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Title of Thesis "OLIGOAMINES AND THEIR METABOLISM IN THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM"

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ABSTRACT

Various aspects of oligoamine metabolism in the cellular slime mould Dictyostelium discoideum are investigated.

The levels of oligoamines in D. discoideum are determined at all stages of the life cycle. The amoebae contain high levels of putrescine and 1,3-diaminopropane whilst spermidine is present but at lower concentrations. Only trace amounts of spermine are present. During development the levels of oligoamines decrease concomitantly with RNA and protein levels. No significant increase in the oligoamine content of spores is detected during germination.

The biosynthetic pathway to putrescine in D. discoideum is typical of eucaryotic organisms, via decarboxylation of ornithine. High levels of ornithine decarboxylase activity are detected in amoebal extracts. Variations in extracellular osmolality result in alterations in the levels of ornithine decarboxylase activity measured. Spermidine is synthesized from putrescine and low levels of S-adenosyl-L-methionine decarboxylase activity are found in amoebal extracts. This enzyme resembles that of Physarum polycephalum and Tetrahymena pyriformis in that it is activated by neither putrescine nor magnesium. During the developmental phase the level of activity of both enzymes decreases.

Amoebae concentrate extracellular putrescine at a rapid rate by an energy dependent process. A model for putrescine uptake is proposed involving adsorptive pinocytosis at low putrescine concentrations and fluid phase pinocytosis at higher concentration.

ACKNOWLEDGEMENTS

I wish to acknowledge my gratitude to Dr W. North for the guidance and patient encouragement he has given me over the years.

The work is dedicated to my parents who encouraged me to embark on the work and my husband who ensured that I completed it.

ABBREVIATIONS

All abbreviations are made in accordance with the recommendations of the Journal of Biological Chemistry with the following additions:-

Dansyl	5-dimethylaminonaphthalene-1-sulphonyl
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
MGBA	Methylglyoxal bis(amidinohydrazone)
PLP	Pyridoxal phosphate
PMSF	Phenylmethylsulphonyl fluoride
SAM	S-adenosyl-L-methionine
$T_{\frac{1}{2}}$	Half life
T_D	Doubling time
TLCK	n- -p-tosyl-L-lysine chloromethyl ketone HCl

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INTRODUCTION

Over the past two decades a growing interest in oligoamines has led to a proliferation in the number of related published papers. These small organic compounds are known to be the end products of a biosynthetic pathway involving several specific enzyme-controlled reactions and yet they neither act as precursors for more complex cellular macromolecules nor has their presence been shown unequivocably to be essential for any biosynthetic cellular process.

At the present time, although a number of naturally occurring oligoamines have been identified in a variety of different cell types, reasearch interests centre around 1,4-diaminobutane (putrescine), 4 azaoctane - 1,8 - diamine (spermidine) and 4,9 - diazadodecane - 1, 12 - diamine (spermine) Figure I.1 , as no cell completely lacking in all three substances has yet been reported. Although found in viruses, bacteria, higher and lower plants, vertebrates and invertebrates the concentrations at which they are present in these cells varies between 10^{-6} and 10^{-2}M (see references in Cohen, 1971; Bachrach, 1973; Tabor & Tabor, 1976a; Stevens & Winther, 1979).

$$^{2}HN - (CH_2)_4 - NH_2 \qquad \text{PUTRESCINE}$$

$$^{2}HN - (CH_2)_4 - NH - (CH_2)_3 - NH_2 \qquad \text{SPERMIDINE}$$

$$^{2}HN - (CH_2)_3 - NH - (CH_2)_4 - NH - (CH_2)_3 - NH_2 \qquad \text{SPERMINE}$$

FIGURE I.1

Most of the effects attributed to oligoamines in a variety of biological systems result from the fact that the pK's of their amine groups are well over 8 and so at physiological pH they are polybasic in nature. Thus they can be

expected to exhibit a high affinity for cellular components with negatively charged groups such as the phosphate groups found on nucleic acids. However much evidence exists which refutes the idea that these compounds act simply as nonspecific intracellular cations.

Since the initial discovery that oligoamines are essential growth factors in many cells (Pohjanpelto & Raina, 1972; Bachrach, 1973), the correlation between rapid cell growth and proliferation and oligoamine content has been well established and strongly supports the physiological importance of these polycations. The activity of ornithine decarboxylase, the enzyme responsible for synthesis of putrescine, has been shown to mirror closely the growth rate in cells. A wide range of conditions associated with rapid growth cause the activity of ornithine decarboxylase to increase dramatically (Jänne et al., 1978), and these changes, together with similar but less marked changes in other enzymes associated with oligoamine synthesis, correlate with an increase in the level of oligoamines in these systems. Numerous attempts have been made to understand at the molecular level the close connection between oligoamine synthesis and the stimulation of cell growth and to determine to what extent the stimulation is dependent on oligoamine synthesis. These studies have provided evidence for the extensive involvement of these compounds in many aspects of macromolecular synthesis in the cell. Recently the interpretation of some of these *in vivo* studies has been complicated by increasing evidence that a number of the inhibitors of oligoamine synthesis used may be involved in a variety of cellular metabolic processes whilst others such as 1,3 - diaminopropane and α - methylornithine as amines may themselves replace some of the functions of oligoamines in the cells (Boynton et al., 1976; Newton & Abdel-Monem, 1977; Pathak et al., 1977).

OLIGOAMINES AND DNA METABOLISM

Oligoamines have been shown to stabilise DNA from several sources against denaturation and shearing (Stevens, 1967; Liquori et al., 1967; Bachrach, 1973).

Models for DNA-oligoamine complexes which have been proposed involve the formation of hydrogen bonds between the protonated amino groups of the higher oligoamines and the phosphate oxygens of the DNA molecules in such a way as to strengthen the binding between the two chains of the helix (Suwalsky et al., 1969; Bachrach, 1973).

A large number of experiments suggest that the enhanced accumulation of oligoamines observed in many systems after growth stimulation may be necessary for stimulation of DNA replication. Thus when oligoamine synthesis is blocked by inhibitors, DNA synthesis is also inhibited by varying degrees whilst no significant alteration in RNA or protein synthesis is observed (Fillingame et al., 1975; Boynton et al., 1976; Mamont et al., 1976; Knutson & Morris, 1978; Pösik et al., 1978). Similar experiments with microbial mutants blocked in the synthesis of putrescine and/or spermidine show that these cells have a decreased growth rate which may result from a reduced rate of DNA replication (Tabor et al., 1978; Whitney & Morris, 1978; Hafner et al., 1979).

Several investigations have attempted to elucidate the mechanism by which lack of oligoamines inhibits DNA synthesis. Brewer & Rusch (1966) showed DNA polymerase activity in Physarum nuclei was enhanced by addition of spermine and they suggested that this was because the oligoamines made the DNA template more accessible to the nuclear enzyme, so increasing chain initiation. Similarly the decreased rate of DNA synthesis observed in MGBA treated human HeLa cells was attributed by Krokan & Eriksen (1977) to a reduction in chain initiation as only a slight reduction in chain elongation was recorded. A different conclusion has been arrived at by other workers. Thus Sunkara et al. (1977) found that Chinese hamster ovary cells deprived of putrescine were inhibited in chain elongation and not initiation. Similarly Geiger & Morris (1980) found that the rate of DNA replication fork movement was markedly reduced in oligoamine dependent mutants of E. coli. As DNA synthesis is believed to be regulated by variations in the frequency of initiation rather than the rate of chain elongation it may be that oligoamines do not directly

regulate DNA replication but rather act as cofactors in the synthetic process.

OLIGOAMINES AND RNA METABOLISM

The majority of studies in this area of oligoamine studies have indicated that RNA synthesis is not primarily affected by inhibition of oligoamine synthesis (Fillingame & Morris, 1973a; Harik et al., 1974; Stevens & Winther, 1979). However previously synthesized oligoamines may still play a role in RNA metabolism.

Oligoamines are believed by some to stabilise RNA *in vivo* (Raina & Jänne, 1968b; Agrell & Heby, 1971; Bolton & Kearns, 1978) by hydrogen bonding to the phosphate oxygen and the 2′ - hydroxide oxygen.

Synthesis of RNA in several organisms appears to be closely linked to oligoamine concentrations (Cohen, 1971; Herbst et al., 1973), and the stimulatory effects of low concentrations of these cationic substances on DNA-dependent RNA polymerases has been reported (Caldarera et al., 1968; Moruzzi et al., 1974). This effect may be similar to that proposed for DNA synthesis i.e. via histone removal and increased availability of template. Alternatively it may result from an effect of oligoamines on the enzyme or the enzyme-template complex. Spermidine and spermine have also been shown to affect other stages of DNA-dependent RNA synthesis including the length of RNA chains (Jänne et al., 1975), the symmetry of transcription (Gumport & Weiss, 1969), and dissociation of RNA polymerase from DNA (Nuss & Herbst, 1973). Pierce & Fausto (1978) found that putrescine stimulates transcription of rat liver chromatin by *E. coli* RNA polymerase, relieving the cessation of RNA chain elongation, the decrease in chain initiation and failure of RNA chains to be released from the polymer - template - RNA-complex found under low ionic strength assay conditions.

Oligoamines are believed to play two roles in the degradation of RNA, depending on the concentration of oligoamines and the nature of the ribonuclease. At low oligoamine concentrations enzymatic degradation of RNA is

stimulated whilst at higher concentrations the oligoamines complex with the RNA and thus protect them from ribonuclease hydrolysis (Karpetsky et al., 1977). Oligoamines also exert effects on the specificity of ribonucleases (Levy et al., 1973; Igarashi et al., 1975).

The increased stability of polyadenylated-mRNA to degradation by ribonuclease activity *in vivo* can be reversed by a concentration of oligoamine proportional to the length of the poly-A segment (Karpetsky et al., 1977). In addition spermine inhibits polyadenylation of various types of RNA (Rose & Jacob, 1976).

Thus oligoamines have been implicated in the regulation of virtually every step in the synthesis and degradation of RNA on the basis of *in vitro* studies. The relative importance of these effects *in vivo* however is not clear and the final RNA content of the cell is likely to depend on the interaction of oligoamine levels with other factors affecting the cellular environment such as ionic strength.

OLIGOAMINES AND PROTEIN METABOLISM

Since oligoamines have been implicated in several aspects of nucleic acid metabolism it is not surprising that protein synthesis may also be affected by these cations, in view of the coupling between the two types of macromolecules. In transformed human lymphocytes the inhibition of protein synthesis, resulting from the use of oligoamine synthesis inhibitors, was reversed by low concentrations of exogenous oligoamines (Hewitt et al., 1979). Similarly addition of putrescine to a slow growing oligoamine-dependent mutant of *E. coli* caused immediate stimulation of protein synthesis and only later RNA and DNA synthesis (Young & Srinivasan, 1972). In several eucaryotic and procaryotic *in vitro* protein synthesizing systems, oligoamines, particularly spermidine and spermine, have been shown to be involved in several intermediate steps of protein synthesis (Cohen, 1971; Bachrach, 1973). Conclusions regarding the importance of these roles *in vivo* are complicated by the observation that in many cases these

functions are nonspecific and the oligoamines are simply replacing Mg^{2+} requirements.

Putrescine, spermidine and spermine have been found in varying combinations and concentrations in ribosomes isolated from both bacterial and animal cells (Stevens, 1970; Tabor & Tabor, 1972). In such experiments isolation techniques are critical as on disruption of the cell the oligoamines present are known to redistribute and binding to ribosomes may occur at this time (Tabor & Kellogg, 1967; Khawaja & Stevens, 1967). Although oligoamines can replace Mg^{2+} in 80% of the cationic binding sites in E. coli ribosomes, so can many other inorganic cations and the remaining 20% of the sites have a critical requirement for Mg^{2+} which is not satisfied by any concentration of oligoamines (Weiss & Morris, 1973; Weiss et al., 1973). Also under optimal Mg^{2+} conditions oligoamines are not required for the in vitro assembly of active 50S ribosomal subunits from dissociated RNA and proteins (Nierhaus & Dohme, 1974). Studies of oligoamine stimulated polypeptide synthesis from synthetic mRNA in procaryotic systems suggest that the 30S ribosomal subunit is involved in the spermidine stimulation (Echandi & Algranati, 1975; Igarashi et al., 1977). Igarashi et al. (1977) provided evidence that spermidine is involved in vitro in the formation of the 30S initiation unit as the oligoamine stimulates binding of phe-tRNA to the P site of ribosomes.

Whether the oligoamines spermidine and spermine have the ability to completely replace Mg^{2+} in the aminoacyl reaction has been the subject of much controversy. Originally it was reported that spermine stimulation of amino acylation of tRNA occurred in a one step process without formation of an amino acid - AMP - enzyme complex intermediate (Igarashi et al., 1971). Later reports failed to confirm these results and it was suggested that addition of spermine to tRNA released sufficient Mg^{2+} to stimulate the aminoacylation reaction (Santi & Webster, 1975; Thiebe, 1975). Takeda (1978) however proposed that the EDTA inhibition of aminoacyl-tRNA formation in the presence of oligoamines reflects the requirement for a metal ion to maintain the amino

acyl-tRNA synthetase in active form.

Isolation of tRNA from several sources containing oligoamines has suggested that this may be the level at which these cations stimulate aminoacylation. Using X-ray crystallography Quigley et al. (1978) located the positions of two spermine and four Mg^{2+} ions in yeast phe-tRNA. Thus spermine by maintaining a stable tertiary tRNA structure may stimulate its aminoacylation by providing a recognition site for the synthetase enzyme. Similarly binding to and release from the ribosome may depend on the conformation of the anticodon region of the tRNA, the site of one of the spermine molecules. A further consequence of spermine stabilization of tRNA structure may be the maintenance of a recognition site for methylase enzymes as evidence exists that methylation of tRNA nucleotide residues is stimulated by oligoamines (Bachrach, 1973). Such methylation reactions are believed to serve a regulatory function in the cell (Bachrach, 1973), and as oligoamine levels increase in rapidly growing cells it may be that these cations are involved in regulation of protein synthesis at this level.

OLIGOAMINES IN CELL DIVISION

The dependence of cell division time on the endogenous concentration of oligoamines has been reported for several cell types (Inouye & Pardee, 1970; Heby et al., 1978). Addition of inhibitors of oligoamine synthesis to various cells causes their progress through the cell cycle to cease - different cells arresting in different phases of the cell cycle (Mamont et al., 1976; Heby et al., 1978; Rupniak & Paul, 1978). Heby et al. (1978) propose that under oligoamine-depletion conditions different cells accumulate in the phase of the cell cycle characteristic for that particular cell type when in a state of general nutritional depletion.

Oligoamines are also known to be involved in cell divisional processes and it is possible that this role entails the division of the cell membrane. The neutralisation of the negatively charged plasma membrane by oligoamines

is believed to account for the ability of these cations to stabilise the protoplasts of several cells (Stevens, 1967; Altman et al., 1977). Despite reports of oligoamines present in isolated membrane preparations there is no evidence that they are located in membranes in vivo (Cohen, 1971; Bachrach, 1973). Sunkara et al. (1979) discovered a high incidence of binucleates amongst oligoamine depleted cells. This may be connected to the report by Oriol-Audit (1978; 1979), that spermidine and spermine promote in vitro polymerisation of actin and its conversion from a globular to a fibrous form. By interaction with actin, a protein with a major role in mitotic spindle formation and cytokinesis, oligoamines may play an important role in cell division.

Thus the functions of the oligoamines remains largely undefined. Further elucidation of their roles in cell metabolism is complicated by:-

(a) their ability to replace and be replaced by Mg^{2+} and other inorganic cations in varying degrees in many functions

(b) the difficulty in determining the distribution of both free and bound intracellular oligoamines

(c) the lack of knowledge of the site of synthesis and subcellular location of oligoamines

Stevens & Winther (1979) propose that the numerous cellular functions of the oligoamines are connected with their stabilizing interactions with nucleic acids. These may be of a transient nature such as the low concentrations of oligoamines that have been observed to stimulate replication, transcription and translation. Higher concentrations however, may inhibit these same reactions by overstabilization. Less transient roles of cellular oligoamines may involve processes such as the maintenance of the tertiary structure of tRNA, ribosomes and condensed chromosomes.

DICTYOSTELIUM DISCOIDEUM

Much of the knowledge concerning the involvement of oligoamines in cell growth is the result of intense work on a relatively few favourable procaryotic

systems such as bacteriophages, E. coli and related bacteria. Progress in understanding their regulatory role in the growth and differentiation of eucaryotic organisms often involves the use of cells maintained in tissue culture which do not usually undergo normal processes of cell differentiation or the use of systems which are experimentally binding due to their inherent complexity. Dictyostelium discoideum, a eucaryotic protist of the Mycetozoa (Olive, 1975) or "cellular slime moulds", offers many advantages for investigating the many facets of oligoamine metabolism in a eucaryotic cell. The primary advantage is that in the life cycle of such organisms growth is separated from differentiation (Figure I.2, Bonner, 1967). In its growth phase D. discoideum is a free-living, unicellular amoebae bounded by a plastic plasma membrane. It locates its prey by a chemotactic response to folic acid liberated by bacteria and feeds phagocytotically (Bonner, 1971). The developmental phase is triggered by starvation and after a period of differentiation the amoebae aggregate into mounds of approximately 10^5 cells (Bonner, 1967). The aggregation is a chemotactic response to cAMP signals emitted by starving amoebae (Konijn, 1972). The signalling is pulsatile and although the cAMP when received by a neighbouring cell is rapidly destroyed by a phosphodiesterase it induces an increase in adenyl cyclase activity in the cell. This is responsible for synthesis of cAMP necessary for signal relay. The cellular response to the chemotactic signal involves moving towards the signal source and formation of cell surface contact sites. These are involved in linking the amoebae into the streams characteristic of late aggregation and finally into the multicellular aggregate (Beug et al., 1973). This aggregate, integrated by deposition of a glycoprotein sheath, may form a fruiting body or sorocarp on the site of aggregation or may become a migrating pseudoplasmodium, grex or slug (Loomis, 1975). Differentiation in the once homogenous cell population can now be detected. Cells in the posterior portion (~66%), of the grex contain characteristic prespore vesicles, whilst the anterior portion consists of cells committed to differentiation into stalk cells (Takeuchi, 1972).

This differentiation is to a certain extent reversible as these cells when disaggregated will divide, after a lag period, when placed in growth medium (Loomis, 1975). Sussman and Schindler (1978) have devised a model of morphogenetic regulation involving cAMP and NH_3 which they use to account for the grex-sorocarp transition and the cytodifferentiation involved in sorocarp construction. The sorocarp consists of a viscous spore mass surmounting a cellulose encased rod of vacuolated stalk cells. Each spore when dispersed and suitably activated, germinates into a vegetative amoebae (Loomis, 1975).

D. discoideum can be propagated under a variety of conditions. Cells grow rapidly either on solid agar substrate seeded with lawns of appropriate bacteria or in shaken liquid salts solution containing washed bacteria. Under both conditions 10^5 - 10^7 spores give rise to 10^9 amoebae virtually free of bacteria in 1 - 2 days and these then construct sorocarps containing approximately 10^5 cells (Sussman, 1966). Several strains of D. discoideum have been isolated which are genetically adapted to growth in an axenic medium (Watts & Ashworth, 1970; Firtel & Bonner, 1972). The enzyme composition of axenically grown cells differs from that of the similar cells or wild type cells grown on bacteria (Ashworth & Quance, 1972). Synchronous differentiation of vegetative cells is achieved by centrifuging the cells away from the growth medium, washing them and placing them on millipore filters. Morphogenetic synchrony is good so that aggregates form in 8h and sorocarps by 24 ± 2h at $22^{\circ}C$ (Figure I.2).

The nuclear DNA genome of D. discoideum is one fiftieth the complexity of that of mammalian cells and eleven times as complex as E. coli (Jacobson & Lodish, 1975), each cell containing 0.2-0.3pg DNA/cell about 2% of which is mitochondrial (Firtel & Bonner, 1972). For reasons not completely understood cells grown on bacteria contain approximately twice as much DNA as axenically grown cells although both are haploid. A possible explanation is that growth conditions affect the time spent by the amoebae in the G_1 and G_2 phases of the cell cycle (Loomis, 1975).

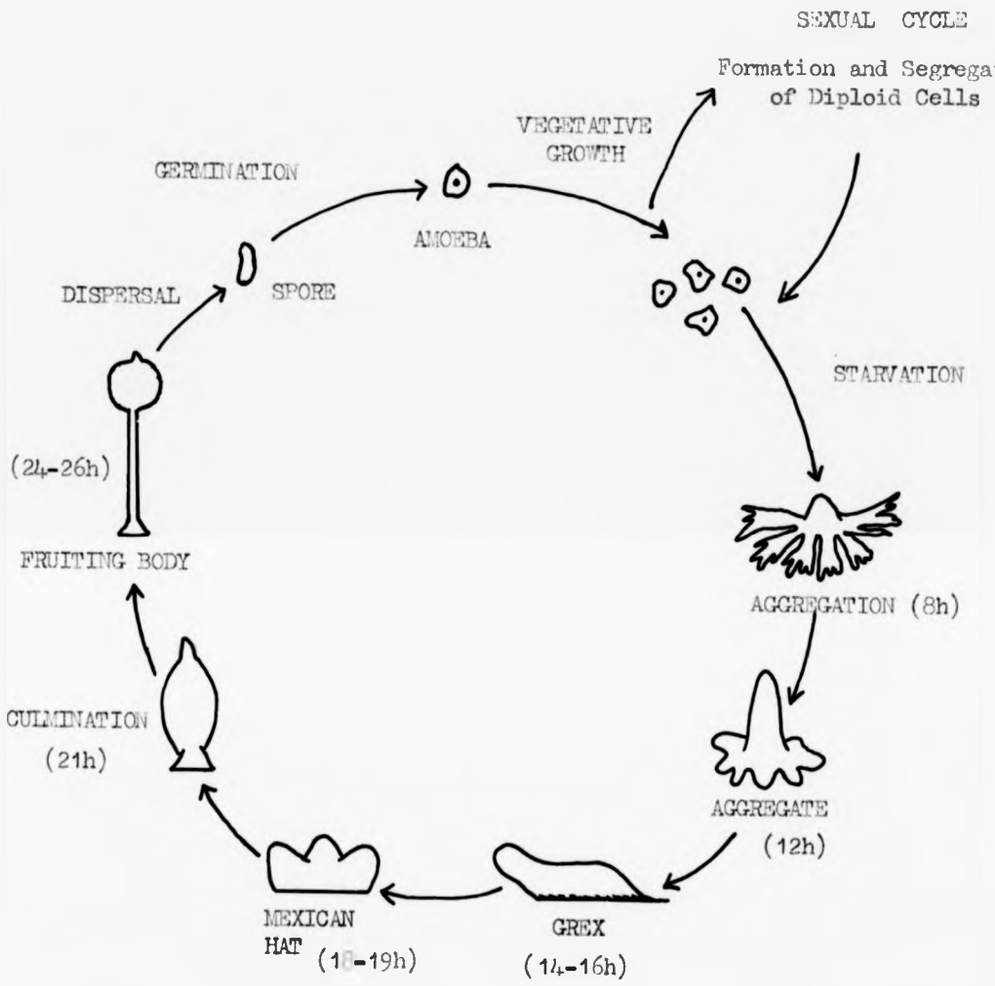


FIGURE I.2

LIFE CYCLE OF DICTYOSTELIUM DISCOIDEUM

D. discoideum possesses typical eucaryotic 80S ribosomes comprising of 60S and 40S subunits (Loomis, 1975). The ribosomal subunits are stable during growth when 90% of the cytoplasmic mRNA and 90% of ribosomes are found in polysomes (Cocucci & Sussman, 1970). After 10 min of development about 60% of ribosomes are present as monosomes (Lodish et al., 1975). During morphogenesis 75% of the vegetative ribosomes are degraded and replaced by newly synthesized developmental ribosomes (Cocucci & Sussman, 1970), although no differences could be detected between the rRNA of growing cells and the rRNA of developing cells (Jacobson & Lodish, 1975).

Development is fuelled by endogenous energy reserves and an extensive turnover of RNA and proteins occurs such that the amount of both in mature sorocarps is approximately half that of amoebal cells (Sussman & Sussman, 1969; Cocucci & Sussman, 1970; Hames & Ashworth, 1974).

The temporal separation of growth and differentiation, along with the changes in the metabolism of nucleic acids and proteins which accompany the developmental phase, suggested that D. discoideum could be a useful eucaryotic system in which to examine the relationship of oligoamine metabolism to the processes of growth and differentiation.

METHODS

CELL GROWTH AND HARVESTING

In the vegetative phase, D. discoideum exists as unicellular amoeboid cells which grow and divide in the presence of a suitable nutrient source.

IN ASSOCIATION WITH BACTERIA (Sussman, 1966)

D. discoideum, strains NC4 (wild type isolate; Raper, 1935) and AX2 (axenic growth isolate; Watts & Ashworth, 1970), were grown in association with Klebsiella aerogenes (Klebsiella pneumoniae: Fulton, 1977) on standard solid medium (SM: 10g D-glucose, 10g bactopeptone, 1g yeast extract, 1g $MgSO_4.7H_2O$ and 10^{-3}M-sodium/potassium phosphate buffer, pH6.4, per litre of 2% (w/v) agar). Stocks of bacteria were maintained in 2.5% (w/v) nutrient broth. Each plate was spread with 0.1ml of bacterial suspension (approx. 10^9 bacteria) and 0.1ml of sterile spore or amoebal suspension (approx. 10^6 cells). The plates were incubated at 22°C and harvested, using a spreader and ice-cold water, when the growing amoebal population had almost cleared the bacterial lawn. The cell suspension was centrifuged at 500g for 5 min, the supernatant discarded and the pellet resuspended in more ice-cold water. The process was repeated until the supernatant was clear of bacteria (Sussman, 1966).

IN AXENIC MEDIUM (Watts & Ashworth, 1970; Franke & Kessin, 1977)

Although wild type isolates of D. discoideum will not grow in the absence of bacteria, several derivatives have been selected for their ability to grow in axenic medium. In this project the strain used was AX2(A.T.C.C. 24397) as isolated by Watts & Ashworth (1970). These cells were grown either in semi-defined liquid axenic medium (HL5), as described by Watts & Ashworth (1970), or in defined liquid minimal medium (FM), as described by Franke & Kessin (1977).

HL5 MEDIUM:- Cells can be grown in this medium (Watts & Ashworth, 1970) in the presence or absence of glucose [Doubling time (T_D) of 8h-10h and 12h-14h respectively]. The medium consists of 7.15g yeast extract, 1.28g $Na_2HPO_4.12H_2O$,

0.486g K H_2PO_4 and 14.3g Oxoid bactopeptone dissolved in 1l of distilled water and the pH adjusted to 6.7. This was sterilized by autoclaving for 15-20 min at 15lb/inch^2 in Erlenmeyer flasks (660ml in a 2l flask, 66ml in a 250ml flask). The 1.5M-glucose solution was autoclaved separately and added where required, such that the final concentration was 86mM.

FM MEDIUM:- The FM medium was constructed from concentrated stock solutions of amino acids, vitamins, salts, trace elements, streptomycin sulphate and glucose as detailed by Franke & Kessin (1977). The complete medium was sterilized by autoclaving at 15lb/inch^2 for 20 min, and stored in the dark. In the absence of glucose there was only limited growth, in its presence the amoebae had a T_D of 20-24h.

Additions of oligoamines and other compounds to growth cultures were made by filter sterilization through Sartorius membrane filters (0.45μm pore size, 13mm diameter). Flasks of media were inoculated with sterile spore or amoebal suspensions and incubated at 22^{o}C in an orbital incubator (130rev/min). Cells growing in either HL5 or FM media grow exponentially to a titre of 5x10^6 cells/ml and then at a decreasing rate to a titre of 2x10^7 cells/ml. The cell density of cultures was monitored microscopically using a Neubauer Haemocytometer. At this stage any contamination of cultures could be detected. Unless otherwise stated, cells used in the determination of cellular processes such as oligoamine content, enzyme activities and rates of putrescine uptake were harvested in mid-log phase by centrifugation at 500g and 4^{o}C for 15 min. The resulting cell pellet was washed twice with copious amounts of ice-cold water.

CELL DEVELOPMENT

When amoebae are deprived of nutrients and deposited on a moist, solid surface they enter the differentiation phase of their life cycle.

Well washed vegetative cells were suspended in ice-cold distilled water to give a cell density of 5x10^7-10^8 cells/ml. Aliquots (0.5ml) of this suspension were evenly dispersed on well washed millipore filters (0.8μm pore

size, 47mm diameter) resting on absorbent glass fibre paper pads saturated with 1.6ml pad diluting fluid (50mM-sodium/potassium phosphate buffer, pH6.5, containing 1.5g KCl, 0.5g $MgCl_2.6H_2O$ and 0.5g streptomycin sulphate per litre, Sussman, 1966). The dishes were incubated on moistened towels in foil covered trays at 22°C and development was completed in 24±2h. To avoid asynchronous development, no more than 1h was allowed to elapse between medium deprivation and the placing of the cells on the millipore filters. The cells were harvested by placing the millipore filter on the inside wall of a beaker and triturating with ice-cold distilled water using a 2ml pipette.

PREPARATION OF CELL EXTRACTS

Cells were harvested into ice-cold distilled water or, for enzyme assays, into the appropriate extraction buffer. Extracts were prepared by sonication (15μm peak to peak) with an M.S.E. ultrasonic disintegrator and continuous cooling in an ice/salt bath. For suspensions of cells up to early culminatation (16h), 4x15s bursts were used, for later developmental stages, 12x15s bursts were used (Hames & Ashworth, 1974). For enzyme assays the crude cell extracts so prepared were centrifuged for 5 min at 1000g and the supernatant used.

GERMINATION

Spores were harvested from millipore filters, 2 days after synchronous fruiting had occurred, by lightly brushing the sori with the edge of a microscope slide. The remaining material, which consisted mainly of stalk cells and a small proportion of spore cells, was harvested by placing the millipore filter on the inside wall of a beaker and triturating with ice-cold distilled water using a 2ml pipette. The spores were washed from the slide with ice-cold distilled water and the resulting spore suspension used immediately or frozen at -20°C in buffer containing 20% (v/v) glycerol. The frozen spores remain viable for 4-6 months (Ennis & Sussman, 1975).

Frozen spores were thawed and washed rapidly by centrifugation twice with

3 volumes of 10mM-sodium phosphate buffer, pH6.7. The spores were suspended, at a density of $3\text{-}5\text{x}10^7$ spores/ml, in buffer containing 20% (v/v) dimethylsulphoxide (DMSO) and shaken for 30 min in an orbital incubator (130rev/min) at 22°C to activate them (Cotter & O'Connell, 1974). After several washings with 10mM-sodium phosphate buffer, pH6.7, the cells were suspended in buffer at a density of $5\text{x}10^7$ spores/ml and incubated at 22°C in an orbital incubator (130rev/min). Changes in cell size and shape characteristic of the germination process (Cotter & Raper, 1966) were followed by periodic microscopic examination using a Neubauer Haemocytometer.

OLIGOAMINE ESTIMATIONS

Oligoamines were obtained from sonicated cell extracts by addition of an equal volume of 4% perchloric acid. This was vortexed and after standing on ice the insoluble material was collected by centrifugation, re-extracted with 2% (v/v) perchloric acid and the soluble fractions combined. The acid insoluble pellet was retained for estimation of RNA, DNA and protein as described later. The oligoamine content of the acid soluble fraction was estimated by one of the following two methods.

METHOD A (Modified from Seiler & Wiechmann, 1967).

This sensitive analytical method allows determination of 10^{-12} to 10^{-11} mol of oligoamine (Bachrach, 1973). It involves conversion of the oligoamines to highly fluorescent 5-dimethylamino-naphthalene-1-sulphonamide (dansyl) derivatives which are separated on silica gel plates. Quantitative analyses of the oligoamines are made by measurement of the fluorescent intensity of the spots after elution from the silica gel.

Dansyl derivatives were prepared from 0.2ml of the acid soluble fraction by addition of 0.4ml dansyl chloride (30mg/ml acetone) solution and 50mg Na_2CO_3. The reaction was allowed to proceed for 16h in the dark at room temperature. Excess dansyl chloride was then converted to dansyl proline by the addition of 0.1ml L-proline (100mg/ml H_2O). This prevents the excess dansyl

chloride being hydrolysed to 1-dimethylamino-naphthalene-5-sulphonic acid, which causes blue-green fluorescent streaks on the chromatogram. After 30 min the dansylated derivatives were extracted from the alkaline phase with 0.5ml benzene.

Microlitre aliquots of the benzene extract (5-20µl) were applied to thin layer silica gel chromatography plates. These were prepared by shaking together 30g silica gel and 60ml of distilled water and applying the slurry to pre-cleaned glass plates (20x20cm) with a Shandon Southern Unoplan variable thickness applicator set at 200µm and activated by heating to 110°C for 1h. Six samples and three oligoamine standards were routinely spotted on each silica plate.

Separation of dansylated oligoamines was effected by development in ethylacetate/cyclohexane (2:3, v/v; Dion & Herbst, 1970) for 45-60 min and, as humidity causes diminished fluorescence, the plates were then dried for 10 min at 130°C (Boulton, 1971). The dansyl amine spots were located by U.V. irradiation, removed by suction and extracted with 4ml benzene/triethylamine (95:5, v/v). The silica gel was removed by centrifugation (approx. 1000g for 10 min) and the fluorescence of the extracts measured with a Perkin-Elmer fluorescence spectrophotometer (Model MPF-3L) at emission maximum 512 nm and excitation maximum 368 nm. A linear relationship between fluorescence and concentration of the oligoamine derivatives exists over the range $10\text{-}10^3$pmol/spot (Seiler & Wiechmann, 1967). Although this linear relationship is reproducible, variations in fluorescence intensity between duplicate plates necessitates inclusion of dansylated oligoamine standards, in the appropriate range, on each plate. By addition of appropriate radioactive internal standards, the recovery of oligoamines by this method was found to be between 87-92%.

METHOD B (Modified from Inoue & Mizutani, 1973).

In this second method the acid soluble fraction was applied to a Dowex-50W column and the oligoamines and basic amino acids separated, after elution from the column, by paper strip electrophoresis. Oligoamines were identified after

reaction with ninhydrin and quantitative estimation made by spectrophotometric determination at 505 nm of the eluted ninhydrin product.

Dowex-50W resin (200-400 mesh, 8% cross linked) was prepared by washing successively, once with 20 vol of 4M-NaOH, twice with 20 vol of 6M-HCl and then once with 50 vol of H_2O. Using this resin, cation exchange columns were prepared (10x1cm).

The acid soluble fraction was applied to the column and the bound material washed with 20ml 0.5M-HCl. The oligoamines and basic amino acids were then eluted with 80ml 6M-HCl and this fraction evaporated to dryness in a rotary evaporator, bath temperature 60°C. The solid material was carefully redissolved in 0.2ml 0.2M-HCl and 10-25µl aliquots of this used in the paper electrophoresis. The columns were regenerated, between runs, by washing with 100ml of water.

The paper strips (3x30cm, Whatman No.3) were dipped in buffer solution and then blotted to remove the excess moisture. A micropipette was used to apply the sample 7cm to the anode side of the centre of the strip, the margins of the strip being avoided. A constant potential of 240V, corresponding to a potential gradient of 8V/cm, was applied giving sufficient resolution within 1½-2h. After completion of electrolysis, the ends of the papers were blotted and the strips allowed to dry at room temperature until stained. The strips were stained by passage through a drip tray containing freshly prepared ninhydrin reagent (100mg cadmium acetate, 1g ninhydrin dissolved in 5ml glacial acetic acid, 10ml water and 85ml acetone). The colour was fully developed by heating the papers at 75°C for 20-30 min. The standard regions were cut out, eluted with 5ml of extractant reagent (0.2g cadmium acetate, 10ml water, 40ml ethanol and 50ml glacial acetic acid), at room temperature for 30 min with shaking and the optical density at 505 nm measured with a Cecil CE 272 U.V. spectrophotometer against an extracting fluid blank. A standard curve of each oligoamine was prepared by inclusion, in each electrophoresis tank, of three strips on which were spotted an appropriate range of standard oligoamine solutions.

The efficiency of this method was measured by addition of internal standards of radioactively labelled oligoamines to the columns. Recovery of label from the strips was 85-90%.

Two buffers were used in the electrophoresis tanks during the course of this study (Raina, 1963).

BUFFER A (0.1M-citric acid buffer, pH3.5).

21g of citric acid and 3.2g NaOH were dissolved in distilled water, the pH adjusted to 3.5 with NaOH and the volume made up to 1l. This buffer can be used on several successive occasions provided it is always remixed between runs.

BUFFER B (0.065M-5-sulphosalicylic acid buffer, pH3.2).

16.5g of 5-sulphosalicylic acid and 4.9g NaOH were dissolved in distilled water, the pH adjusted with NaOH and the solution made up to 1l. During electrophoresis this buffer becomes markedly discoloured especially in the anode side reservoir, thus the buffer must be changed after every run.

CELLULAR PROTEIN, RNA AND DNA ESTIMATIONS

The acid insoluble pellet, retained after oligoamine extraction, was washed twice with 10% (w/v) trichloroacetic acid and then resuspended in 1.2ml 5% (w/v) trichloracetic acid. This suspension was incubated at 90°C for 30 min and the insoluble material collected by centrifugation. The hydrolysate was used for RNA and DNA estimations; the pellet was dissolved in 2M-NaOH and the protein estimated in the resulting solution by the method of Lowry et al.(1951) using bovine serum albumin (Sigma; fraction V) to prepare a standard calibration curve (0-400µg).

DNA was estimated by the method of Giles & Myers (1965) as modified by Hames et al. (1972). Using 0.4ml portions of the hydrolysates, the following reagents were added in order:- 0.1ml 50% (v/v) perchloric acid, 0.5ml 4% (w/v) diphenylamine (recrystallized from ethanol), in glacial acetic acid and 0.05ml 0.2% (v/v) acetaldehyde. These samples, along with standards (0-50µg) of calf thymus DNA (Sigma; type I) prepared in the same manner, were left for 20-24h

at room temperature. The (595-700) nm optical density difference was then measured and the DNA content of the sample determined from a standard calibration curve.

RNA was estimated by the method of Mejbaum (1939). Portions of the hydrolysate (0.05-0.2ml) were made up to a volume of 1.5ml with distilled water and an equal volume of freshly prepared orcinol reagent (1g orcinol, 0.5g $FeCl_3$ and 100ml conc. HCl) added. Standards containing known amounts of D-ribose (0-45μg) were similarly prepared. These solutions were boiled for 30 min, cooled for 20 min and the optical density at 550 nm determined. The ribose content of the samples was determined from a standard calibration curve.

CALCULATION OF APPROXIMATE INTRACELLULAR OLIGOAMINE CONCENTRATIONS

Having determined the oligoamine and DNA content of the various samples, the approximate intracellular oligoamine concentrations were estimated based on the following assumptions:-

(a) that the average DNA content of D. discoideum amoebae grown axenically is 16.8×10^{-8} μg/cell and grown in association with bacteria is 36×10^{-8} μg/cell (Leach & Ashworth, 1972), and remains relatively constant during development (Hames, 1972).

(b) that the mean cell volume of D. discoideum amoebae is 650μm^3 whilst that of spores is 45μm^3 (Cappuccinelli & Ashworth, 1976).

(c) that during the growth and development of D. discoideum amoebae there is an equal distribution of oligoamines throughout the cell.

Whilst the first two assumptions are justified by experimental evidence, the last is not and in view of observations in several experimental systems of oligoamines binding to various cell constituents (Bachrach, 1973), it is thought unlikely that such an equal distribution of these cations should exist *in vivo*.

ENZYME ASSAYS

The activities of ornithine, arginine and S-adenosyl-L-methionine (SAM) decarboxylases were determined by trapping and subsequently measuring the radioactivity of the $^{14}CO_2$ evolved upon incubation of the crude enzyme extract with the appropriate carboxy-labelled substrate and any necessary cofactors. The methods are based on those described by Stevens et al. (1976) for ornithine and SAM-decarboxylase and Morris & Pardee (1966) for arginine decarboxylase.

Crude enzyme extracts were prepared by sonication and centrifugation in 5mM-potassium phosphate buffer, pH7.6, containing 2mM-1, 4-dithiothreitol (DTT), 1mM-$MgCl_2$, 0.1mM-ethylenediaminetetra-acetate (EDTA), 0.1mM-pyridoxal phosphate (PLP) and 10% (v/v) glycerol for both ornithine and SAM-decarboxylase assays and in 0.05M-2-amino-2-hydroxymethylpropane-1,3-diol (Tris/HCl buffer, pH7.6, containing 1mM-EDTA and 1mM-DTT for arginine decarboxylase assays. All operations were carried out at 0-4°C.

The incubation mixture for ornithine decarboxylase was 65mM-Tris/HCl, pH8.1, 0.5mM-L-ornithine, 0.31mM-PLP, 0.125μCi of D, L-[1-^{14}C] ornithine monohydrochloride (58mCi/m mol) and 0.1ml enzyme extract containing less than 0.1mg cell protein, in a total volume of 0.4ml. The incubation mixture for SAM-decarboxylase was 0.1M-sodium phosphate buffer, pH8, 0.025μCi of S-adenosyl-L-[1-^{14}C] methionine (60mCi/m mol) and 0.1ml enzyme extract containing less than 1mg cell protein, in a total volume of 0.4ml. The incubation mixture for arginine decarboxylase was 0.1M-Tris/HCl, pH7.6, 0.033mM-PLP, 3.3mM-$MgCl_2$, 0.125μCi of [U-^{14}C]arginine monohydrochloride (324mCi/m mol) and 0.1ml enzyme extract containing less than 1mg cell protein, in a total volume of 0.3ml.

Duplicate incubations were carried out in test tubes in a shaking water bath at 37°C. Small vials (12mm diameter), containing 50μl of 2-methoxyethanol/ethanolamine (2:1, v/v), were suspended by thread near the top of the test tube and the test tubes sealed with self sealing caps. After 30 min 0.1ml of

50% (w/v) trichloroacetic acid was injected into the test tubes and they were incubated for a further 60 min to trap the liberated CO_2 completely. The small vials were then removed, 2.5ml of scintillation cocktail added, the sealed vials placed in scintillation vials and the radioactivity measured (see page 33). In order to determine the radioactivity arising from non-enzymic decarboxylation during the assays, controls, in which the enzyme extract added had been heat denatured, were carried out routinely as part of each experiment. The values thus obtained were used to correct experimental results during the calculation of enzymic activity. Protein determinations were carried out on trichloroacetic acid precipitated protein pellets. The pellet was dissolved in 2M-NaOH and the protein estimated in the resulting solutions by the method of Lowry et al. (1951), using bovine serum albumin (Sigma; Fraction V) as a standard. Enzyme activity is given in units of n mol or p mol of CO_2 released/min/mg protein.

UPTAKE OF RADIOACTIVE ARGININE, ORNITHINE AND PUTRESCINE BY AMOEBAE

Washed amoebae were incubated in phosphate buffer containing radioactively labelled putrescine, arginine or ornithine and uptake monitored by the measurement, at appropriate time intervals, of the radioactivity present in various cell fractions.

Amoebal cells were harvested, washed and resuspended in 17mM-sodium phosphate buffer, pH6, at a cell density of between $4x10^6$ and $6x10^6$ cells/ml. The cells were pre-incubated in an orbital incubator (130rev/min) at 22°C for 15 min prior to addition of labelled material. At appropriate intervals thereafter 1ml aliquots were removed and the cells pelleted rapidly in a Jobling model 320 microcentrifuge for 1 min. The resulting cell pellet was washed twice with 1ml aliquots of distilled water and finally resuspended in 1ml of 2% (v/v) trichloroacetic acid. After 30 min on ice, the acid insoluble material was collected by centrifugation, re-extracted with a further 1ml of 2% (v/v)

trichloroacetic acid and the soluble fractions combined. Duplicate samples (o.1ml) of the cell suspension, water washing and acid soluble material were transferred to scintillation vials, 10ml of scintillation cocktail added and the radioactivity measured (see page 33). The acid insoluble pellet was filtered onto a Whatman GF/C filter, washed with 5ml of 10% (v/v) trichloroacetic acid and dried at room temperature. These filters were placed in scintillation vials, 10ml of scintillation cocktail added and the radioactivity measured (see page 33).

In some experiments the distribution of radioactivity among the cellular oligoamines was determined by fractionating the trichloracetic acid soluble material on a Dowex-50W resin (200-400 mesh; 8% cross linked) column (see page 25). The unbound material was collected (fraction A), and the column then washed with 20ml of 0.5M-HCl and the eluted material collected (fraction B). The final fraction (fraction C) was obtained after elution of the column with 80ml of 6M-HCl. Samples (o.1ml) of these three fractions in 10ml of scintillation cocktail were used to measure the radioactivity present (see page 33).

Fraction C contains the basic amino acids ornithine and arginine and the oligoamines putrescine, 1,3-diaminopropane, spermidine and spermine and in some experiments the distribution of radioactivity in these substances was determined. This fraction was evaporated to dryness by rotary evaporation at 60°C and the residue redissolved in 200µl of 0.02M-HCl. Portions (20µl) were separated by electrophoresis in 65mM-5-sulphosalicylic acid buffer, pH3.2, as detailed in Method B. The strips were dried at room temperature and the positions of the oligoamines, and where necessary the basic amino acids arginine and ornithine, determined by treatment of a control strip with ninhydrin. The appropriate regions of the experimental strips were then extracted overnight with 1ml 6M-HCl and portions (0.1ml) of this in 10ml of scintillation cocktail used to determine the radioactivity present (see page 33).

MEASUREMENT OF PUTRESCINE UPTAKE

Amoebae, harvested during exponential growth, were resuspended in 17mM-sodium phosphate buffer, pH6, at a cell density of between $1x10^6$ and $6x10^6$ cells/ml. Unless otherwise stated 10ml suspensions were used. The suspension was pre-incubated at 22°C in an orbital incubator (130rev/min) for 15 min after which time a sample of [1,4-^{14}C]putrescine was added. Samples were then withdrawn at regular intervals over a 30 min period, centrifuged in a Jobling model 320 microcentrifuge for 1 min, the cell pellet washed twice with 1ml aliquots of distilled water and finally resuspended in 2ml of distilled water. These cell suspensions were transferred to scintillation vials, 10ml of scintillation cocktail added and the radioactivity measured (see page 33). Rates of putrescine uptake were then determined graphically and are reported as p mol or n mol of putrescine/min/10^7 cells.

PUTRESCINE CATABOLISM

D. discoideum amoebal cells labelled with [1,4-^{14}C]putrescine evolve $^{14}CO_2$ which can be trapped and the radioactivity measured. In this way an approximate rate of putrescine catabolism to CO_2 was obtained.

DURING GROWTH:- AX2 amoebae were harvested during exponential growth from flasks of FM medium, washed and resuspended at a cell density of $8x10^6$ cells/ml in 17mM-sodium phosphate buffer, pH6, containing 0.2μM-[1,4-^{14}C] putrescine monohydrochloride (63mCi/m mol). After 15 min the cells were harvested and resuspended at the same cell density in sterile FM medium, 10ml aliquots of which were then placed in 50ml Erlenmeyer flasks. Carbon dioxide traps, consisting of small vials (12mm diameter) containing 50 l 2-methoxyethanol: ethanolamine (2:1, v/v), were suspended by thread near the top of the flasks which were then sealed with self sealing caps. Incubations were carried out at 22°C in an orbital incubator (130rev/min). At appropriate intervals several flasks were opened, the vials removed, 2.5ml of scintillation cocktail added and the radioactivity determined as usual. From these flasks, 1ml aliquots

were removed and the radioactivity contained in the FM medium and the various cell fractions was determined as before.

DURING DEVELOPMENT:- Vegetative amoebae were labelled as above. After 15 min the cells were harvested, washed and resuspended in ice-cold water at a cell density of $5x10^8$ cells/ml. This suspension was used in the preparation of a modified developmental system, in which the petri dishes normally used were replaced by 50ml Erlenmeyer flasks containing, in addition to the usual millipore filter resting on buffer soaked support pads, a carbon dioxide trap (as above), and sealed with a self sealing cap. The radioactivity in the carbon dioxide trap was determined at intervals over the 24h developmental period as was the radioactivity in the pad diluting fluid and the various cell fractions. Under these conditions the cells developed normally over a 24 ± 2h period.

MEASUREMENT OF RADIOACTIVITY

The scintillation cocktail used throughout consisted of 31.5g 5-(biphenyl)-2-(4-t-butylphenyl)-1-oxa-3, 4-diazole dissolved in 500ml methanol, 3 l toluene and 1.5 l Triton X-100 (Winther & Stevens, 1978). The volumes of sample and scintillation cocktail used in the various experiments are detailed in the appropriate methods section.

Measurements of radioactivity were made using a Philips PW510/01 automatic liquid scintillation analyser counting on appropriate ^{14}C or ^{3}H programmes which made corrections to d.p.m. by the channels ratio method and subtracted background counts (10 min counts).

CHEMICALS

Spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, Dowex-50W resin, PLP, DTT, streptomycin sulphate, protein (fraction V) and DNA were purchased from Sigma (London) Chemical Co., Kingston upon Thames, U.K. 1,3-diaminopropane, D-ribose, 2,4-dinitrophenol, NaN_3, cycloheximide and 5-sulphosalicylic acid were purchased from BDH Chemi-

cals, Poole, Dorset, U.K. Bactopeptone, Yeast Extract, Nutrient Broth and Agar No.3 was purchased from Oxoid. S-adenosyl-L-methionine was purchased from the Boehringer Corporation (London) Ltd. All radioactively labelled chemicals were purchased from the Radiochemical Centre, Amersham, U.K. All other chemicals used were of the highest purity available and purchased from either BDH Chemicals or Fisons, Loughborough, Leics., U.K.

RESULTS AND DISCUSSION - CHAPTER ONE

INTRODUCTION

DISTRIBUTION AND LEVELS OF OLIGOAMINES

The oligoamine levels of a wide variety of organisms have been reported in the literature, however comparison of the data is difficult as different analytical methods and units of calculation are used by the different laboratories. Thus in many ways it is more informative to consider the relative amounts of oligoamines in the different cells.

Since the discovery of spermine and spermidine in human semen, oligoamines have been found in varying relative proportions in numerous human and other mammalian tissues and fluids, where putrescine is present but in much lower concentrations than spermine and spermidine (Bachrach, 1973). In contrast, in the developing embryo of the amphibian Xenopus laevis putrescine and spermidine concentrations are high whilst spermine concentrations are low both in the eggs and embryos (Russell, 1970).

Several studies have confirmed the presence of oligoamines in representatives of all the major invertebrate phyla. Manen et al. (1977) studied oligoamine levels during early development of the mollusc Phestilla sibogae and report the presence, in both larval and adult stages, of large amounts of putrescine and spermidine with little if any spermine. The concentration of spermidine in fertilised Drosophila melanogaster eggs and during early embryonic development, is higher than in subsequent developmental stages. Putrescine concentration is low in fertilised eggs and undergoes only slight elevation during post-embryonic stages (Dion & Herbst, 1970). Spermine is the major oligoamine during the development of sea urchins however (Manen & Russell, 1973).

Most work with plants has been concerned with their putrescine content,

whilst the distribution of spermine and spermidine has tended to be neglected. Moruzzi & Caldarera (1964) found high concentrations of spermine and spermidine throughout the wheat plant with the exception of the roots and anthers. The presence of these oligoamines has also been demonstrated in tomato juice, Phaseolus seedlings, tubers of Helianthus tuberosus and seeds from several higher plants (Bagni, 1966; 1970). Smith (1970a) similarly reported the presence of putrescine, spermidine and spermine in the leaves, roots and fruits of many higher plants. As in other systems, oligoamine levels change during growth and development and are known to function as growth factors in some plants (Bagni, 1966; Bagni et al., 1967).

Oligoamines are also found in coliphages, animal and plant viruses (Bachrach, 1973). In T-even coliphages, which contain approximately 4-6 times as much putrescine as spermidine, the oligoamine content is culture dependent (Ames et al., 1958). This is even more so in the T-odd coliphages which are highly permeable and, as a result, oligoamines exchange freely with extracellular cations resulting in a lower intracellular concentration (Ames & Dubin, 1960). Kelly & Elliott (1977) report that in two densonucleosis viruses (DNV), spermine, spermidine and putrescine are associated specifically with the DNV particles and therefore probably with the viral DNA. By promoting base stacking, oligoamines are thought to aid the condensation of the DNA into a compact form suitable for encapsidation. Oligoamines have also been found in RNA viruses such as tobacco mosaic virus, cucumber virus, polio virus and tomato bushy stunt virus, but in all cases less than 2% of the RNA phosphate could be neutralized by the oligoamines present as compared with 40% neutralization of DNA phage phosphate (Ames & Dubin, 1960).

In general eucaryotes have spermine in addition to spermidine but contain little putrescine, whereas procaryotes have higher concentrations of putrescine than spermidine and lack spermine. Several strains of Escherichia coli have been analysed for their oligoamine contents (Bachrach, 1973), and intracellular concentrations of 25mM-putrescine and 2.5mM-spermidine are typical (Morris et

al., 1970). Gram positive organisms are reputed to contain less oligoamines than gram negative organisms (Bachrach, 1973). Thus in Pseudomonas aeruginosa only putrescine has been detected and at much lower concentrations than in E. coli (Kim, 1966). However, as the oligoamine levels in bacteria are very much dependent on such parameters as pH, nutrient composition and phase of growth, the usefulness of such demarcations is limited. Thus Lactobacillus casei was reported to contain low levels of oligoamines in stationary cultures, but it was found that the organism synthesizes quantities of spermidine in the lag phase and then grows until this is diluted to low levels (Elliott & Michaelson, 1969). The issue is further complicated by the discovery that the growth of L. casei is greatly stimulated by the presence of oligoamines in the media (Guirard & Snell, 1964). Similarly, uptake by bacteria of oligoamines in the media alters the intracellular oligoamine content of these cells. Thus spermine, not usually found in bacteria, is present in cultures of E. coli (Tabor & Tabor, 1966), Staphylococcus (Rosenthal & Dubin, 1962) and L. casei (Guirard & Snell, 1964) grown in spermine rich media. Munro et al. (1972) reported that for E. coli an increase in the osmolarity of the growth medium led to a large increase in potassium uptake accompanied by a rapid excretion of cellular putrescine, while no loss of spermidine or amino acids occurred. They proposed that using putrescine excretion, the cell achieves increases in internal osmolarity whilst controlling the ionic strength and charge balance of the cell.

In an initial survey of the oligoamines present in the other procaryotic phylum, the cyanobacteria, Ramakrishna et al. (1978) were unable to detect spermine but found that all of the organisms were high in spermidine and low in putrescine.

The oligoamine levels reported in some of the lower eucaryotic phylum are more typical of those in procaryotic cells. Thus putrescine and spermidine have been isolated from the unicellular green algae Chlorella fusca and Scenedesmus acutus, where again the intracellular content is dependent on the

age of the culture and environmental parameters (Bachrach, 1973). Similarly spermidine and putrescine are the predominant oligoamines in the Trypanosomatids. The oligoamine content of these parasitic flagellated protozoa fluctuates during growth, peaking in mid log phase and declining to a constant value in stationary cultures (Bacchi et al., 1977). Holm & Heby (1971) found a similar oligoamine distribution in the ciliated protozoa Tetrahymena pyriformis and the absence of spermine in this protozoa has been confirmed (Pösö et al., 1975b).

The oligoamine content of fungal cells resembles more closely that of the higher eucaryotic organisms. The presence of spermine, spermidine and putrescine in various hemiascomycetes has been reported from several sources (Pösö et al., 1976b; Nickerson et al., 1977a). However Nickerson et al. (1977a) failed to detect spermine, "the eucaryotic oligoamine," in fifteen species of filamentous fungi that they examined. They suggest that other workers have incorrectly identified as spermine an unidentified compound with similar Rf value, first reported by Mennucci et al. (1973) as occurring in the water mould, Blastocladiella emersonii. Reports of spermine occurring in Neurospora crassa (Viotti et al., 1971; Bowman & Davis, 1977a) and Aspergillus nidulans (Bushell & Bull, 1974; Winther & Stevens, 1976) have been reaffirmed by Hart et al. (1978), who demonstrated the incorporation of labelled putrescine into spermine in a wide variety of fungi. Thus spermine may be lacking in some fungal species, but is present at varying concentrations in many others. In most fungi however, spermidine is the major oligoamine, with putrescine occurring at much lower concentrations. One exception to this generalization has been reported by Mennucci et al. (1975), who found a high intracellular putrescine/spermidine ratio in B. emersonii. As in other organisms, the concentration of oligoamines varies with the state of growth and the media in which the cells are grown. Several groups have investigated the involvement of oligoamines with the macromolecular synthesis characteristic of the fungal cell differentiation which occurs on spore germination. In Saccharomyces

cerevisiae (Choih et al., 1973), Rhizopus stolonifer, Botryodiplodia theobromae (Nickerson et al., 1977b), A. nidulans (Stevens, 1975), and B. emersonii (Mennucci et al., 1975), a parallel increase in spermidine and RNA levels occurs, such that the spermidine/RNA ratio remains approximately constant.

Slime moulds lie in a transition zone between the major phylogenetic categories. Although some botanists consider these organisms have many features in common with fungal cells, there are outstanding differences between the two. Thus all fungal cells are surrounded by a definite cell wall whereas in the slime moulds a cell wall is found only around spore and stalk cells; vegetative amoebae have only a cell membrane. In addition fungal cells feed saphrophytically in contrast to the phagotrophic feeding methods of the slime moulds. For these reasons many zoologists consider that slime moulds are protozoa, indistinguishable from other soil amoebae. Mitchell & Rusch (1973) have determined that in the true slime mould, Physarum polycephalum, the levels of putrescine and spermidine are high, more typical of the oligoamine content of protozoa than of fungal cells.

BIOSYNTHESIS OF OLIGOAMINES (Figure 1.1)

The pathway for the biosynthesis of oligoamines was established in the procaryote E. coli in which putrescine was found to be formed by decarboxylation of ornithine, an intermediate of the arginine pathway (Morris & Pardee, 1965). This pathway has been shown to be operative in other procaryotes, in plants, fungi and higher eucaryotes (Tabor & Tabor, 1964; Pegg & Williams-Ashman, 1968; Russell & Snyder, 1968; Cohen, 1971; Bachrach, 1973; Stevens & Winther, 1979). In many micro-organisms arginine is not an essential amino acid and its biosynthesis, along with that of ornithine, is regulated by repression and feedback inhibition (Maas, 1961). In some such organisms the action of an arginase has been detected, catalysing the formation of ornithine from arginine and thus allowing synthesis of putrescine under conditions in which ornithine is no longer produced from glutamate. Thus arginase-less

mutants of N. crassa grown in arginine-rich media require putrescine supplements for optimum growth (Davis et al., 1970). In higher eucaryotes, which require arginine as an essential amino acid, arginase exists as a component of the urea cycle and even in organisms lacking a functional urea cycle, arginase activity is retained. presumably because of its role in putrescine biosynthesis (Davis et al., 1970). In micro-organisms such as E. coli, K. aerogenes, B. stearothermophilis and in some plants, arginase activity has not been detected (Morris et al., 1970; Smith, 1970b; Stevens et al., 1978). In these organisms a second pathway of putrescine synthesis exists which involves the decarboxylation of arginine to agmatine. This is converted to putrescine either via agmatine by the action of arginine decarboxylase and agmatine ureohydrolase (Morris & Pardee, 1966), or through the intermediate production of N-carbamylputrescine as is found in some plants (Smith, 1970b). No evidence has yet been obtained for an arginine decarboxylase in mammalian tissues although two arginine decarboxylases and two ornithine decarboxylases have been isolated from E. coli. The biodegradative decarboxylases are induced by acid growth conditions whereas the biosynthetic enzymes are constitutive (Morris & Fillingame, 1974).

Although incorporation of radioactive putrescine into spermidine and spermine in E. coli, A. nidulans and animal tissues (Tabor et al., 1956; Tabor et al., 1958) confirmed that putrescine is a precursor for the other oligoamines, the origin of the propylamine moeity in these compounds remained obscure until Greene (1957) demonstrated that in N. crassa it is derived from methionine. Subsequent reports have shown that putrescine and methionine also act as precursors of spermine and spermidine in vivo in various micro-organisms, fungal cells and animal tissues (Jänne, 1967; Siimes & Jänne, 1967; Bachrach, 1973; Tabor & Tabor, 1976a). Tabor et al. (1958) studied the mechanism of spermidine synthesis in cell free E. coli extracts and found a requirement for Mg^{2+} and ATP. These are involved in the activation of methionine to S-adenosylmethionine by a specific adenosyltransferase. S-adenosylmethionine acts as

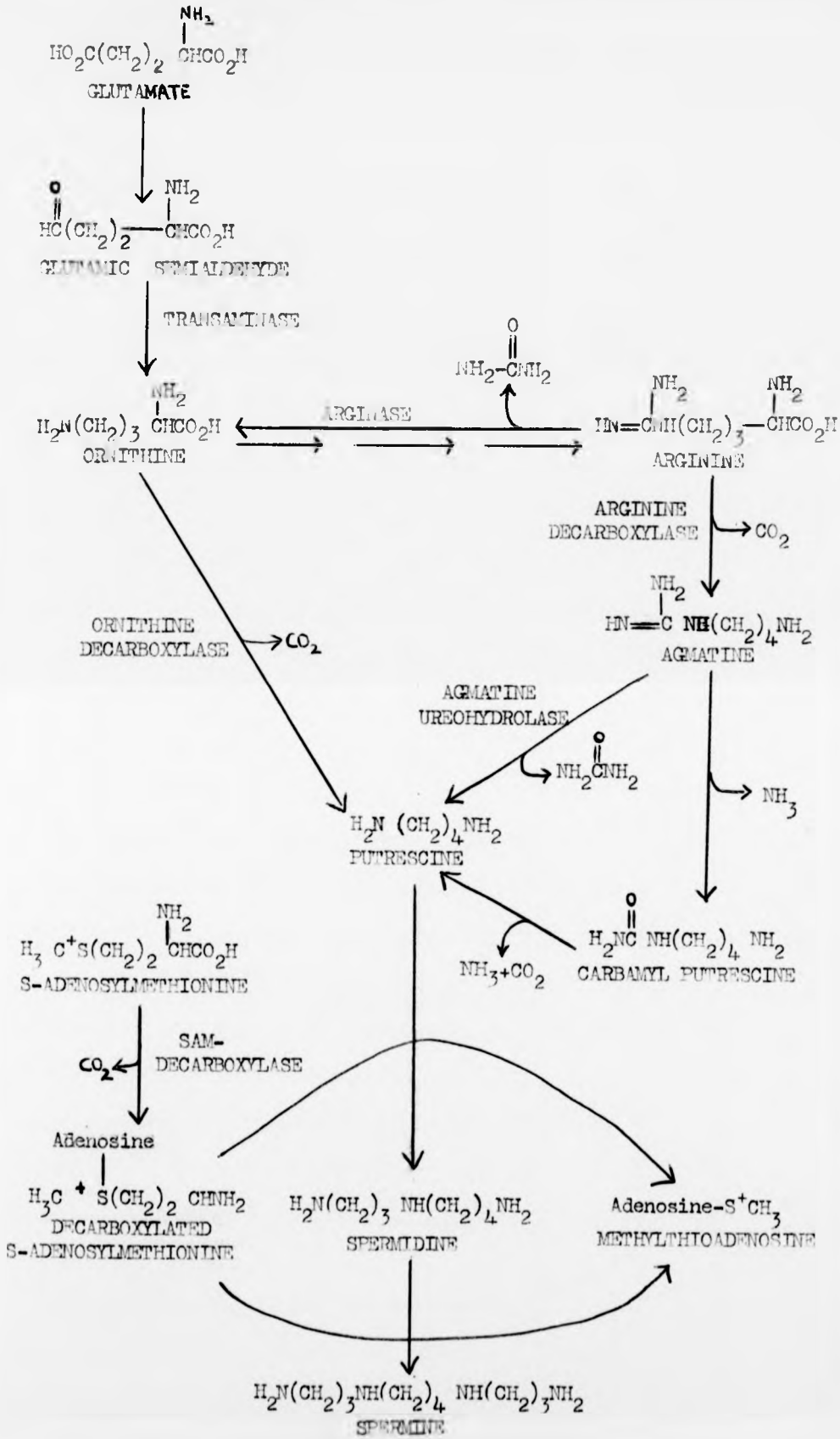


FIGURE 1.1 PATHWAYS OF OLIGOAMINE BIOSYNTHESIS

the methyl group donor in numerous transmethylation reactions and also as the propylamine donor in the biosynthesis of oligoamines. S-adenosylmethionine is decarboxylated by S-adenosylmethionine decarboxylase (SAM-decarboxylase) yielding 5-deoxy-5-S-(3-methylthiopropylamine) sulphonium adenosine (decarboxylated SAM). The transfer of the propylamine group, attached to the sulphonium centre, to putrescine is the final step in the synthesis of spermidine and releases 5-methylthioadenosine (Bachrach, 1973).

5-methylthioadenosine accumulates in certain strains of micro-organisms but is found only in small quantities in mammalian cells, even in tissues rich in oligoamines, due to an efficient catabolic pathway which degrades it to adenine and 5-methylthioribose (Bachrach, 1973). Although eucaryotic synthesis of spermidine follows the same basic pattern of synthesis as that elucidated for E. coli some differences do exist. Thus in mammalian tissues such as rat liver and prostate the decarboxylation of S-adenosylmethionine is activated not by Mg^{2+}, as was the system in E. coli, but by putrescine (Pegg & Williams-Ashman, 1969; Hannonen et al., 1972a). This involvement of putrescine in the decarboxylation of S-adenosylmethionine, from which it eventually accepts a propylamine moiety, caused some workers to suggest that the same enzyme might catalyze both the decarboxylation reaction and the transfer of the propylamine group to putrescine (Pegg & Williams-Ashman, 1969; Feldman et al., 1972; Russell & Potyraj, 1972). More recently however, SAM-decarboxylase has been purified from mouse mammary gland, rat liver and ventral prostate which lacks transferase activity (Hannonen et al., 1972a; Pegg, 1974; Oka et al., 1977). It appears then that separate enzymes are involved, but the existence of a multienzyme complex in vivo is not excluded (Pegg & Williams-Ashman, 1969; Feldman et al., 1972).

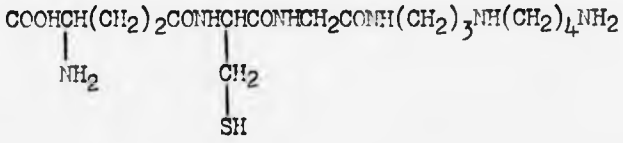
The reported incorporation of labelled spermidine into spermine in vivo in rat liver and chick embryos (Raina, 1963; 1964) suggested that in these systems spermine is formed by the addition of another propylamine group from decarboxylated SAM to spermidine, in a manner analogous to the formation of

spermidine from putrescine. Original attempts to separate the enzymes that catalyse the synthesis of spermidine and spermine were unsuccessful and it was assumed that the two reactions were controlled by the same enzyme (Pegg & Williams-Ashman, 1970). Two different aminopropyltransferases, spermidine and spermine synthetase, have now been partially purified from rat liver (Hannonen et al., 1972a) and rat brain (Raina & Hannonen, 1971).

CATABOLISM OF OLIGOAMINES

Although their biosynthetic pathways and associated enzymes have been studied in both procaryotic and eucaryotic organisms, little is known about the metabolism and elimination of oligoamines. In bacteria such as E. coli little evidence of oligoamine catabolism has yet been reported. Tabor & Dobbs (1970) report that exponentially growing E. coli retain labelled putrescine taken up from the medium. Although intracellular putrescine levels are greatly reduced by an increase in the osmolarity of the medium, Munro et al. (1972) report that this is not the result of catabolism but rather the excretion of putrescine into the medium. During the stationary phase essentially all the intracellular spermidine present in E. coli is converted reversibly to glutathionylspermidine [γ-glutamylcysteinyl-glycylspermidine, Figure 1.2] (Tabor & Tabor, 1975).

FIGURE 1.2



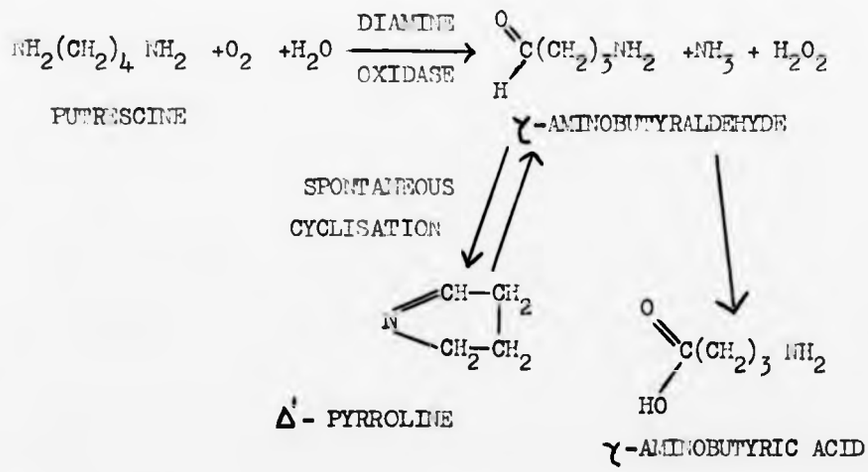
STRUCTURE OF GLUTATIONYLSPERMIDINE

Although a similar glutathionyl conjugate of putrescine was not found in E. coli (Tabor & Tabor, 1976b) evidence has since accumulated that both putrescine and spermidine can be associated covalently with cellular proteins (Canellakis et al., 1981; Chen & Liu, 1981; Haddox & Russell, 1981).

There are numerous reports of amine oxidase activity in various animal tissues and extracellular fluids but the involvement of these enzymes in oligoamine catabolism is unclear, as is the nomenclature and classification of these enzymes used in literature.

Diamine oxidase (EC 1.4.3.6) is an enzyme or group of enzymes catalysing the oxidative deamination of various amines including putrescine, spermidine and spermine (Zeller, 1963). γ-aminobutyrate has been identified as the product of putrescine oxidation in a variety of vertebrate organs (Seiler, 1973; Kremzner et al., 1975; DeMello et al., 1976; Tsuji & Nakajima, 1978; Bachrach, 1980). Putrescine is oxidised by diamine oxidase to γ-aminobutyraldehyde which spontaneously cyclizes to Δ^1-pyrroline (Tabor & Tabor, 1964). Δ^1-pyrroline may be further oxidised to γ-aminobutyrate (Hennings-son & Rosengren, 1976; Andersson et al., 1978) [Figure 1.3].

FIGURE 1.3



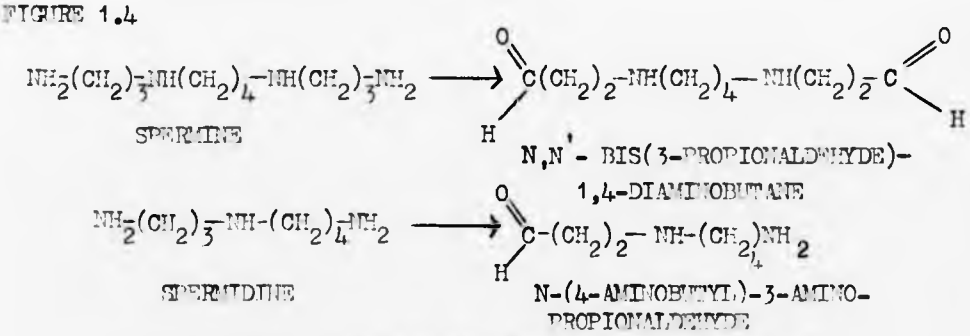
CATABOLISM OF PUTRESCINE TO γ-AMINOBUTYRIC ACID BY DIAMINE OXIDASE

In vivo studies have shown that injection of [^{14}C] putrescine is followed by expiration of large amounts of radioactive carbon dioxide (Jänne, 1967). Later it was found that γ-aminobutyrate is an intermediate in the

catabolism of putrescine to $[^{14}C]$ carbon dioxide (Seiler & Eichentopf, 1975; Henningsson & Rosengren, 1976).

Although tri- and tetraamines, such as spermidine and spermine, are weak substrates of diamine oxidase, these oligoamines are catabolised *in vivo* by polyamine oxidases. The products of these enzymes *in vitro* varies, depending on the source of the enzyme. A polyamine oxidase was isolated from the sera of ruminants which brings about the rapid oxidative deamination of spermidine and spermine (Blaschko, 1962). This enzyme is clearly distinguished from previously reported monamine and diamine oxidases by the absence of significant oxidation of typical monoamines (tyramine and 1-propylamine) and diamines (putrescine and histamine) (Tabor et al., 1954). It acts on the terminal primary amino groups of spermidine and spermine as shown in Figure 1.4 (Tabor et al., 1964).

FIGURE 1.4



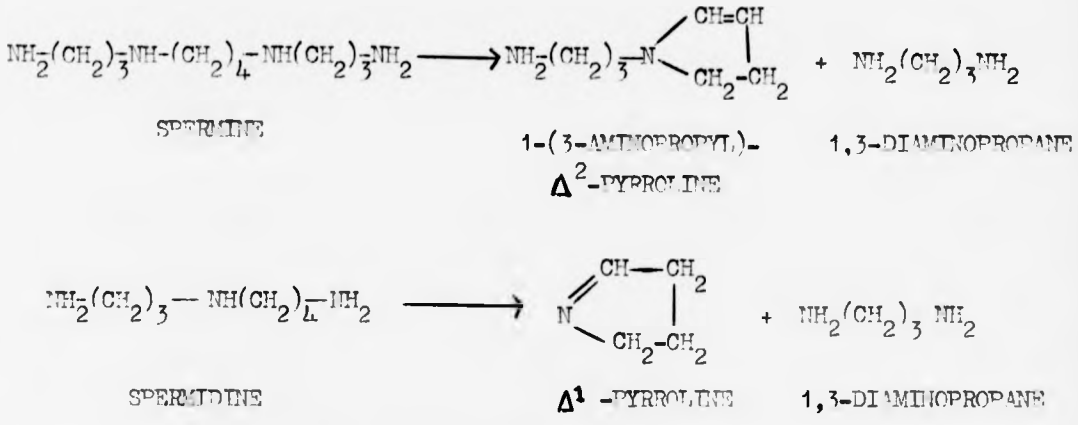
CATABOLISM OF SPERMINE AND SPERMIDINE BY A POLYAMINE OXIDASE FROM RUMINANT PLASMA

In foetal bovine serum the aminoaldehyde produced from spermidine is subsequently degraded to putrescine and acrolein (CH_2=CH-CH=O) (Alarcon, 1970). A polyamine oxidase similar to that found in ruminant plasma has been detected in other vertebrate tissues (Kapeller-Adler, 1970; Cohen, 1971; Tabor & Tabor, 1972; Bachrach, 1973).

Oat, barley and maize seedlings contain an enzyme which acts on both spermine and spermidine yielding 1,3-diaminopropane and either 1-(3-aminopropyl)-

Δ^2-pyrroline or Δ^1-pyrroline respectively (Figure 1.5) (Suzuki & Hirasawa, 1973; Smith 1976; Terano & Suzuki, 1978b).

FIGURE 1.5



CATABOLISM OF SPERMINE AND SPERMIDINE
BY PLANT POLYAMINE OXIDASE

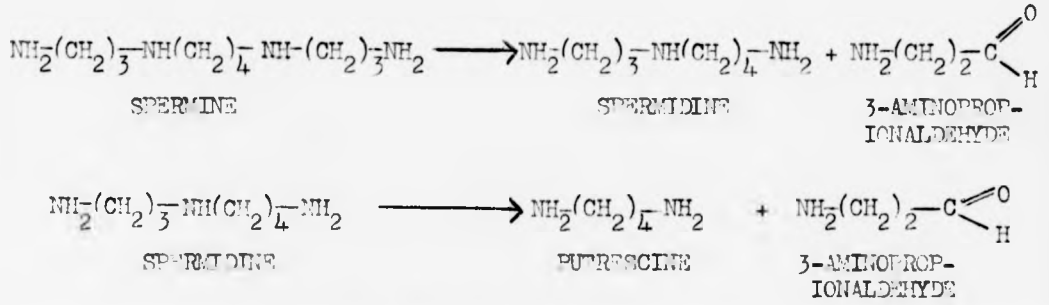
Maize shoots (Terano & Suzuki, 1978a) accumulate β-alanine when incubated with spermine or spermidine, which is thought to result from the oxidation of the 1,3-diaminopropane formed by oligoamine catabolism. Δ^1-Pyrroline and 1-(3-aminopropyl)- Δ^2-pyrroline, the other products of spermine and spermidine oxidation by this plant polyamine oxidase, have been shown to be further oxidised to γ-aminobutyrate in maize seedlings (Terano & Suzuki, 1978b).

Enzymes from Serratia marcescens (Bachrach, 1962a,b) and Micrococcus rubens (Adachi et al, 1966; Yamada, 1971) also degrade spermidine to 1,3-diaminopropane and γ-aminobutyraldehyde, the latter compound subsequently cyclising to Δ^1-pyrroline. Tabor & Kellog (1970) purified the enzyme and showed that it does not react directly with molecular oxygen but requires an oxygen acceptor such as ferricyanide or cytochrome C and as such is more properly classified as a dehydrogenase. Exogenous spermine and spermidine are metabolised by cultures of Anacystis nidulans (Ramakrishna et al, 1978) and

Haemophilus parainfluenzae (Weaver & Herbst, 1958) with the production of 1,3-diaminopropane.

When spermidine is oxidised by Pseudomomas aeruginosa (Padmanabhan & Kim, 1965) or Mycobacterium smegmatis (Bachrach et al.,1960), the oxidation takes place on the other side of the secondary nitrogen as shown in Figure 1.6.

FIGURE 1.6



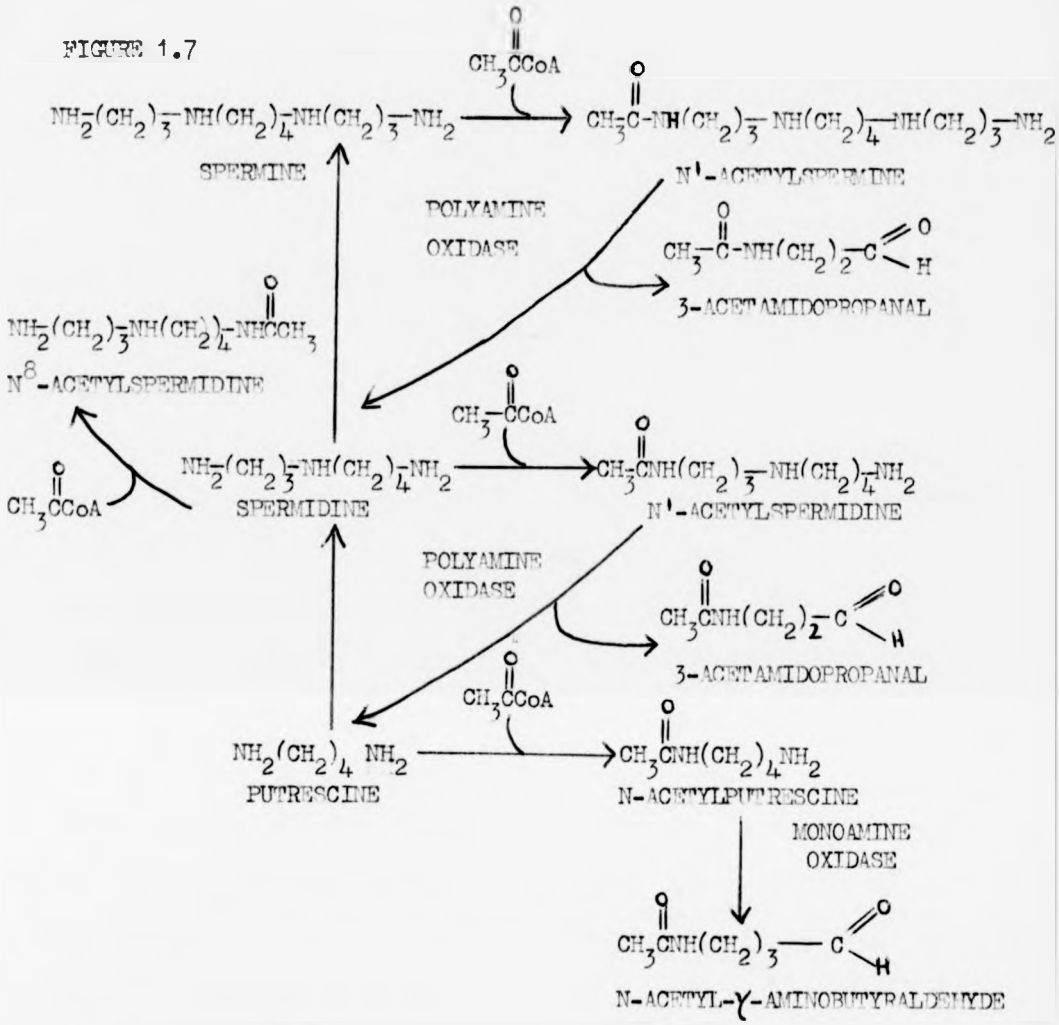
CATABOLISM OF SPERMINE AND SPERMIDINE RESULTING IN INTER-CONVERSION OF OLIGOAMINES

The conversion of spermine to spermidine and spermidine to putrescine has been reported in a variety of vertebrate tissues (Quash & Taylor, 1970; Seiler, 1973; Höltta et al., 1973a; McCormick, 1978). The enzyme responsible for these observed interconversions of oligoamines has been isolated from rat liver peroxisomes by Höltta (1977). The molecules are cleaved at the secondary amino group in a similar manner to that observed in P. aeruginosa (Padmanabhan & Kim, 1965) and M. smegmatis (Bachrach et al., 1960) as shown in Figure 1.6.

Acetylation is an attractive mechanism for the physiological inactivation of oligoamines. Most of the physiological functions suggested for the oligoamines are dependent on the net positive charge carried by these compounds (Cohen, 1971). Acetylation would serve to decrease this net positive charge and this might modify the physiological activity of oligoamines. There are several lines of evidence suggesting that acetylation is involved in oligoa-

mine degradation. The monoacetyled derivative of putrescine is found in various tissues including human brain (Perry et al., 1967) and in several organs of other vertebrates (Seiler et al., 1973) where it is an intermediate in the catabolism of putrescine in those organs which do not contain diamine oxidase (Seiler & Al-Therib, 1974a; Seiler et al., 1979). N^1-acetylspermidine and N^3-acetylspermidine occur in the urine of normal subjects and cancer patients (Abdel-Monem et al., 1975) and in Chinese hamster ovary cells (Prussak & Russell, 1980); N^1-acetylspermidine in rat tissues (Seiler et al., 1980) and N-acetylputrescine and N^1-acetylspermidine in human lymphocytes (Menashe et al., 1980). Although these reports confirm the occurrence of acetylated oligoamines in higher organisms the synthetic pathway of these compounds was unknown until both Seiler & Al-Therib (1974b) and Libby (1973) reported on the nuclear localization of an oligoamine N-acetyltransferase. This has been confirmed by Blankenship & Walle (1977), who also provided evidence for the existence in the cytoplasmic fraction of rat liver and kidney of an enzyme capable of the deacetylation of acetylated oligoamines (Blankenship & Walle, 1978). Seiler & Al-Therib (1974a) found that in rat tissues N-acetylputrescine is oxidatively deaminated by monoamine oxidase to N-acetyl-γ- aminobutyraldehyde. Acetyl derivatives of spermine and spermidine have also been shown to play a role as catabolic intermediates. Blankenship (1979) showed that crude liver extracts are capable of splitting N^1-acetylspermidine to putrescine. Acetyl derivatives of spermine and spermidine are better substrates of polyamine oxidase extracted from rat liver (Höltta, 1977; Bolkenius & Seiler, 1981). This enzyme splits N^1-acetylspermidine and N^1-acetylspermine to 3-acetamidopropanol and putrescine or spermidine respectively (Figure 1.7). Thus the acetylation and subsequent oxidation of oligoamines must be considered to be a major catabolic fate of these compounds (Seiler et al., 1980).

FIGURE 1.7



CATABOLISM OF ACETYLATED OLIGOAMINES

SECTION A - Levels of oligoamines in D. discoideum

Despite the increasing popularity of the cellular slime mould D. discoideum as a model eucaryotic system for studying the molecular and cellular aspects of differentiation, at the commencement of this work no observations had been made of the oligoamine levels in this organism. Thus preliminary experiments were designed to determine whether the relative amounts of putrescine, spermidine and spermine were more typical of fungal cells or those of protozoa.

LEVELS IN AMOEBAE

Wild type isolates of D. discoideum (NC4) can be grown in association

with growing bacteria on solid medium (see methods section). Although it is impossible to grow NC4 in the absence of bacteria, several derivatives of D. discoideum NC4 have been selected for axenic growth e.g. AX1 (Sussman & Sussman, 1967), AX2 (Watts & Ashworth, 1970) and AX3 (Loomis, 1971). There are few differences between NC4 and the axenic strains and all characteristics found in one that have been tested for in the other, have been confirmed (Loomis, 1975). A comparison was thus made of the oligoamine content of D. discoideum amoebae, both NC4 and AX2, grown on different nutrient sources. The oligoamine content of Klebsiella aerogenes, the bacterial nutrient, was also determined for comparative purposes.

The levels of putrescine, spermidine and spermine in each sample were determined using both the methods detailed in the methods section, i.e. the fluorimetric estimation of the dansyl derivatives (Method A) and the spectrophotometric determination of the ninhydrin reaction products after separation by paper strip electrophoresis in 0.1M-citric acid buffer, pH3.6 (Method B).

The separation of dansyl oligoamines obtained on development of silica plates in ethylacetate/cyclohexane (2.3,v/v), was as reported by Dion & Herbst (1970). Dansyl putrescine (Rf 0.43) was clearly resolved from dansyl spermidine (Rf 0.37) and dansyl spermine (Rf0.32). Dansyl ammonia occurred as a fluorescent spot of higher Rf value than dansyl putrescine, whilst a diffuse fluorescent spot of more polar dansyl derivatives remained at the origin (Figure 1.8A).

Five bands of ninhydrin stained material were observed to result on electrophoretic separation of amoebal samples in citric acid buffer, pH3.6. Comparison of the position of these bands with those of commercial compounds on control electrophoresis strips confirmed a mobility order of putrescine > spermidine > spermine > ornithine > arginine as reported by Inoue & Mizutani (1973). The two ninhydrin staining bands nearest the origin, which contained ornithine and arginine, were broad and diffuse due to the possible presence of lysine, histamine, glutamine and other basic intracellular constituents which

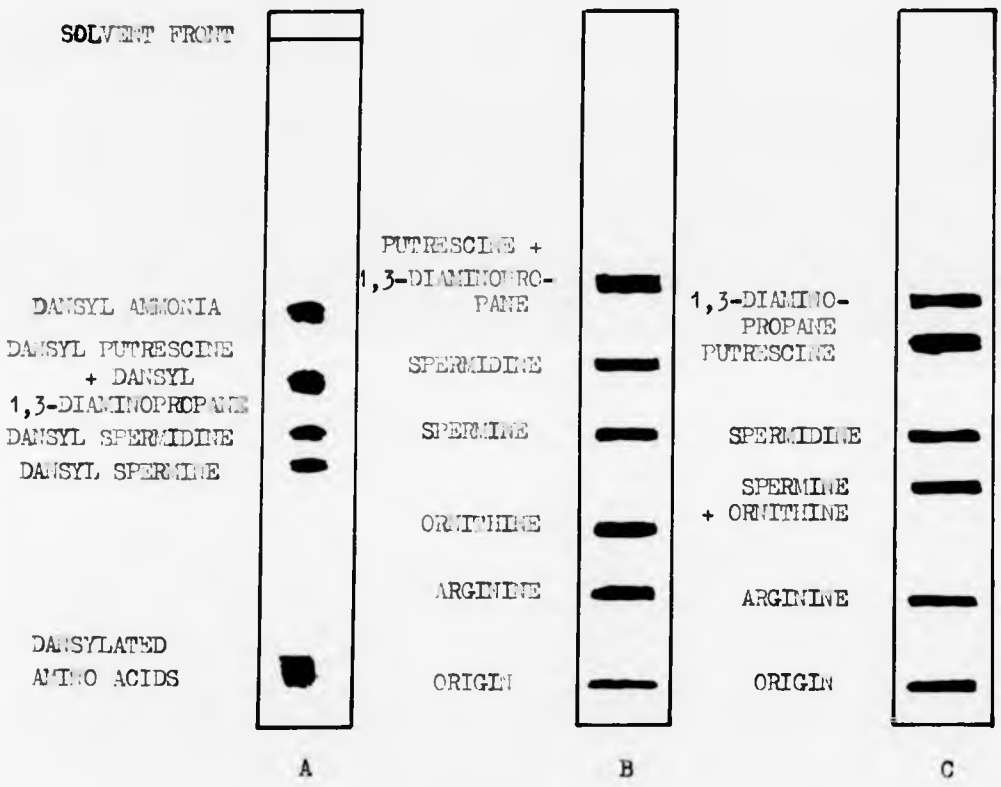


FIGURE 1.8

Separation of standard oligoamine and amino acid solutions by various methods.

(A) Separation of dansylated derivatives on silica plates in ethylacetate/cyclohexane (2:3; v/v).

(B) Separation of oligoamines and amino acids by paper electrophoresis in 0.1M-citric acid buffer, pH3.5.

(C) Separation of oligoamines and amino acids by paper electrophoresis in 65mM-sulphosalicylic acid buffer, pH3.2.

are eluted from the Dowex-50W column by 6M-HCl and are not completely separated by electrophoresis in 0.1M-citric acid buffer, pH3.6 (Dion & Herbst, 1970; Figure 1.8B).

Both methods used in the determination of the oligoamine content of D. discoideum amoebae, yielded very similar results (Table 1.1) Putrescine was the major oligoamine, the amount present being equivalent to an intracellular concentration of 20mM. However, as oligoamines bind to intracellular components such as membranes and nucleic acids, the pool of free putrescine inside the cell was probably very small. Spermidine was present in smaller quantities, equivalent to an intracellular concentration, of 4mM, in the absence of intracellular binding. Spermine, although detected by both methods occurred in quantities too small to allow reliable measurement. The oligoamine content of amoebae was not dependent on the strain of D. discoideum used, since similar values were observed for amoebae of both strains NC4 and AX2 grown in association with K. aerogenes (Table 1.1). The oligoamine content of amoebae was also independent of growth conditions. The DNA content of axenically grown cells is less than half of that of cells grown on bacteria (Ashworth & Watts, 1970; Leach & Ashworth, 1972). This accounted for the apparent difference in oligoamine levels in AX2 amoebae grown in association with K. aerogenes and those grown in HL5 + glucose liquid medium (Table 1.1). The oligoamine content of the bacteria differed markedly from that of D. discoideum (Table 1.1). A significant concentration of cadaverine, a diamine not found in D. discoideum, was detected in K. aerogenes.

In addition to putrescine, spermidine and spermine, the occurrence of several other oligoamines and oligoamine derivatives have been reported in other biological materials. A significant concentration of cadaverine (1,5-diaminopentane) was found in K. aerogenes cells. This diamine, formed by the decarboxylation of lysine, has also been isolated from bacterial and liver ribosomes, fungi and higher plants (Bachrach, 1973). Other oligoamines with an even narrower distribution have been found: putreanine [1-(4-aminobutyl)-

TABLE 1.1

Oligoamine levels in D. discoideum amoebae, strains NC4 and AX2, grown in association with Klebsiella aerogenes and axenically in HL5 medium containing glucose.

Values given ± S.D. for the number of determinations indicated in parenthesis.

STRAIN	NUTRIENT	METHOD	PUTRESCINE μmol/mg DNA	SPERMIDINE μmol/mg DNA
NC4	Klebsiella aerogenes	Dansylation	35.6 ± 2.4 (3)	7.0 ± 2.5 (3)
		Paper strip electrophoresis	32.2 ± 7.0 (2)	7.5 ± 2.6 (2)
AX2	Klebsiella aerogenes	Dansylation	32.7 ± 5.2 (2)	8.6 ± 1.3 (2)
		Paper strip electrophoresis	38.8 ± 6.9 (3)	5.9 ± 1.0 (3)
AX2	HL5 medium + glucose	Dansylation	76.1 ±16.1 (2)	13.0 ± 4.9 (2)
		Paper strip electrophoresis	85.9 ±20.8 (4)	16.8 ± 3.0 (4)
Klebsiella aerogenes	SM agar	Paper strip electrophoresis	1.3 ± 0.4 (3)	Trace

3-aminopropionic acid] is present in mammalian brains (Kakimoto et al., 1969); 2'- hydroxyputrescine is found to be the major oligoamine in Pseudomonas (Kullnig et al., 1970; Tobari & Tchen, 1971); homospermidine [4-azononone-1,9-diamine] is present in plants (Kuttan et al., 1971); glutathionylspermidine is found in stationary E. coli cells (Dubin, 1959; Tabor & Tabor, 1975) and thermine [4,8-diazoundecane-1,11-diamine], an anologue of spermine is found in an extreme thermophile, Thermus thermophilus (Oshima, 1975). 1,3-diaminopropane, a close structural analogue of putrescine, has been detected in relatively few organisms (Herbst et al., 1958; Weaver & Herbst, 1958; Smith, 1970a,b; Nakajima et al., 1967), and usually only in trace amounts. A product of the oxidation of spermidine and spermine by oligoamine oxidase to 1-pyrroline and 1-(3-aminopropyl)-2-pyrroline respectively in higher plants (Smith, 1974), 1,3-diaminopropane has no known function. Recently however this compound has been used in studies on regenerating rat liver (Pösö & Jänne, 1976a,b; Pösö et al., 1977) and Chinese hamster ovary cells (Sunkara et al., 1977) as a potent inhibitor of the induction of ornithine decarboxylase.

The analytical methods employed in preliminary experiments did not allow the separation of putrescine from 1,3-diaminopropane. The differences in mobilities of the dansyl derivatives of cadaverine, putrescine and 1,3-diaminopropane when separated by development of silica plates in ethylacetate/cyclohexane (2:3, v/v), are very small (Dion & Herbst, 1970). Similarly separation of putrescine and 1,3-diaminopropane is not achieved by paper strip electrophoresis in 0.1M-citric acid buffer, pH3.6. Thus further experiments were carried out to determine whether this oligoamine was present in D. discoideum amoebae. The method employed was a modification of Method B involving changing the paper strip electrophoresis buffer to 65mM-5-sulphosalicylic acid buffer, pH3.2. Raina (1963) reported that electrophoresis in this buffer separates 1,3-diaminopropane, putrescine, spermidine and spermine distinctly from each other, although not effectively separating ornithine from spermine (Figure 1.8C).

Electrophoresis of D. discoideum amoebal extracts in this system revealed an additional band not observed with 0.1M-citric acid buffer, pH3.6. This band stained a characteristic brown colour with ninhydrin and had a mobility greater than putrescine. With respect to both colour and mobility, the amoebal material in this band behaved in an identical manner to commercial 1,3-diaminopropane. Cadaverine, which produced a violet colour with ninhydrin, and 1,2-diaminopropane, both had electrophoretic mobilities slower than putrescine and corresponding bands were not detected in amoebal extracts. Thus the material identified in the preliminary experiments as putrescine, was probably a mixture of putrescine and 1,3-diaminopropane.

Bands corresponding to 1,3-diaminopropane were observed in strain AX2 grown axenically on different nutrient sources (Table 1.2). Whilst the HL5 medium consists of yeast extract and bacteriological peptone and may conceivably contain trace amounts of oligoamines, cells grown on defined FM medium, which contains no oligoamines, possessed similar oligoamine levels to those grown in HL5 medium (Table 1.2). Similar results have been obtained with AX2 cells grown in association with K. aerogenes (North & Turner, 1977). The oligoamine content of AX2 amoebae grown in HL5 medium in the absence of glucose, in which the doubling time of amoebae is 10-12h, did not differ from that in AX2 amoebae grown in HL5 medium containing glucose in which the doubling time of amoebae is 8-10h (Table 1.2) The oligoamine content was similarly found to be independent of the phase of growth, since amoebae harvested in either the exponential or stationary phase differed very little in their oligoamine content (Table 1.2).

Although 1,3-diaminopropane made up a significant portion of what had been thought to be entirely putrescine, putrescine remained the major oligoamine present in D. discoideum amoebal extracts, the average amount measured being equivalent to an intracellular concentration of 17mM as compared to a 1,3-diaminopropane concentration of 10mM. The true intracellular diamine concentration however will be much lower than these figures suggest due to

TABLE 1.2

Oligoamine content of D. discoideum amoebae as determined by paper strip electrophoresis using 65mM-5-sulphosalicylic acid, pH3.2.

Values are given ± S.D. for the number of determinations indicated in parenthesis.

NUTRIENT	GROWTH PHASE	PUTRESCINE μmol/mg DNA	1,3-DIAMINO-PROPANE μmol/mg DNA	SPERMIDINE μmol/mg DNA
HL5 medium + G	Exponential	61.6 ±9.7 (6)	33.8 ±2.8 (6)	12.5 ± 1.7 (6)
HL5 medium +G	Stationary	56.5 ±6.1 (2)	28.4 ±8.0 (2)	12.0 ± 2.5 (2)
HL5 medium - G	Exponential	55.2 ±10.4(2)	32.3 ±3.0 (2)	14.8 ± 1.3 (2)
FM medium	Exponential	67.5 ±7.6 (2)	34.3 ±8.2 (2)	15.2 ± 3.3 (2)

widespread intracellular binding. The average spermidine content of amoebal extracts analysed by electrophoresis in 5-sulphosalicylic acid, pH3.2, was in agreement with the results obtained in the previous experiment (Table 1.1 and Table 1.2)

LEVELS DURING DEVELOPMENT

On depletion of the food supply, D. discoideum amoebae enter the developmental phase; cell division ceases, and food vacuoles disappear and subsequent development is sustained by endogenous reserves. The amoebae aggregate (8h), eventually forming a migratory stage called a grex (14-16h). On reaching conditions suitable for sporulation, the grex undergoes the process known as culmination by which the erect sorocarp is eventually produced (24h). This developmental phase is accompanied by dramatic changes in the metabolism of nucleic acids and proteins. Changes in RNA metabolism have been reported including a rapid loss of cellular RNA during development (Hames & Ashworth, 1974); the degradation of 50-75% of ribosomes (Coccucci & Sussman, 1970); the reduction of both rRNA and mRNA synthesis to less than 15% of that in growing cells (Mangiarotti et al., 1981) and alterations in the types of RNA transcripts formed (Firtel, 1972; Blumberg & Lodish, 1980). In view of the association reported between nucleic acids and oligoamines (Bachrach, 1973; Stevens & Winther, 1979), the levels of oligoamines during development of D. discoideum amoebae were monitored as detailed in the legend to Figure 1.9.

During development the levels of putrescine, 1,3-diaminopropane and spermidine decreased from the values observed in amoebae (Figure 1.9a). The putrescine content of the amoebae fell from 61.6 μmol/mg DNA to 7.3 μmol/mg DNA over the 24h period, representing an 88% decrease in putrescine level. Similarly over the same 24h period, the level of 1,3-diaminopropane fell from 33.8 μmol/mg DNA to 6.8 μmol/mg DNA, an 80% decrease; and spermidine from 12.5 μmol/mg DNA to 2 μmol/mg DNA, an 84% decrease.

Development was also accompanied by a fall in both the RNA and protein content of the amoebae (Figure 1.9 b and c). The level of RNA fell from

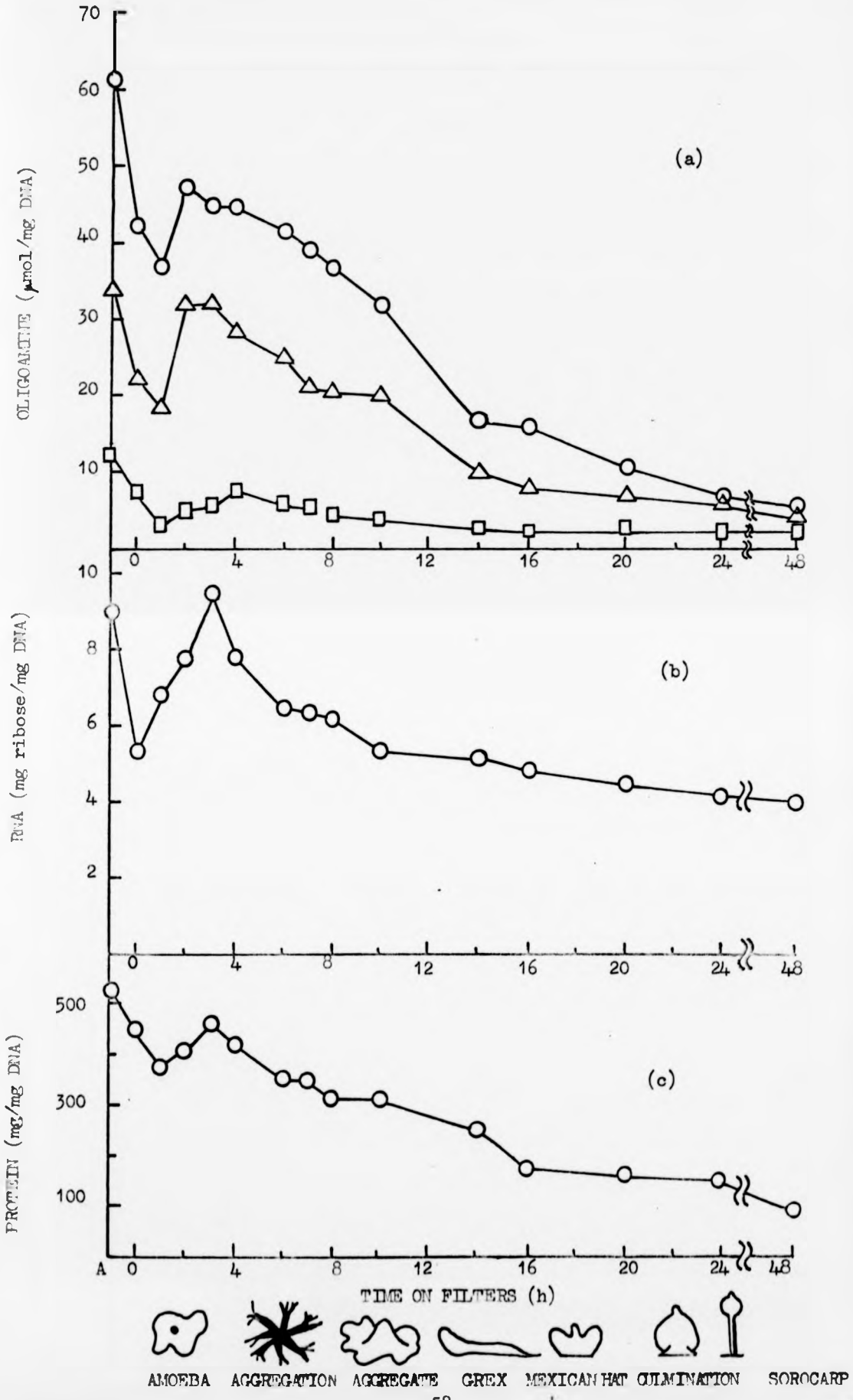

OLIGOAMINE (μmol/mg DNA)
70
60
50
40
30
20
10
(a)
0
4
8
12
16
20
24
48
RNA (mg ribose/mg DNA)
10
8
6
4
2
(b)
PROTEIN (mg/mg DNA)
500
300
100
(c)
A
TIME ON FILTERS (h)
AMOEBA
AGGREGATION
AGGREGATE
GREX
MEXICAN HAT
CULMINATION
SOROCARP

FIGURE 1.9 LEVELS OF PROTEIN, RNA AND OLIGOAMINES DURING DEVELOPMENT

AX2 amoebae were used in the preparation of developmental filters as detailed in the methods section. Samples of these cells were used to prepare cell extracts prior to their deposition on filters (A), and at intervals during the developmental period. The oligoamine contents of these extracts were determined by Method B using 65mM-5-sulphosalicylic acid, pH 3.2, as the electrophoresis buffer. The protein, RNA and DNA content of the extracts were also determined as detailed in the methods section. Relative cellular levels of (a) oligoamines : putrescine, O; 1,3-diaminopropane, Δ; spermidine, □; (b) RNA and (c) protein were calculated using the finding of Hames (1972), that the cellular DNA level remains constant during development on Millipore filters. Each point is the mean of duplicate experiments.

9.05mg/mg DNA to 4.24mg/mg DNA over the developmental period. This 53% decrease in RNA content is comparable with the 60% decrease reported for NC4 amoebae grown in association with K. aerogenes (White & Sussman, 1961) and with the 77% decrease for AX2 amoebae grown in HL5 medium (Hames & Ashworth, 1974). The level of protein fell from 537mg/mg DNA to 151mg/mg DNA, and this 72% decrease is in agreement with the 60% decrease in protein levels reported over the same time period by White & Sussman (1961) for developing NC4 amoebae, and the 67% decrease noted in AX2 amoebae by Hames & Ashworth (1974).

As development, in the case of spore cells at least, results in a decrease in cell volume to approximately ⅓ that of an amoebal cell (Cappuccinelli & Ashworth, 1976), the lower content of oligoamines and macromolecules need not necessarily represent a decrease in the cellular concentration of these cell components.

The level of oligoamines and macromolecules in amoebae fell initially during harvesting and preparation of the developmental dishes and also for the first hour of development (Figure 1.9). This initial downward trend was reversed briefly, and the oligoamine, RNA and protein content of the developing cells rose over the next 2-3h. With the exception of RNA, where the level after 3h development was slightly higher than that in amoebal cells, the levels to which the substances under study rose were still much lower than those in amoebal cells. From 4h onwards the initial downward trend was resumed and the amount of oligoamine, protein and RNA in the cell decreased steadily throughout the remainder of the developmental period.

LEVELS IN SPORE AND STALK CELLS

During culmination, cells from the anterior of the grex vacuolize, expand 3-4 fold in volume and secrete angular cellulose walls. These cells are no longer viable and they migrate to form the stalk cells of the sorocarp. Cells from the posterior of the grex also develop cellulose walls during culmination and their volume decreases by a factor of 10-20 before they become a spherical mass of spore cells embedded in mucilage. After dispersal and dilution of the

germination inhibitor present, each spore germinated liberating one amoebal cell. Several groups have monitored oligoamine synthesis during germination of fungal spores and have reported that the macromolecular synthesis characteristic of this period of rapid growth, is accompanied by a parallel increase in oligoamine levels (Pennucci et al., 1975; Stevens, 1975; Nickerson et al., 1977b; Choih et al., 1978). To determine whether a similar increase occurred on germination of D. discoideum spores, the oligoamine levels were monitored at intervals during germination. To complete the picture of oligoamine levels during the life cycle of the cellular slime mould, cell extracts of spore and stalk cells were also analysed.

From the results shown in Table 1.3 it was concluded that the ratio of spore to stalk cells was greater than 1:1 and closer to the 2:1 ratio reported by Loomis (1975). The oligoamine, RNA and protein levels in spore cells were more than 60% higher than in stalk cells. As the volume of spore cells decreases during culmination whilst that of stalk cells increases (Cappuccinelli & Ashworth, 1976), it is probable that the concentration of oligoamines, RNA and protein was in fact higher in spore cells than in stalk cells. This result would reflect the non-viability of stalk cells after vacuolation during culmination; whereas spore cells retain their viability through extended periods of dehydration, starvation and elevation of temperature (Loomis, 1975).

LEVELS DURING GERMINATION

Cotter & Raper (1966) have described two stages of germination after activation of D. discoideum spores: a swelling of the spore and a thinning of the spore coat followed by the emergence of vegetative amoebae. The activation of D. discoideum spores, strain NC4, grown in association with K. aerogenes, has been brought about by a variety of agents: heat (Cotter & Raper, 1968); exogenous hydrophobic amino acids (Cotter & Raper, 1966); protein denaturing agents such as urea and dimethylsulphoxide (Cotter & O'Connell, 1974) and γ-radiation (Khoury et al., 1970). Germination studies using the spores of axenic strains such as AX2, have not yet been reported. Preliminary attempts

	GERMINATION TIME h	PUTRESCINE µmol/mg DNA	1,3-DIAMINOPROPANE µmol/mg DNA	SPERMIDINE µmol/mg DNA	RNA mg ribose/mg DNA	PROTEIN mg/mg DNA
SOROCARP	-	7.8	6.8	2.2	4.4	151
STALK CELLS	-	5.3	4.3	1.3	3.2	101
SPORE CELLS	-	9.1	7.0	2.6	5.1	164
GERMINATING SPORE CELLS	0	9.4	6.4	2.3	3.6	157
	1	13.3	8.1	2.6	3.1	164
	2	12.3	8.0	2.1	2.8	164
	3	15.0	8.2	1.9	2.7	160
	4	14.5	8.9	2.0	2.4	159

TABLE 1.3 OLIGOAMINE AND MACROMOLECULE LEVELS IN SOROCARPS, STALK CELLS AND IN SPORE CELLS ON HARVESTING AND DURING GERMINATION

Cell extracts were prepared from sorocarps harvested 24h after their formation and from spore and stalk cells, harvested separately, from similarly matured sorocarps as detailed in the methods section. The DNA, RNA and protein levels of these extracts were determined as were the oligoamine levels (Method B and sulphosalicylic-acid electrophoresis buffer). All calculations of intracellular levels were made on the basis of the constancy of the cellular DNA level (Hames, 1972).

to activate these spores using heat shock as detailed by Yagura & Iwabuchi (1976), were unsuccessful. Aberrations in the spore coats of axenic strains have been recently reported (Williams & Welker, 1980), which may account for the difficulty experienced in the germination of these spores. Further attempts to activate the spores using dimethylsulphoxide as described by Cotter & O'Connell (1974) and detailed in the methods section, were successful. Spores started to swell shortly after suspension in 10mM-sodium phosphate, pH6.7, so that 20% were swollen after 0.5h. Emergence of amoebae began around 3h and by 5h over 80% of the spores had released amoebae (Figure 1.10). Only 3% of the spores had failed to show any indication of germination after 5h, compared with 95% in non-activated spores over the same time period. The process of germination observed was slower than that reported by Ennis & Sussman (1975), using dimethylsulphoxide activation of NC4 spores, where emergence of amoebae began at 1.5h and was completed by 3h.

During the microscopic monitoring of germination kinetics, aliquots of germinating spores were removed and oligoamines and macromolecule determinations made (Table 1.3) During the 5h germination period the intracellular levels of 1,3-diaminopropane increased by 26% whilst the level of spermidine decreased slightly (Table 1.3). During the same time period the intracellular putrescine levels increased by more than 50%. The amount of RNA recovered from spores after activation and immediately on suspension in phosphate buffer was 30% lower than in spores prior to this treatment. This downward trend continued throughout the 5h germination period (Table 1.3). Protein levels seemed relatively unaffected by either activating treatments or germination. The oligoamine, protein and RNA levels in newly emerged amoebae are much lower than those found in amoebae harvested during exponential growth (Figure 1.9). The changes observed in these substances during germination represent only slight fluctuations rather than a reversal of the events of development. This may not be the case in spores germinated in a nutrient medium rather than phosphate buffer.

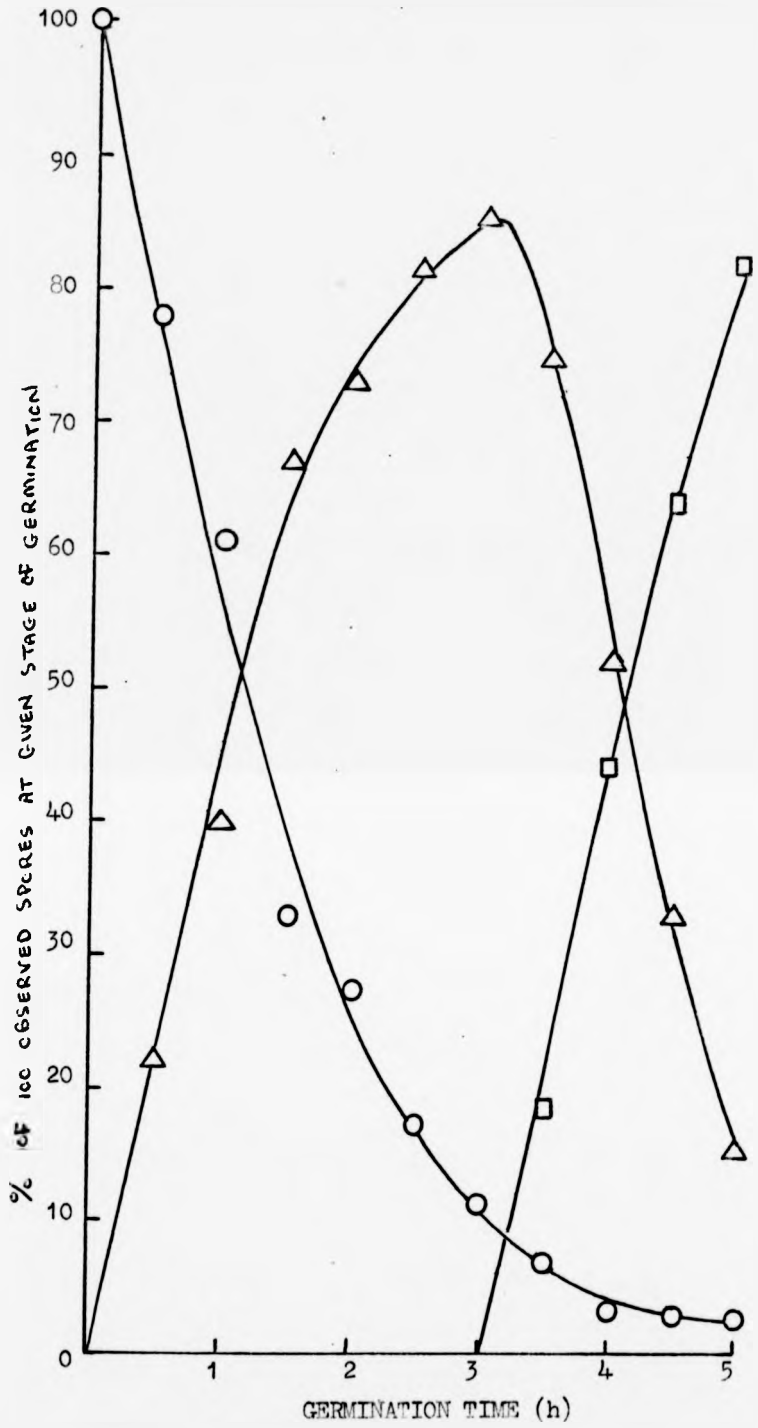


FIGURE 1.10 THE KINETICS OF SPORE GERMINATION

Spores were incubated in 10mM-sodium phosphate buffer, pH6.7, after activation with dimethylsulphoxide as detailed in the methods section. The disappearance of spores (O) and appearance of swollen spores (△) and amoebae (□) was determined microscopically. Each point is the mean of duplicate experiments.

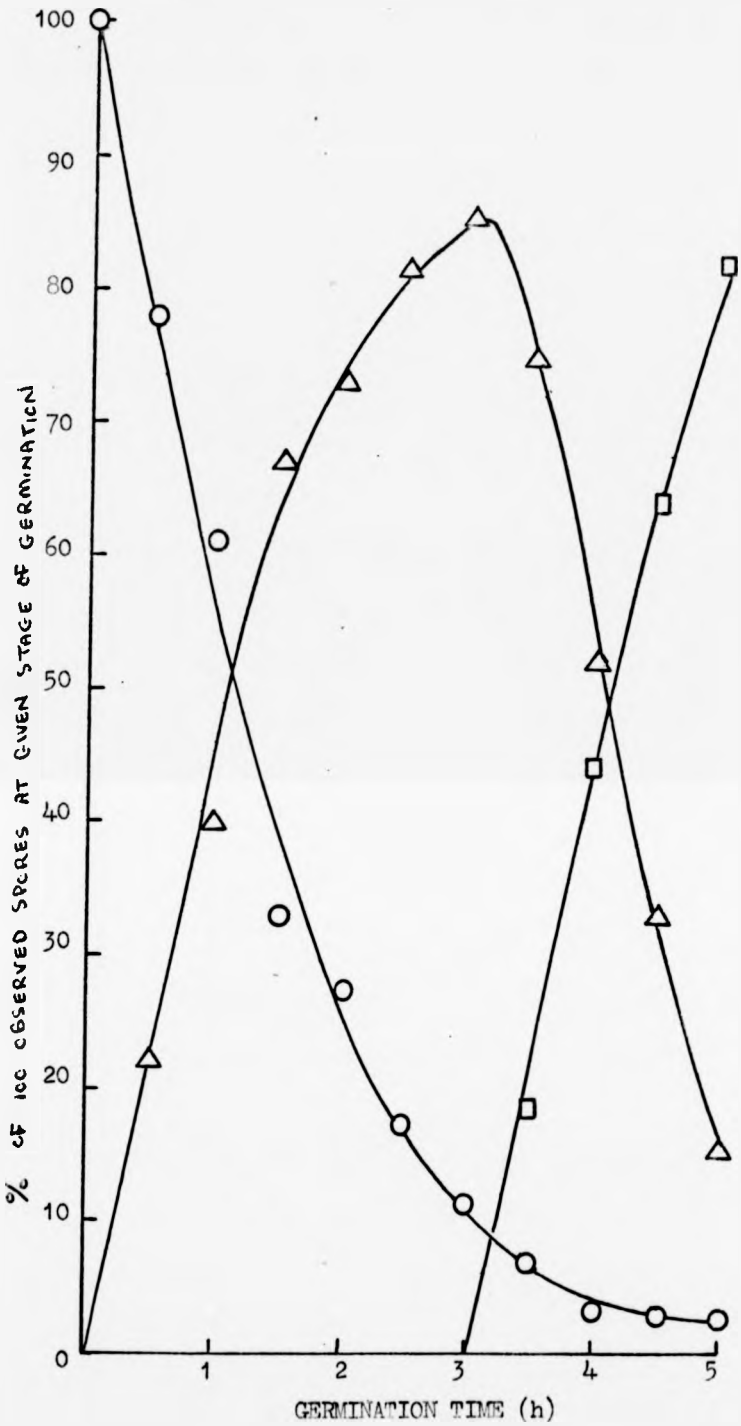


FIGURE 1.10 THE KINETICS OF SPORE GERMINATION

Spores were incubated in 10mM-sodium phosphate buffer, pH6.7, after activation with dimethylsulphoxide as detailed in the methods section. The disappearance of spores (O) and appearance of swollen spores (△) and amoebae (□) was determined microscopically. Each point is the mean of duplicate experiments.

EFFECT OF EXTRACELLULAR OLIGOAMINES ON INTRACELLULAR OLIGOAMINE LEVELS

Having determined the oligoamine levels in D. discoideum amoebae grown on a variety of medium, experiments were carried out to discover whether these levels could be altered by the addition of oligoamines to the growth medium. Defined FM medium, which contains no oligoamines, was used in these experiments in preference to HL5 medium which contains low levels of oligoamines. However the results obtained in preliminary experiments using HL5 medium to which oligoamines were added, were similar to those reported in Table 1.4.

In the presence of low levels of extracellular putrescine (0.1-5mM) the intracellular spermidine content of the cells was unaffected, whilst the putrescine and 1,3-diaminopropane levels were slightly higher than those in the control cells (Table 1.4). Growth inhibition was noted in cells grown in 10mM extracellular putrescine, the doubling time being increased by 1-2h. The intracellular putrescine levels in cells grown in 10mM extracellular putrescine was raised from 61.4 μmol/mg DNA to 107.7 μmol/mg DNA, an increase of more than 75%, whilst the 1,3-diaminopropane level increased from 33.8 μmol/mg DNA to 44.9 μmol/mg DNA, an increase of 32% (Table 1.4). As a result the total amino-nitrogen present in the cellular oligoamines of cells grown in 10mM-putrescine, was 50% higher than in control cells. Intracellular spermidine levels were unaffected by either high extracellular concentrations of putrescine or 1,3-diaminopropane (Table 1.4). As the extracellular concentration of 1,3-diaminopropane was increased the intracellular levels of this diamine increased concomitantly. Cells containing elevated levels of this diamine however had correspondingly low levels of putrescine; as extracellular levels of 1,3-diaminopropane increased intracellular levels of putrescine decreased. As a result, the total amino-nitrogen present in the oligoamines of cells grown in the presence of all levels of extracellular 1,3-diaminopropane, was approximately that of the control cells (Table 1.4). The relative diamine levels of cells grown in the presence of 10mM-putrescine (putrescine: 1,3-diaminopropane, 2.9:1),

TABLE 1.4 EFFECT OF EXTRACELLULAR OLIGOAMINE LEVELS ON THE INTRACELLULAR OLIGOAMINE LEVELS OF D. DISCOIDEUM AMOEBAE

Neutralised and filter sterilized solutions of oligoamines were added to flasks of FM medium to bring them to the desired concentration of oligoamine. After inoculation with AX2 amoebae, the growth rate in the cultures was monitored microscopically over 72h, at which time the amoebae were used for estimation of intracellular oligoamine levels (Method B, sulphosalicylic acid electrophoresis buffer). A small portion of cells were used to prepare duplicate developmental Millipore filters as detailed in the methods section.

EXTRACELLULAR CONCENTRATION OF OLIGOAMINES (mM)	INTRACELLULAR CONCENTRATIONS OF OLIGOAMINES			TOTAL AMINO-NITROGEN IN OLIGOAMINES	
	PUTRESCINE µmol/mg DNA	1,3-DIAMINO-PROPANE µmol/mg DNA	SPERMIDINE µmol/mgDNA	µmol/mgDNA	% OF CONTROL
CONTROL	61.4	33.8	12.4	227.6	100
PUTRESCINE					
0.1	63.9	34.2	9.8	225.6	99
0.5	65.5	36.8	13.5	245.1	108
5.0	62.8	35.3	11.9	231.9	102
10.0	107.7	44.9	12.9	343.9	151
1,3-DIAMINO PROPANE					
0.1	53.0	40.7	12.1	223.7	98
0.5	45.5	34.6	16.3	209.1	92
1.0	34.0	46.5	12.1	197.3	87
5.0	23.4	54.1	10.2	185.6	82
10.0	22.5	66.1	15.8	224.6	99
SPERMIDINE					
0.1	42.1	39.6	11.8	198.8	87
0.5	30.1	27.8	10.3	146.7	64
1.0	28.0	27.6	9.7	140.3	62
SPERMINE					
0.1	32.5	34.2	11.5	167.9	74
0.5	31.1	30.9	14.0	166.0	73

varied little from that of control cells (2.2:1). Cells grown in the presence of 10mM-1,3-diaminopropane however, underwent major changes in their relative diamine levels (0.4:1).

The slight retardation in growth, noted in cells grown in 10mM extracellular putrescine as an increased T_D, was not specific as a similar effect was produced by the presence of 10mM-1,3-diaminopropane in the growth medium. Spermidine and spermine have a more pronounced effect on cell growth. Severe inhibition of growth and cell death was observed in cells grown in medium containing spermidine, in excess of 1mM or spermine in excess of 0.5mM.

In flasks containing spermidine the intracellular levels of spermidine and 1,3-diaminopropane were slightly lower than those of control cells. In contrast the level of putrescine in these cells was greatly reduced. Cells grown in the presence of 0.5mM-spermidine contained 50% less putrescine than was found in control cells (Table 1.4). As the extracellular concentration of spermidine was increased to 1mM the total amino-nitrogen present in the oligoamines of these cells decreased to 62% of the control value. Similar results were obtained with cells grown in the presence of 0.1mM and 0.5mM-spermine. The intracellular levels of spermidine and 1,3-diaminopropane were relatively unaffected, whilst the putrescine level was reduced to 50% of the control value resulting in a correspondingly lower value for the amino-nitrogen present in oligoamines in these cells. An additional electrophoresis of these cell extracts in 0.1M-citric acid buffer, pH3.6, showed that cells grown in the presence of extracellular spermine, contained this oligoamine in significant quantities, unlike control cells.

Despite the inhibition of growth noted in cells grown in the presence of high concentrations of putrescine and 1,3-diaminopropane (10mM) and also in cells grown in the presence of even low extracellular spermidine and spermine, the development of these cells was not effected. All the cells removed from the experimental flasks and placed on developmental filters, developed normally; sorocarp formation being completed within the normal time period.

Cells grown in the presence of high extracellular putrescine contained elevated putrescine and 1,3-diaminopropane levels resulting in a high diamine level in these cells and in inhibition of growth. Growth inhibition also occurred in cells grown in the presence of high extracellular 1,3-diaminopropane in which only intracellular 1,3-diaminopropane levels were raised, putrescine levels being depressed (Table 1.4). The combined effect of these changes was a total diamine level close to that of control cells. Thus the growth inhibition was not just a reflection of altered total diamine levels. Cells grown at low extracellular levels of spermidine and spermine, contained 1,3-diaminopropane in amounts which varied only slightly from those in control cells (Table 1.4) and yet showed severe inhibition of growth. As intracellular putrescine levels in these cells were 50% below those in control cells, it seems probable that growth inhibition was a consequence mainly of altered intracellular putrescine levels.

The intracellular spermidine level remained relatively constant despite variations in the extracellular oligoamines present (Table 1.4), suggesting the existence in the cell of several control mechanisms affecting both the synthesis and degradation of spermidine. The decreased levels of intracellular putrescine in cells grown in medium containing spermidine and spermine was evidence for the existence of end-product inhibition of putrescine synthesis, possibly via ornithine decarboxylase levels (Figure 2.8). The presence of extracellular spermidine and spermine did not result in increased levels of intracellular 1,3-diaminopropane (Table 1.4). If this diamine is a product of the oxidative degradation of these oligoamines (Tabor & Kellogg, 1970; Smith, 1974) its turnover must be rapid. Alternatively the biosynthetic route of 1,3-diaminopropane in D. discoideum amoebae is independent of putrescine and spermidine synthesis i.e. via decarboxylation of diaminobutyric acid or the transamination of 3-aminopropionaldehyde, a possible derivative of β-alanine (Figure 1.11)

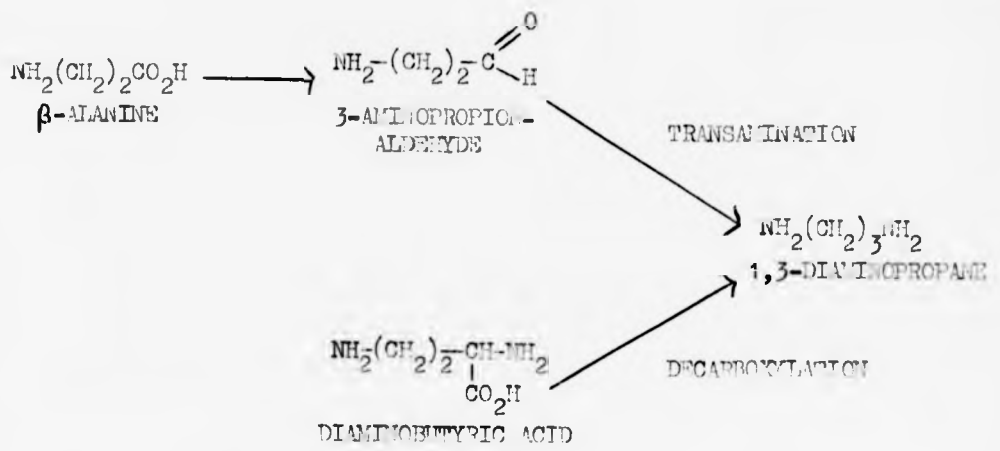


FIGURE 1.11 POSSIBLE BIOSYNTHETIC ROUTES OF 1,3-DIAMINOPROPANE

SECTION B - Biosynthesis of oligoamines in D. discoideum

It was of interest to determine to what extent the pattern of oligoamine biosynthesis in D. discoideum resembled procaryotes, as typified by E. coli (Figure 1.12a), or eucaryotes, as typified by rat tissues (Figure 1.12b) Stevens & Winther, 1979). To examine the biosynthesis of oligoamines in vivo, a series of experiments was undertaken, using labelled ornithine and arginine (possible precursors of putrescine), and putrescine (likely precursor of spermidine). It was not possible to investigate 1,3-diaminopropane synthetic pathways due to the unavailability of suitably labelled precursors.

UPTAKE OF POSSIBLE OLIGOAMINE PRECURSORS

Figure 1.13 shows the time course of labelling of amoebae during incubation with labelled ornithine, arginine and putrescine. The pattern of uptake of the two labelled amino acids was very similar but differed markedly from that of putrescine. The percentage of labelled ornithine and arginine entering the trichloroacetic acid soluble fraction of the cell rapidly reached a small but constant value. As the volume occupied by the cells was approximately 0.3% of the total volume of the suspension, the percentage of label in the acid soluble fraction of the cells was consistent with equilibration of the label in the suspending buffer with the internal pool of the amino acids. A similar

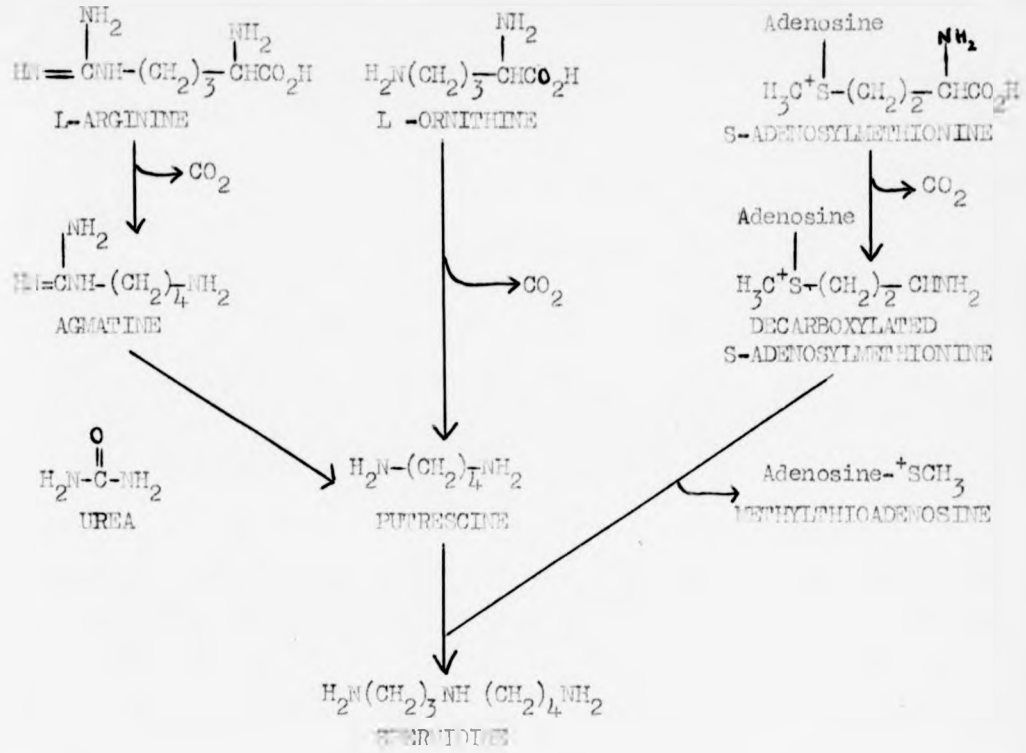


FIGURE 1.12a OLIGOAMINE BIOSYNTHETIC PATHWAY IN *E. COLI*
(Stevens & Winther, 1979)

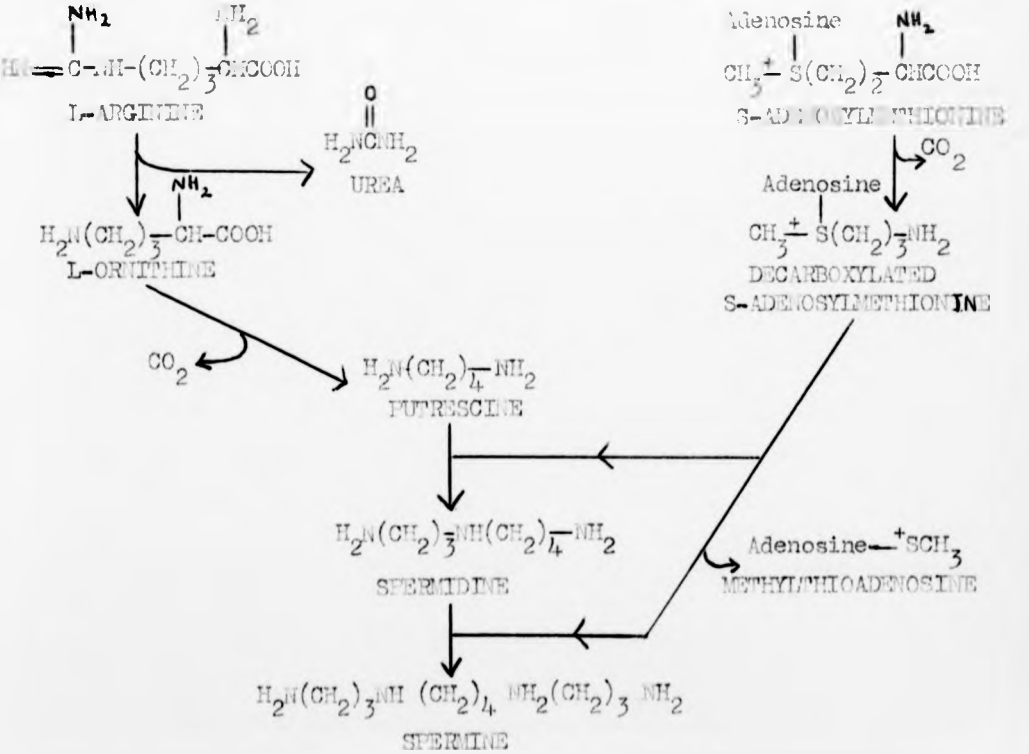


FIGURE 1.12b OLIGOAMINE BIOSYNTHETIC PATHWAY IN RAT LIVER
(Stevens & Winther, 1979)

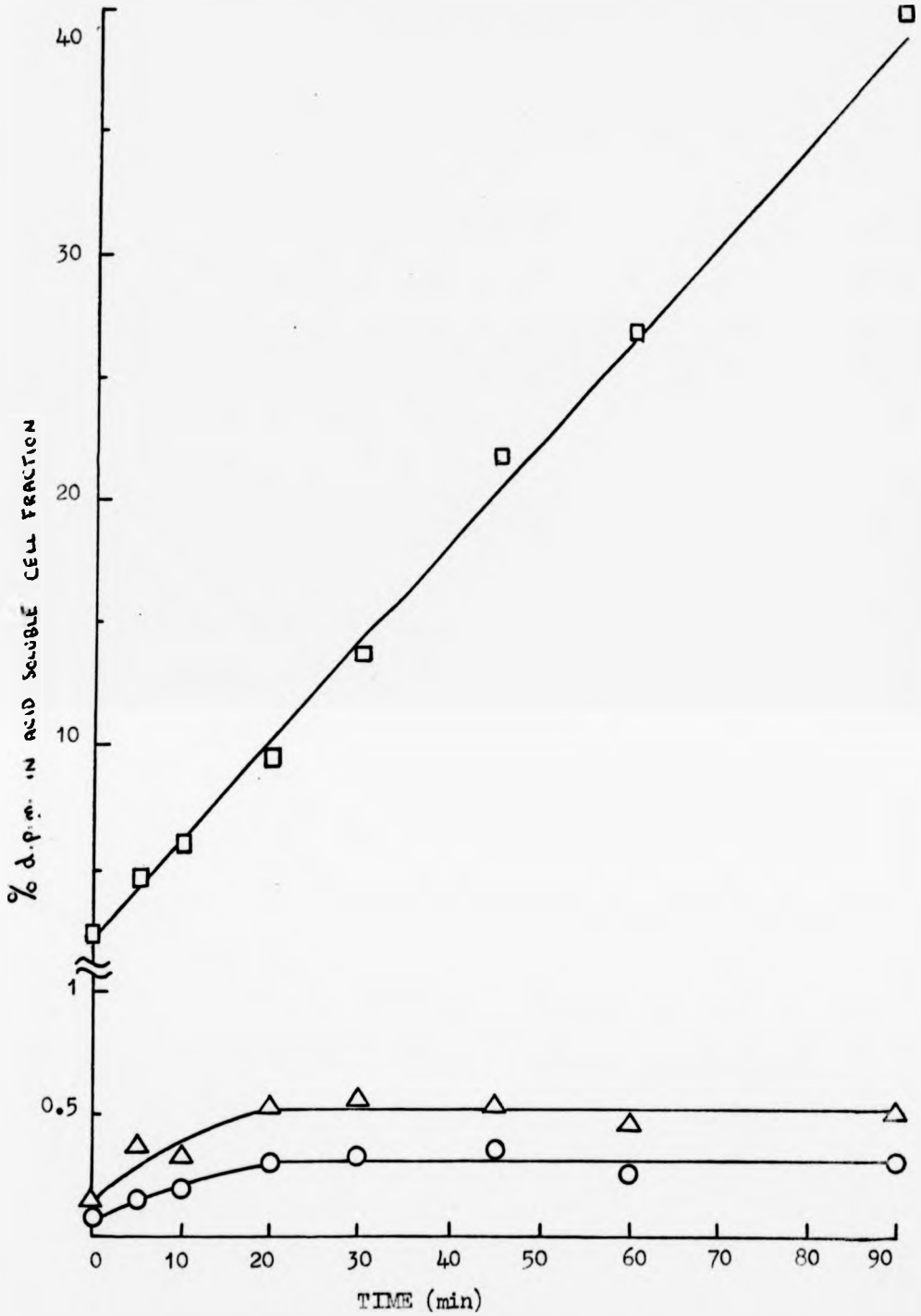


FIGURE 1.13 TIME COURSE OF LABELLING OF AMOEBAE WITH [^{3}H]ORNITHINE, [^{14}C] ARGININE AND [^{14}C] PUTRESCINE

Amoebae were incubated in 17mM-sodium phosphate buffer, pH6, at a density of 5×10^{6} cells/ml. After 15 min D,L-[5-^{3}H]ornithine (O), L-[U-^{14}C] arginine (△) and [1,4-^{14}C] putrescine (□) were added at concentrations of 0.04μM(0.2μCi/ml), 0.6μM(0.2μCi/ml) and 1.27μM (0.08μCi/ml) respectively. The percentage radioactive label present in the acid soluble fraction of the cells was monitored over 1.5h as detailed in the methods section.

equilibration was reported by Lee (1972c) to occur when D. discoideum amoebae are suspended in buffer containg either glutamate or lysine. From studies on the effect of 2,4-dinitrophenol and low temperature on this uptake, Lee concluded that the mechanism of uptake of amino acids by amoebae was passive diffusion. More recent evidence suggests that the uptake mechanism involves pinocytosis (Turner et al., 1979; North & Williams, 1978; North, 1982).

In contrast the uptake of putrescine by amoebae was rapid (Figure 1.13). Within 1.5h over 40% of the putrescine present in the buffer became associated with the trichloroacetic acid soluble fraction of the cells. The amount of putrescine present in amoebae was equivalent to an intracellular concentration of 17mM (Table 1.2). Binding to cellular constituents would make the true free intracellular concentration of putrescine much lower, but it would be unlikely to reduce it to less than the extracellular concentration of putrescine, 1.27μM. Thus it appeared that the uptake of putrescine was concentrative. The properties of this uptake system were investigated further in a later chapter.

EFFECT OF EXTRACELLULAR PUTRESCINE ON THE BIOSYNTHESIS OF PUTRESCINE FROM ORNITHINE

Having determined that the intracellular pools of probable oligoamine precursors can, in amoebae, be radioactively labelled, further experiments were designed to elucidate the pathway of oligoamine biosynthesis in D. discoideum. When putrescine and spermidine were analysed after growth in the presence of 0.2mM-D, L-[5-^{3}H]ornithine, radioactivity was found associated with both compounds (Table 1.5).

Some amoebae were grown in HL5 medium containing, in addition to the labelled ornithine, putrescine at the concentrations shown in Table 1.5. The growth of the amoebae, as monitored microscopically, was not affected by these additions. On analysis of the distribution of radioactivity between the cells and the medium (as detailed in the methods section), the percentage of labelled material found associated with the acid soluble fraction of amoebae averaged 0.5% (Table 1.5). This was comparable to the uptake of ornithine from phosphate

PUTRESCINE mM	RADIOACTIVITY PRESENT IN:- CELLS (% OF TOTAL RECOVERED)	ACID INSOLUBLE MATERIAL (% OF TOTAL IN CELLS)	ACID SOLUBLE MATERIAL (% OF TOTAL IN CELLS)	SPECIFIC RADIOACTIVITY (dpm/nmol OLIGOAMINE) PUTRESCINE	SPERMIDINE
0	0.9	34	66	12.1	6.1
0.05	1.0	32	68	8.7	4.4
0.5	0.5	50	50	1.8	4.0
5	0.7	57	43	1.6	3.1

TABLE 1.5 EFFECT OF PUTRESCINE ON THE DISTRIBUTION OF RADIOACTIVITY AFTER GROWTH OF AMOEBAE IN HL5 MEDIUM CONTAINING 0.2mM-D, L-[5-^{3}H]ORNITHINE

The distribution of radioactivity between cells and HL5 medium was determined on harvesting the cells after 72h growth in medium containing both labelled ornithine, (0.2mM-D,L-[5-^{3}H]ornithine) and varying concentrations of putrescine. The cells were precipitated in trichloroacetic acid, the acid insoluble material fractionated on a Dowex column followed by electrophoresis and the distribution of radioactivity subsequently analysed as detailed in the methods section. Over 98% of the acid soluble radioactivity applied to the Dowex column and over 70% of that applied to electrophoresis strips, was recovered.

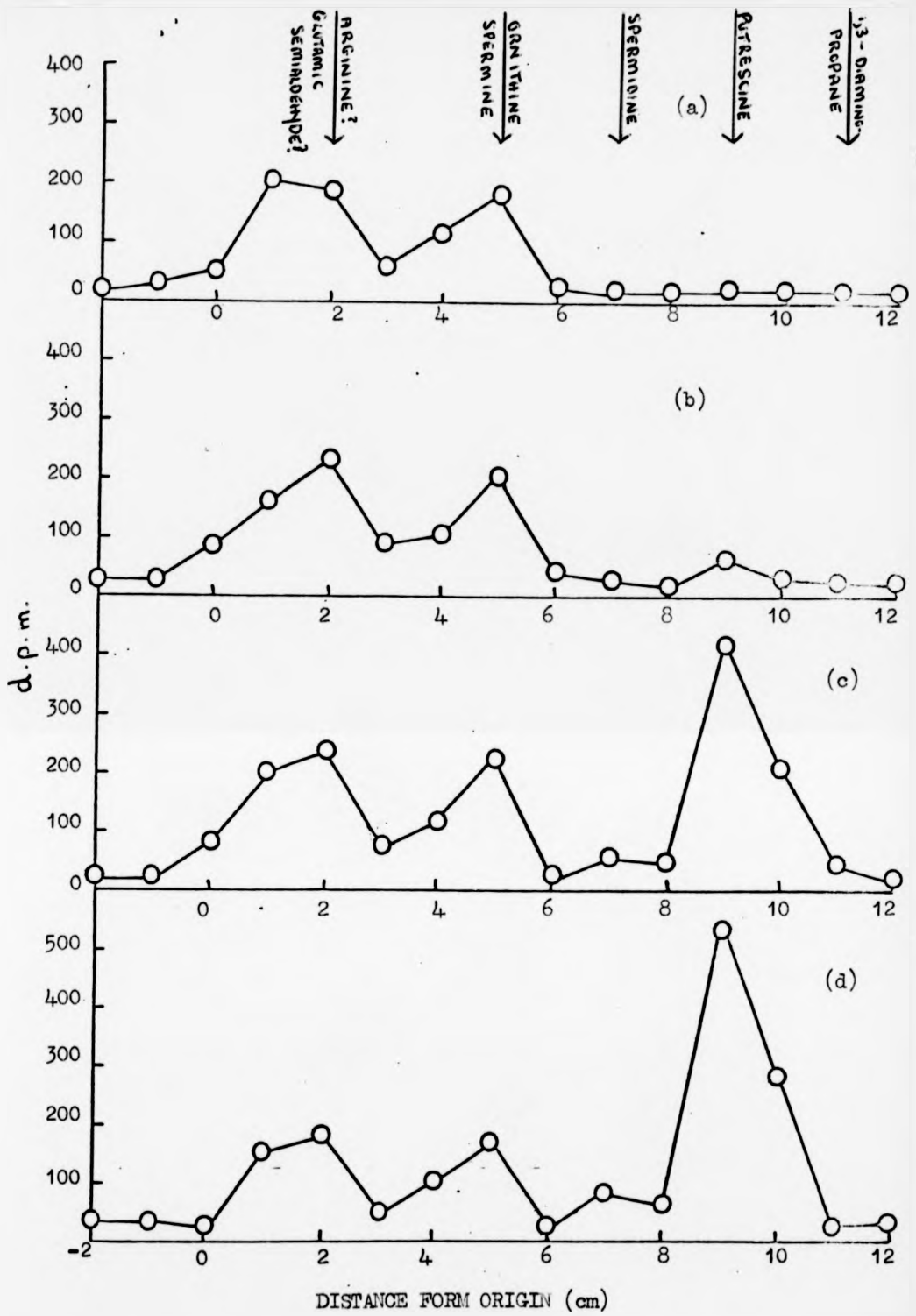


FIGURE 1.14 DISTRIBUTION OF RADIOACTIVITY IN AMOEBAE AFTER GROWTH IN HL5 MEDIUM CONTAINING D,L-[5-^{3}H]ORNITHINE AND THE CONCENTRATIONS OF PUTRESCINE SHOWN

Amoebae were grown in flasks of HL5 medium containing 0.2mM-D,L-[5-^{3}H] ornithine and the following concentrations of putrescine; (a) 5mM, (b) 0.5mM, (c) 0.05mM and (d) no putrescine. The cells were harvested, cell extracts prepared and the distribution of radioactivity in the acid-soluble cell fractions determined as described in the methods section.

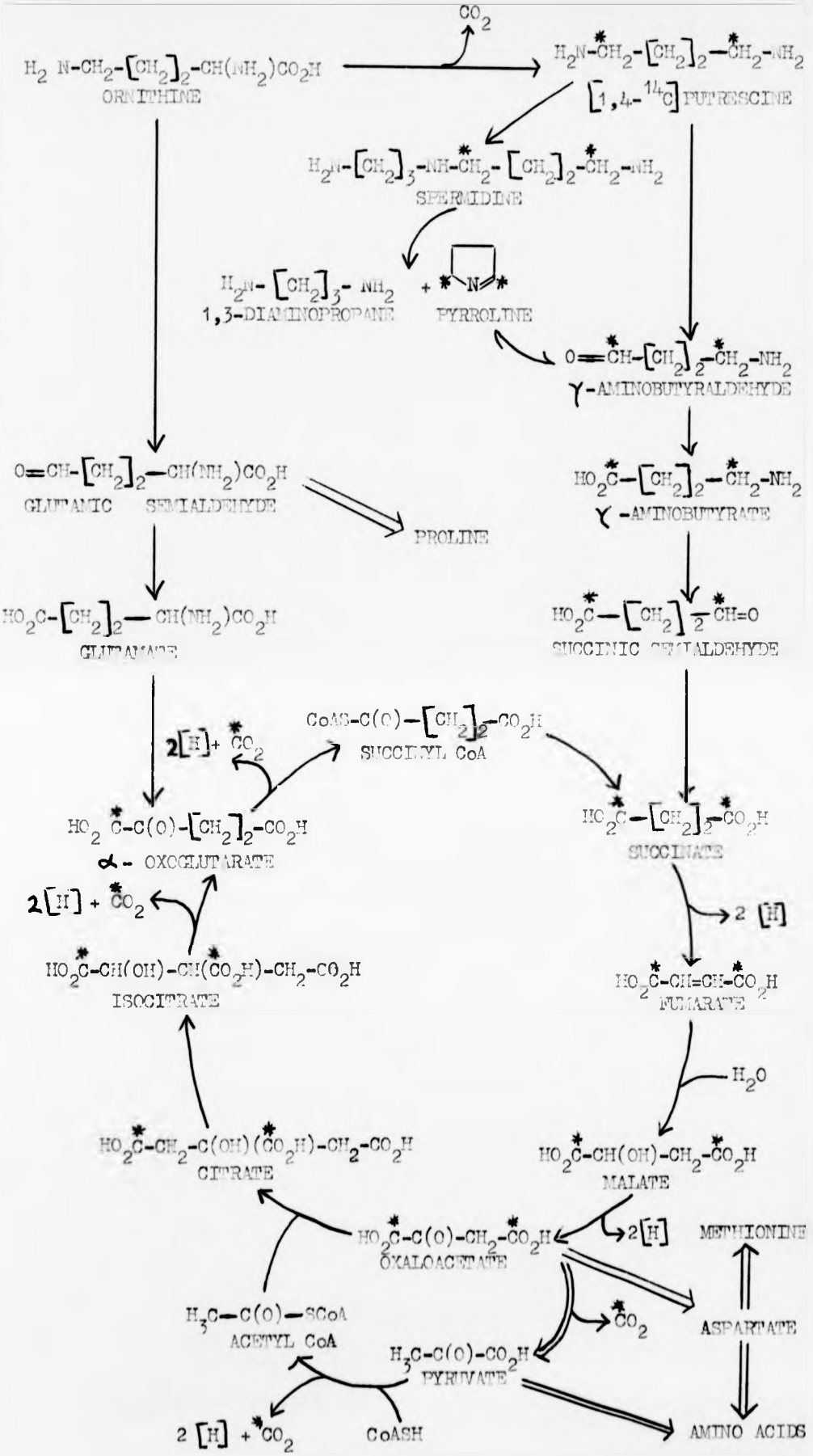


FIGURE 1.15 PATHWAYS FOR [1,4-^{14}C] PUTRESCINE CATABOLISM

buffer (Figure 1.13), i.e. uptake of ornithine was not considerably altered by the switch to growth medium nor by the presence of putrescine. Although the uptake of ornithine was unaffected by the concentration of putrescine in the medium, the intracellular distribution of labelled material was dependent on extracellular putrescine levels. Both in the absence of extracellular putrescine and at low extracellular concentration of putrescine, over 60% of the labelled material in the cell was found in the acid soluble fraction. As the concentration of extracellular putrescine increased to 5mM this figure fell to just over 40%, whilst the percentage in the acid insoluble fraction rose (Table 1.5). The association of radioactivity with the acid insoluble fraction can be accounted for by the conversion of ornithine to other amino acids which are subsequently incorporated into cellular proteins. This alternative metabolism of ornithine may involve its conversion into arginine or alternatively into proline via glutamic semialdehyde (Figure 1.15). The presence of ornithine transaminase has been demonstrated in D. discoideum amoebae (North & Paterson C., unpublished).

Further analysis of the acid soluble cellular fraction revealed that elevated levels of exogenous putrescine depress the conversion of [5-^{3}H] ornithine into putrescine in the cell. Thus in cells grown in HL5 medium containing 0.2mM-ornithine but no added putrescine, 45% of the label recovered from the electrophoresis strip migrated with putrescine and only 16% with ornithine. In cells grown in HL5 medium containing 5mM-putrescine in addition to ornithine, only 4% of the label migrated with putrescine whilst 31% migrated with ornithine (Figure 1.14). The specific activity of both putrescine and spermidine decreased as the extracellular concentration of putrescine increased. Thus exogenous putrescine would appear to be the preferred source of this cellular diamine.

The decrease in the specific activity of spermidine was not as expected. Putrescine is a precursor of spermidine in amoebae, as shown in a later experiment, yet as the amount of unlabelled extracellular putrescine increases, dilut-

ing the intracellular pool and decreasing the specific activity of this diamine, the specific activity of spermidine did not decrease as rapidly (Table 1.5). Thus in cells grown in the presence of 0.5mM and 5mM-putrescine the specific activity of spermidine was greater than that of putrescine. These results would be consistent with the existence of more than one intracellular pool of putrescine in amoebae. If the rate of exchange between the pool containing putrescine derived intracellularly from ornithine, and that containing exogenously derived putrescine was slow, then spermidine synthesized only from the former pool would be of high specific activity. It is however very unlikely that equilibration between intracellular pools would not have occurred during the 72h growth period.

Electrophoretic analysis of the acid soluble fraction revealed, in addition to putrescine, spermidine and ornithine, another positively charged radioactive material which moved, in a diffuse band, behind spermine and ornithine. The electrophoretic mobility of this material is consistent with it being arginine, glutamic semialdehyde or some similar metabolite of ornithine.

In similar experiments carried out with cells grown in the presence of L-[U-^{14}C] arginine, no radioactive label was incorporated into oligoamines. Turner & North (1977), were unable to detect arginine decarboxylase in amoebal extracts (see page 77). It therefore seems that the biosynthetic pathway to putrescine in D. discoideum is typical of eucaryotic organisms, via decarboxylation of ornithine.

DISTRIBUTION OF RADIOACTIVE PUTRESCINE IN AMOEBAE

The biosynthesis of spermidine is from putrescine by the transfer of propylamine from decarboxylated S-adenosylmethionine. On analysis of cells grown in the presence of labelled putrescine, radioactive label was found incorporated in spermidine (Table 1.6). The cells were grown in HL5 medium containing [1,4-^{14}C] putrescine at concentrations of 0.5μM (57mCi/mmol), 5μM (5.7mCi/m mol) and 50μM (0.57mCi/m mol). In all three suspensions 25-35% of the labelled putrescine added to the medium was found associated with the cells,

PUTRESCINE (μM)	RADIOACTIVITY PRESENT IN:- HL5 MEDIUM (% OF TOTAL RECOVERED)	ACID INSOLUBLE MATERIAL (% OF TOTAL IN CELLS)	DOWEX COLUMN FRACTIONS (% OF TOTAL RECOVERED) A	B	C	PUTRESCINE (% OF TOTAL RECOVERED FROM ELECTROPHORESIS STRIPS)	SPERMIDINE	SPECIFIC RADIOACTIVITY (dpm/n mol OLIGOAMINE) PUTRESCINE	SPERMIDINE
0.5	66	4.5	0.8	12.8	86.4	84.9	13.0	81.7	66.7
5	68	3.7	1.2	11.1	87.7	83.5	14.6	139.7	142.1
50	75	5.6	1.7	10.9	87.4	83.1	13.2	134.6	138.9

TABLE 1.6 DISTRIBUTION OF RADIOACTIVITY AFTER GROWTH OF AMOEBAE IN HL5 MEDIUM CONTAINING [1,4-^{14}C] PUTRESCINE

The distribution of radioactivity between cells and medium was determined on harvesting the cells after 72h growth in [1,4-^{14}C] putrescine labelled medium. The cells were precipitated in trichloroacetic acid, the acid soluble material fractionated using a Dowex column followed by electrophoresis, and the distribution of radioactivity within the cells subsequently analysed as detailed in the methods section. Over 95% of the acid soluble radioactivity applied to the Dowex column and over 90% of that applied to the electrophoresis strips, was recovered.

when harvested 72h after inoculation (Table 1.6). Less than 1% of this label was removed by water washing of the cells. On acid precipitation a significant proportion of radioactivity was found in the acid insoluble fractions of all three cell samples, indicating that intracellular metabolism of putrescine had occurred (Table 1.6). Further evidence of this was the elution from the Dowex-50? column of over 10% of the total radioactivity recovered, in fractions A (unbound material) and B (0.5M-HCl) (Table 1.6). The acid insoluble and weakly bound label can be accounted for by the conversion of [1,4-^{14}C] putrescine to labelled amino acids probably via γ-aminobutyrate, succinic semialdehyde and the citric acid cycle (Figure 1.15).

Over 85% of the cellular radioactivity was eluted from the columns with 6M-HCl (Fraction C). These oligoamine containing fractions were subsequently analysed by paper electrophoresis. Whilst about 13-14% of the radioactive material was recovered from the slower moving spermidine position, the largest portion, over 83%, migrated with putrescine. A similar analysis of fractions A and B confirmed the absence of the oligoamines putrescine, spermidine and 1,3-diaminopropane in these fractions. The radioactivity contained in these fractions remained at the origin during electrophoresis in 65mM-5-sulphosalicylic acid, pH3.2.

The specific activity of the putrescine recovered from the cells was similar to that of spermidine, evidence that exogenous putrescine had entered an intracellular pool from which cellular spermidine was derived (Table 1.6). Spermidine is a possible precursor of 1,3-diaminopropane. However spermidine synthesized from 1,4-^{14}C putrescine would not give rise to labelled 1,3-diaminopropane (Figure 1.15).

SYNTHESIS OF OLIGOAMINES DURING DEVELOPMENT

The loss of putrescine, spermidine and 1,3-diaminopropane during the developmental period of amoebae has been recorded (Figure 1.9). It was not known whether this decrease reflected a cessation of synthesis of oligoamines, an increased catabolic rate or a combination of both events. The synthesis

of oligoamines during development was investigated as detailed in the legend to Table 1.7.

During development over 87% of the putrescine and spermidine present in growing amoebal cells was lost (Figure 1.9). Similarly cells labelled with [1,4-^{14}C]putrescine lost over 78% of the radioactivity initially present in the acid soluble cell fraction, over the 24h developmental period. This suggested that during the 30 min labelling period and the time taken to prepare the developmental filters the radioactive putrescine had equilibrated with intracellular pools. This period of incubation was however too short to allow equilibration of label between the putrescine and spermidine pools, thus in initial samples the specific activity of putrescine was greater than that of spermidine (Table 1.7). Equilibration had occurred after 4h on developmental filters, which was consistent with the continued synthesis of spermidine during development.

The specific activity of putrescine decreased throughout development (Table 1.7). Assuming that equilibration between [1,4-^{14}C]putrescine and intracellular putrescine had occurred prior to the start of the experiment, then this decrease could not have been the result of preferential loss of exogenously derived putrescine but may instead have reflected continued synthesis of putrescine during development. The continued increase in the specific activity of spermidine after equilibration at 4h would not have resulted directly from the synthesis of spermidine from a putrescine pool in which the specific activity was decreasing. Newly synthesized spermidine may have been additionally labelled in the propylamine moiety. This could have resulted from the metabolism of labelled putrescine via γ-aminobutyrate succinic semialdehyde and the citric acid cycle into methionine (Figure 1.15). Although during development the levels of oligoamines present in D. discoideum cells decreased, the data in Table 1.7 suggested that putrescine and spermidine had been synthesized during this period.

	SPECIFIC RADIOACTIVITY FOR DIFFERENT TIMES ON DEVELOPMENTAL FILTERS (dpm/n mol)						
	0h	1h	2h	4h	6h	14h	24h
PUTRESCINE	58.7	47.3	39.0	36.9	28.1	22.5	20.9
SPERMIDINE	27.8	33.4	35.0	36.3	38.2	45.8	54.3

TABLE 1.7 CHANGES IN SPECIFIC RADIOACTIVITY IN SPERMIDINE AND PUTRESCINE DURING DEVELOPMENT

Amoebae were incubated for 30 min with 0.2μM-[1,4-^{14}C]putrescine (63mCi/m mol) in 17mM-sodium phosphate buffer, pH6, at a cell density of 8×10^6 cells/ml. The cells were harvested, washed and deposited on development Millipore filters, from which they were subsequently harvested at regular intervals over the 24h developmental period and the specific radioactivity of putrescine and spermidine determined as detailed in the methods section.

SECTION C - CATABOLISM OF PUTRESCINE IN D. DISCOIDEUM

Carbon dioxide is produced during catabolism of putrescine. This diamine is known to be oxidised in some eucaryotic and procaryotic cells to γ-aminobutyrate which enters the citric acid cycle via succinic semialdehyde and is rapidly degraded to carbon dioxide (Figure 1.15, see page 44). Thus in a preliminary investigation into the catabolism of putrescine by D. discoideum, data were obtained by following the conversion of radioactively labelled putrescine to repiratory [^{14}C] carbon dioxide.

EFFECT OF EXTRACELLULAR OLIGOAMINES ON CATABOLISM OF INTRACELLULAR PUTRESCINE TO CARBON DIOXIDE

AX2 amoebae, prelabelled with [1,4-^{14}C] putrescine (as detailed in the methods section), were grown in FM medium containing oligoamines at the concentrations shown in Table 1.8. The concentrations of oligoamine additives in the flasks were at levels known not to cause growth inhibition to AX2 amoebae (page 65). At hourly intervals the radioactivity contained in the carbon dioxide traps, FM medium and trichloroacetic acid soluble and insoluble cell fractions was determined (as detailed in the methods section). Catabolism was monitored over a short time period, 4h, in an attempt to minimize the effects of increasing cell numbers during the course of the experiment. As the doubling time of amoebae in FM medium is 24h, it was estimated that the increase in cell population during the course of the experiment was unlikely to exceed 10%.

In the flask containing no oligoamine additives, less than 1% of the radioactivity was recovered from the FM medium after 4h. This low value suggested that excretion of labelled putrescine and its derivatives during growth was at a very low level. Material released from cells lysed during harvesting and resuspension, or removal of surface bound putrescine may account for the levels of radioactivity recovered from the medium.

The presence in the FM medium of unlabelled putrescine, 1,3-diaminopropane, spermidine and spermine at concentrations of 0.1mM and 0.5mM failed to chase

ADDITION	CONCENTRATION (mM)	RADIOACTIVITY IN:- ACID SOLUBLE FRACTION (% OF TOTAL RECOVERED AFTER 4h)	RADIOACTIVITY IN:- CARBON DIOXIDE TRAP (% OF TOTAL RECOVERED AFTER 4h)	RATE OF CATABOLISM OF 1,4-^{14}C PUTRESCINE TO CARBON DIOXIDE (p mol/h/10^8 cells)	RATE OF CATABOLISM OF CELLULAR PUTRESCINE TO CARBON DIOXIDE (nmol/h/10^8 cells)	% OF CONTROL VALUE
-	-	96.4	2.1	0.46	4.9	100
PUTRESCINE	0.1	96.7	2.1	0.45	4.8	98
PUTRESCINE	0.5	96.2	2.5	0.47	5.8	118
1,3 DIAMINO-PROPANE	0.1	96.4	1.9	0.37	4.5	92
1,3-DIAMINO-PROPANE	0.5	96.8	2.1	0.48	4.9	101
SPERMIDINE	0.1	96.1	2.4	0.31	5.7	116
SPERMIDINE	0.5	95.7	2.9	0.37	6.7	136
SPERMINE	0.1	96.9	1.8	0.41	4.2	87
SPERMINE	0.5	96.4	2.5	0.54	5.9	120

TABLE 1.8 CATABOLISM OF PUTRESCINE DURING GROWTH OF AMOEBAE

Amoebae prelabelled for 15 min with [1,4-^{14}C] putrescine (as detailed in the methods section) were suspended at a cell density of 8×10^6 cells/ml in FM medium containing oligoamine additions as indicated. Carbon dioxide traps were suspended in the flasks and these sealed with self sealing caps as described in the methods section. The distribution of radioactivity was then determined at hourly intervals over a 4h period and the rate of [^{14}C] carbon dioxide production determined graphically. The rate of putrescine catabolism was estimated on the basis of the following assumptions: a) that exogenous and endogenous putrescine pools had equilibrated; b) the ratio of putrescine to DNA in the cells was as determined previously (61.7n mol/μgDNA); c) amoebal cells contain 17×10^{-8} μgDNA per cell (Leach & Ashworth, 1972). Each set of results represents the average of 3 flasks.

radioactivity out of the cells into the medium. When AX2 amoebae, prelabelled with [1,4-^{14}C] putrescine, are suspended in non-nutrient phosphate buffer, the efflux of labelled material is stimulated by the presence of extracellular oligoamines (Turner et al., 1979). Thus putrescine, 1,3-diaminopropane and spermidine, at concentrations of 1mM, chase over 5% of the radioactivity originally present in the cells into the suspending buffer over a 4h period. This figure rises to 12% in the case of 1mM-spermine. The apparent discrepancy in the effect of extracellular oligoamines on the efflux of labelled putrescine probably reflects the fact that the cells used in the latter experiments were under starvation conditions whereas those used in the former, were under growth conditions.

The catabolism of [1,4-^{14}C] putrescine via γ-aminobutyrate, succinic semialdehyde and subsequently the citric acid cycle (Figure 1.15) would probably result in the incorporation of label into the amino acid pool of the cell. These may in turn be utilized in the synthesis of cellular proteins. As less than 0.5% of the radioactivity recovered from the cells was found in the acid insoluble material, the conversion of putrescine into amino acids during growth must be considered to be a minor catabolic pathway. The presence in the HL growth medium of oligoamines at concentrations of 0.5mM, failed to increase the percentage of labelled material in this fraction. In cells prelabelled with [1,4-^{14}C] putrescine and resuspended in non-nutrient phosphate buffer containing no additions, only 0.04% of the radioactivity is recovered in the acid insoluble fraction (Turner et al., 1979). This figure increases to 0.51% in cells resuspended in buffer containing 0.1mM-unlabelled putrescine and to 2.18% in buffer containing 1mM-unlabelled putrescine, suggesting that in cells undergoing starvation extracellular putrescine increases the percentage of labelled putrescine being catabolised via γ-aminobutyrate and the citric acid cycle and hence into amino acids and proteins.

As the results in Table 1.8 clearly show, most of the radioactivity was recovered in the acid-soluble cell fraction. This labelled material would

include putrescine, spermidine synthesized from labelled putrescine and soluble catabolic products of putrescine.

The amount of labelled material present in the FM medium and in both the acid-soluble and acid-insoluble cell fractions was insufficient to account for all the radioactivity lost from the cells. This loss was accounted for by the production of [^{14}C] carbon dioxide by the amoebae during growth. Amoebae suspended in FM medium catabolised [1,4-^{14}C] putrescine to carbon dioxide at a rate of 0.46p mol/h/10^8 cells, a figure representing conversion of over 2% of the [1,4-^{14}C] putrescine taken up by the cells in the 15 min labelling, into [^{14}C] carbon dioxide in 4h (Table 1.8). Assuming free exchange of labelled putrescine with all intracellular pools had occurred during the 1h labelling, harvesting and resuspension period, this value was equivalent to the catabolism of cellular putrescine at a rate of 4.9 n mol/h/10^8 cells (Table 1.8), a turnover of 0.5% of the putrescine present in the cell in an hour.

The presence of putrescine in FM medium at concentrations of 0.5mM or less had little effect on the intracellular oligoamine levels of amoebae (Table 1.4), and similarly these levels of exogenous putrescine had negligible effect on the rate of putrescine catabolism to carbon dioxide (Table 1.8). An alteration was observed in the oligoamine levels of cells grown for 72h in FM medium containing 1,3-diaminopropane at concentrations of 0.1mM and 0.5mM, their intracellular 1,3-diaminopropane content having increased at the expense of their putrescine content (Table 1.4). However the rate of putrescine catabolism to carbon dioxide in cells growing in the same conditions, was similar to that of the control cells (Table 1.8). The presence of 0.5mM-spermidine in FM medium caused intracellular putrescine levels to fall by 48% (Table 1.4). Under the same conditions the rate of putrescine catabolism to carbon dioxide was increased to 136% of control values (Table 1.8). A similar increase in the rate of putrescine catabolism to carbon dioxide was observed in cells grown in FM medium containing 0.1 and 0.5mM-spermine. These concentrations of spermine in the growth medium have been shown to cause intracellular putrescine

levels to fall by 50% whilst other oligoamines were unaffected (Table 1.4). Thus the large decreases in intracellular putrescine levels observed in cells grown in the presence of spermidine or spermine can be accounted for, although only in part, by a concomitant increase in the catabolism of putrescine to carbon dioxide in these cells. The alteration in the diamine levels of cells grown in the presence of 1,3-diaminopropane was however not achieved by catabolism of cellular putrescine to carbon dioxide but may involve control of putrescine synthesis (see page 142).

CATABOLISM OF PUTRESCINE DURING DEVELOPMENT

In amoebae under growth conditions the rate of catabolism of putrescine to carbon dioxide was low. In contrast the level of putrescine in cells undergoing development has been shown to decrease by 88% over the 24h developmental period. Thus the catabolism of putrescine during development was investigated as detailed in the legend to Figure 1.16.

During the 24h developmental period over 75% of the radioactivity initially present in the acid soluble cell fraction was lost (Figure 1.16). This was slightly lower than the 88% decrease in measured putrescine levels during development, possibly due to the presence in the acid-soluble fraction, of labelled metabolites of putrescine.

The percentage of radioactivity recovered in the pad diluting fluid over the 24h developmental period was small and relatively constant at 6.5±0.8% indicating that the loss of putrescine from amoebae during development was not the result of excretion of putrescine or its metabolites. The liberated material detected in the pad diluting fluid was possibly released from cells ruptured during the preparation of the developmental filters or due to release of surface bound putrescine.

In an analysis of the distribution of radioactivity in amoebae immediately after the 15 min labelling period, over 2% of the radioactivity recovered was found to be present in the acid insoluble cell fraction. During the 1h taken to resuspend the cells in water, place them on developmental filters and immed-

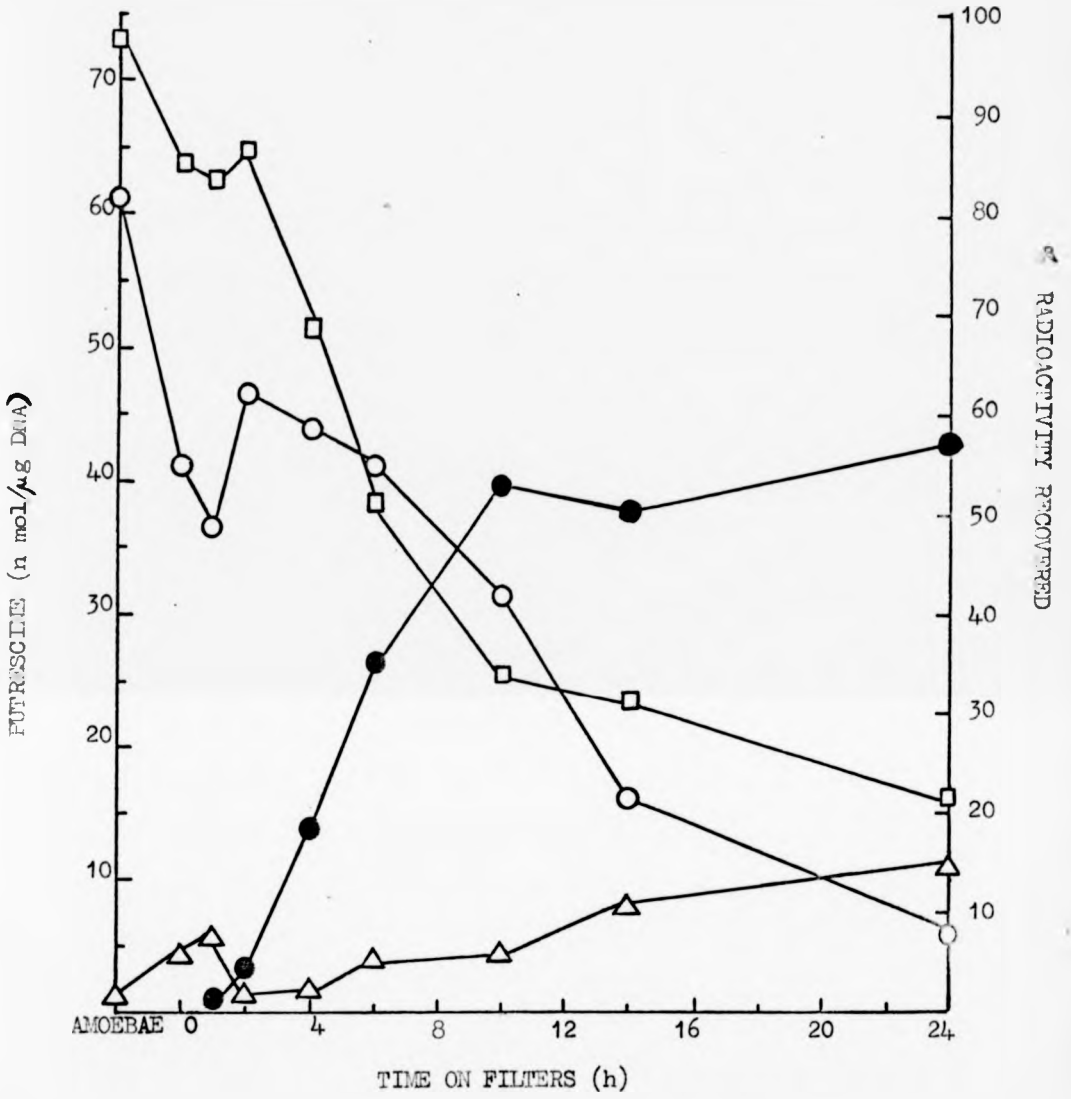


FIGURE 1.16 CATABOLISM OF PUTRESCINE DURING DEVELOPMENT

Amoebae, incubated for 15 min with 0.2µM-[1,4-^{14}C]putrescine in 17mM-sodium phosphate buffer, pH6, were harvested, washed and used in the preparation of a modified developmental system consisting of sealed 50ml-Erlenmeyer flasks containing carbon dioxide traps as detailed in the methods section. The levels of putrescine during development were monitored (O) as was the percentage of radioactivity recovered in the acid insoluble (△) and soluble (□) cell fractions and in [^{14}C] carbon dioxide (●). The results shown represent the average of ten flasks harvested at each time point.

iately reharvest them, the percentage of radioactivity in the acid insoluble cell fraction rose to over 6% and continued to rise during the first hour on developmental filters, reaching a value of 8% (Figure 1.16). In the same cells the percentage of radioactivity recovered in the acid soluble fraction fell sharply from 98% in amoebae newly harvested from labelled buffer, to 84% in cells after 1h on filters. Between 1h and 2h this value increased to 87% but during the remainder of the 24h developmental period the percentage of radioactivity recovered in the acid soluble cell fraction decreased steadily. This pattern corresponded closely to that observed in the levels of oligoamines, RNA and protein during development (Figure 1.9). One possible explanation of these observations is that the amoebae may form putrescine-protein conjugates as an initial response to starvation. This would account for the fall in the radioactivity recovered in the acid-soluble fraction and the rise in the radioactivity recovered in the acid-insoluble fraction during the setting up of developmental filters. After the first 2h on developmental filters the dissociation of these conjugates, releasing putrescine, would cause the levels of radioactivity in the acid-soluble fraction to rise and those in the acid-insoluble fraction to fall. The control of intracellular oligoamine levels by their conjugation has been reported (Rosenblum & Russell, 1977; Melvin & Weir, 1980; Maddox & Russell, 1981). Although the structure of these conjugates has not yet been determined, Rosenblum & Russell (1977) report a spermidine conjugate containing several amino acids and Chen & Liu (1981) are investigating the identity of the Mr 18,000 protein moeity with which putrescine and spermidine associate in neuroblastoma cells.

Data in Figure 1.16 show that cells prelabelled with [1,4-^{14}C] putrescine evolve large amounts of [^{14}C] carbon dioxide during development. After 1h on developmental filters, labelled putrescine was being catabolised to carbon dioxide at a rate of 3.7 p mol/h/10^8 cells; an 8-fold increase in the rate observed in similarly labelled cells suspended in FM growth medium (Table 1.8). During the initial 10h of development the catabolism of labelled putrescine to

carbon dioxide by amoebae was rapid, over 130 p mol/10^8 cells. The rate of catabolism of labelled putrescine to carbon dioxide during this period was determined graphically as 16 p mol/h/10^8 cells, a rate 35 times greater than that observed in cells in FM growth medium (Table 1.8). Although the putrescine levels continued to fall during the last 14h of development the production of labelled carbon dioxide effectively ceased (Figure 1.16). During this period of development however the percentage of radioactivity recovered in the acid insoluble fraction rose steadily (Figure 1.16). One interpretation of these results was that during the first 10h of development putrescine was serving as a substrate via γ-aminobutyrate and succinic semialdehyde for the citric acid cycle and energy production. During the last 14h of development the production of respiratory [^{14}C] carbon dioxide from putrescine ceases, suggesting that during this period the catabolism of putrescine is providing an endogenous supply of carbon for the synthesis of macromolecules; thus causing the radioactivity in the acid insoluble fraction to rise. Alternatively this rise may be accounted for by the reformation of the conjugated putrescine molecule, proposed earlier.

DISCUSSION

Analysis of the oligoamine content of D. discoideum revealed the presence of putrescine, 1,3-diaminopropane and spermidine at all stages of the life cycle. This result has subsequently been independently confirmed, using ion exchange chromatography to determine levels (Mach et al., 1981). The oligoamine content was not dependent on the strain of cells used, since similar values were obtained for amoebae of both strains NC4 and AX2 grown in association with K. aerogenes (Table 1.1). North & Murray (1980) subsequently reported that the oligoamine content of two other strains, X55 and V12 (both non-axenic strains), was similar to that of AX2 and NC4. The growth conditions did not affect the oligoamine composition of amoebae. Although AX2 amoebae grown axenically in either FM medium or HL5 medium (with or without glucose) contained approximately twice as much oligoamine per mg DNA as bacterially grown cells (Table 1.1 and Table 1.2), this apparent variation was a result of a difference in DNA content. Since bacterially grown cells have a DNA content approximately double that of the same cells grown axenically (Teach & Ashworth, 1972), the oligoamine content per cell was independent of the food source. There was a marked difference between the oligoamine composition of D. discoideum amoebae and that of the bacteria on which they were grown, the latter containing only low levels of putrescine and cadaverine (Table 1.1). D. discoideum amoebae must differentially catabolise or excrete cadaverine as this diamine has never been detected in amoebal extracts. North & Murray (1980) have since measured the oligoamine levels of AX2 amoebae grown in association not only with K. aerogenes but also with E. coli and noted that the levels of putrescine, 1,3-diaminopropane and spermidine were slightly lower in the latter cells.

The phase of growth is known to effect oligoamine levels in some systems (Elliott & Michaelson, 1969; Bachrach, 1973; Bacchi et al., 1977). In D. discoideum however, entry of axenically grown cells into stationary growth had

little effect on oligoamine levels (Table 1.2). We have already reported that amoebae harvested at low cell densities ($< 2x10^6$ cells/ml) have a greater 1,3-diaminopropane content than amoebae in denser cultures (Worth & Turner, 1977), but the significance of this is not clear.

The diamine putrescine was present in amoebae at high concentrations (17mM) however the pool of free intracellular putrescine was probably very small as a result of widespread intracellular binding to membranes and nucleic acids (Cohen, 1971; Bachrach, 1973). Similarly high intracellular concentrations of putrescine have been reported in E. coli (25mM) by Morris et al. (1970). Spermidine was present in much smaller quantities whilst spermine was detected in only trace amounts (Table 1.1 and Table 1.2). Nach et al. (1981) also failed to detect spermine in D. discoideum extracts. Such high concentrations of putrescine with lower levels of spermidine and only traces of spermine, resembles the oligoamine composition of other lower eucaryotes such as the true slime mould P. polycephalum (Mitchell & Rusch, 1973), the water mould B. emersonii (Mennucci et al., 1975) and the protozoan T. pyriformis (Pösö et al., 1976b). This pattern is not typical of either fungi or higher eucaryotes, in which spermine and spermidine are the major oligoamines and putrescine is present in only trace amounts and resembles more closely the oligoamine composition of procaryotes (Tabor & Tabor, 1976a; Stevens & Winther, 1979).

D. discoideum was also unusual in possessing significant quantities of the diamine 1,3-diaminopropane. This diamine has been detected only rarely in other organisms (Herbst et al., 1958; Weaver & Herbst, 1958; Smith, 1970a,b; Nakajima et al., 1967) and never at the high levels found in D. discoideum (Table 1.2). The oligoamine content of four other members of the Dictyostelidacea family have subsequently been analysed (Worth & Murray, 1980), but only D. mucoroides possesses 1,3-diaminopropane. Its appearance in the two species of cellular slime mould was of interest not least because this diamine has been shown to inhibit protein synthesis (Tuomi et al., 1970; Kay & Benzie,

1980) and also oligoamine synthesis (Pösö & Jänne, 1976a,b; Pösö et al., 1977; Kallio et al., 1977c,d; Sunkara et al., 1977). The pathway of 1,3-diaminopropane biosynthesis is unknown. By analogy with putrescine synthesis one possible route is via the decarboxylation of 2,4-diaminobutyric acid. An alternative route is via the transamination of aminopropionaldehyde formed possibly by reduction of β-alanine. Both these routes would be of interest as they are independent of the biosynthesis of the other D. discoideum oligoamines and hence might allow independent control of this diamine. In other organisms, 1,3-diaminopropane is reported to be a catabolic product of spermine and spermidine via oxidative degradation by certain polyamine oxidases (Weaver & Herbst, 1956; Bachrach 1962a,b; Adachi et al., 1966; Yamada, 1971; Smith, 1976) and spermidine dehydrogenase (Tabor & Kellogg, 1970). The results reported in Table 1.4 show that in cells grown in the presence of 0.5mM-spermine and 1mM-spermidine, the intracellular levels of these oligoamines were maintained at a constant level whilst the level of 1,3-diaminopropane, a possible catabolic product, remained constant. If the levels of these oligoamines were controlled by an increased rate of catabolism to 1,3-diaminopropane, this diamine must in turn be rapidly degraded. North & Murray (1980) grew both D. discoideum and D. mucoroides in association with K. aerogenes on SM agar containing 5mM-spermidine and found that the intracellular levels of 1,3-diaminopropane were significantly higher in these amoebae. They suggested that this may be due to the presence, in both species, of a diaminopropane-yielding spermidine oxidase. They have also preliminary evidence that extracts of D. discoideum can convert spermidine to 1,3-diaminopropane.

If 1,3-diaminopropane is not simply a catabolic product of spermidine, its role in D. discoideum cells is unclear. Amoebae grown in the presence of 5mM-1,3-diaminopropane had an elevated intracellular level of this diamine and a correspondingly reduced level of putrescine (Table 1.4). Despite the low ratio of putrescine to 1,3-diaminopropane in these cells, growth and development were normal. Growth retardation was noted in cells grown in the

presence of 10mM-1,3-diaminopropane but this inhibition was not specific as a similar effect was noted in the presence of 10mM-putrescine. This observation, that intracellular 1,3-diaminopropane concentration can be increased at the expense of intracellular putrescine without any apparent adverse effects, suggested that much of the putrescine in amoebae has no essential function. Alternatively it may be that 1,3-diaminopropane can substitute for putrescine in some of its less specific cellular functions e.g. osmotic protection or membrane stabilization where the cationic nature of the diamine is critical and the length of the aliphatic chain is less important.

Inhibition of growth was observed in cells grown in the presence of high putrescine levels. As the total amino-nitrogen in these cells was 50% higher than in control cells, this may possibly reflect the inhibition of transcription, translation and synthesis reported as resulting from over-stabilization of nucleic acids (Stevens & Winther, 1979). Growth was also retarded in cells grown in the presence of high concentrations of 1,3-diaminopropane and at all the concentrations of spermidine and spermine tested. The onset of growth retardation in these cells seemed to be related to putrescine levels falling to less than 50% of control values (Table 1.4). This may be an indication of the level of putrescine required for specific cellular functions, in which 1,3-diaminopropane cannot substitute. However spermine and spermidine are known to give rise to toxic catabolic products (Bachrach, 1970; Alarcon, 1970; Pulkkinen et al., 1978) which may cause growth inhibition. Although cells grown in the presence of 0.5mM-spermine and 1mM-spermidine contain very much reduced levels of putrescine, levels of spermine, spermidine and 1,3-diaminopropane remained constant (Table 1.4). Intracellular levels of spermine and spermidine may thus be maintained not by increased catabolism but by decreased synthesis. Control may possibly occur via feedback control of putrescine synthesis or increased putrescine catabolism. An increased rate of catabolism of putrescine to carbon dioxide was noted in cells grown in the presence of exogenous spermine and spermidine (Table 1.10).

Putrescine can be synthesized by D. discoideum amoebae from ornithine but not from arginine (Table 1.5). High levels of ornithine decarboxylase activity have been detected in amoebal extracts (see page 138) but arginine decarboxylase activity was not detected (see page 177). Thus unlike bacteria, where putrescine can be derived not only from ornithine but also from arginine via agmatine, in D. discoideum ornithine was the sole precursor of putrescine. Labelled arginine was not incorporated into putrescine, thus production of ornithine via arginase activity was not of significance in oligoamine synthesis in D. discoideum.

In addition to being able to synthesize putrescine from intracellular ornithine, amoebae were also able to take up putrescine from the medium. A study of uptake (see Results and Discussion Chapter 3) showed that amoebae were capable of concentrating putrescine at a rapid rate. Exogenous putrescine would seem to be the preferred source of cellular putrescine since the addition of putrescine to the growth medium depressed the incorporation of labelled ornithine into putrescine within the cell (Table 1.5). This end product inhibition of putrescine synthesis may have involved direct inhibition of existing ornithine decarboxylase activity, decreased synthesis of this enzyme or its increased catabolism. Further evidence for a control over putrescine synthesis was seen in amoebae grown in the presence of exogenous putrescine in which intracellular putrescine levels were maintained (Table 1.4).

Spermidine was synthesized from putrescine in D. discoideum as shown by the incorporation of the labelled diamine into spermidine (Table 1.6). The propylamine moeity, necessary for the conversion of putrescine into spermidine, is derived from S-adenosyl-methionine decarboxylated by S-adenosyl-methionine decarboxylase. This enzyme activity was detected in amoebal extracts (see page 170).

Development of D. discoideum occurs when the exogenous source of nutrients is removed. Consequently, all subsequent activity and macromolecular synthesis are fuelled by endogenous reserves (Loomis, 1975). The protein and RNA levels of amoebae were observed to decrease during development (Figure 1.9) as

reported by White & Sussman (1961). Putrescine, 1,3-diaminopropane and spermidine levels also decreased over the 24h developmental period (Figure 1.9). Since, in the case of spore formation at least, cell volume decreases significantly during development (Ashworth & Watts, 1970; Leach & Ashworth, 1972), the lower oligoamine content need not necessarily represent a decrease in their cellular concentration.

Growing amoebae contained sufficient oligoamine cationic charge to neutralise more than 350% of the nucleic acid phosphate present. During development the total amino-nitrogen present in the cell fell by 85% but over the same time period RNA levels also fell by over 50%, thus the oligoamine charge remained in excess throughout. Thus it would appear unlikely that the alteration in oligoamine levels observed, played a vital role in nucleic acid metabolism during development. However such a role cannot be ruled out entirely in the absence of any detailed information on the subcellular localization of oligoamines.

Although the total protein and RNA content of amoebae decreases during development, the synthesis of both macromolecules continues (Alton & Lodish, 1977; Blumberg & Lodish, 1980, 1981; Mangiarotti et al., 1981). Despite the overall loss of putrescine and spermidine during development some synthesis of both oligoamines continued. Thus in amoebae labelled with radioactive putrescine the specific radioactivity of this diamine decreased during development, indicating dilution by newly synthesized unlabelled putrescine, whilst the specific radioactivity of spermidine increased as a result of synthesis from labelled putrescine (Table 1.7).

Measurement of the oligoamine content of the sorocarp and of separated spore and stalk cells provided evidence that the ratio of spore to stalk cells in the sorocarp was approximately 2:1. The volume of stalk cells is much greater than that of spore cells (Cappuccinelli & Ashworth, 1976), yet stalk cells contained approximately 40% less oligoamine, RNA and protein than spore cells (Table 1.3). Thus the concentration of these substances was much

greater in spore cells, possibly reflecting the vacuolization which occurs in stalk cells rendering them non-viable (Loomis, 1975).

Studies involving germination of spores of strain AX2 derived from axenically grown cells have not previously been reported. Although attempts to heat activate axenic spores by the method of Yagura & Iwabuchi (1976) were unsuccessful, 97% germination was achieved using dimethylsulphoxide (Cotter & O'Connell, 1974). The germination profile (Figure 1.11) was slightly slower than that reported for a similar activation of NC4 spores, possibly due to a difference in the permeability of axenic spore coats (Williams & Walker, 1980). During the germination of AX2 spores the levels of protein, RNA and oligoamines were monitored (Table 1.3). No quantitative change in protein levels was detected and RNA levels decreased by a further 30% during the 5h germination period. The levels of the diamines putrescine and 1,3-diaminopropane increased during germination whilst spermidine levels decreased slightly. The changes observed represented only slight fluctuations and the losses of RNA, protein and oligoamines which had occurred during development were not reversed during germination. Thus in newly emerged amoebae the levels of these substances were less than 25% of those in exponentially growing amoebae. (Table 1.3 and Figure 1.11). This almost certainly reflected the experimental conditions, as germination was carried out in non-nutrient phosphate buffer and macromolecular synthesis may be limited by precursor pool sizes in dormant spores. Protein, RNA and DNA synthesis have been detected during spore germination in D. discoideum although the changes in macromolecular levels were quantitatively very small (Yagura & Iwabuchi, 1976; Giri & Ennis, 1977; 1978).

Excretion of oligoamines or their acetylated derivatives was not observed in D. discoideum amoebae neither during growth nor development. Thus when cells prelabelled with radioactive putrescine were placed in FM medium or on developmental filters, radioactivity was recovered only at low constant levels from growth medium or pad diluting fluid (Table 1.8 and Table 1.9). The efflux

of labelled putrescine or its labelled metabolites was stimulated by the presence of exogenous oligoamines when amoebae were suspended in non-nutrient buffer (Turner et al., 1979) but not when suspended in nutrient HL5 medium (Table 1.8). Mach et al. (1981) could detect neither monoacetylputrescine nor monoacetylspermidine on analysis of D. discoideum extracts by ion exchange chromatography.

Under growth conditions some labelled putrescine was converted to acid insoluble material (Table 1.6 and Table 1.8). This possibly involved metabolism of putrescine to γ-aminobutyrate, either by direct oxidation or via acetylation and subsequent oxidation and deacylation (Seiler & Al-Therib, 1974a,b; Seiler & Eichentopf, 1975; Seiler et al., 1979; Bolkenius & Seiler, 1981). γ - aminobutyrate has been tentatively identified in D. discoideum amoebal extracts (C.McDonald, Honours Dissertation, Stirling University 1978). γ-aminobutyrate may be further metabolised via succinic semialdehyde and the citric acid cycle, to amino acids which would subsequently be incorporated into proteins. This catabolic pathway was of minor importance in growing amoebae as the percentage of radioactivity recovered in the acid insoluble fraction was small. The presence of exogenous oligoamines had no effect on the percentage conversion of labelled putrescine into acid insoluble materials when cells were suspended in HL growth medium (Table 1.8). However, in cells suspended in non-nutrient phosphate buffer containing 1mM-putrescine, the percentage of labelled putrescine catabolised to acid insoluble products increased (Turner et al., 1979). Similarly, under the starvation conditions of development, the incorporation of radioactivity from labelled putrescine into acid insoluble material increased, particularly during the last 14h of development (Figure 1.16). This possibly reflected the increased catabolism of oligoamines and the use of the catabolites in the synthesis of macromolecules. Alternatively the incorporation of label from putrescine into the acid insoluble cell fraction may have involved the formation of an oligoamine-protein conjugate. Such a conjugate may also explain the anomaly in oligo-

amine levels noted in amoebae during the first 2h on developmental filters (Table 1.2). Thus during development of cells prelabelled with putrescine, the initial fall in putrescine levels which was reversed at 3h was mirrored by an increase and subsequent decrease in the labelled acid insoluble cell fraction.

Putrescine was also catabolised to carbon dioxide probably via γ-aminobutyrate, succinic semialdehyde and the citric acid cycle. In growing cells 0.5 p mol [1,4-^{14}C] putrescine/h/10^8 cells was catabolised to carbon dioxide (Table 1.8) representing a turnover of 0.5% of cellular putrescine per hour. During the first ten hours of development, the rate of catabolism of putrescine to carbon dioxide was significantly increased (Figure 1.16). Thus the decrease in putrescine levels noted during development (Figure 1.9), probably reflected the use of this diamine as an endogenous source of energy. Differentiation in D. discoideum is known to result in the catabolism of approximately 50% of the cellular protein (White & Sussman, 1961; Wright & Anderson, 1960). The resultant amino acids are converted to citric acid cycle intermediates and used in energy production (Kelly et al., 1979).

RESULTS AND DISCUSSION - CHAPTER TWO

INTRODUCTION

The principal precursors of the oligoamines are L-ornithine and S-adenosyl-methionine (Figure 1.1). In all cells putrescine can be formed by the decarboxylation of L-ornithine by ornithine decarboxylase [L-ornithine carboxy-lyase; EC 4.1.1.17]. In some cells where considerable arginase [L-arginine amidinohydrolase; EC 3.5.3.1.] activity is detected, hydrolysis of L-arginine to ornithine and urea may be considered the initial step in oligoamine biosynthesis. In some bacteria and higher plants a second pathway exists in which arginine is decarboxylated by arginine decarboxylase [L-arginine carboxy-lyase; EC 4.1.1.19] to agmatine which is subsequently hydrolysed by agmatine ureohydrolase [agmatine amidinohydrolase; EC 3.5.3.11] to putrescine and urea. The synthesis of the higher oligoamines, spermidine and spermine, is catalyzed by spermidine synthetase [S-methyladenosylhomocysteamine: putrescine aminopropyltransferase; EC 2.5.1.16] and spermine synthetase S-methyladenosylhomocysteamine: spermidine aminopropyltransferase]. In these two reactions putrescine and spermidine respectively, serve as the acceptor of an aminopropyl group transferred from decarboxylated S-adenosyl-L-methionine. The latter is formed by decarboxylation of S-adenosyl-L-methionine, a reaction catalyzed by S-adenosyl-methionine decarboxylase (SAM-decarboxylase) [S-adenosyl-L-methionine carboxy-lyase; EC 4.1.1.50].

The two enzymes believed to be involved in the control of oligoamine biosynthesis in encaryotic and most procaryotic cells are ornithine decarboxylase and SAM-decarboxylase.

ORNITHINE DECARBOXYLASE

Ornithine decarboxylase activity has been shown to increase rapidly in

response to a wide variety of growth stimulating factors and decrease in a similar manner when conditions dictate cessation of growth (Cohen, 1971; Bachrach, 1973; Canellakis et al., 1979). Thus, in addition to its importance in elucidating the function and control of oligoamine biosynthesis, its rapid induction and degradation make it an interesting system for the study of factors involved in control of enzyme turnover in cells.

The generally used assay for ornithine decarboxylase involves following the release of $^{14}CO_2$ from [1-^{14}C]ornithine (Pegg & Williams-Ashman, 1968) but in systems contaminated with mitochondrial enzymes it may be subject to error (Murphy & Brosnan, 1976) and it is advisable to monitor putrescine production where accurate levels of enzyme activity are required.

Ornithine decarboxylase has been purified from several sources including the lower encaryote P. polycephalum (Mitchell et al., 1976); transformed 3T3 mouse fibroblasts (Boucek & Lembach, 1977) and rat tissues (Jänne & Williams-Ashman, 1971a; Friedman et al., 1972a; Ono et al., 1972; Heller et al., 1975). Both the biodegradative and biosynthetic enzyme from E. coli have been purified and their properties determined (Applebaum et al., 1977). The molecular weights of the enzymes range from 65,000 for the rat prostate enzyme to 160,000 for one form of the P. polycephalum enzyme and these differences may reflect variations between species or may be due to in vitro aggregations. The enzymes from different sources do however have a number of common characteristics including a slightly alkaline pH optimum and a requirement for pyridoxal phosphate. Studies on the location of ornithine decarboxylase in rat liver (Jänne & Williams-Ashman, 1971a; Ono et al., 1972; Murphy & Brosnan, 1976) and rat prostate (Pegg & Williams-Ashman, 1968) involved preparation of highly purified cellular fractions and in all cases the enzyme activity was largely localized in the cytosol.

In Neurospora over 95% of cellular ornithine is retained in a metabolic inactive pool in vesicles, about 1% is in the cytosol and 3% in the mitochondria (Bowman & Davis, 1977a). A similar compartmentalization of the amino acid in higher encaryotic cells, in conjunction with subcellular localization

of the enzyme, would provide an effective regulatory control of putrescine biosynthesis.

Ornithine decarboxylase activity is stimulated by a diverse spectrum of events: development in a number of animals; compensatory growth after partial hepatectomy; action of androgens, estrogen, gonadotropins and growth hormones in target tissues; exposure of cell cultures to proliferative stimulus e.g. cold shock, fresh medium, concanavalin A and viral transformation of mammalian cells; and neoplastic growth in various animal tumours (Morris & Fillingame, 1974; Jänne et al., 1978). Some of the effects stimulating ornithine decarboxylase are associated with a prolonged increase in growth rate, others with the transient effects of an inducing hormone on a target tissue, whilst others represent recovery from growth limiting conditions. Whilst in some cases the stimulation is specific in others this is not so. Thus Clark (1974) found that the increase in ornithine decarboxylase activity in 3T3 cells brought about by luteinizing hormone is not mirrored by an increase in any other amino acid decarboxylases. In contrast Hershko et al. (1971) noted that during recovery from growth limiting conditions the activities of several amino decarboxylases and tyrosine aminotransferase increased along with ornithine decarboxylase. It has also been noted that enzyme activity is elevated by procedures other than those generally considered as "growth promoting", e.g. sham operations, feeding, cross circulation between animals, infusions with hypertonic sugar solutions and intraperitoneal injections of chemically inert mechanical irritants (Schrock et al., 1970). In such cases the stimulation in ornithine decarboxylase activity can be explained in terms of a general increase in protein synthesis, as any general increase will manifest itself first in enzymes that turn over rapidly. As ornithine decarboxylase has the shortest half life of any mammalian enzyme so far studied, stimulation of protein synthesis would be expected to result in immediate and dramatic increases in the activity of this enzyme. This may also explain the stimulation of ornithine decarboxylase in cultured cells by addition of fresh medium (Hogan, 1971).

Guanine nucleotides are known to modulate the activity of ornithine decarboxylase in the procaryotic E. coli system. Hölttä et al. (1974) have shown that GTP increases the substrate affinity of the enzyme by a factor of ten and Applebaum et al. (1977) showed that both GTP and GMP activated the biosynthetic ornithine decarboxylase. In eucaryotes control through low molecular weight effectors does not seem to be of physiological importance (Jänne & Williams-Ashman, 1971a).

Fluctuations in ornithine decarboxylase activity have been shown using immunochemical techniques to correlate with the number of enzyme molecules (Hölttä, 1975; Canellakis & Theoharides, 1976; Obenrader & Prouty, 1977b). Inhibitor studies have indicated that protein synthesis is necessary if the elevation of ornithine decarboxylase activity resulting from growth stimulation is to occur. In addition experiments with actinomycin D and α-amanitin suggest that increases in ornithine decarboxylase are at least partially dependent on mRNA synthesis. (Snyder et al., 1970; Clark, 1974; Kallio et al., 1977a). Hogan et al. (1974) found that actinomycin D did not abolish the stimulation of ornithine decarboxylase normally occuring 1-2h after dilution of heptoma cells, but it did abolish the second peak of stimulation occuring at 2-4h. Thus it is possible that the increase in enzyme activity is not the result of an increase in mRNA but rather an increase in translation of a mRNA which turns over rapidly.

There is as yet no evidence supporting the stabilization of existing mRNA as the mechanism of stimulation of ornithine decarboxylase activity. Several groups however have investigated the stabilization of the enzyme itself as the possible mechanism of stimulation of enzyme activity. The methodology of these studies is measurement of activity after treatment with translation inhibitors, e.g. cycloheximide or puromycin. Care must be taken in the interpretation of such experiments as such inhibitors have been shown to affect the rate of degradation of specific enzymes (Kenney, 1967). Using such inhibitors Russell & Snyder (1969) found that the half life of ornithine decarboxylase in hepatect-

omized rats is 10-11 min whilst in rats treated with growth hormone it is approximately 24 min (Russell et al., 1970) In livers of thioacetamide treated rats the half life is substantially longer than in normal rat liver (Obenrader & Prouty, 1977b; Pösö et al., 1978). Similarly Hogan & Murden (1974) observed that the half life of ornithine decarboxylase increases from 5 to 90 min when stationary hepatoma cells are diluted into fresh medium. Prouty (1976) also found variations in the half life of the enzyme in HeLa cells stimulated by glutamine treatment. In 3T3 cells however, an inhibition of growth and concomitant decrease in the half life of ornithine decarboxylase occurs at high cell densities yet a similar decrease in half life occurs when ornithine decarboxylase is induced in the same cells by luteinizing hormone (Clark, 1974). This opposite correlation suggests that in this system at least stabilization of the enzyme does not parallel its activity and thus control is not simply by stabilization/destabilization of the protein.

On the basis of ornithine decarboxylase instability it had been assumed that the rapid fluctuations in enzyme activity are caused by perturbations of a balance between enzyme synthesis and enzyme degradation. Thus investigations have been concerned with the regulation of ornithine decarboxylase via the control of synthesis at the transcriptional or translational level or conversely by controlling degradation through activation of a specific inactivating enzyme or a protein modification rendering the enzyme more susceptible to nonspecific proteases.

A number of studies have indicated that the activity of ornithine decarboxylase is diminished when cultured cells or animal tissues in vivo are exposed to the normal physiological oligoamines (Clark & Fuller, 1975; Fong et al., 1976; Heller et al., 1978; Pegg et al., 1978; Mitchell et al., 1978; Bethell & Pegg, 1979). The administration of 1,3-diaminopropane, not normally present in animal cells, also decreases ornithine decarboxylase activity in various tissues (Pösö et al., 1977, 1978; Sunkara et al., 1977; Pik et al., 1977; Kallio et al., 1979; Heller et al., 1978; Pegg et al., 1978; Persson & Rosengren, 1979; Branca & Herbst, 1980). The decrease in ornithine decarboxylase activity in response to

oligoamines may be an important mechanism for the regulation of oligoamine biosynthesis and the mechanisms by which they suppress the enzyme activity remains an active area of research. Although early evidence suggested that the action of amines on ornithine decarboxylase activity could involve transcriptional control elements (Jänne & Hölttä, 1974; Kallio et al., 1977a) similarities between the action of amines and cycloheximide on the level of the enzyme suggests regulation at some post-transcriptional level of gene expression (Jänne & Höltta, 1974; Clark & Fuller, 1975; Kallio et al., 1977a). Thus putrescine may have a direct or indirect effect on enzyme activity or alter the degradation or synthesis rates of the enzyme. As putrescine is a weak competitive inhibitor of ornithine decarboxylase in most systems, it is unlikely that the low exogenous concentrations of putrescine known to inhibit the enzyme in vivo, directly inhibit intracellular ornithine decarboxylase (McCann et al., 1977). As addition of putrescine and cycloheximide together did not decrease the half life of the enzyme in cells any further than cycloheximide alone, it is unlikely that putrescine has an effect on enzyme degradation (McCann et al., 1977).

The extreme sensitivity and speed of the regulation of ornithine decarboxylase activity by amines, hormones, growth conditions etc. suggests that the control of the enzyme is post-translational. High concentrations of physiological and synthetic diamines lead to the appearance of a heat labile, macromolecular inhibitor of ornithine decarboxylase that has been referred to as ornithine decarboxylase-antizyme (Heller et al., 1976, 1977; Fong et al., 1976; Friedman et al., 1977b; Jefferson & Pegg, 1977; McCann et al., 1977; Pegg et al., 1978; Branca & Herbst, 1980). In addition Kyriakidis et al., (1978) have extracted a non- dialyzable factor that activates E. coli ornithine decarboxylase. The induction of this inhibitor is dependent on protein synthesis and it disappears rapidly on addition of cycloheximide (Fong et al., 1976; Friedman et al., 1977b). The enzyme-antizyme complex is visualized as a means of inactivating the enzyme, either resulting in its more rapid degradation, or

in the creation of a store of enzyme ready for rapid reactivation if required by the cell. Canellakis et al. (1978) have extended this antizyme hypothesis to explain the regulation of oligoamine levels in different cell systems. They postulate the existence of membrane receptors sensitive to low levels of extracellular oligoamines. When intracellular levels of oligoamines are high, Canellakis et al. (1978) propose that they diffuse out of the cells, bind to the oligoamine membrane receptors and cause production of antizyme which in turn inactivates ornithine decarboxylase present in the cell. In this way extracellular concentrations of oligoamines, too low to cause significant changes in the intracellular oligoamine concentrations, could regulate ornithine decarboxylase activity in the cell. Kallio et al. (1977b) were unable to obtain evidence for the formation of macromolecular inhibitors of ornithine decarboxylase in regenerating rat liver after treatment with diamines. They observed that in this system any decrease in enzyme activity is accompanied by a similar decrease in the amount of immunochemical enzyme protein and so suggest that the primary effect of amines on ornithine decarboxylase activity in regenerating rat liver is direct inhibition of enzyme synthesis (Kallio et al., 1977b). The sensitivity of ornithine decarboxylase activity to minute variations in extracellular oligoamines in the absence of significant amounts of antizyme is explained by postulating the existence of two regulatory mechanisms for the control of ornithine decarboxylase levels (Heller et al., 1978; Bethell & Pegg 1979; McCann et al., 1979). Thus at low levels of extracellular oligoamines, ornithine decarboxylase activity may be inhibited via membrane mediated sites. This may involve antizyme production or a decline in the synthesis of the enzyme. As the level of extracellular oligoamines is increased raising the intracellular oligoamine concentration, a second site, either intracellular or membrane associated, is triggered and antizyme production occurs.

An alternative method of post-translational control of ornithine decarboxylase activity has been proposed by Mitchell and co-workers. They report the existence in P. polycephalum of two physically and kinetically distinct

forms of ornithine decarboxylase (Mitchell & Carter, 1977; Sedory & Mitchell, 1977; Mitchell et al., 1978) and multiple forms of the enzyme have subsequently been found in other systems. (Clark & Fuller, 1976; Obenrader & Prouty, 1977a). Mitchell & Sedory (1974) studied the effect of inhibition of protein synthesis in P. polycephalum on two interconvertible forms of ornithine decarboxylase activity both with the same Km for ornithine but with differing Km's for the cofactor pyridoxal phosphate (PLP). They later provided evidence that the two activities are in fact two distinct states of the enzyme ornithine decarboxylase, both catalyzing the synthesis of putrescine (Mitchell et al., 1976). Thus in this organism ornithine decarboxylase exists in either an active (A form) state, which has a high affinity for PLP or a less active (B form) state which is fully activated only by unphysiologically high levels of this coenzyme. Further studies on this system revealed that although total enzyme activity, as measured in the presence of saturating cofactor levels is changed only slightly by factors such as metabolic inhibitors (Mitchell et al., 1976), ionic strength (Mitchell & Kottas, 1979), extracellular oligoamines (Mitchell et al., 1978) or progression through the cell cycle (Sedory & Mitchell, 1977) these changes stimulate a rapid change in the relative proportions of the two enzyme forms. Two separable ornithine decarboxylase forms have been found in rat liver where the two forms differ in their affinity for the substrate (Obenrader & Prouty, 1977a) and in 3T3 cells where they differ in their affinity for the cofactor (Clark & Fuller, 1976). There are however indications that the enzyme and its regulation in P. polycephalum may differ from that in the 3T3 cells, where inhibition of protein synthesis does not result in interconversion of the two forms (Clark & Fuller, 1976). Also the amounts of high Km enzyme extracted from mammalian systems are not large enough to account for the increase in activity which occurs when these systems are stimulated (Clark & Fuller, 1976). This may however merely reflect the instability of this form of the enzyme on extraction. It is interesting to note the recent report by Mitchell etal. (1981) that the interconversion of the two enzyme forms occurred in crude cell lysates in the presence of spermidine or spermine under the influence

of a protein factor resembling the ornithine decarboxylase antizyme.

Studies on cells in culture and an *in vivo* study on rat liver suggest that there is a relationship between increases in cellular cAMP levels and ornithine decarboxylase induction (Byus & Russell, 1975; Bachrach, 1975; Oka & Perry, 1976). When cultured cells are treated with cAMP, cAMP derivatives or compounds known to increase cAMP levels, an increase in ornithine decarboxylase activity occurs (Bachrach, 1975; Russell & Stambrook, 1975; Oka & Perry, 1976). These results, in conjunction with those reporting the increase in ornithine decarboxylase activity caused by both steroidal and protein hormones in their respective target tissues (Bachrach, 1973; Jänne et al., 1978), suggest that cAMP might mediate the hormonal induction of ornithine decarboxylase, and that elevation of this enzyme and the resulting increase in oligoamine levels, may mediate the hormone action on the cell. Changes in ornithine decarboxylase activity during the cell cycle of synchronized baby hamster kidney cells are preceded by increases in the cAMP levels, suggesting that regulation of the enzyme activity by cellular cAMP levels may be a general phenomenon (Hibasami et al., 1977). However such a temporal relationship does not necessarily establish causality. Although elevation of ornithine decarboxylase in the adrenal medulla of the rat is preceded by elevation of cAMP, the latter elevation is rapid, reaching a maximum in 30-60 min, whereas ornithine decarboxylase activity is maximal between 2-6h by which time cAMP has returned to basal levels (Byus & Russell, 1976). This observation combined with a lack of correlation between the degree of change in cAMP levels and the increase in ornithine decarboxylase activity in this system led Byus & Russell (1976) to propose that the increase in ornithine decarboxylase activity is more closely related to the activity of cAMP-dependent protein kinases than to the actual intracellular fluctuation of cAMP. Thus in systems where the action of the stimulus has been shown to produce barely detectable increases in cAMP concentration (Russell & Snyder, 1969; Byus et al., 1976), they propose that a close correlation exists betwen the degree of activation of cAMP-depen-

dent protein kinase and that of ornithine decarboxylase (Byus & Russell, 1976). The intermediate steps between the stimulation of cAMP-dependent protein kinase and the increase in ornithine decarboxylase are still unclear. It appears that the activation of ornithine decarboxylase is not regulated by direct phosphorylation catalyzed by cAMP-dependent protein kinase, as experiments with actinomycin D and cycloheximide implicate a transcriptional mechanism (Byus & Russell, 1976; MacDonnell et al., 1977). Regulation of gene activity via phosphorylation of non-histone nuclear protein by cAMP-dependent protein kinase is one possible mechanism whereby cAMP might control ornithine decarboxylase synthesis at a transcriptional level. An additional modulation of this system is the observation that oligoamines inhibit the activity of protein kinases in several systems (Takai et al., 1976; Bachrach et al., 1978). Thus the regulation of ornithine decarboxylase by cyclic nucleotides represents a further aspect of an already complex regulatory scheme, the final solution of which will require further work and technical development.

SAM-DECARBOXYLASE

SAM-decarboxylase catalyzes the decarboxylation of S-adenosyl-L-methionine yielding S-methyladenosylhomocysteamine which in turn donates its propylamine moeity for the biosynthesis of both spermidine and spermine. Although factors causing stimulation of ornithine decarboxylase activity generally result in an increased SAM-decarboxylase activity e.g. partial hepatectomy of rat liver (Hannonen et al., 1972a,b) the inducibility of the latter enzyme is much less pronounced than that of the former (Tabor & Tabor, 1976a).

The enzyme has been extensively purified from E. coli (Wickner et al., 1970); rat liver (Feldman et al., 1971; Hannonen et al., 1972b; Pegg, 1974, 1977; Demetriou et al., 1978); human tissues (Zappia et al., 1972; Porta et al., 1977) mouse mammary gland (Oka et al., 1978) and S. cerevisiae (Pösö et al., 1975a; Cohn et al., 1977). This purification of SAM-decarboxylase from several sources established that, contrary to earlier reports (Feldman et al.,

1972; Manen & Russell, 1974), the decarboxylation of S-adenosyl-L-methionine and the transfer of the propylamine group to putrescine are catalyzed by different enzymes (Jänne et al., 1971a, Hannonen et al., 1972a, Pegg, 1974).

The procaryotic and eucaryotic enzymes differ considerably, in that the enzymes from the latter source are intensively and specifically stimulated by putrescine even at low concentrations whereas the bacterial enzyme is totally insensitive to putrescine but instead requires Mg^{2+} for maximum activity (Pösö et al., 1975b). SAM-decarboxylase has been found to be putrescine activated in the livers of reptiles, amphibia, birds, crustacea and mammals (Coppoc et al., 1971). Similarly the fungal enzyme is putrescine activated (Coppoc et al., 1971; Stevens et al., 1976; Hart et al., 1978), in contrast to the enzymes isolated from E. coli and Azotobacter vinelandii which have an absolute requirement for divalent cations such as Mg^{2+} (Wickner et al, 1970; Coppoc et al., 1971). This division of SAM-decarboxylase, into putrescine-insensitive procaryotic enzyme and putrescine-activated eucaryotic enzyme, is not clear cut as later reports have shown. Thus SAM-decarboxylase isolated from Lathyrus sativus (Suresh & Adigo, 1977) and from mung bean sprouts (Coppoc et al., 1971) was putrescine insensitive but enhanced by Mg^{2+} whilst the enzyme from P. polycephalum (Mitchell & Rusch, 1973) and T. pyriformis (Pösö et al., 1975b) is stimulated by neither putrescine nor by Mg^{2+}. It has been suggested that the activation of SAM-decarboxylase by putrescine in higher eucaryotes is an evolutionary change associated with the appearance of a specific spermine synthetase, as the presence of a putrescine activated SAM-decarboxylase correlates with the presence in the cell of spermine (Pösö et al., 1976b).

SAM-decarboxylase from both eucaryotic and procaryotic systems are subject to product inhibition by S-methyladenosylhomocysteamine (Pösö et al., 1976a), but differ in their sensitivity to the inhibitor methylglyoxal bis (amidinohydrazone) [MGBA]. Williams-Ashman & Schenone (1972) report that this compound is a specific, reversible, competitive inhibitor of putrescine-activated SAM-decarboxylase from rat ventral prostate and S. cerevisiae whereas relatively

high concentrations are needed for a similar inhibition of the putrescine-insensitive E. coli enzyme. Diamine oxidase from rat liver is also extremely sensitive to inhibition by MGBA (Höltta et al., 1973b), which is further evidence that the mechanism of inhibition involves interaction with the putrescine binding site, especially as the inhibitor diminishes the degree of putrescine stimulation of the enzyme. MGBA also inhibits the putrescine-insensitive enzyme from P. polycephalum (Mitchell & Rusch, 1973) and T. pyriformis (Pösö et al., 1975b) although the latter is less sensitive than the higher eucaryotic enzyme. Interaction with MGBA stabilises SAM-decarboxylase against degradation by proteolytic enzymes and results in an increase in the total amount of enzyme in the cell (Fillingame & Morris, 1973b; Pegg et al., 1973).

Inhibition studies with hydroxylamine phenylhydrazine, semicarbazide and borohydride indicate that both the procaryotic and eucaryotic SAM-decarboxylase contain a carbonyl function. Although evidence for a pyruvoyl residue in the E. coli enzyme was reported by Wickner et al. (1970), attempts to characterize the cofactor in the eucaryotic enzyme were for many years inconclusive. Initial reports that pyridoxal was present or involved in the reaction were based on the inhibition of SAM-decarboxylase from rat ventral prostate, but not of the pyruvate-requiring bacterial enzyme, by a potent inhibitor of PLP-requiring enzymes (Pegg & Williams-Ashman, 1969; Tabor & Tabor, 1972). Subsequently it was reported that the rat liver enzyme could be stimulated by addition of PLP (Feldman et al., 1972; Sturman & Kremzner, 1974), but these results were not confirmed by other groups (Williams-Ashman et al., 1972; Pegg, 1974; Hannonen, 1976). Pegg (1977a) examined SAM-decarboxylase from rats fed on a Vitamin B6-deficient diet and found no stimulation of activity by addition of PLP. Further studies using purified enzyme and acid hydrolysates of the enzyme have confirmed the presence of covalently bound pyruvate in the eucaryotic enzyme as well as the procaryotic enzyme (Cohn et al., 1977; Pegg, 1977b; Demetriou et al., 1978). Suresh & Adiga (1977) isolated an artifactual SAM-decarboxylating activity from L. sativus seedlings which is non-

enzymic and thought to involve hydrogen peroxide. A similar non-enzymic decarboxylation may explain the anomalous PLP-activation of rat liver SAM-decarboxylase (Feldman et al., 1972; Sturman & Kremzner, 1974), as spontaneous decarboxylation of S-adenosyl-L-methionine has been observed to occur in the presence of PLP (Coppoc et al., 1971).

In the rat brain and ventral prostate SAM-decarboxylase activity is found largely in the supernatant fraction (Pegg & Williams-Ashman, 1969; Schmidt & Cantoni, 1973). Sturman (1976) whilst confirming this subcellular location of SAM-decarboxylation in several rat tissues and the liver homogenates of several vertebrates, observes that in some preparations the enzyme activity is increased by the inclusion of Triton X-100 in the homogenizing buffer. This detergent-released activity is localized in the crude nuclear fraction and as little or no activity is found associated with purified nuclei, it is suggested that this activity is associated with membrane fragments Further investigation suggests that this membrane-associated SAM-decarboxylase activity is not associated with spermidine synthesis (Sturman, 1976). Symonds and Brosnan (1977) however carried out a systematic subcellular fractionation of rat liver in the presence and absence of Triton X-100 and report that putrescine-dependent SAM-decarboxylase is exclusively located in the cytosol of rat liver cells. Thus SAM-decarboxylase activity would be subject to control by fluctuations in the concentrations of substrate, cofactor, activator and inhibitor substances occurring in this cell compartment.

The half life of SAM-decarboxylase, although longer than that of ornithine decarboxylase, is still much shorter than that of most other enzymes and this suggests that it too may have a regulatory role in oligoamine synthesis (Hannonen et al., 1972b; Mitchell & Rusch, 1973; Pegg et al., 1973; Grillo et al., 1978).

In view of the position occupied by both ornithine decarboxylase and SAM-decarboxylase as key enzymes in oligoamine biosynthesis the two enzymes were extracted from D. discoideum amoebae and a preliminary investigation of their properties was made.

SECTION A:- Optimization of extraction and storage of ornithine decarboxylase activity from D. discoideum

Difficulties were experienced in preliminary attempts to determine the level of ornithine decarboxylase activity in D. discoideum as large fluctuations in the activity of the enzyme were observed. For this reason a series of experiments were carried out in an attempt to stabilize the ornithine decarboxylase activity recovered in cell extracts.

PREPARATION AND STORAGE OF ENZYME EXTRACTS

Three amoebal preparations were obtained by suspending cells in extraction buffer for 15 min, by sonicating a suspension of cells in extraction buffer and by sonicating and subsequently centrifuging a suspension of cells in extraction buffer. The levels of ornithine decarboxylase activity in these preparations were determined immediately. Aliquots of each preparation were then stored under a variety of conditions (Table 2.1) and the level of enzyme activity in these determined after 24h.

The highest level of ornithine decarboxylase activity was obtained from the sample in which amoebae had been suspended in extraction buffer for 15 min prior to the assay (Table 2.1). The release of considerable enzyme activity from these non-sonicated cells suggests that a number of amoebae were lysed either by the extraction or assay buffer. The level of ornithine decarboxylase activity in cell extracts which had been sonicated was 40% less than those in unsonicated preparations (Table 2.1), an indication that the enzyme was unstable in vitro. The instability of ornithine decarboxylase both in vivo and in vitro has been reported from several sources (Russell & Snyder, 1969; Jänne & Williams-Ashman, 1971a; Clark, 1974; Boucek & Lembach, 1977). If sonication was followed by centrifugation at 1000g for 5 min, the loss of ornithine decarboxylase activity was reduced (Table 2.1), reflecting a slight purification of the enzyme extract effected by removal of cell debri.

TABLE 2:1 PREPARATION AND STORAGE OF ORNITHINE DECARBOXYLASE ACTIVITY IN CELL EXTRACTS

Amoebae in exponential growth were harvested (see methods section) and resuspended at a cell density of 10^8 cells/ml in extraction buffer (5mM-phosphate buffer, pH7.6, 2mM-DTT, 1mM-$MgCl_2$, 0.1mM-EDTA, 0.1mM-PLP). A portion of this cell suspension was sonicated (as detailed in the methods section) and some of this sonicated extract centrifuged at 1000g for 5 min. The level of ornithine decarboxylase activity in these three preparations (unsonicated, sonicated and sonicated and centrifuged) was determined immediately (as detailed in the methods section). Portions of each preparation were then stored at room temperature, 4°C, -18°C or dialysed at 4°C against two 100ml portions of extraction buffer and the level of ornithine decarboxylase activity in each determined after 24h. The levels of enzyme activity are reported as percentages of that obtained in the fresh, unsonicated preparation (381 pmol CO_2/min/mg protein = 100%)

STORAGE CONDITIONS	% ORNITHINE DECARBOXYLASE ACTIVITY		
	UNSONICATED PREPARATION	SONICATED PREPARATION	SONICATED AND CENTRIFUGED PREPARATION
CONTROL	100	57	73
ROOM TEMPERATURE	14	5	8
4°C	22	11	16
-18°C	30	22	32
DIALYSED AGAINST EXTRACTION BUFFER AT 4°C	16	15	10

As had been previously observed, considerable losses in ornithine decarboxylase activity occurred on storage of the enzyme extract. The enzyme was more than simply cold labile, as storage of all three extracts at room temperature for 24h resulted in loss of approximately 90% of the activity present in fresh extracts (Table 2.1). Storage at 4°C resulted in retention of 20% of the original enzyme activity in all three extracts (Table 2.1). Dialysis against extraction buffer at 4°C, failed to substantially protect the ornithine decarboxylase activity remaining after 24h storage. Thus it seemed unlikely that the loss of activity was due to the presence of any diffusible material e.g. unchelated metal ions or small inhibitory molecules. The loss of ornithine decarboxylase activity was minimised by storage of the extract at -18°C, the greatest retention occurring in the sonicated and centrifuged cell extract. The results in Table 2.1 show that cell extracts prepared by sonication of cells in extraction buffer followed by centrifugation at 1000g for 5 min, retained 40% of their ornithine decarboxylase activity after 24h storage at -18°C.

STABILIZATION OF ENZYME ACTIVITY IN VITRO

The loss of ornithine decarboxylase activity was considered unacceptable and in an attempt to stabilize the enzyme activity amoebae were suspended in a variety of extraction buffers as detailed in Table 2.2. Aliquots of these cell suspensions were immediately sonicated, centrifuged and the levels of ornithine decarboxylase activity determined. These extracts, along with the corresponding unsonicated preparations, were stored at -18°C and the levels of enzyme activity in both determined after 24h.

Over 80% of ornithine decarboxylase activity was lost when cells in standard extraction buffer were stored at -18°C for 24h (Table 2.2). As in the previous experiment, in standard extraction buffer the retention of enzyme activity in the sonicated and centrifuged extract was slightly greater than in the unsonicated sample. Phenylmethylsulphonyl fluoride (PMSF), a serine proteinase inhibitor (Gold, 1967), and n-α-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK), an inhibitor of some serine and most thiol proteinases (Shaw et al.,

TABLE 2:2 STABILITY OF ORNITHINE DECARBOXYLASE ACTIVITY IN MODIFIED EXTRACTION BUFFERS

Exponentially growing amoebae were harvested and suspended in the various extraction buffers at an approximate cell density of 10^8 cells/ml. An aliquot of each cell suspension was sonicated and centrifuged (as detailed in the methods section) and the level of ornithine decarboxylase activity in the resulting cell extracts determined immediately (see methods section). The sonicated extracts and the corresponding unsonicated preparations were stored at -18°C and the levels of enzyme activity in both determined after 24h. The levels of enzyme activity are reported as percentages of that obtained in the extract prepared in standard extraction buffer and assayed immediately (798 pmol CO_2/min/mg protein = 100%).

ADDITIONS TO STANDARD EXTRACTION BUFFER	% ORNITHINE DECARBOXYLASE ACTIVITY		
	SONICATED EXTRACT ASSAYED IMMEDIATELY	SONICATED EXTRACT ASSAYED AFTER 24h	UNSONICATED EXTRACT ASSAYED AFTER 24h
NONE	100	19	17
PMSF (2.5mM)	114	16	22
TLCK (0.1mM)	96	17	18
BOVINE SERUM ALBUMIN (1mg/ml)	60	36	12
SUCROSE (2.5mM)	51	38	16
GLYCEROL			
5% (v/v)	62	53	29
10% (v/v)	79	81	54
20% (v/v)	74	67	51
30% (v/v)	68	74	52
DIMETHYLSULPHOXIDE			
5% (v/v)	80	25	14
10% (v/v)	68	58	32
20% (v/v)	64	52	35
30% (v/v)	70	59	43

1965), were included in extraction buffers. Neither substance increased the amount of ornithine decarboxylase activity remaining in either sonicated or unsonicated samples (Table 2.2). If loss of activity is due to proteolysis, the proteinase involved is insensitive to these inhibitors. Such proteinases are known to be present in D. discoideum but may not operate at pH 7.6 (North & Harwood, 1979). Some enzyme activities are stabilized by raising the osmolality of the extraction buffer, and ornithine decarboxylase from several sources has been reported to be sensitive to changes in osmolality (Munro et al., 1975; Friedman et al., 1977a; Mitchell & Kottas, 1979; Perry & Oka, 1980). The osmolality of the extraction buffer was increased by the addition of bovine serum albumin, sucrose, glycerol and dimethylsulphoxide. The level of ornithine decarboxylase activity measured in fresh extracts containing any of these substances was between 20% and 40% lower than that measured in standard extraction buffer. However the retention of enzyme activity on storage was significantly improved in these high osmolality extraction buffers. This stabilization of enzyme activity was greater in sonicated extracts, most probably as release of the enzyme from the cell allowed it to associate more closely with the stabilizing molecules in the extraction buffer. In sonicated samples after 24h storage, the presence of 0.25M-sucrose in the extraction buffer increased the level of ornithine decarboxylase activity remaining to over 70% that of the fresh extract, slightly greater than the 60% retention noted in the presence of 1mg/ml-bovine serum albumin. Dimethylsulphoxide also improved the stability of ornithine decarboxylase activity in vitro: concentrations of 10% (v/v) or higher in the extraction buffer resulted in retention of over 80% of the enzyme activity recorded in fresh samples. Dimethylsulphoxide is known to have protein denaturing effects (Henderson et al., 1975); thus the stabilization of enzyme activity may be complex, involving more than the increased osmolarity of the extraction buffer containing dimethylsulphoxide. Glycerol added to the extraction buffer had the greatest stabilizing effect. At concentrations of 10% (v/v) or more, approximately 100% of ornithine decarboxylase activity was ret-

ained after storage for 24h at -18°C. As glycerol also increased the viscosity of the extraction buffer, accurate volume additions to the assay mixture were difficult. So it was decided to include 10% (v/v) glycerol in the standard extraction buffer thus maximising the stabilization of ornithine decarboxylase activity whilst minimising the problems associated with the increased viscosity of a glycerol-containing extraction buffer.

EFFECT OF PROTEIN CONCENTRATION IN ENZYME EXTRACT

Although the presence of 10% (v/v) glycerol in the extraction buffer increased the stability of stored ornithine decarboxylase activity, large fluctuations were still observed in the enzyme activity extracted from D. discoideum amoebae. As these appeared to be related to the protein concentration of the extracts used, this feature of the ornithine decarboxylase assay was investigated as detailed in the legend to Figure 2.1.

The level of enzyme activity obtained at low concentrations of protein were approximately four times greater than those observed in previous experiments (Figure 2.1). However as Figure 2.1 shows, the specific activity of ornithine decarboxylase recorded was constant only at these low concentrations of protein; less than 0.16mg protein per assay. At higher concentrations of protein the specific activity of ornithine decarboxylase was inversely related to the concentration of protein used in the assay (Figure 2.1). This phenomenon may have been the result of either polymerisation of the enzyme or interconversion of multiple forms of ornithine decarboxylase in concentrated cell extracts.

To determine whether the loss of ornithine decarboxylase activity in assays containing high concentrations of protein, was merely an artifact resulting from the assay system used or reflected a change in the properties of the enzyme the dilution phenomenon was further investigated as detailed in the legend to Table 2.3.

As in the previous experiment, the ornithine decarboxylase specific acti-

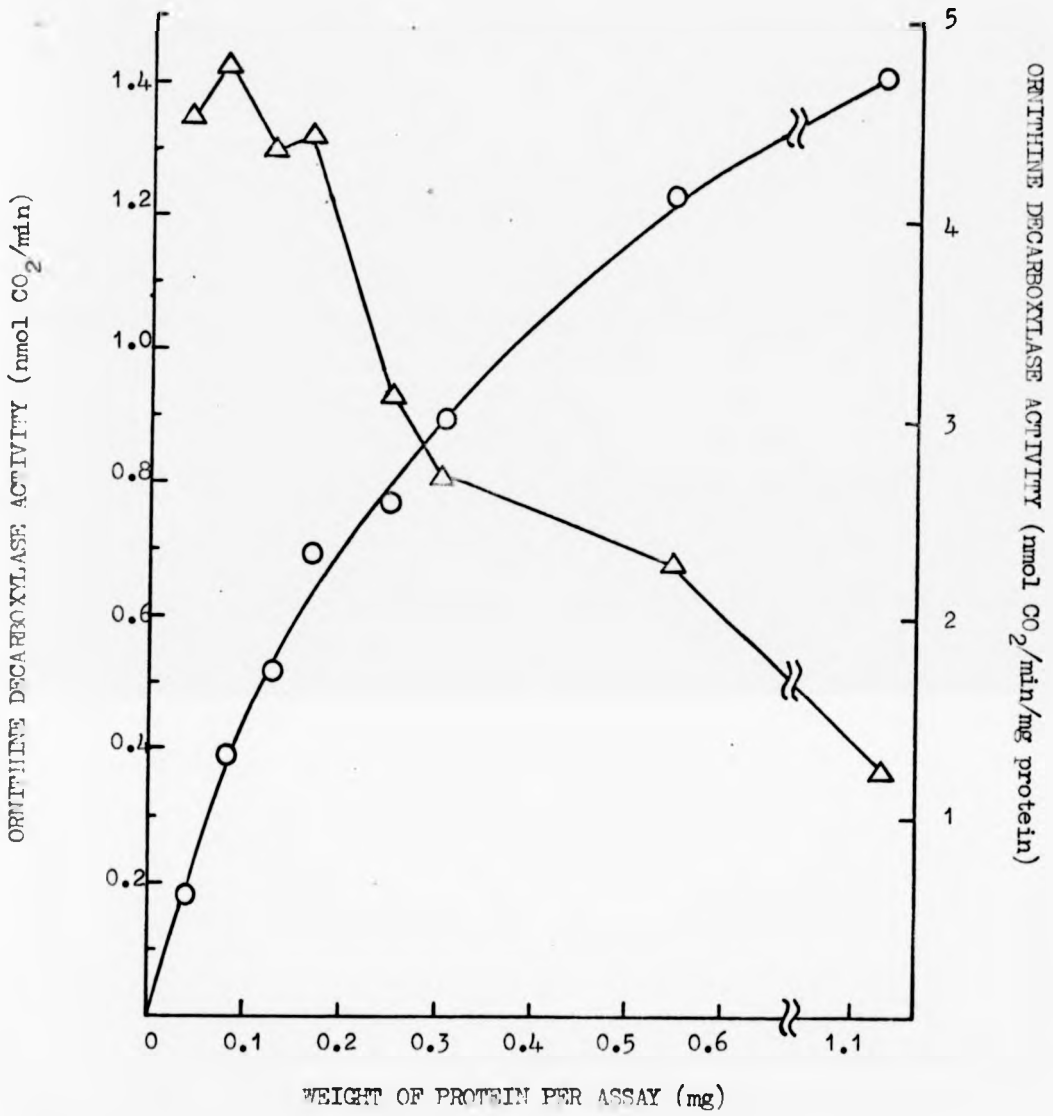


FIGURE 2.1 ORNITHINE DECARBOXYLASE ACTIVITY AS A FUNCTION OF PROTEIN CONCENTRATION

Amoebae were harvested and suspended in 10% (v/v) glycerol extraction buffer at a density of approximately 10^6 cells/ml. After sonication and centrifugation the cell extract was diluted with the extraction buffer to give a range of protein concentrations. These extracts were assayed for ornithine decarboxylase activity as previously. Each assay was carried out in duplicate and the values shown represent the average of two experiments where O= enzyme activity in nmol CO_2/min and Δ= enzyme activity in nmol CO_2/min/mg protein.

vity measured was constant only in assays containing low concentrations of protein (0.15mg protein/assay), whilst at higher protein concentrations the specific activity decreased sharply (Table 2.3). When diluted extracts were stored for 24h at -18°C the retention of enzyme activity varied. In extracts containing low levels of protein 90% of ornithine decarboxylase activity was retained, but as the concentration of protein in the extract increased so the enzyme activity remaining after 24h decreased. In enzyme assays containing 0.5mg protein/assay only 40% of the enzyme activity determined in fresh extracts remained after 24h storage (Table 2.3). The most dramatic loss of activity, over 95%; occurred when the concentrated enzyme extract was stored and diluted just prior to the enzyme assay (Table 2.3). These results suggested that the fluctuations in ornithine decarboxylase activity *in vitro* resulted from changes in the enzyme molecule such as polymerisation or interconversion of multiple forms.

The two forms of ornithine decarboxylase detected in *P. polycephalum* by Mitchell et al. (1976), differ in their ability to bind PLP. Both forms of the enzyme were active only at unphysiologically high coenzyme levels (200μM-PLP). Mitchell et al. (1976) concluded that fluctuations in enzyme activity noted in extracts assayed at intermediate coenzyme concentrations were due to interconversions between the two forms. Although assays of *D. discoideum* ornithine decarboxylase activity were carried out at much higher concentrations of PLP (0.31mM), it was possible that in concentrated extracts a high Km form of the enzyme was not fully activated resulting in fluctuations in activity. Similarly ornithine decarboxylase activity would be depressed at high protein concentrations if the level of ornithine in the assay became limiting. Thus in extracts containing a range of protein concentrations the specific activity of ornithine decarboxylase was measured at both normal and elevated ornithine and PLP levels.

As before, using the standard assay mix the specific activity of ornithine decarboxylase was constant only when extracts containing low levels of protein

TABLE 2:3 EFFECT OF DILUTION AND STORAGE ON ORNITHINE DECARBOXYLASE ACTIVITY

A concentrated amoebal extract (approx. $5x10^8$ cells/ml) was prepared in 10% (v/v) glycerol extraction buffer (see methods section). An aliquot of this extract was diluted with 10% (v/v) glycerol extraction buffer to give the range of protein concentrations shown. The ornithine decarboxylase activity in these diluted extracts was assayed immediately. The diluted extracts and the remainder of the concentrated extract were stored at -18°C and 24h later the latter extract was diluted as the former extract had been on the previous day. The ornithine decarboxylase activity of the "stored and diluted" and "diluted and stored" extracts was then determined.

WEIGHT OF PROTEIN PER ASSAY (mg)	ORNITHINE DECARBOXYLASE ACTIVITY IN:- (nmol CO_2/min/mg protein)		
	DILUTED AND ASSAYED IMMEDIATELY	DILUTED EXTRACTS STORED FOR 24h AND THEN ASSAYED	CONCENTRATED EXTRACT STORED FOR 24h, DILUTED AND THEN ASSAYED
0.046	4.20	3.68	0.02
0.107	3.72	4.03	0.06
0.157	3.83	3.45	0.06
0.207	2.92	2.28	0.05
0.252	2.59	1.64	0.11
0.525	1.59	0.64	0.05

were used in the assay; at high concentrations the specific activity of the enzyme was reduced (Table 2.4). Increasing the concentration of coenzyme from 0.31mM to 1mM had little effect on the level of ornithine decarboxylase activity measured either at normal concentrations of ornithine (0.5mM) or elevated concentrations (50mM) (Table 2.4). As elevated levels of PLP failed to increase the level of enzyme activity in concentrated enzyme extracts, the lowering of activity observed in these samples would not seem to be the result of limiting coenzyme levels.

A significant increase in the level of ornithine decarboxylase activity in all extracts was noted on increasing the concentration of ornithine in the incubation mixture from 0.5mM to 50mM (Table 2.4). Thus the concentration of ornithine in the standard incubation mixture is limiting even at low protein levels. At saturating ornithine levels (50mM), the ornithine decarboxylase activity measured in dilute cell extracts was 3.7-fold greater than when measured in limiting ornithine concentrations (0.5mM) (Table 2.4). Even at saturating ornithine concentrations however, the specific activity of ornithine decarboxylase measured in extracts containing high levels of protein, was 70% lower than those of extracts containing low levels of protein (Table 2.4). These results, together with those of the previous experiment, suggest that although the standard assay was carried out in conditions of limiting substrate, this factor alone was not responsible for the lowering of enzyme activity observed in concentrated extracts. This phenomenon was possibly a result of modifications of ornithine decarboxylase in concentrated D. discoideum amoebal extracts.

In subsequent assays the concentration of ornithine in the incubation mixture was maintained at 0.5mM. Using non-saturating levels of ornithine has several disadvantages; for example apparent changes in the level of ornithine decarboxylase activity may be due to minor fluctuations in endogenous concentrations of non-radioactive ornithine. On the other hand, assays performed at high ornithine concentrations reduce the level of free PLP in the assay due to

TABLE 2:4 ORNITHINE DECARBOXYLASE ACTIVITY AS A FUNCTION OF PROTEIN, COENZYME AND SUBSTRATE CONCENTRATION

A concentrated amoebal extract (approx. $5x10^8$ cells/ml)was prepared in 10% (v/v) glycerol extraction buffer (see methods section). An aliquot of this extract was diluted with 10% (v/v) glycerol extraction buffer to give the range of protein concentrations shown. Each extract was assayed in the standard incubation mixture (0.5mM-ornithine and 0.31mM-PLP) and in incubation mixtures containing elevated levels of PLP and ornithine.

WEIGHT OF PROTEIN PER ASSAY (mg)	ORNITHINE DECARBOXYLASE ACTIVITY ASSAYED IN:- (nmol CO_2/min/mg protein)			
	0.5mM-ORNITHINE 0.31mM-PLP	0.5mM-ORNITHINE 1mM-PLP	50mM-ORNITHINE 0.31mM-PLP	50mM ORNITHINE 1mM-PLP
0.034	4.42	3.99	16.62	16.07
0.116	3.69	3.61	15.23	11.88
0.148	3.61	3.30	14.82	13.50
0.209	2.59	3.17	13.22	12.15
0.241	2.25	3.00	11.55	14.10
0.516	1.59	1.47	4.47	5.23

non-enzymatic Schiff base formation (Mitchell & Sedory, 1974; Clark & Fuller, 1976). In addition, an elevation of the level of non-radioactive ornithine present in the assay mixture would have meant either a comparable increase in the amount of [1-^{14}C] ornithine used, thus increasing significantly the cost of the assay, or a sacrifice in the accuracy of enzyme activity measurements due to over-dilution of the radioactive substrate. As this study was concerned mainly with the properties of ornithine decarboxylase from D. discoideum and factors altering the activity of this enzyme, rather than the measurement of total enzyme activity, assays continued to be performed with non-saturating substrate levels and low levels of protein.

TIME COURSE OF ASSAY

Since non-saturating levels of substrate were being used in the ornithine decarboxylase assay, it was important to determine the time period and the protein concentration over which the enzyme activity was linear.

The results in Figure 2.2 show that in assays carried out in 0.5mM-ornithine and containing less than 0.23mg protein/assay, ornithine decarboxylase activity was linear for 30 min; the standard assay time. Under these conditions the specific activity of the enzyme was 3.9$\pm$ 0.23 nmol CO_2/min/mg protein, within the range of ornithine decarboxylase activity found in many other eucaryotes (Jänne & Williams-Ashman, 1971a; Friedman et al., 1972a; Ono et al., 1972; Mennucci et al., 1975; Mitchell et al., 1976; Stevens et al., 1976; Boucek & Lembach, 1977).

SONICATION

In earlier experiments, using concentrated enzyme extracts, the level of ornithine decarboxylase activity in unsonicated samples was greater than those which were sonicated in non-glycerol buffer (Table 2.1). This observation may have resulted from the lability of ornithine decarboxylase to sonication. Alternatively at high concentrations of protein, sonication may have released

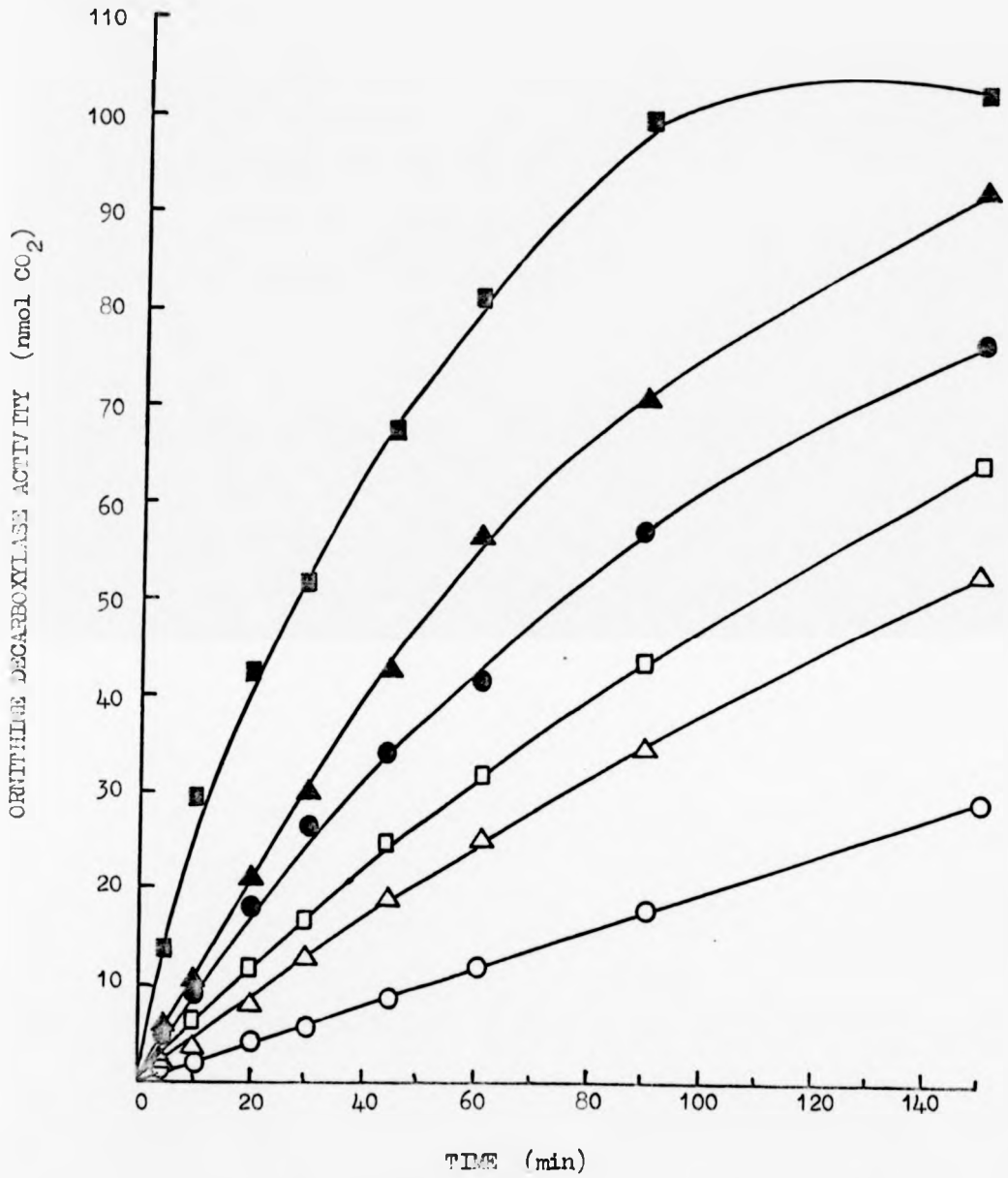


FIGURE 2.2 ORNITHINE DECARBOXYLASE ACTIVITY AS A FUNCTION OF TIME AT DIFFERENT PROTEIN CONCENTRATIONS

Amoebal enzyme extracts, containing a range of protein concentrations, were prepared in 10% (v/v) glycerol extraction buffer as in the previous experiment. Ornithine decarboxylase activity was assayed under standard conditions (see methods section), the reaction being interrupted after various time intervals. Protein concentrations of 0.05mg/assay (○), 0.11mg/assay (△), 0.14mg/assay (□), 0.21mg/assay (●), 0.23mg/assay (▲) and 0.53mg/assay (■) were used.

an inhibitory system possibly activating polymerisation of ornithine decarboxylase or interconversion of active and less active forms. The addition of glycerol to the extraction buffer was subsequently shown to reduce the level of enzyme activity in concentrated and sonicated extracts by approximately 20%, yet sonication significantly increased the stability of ornithine decarboxylase during storage (Table 2.2). This reduction in the initial enzyme activity in the presence of glycerol may however have resulted from the use of extracts containing slightly higher protein concentrations. Further clarification was required on the importance of sonication and glycerol in the determination of ornithine decarboxylase activity in extracts containing low levels of protein.

In unsonicated preparations comparable levels of ornithine decarboxylase activity were realeased into both glycerol and non-glycerol extraction buffer (Table 2.5). Thus glycerol failed to prevent the lysis of amoebae which occurred either in the extraction buffer or assay mixture. Comparison of the data in Table 2.1 and Table 2.5 shows that, irrespective of the concentration of protein in cell extracts, sonication in non-glycerol extraction buffer resulted in loss of ornithine decarboxylase activity. Sonication for 1 min in 10% (v/v) glycerol extraction buffer released 1.8 times more enzyme activity. Thus the loss of activity noted in concentrated extracts on addition of glycerol to the extraction buffer was not observed in extracts containing low levels of protein. This suggests that irrespective of protein concentration, glycerol is required in the extraction buffer to stabilise ornithine decarboxylase activity when released from the amoebae by sonication. Maximum activity was observed in extracts containing 10% (v/v) glycerol after 1 min sonication i.e. 4 @ 15s bursts (Table 2.5).

TEMPERATURE AND pH OPTIMA

The temperature and pH optima for the *in vitro* assay of ornithine decarboxylase activity from *D. discoideum* were 37°C and pH 8.25 respectively (Figures 2.3 and 2.4). This temperature optimum was higher than that for the

TABLE 2:5 ORNITHINE DECARBOXYLASE ACTIVITY RELEASED INTO GLYCEROL AND NON-GLYCEROL EXTRACTION BUFFER BY SONICATION

Amoebae were suspended in either 10% (v/v) glycerol extraction buffer and non-glycerol extraction buffer at cell densities of approximately 5×10^6 cells/ml. Ornithine decarboxylase assays were carried out using unsonicated extracts or extracts sonicated (as detailed in the methods section) for the time periods indicated.

PERIOD OF SONICATION (Number of 15s bursts)	ORNITHINE DECARBOXYLASE ACTIVITY (nmol CO_2/min/mg protein)	
	10% (v/v)-GLYCEROL EXTRACTION BUFFER	NON-GLYCEROL EXTRACTION BUFFER
0	2.38	2.50
2	4.29	2.24
4	4.30	1.83
6	3.20	1.04
8	2.11	0.48

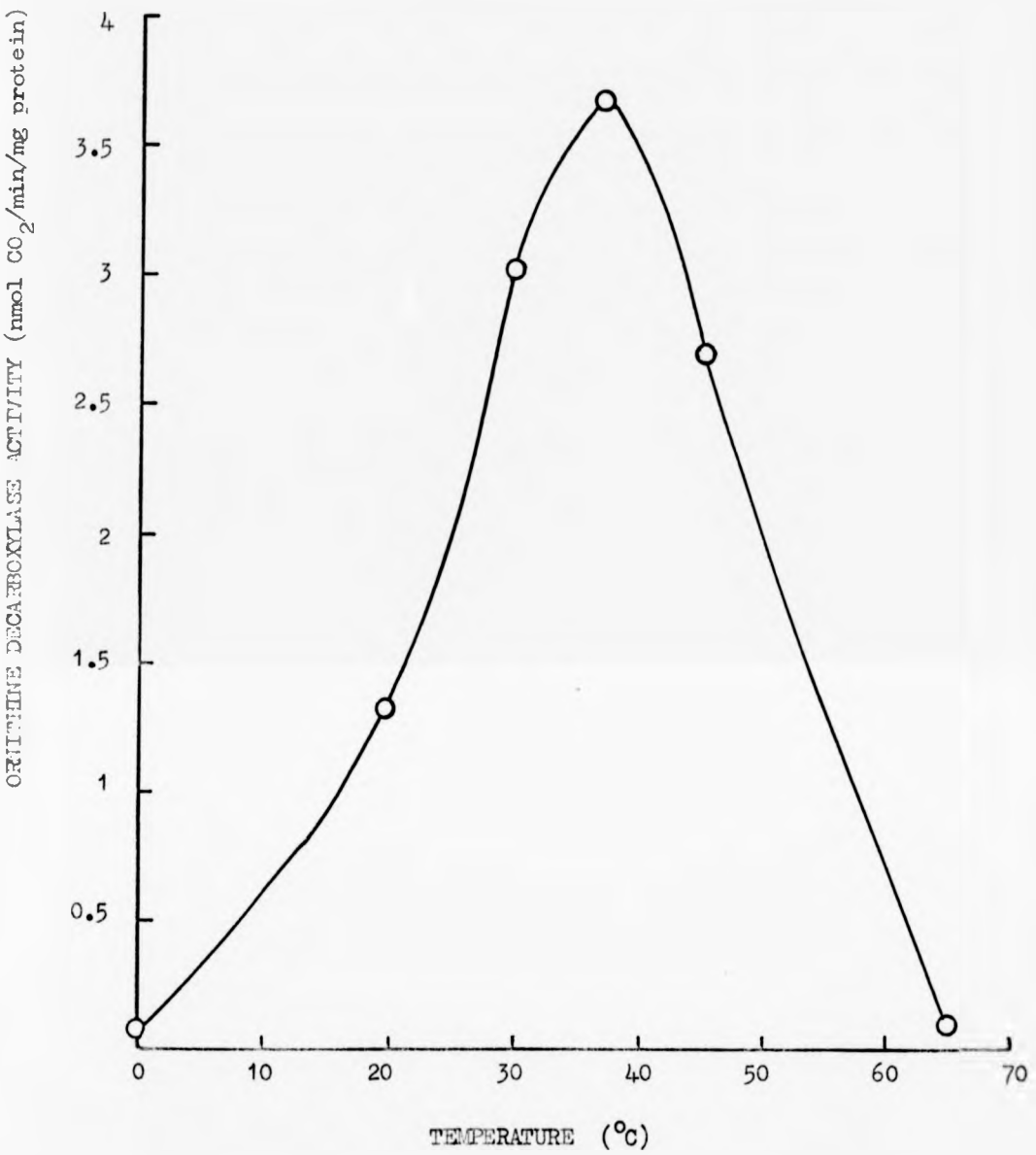


FIGURE 2.3 TEMPERATURE OPTIMUM OF ORNITHINE DECARBOXYLASE ACTIVITY

Assays were carried out under standard conditions (see methods section) using an amoebal extract which gave a protein concentration of less than 0.1mg protein/assay. The incubations were carried out at the temperatures indicated.

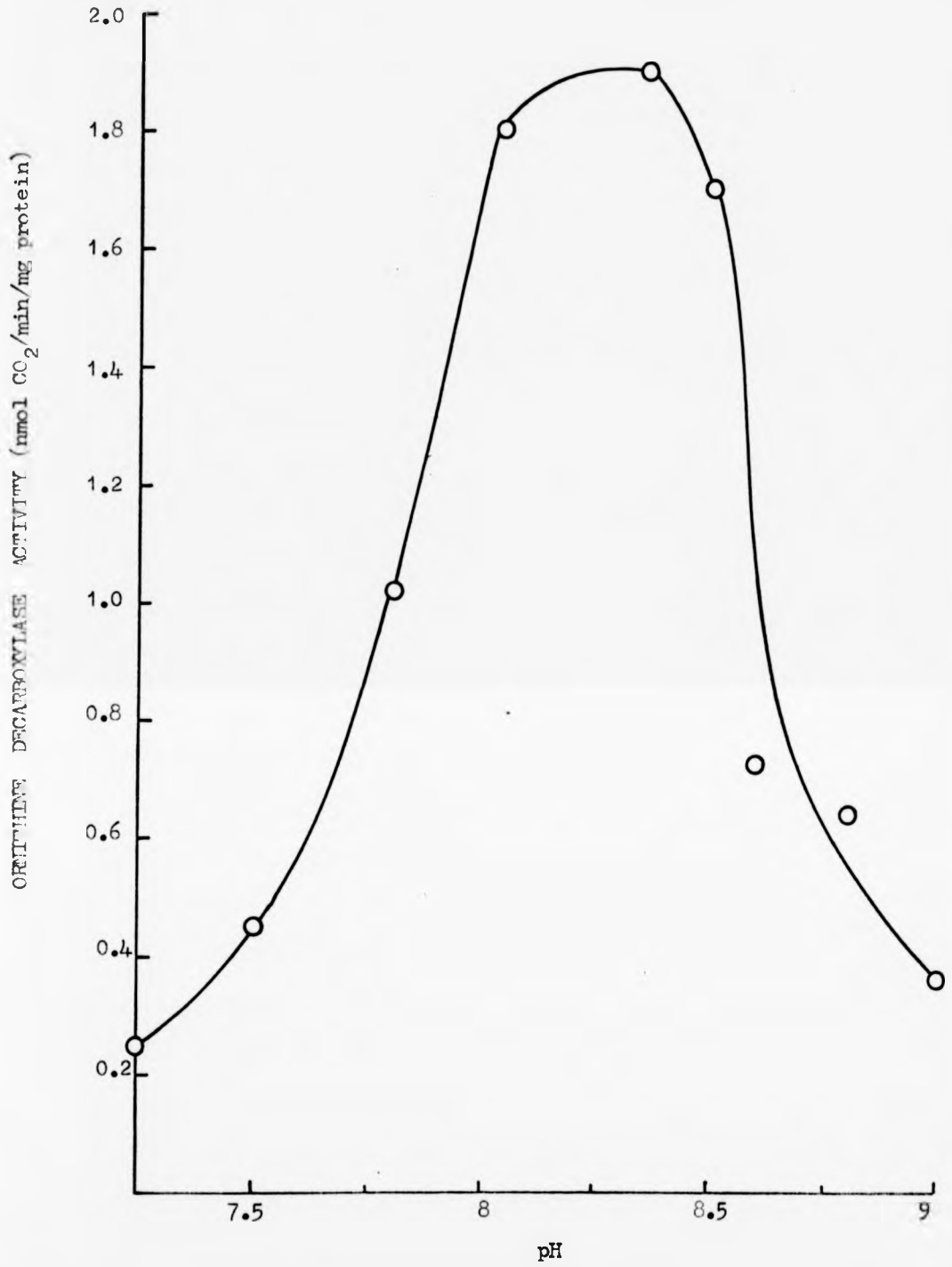


FIGURE 2.4 pH OPTIMUM OF ORNITHINE DECARBOXYLASE ACTIVITY

Assays were carried out under standard conditions (see methods section) at less than 0.1mg protein/assay, except that the pH of the 65mM-Tris/HCl assay buffer was adjusted such that the final pH of the assay mixture were as shown

growth and development of D. discoideum (22°C-27°C). The pH optimum was similar to that of ornithine decarboxylases from other systems (Morley & Ho, 1976; Stevens et al., 1976; Applebaum et al., 1977). As the ornithine decarboxylase activity measured at pH 8.1 (the pH of the standard assay), was only slightly lower than that at the optimum, pH 8.25, no change was made in the conditions of the standard assay.

COMPOSITION OF THE EXTRACTION BUFFER

The standard extraction buffer used in the preparation of D. discoideum ornithine decarboxylase extracts, was that used by Stevens et al. (1976) in a study of this enzyme in A. nidulans. Thus it was necessary to investigate the effect of the various components of the extraction buffer on the D. discoideum enzyme. The level of ornithine decarboxylase activity was determined in enzyme extracts prepared in the various extraction buffers described in Table 2.6, all of which contained 10% (v/v) glycerol.

As the data in Table 2.6 show , over 50% of ornithine decarboxylase activity was lost when the enzyme was extracted into 5mM-phosphate buffer containing only 10% (v/v) glycerol. In enzyme extracts prepared in buffer containing 2mM-DTT the level of ornithine decarboxylase activity measured was 98% of extracts prepared in standard extraction buffer. Increasing the concentration of DTT in the extraction buffer to 4mM raises the enzyme activity measured to a level 53% higher than that in standard extraction buffer (Table 2.6). The dependence of ornithine decarboxylase activity on the presence of DTT was not unexpected as in all enzyme extracts so far investigated, the presence of a thiol-protecting agent has been found necessary for maximum ornithine decarboxylase activity. The inactivation of the enzyme by the modification of thiol groups has been reported for a variety of ornithine decarboxylases (Jänne & Williams-Ashman, 1971a; Ono et al., 1972; Friedman et al., 1972a; Clark, 1974). Removal of DTT from purified 3T3 mouse fibroblast ornithine decarboxylase resulted not only in almost complete loss of enzyme activity but also in dimerisation of the

TABLE 2.6 COMPONENTS OF EXTRACTION BUFFER REQUIRED FOR ORNITHINE DECARBOXYLASE ACTIVITY

Enzyme extracts were prepared as usual (see methods section), in the various extraction buffers described below. All extracts contained 10% (v/v) glycerol and unless otherwise indicated, 5mM-phosphate buffer, pH7.6. Ornithine decarboxylase activity was assayed under standard conditions (see methods section) and the results are reported as percentages of the level of enzyme activity measured in standard extraction buffer i.e. 1mM-$MgCl_2$, 2mM-DTT, 0.1mM-PLP, 0.1mM-EDTA (9 nmol CO_2/min/mg protein = 100%)

COMPOSITION OF EXTRACTION BUFFER	% ORNITHINE DECARBOXYLASE ACTIVITY
1mM-$MgCl_2$, 2mM-DTT, 0.1mM-PLP, 0.1mM-EDTA	100
NO ADDITIONS	44
2mM-DTT	98
4mM-DTT	153
4mM-cysteine	52
10mM-cysteine	57
4mM-mercaptoethanol	56
10mM-mercaptoethanol	77
0.1mM-EDTA	52
1mM-$Mg\ Cl_2$	67
0.1mM-PLP	51
0.1mM-EDTA, 1mM-$Mg\ Cl_2$	68
0.1mM-PLP, 2mM-DTT	63
0.1mM-EDTA, 2mM-DTT, 1mM-$Mg\ Cl_2$	86
0.1mM-PLP, 2mM-DTT, 1mM-$Mg\ Cl_2$	87
0.05mM-borate buffer, pH7.6, 0.1mM-PLP, 2mM-DTT, 1mM$g\ Cl_2$, 0.1mM-EDTA	27

enzyme (Boucek & Lembach, 1977). This was also shown to occur with ornithine decarboxylase from rat prostate (Jänne & Williams-Ashman, 1971a). Other thiol-protecting agents were not as effective as DTT in stabilizing ornithine decarboxylase activity. The level of enzyme activity in phosphate buffer containing 4mM-cysteine was 4.7 nmol CO_2/min/mg protein as compared with 8.8 nmol CO_2/min/mg protein in phosphate buffer containing 2mM-DTT. Increasing the cysteine concentration to 10mM had little additional effect (Table 2.6). Similarly mercaptoethanol, even at concentrations of 10mM, was not as effective as 2mM-DTT at stabilising ornithine decarboxylase activity (Table 2.6). Mercaptoethanol has been found to be less effective than DTT at stabilising ornithine decarboxylase activity in other systems (Clark & Fuller, 1976; Stevens et al., 1976). Extracts containing DTT retained approximately 100% of their activity on storage at -18°C for 24h, whilst after a similar period of storage the ornithine decarboxylase activity of all other extracts had almost completely disappeared.

The addition of EDTA to the phosphate buffer, resulted in only a very slight increase in the level of enzyme activity, probably resulting from the chelation of heavy metal ions (Table 2.6). Although the level of ornithine decarboxylase activity in extracts in phosphate buffer containing 1mM-$MgCl_2$ was 35% lower than those in standard extraction buffer, it was 23% greater than those in phosphate buffer only. The addition of EDTA to the magnesium-containing phosphate buffer, failed to cause a further increase in the level of ornithine decarboxylase activity (Table 2.6).

PLP is a known cofactor of many decarboxylases including ornithine decarboxylase. When the enzyme extract was prepared in buffer lacking PLP and assayed in the absence of additional cofactor, no ornithine decarboxylase activity was detected. However the addition of 0.1mM-PLP to the phosphate buffer resulted in only a slight increase in the level of ornithine decarboxylase activity in this extract. Thus PLP was involved in the activation of ornithine decarboxylase but not in its stabilisation. The inclusion of this cofactor in

the extraction buffer is reported as necessary for the stabilisation of some ornithine decarboxylases (Pegg & Williams-Ashman, 1968; Clark & Fuller, 1976). Other eucaryotic enzymes, whilst requiring the cofactor for activity, do not need it for stabilisation of the enzyme in storage (Mitchell & Sedory, 1974; Murphy & Brosnan, 1976; Morley & Ho, 1976; Friedman et al., 1977a). The level of ornithine decarboxylase activity in the extract in phosphate buffer containing 2mM-DTT, was 8.8 nmol CO_2/min/ mg protein. This activity fell to 5.73 nmol CO_2/min/mg protein when 0.1mM-PLP was added to this DTT-containing extraction buffer. (Table 2.6). This probably resulted from the formation of an adduct between DTT and PLP (Mitchell & Sedory, 1974; Clark & Fuller, 1976), thus reducing the effective concentration of the thiol-protecting group in the extraction buffer.

Mitchell & Sedory (1974) used 0.05mM-borate buffer in preference to Tris buffer when extracting ornithine decarboxylase from P. polycephalum. They suggest that Tris buffer reduces ornithine decarboxylase activity by forming Schiff bases with PLP thus lowering the effective concentration of this coenzyme. The level of ornithine decarboxylase activity in D. discoideum extracts prepared in 0.05mM-borate buffer containing all the other components of the standard extraction buffer was less than 30% of that in standard phosphate extraction buffer (Table 2.6). Similarly extracts, prepared in the borate buffer and assayed in an incubation mixture in which the Tris buffer had been replaced with borate buffer, had lowered levels of ornithine decarboxylase activity. Thus the concentration of pyridoxal phosphate present in the assay mixture must be sufficient to saturate the cofactor requirement of the enzyme despite Schiff base formation with Tris buffer.

Thus in 2mM-DTT-containing phosphate extraction buffer, PLP, EDTA and $MgCl_2$ were not essential for the stability of ornithine decarboxylase. However, as their presence caused only a slight depression in the level of enzyme activity measured, the composition of the standard extraction buffer was not changed. Although higher concentrations of dithiothreitol increased both the specific

activity of ornithine decarboxylase and the stability of the enzyme on storage, the concentration of this thiol-protecting agent in the standard extraction buffer was maintained at 2mM as it is an expensive chemical.

SECTION B - Kinetic parameters of ornithine decarboxylase from D. discoideum

Having determined the conditions necessary for the extraction and measurement of ornithine decarboxylase activity from D. discoideum, some of the in vitro properties of the enzyme were investigated in an attempt to characterise the enzyme.

The binding of PLP to ornithine decarboxylase has been postulated to be the first step in the conversion of apoenzyme to holoenzyme (Clark & Fuller, 1976; Morley & Ho, 1976; Boucek & Lemback, 1977). Compared with other PLP-requiring enzymes however, the apoenzyme of ornithine decarboxylase has a relatively low affinity for the cofactor (Janne & Williams-Ashman, 1971a; Morley & Ho, 1976; Boucek & Lembach, 1977). To determine the ease of activation of D. discoideum ornithine decarboxylase by this cofactor two amoebal extracts, differing in their concentration of protein, were assayed at a range of PLP concentrations. Although assays were at elevated concentrations of ornithine, the level of substrate was still non-saturating thus minimizing alterations in the level of coenzyme due to non-enzymatic Schiff base formation. With both extracts normal hyperbolic saturation kinetics were observed producing linear Lineweaver-Burk plots (1934), (Figure 2.5). These were extrapolated to estimate Km values for PLP.

At both concentrations of protein, ornithine decarboxylase was saturated at 120μM-PLP. This confirmed the conclusions drawn from the data in Table 2.4, i.e. that the standard assay was carried out at saturating cofactor levels. The cofactor affinity of ornithine decarboxylase in both extracts was the same, both having a Km value for PLP of 23μM. Similarly there was little difference in V max values (16.1 and 17.2 nmol CO_2/min/mg protein). These values were within the range found for ornithine decarboxylases from other organisms (Clark

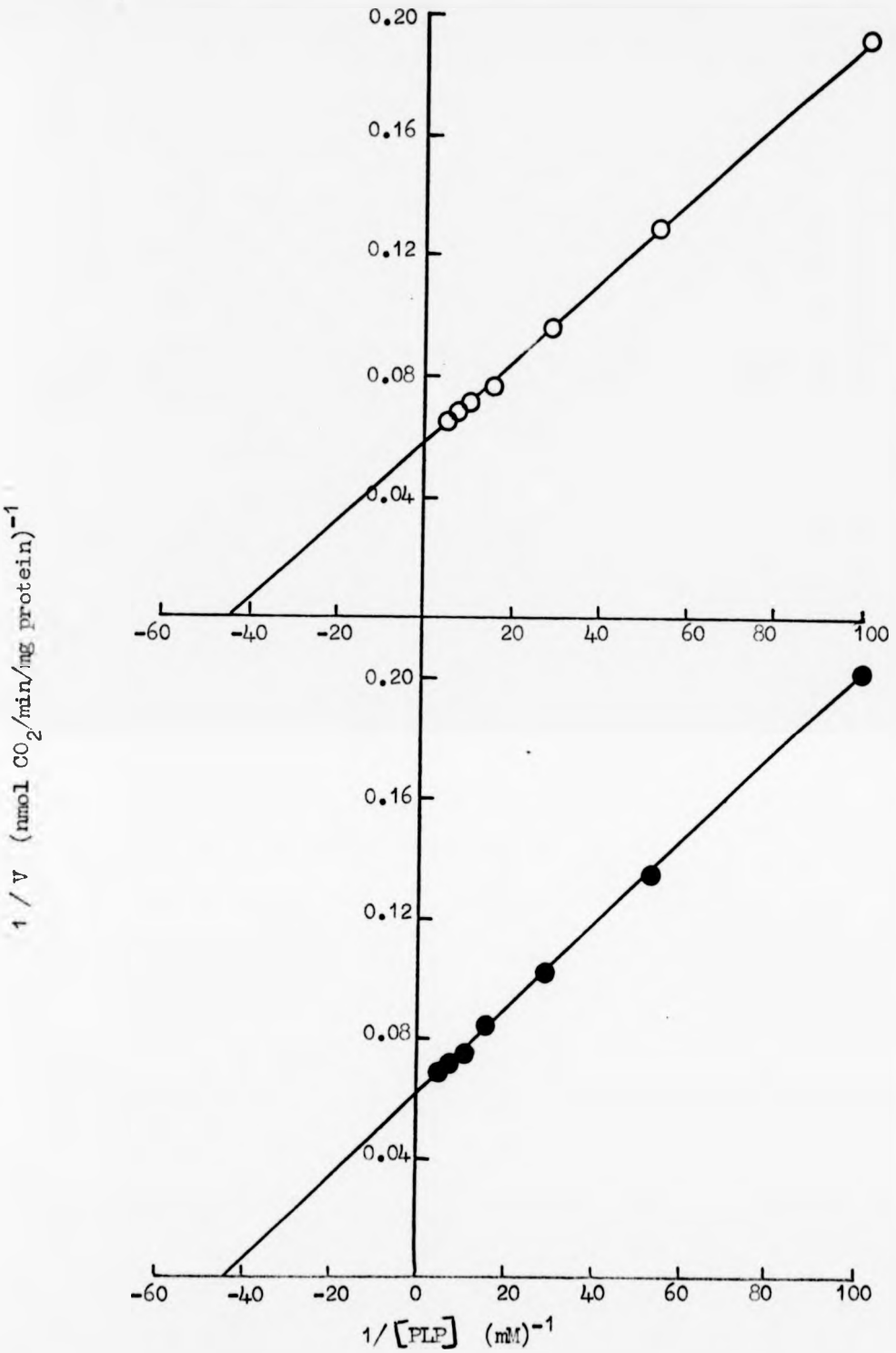


FIGURE 2.5 THE EFFECT OF VARYING PLP CONCENTRATIONS ON ORNITHINE DECARBOXYLASE ACTIVITY

An enzyme extract was prepared in 10% (v/v) glycerol buffer (see methods section), and diluted to give protein concentrations of 0.11mg protein/assay (O) and 0.56mg protein/assay (●). The level of ornithine decarboxylase activity in these extracts was assayed as usual at the indicated PLP concentrations and at an elevated ornithine concentration (5mM). The data were plotted by the method of Lineweaver & Burk (1934). Assays with boiled enzyme were used to correct for non-enzymic carbon dioxide formation at each concentration of PLP.

& Fuller, 1976; Mitchell et al., 1976; Applebaum et al., 1977; Boucek & Lembach, 1977; Obenrader & Prouty, 1977a). The activity in both extracts exhibited the same Km, thus if the inhibition of activity observed at high protein concentrations was due to polymerisation or interconversion of forms these would not seem to have been distinct with respect to their PLP affinities.

The substrate dependence of ornithine decarboxylase was also investigated using several amoebal extracts of differing protein concentrations. With all extracts normal hyperbolic saturation kinetics were observed producing linear Lineweaver-Burk plots (Figure 2.6) which were extrapolated to estimate Km values for ornithine.

Ornithine was saturating in all extracts at 10mM. This confirmed the conclusions drawn from Table 2.4, that standard assays were carried out under non-saturating conditions. The substrate affinity of the activity in cell extracts, as measured by the Km value, was 1.7mM. Obenrader & Prouty (1977a) report the separation from rat liver of at least two forms of ornithine decarboxylase activity which differ in their Km with respect to ornithine. The results of the experiment reported here however, did not support the existence of such a system in D. discoideum. The V max values varied between 10 nmol CO_2/min/mg protein for the concentrated extract and 17 nmol CO_2/min/mg protein for thedilute extract (Table 2.6), a value which was identical to that obtained in the previous experiment. The Km for ornithine observed in D. discoideum extracts was slightly higher than those from other eucaryotic systems where Km values range between 0.01 and 0.5mM (Abdel-Monem et al., 1975; Clark & Fuller, 1976; Mitchell et al., 1976; Obenrader & Prouty, 1977a). The low substrate affinity observed in D. discoideum resembled more closely that of the ornithine decarboxylases from E. coli for which Km's of 2 and 5.6mM have been reported (Applebaum et al., 1977; Kyriakidis et al., 1978). The most dilute enzyme extract was assayed at two concentrations of PLP, 0.31mM and 10mM, but no difference in the level of enzyme activity was observed. Such high concentrations of cofactor in the assay mix would be expected to reduce the level of free ornithine in the

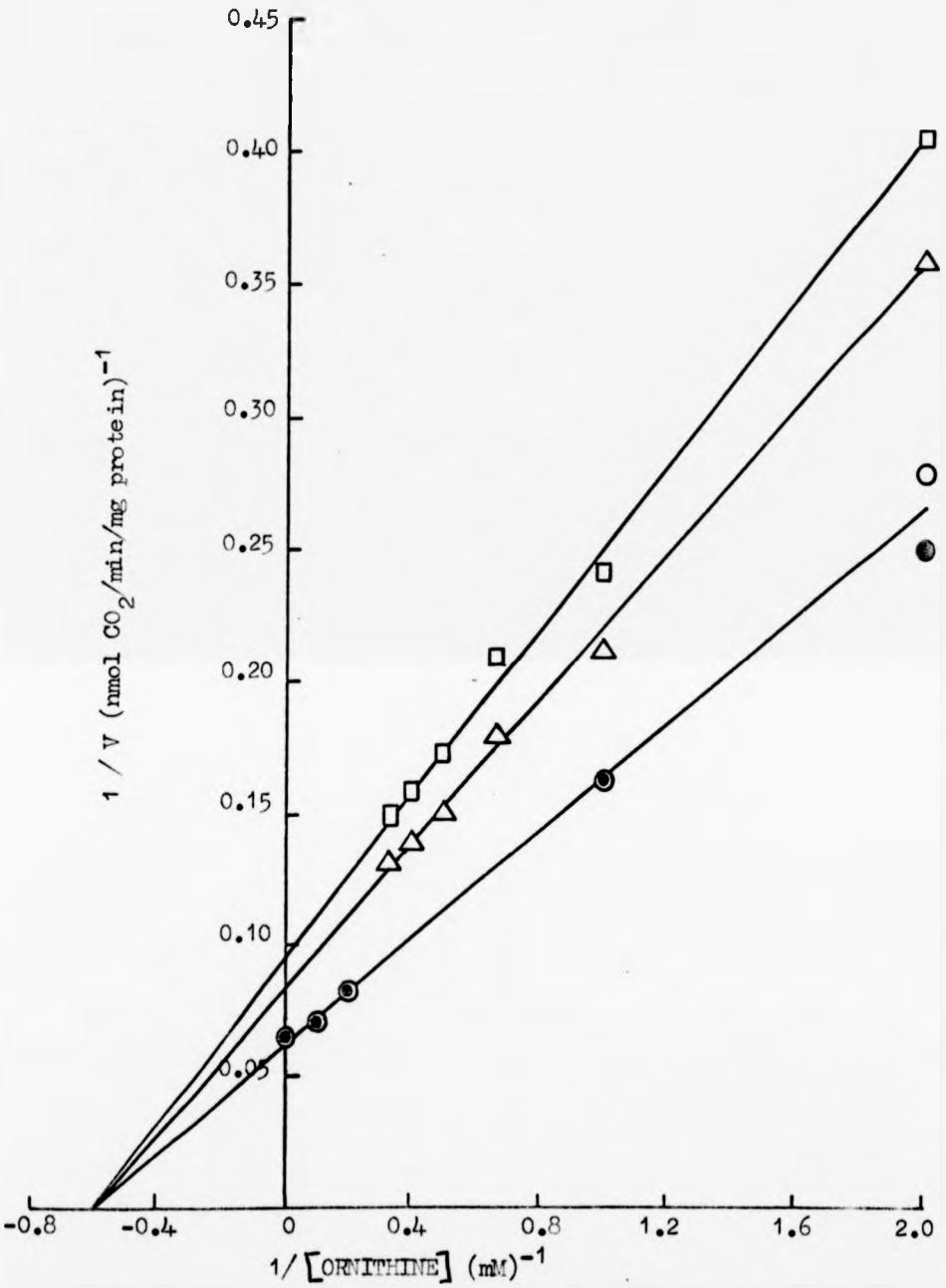


FIGURE 2.6 THE EFFECT OF VARYING SUBSTRATE, PLP AND PROTEIN CONCENTRATIONS ON ORNITHINE DECARBOXYLASE ACTIVITY

Enzyme extracts in 10% (v/v) glycerol extraction buffer were prepared (see methods section), containing 0.09mg protein/assay (○), 0.18mg protein/assay(△) and 0.55mg protein/assay (□). The level of ornithine decarboxylase activity in these extracts was assayed as usual at the indicated ornithine concentrations and 0.31mM-PLP. In addition the extract containing 0.09mg protein/assay was assayed at 10mM-PLP (●). The data were plotted by the method of Lineweaver & Burk (1934). Assays with boiled enzyme were used to correct for non-enzymic carbon dioxide formation at each concentration of ornithine and PLP.

assay mix and increase the concentration of Schiff base between ornithine and PLP. As the data in Figure 2.6 show, such elevated levels of cofactor failed to affect the V max of the enzyme. This suggested that the form of ornithine decarboxylase being assayed was possibly binding the Schiff base complex.

Similar kinetic studies were carried out with non-glycerol enzyme extracts. The resulting Km values for PLP and ornithine were much reduced but were subject to fluctuations. This was evidence for the possible existence in D. discoideum of more than one form of ornithine decarboxylase with differing cofactor and substrate affinities. The presence of glycerol in the extraction buffer would seem to have stabilised the less active form which has high Km values for both ornithine and PLP. Boucek & Lembach (1977) found it necessary to stabilise purified 3T3 mouse fibroblast ornithine decarboxylase with albumin and they too note that this results in variations in the Km values for substrate and cofactor.

SECTION C - Levels of ornithine decarboxylase activity in D. discoideum

GROWTH CONDITIONS

It has been shown in a number of tissues and organisms that an increase in ornithine decarboxylase activity may be evoked by a wide variety of conditions, yet the total increase in oligoamine levels in such cells is often small. The level of ornithine decarboxylase activity was determined in D. discoideum amoebae grown in a variety of nutritional conditions, although no variation had been observed in the oligoamine levels of similar cells (Table 1.2).

AX2 amoebae were grown both axenically and in association with K. aerogenes. Axenic cells were grown in HL5 medium, either with or without the addition of glucose, or in FM medium. Despite large variations in the growth rate of amoebae in these various nutrients, little variation was observed in the level of ornithine decarboxylase activity in the cell extracts (Table 2.9).

Growth rate is one of the conditions reported to affect ornithine decarboxylase activity, increases in enzyme activity occurring after dilution into fresh medium (Hogan, 1971; Hogan & Murden, 1974; McCann et al., 1975). The

TABLE 2.9 ORNITHINE DECARBOXYLASE ACTIVITY IN *D. DISCOIDEUM* AMOEBAE, STRAIN AX2, GROWN IN VARIOUS NUTRIENTS

Amoebae, grown in the various nutrients indicated, were harvested during exponential growth and enzyme extracts prepared in 10% (v/v) glycerol extraction buffer as detailed in the methods section. The level of ornithine decarboxylase activity was measured using the standard assay mixture.

NUTRIENT	ORNITHINE DECARBOXYLASE ACTIVITY (nmol CO_2/min/mg protein)	AVERAGE DOUBLING TIME (h)
HL5 MEDIUM + GLUCOSE	9.7	8
HL5 MEDIUM - GLUCOSE	6.3	12
FM MEDIUM	8.8	24
KLEBSIELLA AEROGENES	7.6	4

level of ornithine decarboxylase activity was monitored at intervals during both exponential and stationary growth of AX2 amoebae. As the data in Table 2.10 indicate, the level of enzyme activity in amoebae was not significantly altered by the change in growth rate associated with cells entering a stationary growth phase.

DEVELOPMENT

Oligoamine levels were observed to decrease substantially during the 24h developmental period which follows the removal of D. discoideum amoebae from nutrients (Figure 1.9). As ornithine decarboxylase is thought to be the rate-controlling enzyme in the biosynthesis of oligoamines, the activity of this enzyme was monitored during the development of amoebae into sorocarps.

The results shown in Figure 2.7 suggest that the level of ornithine decarboxylase activity in amoebae increased by 72% during the 30 min (approx) taken to place the cells on developmental filters and immediately reharvest them. In addition this apparent increase was subsequently lost during the first hour of development. The results of later experiments (see page 141) suggest that this anomoly in ornithine decarboxylase activity was due to the exposure of the cells to low osmotic strength solutions during harvesting. The level of ornithine decarboxylase activity decreased sharply during the first 6h of development, falling to 42% of the amoebal value (Figure 2.7). During the last 18h of the developmental period the level of ornithine decarboxylase activity in sorocarp cells had recovered to 80% of amoebal values (Figure 2.7).

SECTION D - Control of ornithine decarboxylase activity

IN VIVO EFFECT OF OLIGOAMINES

Reports from numerous sources suggest that control of oligoamine synthesis is achieved by sensitive modulation of the activity of ornithine decarboxylase, which catalyzes the rate limiting step in this biosynthetic pathway. Although the activity of this enzyme has been shown to vary sharply in response to many stimuli, the control of ornithine decarboxylase activity by variations in the

TABLE 2.10 ORNITHINE DECARBOXYLASE ACTIVITY IN AMOEBAE DURING GROWTH

AX2 amoebae were harvested from flasks of HL5 medium at intervals during both exponential growth (cell densities $< 8 \times 10^6$ cell/ml) and stationary growth (cell densities $> 8 \times 10^6$ cell/ml). Enzyme extracts were prepared in 10% (v/v) glycerol extraction buffer as detailed in the methods section and the level of ornithine decarboxylase activity measured using the standard assay mixture.

CELL DENSITY ($\times 10^6$ cells/ml medium)	ORNITHINE DECARBOXYLASE ACTIVITY (nmol CO_2/min/mg protein)
0.5	4.1
2.4	4.6
5.6	4.9
7.7	5.03
13.1	5.0
14.5	4.6
20.0	4.8

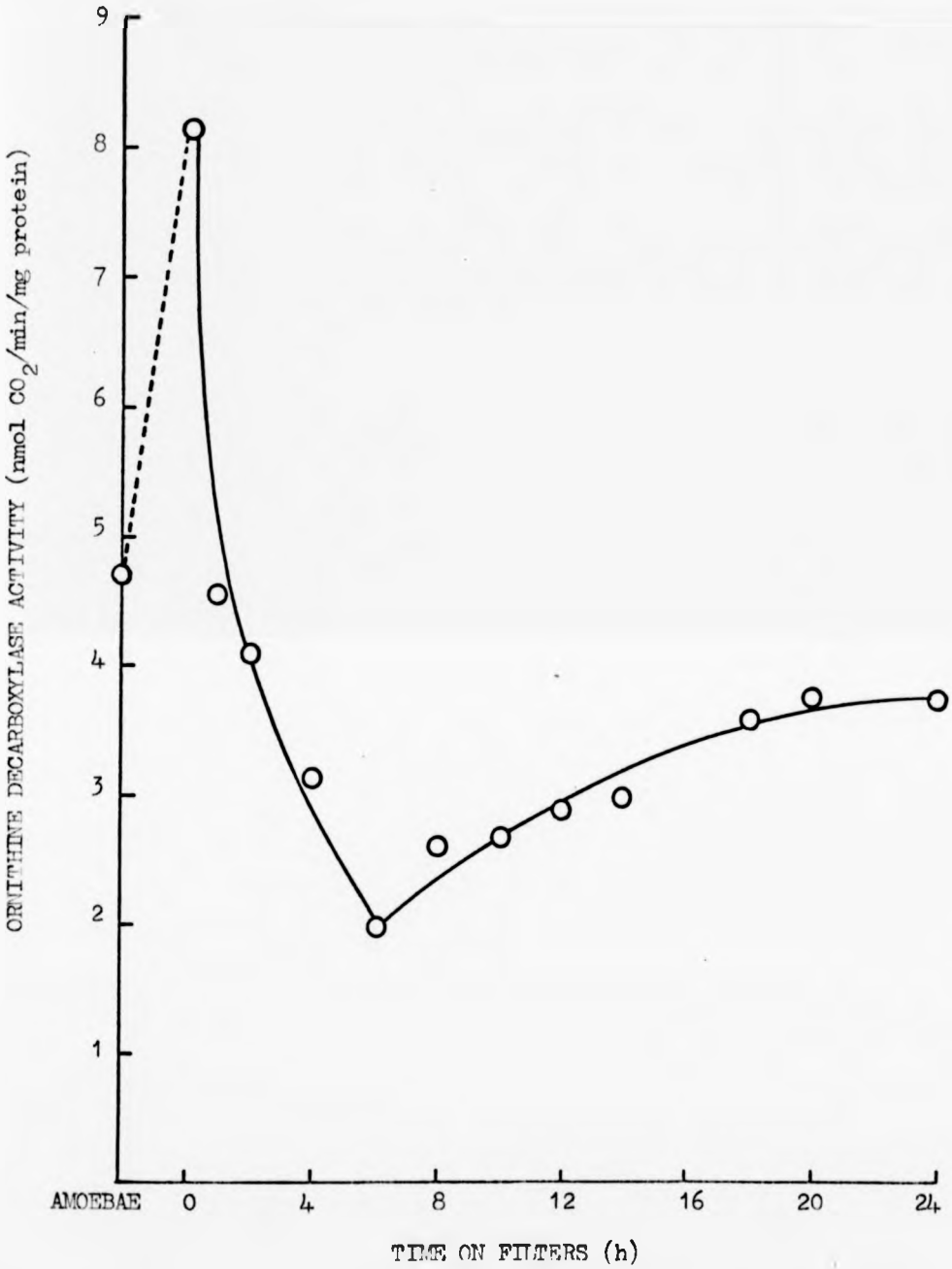


FIGURE 2.7 ORNITHINE DECARBOXYLASE ACTIVITY DURING DEVELOPMENT OF D. DISCOIDEUM

Amoebae, harvested during exponential growth, were used to prepare developmental Millipore filters. At intervals during the 24h developmental period the level of ornithine decarboxylase activity in the developing cells was determined in the usual method. A portion of the amoebal suspension used in the preparation of the developmental filters was retained, and the level of ornithine decarboxylase activity present in these cells was also determined.

concentration of its products, the oligoamines, is at present unclear.

The *in vivo* effect of oligoamines on the levels of ornithine decarboxylase activity in *D. discoideum* amoebae was investigated as detailed in the legend to Figure 2.8. The effect of extracellular putrescine, spermidine and 1,3-diaminopropane was very similar. The amoebae were slightly more sensitive to low concentrations of spermidine and putrescine, 30% reductions in the level of enzyme activity occurring at concentrations of 1mM, whilst similar concentrations of 1,3-diaminopropane resulted in only a 10% decrease in the level of ornithine decarboxylase activity. Amoebae grown in medium which was 5mM in putrescine, spermidine or 1,3-diaminopropane had ornithine decarboxylase levels 50% lower than control cells and at 10mM these same oligoamines reduced the level of enzyme activity by 80% (Figure 2.8). The addition of spermine to the amoebal growth medium had a less marked effect on ornithine decarboxylase levels; over 60% of the enzyme activity measured in control cells remaining in cells grown in the presence of 10mM-spermine (Figure 2.8). Spermidine and spermine at extracellular concentrations of 0.1mM caused substantial reductions in the putrescine content of amoebae (Table 1.4). Yet this concentration of these oligoamines had little effect on the ornithine decarboxylase levels of these cells. Regulation of putrescine levels by spermidine and spermine must operate at another level possibly involving an increase in catabolism of the diamine as indicated by the results in Table 1.8.

IN VITRO EFFECT OF OLIGOAMINES

Very little consideration has been given to the direct effect of endogenous oligoamines on ornithine decarboxylase. Although putrescine and spermidine have been shown to inhibit the enzyme with a K_i in the 1-5mM range (Pegg & Williams-Ashman, 1968; Kay & Lindsay, 1973; Morley & Ho, 1976), this effect has been thought by many to be insignificant in contrast to the marked decay of enzyme activity induced by the addition to the medium of oligoamine concentrations 2-3 orders of magnitude less. Yet the intracellular oligoamine concentrations of most cells are maintained in the millimolar range and therefore it

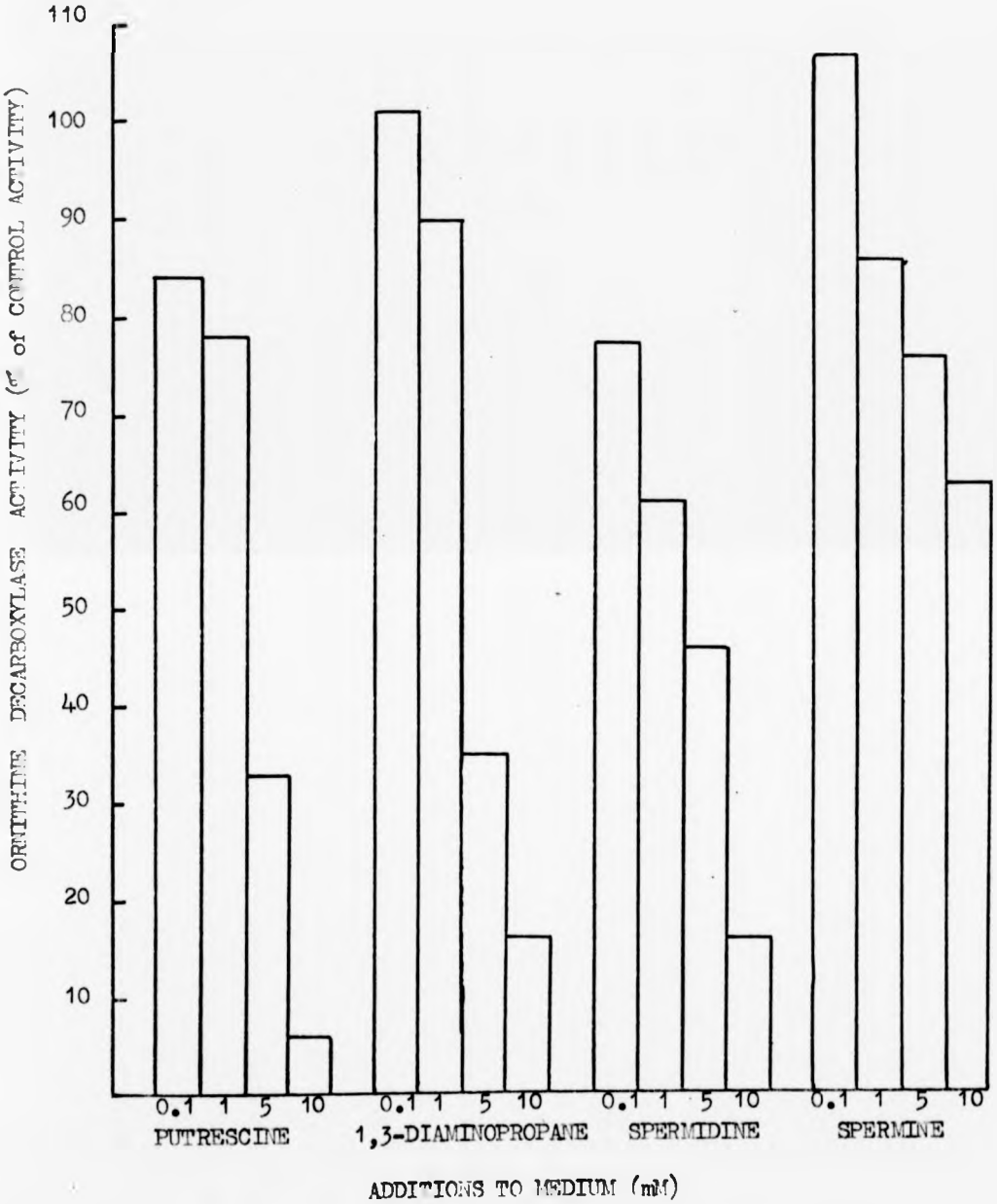


FIGURE 2.8 THE IN VIVO EFFECT OF OLIGOAMINES ON THE LEVEL OF ORNITHINE DECARBOXYLASE ACTIVITY

Amoebae were grown for 72h in HL5 medium containing the concentrations of oligoamines shown. The cells were harvested in exponential growth and the level of ornithine decarboxylase activity in the cell extracts determined as detailed in the methods section. Each value shown represents the average of five experimental flasks.

is this concentration of oligoamines to which ornithine decarboxylase must be sensitive. Consistent with this idea is the recent report by Mitchell et al. (1978) that in P. polycephalum millimolar levels of oligoamines have a direct effect on both the catalytic activity and the physical conformation of purified preparations of ornithine decarboxylase.

The in vitro effect of oligoamines on the level of ornithine decarboxylase activity measured in D. discoideum amoebae was investigated as detailed in the legend to Figure 2.9. As reported for other systems, oligoamines were more effective in reducing the level of ornithine decarboxylase activity in D. discoideum amoebal extracts when added to the growth medium as opposed to the enzyme assay. Thus putrescine, spermidine, 1,3-diaminopropane and spermine at 0.1mM concentrations had no effect on the level of enzyme activity measured. At 1mM, all four of the oligoamines tested caused less than a 20% reduction in measured ornithine decarboxylase activity whilst even at 10mM more than 20% of the enzyme activity measured in control tubes remained (Figure 2.9). Although the intracellular concentration of oligoamines in D. discoideum amoebae was high, the concentration of free amines was unlikely to be at a level where feedback inhibition of ornithine decarboxylase by its direct product putrescine or its less immediate products spermidine, spermine and possibly 1,3-diaminopropane, would play an important role in the control of cellular amine levels.

EFFECT OF 1,4-DIAMINOBUTANONE

The development of substances which inhibit ornithine decarboxylase and thus deplete endogenous cellular oligoamine levels has been the object of much recent research. Stevens et al. (1977) report that while 1,4-diaminobutanone, an analogue of putrescine, was a powerful competitive inhibitor of ornithine decarboxylase activity in A. nidulans, regenerating rat liver and prostate (Stevens et al., 1978), when added to the growth medium of A. nidulans the level of enzyme activity was increased as a result of the stabilization of the enzyme molecule against degradation. The effect of 1,4-diaminobutanone on the level of ornithine decarboxylase activity in D. discoideum amoebal extracts was investigated as detailed in the legend to Figure 2.9. As the data in Fig-

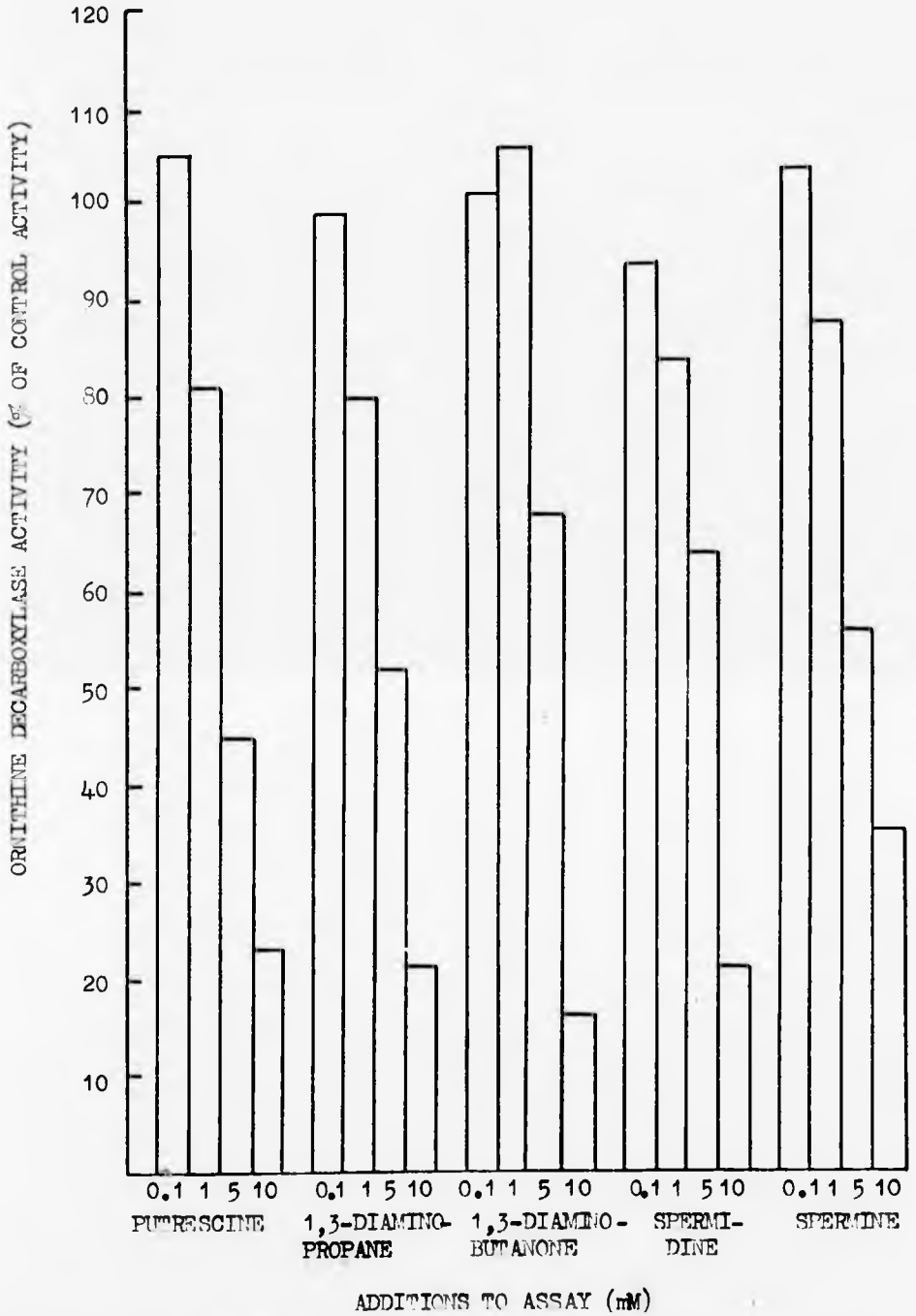


FIGURE 2.9 THE IN VITRO EFFECT OF OLIGOAMINES ON THE ACTIVITY OF ORNITHINE DECARBOXYLASE

Amoebae were harvested in exponential growth and the level of ornithine decarboxylase activity in the cell extracts was determined using the standard assay mix (see methods section), to which appropriate solutions of oligoamines had been added such that the final concentrations were as indicated above.

ure 2.9 show, concentrations of the inhibitor below 1mM had no measurable effect on the level of ornithine decarboxylase activity in amoebal extracts. A 30% decrease in the level of enzyme activity is noted at 1mM concentrations of the inhibitor whilst at 5mM over 80% inhibition of the *in vitro* ornithine decarboxylase activity has occurred. These results are in sharp contrast to those obtained from *A. nidulans* extracts, where 0.13mM-1,4-diaminobutanone reduces ornithine decarboxylase activity to undetectable levels. Stevens et al. (1978) report that ornithine decarboxylase from *E. coli* which, like the *D. discoideum* enzyme has a low affinity for ornithine as judged by the Km, also resembles the *D. discoideum* enzyme in that it is less sensitive to the inhibitor than enzymes from *A. nidulans*, rat liver and prostate which have a high affinity for ornithine. 1,4-diaminobutanone was unsuitable for use as an inhibitor of ornithine decarboxylase activity in *D. discoideum in vitro* and thus *in vivo* is unlikely to cause a significant decrease in intracellular oligoamine levels.

NUCLEOTIDES

In mammalian systems control of ornithine decarboxylase activity by low molecular weight effectors does not seem to be of major physiological importance (Boucek & Lembach, 1977; Friedman et al. 1972a; Morley & Ho, 1977). In contrast, the activation of biosynthetic ornithine decarboxylase from *E. coli* by GTP and other nucleotides has been reported (Höltta et al., 1972; Sakai & Cohen, 1976; Applebaum et al., 1977). UTP, UDP, GTP, GMP, CTP, CMP, ATP, ADP, AMP and cAMP were added to the standard ornithine decarboxylase assay mixture such that the final concentrations of the nucleotides in the assay were 1mM. The enzyme activity of an amoebal extract was then determined as detailed in the methods section. No activation of *D. discoideum* ornithine decarboxylase was observed by any of the tested nucleotides.

SECTION E:- Experimentally induced changes in the levels of ornithine decarboxylase in *D. discoideum*

The activity of ornithine decarboxylase in many tissues has been found

to respond to changes in various growth parameters including nutritional upshift (Hogan & Murden, 1974; Lembach, 1974; McCann et al., 1975; Russell & Stambrook, 1975; Bachrach, 1976; Yamasaki & Ichihara, 1977), and the osmolality of the medium (Munro et al., 1972, 1975; Chen et al., 1976; Friedman et al., 1977a; Mitchell & Kottas, 1979; Perry & Oka, 1980).

NUTRITIONAL UPSHIFT

The induction of ornithine decarboxylase activity by fresh medium has been reported for several cell lines and is thought to result from the stimulation of new enzyme synthesis (Bachrach, 1976). Preliminary experiments were carried out to discover if changing the growth state of D. discoideum resulted in any change in the enzyme levels measured in vitro. The level of ornithine decarboxylase activity did not change when stationary cells were resuspended at a lower cell density either in old HL5 or fresh HL5 medium. The level of enzyme activity in exponentially growing cells was similarly unaffected by resuspension in either growth media (Table 2.11). Although seemingly unaffected by nutritional upshift, the measurable ornithine decarboxylase activity of both stationary and exponential cells was increased by a factor of five when these cells were resuspended in 17mM phosphate buffer (Table 2.11). Removal of amoebae from HL5 growth medium and resuspension in phosphate buffer is known to trigger developmental changes in D. discoideum. However as ornithine decarboxylase levels have been shown to fall during development (Figure 2.7), the increase in enzyme activity observed must reflect either a transitory rise occurring during the onset of development or the effect on the level of ornithine decarboxylase activity of some other difference in the resuspension media.

OSMOTIC EFFECTS

The data in Table 2.11 suggested that the levels of ornithine decarboxylase activity in D. discoideum amoebae were sensitive to changes in the suspension media, consistent with osmotic regulation. Variations in the levels of enzyme activity resulting from suspension and washing of amoebae in different media were further investigated as detailed in the legend to Table 2.12. Com-

TABLE 2.11 THE EFFECT OF RESUSPENSION IN BUFFER, OLD HL5 MEDIUM, AND NEW HL5 MEDIUM ON THE LEVEL OF ORNITHINE DECARBOXYLASE ACTIVITY IN BOTH STATIONARY AND EXPONENTIALLY GROWING AMOEBAE

A culture of exponentially growing amoebae and one of stationary cells was harvested as usual and media from the latter flask retained (old HL5 medium). Aliquots of cells from both cultures were resuspended in old HL5, new HL5, and phosphate buffer at a cell density of $4x10^6$ cells/ml. After 15 min the amoebae were harvested and the cell pellets resuspended without washing in extraction buffer. Cell extracts were prepared and the levels of ornithine decarboxylase activity determined as detailed in the methods section.

GROWTH STATE OF AMOEBAE	RESUSPENSION MEDIUM	ORNITHINE DECARBOXYLASE ACTIVITY (nmol CO_2/min/mg protein)
STATIONARY	OLD HL5 MEDIUM	0.6
STATIONARY	NEW HL5 MEDIUM	0.5
STATIONARY	17mM-PHOSPHATE BUFFER	2.4
EXPONENTIAL	OLD HL5 MEDIUM	0.5
EXPONENTIAL	NEW HL5 MEDIUM	0.5
EXPONENTIAL	17mM-PHOSPHATE BUFFER	2.7

parison of the enzyme activities of extracts prepared from cells which had been suspended either in HL5 medium or phosphate buffer and subsequently exposed to the same washing media, revealed that the level of ornithine decarboxylase activity was always higher in amoebae which had been suspended for 15 min in buffer prior to washing (Table 2.12). This higher activity may have resulted directly from the lower osmolality of the buffer and/or a relief of repression of enzyme activity caused by the higher osmolality of the HL5 medium. Alternatively cells suspended in HL5 medium were in a growth situation unlike those in non-nutrient phosphate buffer. Thus the consistently higher levels of ornithine decarboxylase activity in the latter cells may have reflected a transitory rise in enzyme activity during the initial stages of development. The lowest levels of ornithine decarboxylase activity were observed in extracts prepared from cells which had been resuspended for 15 min in HL5 medium and subsequently either washed in the same medium or not washed at all (Table 2.12). The highest activity was recorded in extracts prepared from cells which had been suspended in buffer and then washed in water. Similarly water washing of HL5 suspended cells resulted in an increase in enzyme activity to a level ten fold greater than that in non-washed cells (Table 2.12). In both HL5 and buffer suspended cells the ornithine decarboxylase activity of extracts prepared from buffer washed cells was greater than from those washed in 1M-NaCl solution. Thus, with the exception of HL5 washed cells, the level of ornithine decarboxylase activity in cell extracts was inversely related to the osmolality of the washing media. The incongruity of the enzyme activity in HL5 washed cells may reflect the fact that although HL5 had an osmolality less than either of the salt solutions, it was a growth medium whereas the other washing media were not.

The effect of the washing media on the levels of ornithine decarboxylase activity measured in vitro was a very rapid one. The process of washing amoebal pellets with 2x10ml aliquots of the appropriate washing medium took approximately 5 min, yet the variation in the enzyme activity resulting from this 5 min exposure were very large. However 5 min was not sufficient time for the effect

TABLE 2.12 THE EFFECT OF VARIOUS RESUSPENSION AND WASHING MEDIA ON THE LEVELS OF ORNITHINE DECARBOXYLASE ACTIVITY IN AMOEBAE

Amoebae were harvested during exponential growth, washed in water and resuspended at a cell density of $4x10^6$ cells/ml in either 17mM-phosphate buffer or HL5 growth medium (see methods section). After 15 min the contents of the flasks were split into 10ml aliquots and the amoebae harvested by centrifugation at 500g for 1 min in a bench centrifuge. Each cell pellet was then washed with two 10ml aliquots of the wash media shown below. The resulting pellets along with two unwashed pellets, were quickly resuspended in extraction buffer, cell extracts prepared and the levels of ornithine decarboxylase activity in the extracts determined as detailed in the methods section.

WASH MEDIUM	RESUSPENSION MEDIUM	
	HL5 MEDIUM ORNITHINE DECARBOXY-LASE ACTIVITY (nmol CO_2/min/mg protein)	17mM-PHOSPHATE BUFFER ORNITHINE DECARBOXY-LASE ACTIVITY (nmol CO_2/min/mg protein)
-	0.3	2.9
17mM-PHOSPHATE BUFFER	2.1	3.2
HL5 MEDIUM	0.2	0.6
WATER	2.9	4.8
0.5M-NaCl	1.1	1.5
1M-NaCl	0.9	1.2

of the washing media to reverse completely the effect on measured ornithine decarboxylase levels brought about by the prior incubation of amoebae in buffer or HL5 medium. Thus for all washing media used the enzyme activity was consistently higher in extracts prepared from buffer suspended cells (Table 2.12). It is quite likely that the change in the levels of enzyme activity measured in the various cell extracts after 5 min exposure to the washing media did not represent maximal response of the enzyme to the various osmotic changes. The rapidity of the response of ornithine decarboxylase levels to osmotic perturbation did however resemble the response time of P. polycephalum ornithine decarboxylase, where the time taken for maximal response to changes in the osmotic environment is 4 min (Mitchell & Kottas, 1979). This is in contrast to mammalian cells where the time taken for maximal increases/decreases in ornithine decarboxylase activity is in terms of hours not minutes (Munro et al., 1975; Friedman et al., 1977a; Perry & Oka, 1980). The fluctuations in enzyme activity observed in mammalian cells as a result of osmotic shock are much greater than the 2-4 fold variations observed in P. polycephalum ornithine decarboxylase activity. The amplitude of the response to changes in the osmolality of the media observed in D. discoideum amoebae, whilst larger than that observed in P. polycephalum cells, was not as great as the 1000-fold stimulation reported in mammalian cells (Friedman et al., 1977a; Perry & Oka, 1980).

The ornithine decarboxylase activity of a 1:1 mixture of the extracts prepared from unwashed HL5 and unwashed phosphate buffer suspended cells was determined. The level of enzyme activity was the average of the activities of the two individual extracts. Thus there was no evidence of inhibitory activity present in the HL5 extract. This result did not however rule out the presence of an inhibitory molecule in extracts prepared from HL5 suspended cells. As some active enzyme was still observable in these extracts the presence of free antizyme could perhaps not be expected. It has since been reported that in P. polycephalum changes in osmolality effect a reversible interconversion of two forms of ornithine decarboxylase which do not change state once the cell

has been disrupted (Mitchell et al., 1981). The rapidity of the increase in ornithine decarboxylase activity in D. discoideum by changes in osmolality suggest a post-translational control. This may involve interconversion of enzyme forms or alternatively the involvement of an antizyme type molecule which binds to the enzyme rendering it inactive.

The incongruity of the levels of ornithine decarboxylase activity in extracts prepared from cells which had been suspended and washed in HL5 medium was further investigated. Water washed amoebae were resuspended in a range of media comprising of components of HL5 medium and HL5 medium containing additional salts. After 15 min suspension the level of ornithine decarboxylase activity in the various amoebae was determined. The highest activity was measured in extracts prepared from cells which had been suspended in 6.25mM-phosphate buffer, the buffer used in HL5 medium (Table 2.13). The addition of yeast extract to the buffer reduced the level of ornithine decarboxylase activity in the amoebal extract by 36% whilst the presence of bactopeptone reduced it by 42% (Table 2.13). The greatest reduction in the level of enzyme activity, 68%, resulted from the addition of glucose to the buffer (Table 2.13). The level of ornithine decarboxylase in extracts prepared from cells suspended in HL5 medium containing all the constituents with the exception of glucose (86mM), was approximately three fold higher than in complete HL5 medium (Table 2.13). This most probably reflects the great increase in the osmolality of the medium resulting from the addition of 86mM-glucose. Thus, as would be predicted, 50% dilution of complete HL5 medium with water increased the measurable ornithine decarboxylase activity by 100% (Table 2.13). Attempts to further increase the osmolality of HL5 medium by the addition of sodium and potassium salts, failed to further depress the levels of ornithine decarboxylase activity measured *in vitro*. It was possible that, as observed by Mitchell and coworkers in P. polycephalum (1976), the low enzyme activity observed in these cell extracts was due to a less active form of ornithine decarboxylae not normally active *in vivo* but active *in vitro* apparently as a result of the elevated level

TABLE 2.13 THE EFFECT OF VARIOUS COMPONENTS OF HL5 GROWTH MEDIUM ON THE LEVELS OF ORNITHINE DECARBOXYLASE ACTIVITY IN AMOEBAE

Amoebae were harvested during exponential growth, washed in water and resuspended at a cell density of $4x10^6$ cells/ml in the media shown below. After 15 min the cells were harvested and the cell pellet resuspended, without washing, in extraction buffer. Cell extracts were prepared and the levels of ornithine decarboxylase activity in the cell extracts determined as detailed in the methods section.

RESUSPENSION MEDIUM	ORNITHINE DECARBOXYLASE ACTIVITY (nmol CO_2/min/mg protein)
6.25mM-PHOSPHATE BUFFER	3.8
6.25mM-PHOSPHATE BUFFER + YEAST EXTRACT	2.8
6.25mM-PHOSPHATE BUFFER + BACTOPEPTONE	2.2
6.25mM-PHOSPHATE BUFFER + GLUCOSE	1.2
HL5 MEDIUM - GLUCOSE	2.2
HL5 MEDIUM	0.8
HL5 MEDIUM (DILUTED 50% WITH WATER)	1.7
HL5 MEDIUM + 0.2M-KCl	0.9
HL5 MEDIUM + 1M-KCl	0.7
HL5 MEDIUM + 0.2M-NaCl	0.8
HL5 MEDIUM + 1M-NaCl	0.7

of cofactor present in the assay mixture.

In order to determine whether the ornithine decarboxylase activity in extracts exhibiting low enzyme levels had an altered dependence on *in vitro* PLP concentrations, extracts prepared from cells which had been suspended in various media were assayed at two different concentrations of PLP. As the data in Table 2.14 show, a ten fold reduction in the concentration of PLP in the assay mixture had a negligible effect on the measured enzyme activity of any of the extracts. As before the lowest activity was observed in the extracts prepared from cells which had been suspended in HL5 medium. Only a slight decrease in the level of ornithine decarboxylase activity, albeit equivalent to a 25% reduction, resulted when this extract was assayed at the lower PLP concentration (Table 2.14). Further investigations would be necessary before any conclusions could be reached as to the cofactor dependence of the measured *in vitro* ornithine decarboxylase activity of these low activity extracts.

The highest ornithine decarboxylase activities were measured in extracts prepared from cells which had been washed in water (Table 2.12). The effect of washing the amoebae with water on the measurable enzyme activity was thus further investigated as detailed in the legend to Table 2.15.

As before, the level of enzyme activity was very low in extracts prepared directly from unwashed cells harvested from HL5 medium (Table 2.15). Washing the cells with one 10ml portion of water resulted in a sixteen-fold increase in the level of enzyme activity detected *in vitro*. As this washing process was completed within 3 min, the changes in ornithine decarboxylase levels were extremely rapid. The level of enzyme activity measured continued to increase with further washing of the cells. Thus even after 10x10ml water washes the level of ornithine decarboxylase activity detected in the extracts was thirty-fold greater than in the unwashed cells and still increasing. The effect was believed to be a response to osmotic changes rather than the result of removing components of the HL5 medium from the cells, as HL5 had been shown not to inhibit ornithine decarboxylase activity when included in the assay mixture. Perry &

TABLE 2.14 THE EFFECT OF VARIATIONS IN THE ASSAY CONCENTRATION OF PLP ON THE LEVEL OF ORNITHINE DECARBOXYLASE ACTIVITY IN AMOEBAE HARVESTED FROM DIFFERENT MEDIA

Amoebae were harvested during exponential growth, washed in water and resuspended at a cell density of $4x10^6$ cells/ml in the media shown below. After 15 min the cells were harvested and the cell pellet resuspended, without washing, in extraction buffer. Cell extracts were prepared and the levels of ornithine decarboxylase activity in each extract determined both in the standard assay mixture containing 0.31mM-PLP and in an assay mixture containing 0.031mM-PLP.

RESUSPENSION MEDIUM	ORNITHINE DECARBOXYLASE ACTIVITY (nmol CO_2/min/mg protein)	
	0.31mM-PLP	0.031mM-PLP
HL5 MEDIUM	0.8	0.6
HL5 MEDIUM - GLUCOSE	1.5	1.6
17mM-PHOSPHATE BUFFER	2.9	3.0
WATER	5.3	5.9

TABLE 2.15 THE EFFECT OF WATER WASHING ON THE LEVEL OF ORNITHINE DECARBOXYLASE ACTIVITY IN AMOEBAE

A culture of exponentially growing amoebae was split into several aliquots and the cells harvested by centrifugation. The pellets were washed with 10 ml portions of water and the cells repelleted for the number of times indicated below. The pellets were then resuspended in extraction buffer, cell extracts prepared and the level of ornithine decarboxylase activity in the extracts determined using the standard assay mixture.

NUMBER OF WATER WASHES	ORNITHINE DECARBOXYLASE ACTIVITY (nmol CO_2/min/mg protein)
0	0.2
1	3.3
2	3.9
3	6.1
4	9.4
5	12.4
10	15.3

Oka (1970), reported that dilution of the incubation medium of mouse mammary gland cells by 53% with water results in approximately a thousand fold stimulation of ornithine decarboxylase activity over the initial level. Thus the response noted in D. discoideum amoebae may not represent a maximal value.

These experiments confirm that variations in ornithine decarboxylase activity measured in vitro can result from exposure of the amoebae to changes in their osmotic environment.

SECTION F - Optimization of the extraction, storage and assay of SAM-decarboxylase activity

Although putrescine was the major oligoamine present in D. discoideum amoebae (Table 1.2), these cells also contain considerable quantities of spermidine. Thus in addition to ornithine decarboxylase activity, amoebal extracts were expected to contain SAM-decarboxylase activity. Preliminary attempts to determine the level of SAM-decarboxylase activity in these extracts revealed a level of activity which was lower than anticipated. In addition the enzyme activity was unstable in vitro. Thus a series of experiments were carried out in an attempt to optimize the extraction, storage and assay of SAM-decarboxylase activity from D. discoideum amoebae.

PREPARATION AND STORAGE OF ENZYME EXTRACTS

Three amoebal preparations were obtained by suspending cells in extraction buffer for 15 min, by sonicating a suspension of cells in extraction buffer and by sonicating and subsequently centrifuging a suspension of cells in extraction buffer. The levels of SAM-decarboxylase activity in these preparations were determined immediately. Aliquots of each preparation were then stored either at 4°C or -18°C and the level of enzyme activity in these determined after 24h.

The highest level of SAM-decarboxylase activity was observed in the preparation in which amoebae had been suspended in extraction buffer for 15 min prior to the assay (Table 2.16). This result was similar to that observed in a parallel experiment involving ornithine decarboxylase activity (Table 2.1), and

TABLE 2.16 PREPARATION AND STORAGE OF SAM-DECARBOXYLASE ACTIVITY IN CELL EXTRACTS

Amoebae in exponential growth were harvested (see methods section), and resuspended at a cell density of 10^8 cells/ml in extraction buffer (5mM-phosphate buffer, pH7.6, 2mM-DTT, 1mM-$MgCl_2$, 0.1mM-EDTA, 0.1mM-PLP). Aportion of this cell suspension was sonicated (as detailed in the methods section) and some of this sonicated extract subsequently centrifuged at 1000g for 5 min. The level of SAM-decarboxylase activity in these three preparations (unsonicated, sonicated, and sonicated and centrifuged) was determined immediately (as detailed in the methods section). Portions of each extract were then stored either at 4°C or -18°C and the level of SAM-decarboxylase activity in each determined after 24h. The levels of enzyme activity are reported as percentages of that obtained in the fresh unsonicated preparation (0.4 pmol CO_2/min/mg protein = 100%)

CELL PREPARATION	% SAM-DECARBOXYLASE ACTIVITY		
	ASSAYED IMMEDIATELY	ASSAYED AFTER 24h STORAGE AT -18°C	ASSAYED AFTER 24h STORAGE AT 4°C
UNSONICATED	100	45	38
SONICATED	53	25	18
SONICATED AND CENTRIFUGED	58	25	23

suggested that in these non-sonicated cells considerable enzyme activity was released due to lysis of amoebae either by the extraction or assay buffer. Sonication reduced the level of SAM-decarboxylase activity in cell extracts by 50% (Table 2.16), indicating that the enzyme activity was, like ornithine decarboxylase activity, unstable *in vitro*. Cohn et al. (1977) found that storage of the purified enzyme from *S. cerevisiae* at 2-4°C results in no loss of activity over a 3-month period. The same preparation rapidly loses activity at 37°C unless protected by the addition of bovine serum albumin. However the majority of SAM-decarboxylase enzymes reported in literature have been found to be relatively stable *in vitro*. Centrifugation of the sonicated extract at 1000g for 5 min resulted in only a slight increase in the level of enzyme activity measured. Measurable SAM-decarboxylase activity decreased by about 50% on storage of the enzyme extract either at 4°C or -18°C. The highest level of enzyme activity was retained in unsonicated cell preparations and slightly less activity was lost when the preparation was stored at -18°C rather than 4°C (Table 2.16). Although considerable loss of SAM-decarboxylase activity occurred during storage (Table 2.16), the percentage loss was not quite as large as that which occurred when the ornithine decarboxylase activity of a similarly stored extract was determined (Table 2.1). However in view of the very low level of SAM-decarboxylase activity measured *in vitro* in *D. discoideum* amoebal extracts, such a high loss of activity was unacceptable.

STABILIZATION OF ENZYME ACTIVITY *IN VITRO*

In an attempt to stabilize the *in vitro* enzyme activity amoebae were suspended in a variety of extraction buffers as detailed in Table 2.17. These cell suspensions were immediately sonicated, centrifuged and the levels of SAM-decarboxylase activity determined. These extracts were stored at -18°C and the levels of enzyme activity determined after 24h.

As in the previous experiment, over 50% of SAM-decarboxylase activity was lost when cells in standard extraction buffer were stored at -18°C for 24h. Like ornithine decarboxylase activity (Table 2.2), SAM-decarboxylase activity

TABLE 2.17 STABILITY OF SAM-DECARBOXYLASE ACTIVITY IN MODIFIED EXTRACTION BUFFERS

Exponentially growing amoebae were harvested and suspended in the various extraction buffers at an approximate cell density of 10^8 cells/ml. Each cell suspension was sonicated and centrifuged (as detailed in the methods section) and the SAM-decarboxylase activity of the resulting cell extracts assayed immediately (as detailed in the methods section). The extracts were stored at -18°C and the level of enzyme activity in each extract determined after 24h. The levels of enzyme activity are reported as percentages of that obtained in the extract prepared in standard extraction buffer and assayed immediately (0.51 pmol CO_2/min/mg protein = 100%).

ADDITIONS TO STANDARD EXTRACTION BUFFER	% SAM-DECARBOXYLASE ACTIVITY	
	ASSAYED IMMEDIATELY	ASSAYED AFTER STORAGE AT -18°C
CONTROL	100	45
PMSF (2.5mM)	88	53
TLCK (0.1mM)	92	47
BOVINE SERUM ALBUMIN (1mg/ml)	86	43
SUCROSE (0.25M)	98	49
GLYCEROL		
5% (v/v)	114	76
10% (v/v)	100	106
20% (v/v)	98	106
30% (v/v)	110	108
DIMETHYLSULPHOXIDE		
5% (v/v)	78	37
10% (v/v)	94	88
20% (v/v)	92	96
30% (v/v)	96	106

TABLE 2.17 STABILITY OF SAM-DECARBOXYLASE ACTIVITY IN MODIFIED EXTRACTION BUFFERS

Exponentially growing amoebae were harvested and suspended in the various extraction buffers at an approximate cell density of 10^8 cells/ml. Each cell suspension was sonicated and centrifuged (as detailed in the methods section) and the SAM-decarboxylase activity of the resulting cell extracts assayed immediately (as detailed in the methods section). The extracts were stored at -18°C and the level of enzyme activity in each extract determined after 24h. The levels of enzyme activity are reported as percentages of that obtained in the extract prepared in standard extraction buffer and assayed immediately (0.51 pmol CO_2/min/mg protein = 100%).

ADDITIONS TO STANDARD EXTRACTION BUFFER	% SAM-DECARBOXYLASE ACTIVITY	
	ASSAYED IMMEDIATELY	ASSAYED AFTER STORAGE AT -18°C
CONTROL	100	45
PMSF (2.5mM)	88	53
TLCK (0.1mM)	92	47
BOVINE SERUM ALBUMIN (1mg/ml)	86	43
SUCROSE (0.25M)	98	49
GLYCEROL		
5% (v/v)	114	76
10% (v/v)	100	106
20% (v/v)	98	106
30% (v/v)	110	108
DIMETHYLSULPHOXIDE		
5% (v/v)	78	37
10% (v/v)	94	88
20% (v/v)	92	96
30% (v/v)	96	106

was not protected by the presence in the extraction buffer of the proteinase inhibitors PMSF (Gold, 1967) or TLCK (Shaw et al., 1965). Thus if loss of activity is due to proteolysis, the enzyme involved must be insensitive to these inhibitors. A variety of substances were used to raise the osmolality of the extraction buffer in an attempt to stabilize the level of SAM-decarboxylase activity.

The data in Table 2.17 show that the percentage of SAM-decarboxylase activity remaining after 24h storage in extraction buffer containing 0.25M-sucrose or bovine serum albumin (1mg/ml) was not significantly greater than in the extract prepared in standard extraction buffer. Both dimethylsulphoxide and glycerol at concentrations of 10% (v/v) and greater, stabilized SAM-decarboxylase activity during storage at -18°C. The data in Table 2.17 show that approximately 100% of the enzyme activity recorded in the fresh extract was retained after 24h storage. Thus in amoebal extracts SAM-decarboxylase activity resembled ornithine decarboxylase activity in as much as the stability of the enzyme activity *in vitro* was improved by extraction buffers containing glycerol or dimethylsulphoxide. As high retention of enzyme activity after storage was observed in extracts containing 10% (v/v) glycerol, in all subsequent SAM-decarboxylase experiments this extraction buffer was used in the preparation of cell extracts.

SONICATION

The results shown in Table 2.16 suggested that the SAM-decarboxylase activity in extracts prepared in non-glycerol extraction buffer, was destroyed by sonication. Thus the enzyme activity was almost 50% higher in unsonicated preparations. This apparent lability of SAM-decarboxylase to sonication was not however observed if 10% (v/v) glycerol was included in the extraction buffer. Rather, after 1 min sonication (4 @ 15s bursts) in 10% (v/v) glycerol extraction buffer the level of SAM-decarboxylase activity was 50% higher than in unsonicated preparations in either glycerol or non-glycerol extraction buffer. Thus in all subsequent experiments SAM-decarboxylase extracts were

prepared by sonication and centrifugation in 10% (v/v) glycerol extraction buffer as detailed in the methods section.

EFFECT OF PROTEIN CONCENTRATION IN ENZYME EXTRACT

Experiments on D. discoideum amoebal extracts had shown that the level of ornithine decarboxylase activity measured in vitro was inversely proportional to the protein concentration present in the extract (Figure 2.1 and Table 2.3). Thus parallel experiments were carried out to determine whether the protein concentration of cell extracts and the level of SAM-decarboxylase activity were similarly related.

The data in Figure 2.10 show that unlike ornithine decarboxylase activity, the level of SAM-decarboxylase activity measured in vitro is directly proportional to the protein concentration of the extract. Over the range 0.1-1.4mg protein/assay the specific activity of SAM-decarboxylase varied only slightly. Thus in all subsequent experiments concentrated amoebal extracts were used (0.5-1mg protein/assay).

TIME COURSE OF ASSAY

Since non-saturating levels of substrate were being used in the SAM-decarboxylase assay, it was important to determine the time period over which the enzyme activity measured was linear. The results in Figure 2.11 show that in assays carried out in 1μM-S-adenosyl-L-methionine the reaction was linear for 45 min, thus the standard assay time of 30 min is within the linear period of the assay curve.

TEMPERATURE AND pH OPTIMA

The temperature optimum for the in vitro assay of SAM-decarboxylase activity in D. discoideum amoebal extracts was 37°C. Thus, like ornithine decarboxylase activity the in vitro temperature optimum of SAM-decarboxylase activity in D. discoideum amoebal extracts is higher than the optimum temperature for growth and development (22-27°C).

The pH optimum for the in vitro assay of SAM-decarboxylase activity was determined. The two assay buffers used, 0.1M-phosphate buffer and 65mM-Tris HCl,

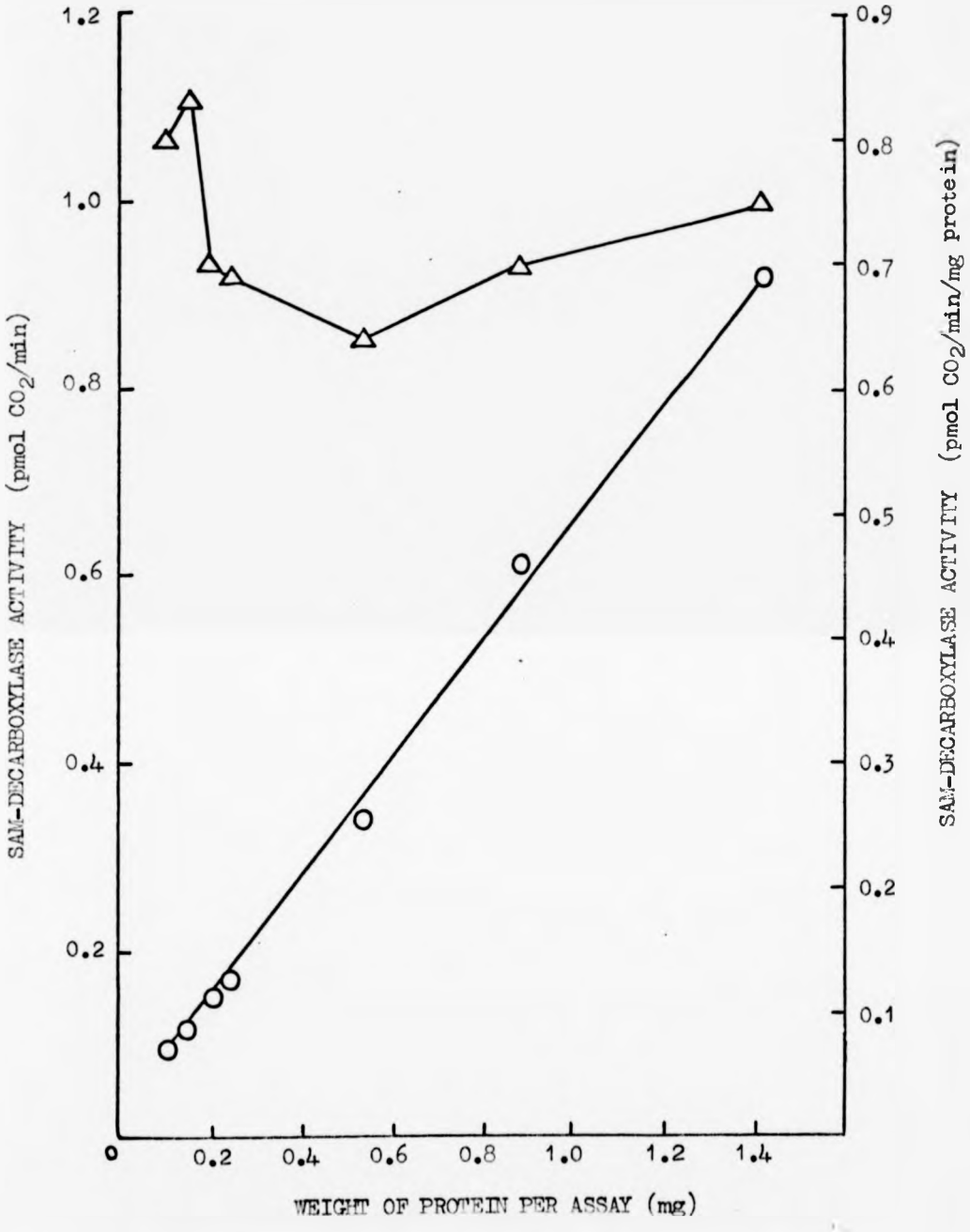


FIGURE 2.10 SAM-DECARBOXYLASE ACTIVITY AS A FUNCTION OF PROTEIN CONCENTRATION

Amoebae were harvested and suspended in 10% (v/v) glycerol extraction buffer at a density of approx. 10^9 cells/ml. After sonication and centrifugation as detailed in the methods section, the cell extract was diluted with extraction buffer to give a range of protein concentrations. These extracts were assayed for SAM-decarboxylase activity as detailed in the methods section. O = SAM-decarboxylase activity (pmol CO_2/min). Δ = SAM-decarboxylase activity (pmol CO_2/min/mg protein).

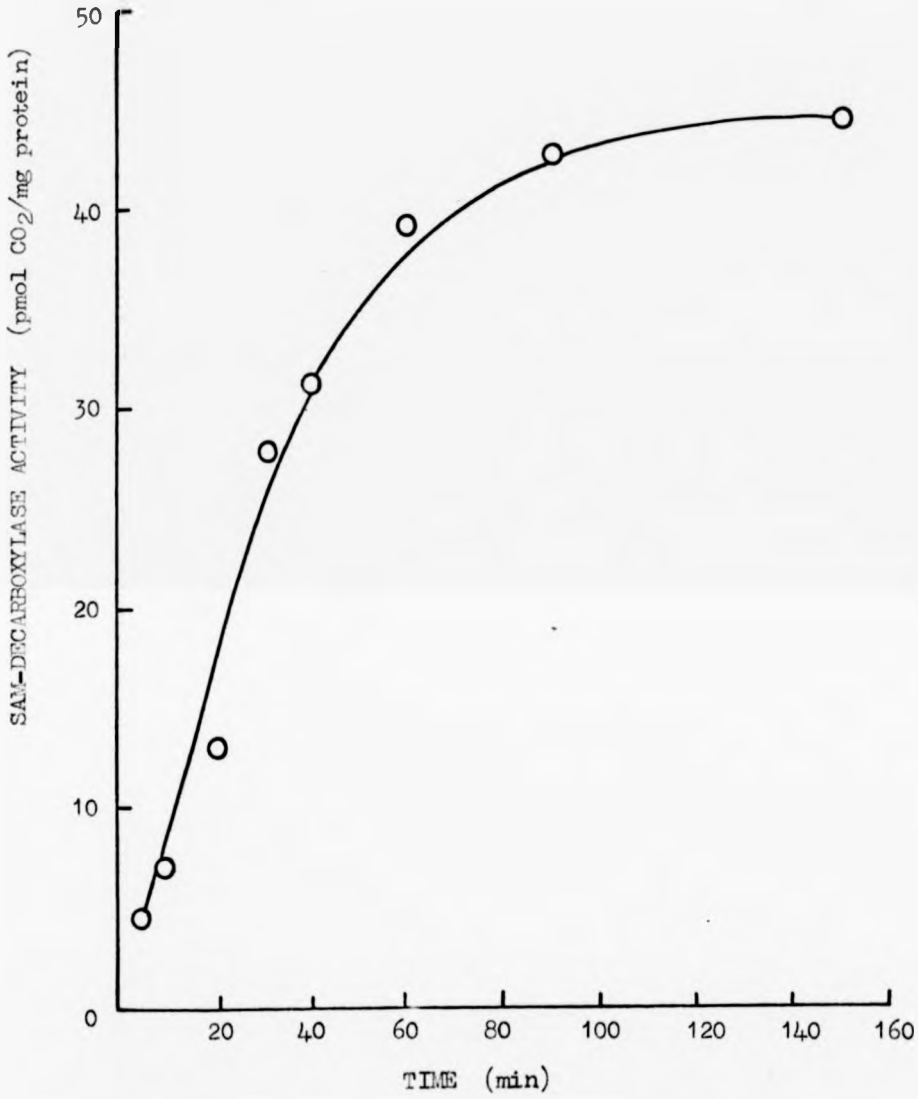


FIGURE 2.11 SAM-DECARBOXYLASE ACTIVITY AS A FUNCTION OF TIME

Assays were carried out under standard assay conditions (see methods section) except that the reaction was interrupted at the various time intervals shown.

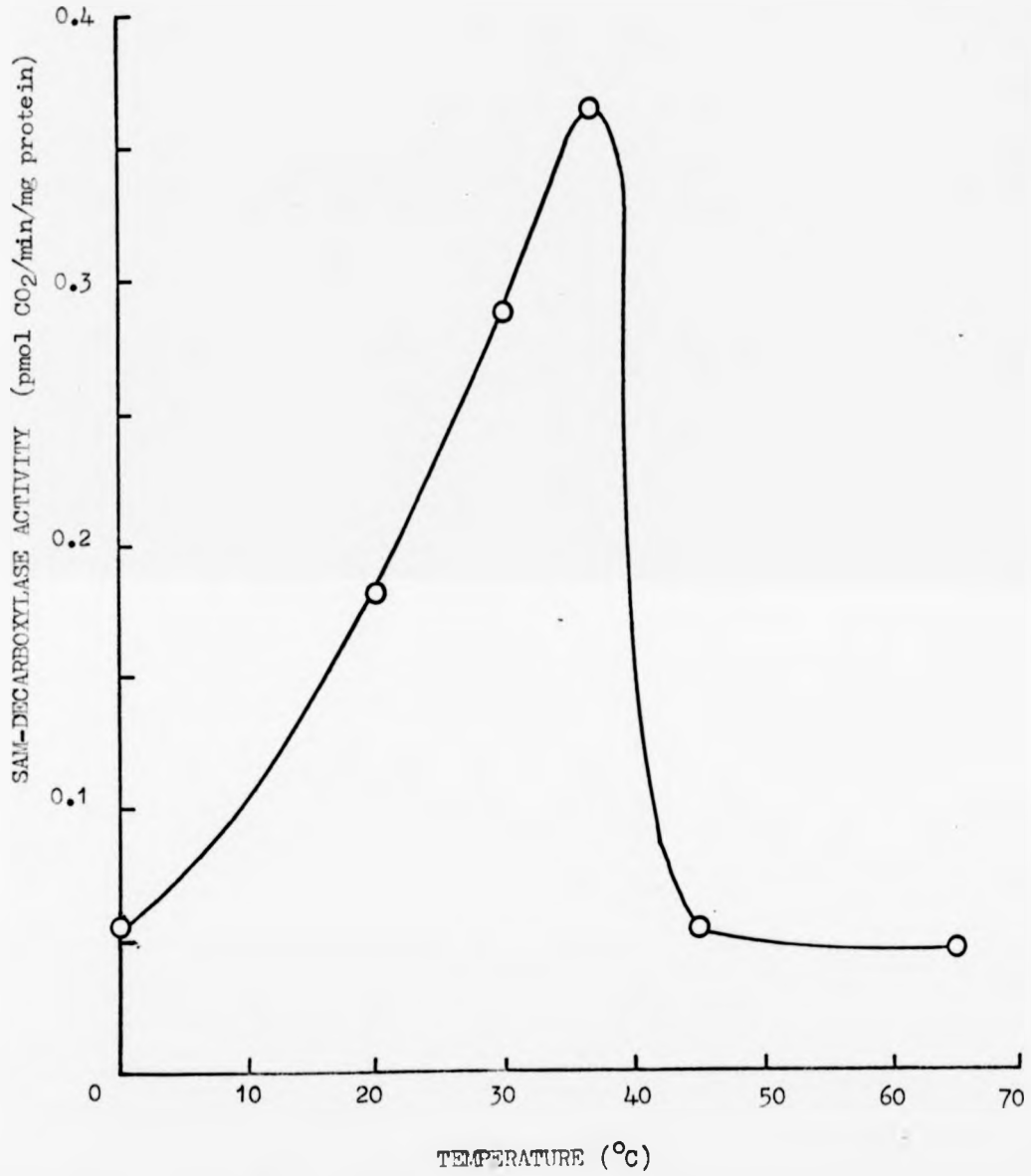


FIGURE 2.12 TEMPERATURE OPTIMUM OF SAM-DECARBOXYLASE ACTIVITY

Assays were carried out under standard conditions (see methods section) except that incubations were carried out at the temperatures indicated.

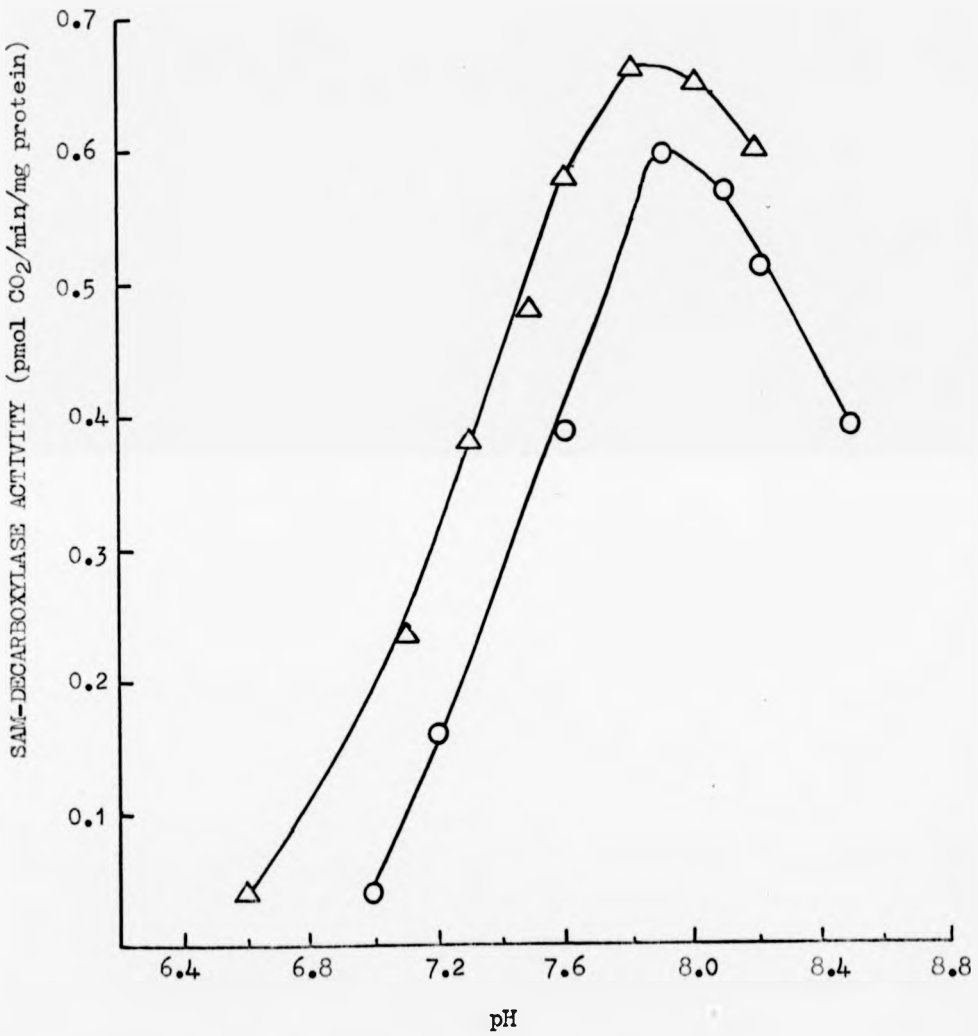


FIGURE 2.13 pH OPTIMUM OF SAM-DECARBOXYLASE ACTIVITY

Assays were carried out under standard assay conditions (see methods section), except that the pH of the 0.1M-sodium phosphate assay buffer (O) was adjusted such that the final pH of the assay mixture was as shown. A series of assays were also carried out in 65mM-Tris/HCl assay buffer (Δ) at the various pH values shown.

are those used by other workers in SAM-decarboxylase experiments. In both buffers maximal SAM-decarboxylase activity was obtained at pH values between 7.8 and 8. This was only slightly higher than the pH optima of SAM-decarboxylase from other systems e.g. pH optimum for A. nidulans is 7.6 (Stevens et al., 1976), for human placenta 7.2, (Torta et al., 1977) and for rat liver 7-7.8 (Demetriou et al., 1978).

COMPOSITION OF THE EXTRACTION BUFFER

The standard extraction buffer used in the preparation of D. discoideum SAM-decarboxylase extracts was the same as used for the extraction of ornithine decarboxylase activity. As part of the series of experiments designed to optimize the extraction and assay conditions of SAM-decarboxylase activity, the effect of the various components of the extraction buffer on the level of SAM-decarboxylase activity measured in vitro was investigated. Enzyme extracts were prepared in the various extraction buffers described in Table 2.18, all of which contained 10% (v/v) glycerol, and the level of SAM-decarboxylase activity determined.

As the data in Table 2.18 show, 50% of the SAM-decarboxylase activity present in normal extraction buffer was lost when the enzyme was extracted into 5mM-phosphate buffer containing only 10% (v/v)-glycerol. In enzyme extracts prepared in buffer containing 2mM-DTT the level of SAM-decarboxylase activity measured was the same as in extracts prepared in standard extraction buffer (Table 2.18). This result was not unexpected as a thiol-protecting agent has been found to be a necessary inclusion in the extraction buffers of SAM-decarboxylase activity from many other systems (Pösö et al., 1975b; Stevens et al., 1976; Pegg, 1977a; Suresh & Adiga, 1977; Cohn et al., 1977; Demetriou et al., 1978). This requirement of SAM-decarboxylase stability for DTT suggests that, like ornithine decarboxylase (Table 2.6), thiol groups are involved in the catalytic process of SAM-decarboxylase.

PLP is a known cofactor of many decarboxylases including D. discoideum ornithine decarboxylase. No ornithine decarboxylase activity was detected in

TABLE 2.18 COMPONENTS OF EXTRACTION BUFFER REQUIRED FOR SAM-DECARBOXYLASE ACTIVITY

Enzyme extracts were prepared as usual (see methods section) in the various extraction buffers described below. All extracts contained 10% (v/v) glycerol and unless otherwise stated, 5mM-phosphate buffer, pH7.6. SAM-decarboxylase activity was assayed under standard conditions (see methods section) and the results are reported as percentages of the level of enzyme activity measured in standard extraction buffer i.e. 1mM-$MgCl_2$, 2mM-DTT, 0.1mMPLP, 0.1mM EDTA (0.95 pmol CO_2/min/mg protein = 100%)

COMPOSITION OF EXTRACTION BUFFER	% SAM-DECARBOXYLASE ACTIVITY
1mM-$MgCl_2$, 2mM-DTT, 0.1mM-PLP, 0.1mM-EDTA	100
NO ADDITIONS	49
1mM-$MgCl_2$	36
0.1mM-EDTA	51
0.1mM-PLP	36
2mM-DTT	100
1mM-$MgCl_2$, 0.1mM-EDTA	56
0.1mM-PLP, 2mM-DTT	91
1mM-$MgCl_2$, 0.1mM-EDTA, 2mM-DTT	79
1mM-$MgCl_2$, 0.1mM-PLP, 2mM-DTT	77
0.05mM-borate buffer, pH7.6, 1mM-$MgCl_2$, 2mM-DTT, 0.1mM-PLP, 0.1mM-EDTA	60

extracts prepared in extraction buffer lacking PLP and assayed in its absence. PLP was not a constituent of the standard SAM-decarboxylase assay mixture and its addition to the 5mM-phosphate extraction buffer was slightly inhibitory. The addition of 0.1mM-PLP to DTT-containing phosphate extraction buffer decreased the level of SAM-decarboxylase activity by 10%. This probably resulted from the formation of an adduct between DTT and PLP which effectively reduced the concentration of the thiol-protecting agent present in the extraction buffer. These results suggested that PLP was not a cofactor for D. discoideum SAM-decarboxylase.

The chelation of heavy metal ions which may result from the addition to the 5mM-phosphate buffer of 0.1mM-EDTA, similarly resulted in no significant increase in the level of SAM-decarboxylase activity. The enzyme activity observed in extracts in which the extraction buffer contained 1mM-$MgCl_2$ was lower than in those extracts prepared in 5mM-phosphate buffer only (Table 2.18). This suggests that D. discoideum SAM-decarboxylase is not activated by Mg^{2+} ions (see page 175). In extracts prepared in 5mM-phosphate buffer containing both Mg^{2+} and EDTA the slight inhibition caused by the presence of Mg^{2+} was relieved. This possibly results from chelation of the metal ions by the EDTA thus lowering the effective concentration of the cation. Addition to the phosphate buffer of other combinations of the normal components of the standard extraction buffer confirmed that only DTT was necessary for extraction of maximal SAM-decarboxylase activity (Table 2.18). When the extracts shown in Table 2.18 were stored overnight only those in buffer containing DTT retained SAM-decarboxylase activity.

The level of enzyme activity detected in extracts prepared in 0.05mM-borate buffer containing all the other components of the standard extraction buffer, was 40% lower than in similar extracts prepared in standard phosphate extraction buffer. Similarly extracts prepared in borate buffer and assayed in an incubation mixture in which the 0.1M-phosphate buffer, pH8, had been replaced by borate buffer of the same pH, had lowered levels of SAM-decarboxylase activity. Thus

for maximal extraction of SAM-decarboxylase activity from D. discoideum amoebae only DTT was necessary in the phosphate extraction buffer. However as the enzyme activity was not lowered by the inclusion of PLP, Mg^{2+} and EDTA to the DTT-containing phosphate extraction buffer, no change was made in subsequent experiments to the composition of the standard extraction buffer.

Despite this series of experiments, even under the optimum conditions determined, the specific activity of SAM-decarboxylase in D. discoideum amoebal extracts was much lower than have been reported for other systems containing comparable oligoamine levels. This suggested that the extraction and assay of this enzyme require further study.

SECTION G - The kinetic parameters of SAM-decarboxylase activity

The substrate dependence of SAM-decarboxylase activity from D. discoideum was investigated as detailed in the legend to Figure 2.14. Normal hyperbolic saturation kinetics were observed producing a linear Lineweaver-Burk plot (1934), (Figure 2.14). This was extrapolated to estimate a Km value for S-adenosyl-L-methionine.

Under these conditions S-adenosyl-L-methionine was saturating at 2μM. As the concentration of substrate in the standard assay was only 1μM, enzyme activity was being routinely assayed at non-saturating substrate concentrations. From the Lineweaver-Burk plot (1934), (Figure 2.14), a Km of 1.7μM SAM and a V max of 1.5 pmolCO_2/min/mg protein was determined. The Km values for SAM-decarboxylase reported in literature for most other eucaryotic systems are higher 20-160μM, (Stevens et al., 1976; Porta et al., 1977, Demetriou et al., 1978). Mitchell & Rusch (1973) determined a Km of 5μM for SAM-decarboxylase from P. polycephalum. The V max value was also much lower than in other eucaryotic systems possibly reflecting a difference between the level of SAM-decarboxylase activity present in vivo and that measured under standard assay conditions in amoebal extracts.

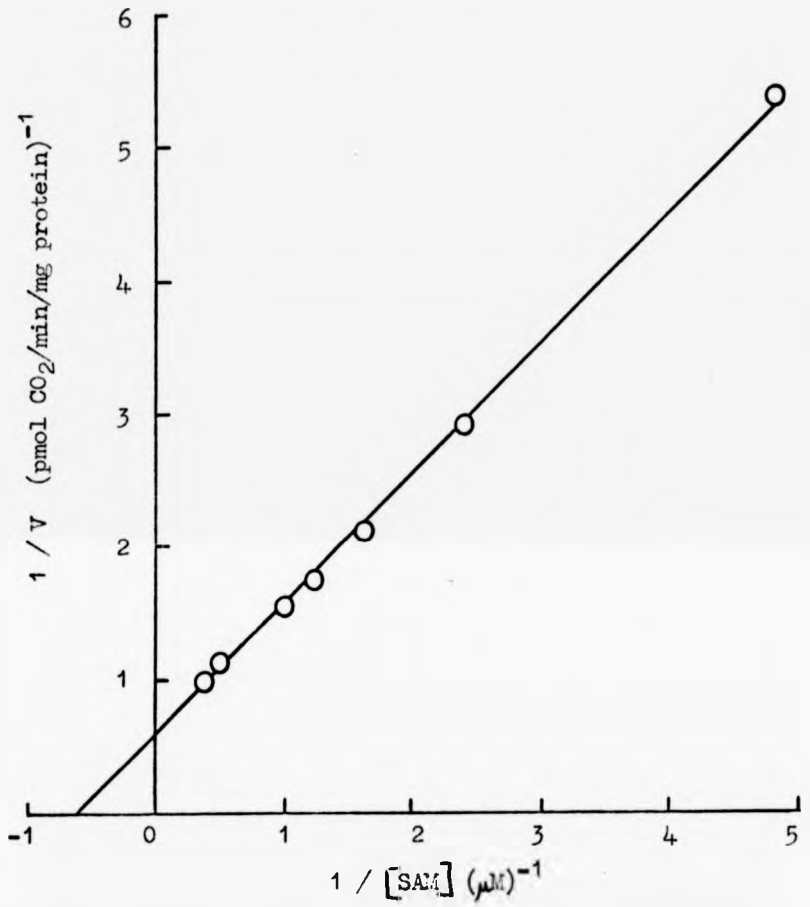


FIGURE 2.14 THE EFFECT OF VARYING SUBSTRATE CONCENTRATIONS ON SAM-DECARBOXYLASE ACTIVITY

The SAM-decarboxylase activity of amoebal extracts was assayed as usual (see methods section) at the indicated SAM concentrations. The data were plotted by the method of Lineweaver & Burk (1934). Assays with boiled enzyme were used to correct for non-enzymic decarboxylation at each concentration of substrate.

SECTION H - Levels of SAM-decarboxylase activity during development

Spermidine levels were observed to decrease substantially during the 24h developmental period which follows the removal of D. discoideum from nutrients (Figure 1.9). As SAM-decarboxylase is believed to be the rate limiting enzyme in the biosynthesis of spermidine, the activity of this enzyme was monitored during the development of amoebae into sorocarps.

The results shown in Figure 2.15 suggest that the level of SAM-decarboxylase activity in amoebae fell by 27% during the 30 min (approx.) taken to place the cells on developmental filters and immediately reharvest them. The level of SAM-decarboxylase activity continued to decrease sharply and after 1h on developmental filters it had fallen to 29% of that detected in amoebal extracts. The level of enzyme activity continued to decrease steadily and at 14h only 8% of the SAM-decarboxylase activity present in amoebal extracts, remained. During the final 10h of development the SAM-decarboxylase activity appeared to level off. However, as cellular protein levels fall throughout development this apparent levelling off of enzyme activity may in fact mask a continuing decrease in the level of the enzyme in the developing cells.

SECTION I:- Control of SAM-decarboxylase activity

IN VIVO EFFECT OF OLIGOAMINES

The in vivo effect of oligoamines on the levels of SAM-decarboxylase activity in D. discoideum amoebae was investigated as detailed in the legend to Figure 2.16. The presence of extracellular putrescine, 1,3-diaminopropane, spermidine or spermine at concentrations between 0.1 and 10mM, had no significant effect on the level of SAM-decarboxylase activity measured in the cell extracts (Figure 2.16).

IN VITRO EFFECT OF OLIGOAMINES

The endogenous levels of oligoamines in D. discoideum amoebae had been determined (Table 1.2) and putrescine, 1,3-diaminopropane and spermidine were found to be present in millimolar concentrations. Thus endogenous oligoamines

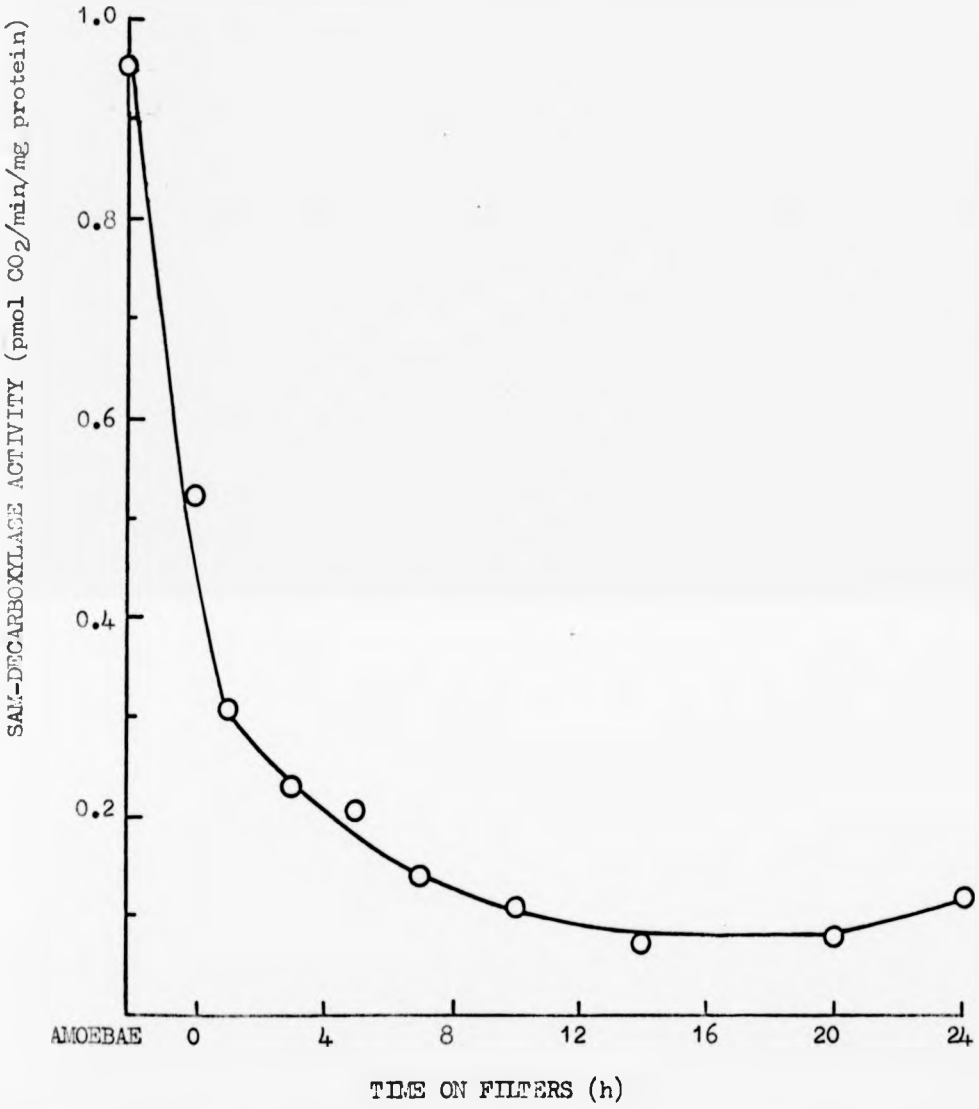


FIGURE 2.15 SAM-DECARBOXYLASE ACTIVITY DURING DEVELOPMENT OF D. DISCOIDEUM

Amoebae, harvested during exponential growth, were used to prepare developmental Millipore filters. At intervals during the 24h developmental period, the level of SAM-decarboxylase activity in the developing cells was determined in the usual manner (see methods section). A portion of the amoebal suspension used in the preparation of the developmental filters was retained, and the level of enzyme activity present in these cells was also determined.

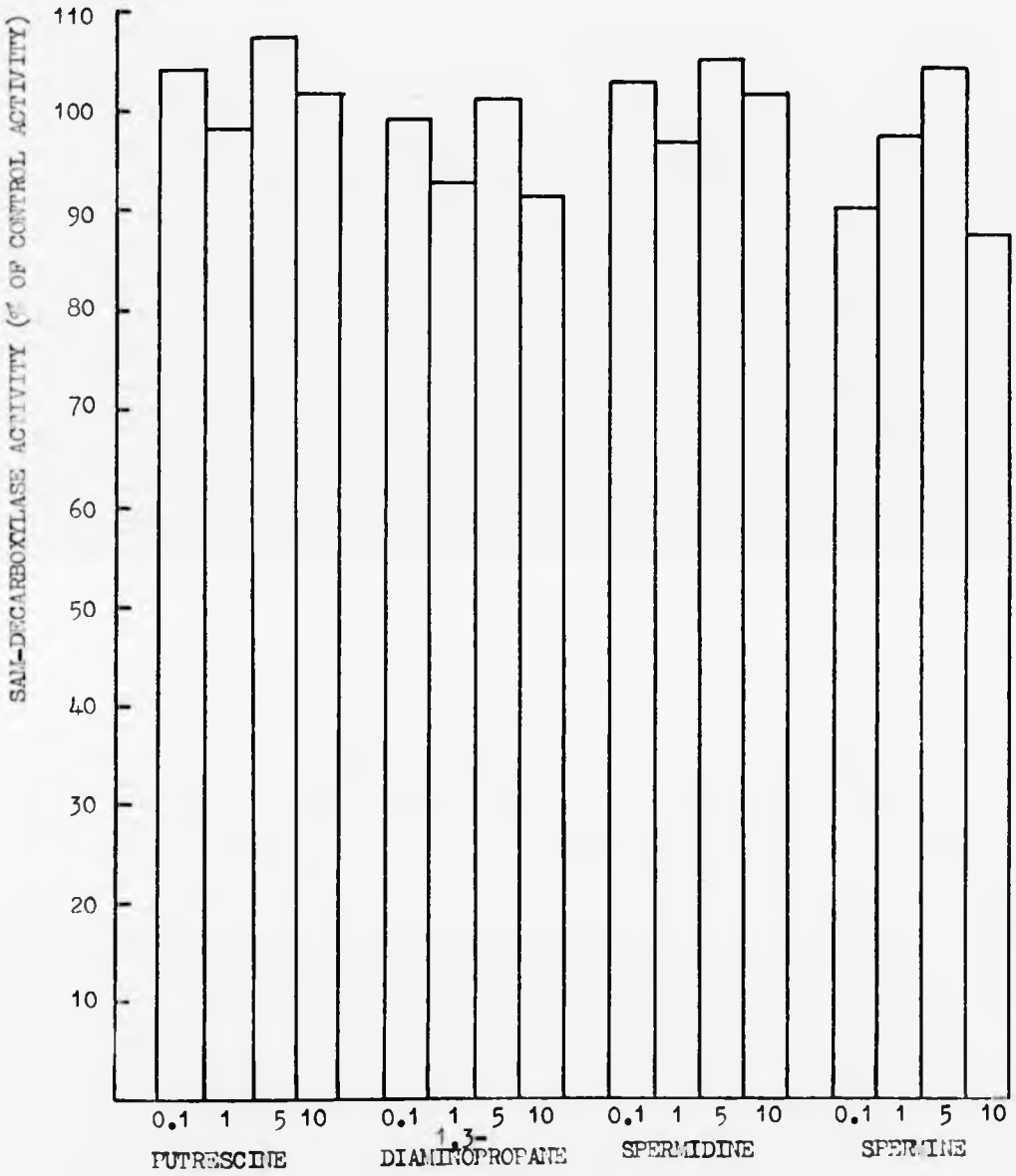


FIGURE 2.16 THE IN VIVO EFFECT OF OLIGOAMINES ON THE LEVEL OF SAM-DECARBOXYLASE ACTIVITY

Amoebae were grown for 72h in HL5 medium containing the concentrations of oligoamines shown. The cells were harvested in exponential growth and the level of SAM-decarboxylase activity in the cell extracts determined as usual (see methods section). Each value shown represents the average of five experimental flasks.

may have a direct effect on SAM-decarboxylase activity. The *in vitro* effect of oligoamines on the levels of SAM-decarboxylase activity in *D. discoideum* amoebae was investigated as detailed in the legend to Figure 2.17.

The effect on the level of SAM-decarboxylase activity of adding putrescine, 1,3-diaminopropane, spermidine and spermine to the assay mixture is shown in Figure 2.17. None of the four oligoamines tested, significantly reduced the activity of SAM-decarboxylase activity measured in amoebal extracts. Even at high concentrations, 10mM, inhibition of enzyme activity by any of the oligoamines tested was less than 35% (Figure 2.17). Although the intracellular concentration of oligoamines in *D. discoideum* amoebae was high, the concentration of free amines was unlikely to reach such levels. Thus a control of spermidine synthesis by oligoamines which involves decreasing the supply of decarboxylated-SAM is unlikely.

EFFECT OF PUTRESCINE AND MAGNESIUM

The SAM-decarboxylases investigated so far generally fall into one of three classes; (a) procaryotic enzymes which are Mg^{2+} dependent but putrescine insensitive e.g. *E. coli* and *Azobacter Vinelandii* (Wickner et al., 1970; Coppoc et al., 1971), (b) lower eucaryotic enzymes which are not influenced either by divalent cations or putrescine e.g. *Tetrahymena pyriformis* and *P. polycephalum* (Mitchell & Rusch, 1973; Pösö et al., 1975b) and (c) the enzymes from animals, some higher plants and *S. cerevisae* which are stimulated by putrescine (Coppoc et al., 1971; Jänne et al, 1971b; Jänne & Williams-Ashman, 1971b; Suresh & Adiga, 1977). Addition of putrescine and Mg^{2+}, at a range of concentrations, to the SAM-decarboxylase assay mixture failed to increase the level of enzyme activity measured in *D. discoideum* amoebal extracts (Figure 2.17). Thus *D. discoideum* SAM-decarboxylase seems to fall into the final class of enzymes, maximum activity being observed in the absence of either putrescine or Mg^{2+}.

EFFECT OF 1,4-DIAMINOBUTANONE

1,4-diaminobutanone is a powerful competitive inhibitor of ornithine decarboxylase activity from eucaryotic sources e.g. *A. nidulans* and rat liver

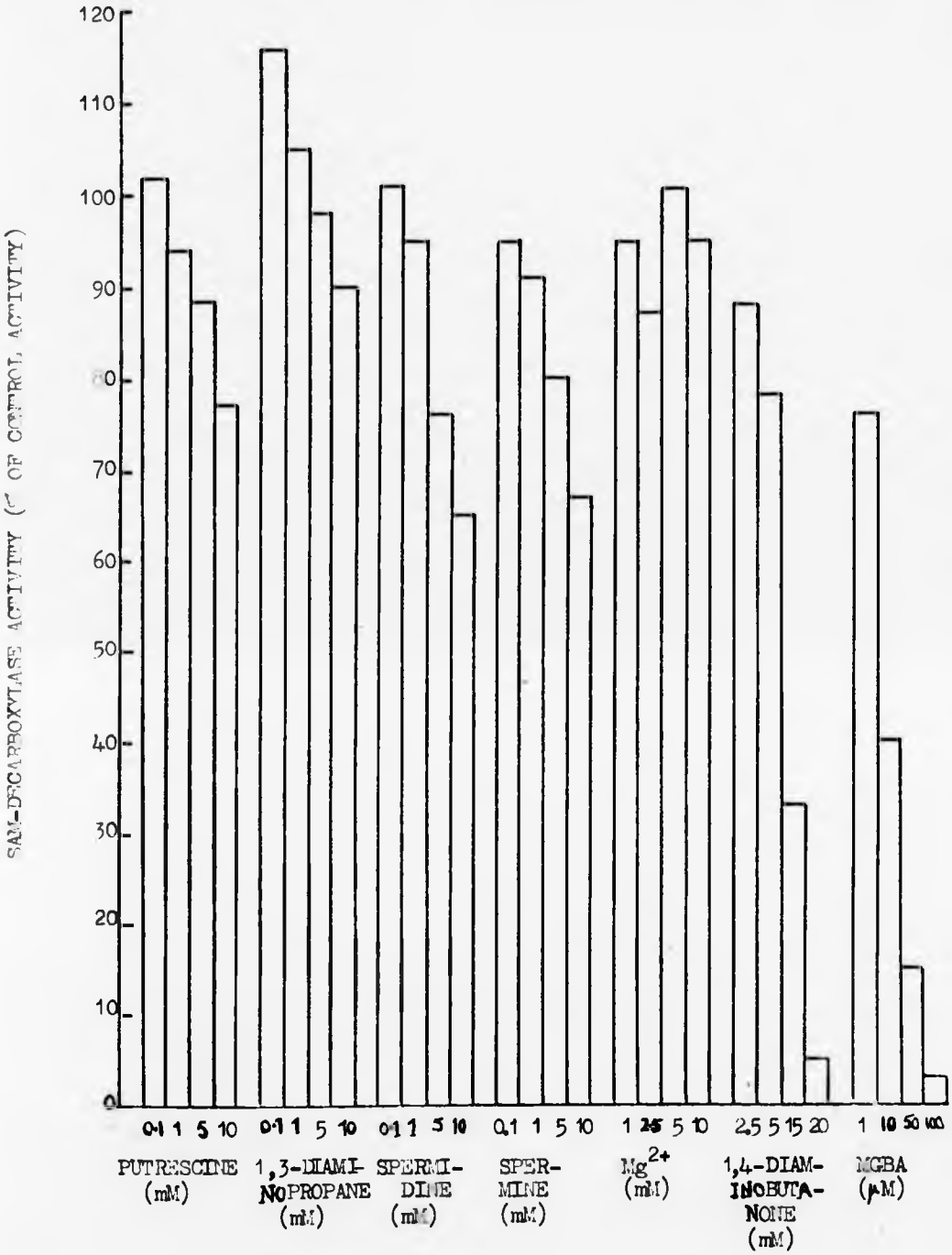


FIGURE 2.17 THE IN VITRO EFFECT OF OLIGOAMINES, Mg^{2+} AND MGBA ON SAM-DECARBOXYLASE ACTIVITY

Amoebae were harvested in exponential growth and the SAM-decarboxylase activity of the cell extracts determined as usual (see methods section), in which the water had been replaced by appropriate solutions of oligoamines, $MgCl_2$, and MGBA such that the final concentrations were as shown below.

(Stevens et al., 1978). When the same workers investigated the effect of the inhibitor on SAM-decarboxylase from several sources, they found that only those enzymes activated by putrescine e.g. rat liver and prostate and A. nidulans, were also activated by 1,4-diaminobutanone but to a lesser extent. As the data in Figure 2.17 show putrescine-insensitive SAM-decarboxylase from D. discoideum was not activated by 1,4-diaminobutanone. Rather this substance at high concentrations caused inhibition; at 15mM 1,4-diaminobutanone inhibited SAM-decarboxylase activity measured in vitro by 67%, and at 20mM, 95% inhibition was observed (Figure 2.17). At such high concentrations this inhibition is unlikely to be specific, but rather the result of binding between the reactive carbonyl group on 1,4-diaminobutanone and various function groups on the enzyme.

EFFECT OF METHYLGLYOXAL BIS(AMIDINOHYDRAZONE)

Williams-Ashman & Schenone (1972), discovered that methylglyoxal bis(amidinohydrazone) [MGBA], is a potent and specific inhibitor of eucaryotic putrescine-stimulated SAM-decarboxylase. As such it is potentially useful as a means of decreasing cellular spermidine levels. Thus 1.5μM-MGBA causes 50% inhibition of purified rat prostate SAM-decarboxylase whereas the putrescine-insensitive enzyme from E. coli is approximately two orders of magnitude less sensitive (Williams-Ashman & Schenone, 1972). As anticipated, relatively high concentrations of MGBA were required to cause inhibition of putrescine-insensitive SAM-decarboxylase from D. discoideum. As can be seen from Figure 2.17, 10μM-MGBA was required to cause 60% inhibition of SAM-decarboxylase activity and 95% inhibition occurs only in the presence of 0.1mM-MGBA.

SECTION J - Arginine decarboxylase activity in D. discoideum amoebae

In addition to the direct decarboxylation of L-ornithine by ornithine decarboxylase, certain bacteria and higher plants possess an additional pathway for putrescine biosynthesis involving the decarboxylation of L-arginine by arginine decarboxylase (EC4.1.1.19) to agmatine and the subsequent hydrolysis of agmatine to putrescine and urea by agmatine ureohydrolase (EC3.5.3.11) [Fig-

ure 1.1; Tabor & Tabor, 1964; Morris & Pardee, 1966]. In some organisms e.g. B. stearothermophilus and K. aerogenes, this latter pathway predominates. Such bacteria possess very low ornithine decarboxylase activities but high arginine decarboxylase activities. (Stevens et al., 1978). In many eucaryotes the ornithine pathway is the only synthetic route for putrescine. In some organisms however, e.g. E. coli both pathways are present, although decarboxylation of ornithine is the major source of cellular oligoamines. Although high levels of ornithine decarboxylase have been detected in D. discoideum extracts, experiments were carried out to determine whether the second pathway also existed in these cells.

The extraction buffer and assay conditions used were essentially those detailed by Wu & Morris (1973a,b). Amoebae were harvested and resuspended in extraction buffer (0.05M-Tris HCl,pH 7.6, 1mM-EDTA, 1mM-DTT), sonicated and the crude cell extract centrifuged for 5 min at 1000g. The arginine decarboxylase activity was then determined by incubation of 0.1ml cell extract (0.5-1mg protein) with 1.3μM-[U-^{14}C]arginine (324mCi/mmol), 0.04mM-PLP, 4mM-$MgSo_4$, 0.2M-Tris HCl, pH7.6, in a total volume of 0.3ml.PLP and Mg^{2+} were included as Wu & Morris (1973b) reported an absolute requirement by the E. coli enzyme for these cofactors. Control tubes containing boiled enzyme were used to determine the level of non-enzymatic decarboxylation of arginine in the cell extracts. Comparison of these tubes with the experimental tubes suggested that the level of arginine decarboxylase activity in D. discoideum, if present at all, exists at such low levels as to be of little importance in the synthesis of cellular oligoamines. Further experiments would however be necessary before the existence of arginine decarboxylase activity in D. discoideum cells could be completely ruled out.

DISCUSSION

Amoebal extracts of D. discoideum cells were found to contain high levels of ornithine decarboxylase activity and considerably lower levels of SAM-decarboxylase activity. The presence of ornithine decarboxylase, along with the absence of any detectable level of arginine decarboxylase activity suggested that in these cells the synthesis of putrescine resembled that found in eucaryotic cells being via ornithine rather than directly from arginine (Figure 1.12). This confirmed previous observations that amoebae could incorporate radioactively labelled ornithine, but not arginine, from the growth medium into putrescine and spermidine (Table 1.5).

AX2 amoebae grown in HL5 medium have a doubling time of 8h. The level of ornithine decarboxylase activity required by these cells in order to maintain the cellular oligoamine levels (Table 1.2) during successive divisions would be 0.35 nmol CO_2/min/mg protein. This value is based on the assumption that the 1,3-diaminopropane present in the cell originates via spermidine from putrescine. If the biosynthesis of 1,3-diaminopropane was independent of these oligoamines, the level of ornithine decarboxylase activity required would be reduced to 0.24 nmol CO_2/min/mg protein. Although the level of ornithine decarboxylase detected in amoebal extracts was subject to fluctuations, the level of enzyme activity measured *in vitro* ranged between 0.5-15 nmol CO_2/min/mg protein i.e. always in excess of that required.

The level of SAM-decarboxylase activity required to maintain the cellular content of spermidine only (Table 1.2) during successive divisions would be 0.04 nmol CO_2/min/mg protein. If in addition the 1,3-diaminopropane in the cell is derived from spermidine the level of enzyme activity required would rise to 0.15 nmol CO_2/min/mg protein. The highest level of SAM-decarboxylase activity measured in amoebal extracts was however only 0.001 nmol CO_2/min/mg protein. This indicated that the level of SAM-decarboxylase activity measured *in vitro* was less than that present *in vivo*. Thus further experiments appear to be

necessary to optimize the extraction and assay conditions if the true level of SAM-decarboxylase activity in D. discoideum amoebal cells was to be determined.

Both ornithine decarboxylase and SAM-decarboxylase activity were labile to sonication in standard extraction buffer and the enzyme activity in these extracts was unstable during storage (Table 2.1 and Table 2.16). This instability did not appear to be the result of proteinase activity as the addition of PMSF and TLCK, two proteinase inhibitors, to the extraction buffer failed to improve the retention of either enzyme activity (Table 2.2 and Table 2.17). Increasing the osmolality of the extraction buffer by the addition of substances such as bovine serum albumin, surcrose, gylcerol or dimethylsulphoxide did however stabilise both decarboxylase activities. Thus 10% (v/v) glycerol became a permanent component of the standard extraction buffers for both decarboxylase enzymes. Glycerol not only stabilized the enzymes during storage but also during sonication (Table 2.5).

Glycerol on its own was not sufficient to stabilize either decarboxylase activity during extraction or storage (Table 2.6 and Table 2.18). In extracts prepared in the absence of a thiol-protecting agent and assayed immediately, the levels of both decarboxylase activities measured were substantially lowered and after storage the levels of enzyme activity fell to undetectable levels (Table 2.6 and Table 2.18). The presence of 2mM-DTT in the extraction buffer restored the stability of both enzymes during extraction and storage.

Thiol groups would seem to be involved in the catalytic process of both ornithine and SAM-decarboxylase from D. discoideum. The importance of thiol groups in the decarboxylase enzymes of oligoamine synthesis has been generally accepted (Jänne & Williams-Ashman, 1971a; Friedman et al., 1972a; Morley & Ho, 1976; Stevens et al., 1976; Boucek & Lembach, 1977; Demetriou et al., 1978) and enzyme inactivation in the absence of suitable protecting agents is believed to involve dimerisation of the enzyme (Jänne & Williams-Ashman, 1971a; Boucek & Lembach, 1977).

There was little evidence to support either the activation or inhibition

of either decarboxylase enzyme by metal ions (Table 2.6 and Table 2.18). The two enzymes differed however in their requirement for PLP, a known cofactor of many decarboxylase enzymes. Although PLP was not required for the stabilization of either decarboxylase enzyme nor for the activation of SAM-decarboxylase from D. discoideum, in the absence of added cofactor no ornithine decarboxylase activity was detected. Whilst PLP is believed to be the cofactor for ornithine decarboxylase from all other systems so far studied (Pegg & Williams-Ashman, 1968; Friedman et al, 1972a; Clark & Fuller, 1976; Mitchell & Sedory, 1974; Morley & Ho, 1976), reports suggest that the cofactor for both procaryotic and eucaryotic SAM-decarboxylase enzymes is covalently bonded pyruvate (Wickner et al., 1970; Pegg, 1977b; Cohn et al., 1977; Demetriou et al., 1978).

Neither putrescine nor magnesium increased the level of SAM-decarboxylase in vitro (Figure 2.17). This suggests that the D. discoideum enzyme resembles the SAM-decarboxylases of other lower eucaryotes e.g. T. pyriformis and P. polycephalum (Mitchell & Rusch, 1973; Pösö et al., 1975b) in that it is activated by neither putrescine, as are the higher eucaryote enzymes, nor by magnesium as are the procaryotic enzymes.

The temperature optimum of both enzymes in vitro was 37°C (Figure 2.3 and Figure 2.12) which is higher than the optimum temperature for the growth and development of D. discoideum (22-27°C). Whilst the pH optimum in vitro of ornithine decarboxylase activity (8.25, Figure 2.4) was similar to that of ornithine decarboxylases from other sources (Morley & Ho, 1976; Stevens et al., 1976; Applebaum et al., 1977), that of D. discoideum SAM-decarboxylase 7.8-8, Figure 2.13), was slightly higher than reported for other systems (Stevens et al., 1976; Porta et al., 1977; Demetriou et al., 1978).

As expected SAM-decarboxylase activity in vitro was proportional to the protein concentration of the extract (Figure 2.10). This was not the case with ornithine decarboxylase activity (Figure 2.1 and Table 2.3). Increasing the concentration of PLP and/or ornithine in the assay mixture failed to relieve the inhibition brought about by high protein concentrations (Table 2.4).

Experimental evidence suggested that the protein effect was not an artifact resulting from the assay conditions but rather reflected a change in the enzyme occurring in the extraction buffer only in concentrated extracts (Table 2.3). The activation and inactivation of mammalian ornithine decarboxylase by monomeric and dimeric form conversion was suggested by Jänne & Williams-Ashman (1971a), and evidence for the polymerisation of the enzyme from P. polycephalum has also been reported (Mitchell et al., 1976; Mitchell & Carter, 1977). It is not known however whether this polymerisation occurs in vivo. Alternatively ornithine decarboxylase in D. discoideum may exist in multiple forms such as have been reported in several other eucaryotic systems (Abdel-Monem et al., 1975; Clark & Fuller, 1976; Obenrader & Prouty, 1977a,b; Mitchell & Carter, 1977). It is possible that in amoebal extracts DTT, by protecting thiol groups, prevents a polymerisation/interconversion type reaction which inactivates the ornithine decarboxylase. If this were the case, then in concentrated extracts there may be insufficient DTT present to protect all the enzyme present. It has been shown that increasing the concentration of DTT in the standard extraction buffer resulted in an increase in the level of ornithine decarboxylase activity measured in vitro (Table 2.6).

Irrespective of the concentration of protein in amoebal extracts the ornithine decarboxylase activity exhibited the same Km value for the cofactor PLP (Km 23μM) and for the substrate ornithine (Km 1.7mM) [Figure 2.5 and Figure 2.6]. However some variation in the V max was observed as the protein concentration of the extract increased (Figure 2.6). Thus if the lower activity observed in extracts containing high concentrations of protein was due to a change in the form of the enzyme, whether resulting from the binding of an inhibitor, polymerisation or a reversible interconversion of forms, these must have been very similar in respect to their cofactor and substrate affinities. It may be that in concentrated extracts whilst some of the enzyme was completely inactivated, possibly due to a lack of the thiol-protecting agent DTT, the cofactor and substrate affinity of the remaining enzyme would be unaffected

whilst the V max would be reduced. Alternatively the change in the enzyme occurring in extracts containing high levels of protein, whilst not affecting the binding of the cofactor or the substrate may still reduce the rate at which the intermediate complex dissociates to form the products. In the absence of glycerol both Km values were reduced. Thus if two forms of ornithine decarboxylase activity exist in amoebal extracts, it may be that glycerol stabilizes the form with a low affinity for both substrate and cofactor.

The substrate affinity of ornithine decarboxylase, as measured by the Km value, was lower than has been reported for other eucaryotic systems. Thus the possible importance of *in vivo* levels of ornithine in the control of intracellular oligoamine levels must not be overlooked.

Although no evidence of an induction of ornithine decarboxylase activity resulting from a change in the growth state of *D. discoideum* was obtained (Table 2.11), a number of observations did suggest that the enzyme can respond to changes in osmolality. A correlation has since been reported between the osmolality of the cell's environment and the ornithine decarboxylase activity of eucaryotic cells (Munro et al., 1975; Friedman et al., 1977a; Perry & Oka, 1980). Thus in *D. discoideum* amoebae decreasing the osmolality of the culture medium resulted in an increase in the level of ornithine decarboxylase activity measured *in vitro* whilst increasing it caused the level of enzyme activity to fall rapidly to undetectable levels. Similarly Mitchell & Kottas (1979) report that in the lower eucaryote *P. polycephalum*, although only a small alteration in the total ornithine decarboxylase activity occurs in response to osmotic changes, the proportion of this total activity attributable to the active form is extremely sensitive to small changes in the osmolality of the medium. Unlike mammalian cells the response in *P. polycephalum* is rapid and transitory involving interconversion of the more active form with the less active form. In both *P. polycephalum* and mammalian cells the response is evoked equally by the addition of salts or sugar to the growth medium, thus it is believed to be a general osmotic effect rather than the result of changing ion concentrations (Mitchell &

Kottas, 1979; Perry & Oka, 1980). Putrescine levels have been found to be related to the extracellular osmolality in mouse mammary glands (Perry & Oka, 1980), and other mammalian systems (Munro et al., 1975). In E. coli intracellular putrescine levels are also affected by extracellular osmolality (Munro et al., 1972) and at high osmolality putrescine is rapidly excreted (Günther & Peter, 1979). Further investigations by Harris & North (1982) have since confirmed the effect of osmotic perturbations on ornithine decarboxylase activity in D. discoideum. A more detailed analysis of ornithine decarboxylase kinetics revealed two forms of activity with differing Km values; a high activity, low Km form observed at low osmolalities and a low activity, high Km form at high osmolalities. That these forms were not observed in the studies under discussion probably reflects differences in the extraction processes used, as Harris & North (1982) used an increased level of DTT and no glycerol in the extraction buffer.

The significance of this osmotically induced response in D. discoideum is unknown. The speed and amplitude of the response may possibly be sufficient to cause major changes in amoebal oligoamine levels. If this was the case then the response observed may represent an attempt by the cells to maintain osmotic balance by varying the intracellular oligoamine concentrations. The time taken for the enzyme activity to return to steady state levels in D. discoideum is not known. Thus, if like P. polycephalum the response is short lived, it is more probable that the phenomenon reflects the sensitivity of some part of the ornithine decarboxylase controlling mechanism to osmotic shock. It is possible that the mechanism for interconverting the two forms of ornithine decarboxylase is located at the cell membrane and thus susceptible to changes in extracellular osmolality.

No significant change in measured ornithine decarboxylae activity was observed when amoebae were subjected to changes in growth conditions such as occur when the composition of the growth medium is altered (Table 2.9), when exponentially growing cells enter stationary phase (Table 2.10) or stationary phase

cells are resuspended in fresh HL5 medium (Table 2.11). This is in agreement with the observation that cellular oligoamine levels in amoebae were unaffected by similar treatments (Table 1.2). Although both ornithine decarboxylase and SAM-decarboxylase levels fell during development, the pattern of enzyme decrease differed. Thus during the first half of the 24h developmental period SAM-decarboxylase activity fell rapidly reaching a level only 8% of that in amoebal cells (Figure 2.15). This level was then apparently maintained during the latter stages of development, although as cellular protein levels fall throughout development (Table 1.2), it may be that during this period the rate at which SAM-decarboxylase activity was decreasing was approximately that at which other cell proteins was being catabolised. The level of SAM-decarboxylase activity during development mirrored quite closely the decreasing level of spermidine within the developing cells (Figure 1.9 and Figure 2.15). Some SAM-decarboxylase activity may be functioning during development as the specific radioactivity of spermidine in developing amoebae, prelabelled with ^{14}C- putrescine, was observed to decrease (Table 1.6). In contrast the ornithine decarboxylase activity measured in amoebae increased substantially during the time required to prepare developmental filters. This is thought to be due to exposure of amoebae to the low osmotic strength washing and suspension media. That a similar increase did not occur in SAM-decarboxylase levels during this period, suggests that this enzyme was not as susceptible to osmotic perturbations. After this initial increase ornithine decarboxylase levels fell but not as rapidly as SAM-decarboxylase levels (Figure 2.7), and from 6h onwards the trend was reversed and during the latter stages of development the level of enzyme activity was apparently restored to 80% of amoebal levels. This increase was not reflected in intracellular oligoamine levels, as putrescine levels have been found to fall throughout the 24h developmental period (Figure 1.9). Thus the ornithine decarboxylase activity measured *in vitro* may not represent the enzyme activity *in vivo* during development. This might result if ornithine or PLP levels became limiting during the latter stages of development. Alterna-

tively as this apparent increase in enzyme activity occurred during a period of falling cellular protein levels (Figure 1.9), it may be that during the last 18h of development catabolism of ornithine decarboxylase was less than that of the majority of cellular proteins. Some ornithine decarboxylase may be functioning during development as the specific radioactivity of putrescine in developing amoebae prelabelled with ^{14}C-putrescine was observed to decrease (Table 1.7). Alternatively the continued decrease in putrescine levels may have resulted from an increased rate of catabolism of the diamine during the latter stages of development. The data in Figure 1.9 showed that although catabolism of putrescine to CO_2 decreases at this time, the oligoamine became associated with the acid insoluble material in these cells. Thus the control of oligoamine levels during development would seem to involve both their synthesis and degradation.

Whilst the presence of the oligoamines putrescine, spermidine, spermine and 1,3-diaminopropane either *in vivo* or *in vitro* had no significant effect on the measurable SAM-decarboxylase activity of amoebal extracts (Figure 2.16 and Figure 2.17), ornithine decarboxylase activity was significantly reduced under the same conditions. Even so, comparison of the data in Figure 2.8 with those in similar experiments reported in literature, suggest that in *D. discoideum* the level of ornithine decarboxylase is less sensitive to control by extracellular oligoamine levels than other systems where the activity of the enzyme is diminished by micromolar concentrations of oligoamines *in vivo* (McCann et al., 1977; Heller et al., 1978; Bethall & Pegg, 1979; Branca & Herbst, 1980). *D. discoideum* ornithine decarboxylase does however resemble that from other systems in that the level of enzyme activity was more effectively reduced by the presence of oligoamines in the growth medium than in the enzyme assay, where even at concentrations in excess of those liable to exist in a free state in the cell, the reduction in enzyme activity was slight (Figure 2.8 and Figure 2.9). Thus direct feedback inhibition of ornithine decarboxylase is not a major method of control of intracellular oligoamine levels. Although 1,3-dia-

minopropane has been found to be a potent inhibitor of ornithine decarboxylase in a variety of systems (Pösö & Jänne, 1976a,b; Guha & Jänne, 1977; Hallio et al., 1977b,c; Piik et al., 1977; Pösö et al., 1977), in D. discoideum this diamine both in vivo and in vitro had an inhibitory effect similar to that of spermidine or spermine (Figure 2.8 and Figure 2.9). This possibly reflects the fact that in D. discoideum 1,3-diaminopropane is a physiological amine. Levels of extracellular spermine, spermidine and 1,3-diaminopropane, known to cause substantial reductions in intracellular putrescine levels (Table 1.4), had little effect on the level of ornithine decarboxylase activity (Figure 2.8). This control of the intracellular level of putrescine was not solely by means of decreased synthesis but must also involve some other mechanism such as catabolism of putrescine which is known to be increased by extracellular spermine and spermidine (Table 1.3). Similarly levels of spermidine remained constant in cells grown in the presence of a range of extracellular oligoamines (Table 1.4). As SAM-decarboxylase activity is known to be unaffected under similar conditions (Figure 2.16) maintenance of intracellular spermidine levels by extracellular oligoamines, if it occurs, must operate at an alternative level involving for example the activity of ornithine decarboxylase (Figure 2.8), the rate of catabolism of putrescine (Figure 1.8), and/or spermidine or the activity of spermidine synthetase.

1,4-diaminobutanone in vitro is a powerful competitive inhibitor of ornithine decarboxylase activity in A. nidulans, regenerating rat liver and prostate (Stevens et al., 1978) and in these same organisms can substitute for putrescine in the activation of SAM-decarboxylase. Yet the response of D. discoideum ornithine decarboxylase and SAM-decarboxylase to 1,4-diaminobutanone resembled that of the procaryote E. coli (Stevens et al., 1978). Thus like bacterial ornithine decarboxylase the enzyme in D. discoideum was relatively insensitive to the presence of the inhibitor in vitro (Figure 2.9), possibly a reflection of the low affinity, as judged by the Km values, of both enzymes for the substrate ornithine in contrast to eucaryotic enzymes. Similarly the putrescine-insensitive SAM-decarboxylase from both E. coli (Stevens et al.,

1978), and D. discoideum (Figure 2.17), were not activated by 1,4-diaminobutanone. D. discoideum SAM-decarboxylase also resembled the E. coli enzyme Williams-Ashman & Schenone, 1972), in its response to the inhibitor MGBA, the potent specific inhibitor of eucaryotic putrescine-activated SAM-decarboxylase (Figure 2.17). Whilst the level of enzyme activity measured in higher eucaryotic cells was substantially reduced by the presence of micromolar concentrations, a ten fold increase in inhibitor concentration was necessary to bring about the same decrease in the level of D. discoideum enzyme. Thus an inhibitor of either ornithine decarboxylase or SAM-decarboxylase which could be used to deplete the level of oligoamines in D. discoideum amoebae was not found.

INTRODUCTION

UPTAKE OF PUTRESCINE

Intracellular levels of putrescine can be maintained through two mechanisms; synthesis from ornithine and/or arginine or uptake of exogenous putrescine. In mammalian systems this latter source has received relatively little attention. Mouse brain slices accumulate putrescine by a different system than they use to accumulate basic amino acids and monoamines (Lajtha & Shershen 1974). Regenerating rat livers also take up putrescine at a higher rate than non-liver cells (Dykstra & Herbst, 1965). Mouse LS cells (McCormick, 1975) and mouse mammary glands (Kano & Oka, 1976) also possess a mechanism for obtaining putrescine, spermidine and spermine from extracellular sources. In human fibroblasts putrescine, spermidine and spermine are actively transported into the cell and appear to share the same transport mechanism (Di Pasquale et al., 1978). Data from these various studies suggest the involvement of specific energy-dependent transport systems. More attention has been applied to the uptake of oligoamines by bacterial systems such as E. coli (Tabor & Tabor, 1966; Simon et al., 1970; Munro et al., 1974). Exogenous putrescine, spermidine and spermine are known to accumulate in E. coli and the Km values for uptake range between 80 nM and 200 nM (Tabor & Tabor, 1966). These values are low compared to amino acid uptake systems in E. coli and may reflect the low concentration of putrescine required to sustain growth in the absence of endogenous putrescine synthesis.

D. discoideum is known to be permeable to various substances such as nutrients and labelled precursors and inhibitors, during both the vegetative and developmental phases of its life cycle. Although the amoebae are believed to obtain food substances by pinocytosis and phagocytosis (Ryter & Chastellier, 1977), very little has been reported on the mechanism of uptake of small

molecules. Lee (1972c) using low temperature studies and the inhibitor 2,4-dinitrophenol, showed that the uptake of inulin by D. discoideum amoebae is by means of pinocytosis. In contrast the amount of lysine and glutamate entering the cells is comparable with that expected for equilibration of the internal pool with the suspending medium, suggesting passive diffusion. Pinocytosis is also implicated in the uptake of 6-aminohexanoic acid (ε-amino-caproic acid). North & Williams (1978) demonstrated that the uptake of this developmental inhibitor is sensitive to 2,4-dinitrophenol, sodium azide and cycloheximide.

During an investigation of the biosynthetic pathways to spermidine in D. discoideum amoebae, it was discovered that the uptake of putrescine by these cells was very different from that of 6-aminohexanoic acid and other amino acids (North & Williams, 1978), in that extracellular putrescine was concentrated by the cells at a rapid rate (see page 72). This phenomenon was further investigated and some of the properties of the uptake system determined.

SECTION A -
FACTORS AFFECTING MEASUREMENT OF RATE OF [1,4-^{14}C] PUTRESCINE UPTAKE

Figure 3.1 shows the time course of labelling of AX2 amoebae during their incubation in 17mM-sodium phosphate buffer, pH6, containing [1,4-^{14}C] putrescine at either 5μM or 1mM. With 5μM-putrescine radioactive labelling was rapid (0.024 n mol/min/10^7 cells) and within 1h 16% of the putrescine present in the buffer had become associated with the cells. Although at 1mM-putrescine the rate of uptake was increased (0.32 n mol/min/10^7 cells) it did not increase directly in proportion to the concentration of putrescine. Thus at the higher putrescine concentration the proportion of extracellular putrescine associated with the cells after 1h labelling, was less than 2%. At the lower concentration (5μM) the labelling was proportional to incubation time up to 1.5h and at higher concentrations (1mM) up to 1h (Figure 3.1).

The data in Figure 3.2 indicate that labelling was proportional to the cell

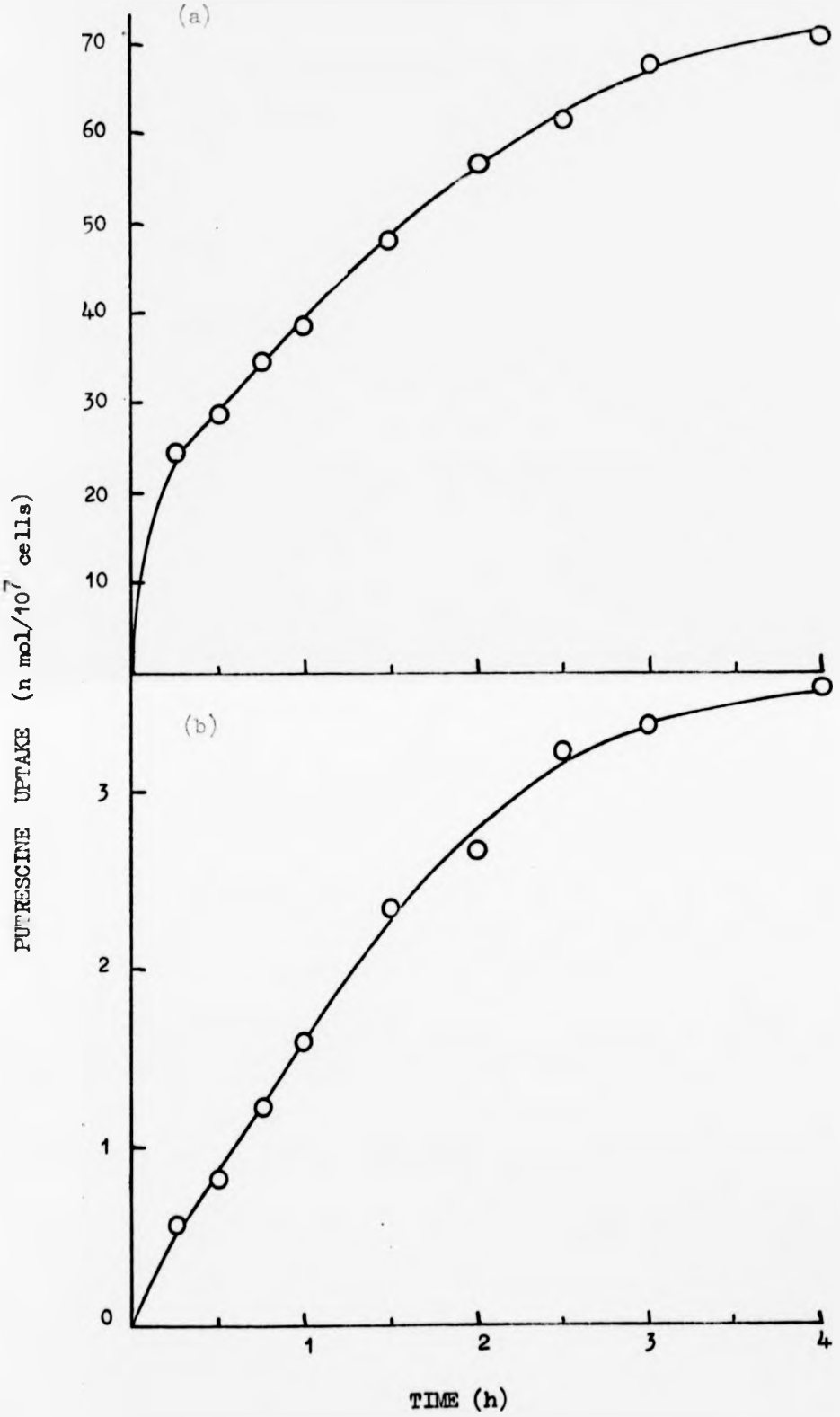


FIGURE 3.1 TIME COURSE OF LABELLING OF AMOEBAE WITH [1,4-^{14}C] PUTRESCINE

Amoebae were incubated in 0.17mM-sodium phosphate buffer, pH6, at a density of 4.5×10^6 cells/ml. After 15min [1,4-^{14}C] putrescine was added to give a concentration of (a) 1mM(0.25μCi/μmol) or (b) 5μM (2.5μCi/μ mol). Labelling was measured as detailed in the methods section.

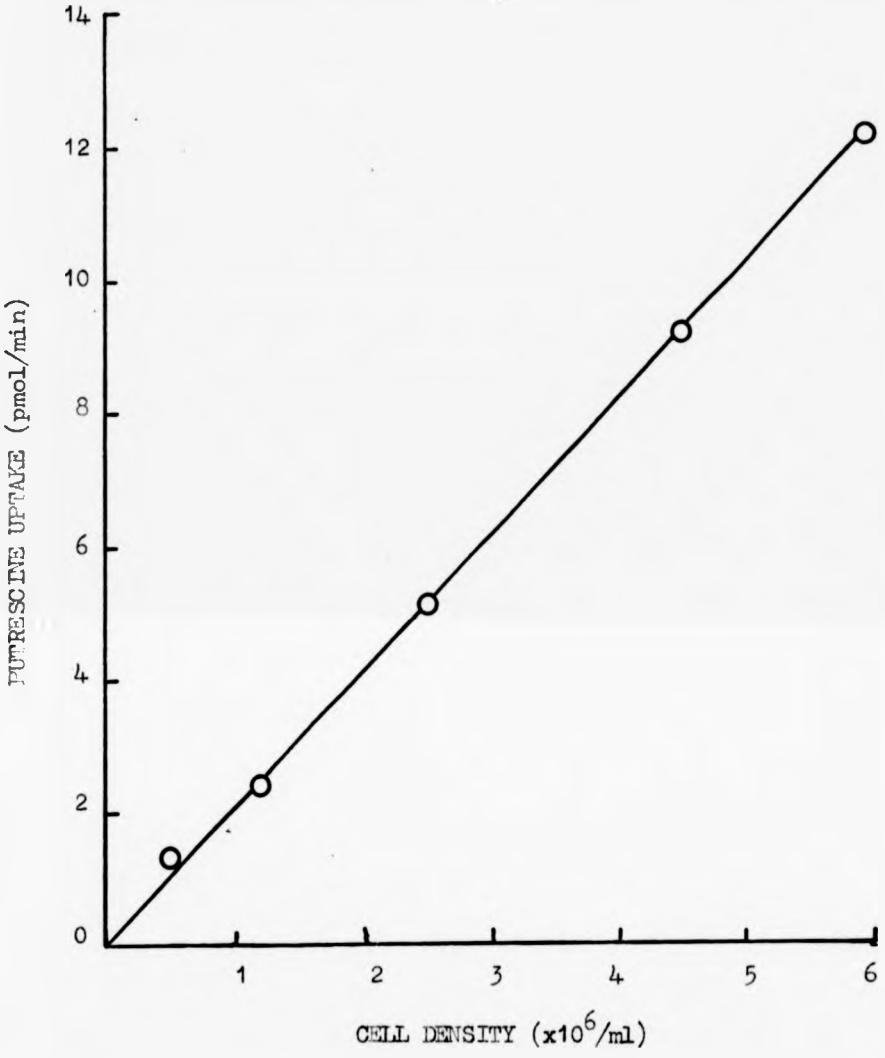


FIGURE 3.2 INITIAL RATE OF PUTRESCINE UPTAKE AS A FUNCTION OF CELL DENSITY

Amoebae were incubated in 0.17ml-sodium phosphate buffer, pH6, at the cell densities indicated. After 15 min 5μM-[1,4-^{14}C] putrescine (2.5μCi/μmol) was added. Labelling was monitored over 0.5h as detailed in the methods section and the initial uptake rate determined graphically.

density up to densities of $6x10^6$ cells/ml. Thus in all subsequent experiments the rate of putrescine uptake was calculated by determining the amount of labelled putrescine present in the washed cells at intervals during a 0.5h incubation period and at cell densities less than $6x10^6$ cells/ml. From these figures the amount of exogenous putrescine having entered the cell was calculated and the uptake rates were then determined graphically.

The pH of the suspension medium had little effect on the rate of putrescine uptake in the range 6-7, although at higher pH values a slight depression of uptake rate was observed (Figure 3.3). Uptake rates were measured routinely at pH6.

At both concentrations of putrescine shown in Table 3.1, the rate of putrescine uptake from phosphate buffer was slightly lower (20-36%) when using amoebae grown in HL5 medium containing no glucose rather than those grown in the standard glucose - containing HL5 medium. These cells are known to vary in other ways causing differences in their growth rates (T_D glucose cells = 8h, T_D glucose absent cells = 12h). In HL5 + glucose medium (T_D= 8h) the rate of uptake was higher than in FM medium (T_D= 24h) [Table 3.1]. The uptake of putrescine from either HL5 or FM growth medium was 40-70% slower than from phosphate buffer. This may reflect the complexity of the growth medium and thus a competition between nutrients and putrescine for uptake. An alternative explanation was that the higher rate of uptake in buffer was caused by withdrawal of nutrients and entry into the developmental phase, however the results of subsequent experiments showed that this was unlikely (see page204).

At both high and low concentrations of putrescine, uptake was considerably reduced at low temperatures. Thus at 0°C with 5μM-putrescine the rate of uptake measured was 17% of that measured at 22°C and at 0.1mM-putrescine it was reduced to 15% of the control value.

As the data in Figure 3.4 show, the addition of sodium azide (1mM), 2,4-dinitrophenol (0.1mM) or cycloheximide (500 g/ml) 15 min after putrescine, resulted in the cessation of putrescine uptake from the phosphate buffer.

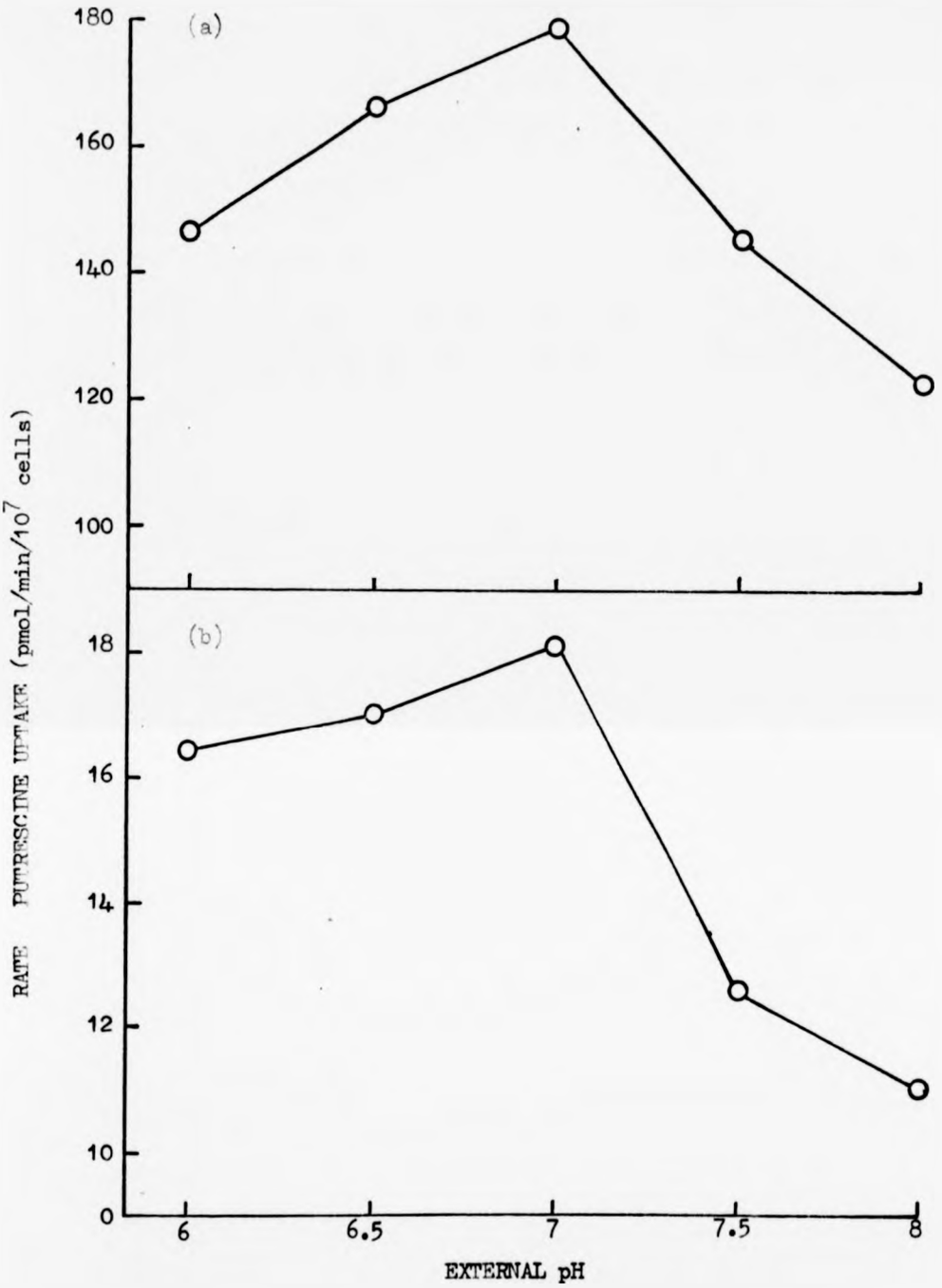


FIGURE 3.3 INITIAL RATE OF PUTRESCINE UPTAKE AS A FUNCTION OF EXTERNAL pH

Amoebae were incubated at a cell density of 5.6×10^6 cells/ml in 17mM-sodium phosphate buffer at the pH values indicated. After 15 min [1,4-^{14}C] putrescine was added to give a concentration of (a) 0.1mM (0.25μCi/μmol) (b) 5μM (2.5μCi/μmol). Labelling was monitored over 0.5h as detailed in the methods section and the initial uptake rate determined graphically.

TABLE 3.1 COMPARISON OF PUTRESCINE UPTAKE FROM DIFFERENT SUSPENSION MEDIA

Amoebae, harvested from either HL5 + glucose (G) or HL5 + no glucose (NS) medium, were incubated at cell densities of $5\text{-}6\times10^6$ cells/ml in the suspension media indicated below. After 15 min [1,4-^{14}C] putrescine was added to give final concentrations of either 5μM(2.5μCi/μmol) or 0.1mM (0.25μCi/μmol). Labelling was monitored over 0.5h as detailed in the methods section and the initial uptake rate determined graphically.

SUSPENSION MEDIUM	GROWTH CONDITION OF CELLS	UPTAKE RATE (p mol/min/10^7 cells) 5μM-PUTRESCINE	0.1mM-PUTRESCINE
PHOSPHATE BUFFER	G	19.2	143.1
PHOSPHATE BUFFER	NS	15.0	91.1
HL5 GROWTH MEDIUM	G	11.4	73.8
FM GROWTH MEDIUM	G	8.4	51.1

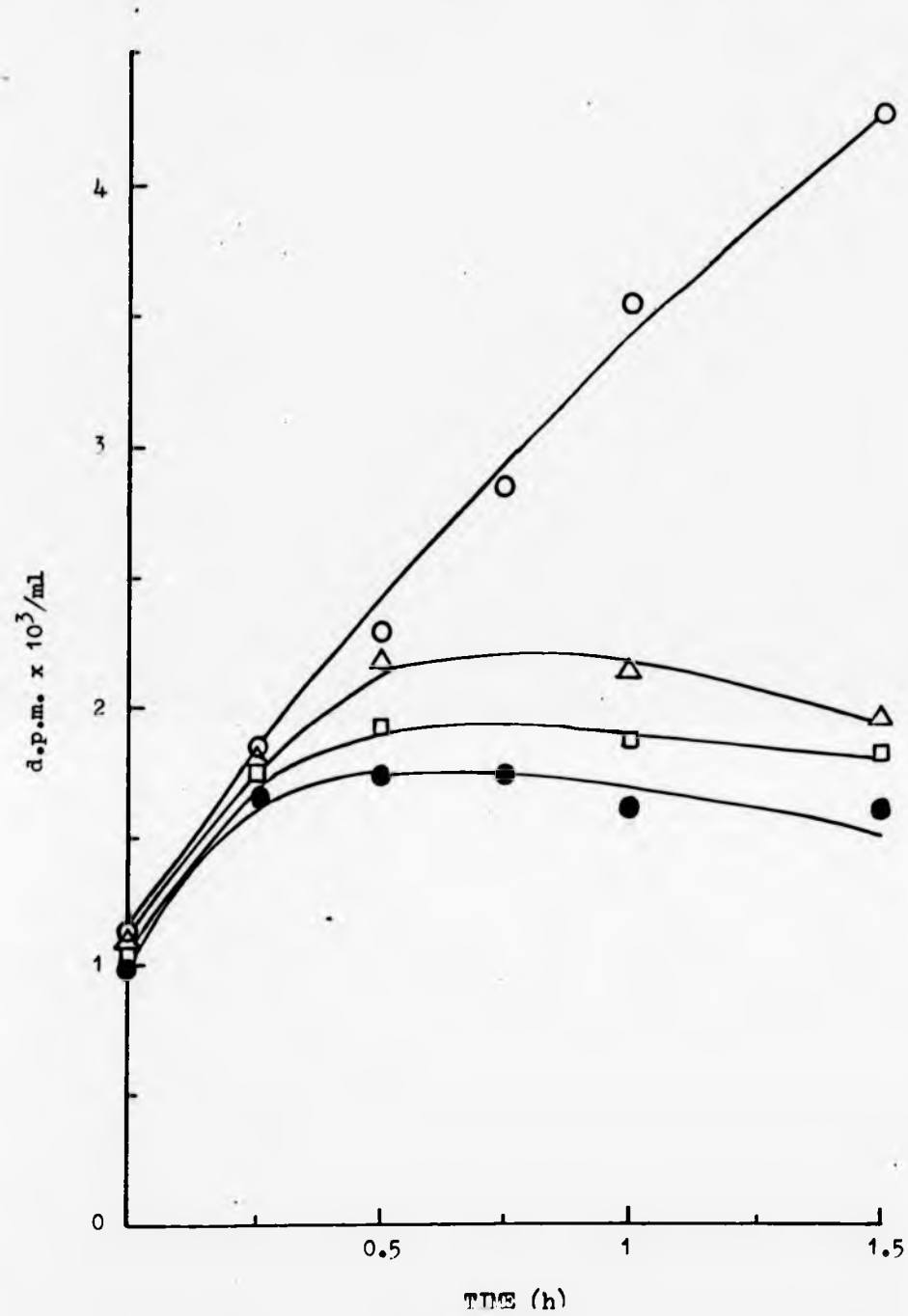


FIGURE 3.4 EFFECTS OF METABOLIC INHIBITORS ON PUTRESCINE UPTAKE

Amoebae were incubated at cell densities between 4.6×10^6 and 5.1×10^6 cells/ml in 17mM-sodium phosphate buffer, pH6. After 15 min 5μM-[1,4-^{14}C] putrescine (2.5μCi/ml) was added. At regular intervals from this time labelling of the cells was determined as detailed in the methods section. Additions of metabolic inhibitors were made 15 min later. Control (○); 500μg/ml-cycloheximide (△); 1mM-sodium azide (□); 0.1mM-dinitrophenol (●).

Putrescine uptake is similarly affected by sodium azide and 2,4-dinitrophenol when these compounds were added along with putrescine to the phosphate buffer (Turner et al., 1979). Whilst the inhibition of uptake by these two compounds was maximal within a few minutes, cycloheximide took slightly longer to cause complete inhibition of uptake (Figure 3.4). This could be interpreted in terms of the time taken for cycloheximide to enter the cell and /or inhibit synthesis of a membrane protein, which when not continually replaced, results in cessation of putrescine uptake. The inhibition by 2,4-dinitrophenol was pH dependent being effective at pH6 but not at pH7 (Turner et al., 1979). A reversal of 2,4-dinitrophenol inhibition by increased intracellular pH has also been reported by Geller & Brenner (1978) in studies on oscillations in D. discoideum.

The temperature dependence of putrescine uptake and the results in Figure 3.4 were evidence that the labelling of the D. discoideum amoebae with [1,4-^{14}C] putrescine involved an active process and could not have been due solely to binding of the radioactive material to the cell surface. Further evidence for an active component in putrescine uptake was obtained by the following experiments. Amoebae were preincubated with high levels of unlabelled putrescine (1mM) to saturate any surface binding sites. The cells were then harvested, washed and incubated at a cell density of 5.1×10^6 cells/ml in 17mM-phosphate buffer, pH6, containing 5μM-[1,4-^{14}C] putrescine (2.5μCi/ml). The rate of putrescine uptake observed in these cells did not differ significantly from that of untreated controls. Thus if labelling of amoebae was just the result of binding of putrescine to the cell surface, the unlabelled material bound during the preincubation must have either been removed during the washing of the cells prior to placing them in buffer containing labelled putrescine, or exchanged very rapidly with the labelled putrescine. The former was shown to be unlikely as a relatively small percentage of the radioactivity associated with similarly labelled amoebae, was removed during a 3h incubation in 17mM-phosphate buffer (page204). Washing the labelled cells with 10mM solutions of putrescine, NaCl, $MgCl_2$ or $CaCl_2$ failed to remove the associated radioacti-

vity as did incubation of labelled cells for 2h in buffer containing unlabelled putrescine (10mM). These observations suggested that once putrescine had become associated with the amoebae it was neither rapidly exchanged nor washed off. The labelled putrescine seemed to have entered a pool from which rapid exchange with extracellular putrescine was not possible. This pool was believed to be intracellular and thus labelling of cells with radioactive putrescine must result from its uptake into the cell from the buffer. Analysis of the TCA soluble material in similarly labelled amoebae confirmed that the major portion of radioactive label in the cells was in the form of putrescine or spermidine (page 79).

Figure 3.5 shows the results of a more detailed study of the dependence on putrescine concentration of the rate of its uptake. Plotting the results by the method of Lineweaver & Burk (1934), a straight line double reciprocal plot was obtained when using uptake data for the low range of putrescine concentrations (1-20μM). At these concentrations of extracellular putrescine the uptake system involved had an apparent Km for putrescine of 10μM and a V max of 0.1 n mol/min/10^7 cells (Figure 3.5b). The uptake data for the higher range of putrescine concentrations (25μM-20mM) was not compatible with the kinetic parameters determined at the lower concentrations of putrescine. The double reciprocal plot of this data was not a straight line and at millimolar concentrations of putrescine these plots curved towards the origin (Figure 3.5a). These results could be interpreted in terms of uptake of putrescine by D. discoideum amoebae involving at least two systems: one which is saturable at low concentrations of putrescine (<20μM) and at higher concentrations of putrescine (>50μM) a second non-saturable system.

SECTION B - INHIBITION OF PUTRESCINE UPTAKE

Putrescine uptake was effected by the inclusion in the suspension buffer of a variety of compounds. At concentrations of less than 10μM the bivalent metal ions calcium, magnesium and manganese stimulated the rate of uptake of

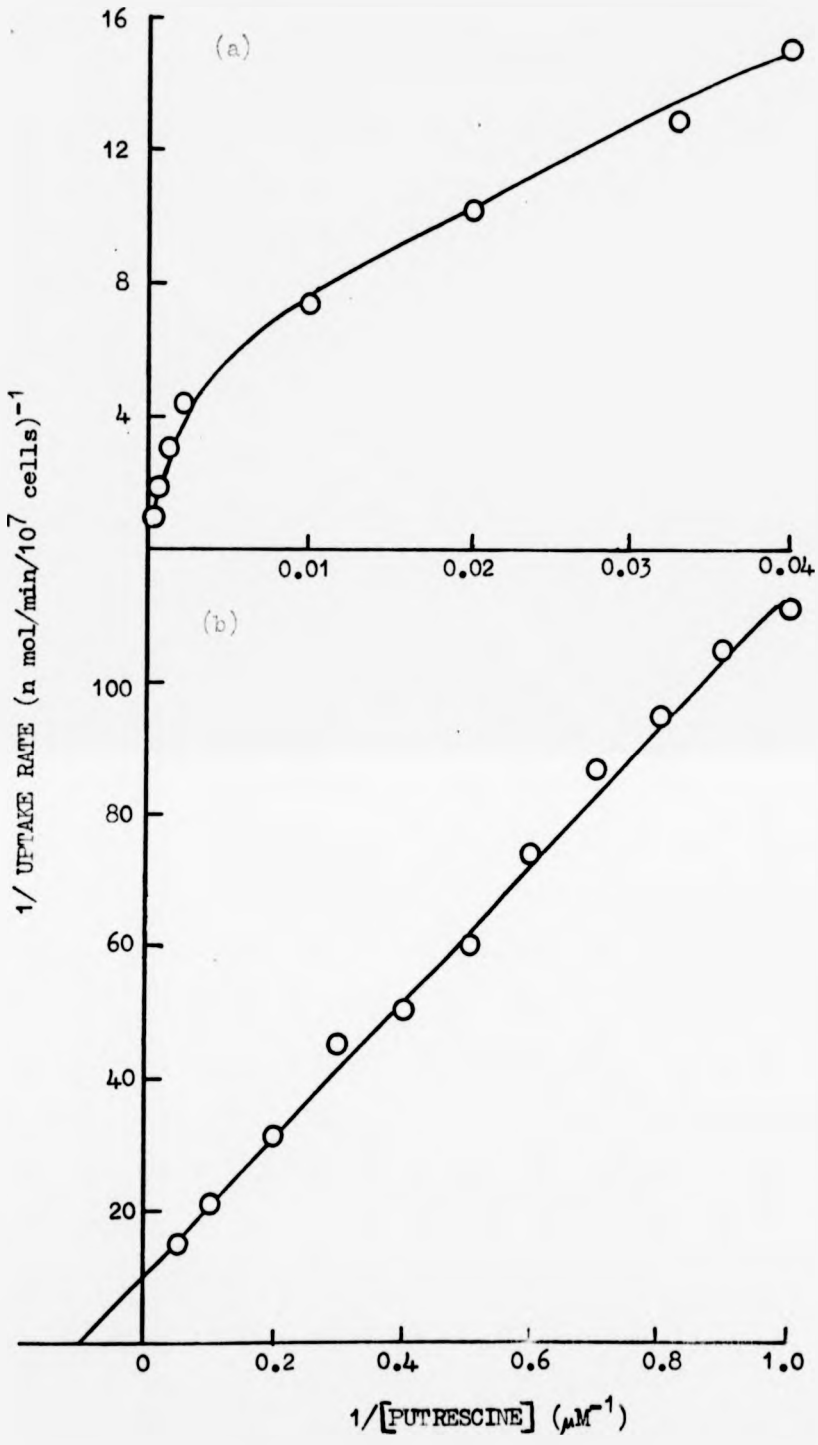


FIGURE 3.5 LINEWEAVER-BURK PLOTS FOR PUTRESCINE UPTAKE

Amoebae were incubated in 17mM-sodium phosphate buffer, pH6, at 3.9×10^6 cells/ml. After 15 min [1,4-^{14}C] putrescine was added at concentrations between (a) 25μM and 25mM (2.5μCi/n mol) or (b) 1μM and 20μM (2.5μCi/μmol) and the uptake rate determined as detailed in the methods section. The data was plotted by the method of Lineweaver & Burk (1934).

5μM-putrescine to a level 60-70% higher than that observed in control cells (Table 3.2). Above 10μM however, the same ions had an inhibitory effect and at concentrations of 1mM the rate of putrescine uptake was reduced to between 10-30% of that observed in control cells (Table 3.2). This inhibitory effect is non-specific since the uptake of 6-amino-[^{14}C]-hexanoic acid by D. discoideum amoebae is also inhibited by $CaCl_2$ and $MnCl_2$ and to a lesser extent by $MgCl_2$ (Turner et al., 1979). Monovalent metal ions when present in the suspension buffer at concentrations between 1μM and 1mM enhanced the rate of putrescine uptake (Table 3.2). The inhibitory effect of bivalent metal ions may possibly have resulted from competition between putrescine and the metal ions for extracellular or intracellular binding sites thus causing decreased putrescine uptake or increased putresine efflux. The stimulatory effect was possibly due to a secondary effect of low levels of metal ions on amoebal metabolism resulting in an increased rate of energy dependent putrescine uptake.

The oligoamines spermidine, spermine and 1,3-diaminopropane all inhibited putrescine uptake, the polyamines having a more severe inhibitory effect than the diamine. At low concentrations of putrescine (5μM) the presence of either 1mM-spermidine or spermine in the suspension buffer completely inhibited putrescine uptake (Figure 3.6). Spermidine and spermine have less effect on the non-saturable component of putrescine uptake operating at higher concentrations of putrescine. Thus at 1mM-putrescine the addition of 10mM-spermidine or spermine to the buffer decreases the uptake rate by 65% and at 10mM-putrescine the same concentration of spermidine and spermine had no effect at all (Turner et al., 1979). The inhibitory effect of spermidine and spermine are specific for putrescine uptake, neither compound affecting the uptake of 6-amino-[^{14}C]-hexanoic acid (Turner et al., 1979). Plotting the data for putrescine uptake in the presence of 1,3-diaminopropane by the method of Dixon (1953) did not give the intersecting lines expected for simple competitive inhibition but showed that complex inhibition kinetics were involved (Figure 3.7). The inhibition of putrescine uptake by 1,3-diaminopropane resembles that by sper-

TABLE 3.2 EFFECT OF METAL IONS ON PUTRESCINE UPTAKE

Amoebae were incubated at cell densities between 4.8×10^6 and 5.2×10^6 cells/ml in 5mM-Mes, adjusted to pH6 with NaOH, with additions made as indicated. After 15 min 5μM-[1,4-^{14}C] putrescine (2.5μCi/μmol), was added and the uptake rates determined as detailed in the methods section. Uptake rates are given as percentages of that measured in a control suspension to which no additions had been made (17.7 p mol/min/10^7 cells = 100%).

CONCENTRATION OF ADDITIONS	ADDITIONS				
	$MgCl_2$	$CaCl_2$	NaCl	K Cl	$MnCl_2$
1μM	171	171	163	191	163
10μM	105	108	148	173	111
0.1mM	64	56	127	156	44
1mM	33	34	116	142	10

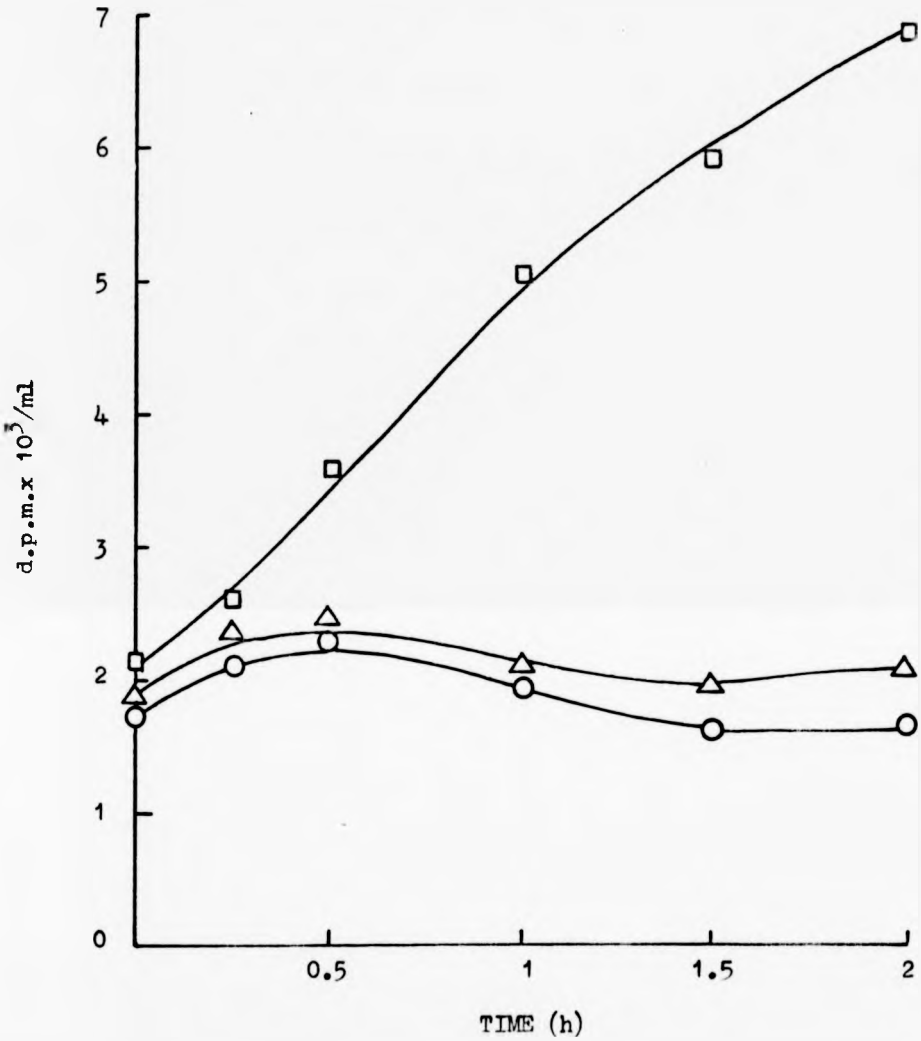


FIGURE 3.6 INHIBITION OF PUTRESCINE UPTAKE BY SPERMIDINE AND SPERMINE

Amoebae were incubated at 4.9×10^6 cells/ml in 17mM-sodium phosphate buffer, pH6. After 15 min 5μM-[1,4-^{14}C] putrescine (2.5μCi/μmol) was added to all the flasks. At the same time 1mM-spermidine (○) or 1mM-spermine (△) were added to all the flasks with the exception of the control flask (□). At regular intervals from this time labelling of the cells was determined as detailed in the methods section.

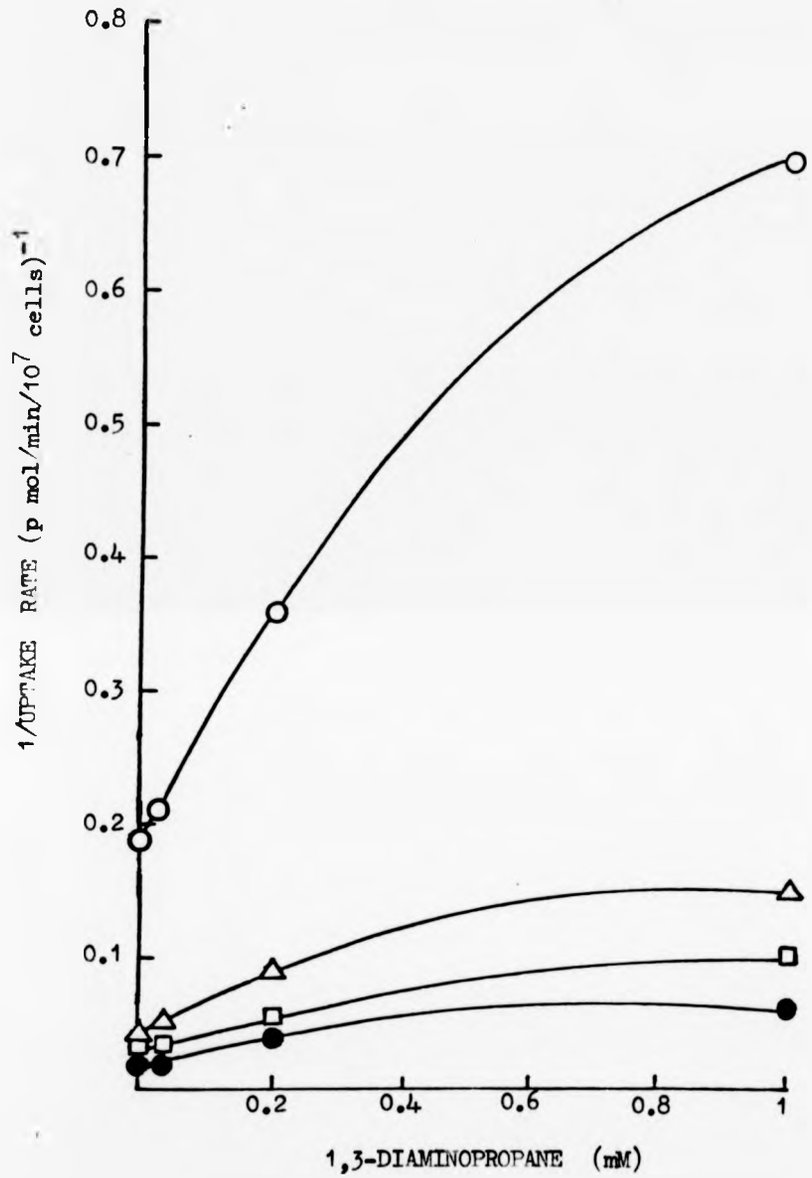


FIGURE 3.7 INHIBITION OF PUTRESCINE UPTAKE BY 1,3-DIAMINOPROPANE

Amoebae were incubated at a density of 4.3×10^6 cells/ml in 17mM-sodium phosphate buffer, pH6. After 15 min [1,4-^{14}C] putrescine and 1,3-diaminopropane were added and the uptake rate determined as detailed in the methods section. Putrescine concentrations were 1μM(○), 5μM(△), both at 2.5μCi/μmol, 10μM (□) and 20μM (●) both at 0.25μCi/μmol. The data was plotted by the method of Dixon (1953).

midine and spermine, in that it is less effective at higher concentrations of putrescine (Turner et al.,1979). Putrescine uptake in D. discoideum has been shown to be inhibited by ω-aminocarboxylic acids, the degree of inhibition being dependent on the chain length of the amino acid, but not by the α-amino acids ornithine or lysine (Turner et al., 1979).

SECTION C - EFFLUX OF PUTRESCINE FROM AMOEBAE

When amoebae prelabelled with [1,4-^{14}C] putrescine were suspended in 17mM-phosphate buffer, pH6, containing 10mM-$MgCl_2$, $CaCl_2$ or $MnCl_2$, no efflux of radioactively labelled material was observed. However the presence of oligoamines in the suspension buffer at 1mM concentrations stimulated efflux of label from similarly prelabelled cells (Figure 3.8). At 0.5mM-extracellular putrescine the net efflux of labelled material from the cells was negligible, whilst at 1mM-extracellular putrescine, spermidine, spermine or 1,3-diaminopropane efflux rates corresponding to a loss of 5-6% of the initial cell radioactivity/h were measured. Electrophoretic analysis of the suspension buffers after 3h indicated that the major labelled material present was putrescine with small amounts of spermidine particularly in those flasks containing extracellular spermidine and spermine.

SECTION D - EFFECT OF STARVATION ON THE RATE OF PUTRESCINE UPTAKE

When suspended in 17mM-phosphate buffer, amoebae are under starvation conditions and thus undergo many of the changes characteristic of early development. The effect of such treatment on the ability of amoebae to take up putrescine was investigated (Figure 3.9). At both 5μM and 0.1mM-putrescine the rate of putrescine uptake decreased with the time of starvation. The effect was slightly greater at 5μM-putrescine than at 0.1mM-putrescine (Figure 3.9). Thus after 5h suspension in phosphate buffer no uptake was detected from buffer containing 5μM-putrescine (Figure 3.9).

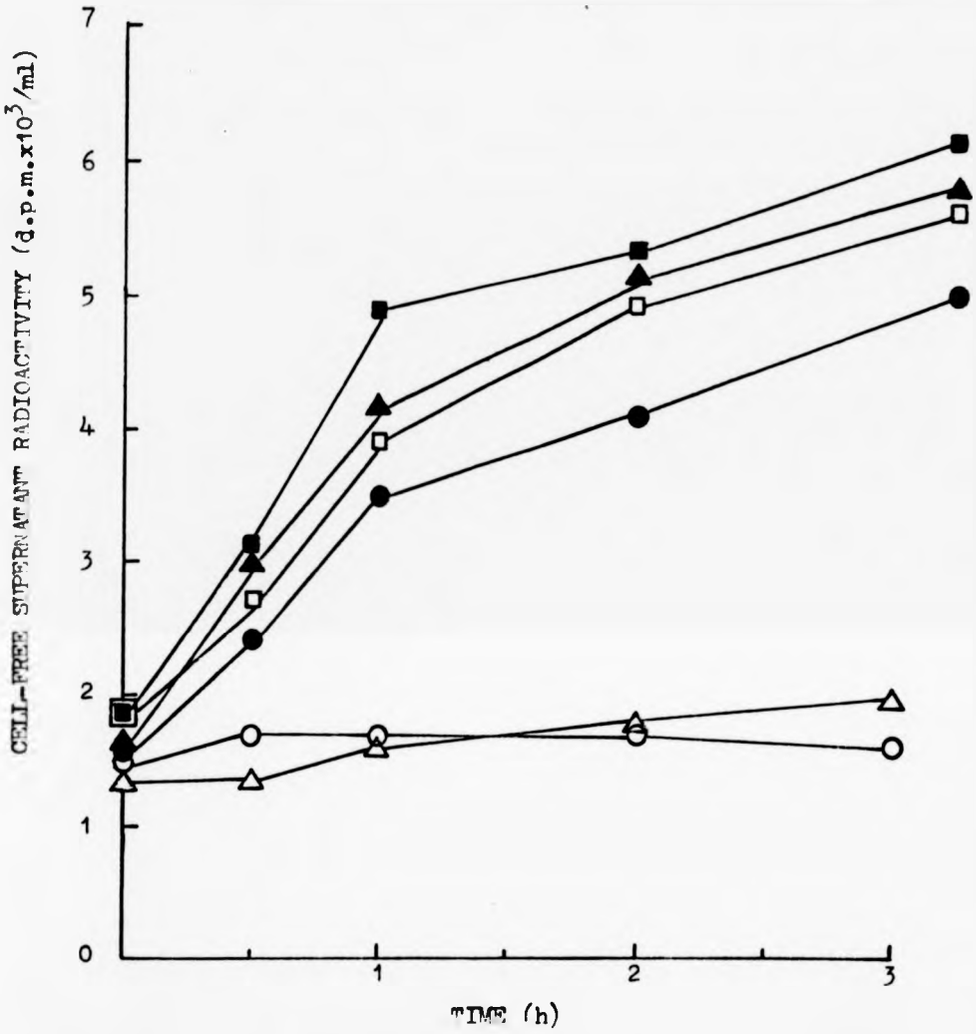


FIGURE 3.8 EFFLUX OF RADIOACTIVELY LABELLED MATERIAL FROM AMOEBAE PRELABELLED WITH [1,4-^{14}C] PUTRESCINE

Amoebae were incubated at 4.5×10^6 cells/ml in 17mM-sodium phosphate buffer, pH6. After 15 min 5μM-[1,4-^{14}C] putrescine (12.5μCi/μmol) was added and incubation continued for 30 min. The cells were harvested, washed twice with water and resuspended at a density of 4.3×10^6 cells/ml in 17mM-sodium phosphate buffer, pH6, containing no additions (○); 0.5mM-putrescine (△); 1mM-putrescine (□); 1mM-1,3-diaminopropane (●); 1mM-spermidine (▲) or 1mM-spermine (■). At intervals 1ml samples were withdrawn and centrifuged for 5 min at 500g. The supernatant was transferred to a scintillation vial and counted for radioactivity as detailed in the methods section.

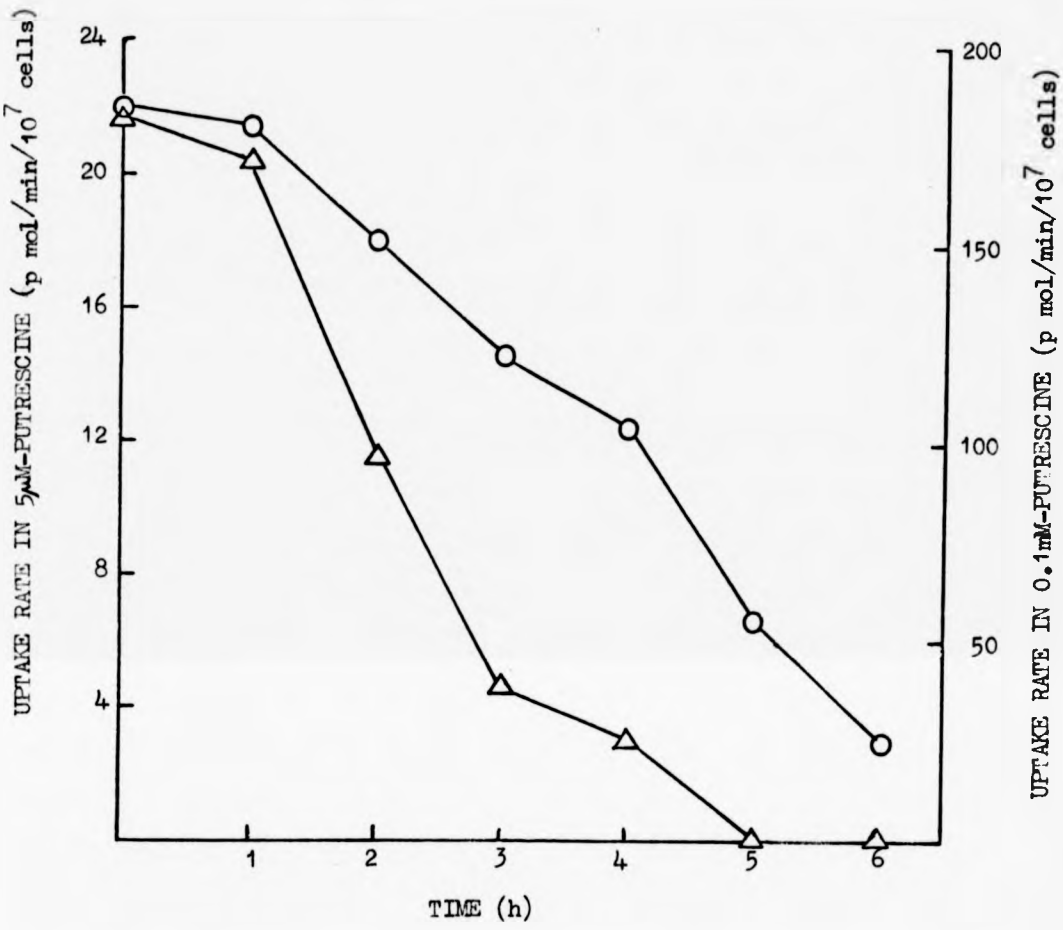


FIGURE 3.9 EFFECT OF TIME OF STARVATION ON PUTRESCINE UPTAKE RATES

Amoebae were incubated in 17mM-sodium phosphate buffer, pH6, at a density of 1.9×10^6 cells/ml. At intervals 10ml aliquots were withdrawn, [1,4-^{14}C] putrescine added and the uptake rate determined over 30 min as detailed in the methods section. Putrescine concentrations were 5μM [2.5μCi/μmol, O] and 0.1mM [0.25μCi/μmol, Δ].

DISCUSSION

When D. discoideum amoebae were incubated in medium containing [1,4-^{14}C] putrescine, radioactivity was recovered associated with cells well washed with unlabelled putrescine and this was believed to be due to the uptake of diamine into the cells. Over 90% of the radioactive material associated with the cells after 72h was soluble in 2% (w/v)-trichloroacetic acid and on further analysis by paper electrophoresis, the major portion of this label was found to migrate with putrescine (Table 1.6). The uptake was a time-dependent process and appeared to require active metabolic processes as it was dependent on incubation temperature and sensitive to inhibition of oxidative metabolism and protein synthesis (Figure 3.5). Neither preincubation nor postincubation of the cells with unlabelled putrescine significantly altered the labelling of the cells with [1,4-^{14}C] putrescine and the label was not removed by washing the cells with water nor with solutions of monovalent or divalent metal ions.

Studies of oligoamine uptake in other systems suggest the involvement of specific energy-dependent transport systems. Putrescine uptake by mouse mammary gland cells resembles that in D. discoideum as it too is sensitive to inhibition by metabolic inhibitors such as 2,4-dinitrophenol and cycloheximide (Kano & Oka, 1976). As a soil amoebae D. discoideum ingests food by phagocytosis whilst pinocytosis is believed to be the feeding mechanism which enables the amoebae to be cultured axenically in soluble culture medium (Ryter & Chastellier, 1977). Thus it would seem unlikely that the amoebae would possess specific systems for transporting substances across the cell membrane. As D. discoideum amoebae have the capacity to synthesize putrescine intracellularly (Table 1.5) and it is not a growth requirement (Fanke & Kessin, 1977), possession of a specific putrescine transport system by these cells would seem to be unnecessary. In Acanthamoeba castellani the uptake of a variety of solutes from the medium is believed to occur by pinocytosis and has been

shown to be a temperature-dependent process which is affected by the inhibition of oxidative metabolism (Bowers & Olszewski, 1972; Bowers, 1977). Although a few substances enter D. discoideum amoebae by passive diffusion (Lee, 1972c), pinocytosis is believed to be the mechanism by which many solutes such as inulin, 6-aminohexanoic acid and perhaps lysine enter the cells and the uptake of these substances has been shown to be sensitive to temperature and metabolic inhibitors (Lee, 1972c; North & Williams 1978). Thus pinocytosis is probably involved in the entry of putrescine into D. discoideum amoebae; a process which is non specific yet energy requiring.

If a variety of different solutes enter the amoebae by the same bulk transport mechanism, i.e. pinocytosis, then it would be expected that comparison of their uptake rates when expressed as the volume of medium ingested by a standard number of amoebae per unit time, would be within the same range. Thus, despite differences in properties and intracellular fates, the endocytotic indices of 6-aminohexanoic acid, glucose, β-alanine, inulin, uracil, and protein hydrolysate in D. discoideum amoebae are between 2μl and 8μl medium/h/10^7 cells (North, 1982). Thilo & Vogel (1980) report a similar rate of FITC-dextran uptake by D. discoideum from HL5 medium (8.4μl medium/h/10^7 cells). The similarity of these rates is evidence in support of a common uptake mechanism, probably fluid phase pinocytosis. Had diffusion or specific transport systems contributed significantly to the entry of these solutes, the rates of uptake would have shown more variation, dependent on properties such as solute size, charge and intracellular concentration. Putrescine uptake differed however from that of other compounds,in that it appeared to be concentrative and its uptake rate was always considerably faster than that of solutes taken up by fluid phase pinocytosis. Thus at a concentration of 5μM it was taken up from phosphate buffer at a rate equivalent to 300μl medium/h/10^7 cells, whilst at 1mM-putrescine the rate fell to the equivalent of 19μl medium/h/10^7 cells. (From data in Figure 3.1). These faster rates have been interpreted in terms of adsorptive pinocytosis (Silverstein et al., 1977). In this process material

enters the cell bound to the inner surface of the pinosome membrane. Although the total volume of medium ingested by pinocytosis is not large, in adsorptive pinocytosis the uptake rate is also affected by the number and affinity of cell surface sites to which the solute can bind. Thus the complexity of putrescine uptake kinetics (Figure 3.6) is believed to result from the involvement of several different binding sites on the cell surface of amoebae, each having a different affinity for binding putrescine. Closer scrutiny of the Lineweaver-Burk plots (Figure 3.6), suggested that a number of systems were involved, at least one of which was saturable. Thus in the concentration range 1-20μM, a binding site with an apparent Km for putrescine of 10μM was implicated (Figure 3.6a). This value is very similar to that observed for putrescine uptake by cultivated human fibroblasts (approx. $1x10^{-6}$M, Pohjanpelto, 1976; DiPasquale et al., 1978). At higher concentrations of putrescine (>20 M), the rate of putrescine uptake was increased and the shape of the Lineweaver-Burk plot suggested that at these concentrations a non-saturable process was making a significant contribution to the overall uptake. This would be consistent with the saturation of cell surface sites, so that as the concentration of putrescine increased further the contribution to putrescine uptake by adsorption to the cell-surface sites becomes less significant and pinocytotic uptake of putrescine in the fluid phase would predominate. The involvement of adsorptive pinocytosis would account for the difference in uptake rate noted between putrescine and other solutes whose endocytotic indices were much lower than that of the diamine. Thus in a medium containing both types of solutes although the amount of each entering the cell in fluid phase via a pinosome, depends only on the volume of extracellular fluid internalized, additional putrescine would enter the cell as a consequence of its adsorption to the internal surface of the pinocytotic vesicle. The existence of such a mechanism makes it unnecessary to propose the existence of specific putrescine cell surface binding sites on D. discoideum amoebae, rather adsorption is seen as a fortuitous consequence of the presence of negatively charged groups on the cell surface.

The decreasing ability of amoebae to take up putrescine after increasing periods of suspension in non-nutrient phosphate buffer (Figure 3.10) is believed to be due to a decrease in the number of putrescine adsorption sites available. Cell electrophoresis studies on D. discoideum have shown that under conditions of starvation the cell surface charge decreases (Garrod & Gingell, 1970; Yabuno, 1970; Lee, 1972a,b). Consistent with this hypothesis is the observation that at high concentrations of putrescine (2.5mM), where putrescine uptake is believed to be less dependent on adsorption, uptake of the diamine is less affected by exposing the cells to starvation conditions (Turner et al., 1979). Kinetic studies reveal that in cells subjected to such conditions the loss of uptake ability at low concentrations of putrescine is the result of a decrease in the Vmax and not to a change in Km (Turner et al., 1979). This would result if the number of available putrescine adsorption sites on the cell surface decreased whilst the energy of binding to the remaining sites was unaltered.

Other oligoamines would be expected to compete with putrescine for negatively charged cell surface adsorption sites. Thus the inhibition of putrescine uptake by substances such as 1,3-diaminopropane, spermidine and spermine would depend on the relative binding affinity of the different sites for the various oligoamines. At low concentrations of putrescine 1mM-spermidine and spermine completely inhibit putrescine uptake (Figure 3.7). At millimolar concentrations of putrescine where adsorptive pinocytosis is believed to be less significant, spermidine and spermine are found to be less effective at inhibiting putrescine uptake (Turner et al., 1979). The kinetics of inhibition of putrescine uptake by 1,3-diaminopropane was complex (Figure 3.8), but again the diamine was a more effective inhibitor at low concentrations of putrescine (5μM). Inhibition by the diamine was less severe than with spermidine and spermine possibly due to the greater concentration of cationic charge on these oligoamines. The uptake of putrescine was not inhibited by the basic amino acids ornithine, the metabolic precursor of putrescine, nor lysine (Turner et al., 1979). Uptake

of ornithine by D. discoideum amoebae was much slower than that of putrescine (Figure 1.13) and probably enters the cell mainly by fluid phase pinocytosis.

The quantities of putrescine extracted from D. discoideum amoebae were equivalent to an intracellular concentration of 17mM (from data in Table 1.2). Thus it would appear that at all the extracellular concentrations used in this study, uptake of putrescine was a concentrative process. However the cationic nature of putrescine prevents such a conclusion, since binding of the diamine to intracellular components such as nucleic acids and membranes most probably reduces considerably the pool of free intracellular putrescine. Whilst labelled putrescine was not lost from prelabelled amoebae subsequently incubated in buffer containing bivalent metal ions, efflux of the diamine was stimulated by the presence in the suspension buffer of 1mM-putrescine (Figure 3.9). This was probably due to an influx of unlabelled diamine into the amoebae, enlarging the intracellular pool of free putrescine which subsequently exchanged with bound labelled putrescine. The result was increased levels of excretion (Figure 3.9). Extracellular 1,3-diaminopropane, spermidine and spermine resulted in a similar stimulation of labelled putrescine efflux from amoebae (Figure 3.9). This suggested that these oligoamines can exchange with bound putrescine in at least some of its cellular roles.

The rate of putrescine uptake from phosphate buffer by cells grown in the presence of glucose was higher than by cells grown in the absence of glucose (Table 3.1). Similarly uptake of putrescine from HL5 medium was faster than from FM medium. The rates of putrescine uptake show a correlation with the doubling time of the amoebae used. Differences in the rate of putrescine uptake may simply reflect a difference in the cell size of amoebae grown in the different media and therefore differences in the surface area available for adsorption. Alternatively the higher rate of putrescine uptake and the rapid doubling time observed in cells grown in glucose-containing HL5 medium may both result from an increased permeability of these cells to nutrients due to an increased rate of pinocytosis. Studies on 6-aminohexanoic acid uptake (North & Williams,

1978) have demonstrated a difference in permeability between axenic strains (used in the putrescine uptake studies reported here), and all non-axenic and slow growing axenic strains. This is believed to reflect differences in their ability to carry out pinocytosis. The rate of putrescine uptake from phosphate buffer was higher than from HL5 medium (Table 3.1). This could result from an increased rate of pinocytosis when cells are suspended in non-nutrient buffer or alternatively it may reflect an increased competition for cell surface binding sites in the relatively complex HL5 growth medium. A detailed comparative study of putrescine uptake by different strains of D. discoideum grown under different conditions would be necessary to determine the exact relationship between the rate of putrescine uptake, the doubling time of a given culture of amoebae and the rate of pinocytosis.

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