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Title of thesis "Biochemical Studies of the Germinating Conidia of *Aspergillus nidulans*"

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Biochemical investigations of the germinating conidia of Aspergillus nidulans were carried out to elucidate the mechanisms controlling macromolecular synthesis during germ-tube emergence. Conidial germination of the wild type strain of A. nidulans, BWB 272, is accompanied by a hundred-fold increase in a putrescine-stimulated S-adenosylmethionine decarboxylase activity and a rapid accumulation of spermidine and spermine. Putrescine levels remain low at all stages of growth. The highest specific rates of protein and nucleic acid synthesis occur coincident with germ-tube emergence. Use of metabolic inhibitors demonstrates the necessity of RNA and protein synthesis, but not DNA synthesis, for conidial germination.

Conidia of the putrescine auxotroph, A. nidulans puA_1, require putrescine for optimal rates of germination and growth. Putrescine starvation is a slow process resulting in a gradual run-down of a wide variety of metabolic processes. However a rapid change in the intracellular levels of spermidine and spermine occurs following the addition of putrescine to cultures starved for 12 hours. This results in immediate increases in the rates of protein and nucleic acid biosynthesis, oxygen consumption and polypeptide chain elongation. Also the average size of the isolated polysomes is three-fold larger and the proportion of ribosomal RNA synthesized increases two-fold. It is concluded that putrescine, spermidine and spermine are likely to have essential roles in macromolecular synthesis.

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ABBREVIATIONS

All abbreviations are made in accordance with the recommendations of the Biochemical Journal 153, 1 - 21 (1976) with the following additions:

MGBG methyl glyoxal bis(Guanylhydrazone)

SAM S-adenosyl-L-methionine

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INTRODUCTION

Preface

Cell differentiation is the process in which cells develop different characteristics by unequal expression of the same genetic material. The production of morphologically, physiologically and biochemically distinct cells by genetically programmed events is dependent on differential gene expression which often proceeds as a direct or indirect response to extracellular signals acting on the cell. Thus the development of any organism depends on the product of the interaction between its genetic complement and environmental stimuli and resources. In multicellular organisms differentiation can lead to the formation of complex structures or tissues but differentiation exists among unicellular organisms as well, such as in spore production and the temporal differentiation that all cells undergo during the cell cycle.

A major development in modern biological research is directed towards developing an understanding of morphogenesis and differentiation in molecular terms. In those cases such as spore germination, where development involves a sequence of differential gene activations, the first step in understanding this process is to identify the developmentally significant gene products. Then it may be possible to determine the mechanisms controlling the activity of the genes involved. In order to be classified as developmentally significant the event must be both unique and essential to the process under consideration. Similar criteria have been applied successfully to the study of bacterial sporulation (Hanson et al, 1970) and development of Dictyostelium discoideum (Killick and Wright, 1974).

Changes in cell structure and properties can be attributed to differences in the chemical composition of the cell which are largely determined by the nature of the cell's enzymatic activities. Enzyme activity can be controlled at three or more levels. First, the activity of an enzyme protein can be affected by small molecular weight compounds such as substrates, products, cofactors, acitvators and inhibitors. Secondly, the amount of enzyme protein can be modulated through alteration of the rates of translation, protein degradation, post-translational modification or compartmentalization. Lastly, the changes in enzyme protein may be caused by variations in the mRNA population due to altered specificity of the RNA polymerases or through increased rates of synthesis or degradation of specific mRNA species.

Synthesis of structural proteins and enzymes usually involves both RNA and protein synthesis, so the control of macromolecular synthesis is fundamental to development. Although the in vivo functions of the naturally occurring oligoamines, spermine, spermidine and putrescine are unknown, they may play an important role in controlling nucleic acid and/or protein synthesis (Stevens, 1970; Tabor and Tabor, 1972 and 1976).

The aim of this project was to investigate some aspects of macromolecular synthesis including possible control mechanisms in the germinating conidia of the ascomycete mould Aspergillus nidulans. In particular it was intended to examine the possibility that oligoamines have a role in bringing about the increases in nucleic acid and protein synthesis which accompany germination (Bainbridge, 1971). A. nidulans was chosen for this study because it can be easily manipulated both biochemically and genetically, with a large stock of mutants available, yet is a eukaryotic organism with a true nucleus containing histones (Morris et al, 1977). Furthermore, A. nidulans, in common with other filamentous fungi,

is a valuable link between the unicellular state of the abundantly investigated bacteria and the more complex multicellular organization found in higher organisms. Thus, study of this relatively simple organism has considerable relevance to the study of differentiation in other eukaryotes.

In order to consider fully the possibility that oligoamines may have a role in controlling macromolecular synthesis during germination it will be necessary to discuss in some detail: (i) the various control points of nucleic acid and protein synthesis to understand better how oligoamines may be involved in these processes. The role of oligoamines in fungal development has been reviewed by Stevens and Winther (1978), (ii) germination of fungal spores and (iii) oligoamine metabolism and *in vivo* and *in vitro* studies of oligoamine functions.

A The Control of Macromolecular Synthesis

The control of macromolecular synthesis is intimately concerned with germination and conidiogenesis. Our knowledge of control mechanisms in eukaryotes is fragmentary, especially when contrasted with the detailed knowledge about prokaryotes such as E. coli. In part this is a reflection of the greater complexity of the control of macromolecular synthesis in eukaryotes. This section will review recent advances in understanding fundamental control mechanisms, especially where relevant to the control of dormancy and subsequent resumption of active growth. The possible role of oligoamines in regulating macromolecular synthesis is considered further in section C.

(i) DNA Synthesis and Nuclear Replication

Nucleus: structure and replication

Fungi, like all other eukaryotes, contain the bulk of their genetic material in a special organelle, the nucleus, which is bounded by a membrane. Thus there is a spatial separation between protein synthesis (cytoplasmic) and DNA and RNA synthesis (nucleoplasmic). This very fundamental difference in structure between prokaryotes and eukaryotes is likely to be paralleled by different means of regulating the expression of genetic material. Fungal nuclei are generally small and the events of mitosis in many fungi have only relatively recently been described. The morphology of the nucleus of Aspergillus nidulans and its changes during the cell cycle have been described by Robinow and Caten (1969). The nucleus is bounded by a membrane and has a true nucleolus. At metaphase the nucleolus disappears as the chromatin condenses into densely-staining chromosome-like structures. The chromosomes are apparently organized by a microtubular spindle apparatus which, in anaphase, separates the chromosomes, moving them to either end of the

spindle. Lastly, the nucleus divides and the chromatin again becomes diffuse. Some features of mitosis in A. nidulans are unlike those of typical higher eukaryotes. The chromosomes are oriented along a spindle rather than a metaphase plate and centrioles are replaced by spindle plaques (Moens and Rapport, 1971). The nuclear membrane does not break down and disappear in mitosis. Furthermore, in coenocytic fungi such as Aspergillus, mitosis is not followed by septation and cell division. There is not a rigid mechanism interlocking the nuclear and cell division cycles. However A. nidulans does maintain a nearly constant ratio of nuclear to cytoplasmic volume (Clutterbuck, 1969) and it has been suggested that septation is coupled to nuclear division (Clutterbuck, 1970).

From studies on yeast (Franco et al, 1974; Thomas and Furber, 1976), Neurospora (Goff, 1976; Noll, 1976) and A. nidulans (Morris, 1976c) it now seems certain that most fungi have a full complement of histones as well as non-histone proteins complexed with nuclear DNA. The electrophoretic properties and amino acid composition of histones from A. nidulans resemble those of calf-thymus histones. Digestion with nucleases indicates that chromatin in A. nidulans, like that of yeast and rat liver, has a regular repeating structure based on nucleosomes. The nucleosome repeat length of A. nidulans chromatin is about 150 base pairs, which is significantly shorter than the 200 base pair repeat length of rat liver chromatin (Morris, 1977). This is due to the DNA "linker" between nucleosomes being shorter; only 10 to 20 base pairs long. The shorter "links" may reflect the less basic charge found in the A. nidulans histone H1. The nucleosome cores of A. nidulans and rat liver are both 140 base pairs long and have similar structural organizations as shown by digestion with DNAse I. Sheir-neiss et al, (1976) have demonstrated the presence of tubulin, the major protein

component of microtubules, in A. nidulans.

Recently a number of temperature-sensitive mutant strains of A. nidulans deficient in mitosis have been isolated (Morris, 1976a,b; Orr and Rosenberger, 1976a,b). Morris (1976a) identified over 35 genes required for nuclear division, septation or nuclear distribution. Mutations could block mitosis early in the nuclear replication cycle, in the middle , or close to or during mitosis (Orr and Rosenberger, 1976b). Some of the mutants could continue synthesizing DNA, resulting in abnormally large nuclei with a single nucleolus (Orr and Rosenberger, 1976a). Apparently the entire genome can be duplicated a number of times. This class of mutation has not been reported to occur in other organisms (Hartwell, 1974).

Composition of the fungal genome

Fungi are among the simplest of organisms with a true nucleus, possessing some of the smallest genomes of any differentiating eukaryotes. The haploid fungal genome varies in size from 10^7 nucleotide base pairs, or only twice that of the average bacterial genome, to 5×10^7 base pairs (Ojha et al, 1977; Storck, 1974). This is still small compared to the mammalian genome of roughly 3×10^9 base pairs or the genome of various amphibia which may range in size up to 8×10^{10} base pairs (see Southern, 1974). The necessity for these vast quantities of DNA in the genome is not explained simply by differences in the complexity of the organisms. In part the increase in the amount of DNA in eukaryotic cells when compared with prokaryotes represents an increase in the regulatory complexity of the cell rather than an increase in the number of structural genes. According to one estimate not much more than 5% of the DNA in amphibian chromosomes is transcribed into RNA sequences (Miller and Bakken, 1972), leaving the functions of the

"excess" 95% a mystery.

The nuclear DNA of eukaryotes is not homogeneous but composed of unique, or single copy sequences and repeated sequences (Davidson et al, 1973). In general, more than one-third of the DNA of higher organisms is made up of sequences which recur anywhere from a thousand to a million times per cell (Britten and Kohne, 1968). A slightly smaller portion of the total cellular DNA is found to be reiterated in fungi, from 5 to 25%, which can only be partly accounted for by reiterated rRNA and tRNA cistrons (Christiansen et al, 1971; Brooks and Huang, 1972; Dutta, 1973; Ojha et al, 1977). Some of the repeated DNA sequences may be transcribed. The hnRNA from HeLa cells contains intermediate repetitive and unique sequences interspersed with one another (Southern, 1970; Burden and Shenkin, 1972).

The guanosine plus cytosine content (moles % GC) of fungal DNAs ranges from 27.5 to 70% with only a low proportion of unusual bases (Storck and Alexopoulos, 1970). A. nidulans has a GC content of about 50% (Pontecorvo, 1967). Some 10-20% of the nuclear DNA can occur as satellite species with distinct buoyant densities in CsCl. In Saccharomyces cerevisiae the majority of the rRNA cistrons are found in the satellite bands (Finkelstein, Blamire and Marmur 1972). The haploid conidia of A. nidulans contain 1.3×10^{10} daltons of DNA per spore, or about 2×10^{7} base pairs per haploid genome (Bainbridge, 1971). A. nidulans has eight chromosomes (Elliot, 1960) represented by eight well-marked linkage groups (Kafer, 1958). Therefore the size of the average chromosome for this organism is approximately one-half that of the E. coli genome. Although chromosomal content of DNA in fungi is normally considered to be stable and unchanged throughout the life cycle and differentiation (Berry and Berry, 1976) it now appears that

under certain circumstances ribosomal DNA magnification can occur in S. cerevisiae (Kaback and Halvorson, 1978).

Part of the fungal genome is extra-nuclear DNA. From 0.5 to 20% of the fungal DNA may be mitochondrial and additional species of DNA have been found in the cytoplasm of some fungi (Myers and Cantino, 1971; Sinclair et al, 1967). The mitochondrial DNA of A. nidulans is a single, twisted circular form with a contour length of 10μm, and contains approximately 30,000 base pairs (Lopez and Turner, 1975). This is considerably smaller than that found in yeast (26μm contour length) or N. crassa (20μm) but twice as large as mouse or human mitochondrial DNA (Jones and Walker, 1964). Extra-nuclear mitochondrial mutants have been isolated from many fungal species (see Turner and Rowlands, 1977).

DNA polymerases

DNA-dependent DNA polymerases have been isolated from only a few fungi including Ustilago maydis (Jeggo et al, 1973), S. cerevisiae (Wintersberger and Wintersberger, 1970), Rhizopus stolonifer (Gong et al, 1973), Neurospora crassa (El-Assouli and Mishra, 1978) and the slime moulds D. discoideum (Loomis et al, 1976) and Physarum polycephalum (Baer and Schiebel, 1978; Zänker and Schiebel, 1978).

The most extensively studied DNA polymerase activities are those of S. cerevisiae which contains at least two non-mitochondrial DNA polymerase activities, A and B, both with molecular weights of 150,000-170,000 daltons. Yeast DNA polymerase A has many properties in common with the DNA polymerase α of animal cells (Wintersberger, 1977); the enzyme thought to be responsible for DNA replication. An enzyme corresponding in size to the nuclear DNA polymerase β is lacking in yeast (Wintersberger, 1974) and other lower eukaryotes (Chang, 1976). The yeast DNA polymerases A and B are antigenically distinct. Only

DNA polymerase A can use oligoribonucleotide primers, suggesting a role in DNA replication (Wintersberger, 1978). Yeast DNA polymerase B exhibits a 3'-exonuclease activity which is consistent with a role in repair processes (Chang, 1977).

A DNA polymerase from Ustilago maydis is also of the α type (Banks et al, 1976). The pol 1-1 mutant strain is temperature sensitive for nuclear DNA synthesis in vivo, and its DNA polymerase activity in crude extracts and partially purified is thermolabile (Jeggo et al, 1973). Thus the temperature sensitive DNA polymerase appears to be the one involved in chromosome replication. The purified polymerase has associated a 3'-5' exonuclease activity (Banks and Yarrington, 1976). This association of exonuclease activity in fungal DNA polymerases resembles the behaviour of prokaryotic polymerases (Livingstone and Richardson, 1975) rather than eukaryotic DNA polymerases (Brun et al, 1974).

The DNA polymerase activities of R. stolonifer purified from dormant and germinating conidia were found to be physically and chromatographically identical (Gong et al, 1973). However, clearly differences between the two enzymes or the substrates do exist as only the activity from the dormant conidia was dependent on an exogenous DNA template.

The replication of DNA involves much more than just DNA polymerase. T_4 bacteriophage has at least six genes in its chromosome that code for proteins absolutely essential in DNA replication (Barry and Alberts, 1972). The more complicated chromosomes of bacteria and eukaryotes must require at least as many. Indeed, six distinct loci involved in DNA replication are known in E. coli (Kornberg, 1977). These would include RNA polymerases for initiation, DNA polymerases for chain extension and completion, unwinding protein for fork movement and ligases for sealing nicks. Hartwell (1973) could find five genes thought to

have a role for initiation and/or elongation of DNA synthesis in yeast, and more are likely to exist. The formation of small denatured regions of DNA (300 base pairs long) occurs during meiosis in S. cerevisiae at the time of DNA replication and recombination (Klein and Byers, 1978). Conditional mutants can be obtained trapped at this stage, unable to complete the replication cycle.

Studies of histone biosynthesis and its interruption by cycloheximide have suggested a coupling of the rate of DNA chain elongation with the availability of histones (Weintraub and Holtzer 1972). DNA replication must be dependent on protein synthesis as it also involves the doubling of histone and non-histone chromosomal proteins with which it is associated.

Although there is a good correlation between DNA synthesis in vivo and DNA polymerase activity in extracts of rat tissues (Baril and Laszlo, 1971) the rate of DNA synthesis in vivo is not likely to be controlled solely by the levels of DNA polymerase available (Weissbach, 1977). Although DNA polymerase activity and the rate of DNA synthesis both increased during the germination of R. stonifer spores the complete lack of DNA synthesis in early germination is not simply a result of an absence of DNA polymerase, since an active enzyme can be isolated from ungerminated spores (Gong et al, 1973). DNA polymerase activity in D. discoideum is higher in stationary phase cells than in log phase cells, which does not reflect the in vivo rates of DNA synthesis (Loomis et al, 1976).

(ii) RNA Synthesis

General

The major species of RNA found in fungi are typical of those occurring in other eukaryotes (Berry and Berry 1976). Normally ribosomal RNA comprises approximately 80% of the total RNA with transfer RNA, 5S RNA

amounting to 15 to 20% of the total. Messenger RNA species make up less than 5% of the bulk RNA (see Van Etten et al, 1976). Mitochondria from fungi contain rRNAs, tRNAs and mRNAs which are transcribed from mitochondrial DNA. Mitochondrial ribonucleic acids, in general, resemble those of prokaryotes rather than those of the (non-mitochondrial) cytoplasm as shown by studies of A. nidulans (Edelman et al, 1970; Verma et al, 1970) and S. cerevisiae (Reijnders et al, 1973). The cytoplasmic ribosomes of all eukaryotes, including fungi, contain four distinct RNA classes. In yeast the 25S (1.3×10^6 daltons) and 18S (0.72×10^6 daltons) rRNAs are synthesized as a 35S (2.5×10^6 daltons) precursor (Warner, 1974). Cleaved from a larger common precursor, 5.8S rRNA (0.6×10^5 daltons) remains attached by hydrogen bonds to the mature 25S rRNA (Trapman et al, 1975). The genes for 5S RNA, though located near the other ribosomal genes are synthesized as separate transcripts (Gilbert et al, 1977).

The nucleus contains a well defined group of relatively small molecular weight RNAs (smwRNAs) of 80 to 200 nucleotides length (Ziere and Penman, 1976). These macromolecules are precisely located, as is hnRNA, within the nuclear skeletal framework (Miller et al, 1978). The functions of the smwRNAs is unknown.

Messenger RNA

The only definitive means of establishing a class of RNA as messenger RNA is by using it to direct protein synthesis in a cell free system. Total cellular RNA and poly A-containing RNA have been used successfully to direct polypeptide synthesis in vitro using RNA preparations from N. crassa (Mirkes and McCalley, 1976; Hopper et al, 1978) Botryodiplodia theobromae (Wenzler and Brambl, 1978) and the cellular slime mould Dictyostelium discoideum (Jacobson et al, 1977).

Furthermore the translation of specific proteins, identified electrophoretically and immunologically, has been achieved using yeast RNA to direct ribosomal protein synthesis (Gorenstein and Warner, 1977) or uridyl transferase (Hopper et al, 1978), N. crassa RNA to direct glutamine synthase (Palacios et al, 1977) or D. discoideum RNA to direct actin synthesis (Kindle and Firtel, 1977). Holland et al, (1977) found that 25% of the yeast polyadenylated mRNA consists of about ten specific mRNA species coding for enzymes of the glycolytic pathway.

Studies of animal cells have established that a large portion of the mRNA population is associated with a specific class of proteins, forming complexes termed messenger ribonucleoproteins or mRNPs (Greenberg, 1975). EDTA treatment of polysomes causes the release of a heterogeneous population of particles ranging in size from 4S to 120S with a ratio of protein to RNA of about 4:1 (wt/wt). The cytoplasmic mRNA not associated with polysomes also occurs as mRNP complexes. The association of specific proteins with mRNA begins in the nucleus during transcription (Lukanidin, 1972) and continues on until polysome assembly (Irwin et al, 1975; Schochetman and Perry, 1972). It seems likely that mRNPs are common in fungi as Mirkes (1977) found that the mRNA of N. crassa is associated with six polypeptides which were clearly not ribosomal proteins. The protein constituents in eukaryotic mRNAs may protect the RNA from nuclease degradation, aid in transport or regulate the translation of specific messengers (Spirin, 1969). A direct role in protein synthesis is unlikely as the translation of globin mRNP is no better than that of the isolated 9 S mRNA (Sampson et al, 1972).

Most eukaryotic mRNA molecules including those of fungi are now known to contain blocked and methylated 5'-terminal structures called "caps" (see Shatkin, 1976; Rottman, 1976). Muthukrishnan et al (1975) demonstrated the necessity of the 5'-terminal 7-methylguanosine for the

translation of eukaryotic mRNAs in in vitro protein synthesizing systems. Methylation may not be necessary for transport of mRNA to the cytoplasm (Kaehler et al, 1977) but the timing of capping does appear to be important (Friderici, et al, 1976). The functional role of the 5'-cap seems to be as an allosteric binding site for one or more proteins involved in the initiation reactions in protein synthesis (Busch et al, 1976). Thus the translation of mRNA can be specifically inhibited by pm^7G (Hickey et al, 1976).

In common with higher eukaryotes a large portion of the mRNA population in fungi contain polyadenylate segments at the 3' end of the molecule (see Van Etten et al, 1976). Approximately one-half of the yeast (McLaughlin et al, 1973) and N. crassa (Mirkes and McCalley, 1976) messengers contain poly-adenylate tracts and those that do are from 40 to 60 nucleotides long. This is significantly shorter than the 150 to 200 long nucleotide residues found in mammalian cell mRNAs (Darnell et al, 1971). The sequence A-A-U-A-A-A has been found to occur in the 3' non-coding region about 20 nucleotides in from the poly A tract in all eukaryotic mRNAs which have been sequenced in this region (Ferrier et al, 1977). The absence of poly A tracts in a portion of the mRNA in fungi and animal cells (Milcarek et al, 1974; Nemer et al, 1974) suggests that the sequence is not essential for mRNA functions. The removal of poly A from messengers does not alter their translation in cell free systems (Munoz and Darnell, 1974) but they are less effectively translated in Xenopus oocytes, possibly as a result of a more rapid degradation (Marbaix et al, 1975). Despite a measurable shortening of mRNA poly A tracts with ageing of the messenger, this cannot as yet be correlated with a regulatory role in protein synthesis (Sheirneiss and Darnell, 1973; Brawerman, 1974). In this respect it is interesting to note that histone mRNAs, which lack poly A tracts (Adesnick et al, 1972),

are degraded very rapidly after S-phase during the cell cycle (Stein et al, 1976; Perry and Kelley, 1973).

It has been assumed that eukaryotic genes and their mature RNA products are strictly colinear (Watson, 1976). Results from use of the techniques of gene cloning and hybridization have dramatically altered this view. Within the diverse structural gene sequences coding for rabbit and mouse γ-globin (Jeffreys and Flavell, 1977; Tilghman et al, 1978), mouse immunoglobin light chain (Brack and Tonegawa, 1977), Drosophila ribosomal genes (White and Hogness, 1977) and yeast transfer RNA (Valenzuela et al, 1978) regions of DNA sequences have been found which are not represented in the mature RNA product. A detailed map of the natural gene for ovalbumin has shown that one half of the structural gene is interrupted by at least seven large intervening DNA sequences (Dugaiczyk et al, 1978). The entire ovalbumin gene appears to be transcribed into a precursor RNA three times the size of the mature mRNA with the intervening sequences being subsequently enzymatically processed away from the mature RNA by precise excision and ligation. Much of the extra DNA in eukaryotes may be involved in coding for sequences which are never part of mature RNA but may have a role in mRNA processing or transport. This may account for some of the large heterogeneous nuclear RNA (hnRNA) found in eukaryotes which is thought to include precursors of the smaller cytoplasmic mRNAs (Darnell et al, 1971). 90% of the RNA of poly A-bearing hnRNA molecules is degraded within the nucleus. A similar pattern might be expected in fungi, though Firtel and Lodish (1973) reported that the mRNA precursor in the slime mould D. discoideum is only 20% larger than the mature RNA. Large hnRNAs have not been detected in Phycomyces blakesleeanus (Gamow and Prescott, 1972), Rhizopus stolonifer (Roheim et al, 1974) and D. discoideum (Lodish, 1977). As the yeast genes coding for tyrosine

and phenylalanine tRNAs contain intervening sequences not present in the mature tRNA (Goodman et al, 1977; Valenzuela et al, 1978) it seems likely that the primary mRNA transcripts will contain some non-coding, intervening sequences as well. However a number of yeast genes are capable of producing functional protein products when cloned in E. coli, suggesting that the structural genes are not "split", that is they do not contain intervening sequences (Ratzin and Carbon, 1977; Hicks and Fink, 1977; Nasmyth, 1978). Thus the structure of mRNA in fungi may not resemble that of higher eukaryotes with regards to the presence of "split" genes.

RNA Polymerases

The existence of multiple DNA-directed RNA polymerases have been described for many eukaryotic organisms (Biswas et al, 1975). Multiple RNA polymerase activities have been isolated from Physarum polycephalum (Gornicki et al, 1974), Dictyostelium discoideum (Pong and Loomis, 1973), Mucor rouxii (Young and Whitley, 1975) and a variety of other fungi (see Van Etten et al, 1976). It is difficult to assign functions to these various polymerase activities for a number of reasons. In higher eukaryotes three classes of polymerases are recognized (Chambon, 1974) with class A insensitive to α-amanitin, class B sensitive to low concentrations of α-amanitin (0.001-0.01μg/ml) and class C sensitive only to high concentrations of α-amanitin (10-100μg/ml). There is good evidence that RNA polymerase class B is largely responsible for hnRNA and mRNA synthesis (Chambon, 1974). In fungi, however, there is no polymerase activity as sensitive to α-amanitin as class B RNA polymerases. Levels of 1-33μg/ml α-amanitin are required for inhibition (Young and Whiteley, 1975; Pong and Loomis, 1973; Gornicki et al, 1974; Tellez de Inon et al, 1974; and Gong and Van Etten, 1972) which is closer to RNA polymerase class C in character. Also it has not been possible to definitely locate the fungal

RNA polymerases in specific regions of the nucleus as has been shown with higher eukaryotes (Chambon, 1974), although there is an enrichment of α-amanitin resistant RNA polymerase activity in nucleolar fraction obtained from Physarum polycephalum (Grant, 1972; Hildebrant and Sauer, 1973). Recently Hager et al, (1977) purified three polymerases from S. cerevisiae and presented evidence that polymerase I selectively transcribes ribosomal cistrons (Holland et al, 1977). Thus hybridization analysis of transcriptional products using natural chromatin templates may help identify specific RNA polymerase functions. This has been done successfully with Xenopus (Birkenmeier et al, 1978) to identify specific RNA polymerases. Variation in the proportions of the various main classes of RNA that are synthesized could result from changes in the relative levels of the respective polymerases. However, in some systems it would appear that RNA polymerase levels are not rate limiting factors in RNA synthesis (Gross and Pogo, 1976a). The control of RNA polymerase I activity (α-amanitin resistant) appears to be mediated by short-lived proteins. The amount of RNA polymerase I activity does not itself change during amino acid starvation or protein synthesis inhibition (Cereghini and Franz-Fernandez, 1974). The regulatory capacity of the rapidly turning over protein(s) is expressed only in intact nuclei.

The occurrence in mitochondria of RNA polymerases consisting of only a single, relatively small polypeptide (Kuntzel and Schäfer, 1971; Wu and Dawid, 1972) which can accurately transcribe certain templates demonstrates that transcription does not inherently require a complex multisubunit polymerase. This suggests that the structural complexity of RNA polymerases reflects a complexity of transcriptional control mechanisms. Progress has been made in assigning functions to various subunits of DNA-dependent RNA polymerase in E. coli (Saitoh et al, 1977) but we are still far from a full understanding of the structure-

function relationships in eukaryotic non-mitochondria DNA-dependent RNA polymerases.

Selectivity in Transcription

The multiplicity of RNA polymerases in eukaryotic organisms allows independent control of the various classes of RNA. The relative proportions of rRNA, tRNA and mRNA can be separately regulated as shown in developing embryos (Woodland and Gurdon, 1968), serum stimulated mouse fibroblasts (Zardi and Baserga, 1974) and during germination of fungal spores (Van Etten et al, 1976). The amount of the various RNA polymerase activities can act to control quantitatively RNA synthesis (Kohl et al, 1969), though other controlling elements exist which modulate transcription in eukaryotic cells (Biswas et al, 1975). For example, the unique structure of the rRNA cistrons may be significant in controlling the production of rRNA. Some plants and amphibians may have as many as 20,000 rRNA cistrons per haploid genome, though the levels found in fungi are much smaller at 100 to 200 per haploid genome (see Van Etten et al, 1976).

Again, because they are understood in such detail, prokaryotic control systems are useful model systems for trying to study eukaryotic regulatory mechanisms. The control of protein synthesis in bacteria is exerted primarily at the level of mRNA transcription (Chamberlin, 1970). Genetic and biochemical techniques have identified a number of non-structural regulator genes which control the rate of transcription of structural genes (Jacob and Monod, 1961; Zubay, 1974). In prokaryotes the selectivity of transcription is largely conferred by the σ subunit which can alter the *in vitro* selectivity of *E. coli* RNA polymerase to parallel RNA synthesis *in vivo* (Travers et al, 1978). Bacteriophage infection in *E. coli* or *Bacillus subtilis* (Fox, 1976; Tjian and Pero,

1976) and sporulation in B. subtilis (Greenleaf et al, 1973) are associated with the production of new proteins which interact with the RNA polymerase to alter the transcriptional specificity.

The control of mRNA and protein synthesis in eukaryotes is considerably more complex. However examples of apparent transcriptional control over the production of specific mRNA molecules in animal cells do occur with the production of hemoglobin by erythroleukemic cells (Friend et al, 1971) and production of ovalbumin and avidin by oviduct in response to oestrogen (Means et al, 1972). Holland et al, (1977) found that the high levels of glycolytic enzymes in S. cerevisiae are paralleled by high levels of the corresponding mRNA species. A detailed analysis of uridyl transferase, an inducible enzyme involved in galactose utilization in S. cerevisiae, has demonstrated that the induction of enzyme protein and activity is associated with the appearance of a functional mRNA species for this enzyme (Hopper et al, 1978). The use of RNA synthesis inhibitors in D. discoideum has established that the translation of mRNA follows closely the synthesis of the mRNA, again suggesting that the primary control of protein synthesis is at the level of transcription (Firtel et al, 1973). The induction of enzyme synthesis in A. nidulans also requires both protein and RNA synthesis (Weiss and Anterasian, 1977) though without more measurements of enzyme protein and specific mRNA levels, conclusions regarding the nature of specific control mechanisms can only be tentative.

A major role in determining transcriptional specificity is attributed to non-histone chromosomal proteins. Transcriptionally active chromatin is enriched in non-histone proteins and impoverished in histones as compared to whole chromatin (Bonner, 1977). In common with other eukaryotes, fungi possess a large population of non-histone chromosomal proteins,

a heterogeneous collection of proteins ranging in size from 20,000 to 200,000 daltons (Jacobson et al, 1977; Wintersberger et al, 1973; Hsiang and Cole, 1973). It has been observed that both of the putative regulatory gene products identified in fungi are proteins that bind strongly to DNA and could thus be classified as "non-histone chromosomal proteins" (Jacobson et al, 1977 ; D Philippides and C Scazzocchio, unpublished observations).

One role of the regulatory molecules, presumably found in the chromatin, is to coordinate the synthesis of specific genes located on separate chromosomes. Yeast mRNAs, like those of other eukaryotes, are predominantly, if not exclusively, monocistronic (Petersen and McLaughlin, 1973). Thus a versatile regulatory mechanism is required to coordinate ribosomal protein synthesis in yeast which involves over fifty unlinked genes. This control is exerted at the transcriptional level, as shown by the use of cell free protein synthesizing systems (Gorenstein and Warner, 1977). However not all non-histone chromosomal proteins have regulatory functions. In rat liver over half of the mass of the non-histone chromosomal proteins is composed of structural proteins including actin, myosin and tubulin (Garrard et al, 197). Tubulin has been isolated from A. nidulans, though its intracellular localization was not established (Morris et al, 1977).

Stringent Control of RNA Synthesis

Stringency is a genetically controlled process first studied in E. coli which enables an organism to cope with changing environmental conditions. During amino acid starvation in E. coli there is a stepdown in the rate of protein synthesis followed immediately by a decrease in rRNA synthesis (Edlin and Broda, 1968). This co-ordinate regulation prevents the unbalanced growth which would result if protein synthesis ceased due to lack of

precursors, and RNA synthesis continued unabated. During stringent control there is an increase in guanosine tetraphosphate (ppGpp) which in some way decreases the level of rRNA biosynthesis (Cashel, 1969 and 1975). "Relaxed" strains of E. coli can be obtained by mutation, which has led to a genetic study of the phenomenon. Mutations at three genetic loci, specifying three separate ribosome associated or ribosomal proteins, can affect RNA synthesis and the levels of guanosine tetraphosphate (Block and Haseltine, 1975; Parker et al, 1976; Mosteller, 1978).

Stingency is observed also in eukaryotic organisms including A. nidulans (Arst and Scazzocchio, 1972), ascites cells and yeast (Franze-Fernandez and Pogo, 1971; Gross and Pogo, 1974). The genetic basis for this control is illustrated by the phenotypic relaxation observed in various mutant strains of yeast (Foury and Goffeau, 1973; Gross and Pogo, 1974). The studies with yeast suggest the presence of two factors, presumably polypeptides, one of which switches RNA synthesis on, the other switching it off. These two factors are under the influence of the protein synthetic machinery (Gross and Pogo, 1976b). As a slight inhibition of protein synthesis produces nuclear template restriction it seems likely that the promoter-like factor(s) have a high rate of turnover.

Guanosine tetraphosphate (ppGpp) could not be detected in a number of studies of fungi (Alberghina et al, 1973; Kudrna and Edlin, 1975) and other eukaryotes (Fan et al, 1973; Gross and Pogo, 1976b; Pirrone et al, 1976) under conditions it would be present in prokaryotes (Harshman and Yamazaki, 1971). However using more sensitive techniques small quantities of ppGpp have been detected in S. cerevisiae subjected to heat shock (Pao et al, 1977). If this compound has a role in controlling RNA synthesis in yeast its effects may be restricted to mitochondrial functions. Specific inhibition of yeast mitochondrial ribosomes by oxytetracycline inhibits ppGpp formation presumably much in the same

way that the ribosome-linked synthesis of ppGpp in bacteria is inhibited by the tetracycline (Kaplan et al, 1973).

Any discussion about the regulation of rRNA would be incomplete without reference to ribosome assembly. The bulk of the evidence supports the view that the growth rate of mammalian cells (Luck and Hamilton, 1972; Liau et al, 1975) and fungi (Bull and Trinci, 1977) can be correlated with the rate of ribosome production. Regulation of ribosome production apparently operates primarily through control of RNA maturation processes (Craig, 1973). In the nucleolus the pre-rRNA unites with ribosomal proteins (Prestayko et al, 1974) and certain non-ribosomal proteins (Kumar and Warner, 1972; Kuter and Rogers, 1976) to form a precursor complex. Maturation processes occur in the nucleolus and possibly the cytoplasm (Kumar and Warner, 1972). As methylation of rRNA and a supply of one or more specific proteins is required for normal rRNA processing these may be key targets for regulation (Maden, 1972; Pederson and Kumar, 1971; Luck and Hamilton, 1975).

The dependence of rRNA synthesis upon simultaneous protein synthesis is a significant regulatory mechanism in both prokaryotes and eukaryots (Penman et al, 1976). In yeast the synthesis of pre-rRNA, while dependent on total protein synthesis, is not dependent on the synthesis of ribosomal proteins specifically (Gorenstein and Warner, 1977). Several inhibitors of protein synthesis inhibit pre-rRNA synthesis and processing. In yeast the primary block is on pre-rRNA processing, with subsequent accumulation of 35S, 27S and 20S pre-rRNA intermediates (Udem and Warner, 1972). The block in ribosome biogenesis caused by cycloheximide could be due to the rapid limitation in the supplies of proteins critical for the formation of mature ribosomal subparticles, or by a more direct inhibition of RNA synthesis. The RNA polymerase activities in extracts of Blastocladiella emersonii (Horgen and Griffin 1971) and Achlya bisexualis

(Timberlake et al, 1972b) can be inhibited by cycloheximide or rifampin. However, whether these protein synthesis inhibitors inhibit RNA synthesis in vivo is questionable as the fungal RNA polymerases in question are only sensitive to cycloheximide or rifampin when isolated by a specific technique and so may be simply an artifact of the extraction process (Timberlake et al, 1972a).

(iii) Protein Synthesis

General

Although there is obviously an increasing need for translational control mechanisms in nucleated cells as compared to prokaryotic cells, the mechanisms and extent of this type of control are still controversial. The known involvement of spermidine and spermine in protein synthesis (Tabor and Tabor, 1976; Anderson et al, 1977) suggests that oligoamines may be one factor in controlling translation. However, difficulty in elucidating the means by which cells effect the control of protein synthesis in vivo has hindered the realization of this goal. One approach has been to develop in vitro protein synthesizing systems with the aim of duplicating in vivo conditions, particularly those in which translational control is known to occur (Bielka, 1977). The role of translational control during fungal spore germination is considered in section B.

The translation of poly (U) by cell free extracts of S. cerevisiae (Bretthauer et al, 1963; So and Davie, 1963) was the first demonstration of an in vitro protein synthesizing system derived from a eukaryotic organism. Subsequently work on protein synthesis in fungi lagged behind that of mammalian systems and has been generally directed towards establishing the similarity of the processes in fungi and higher eukaryotes (Berry and Berry, 1976; Lovett, 1976). In vitro protein

synthesizing systems capable of the poly (U) directed synthesis of polyphenylalanine have been described for Penicillium cyclopium (Van Etten, Parisi and Ciferii, 1966), Botryodiplodia theobromae (Van Etten, 1968), Fusarium solani (Rado and Cochrane, 1971), Trichoderma viridie (Stavy, Stavy and Galun, 1970) and Neurospora crassa (Alberghina, Sturani and Ursino, 1969), though none of the systems were rigorously characterized. Gallis et al (1975) demonstrated that the activity of the in vitro protein synthesizing system of S. cerevisiae was due entirely to the elongation of existing chains and was incapable of initiating the synthesis of new chains. To characterize a system in depth it is necessary to translate natural mRNA, as poly (U) translation does not require all the components of normal initiation (Wigle and Smith, 1973). By carefully isolating intact polyribosomes a protein synthesizing system capable of initiating new chains has been established with an extract of S. cerevisiae (Schulz-Harder and Lochmann, 1976). The faithful translation of mRNA to produce complete polypeptides has not been demonstrated for any fungal system as it has for rat liver cell free extracts or reticulocyte lysates (see Lingrel, 1974).

Polypeptide synthesis in vivo is a very complex process requiring the participation of over fifty different elements including small molecules, various polypeptides, RNA species and macromolecular aggregates (see Haselkorn and Rothman-Denes, 1973). Methionyl-$tRNA_f$ is the specific aminoacyl-tRNA that initiates protein synthesis in fungi and other eukaryotes. However many other factors are required for maximum rates of protein synthesis. Salt-washed ribosomes from mammalian cells are unable to initiate polypeptide synthesis on natural mRNAs unless supplemented with the wash. Analysis of the factors present in the wash has revealed at least six proteins required for

initiation, two for elongation and one for termination (Weissbach and Ochoa, 1976). Recent extension of this work has detailed the requirements for the initiation of natural eukaryotic mRNAs. Seven initiation factors, ATP, GTP, Met-tRNA$_f$, 40S and 60S ribosomal subunits and spermidine are required for maximal incorporation of globin mRNA into an 80s initiation complex (see Anderson et al, 1977).

The trinucleotide sequence AUG is the only recognizable signal sequence at the 5'-end of mRNA molecules (Baralle and Brownlee, 1978) unlike prokaryotes where the mRNA sequence at the 5'-end contains a region of complementarity with a portion of the 3'-end of the 16S rRNA (Shine and Dalgarno, 1975; Steitz, 1978). Although AUG is the only essential feature needed for ribosome binding (Kozak and Shatkin, 1978) eukaryotic ribosomes are unable to initiate protein synthesis on internal AUG initiation sequences that are open to bacterial ribosomes (Sherman and Stewart, 1975; Anderson et al, 1977). This may account for the lack of polycistronic messengers in eukaryotes (Petersen and McLaughlin, 1973; Nakamoto and Vogl, 1978).

When extracted carefully to avoid ribonuclease activity and mechanical shear, isolated ribosomes are found to occur in clusters of up to 100 when analysed after sucrose density gradient centrifugation. These polyribosome or polysome complexes consist of a number of ribosomes attached to and actively translating a single mRNA molecule. The quantity and size distribution of polysomes, the "polysome profile", is a sensitive measure of the activity of the protein synthesizing machinery in vivo. The percentage of ribosomes occurring as polysomes increases with the rate of cellular protein synthesis (Hogan and Korner, 1968). As well as reflecting the overall rate of protein synthesis the polysome profile depends on the relative rates of

initiation and elongation. If elongation is preferentially inhibited, as for example by cycloheximide, the average size of the polysomes will increase as the ribosomes crowd together on the mRNA (Lodish, 1971). A defect in polypeptide chain initiation, as occurs in temperature-sensitive mutants of S. cerevisiae and N. crassa results in a rapid conversion of polysomes to monosomes and ribosomal subunits (Hartwell et al, 1970; Loo, 1975). Other factors that can decrease the rate of polypeptide chain initiation are double-stranded RNA, oxidized glutathione, interferon and haemin-deficiency (Jackson, 1974; Anderson et al, 1977).

Selectivity of Translation

Selective protein synthesis is at the heart of cellular differentiation. While some highly differentiated cells (e.g. erythrocytes) may produce predominantly one or a few specific proteins, other forms of cell differentiation may involve synthesis of only minor amounts of certain proteins. Sporulation in Neurospora and related species is associated with a major phase-specific perithecial protein (Nasrallah and Srb, 1977). There is also a program of differential protein synthesis evident by the pattern of polypeptide labelling in the development of Polysphondylium pallidum and other organisms (Francis, 1977). The control of protein synthesis, whether at the transcriptional of translational level, requires a mechanism with great powers of discrimination and selectivity.

There is little evidence to support the view that the cellular machinery of protein synthesis in eukaryotes is able to select specific classes of mRNAs for translation. Messenger RNAs from one cell type can be translated at high efficiency in cell extracts or intact cells of a different type or even from different species (Lodish, 1976). Thus the protein synthesizing system obtained from rabbit reticulocytes, which is a highly specialized cell producing predominantly globin,

can translate at high efficiency mRNAs as diverse as collagen mRNA (Boedtker et al, 1974), Dictyostelium actin mRNA (Lodish, 1976) and feather keratin mRNA (Partington et al, 1973). A very detailed study by Palmiter (1974) concluded that rabbit reticulocytes contain all factors required for translation of any eukaryotic mRNA. Similarly Xenopus oocytes can translate a wide variety of mRNAs without any further added factors (Gurdon, 1974) and even carry out the correct post-translational modifications such as hydroxylation, acetylation and proteolytic cleavage.

Evidence for mRNA-specific factors affecting the rate of synthesis of particular proteins has been found for a few systems. Using ascites cell-free extracts, Nudel et al, (1973) isolated a factor specific for α-globin mRNA, relative to β-globin mRNA, but due to their different rate constants for ribosome attachment a non-specific factor increasing overall protein synthesis could accomplish this as well (McKeehan, 1974). Other reports of mRNA-specific factors must be treated with caution due to defects in the experimental systems. Most cell-free systems have low rates of initiation and chain elongation, producing less than two molecules of protein per mRNA added (Metafora et al, 1972). Only about 1% of the ribosomes can bind to mRNA and participate in protein synthesis (Ensminger and Henshaw, 1973). This is far from what occurs in vivo or in reticulocyte lysates which can use mRNA templates for globin over 200 times (Adamson et al, 1969) and initiate and elongate polypeptide chains at rates approximating those occurring in vivo (Palmiter, 1973). The fact that protein synthesis in vivo is more sensitive to inhibitors such as cycloheximide and emetine than the in vitro systems suggests that the rate limiting steps in the two conditions are different (Emmerlich et al, 1976). Thus control

mechanisms operating *in vivo* may not be easily detectable by *in vitro* techniques alone.

Translational Control Mechanisms

The existence of control mechanisms at the level of transcription does not rule out additional controls at the level of translation. Indeed this second tier of control seems likely for two reasons: i) unlike bacterial mRNA the majority of animal cell mRNA is relatively stable, and ii) eukaryotic mRNA transcripts must undergo extensive post-transcriptional processing and modification, including transport across the nuclear membrane to the cytoplasmic ribosomes for translation. Thus there is a temporal and spatial separation of transcription and translation that would result in a slow and cumbersome metabolic response if control was exerted only at the level of RNA synthesis. There are two distinct classes of control that might regulate polypeptide chain initiation and elongation. Cells might regulate the overall rate of polypeptide chain initiation affecting translation of all mRNAs. Alternatively cells could utilize message-specific components leading to changes in the specific species of mRNAs being translated.

It seems clear that the overall rate of protein synthesis can be regulated. During mitosis in Chinese hamster ovary cells, the rate of polypeptide synthesis is greatly reduced without apparent loss of either functional ribosomes or mRNA (Fan and Penman, 1970). A return to the interphase rate of protein synthesis follows mitosis even in the presence of actinomycin D (Hodge et al 1969). Spore senescence in *Uromyces phaseoli* is accompanied by a decreasing rate of protein synthesis and possibly an alteration of the ribosomal proteins (Yaniv and Staples, 1975). Other examples of this gross control of protein synthesis are known in eukaryotes, including fungi (see Molloy and Puckett, 1973;

Orlowski and Sypherd, 1978a,b). An even more dramatic example of translational control is found with embryonic muscle cells. In this system muscle-specific messengers are transcribed and stored in the cytoplasm without being translated. These stored mRNAs are subsequently activated and translated (Heywood and Kennedy, 1976). Sea Urchin development (Paul, 1974), Amphibian embryonic development (Denis, 1974) and the germination of certain fungal spores (Knight and Van Etten, 1976) can proceed with new protein synthesis in the absence of RNA synthesis so some control of translation must be exerted in these systems as well.

Studies with enucleated cells have also demonstrated the existence of post-transcriptional control mechanisms. In the giant unicellular alga, Acetabularia growth of the stalk and complete differentiation of the species-specific cap can take place long after the cell nucleus has been removed (see Harris, 1974). Mouse L-cells can be enucleated with cytochalasin B to produce "cytoplasts" which remain active for at least twenty-four hours. The addition of fresh medium to the cytoplasts induces a dramatic increase in ornithine decarboxylase activity much as it does in whole cells (McCormick, 1977). Again this implies the existence of post-transcriptional control mechanisms.

The absence or low levels of specific aminoacyl-tRNAs will slow down the overall rate of protein synthesis simply by blocking the completion of polypeptide chains. But the absence of specific minor leu-tRNAs may act more selectively as occurs with the translation of Mengo virus RNA and globin mRNA (Content, et al, 1974). Thus different species of tRNA may be necessary for the translation of different mRNAs (Revel et al, 1977). Changes in isoaccepting tRNA species occurs during many types of development (Littauer and Inouye, 1973).

Translation may be controlled simply by the efficiency of the mRNA itself to initiate protein synthesis. As there is an excess of messengers over ribosomal capacity to translate (Brawerman, 1974) competition for limited initiation sites will follow. Lodish (1976) has established that mRNAs with greater affinities for attachment to ribosomes or higher rates of polypeptide chain initiation will produce more polypeptide products than mRNAs with low initiation rates. A kinetic analysis of initiation and elongation demonstrates that a nonspecific reduction in the rate of polypeptide chain initiation steps at or before binding of mRNA will result in preferential inhibition of translation of mRNAs with lower rate constants for polypeptide chain initiation (Lodish, 1974). Thus without any changes in the specificity of the ribosomal apparatus a rapid change in the overall rate of protein synthesis will bring about a qualitative change in the proteins synthesized.

(iv) Genetic Analysis of Control Mechanisms

The combination of biochemical and genetical investigations which proved so successful in understanding control mechanisms in E. coli have been useful in analyzing eukaryotic microorganisms as well. Extensive genetic analysis has been carried out on A. nidulans beginning with the work of Pontecorvo et al, (1953) and subsequently carried out by researchers around the world to establish this organism as one of the most thoroughly understood eukaryotes on a genetic level (see Smith and Pateman, 1977). In general, evidence suggests that there is an increase in the rate of synthesis of enzyme protein during enzyme induction and a decreased rate of synthesis during repression. That the control of enzyme activity probably occurs at the transcriptional level in A. nidulans is shown by the work of Cybis and Weglenski (1972) and Bartnick et al (1973) for arginase, Dunsmuir and Hynes (1973) for acetamidase, and Scazzocchio

(unpublished observations) for urate oxidase. However these conclusions are based almost exclusively on the use of metabolic inhibitors so there is still considerable uncertainty in establishing precisely the level(s) at which control is exerted. Final resolution of this problem requires the ability to measure enzyme protein, as opposed to just enzyme activity, and to assay for the presence of specific messenger RNAs. Techniques for doing these measurements may now be available (e.g. Kindle and Firtel, 1977; Hopper et al, 1978).

The extensive work on gene regulation in prokaryotes has influenced approaches used to study control in eukaryotes. The bacterial genome organization includes clusters of genes called operons, the expression of which is regulated coordinately, and which control the simultaneous formation of sets of proteins related in function. The number of genes comprised by the individual operons vary from three as in the lactose operon (Martin, 1969) to the nine genes comprising the histidine operon (Garrick-Silversmith and Hartman, 1970). The operon concept as expressed by Jacob and Monod (1961) postulates that the operon as a whole is transcribed as a single large, polycistronic messenger which carries the information for more than one protein. The actual expression of the various structural genes in the operon is controlled by a small region in one end, called the operator, which exerts its action at the level of transcription (Gilbert and Müller-Hill, 1966). This region can be recognized genetically by the occurrence of cis-dominant mutations affecting the entire set of structural genes.

In the fungus A. nidulans, as in higher eukaryotes, functionally related genes are seldom clustered (Clutterbuck, 1974). This is consistent with the finding that the mRNAs of yeast (Petersen and McLaughlin, 1973) and animal cells (Staehelin et al, 1964; Matthews, 1973) are largely if

not entirely monocistronic, that is, code for a single polypeptide. Thus gene clustering in eukaryotes is probably not to be understood in terms of coordinate regulation of the corresponding enzymes. Instead, the gene clustering that does occur in fungi may facilitate the subsequent assembly of related proteins into a multienzyme complex or it may provide a selective advantage for inheriting a set of metabolically related genes. True gene clustering may be even less common than has been previously supposed as some genetically identified "gene clusters" are now known to code for single multifunctional polypeptide chains (Minson and Creaser, 1969; Bigelis et al. 1977).

The existence of gene clusters and cis-dominant control regions in fungi is taken as evidence of transcriptional control, in analogy with the control of polycistronic messengers of E. coli as discussed above. Such comparisons ignore some fundamental differences between prokaryotes and eukaryotes. Unlike bacterial mRNA the majority of animal cell mRNA is relatively stable and is subject to a much greater degree of post-transcriptional modification. The presence of the nuclear membrane which physically separates transcription and translation prevents the direct, intimate coupling of these processes as found in E. coli

The Britten-Davidson model of gene regulation (Britten and Davidson, 1969; Davidson and Britten, 1973) has had a large impact on our thinking about the molecular aspects of gene regulation in higher eukaryotes. Gene regulation is the coordinated activation (or repression) of a battery or set of structural genes characteristic of a particular developmental state. This coordinate activation may involve a number of genes distantly located on several chromosomes. The model proposes that each structural gene in a given battery is preceded by a characteristic repetitive sequence, the "receptor" sequence, for recognition and binding

of regulatory molecules. The positively acting regulatory molecules, which could be RNA or protein, are coded for by regulatory genes. One type of regulatory gene is the "integrator" gene whose role is to achieve the concomitant expression of non-contiguous structural genes through specific interactions at receptor sequences for the integrator gene products. Coordinated expression thus requires either a redundancy of receptor sites or a redundancy of integrator genes (or both). Individual genes that may be required by more than one developmental sequence would be subject to parallel positive controls by separate regulatory activators or integrators. Support for this model in which the DNA sequence organization provides the structural basis for higher cell gene regulation comes from the finding that the DNAs of at least two animals display an ordered interspersion of non-repetitive sequences (presumptive structural genes) and repetitive sequences (presumptive regulatory genes).

Genetic analysis has also identified potential receptor sites for regulatory genes in A. nidulans, where several cis-acting regulatory mutations tightly linked to structural genes under their control have been identified (Hynes, 1975; Arst and MacDonald, 1975; Arst and Scazzocchio, 1975; Lukaszkiewicz and Paszewski, 1976). The majority of the regulatory genes found in fungi have positively acting regulatory products. This may allow greater possibilities for a hierarchy of interaction within a particular control system as well as interactions betwen control systems. The presence of multiple tiers of control is a significant aspect of regulation in eukaryotes. In catabolic pathways the expression of a gene usually requires at least two regulatory signals acting simultaneously, including specific induction signals, usually the substrate involved, and general induction signals such as carbon or nitrogen metabolite repression. Furthermore, Arst (1976)

has identified a positive regulatory gene involved in acetamidase synthesis, designated int A, which appears to be an integrator gene of the type described by the Britten-Davidson model. A hypothetical model for the action of several regulatory genes based on alterations in transcriptional specificity by addition of subunits to a core RNA polymerase has been proposed by Pateman and Kinghorn (1977). Alternatively the regulatory genes products may interact directly with the chromatin to make specific genes transcriptionally accessible.

Some progress has been made in identifying the proteins coded for by formally defined regulatory genes. One of these is the positively acting product of the uaY gene which controls uric acid-xanthine permease in A. nidulans (Scazzocchio and Arst, 1978; D Philippides and C Scazzocchio, unpublished observations). In Neurospora crassa two structural genes have been tentatively identified, the positive regulatory effectors controlling quinic acid catabolism (Jacobson et al, 1977) and phosphorus metabolism (Metzenberg and Nelson, 1977). A detailed knowledge of the mode of action of these regulatory molecules will be of immense importance in understanding developmental processes in eukaryotes.

B Fungal Spore Germination

(i) General

The fungal spore is a unique stage in the life cycle of most fungi, highly specialized for reproduction, dispersal and survival. The spore is physiologically quite different from the mycelial growth phase. Characteristically fungal spores have low water content, minimal metabolic activity and a lack of cytoplasmic movement (Gregory, 1966). There is an amazing diversity in size, shape, longevity and function among fungal spores. The various roles performed by spores may require different and possibly incompatible structures. Thus a number of fungi produce a diversity of spore types by both sexual and asexual processes. Spores can be generally characterized as being xenospores, short lived and designed for dispersal or memnospores, remaining at the place of origin and requiring a specific mechanism to break dormancy (Gregory, 1966).

Though spores are considered metabolically inert this is not strictly so and they are capable of activity in metabolizing a variety of compounds (Vezina et al, 1968). This is probably related to the necessity of maintaining a basal metabolic rate to preserve structural integrity and viability. Prolonged storage will usually lead to a loss of viability depending on the environmental conditions and type of spore. A cool, dry environment usually allows a longer survival (Cochrane, 1958; Clerk and Madelin, 1965).

Four distinct but overlapping stages can be discerned during the germination of most fungal spores: i) hydration and swelling of the spore, ii) physiological and morphological changes within the intact spore dependent on metabolism of endogenous or exogenous energy sources,

iii) protrusion of the germ-tube through the external cell wall and the polarization of growth (this is often used as a visual criterion of germination) and iv) germ-tube elongation and apical growth of hyphae.

The definition of "germination" as the formation of a germ-tube from the dormant spore (Manners, 1966) is most commonly used for fungal spores, largely due to the simplicity of the determination. However as a vast amount of metabolic and biochemical changes occur before germ-tube emergence, Sussman (1966) has proposed that any measureable irreversible change can be an acceptable criteria for the definition of "germination". This has not gained wide acceptance due to the lack of wide spread easily measurable, biochemical or morphological landmarks in the early stages of fungal spore germination. Changes do occur in germinating conidia within twenty minutes after hydration including loss of heat resistance, polysome formation (Kobayashi, 1972) and the development of cyanide insensitive respiration (Doi and Halvorson, 1961) and in this respect resemble the "activation" stage of bacterial spore germination.

The nature of dormancy and the process of germination may be quite variable but fungal spore germination is still a valuable model system for studying the resumption of an active metabolic state and cellular differentiation in general. This is enhanced by the simplicity of the development, especially in unicellular, uninucleate spores, by the ability to obtain uniform and synchronous changes in a homogeneous population of cells, and the universality of the dormant phase among living organisms. Thus similarities may well be found between the onset of rapid growth in fungal spores and other tissues or cells undergoing active growth such as embryo development, tissue regeneration and tumour growth. As fungal spores usually contain the haploid genome content

(i.e. they are in G_1) and proceed uniformly through the nuclear division cycle during germination they can be used to study cell cycle events. In fact germination studies are complicated by this superimposition of cell cycle events (particularly those involved with nucleic acid synthesis) and events unique to germination.

By entering a dormant state many fungal spores are capable of remaining viable for long periods of time. Dormancy can be due to one of two factors. Where the resting period is simply a response to unfavourable environmental conditions this is termed "exogenous dormancy". However some spores will not germinate when placed under conditions favourable to subsequent hyphal growth but first require an activation process to overcome some block. This "constitutional dormancy" can arise from the presence of self-inhibitors, metabolic blocks or diffusion barriers. Only a few self-inhibitors have been identified. The mode of action of such self-inhibitors *in vivo* is not clear but some are inhibitors of protein synthesis (Leppik et al, 1972; Lingappa et al, 1973 and Adelman and Lovett, 1974). Another compound inhibits digestion of the germ-pore plug (Hess et al, 1975). Fungal spores may be activated by detergents, organic solvents, lipids, heat shock and other relatively extreme environmental conditions (Sussman,1966). Partial protein denaturation (Cotter, 1975) or rearrangement of the lipid components of membranes are plausible sites of action for these activators. It has been proposed that the primary effect of many of the organic and inorganic activators may be to initiate the availability and metabolism of storage compounds (Foerster and Foerster, 1973).

The conidia of *A. nidulans* germinate readily, with a high degree of synchrony, when placed in a minimal media of carbon and nitrogen source with salts (Rosenberger and Kessel, 1967; Shepherd, 1957). Spore

concentrations at densities greater than 2 X 10^7 spores/ml result in auto-inhibition of germination (Bainbridge, 1971). This suggests that self-inhibitors are present in the dormant conidia which diffuse outwards early in germination. The rapidity of germ-tube formation can also be affected by culture conditions and strain differences.

(ii) Morphological and Ultrastructural Changes Accompanying Germination

One of the first visible signs of germination is a swelling of the spore, with increases in volume from 2 to 30-fold, the larger increases occurring in those spores requiring exogenous nutrients (Smith and Anderson, 1973). In A. nidulans the diameter of the dormant conidia is 3uM which increases to about 5uM prior to germ-tube emergence (Border and Trinci, 1970). The initial swelling is simply a rehydration process which is usually followed by a metabolic swelling, or spherical growth. Spherical growth normally ceases at the start of germ-tube emergence when growth becomes polarized (unidirectional apical growth). During spherical growth the new cell wall incorporation is deposited uniformly around the spore wall but once growth becomes polarized incorporation is limited to one area from which the germ-tube will arise. Anderson and Smith (1972) have shown that this transition to polarized growth may be retarded or prevented in A. niger by growth at elevated temperatures resulting in the production of giant spores up to 25um in diameter.

Marked changes in the morphology of the spore wall occur early in germination. The cell wall of dry, dormant conidia of A. nidulans is three-layered. During hydration two new layers appear in the conidial wall within 30 minutes (Florance et al, 1972). This layering is thought to result from changes within the existing wall brought about by imbibition of water and macromolecular rearrangement during development rather than de novo synthesis of new wall material (Bartnicki-Garcia, 1968).

In A. nidulans the new hyphal wall of the germ-tube is continuous with the innermost layer of the conidial wall, which appears only after hydration. This method of germ-tube formation is found in a variety of fungi (Smith et al, 1976; Hawker, 1966; Bracker, 1967; Marchant, 1966). Reports that Penicillium frequentans (Hawker, 1966) and A. oryzae (Tanaka, 1966) produce germ-tube walls continuous with a wall already present in the dormant spore, may be in error as the "dormant" spores were exposed to water and may have rapidly formed new wall layers before the onset of fixation.

There is a unique localization of organelles on the side of the conidium from which the germ-tube arises (Florance et al, 1972; Bracker, 1967). The wall in the area of the emergent germ-tube is first softened by enzymatic degradation (Marchant, 1966; Leighton and Stock, 1970). The number of mitochondria and the amount of endoplasmic reticulum increases during germination. The size and number of vacuoles increase on the side of the conidium away from the developing germ-tube (see Smith et al, 1976). An increase in nuclear volume, followed by nuclear division usually occurs before or during germ-tube emergence (Van Etten et al, 1976), though in the case of A. nidulans nuclear division follows germ-tube emergence (Bainbridge, 1971). Furthermore, mutants of A. nidulans defective in nuclear division can germinate, though subsequent apical growth is restricted (Orr and Rosenberger, 1976a). Thus nuclear division is not a prerequisite for germination in this organism.

The mitochondria in the dormant spores can appear morphologically normal as in N. crassa and Trichoderma viride (Manocha, 1968; Rosen et al, 1974) or in a more rudimentary condition as in anaerobic S. cerevisiae cells (Criddle and Schatz, 1969). Some mitochondria in dormant spores appear to lack a complete cytochrome c oxidase (Brambl, 1977). An

increase in the numbers and activity of mitochondria is an important step in the resumption of active growth. As well as supplying energy it has been proposed that the localization of mitochondria could influence the establishment of apical growth (Zorzopulos et al, 1973).

The respiration level of fungal spores increases substantially during germination (Sussman and Halvorson, 1966) and inhibitors of respiratory function (e.g. cyanide, dinitrophenol) can inhibit germination (Niederpruem and Dennen, 1966). Although the "energy charge", $(ATP+\frac{1}{2}ADP)/(ATP+ADP+AMP)$ see Atkinson and Walton (1965), in conidia is 0.7, the same as that for the mycelia, continued respiratory activity is necessary to maintain this high level (Slayman, 1973). The ungerminated conidiospores of Botryodiplodia theobromae contain a potentially functional aerobic respiratory system which becomes active upon inoculation of the spores into a liquid media. The action of cytoplasmic ribosomes is required for the cyanide-sensitive respiration and germination of the spore, although functioning of the mitochondrial genome is dispensable (Brambl, 1975). Dormant conidia of N. crassa have a low rate of oxygen consumption, respiring predominantly via a cyanide-resistant pathway with a P/O ratio of zero (Colvin et al, 1973; Greenawalt et al, 1972). In the later stages of germination a ten-fold increase in oxygen consumption is correlated with the appearance of cyanide-sensitive respiration (Slayman, 1973) with an increased P/O ratio indicating an increased activity of oxidative phosphorylation. A similar process occurs in other resting cells resuming active growth such as seeds (Bendall and Bonner, 1971) and bacterial spores (Keynan, 1973).

Not all spores germinate in the manner just described and many unusual features can be found in the germination of aquatic zoospores (Leaver and Lovett, 1974, Turian, 1975). For example, the ribosomes of zoospores

of Blastocladiella are contained in a membrane bound structure or nuclear cap which later fragments. Another membrane bound organelle, the "gamma particle" is present in the zoospores and may be involved in wall synthesis (Myers and Cantino, 1971). Cell wall growth in budding yeasts is comparable to the spherical growth phase (Cortat et al, 1972) but it is not followed by a polarized growth phase.

(iii) Biochemical Changes in Germination

General

Even before any changes are observable on a morphological level, changes in the biochemical composition of germinating spores are detectable. In order to study the very earliest changes it is necessary to use dry harvested spores as a number of events quickly follow hydration. Many of the earliest events detectable involve rapid fluctuations in the levels of small molecular weight compounds. The most detailed studies of this type have been carried out on N. crassa (see Schmit and Brody, 1976). Dormant conidia have a large pool of glutamic acid (2.5% of the dry weight) which is metabolized rapidly following hydration. This may act as a reservoir for the production of reduced coenzymes necessary for biosynthesis (NADH and NADPH) which increase threefold in the earliest stages of germination (Schmit et al, 1975). While the concentrations of most amino acids remain steady during germination, very large increases occur in the amounts of arginine, citrulline and ornithine (Schmit and Brody, 1975). The accumulation of citrulline, but not ornithine or arginine, is inhibited by cycloheximide (10ug/ml) suggesting that the enzymes required for the synthesis of these latter compounds are present in the conidia, presumably synthesized during conidiation. The increase in ornithine may be necessary for subsequent oligoamine synthesis during germination (see Stevens and Winther, 1978).

Conidia contain high levels of oxidized glutathione (GSSG) and protein bound glutathione (PSSG) which rapidly diminish in the first few minutes of germination of N. crassa (Fahey et al, 1975). The high levels of disulphide compounds in conidia, with 2% of the protein sulphydryls bound to glutathione residues, are thought to play an important role in maintaining conidial dormancy. For germination to precede these disulphide linkages may need to be reduced. The high levels of NADPH may participate in reducing the disulphide linkages. A possible model for glutathione induced dormancy will be considered later.

A variety of storage compounds are present in fungal spores which may enable them to germinate without added nutrients. Carbohydrates are present in some spores, e.g. trehalose in Neurospora (Lingappa and Sussman, 1959) and Phycomyces (Rudolph and Ochsen, 1969) and glycogen in Blastocladiella (Suberkrapp and Cantino, 1972). Lipids are also present in fungal spores comprising from 1 to 30% of the dry weight (Steiner, 1957). In many species both carbohydrates and fats are actively metabolized at the onset of germination (Daly et al, 1967) and the activities of respiratory enzymes undergo increases of up to 210-fold during germination (Ohmori and Gottlieb, 1965). The degradation of lipid reserves provides not only energy but also building blocks for membrane cell wall biosynthesis (Weber and Hess, 1974; Jackson and Frear, 1967).

DNA Synthesis

DNA synthesis is a requirement for cell growth and division in higher organisms where discrete cells with individual nuclei occur. In coenocytic fungi, however, many nuclei are found in a common cytoplasm and growth is not rigidly linked to nuclear replication though a near constant ratio of nuclei per volume of cytoplasm is normally found in A. nidulans

(Clutterbuck, 1969). In most fungi examined, DNA synthesis begins prior to or at the time of germ-tube emergence (see Van Etten et al, 1976). Notable exceptions to this are found in S. cerevisiae ascospores (Rousseau and Halvorson, 1973) and Blastocladiella emersonii zoospores (Lovett, 1968) where germination precedes any increase in DNA synthesis. In any case it is clear that extensive transcription and translation can occur prior to, and thus independent of, DNA synthesis in the early stages of germination, though later stages particularly germ-tube emergence, may require DNA synthesis either as a means of "unlocking" or de-repressing specific genes or simply because of the increased genome made available for increased transcription. Experiments with inhibitors of DNA synthesis which have been designed to access the role of DNA synthesis in germination have proven inconclusive since they have not demonstrated both that the inhibitor penetrates the spores and that the inhibition is specific for DNA. Nevertheless the evidence as it stands supports the view that nuclear DNA synthesis is not essential for germination. Similar studies have demonstrated that mitochondrial DNA synthesis is not required for germination of yeast ascospores (Tingle et al, 1974) or Botryodiplodia theobromae conidiospores (Dunkle et al, 1972).

RNA Synthesis

The synthesis of macromolecules, particularly RNA and protein, is an important event in the germination of fungal spores and rapily growing tissues. An indication of the rapidity of macromolecular synthesis in germinating conidia is shown by the incorporation of labelled precursors into rRNA, assembly into ribosomes and formation of polysomes in the cytoplasm within fifteen minutes of hydration (Mirkes and McCalley, 1976). The synthesis of RNA, whether detected by labelled precursor incorporation

or increases in the total quantity present, occurs at an early stage in the germination of most all fungal spores (see Van Etten et al, 1976). Using such techniques it can be shown that germinating spores can be placed into three separate categories depending on whether RNA synthesis precedes, follows or is coincident with the initiation of protein synthesis. The important conclusions that can be drawn from this are that where RNA synthesis clearly precedes protein synthesis, then preformed RNA polymerases must be present and where protein synthesis is first detectable, the spore must contain pre-existing mRNA. In all cases a fully formed protein synthesizing system is indispensible for the spore to emerge from the dormant state.

Bearing in mind the lack of data on uptake and specificity of RNA synthesis inhibitors, conclusions on the necessity of RNA synthesis for germination can only be tentative. In a number of fungal species. Botryodiplodia theobromae (Brambl and Van Etten, 1970), S. cerevisiae (Rousseau and Halvorson, 1973), Alternaria solani and Peronospora tabacina (Hollomon, 1970) it appears that RNA synthesis is not required for germination. RNA synthesis does seem to be required for germination in A. nidulans, N. crassa (Hollomon, 1970) R. stolonifer and other fungal species (see Van Etten et al, 1976). The class of RNA required for germination is unknown but could include mRNA which is necessary for early protein synthesis.

This possibility has lead a number of researchers to attempt to verify the presence or absence of mRNA in dormant spores. The existence of polysomes in resting spores is one type of evidence indicating the presence of mRNA. Investigations have revealed a complete absence or only low levels of polysomes in the dormant state in fungi (Van Etten et al, 1976). Though good evidence does exist for polysomes in uredospores of Uromyces

phaseoli (Staples et al, 1963), pycnidiospores of B. theobromae (Brambl and Van Etten, 1970) and sporangiospores of R. stolonifer (Freer and Van Etten, unpublished observations), the work with N. crassa (Mirkes, 1974) emphasizes the need to work with dry harvested spores since polysome formation occurs within minutes of hydration. Evidence of a more direct kind points to the existence of mRNA in dormant spores. Presumptive messenger RNA fractions have been isolated from the dormant zoospores of B. emersonii (Van Etten et al, 1976), Uromyces phaseoli uredospores (Ramakrishnan and Staples, 1970), N. crassa conidia (Bhagwat and Mahadevan, 1970), Botryodiplodia theobromae conidia (Wenzler and Brambl, 1978) and sporangiospores of Rhizopus stolonifer (Van Etten and Freer, 1977).

As discussed earlier the various classes of RNA are separately regulated in many systems. In germinating spores the initiation of synthesis of each class of RNA may occur simultaneously or sequentially, though the sequence of events may differ for each spore type. A common pattern of RNA synthesis in germinating spores is for the initiation of rRNA synthesis to occur first, early on in germination, followed by tRNA and mRNA synthesis (Van Etten et al, 1976). The synthesis of rRNA and assembly of ribosomes may be essential for enabling a more rapid growth rate subsequent to germination.

Protein Synthesis

Macromolecular synthesis is necessary for the dry weight increases preceding germ-tube emergence. Protein synthesis, involving both structural and catalytic elements, has been found to occur during the germination of all spores investigated to date. Furthermore, on the basis of studies using inhibitors of protein synthesis there appears to be a universal requirement for some protein synthesis before germ-tube emergence can

be completed (Lovett, 1976). Although synthesis of protein occurs at this time, a net accumulation may not. During germination of spores of Puccinia graminis (Trocha and Daly, 1970) and Blastocladiella emersonii (Lovett, 1968) extensive turnover provides the precursors for new protein synthesis. The process of synthesizing new proteins at the expense of non-essential proteins may be a common phenomena, especially with germinating spores of obligate parasites, and may explain the importance of proteolysis in the germination of Microsporum gypseum (Leighton and Stock, 1970).

A wide range of proteins are synthesized by germinating fungal spores (Van Etten et al, 1976; Silverman et al, 1974) only a small number of which may be essential for further development. It is not yet possible to identify the essential proteins or the stages of germination at which they are required. A significant number of the proteins synthesis in germinating Blastocladiella zoospores are ribosomal proteins (Lovett, 1976). A number of enzymes increase in specific activity in germination though none of the increased activities could be shown necessary for the completion of germination (Ohmori and Gottlieb, 1965). Structural proteins would be necessary for cell wall and membrane extension, though the specific proteins involved have not been identified.

A gentic analysis of germination may identify key steps and essential functions for the completion of this developmental process. There are three distinct genetic approaches to studying germination, Firstly, mutant strains can be isolated which are specifically affected in the germination process. This would reveal the number of unique loci involved in germination and define those steps essential for germination. Unfortunately this has not been a successful approach with N. crassa as it has for some other organisms (see Schmit and Brody, 1976).

A second approach involves the characterization of the germination process in those mutant strains already available. Most unsupplemented auxotrophs of N. crassa are able to germinate, possibly because the existing pools of conidial compounds are at least sufficient for the early stages of germination, including germ-tube formation (Schmit, and Brody, 1976). In general, defects in the synthesis of aromatic or basic amino acids leads to inability to form germ-tubes. An unsupplemented putrescine auxotroph of A. nidulans, deficient in ornithine decarboxylase activity, also fails to germinate, suggesting that oligoamines have an essential role in this developmental process. Thus ornithine decarboxylase may be one enzyme whose synthesis is necessary for germination in minimal media with this organism.

With the basic knowledge of potential control points in germination a third genetic approach is available. This involves isolating mutants for specific functions which occur at germination to determine whether or not they are essential. Using this type of analysis, Nelson et al, (1975) determined that nicotinamide adenine dinucleotide glycohydrolase (NADase) was not essential for conidiation or conidial germination in N. crassa.

Protein synthesis occurs at a low or undetectable rate in dormant spores. The resumption of active protein synthesis is possibly the most dramatic event in germination, and understanding the mechanism by which this comes about is fundamental to a knowledge of this developmental process. It has been demonstrated that most, if not all, of the components for a complete protein synthesizing machinery are present in the dormant spore (see Lovett, 1976), though the lack of specific initiation or elongation factors may still be a possibility. Assuming that all the necessary components are present there are at least five mechanisms for inhibiting

protein synthesis in the spore: i) the mRNA may be sequestered in an inactive form (Spirin, 1969), ii) the presence of diffusible self-inhibitors (Macko et al, 1976) which are readily removed in a liquid media, iii) the presence of glutathione at concentrations preventing protein synthesis (Fahey et al, 1975), iv) reversible modification of isoacceptor tRNA species and v) the localization of various components in separate organelles. Sufficiently detailed studies have not been made to allow the identification of any of these mechanisms as being significant controlling factors in vivo.

(iv) Conidiogenesis

Insights into the mechanism of germination can be obtained not only by studying details of the process itself, but also by analysis of the dormant spore and the means of its production. Many of the enzymes necessary for germination, especially the earliest stages, are probably synthesized during conidiation. Within the ascomycetes a wide variety of spore types are produced. Sexual spores (ascospores) are the result of a more or less uniform pattern of internal reorganization within the ascus. Conidia, a type of asexual spore, differentiate at the ends of mycelia or conidiophores, within thin walls. The conidiophores vary considerably in their complexity, from being only slightly modified hypae as in Neurospora to the complex structures present in Aspergillus and Penicillium. An indication of the complexity of structure and function of the conidiophore in A. nidulans comes from genetic studies in which it has been found that as many as 150 loci may specifically affect conidiogenesis (Martinelli and Clutterbuck, 1971). Several mutants have been isolated which are able to initiate conidiophore development but are blocked before the formation of conidia (Clutterbuck, 1969) suggesting that sequential gene activation occurs.

Conidiation involves formation of at least three wall types of varying morphology and composition (Oliver, 1972) and presumably different properties which may be important in isolating the conidia from the mycelial metabolic environment. Unfortunately no enzymes specifically involved in the morphogenetic events of conidiation have been identified though two enzymes responsible for pigment formation are known to be under developmental control (Clutterbuck, 1977). Proteases may play an important role both in providing substrates for macromolecular synthesis where conidiation is induced by starvation and through the specific degradation of key enzymes to induce dormancy. During ascospore formation in S. cerevisiae a major portion of the cellular proteins are degraded and turnover as protease activity increases. Degradation of the cytochrome components contributes to the acquisition of metabolic dormancy in this organism (Betz, 1977).

Once they are produced, conidia may require a period of maturation to achieve maximum viability. In the case of N. crassa this may require three days (Stine, 1969). Thus the older, more pigmented conidia of A. melleus are more resistant to ultraviolet light (see Sussman and Halvorson, 1966). Conidial viability and resistance to environmental extremes are influenced by the environmental conditions during conidiogenesis (Darby and Mandels, 1955), strain differences and mutations. Martinelli and Clutterbuck (1971) identified a number of loci, mutations in which result in the production of non-viable spores. Sporulation of filamentous fungi in continuous culture may often show considerable reduction in the complexity of the conidiophore (Ng et al, 1973). Detailed studies of the conidia produced under such conditions are lacking.

Many of the mutations affecting mycelial growth rates will allow conid-

iation after the point of competence has been reached (Clutterbuck, 1977). In contrast, an absolute requirement for spermidine or spermine exists in the sporulation of yeast (Cohn et al, 1978). Why the successful completion of sporulation has this requirement for oligoamines is uncertain, as sporulation is not a proliferative process in this organism. A similar necessity for oligoamines may explain the inability of an ornithine non-utilizing strain to produce conidia in N. crassa (Davis and Mora, 1968).

C Spermine, Spermidine and Putrescine: Metabolism and Functions

(i) General

The aliphatic amines spermine, spermidine and putrescine, together with their derivatives, are commonly referred to as "polyamines" though the name "oligoamines" is more accurate and will be used here. Oligoamines occur in all living organisms examined to date, though their functions *in vivo* have not been clearly established. Increasingly, evidence points to a clear correlation between oligoamine synthesis and the onset of rapid growth (see Tabor and Tabor, 1972 and 1976; Jänne et al, 1976). Changes in the rate of synthesis of oligoamines are among the earliest events detectable when cells move from a non-dividing to a dividing condition, which implies that oligoamines have an important role relating to cell growth. As the germination of fungal spores involves a similar transition from dormancy to active growth, oligoamines may have a significant role in this process as well (see Stevens and Winther, 1978).

Spermine, spermidine and putrescine are small cations very soluble in aqueous solution with approximately 3, 2½ and 2 positive charges per molecule, respectively, at neutral pH values. Oligoamines are weak chelators of transition metals (Cox et al, 1976) but their biochemical functions are almost certainly related to their ability to bind strongly to the phosphate groups of nucleic acids and lipids. The binding of oligoamines to nucleic acids decreases with increasing ionic strength (Stevens, 1969; Rubin, 1977) thus *in vitro* experiments designed to test functions of oligoamines should approximate such physiological conditions. Ribosomes and RNA have two types of sites which bind to oligoamines, a small number of high affinity sites and a larger number with weaker binding characteristics. The localization of the oligoamines bound to ribosomes is unknown though spermine is known to bind preferentially

to double stranded RNA (Ikemura, 1969; Stevens and Pascoe, 1972). The location of the two spermine molecules tightly bound to tRNA has been precisely determined by means of X-ray crystallography (Quigley et al, 1978).

Oligoamines are not normally precursors of macromolecules or other compounds. Spermidine may be modified by being covalently linked to glutathione forming glutathionyl spermidine (Tabor and Tabor, 1975) or to a specific pentapeptide forming Edeine A (Hettinger and Craig, 1968). Other complex materials containing oligoamine structures can be found, especially in plants (Smith, 1975). The formation of conjugates of oligoamines by E. coli is presumably a means of lowering the active pool of oligoamines (Tabor, 1968).

(ii) Biosynthesis and Degradation

Oligoamines lie at the end of an anabolic pathway beginning with the amino acids methionine and ornithine or arginine (Figure 1). E. coli is capable of synthesizing putrescine by the decarboxylation of either ornithine or arginine, the latter via agmatine (Morris and Fillingame, 1974). Only the former pathway is known to exist in higher eukaryotes and fungi. Arginine decarboxylase activity is not detectable in A. nidulans as it is in E. coli (Stevens, 1975). Moreover seven separate mutations in A. nidulans leading to complete putrescine auxotrophy all map at a single locus designated puA (Clutterbuck, 1974). As puA is presumably the structural gene for ornithine decarboxylase (Stevens, 1975) this demonstrates that the enzyme is indispensible for oligoamine biosynthesis, which is consistent with the view that only one pathway is available in A. nidulans. Additionally, auxotrophy for putrescine or ornithine occurs in double mutants of A. nidulans (Arst, 1977), S. cerevisiae (Whitney and Morris, 1978) and N. crassa (Davis et al, 1970)

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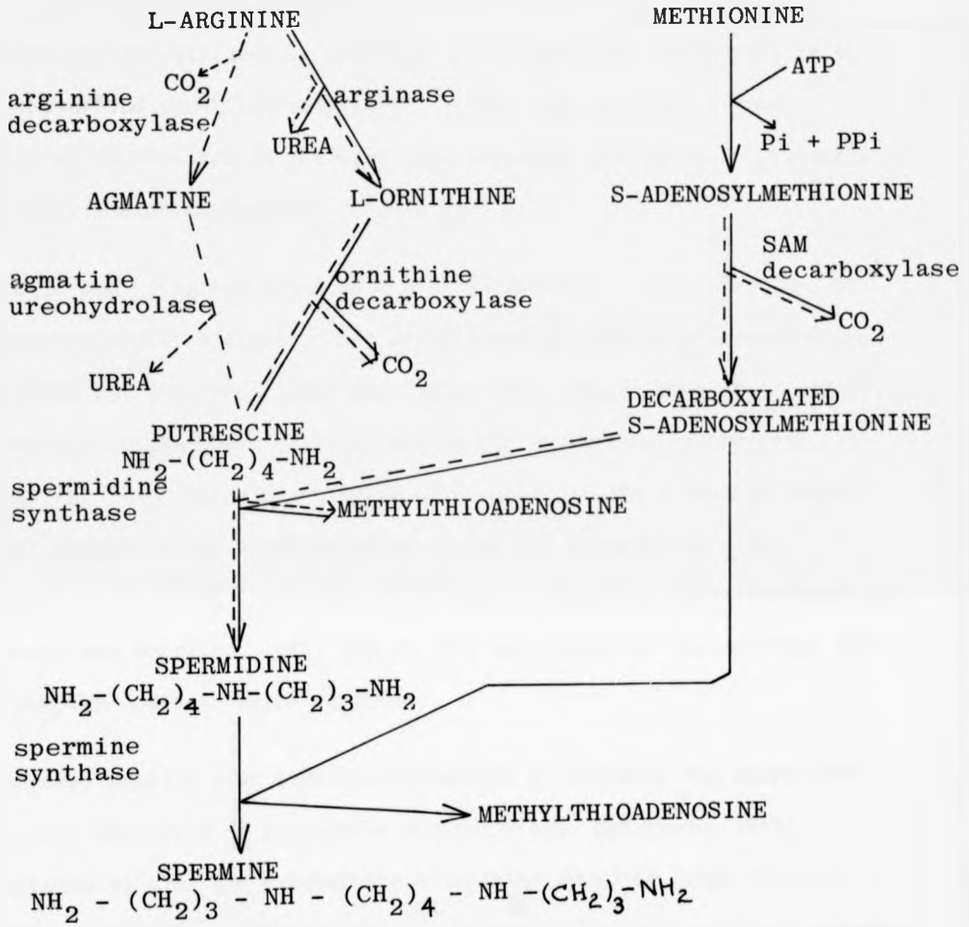

Figure 1 Biosynthesis of Oligoamines

The reactions occurring in prokaryotes, as exemplified by E. coli (-----→) and eukaryotes, as exemplified by rat liver (⟶) are illustrated (Tabor and Tabor, 1976).

lacking arginase and blocked before ornithine in the arginine biosynthetic pathway. Some residual ornithine synthesis in such double mutants can be catalysed by ornithine δ-transaminase, especially if it is synthesised constitutively (Arst, 1977). The majority of the ornithine synthesized in the wild type organism is from the L-glutamate via N-acetylated derivatives (Figure 2).

Bacteria and bluegreen algae have high intracellular concentrations of putrescine and/or spermidine but significant quantities of spermine are generally not observed (Tabor and Tabor, 1972; Ramakrishna et al, 1978). In general, prokaryotic cells do not appear to synthesize spermine even though the propylamine transferase of E. coli can, to a limited extent, accept spermidine as a substrate to synthesize spermine in vitro (Bowman et al, 1973). Spermine is present in Bacillus stearothermophilus (Stevens and Morrison, 1968) though this may simply be incorporated from the complex media in which it grows.

Eukaryotes usually have high concentrations of spermine and spermidine with only low levels of putrescine present (Tabor and Tabor, 1972). Spermidine is also the predominant oligoamine found in fungi (Stevens and Winther, 1978). Nickerson et al (1977) were unable to detect spermine in a variety of filimentous fungi though other investigators have detected spermine in A. nidulans (Bushell and Bull, 1974; Winther and Stevens, 1976) and N. crassa (Viotti et al, 1971). The ability of fungi to synthesize spermine has been confirmed by the observation that labelled ornithine or putrescine is incorporated into spermine in a variety of fungi (Winther and Stevens, 1976; Hart et al, 1978).

Pösö et al, (1976) have suggested, on the basis of measurements made in a variety of prokaryotes and eukaryotes, that the absence of spermine, or its presence in very low concentrations in an organism may be related

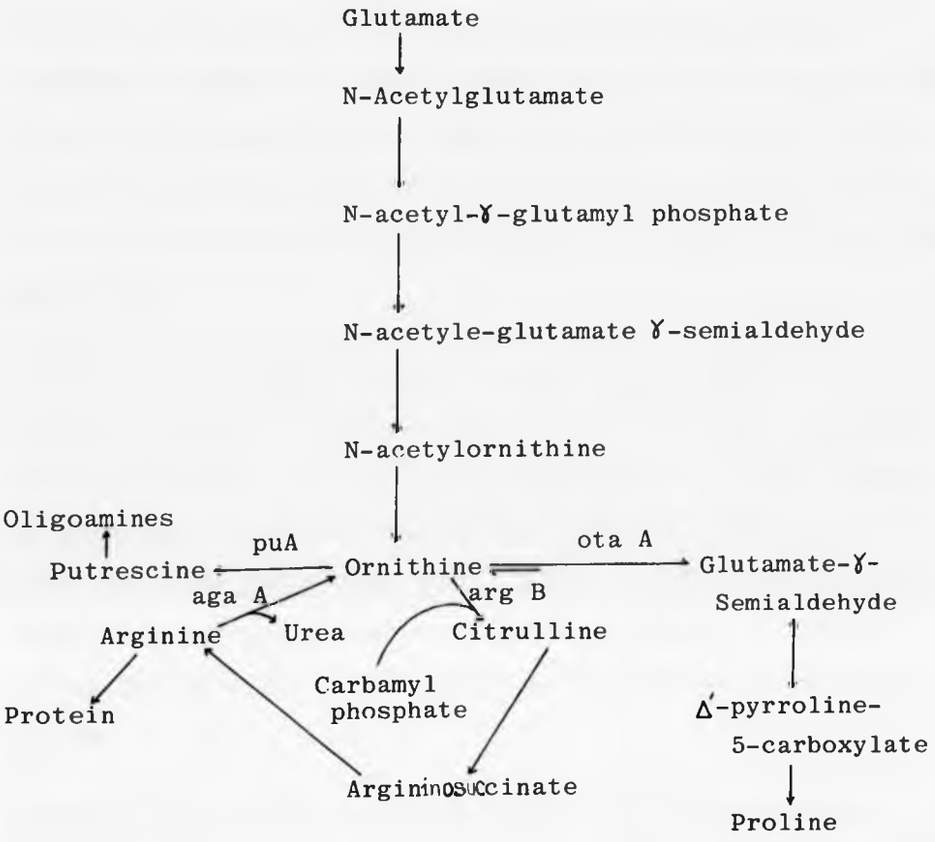

Figure 2 Ornithine Metabolism in Aspergillus nidulans.
The figures in brackets indicate mutations affecting the metabolic step.

gene symbol	enzyme activity
puA	ornithine decarboxylase
aga A	arginase
arg B	ornithine transcarbamylase
ota A	ornithine transaminase

to the ability of putrescine to stimulate S-adenosyl methionine decarboxylase. Those organisms which have a putrescine sensitive S-adenosyl methionine decarboxylase contain spermine, whereas those having putrescine insensitive S-adenosylmethionine decarboxylase activities lack spermine. Fungi fit into the eukaryotic pattern of possessing spermine and having putrescine-stimulated S-adenosylmethionine decarboxylase activities (Hart et al, 1978).

Ornithine decarboxylase, the first enzyme in the oligoamine biosynthetic pathway, has been measured in a number of fungi including S. cerevisiae (Whitney and Morris, 1978), N. crassa (Weiss and Davis, 1973), Physarum polycephalum (Mitchell and Rusch, 1973), B. emersonii (Mennucci et al, 1975) and A. nidulans (Stevens et al, 1976). In common with ornithine decarboxylases from higher organisms pyridoxal phosphate is necessary for maximal activity and putrescine is a weak inhibitor of the fungal enzymes.

Spermidine synthase has been partially purified from S. cerevisiae (Jänne et al, 1971) and occurs in a six-fold excess over S-adenosylmethionine decarboxylase activity. Spermidine synthase activity has also been measured in A. nidulans (L, Stevens, unpublished observations). The evidence from experiments with rat liver is that spermidine and spermine synthase activities are separate enzymes (Raina and Hannonen, 1971; Hannonen et al, 1972).

Though the biosynthetic pathways have been extensively studied, little is known about oligoamine catabolism. Oligoamines turn over slowly in animal tissues, the half-life of spermidine has been determined in rat brain and regenerating liver and ranges from four to nineteen days with the half-life of spermine being even longer (Shaskan and Snyder, 1973; Russell et al, 1970). E. coli does not degrade spermidine to any significant extent but instead forms conjugates of the triamine

which are excreted (Tabor, 1968; Tabor and Tabor, 1976b). Such conjugates are not formed in the blue-green algae Anacystis nidulans which can catabolize both spermine and spermidine (Ramakrishna et al, 1978). Both the rat (Jänne, 1967) and Dictyostelium discoideum (Turner and North, 1977) excrete large amounts of exogenously supplied [^{14}C]-putrescine as respiratory $^{14}CO_2$. The mechanisms of oligoamine degradation remain unknown. Various amine oxidases can accept putrescine as a substrate (see Kapeller-Adler, 1970) and a novel enzyme actively termed "polyamine oxidase", which could have a role in oligoamine turnover (Hölttä, 1977).

Genetic studies of Aspergilius nidulans have produced preliminary information concerning oligoamine catabolism in this organism. While spermidine and spermine are not effective as sole nitrogen sources, diamines such as putrescine are good nitrogen sources (Page, 1973). Mutations which result in reduced amine catabolism and poor growth on monoamines (small, N-alkylamines) as sole nitrogen source, designated mau, are still able to utilize diamines. Recessive mutations in the unlinked mauA and mauB genes lead to impaired monoamine oxidase activity which suggests that this enzyme is not essential for putrescine catabolism. Results from mauC mutants are harder to explain as they have near normal monoamine oxidase levels, are semi-dominant, and are expressed only in the presence of a puA mutation background (Page, 1973). This suggests that ornithine decarboxylase may be involved in one pathway of monoamine utilization. Arst (1977) has proposed that a possible minor route of ornithine catabolism in A. nidulans might begin with the ornithine decarboxylase reaction and end with the succinic semialdehyde dehydrogenase reaction. This would involve the catabolism of putrescine by an unknown diamine oxidase activity to form γ-amino-n-butyraldehyde. This could conceivably

be a pathway for putrescine catabolism as well, which then proceeds via γ-aminobutyric acid, succinic semialdehyde and succinic acid (see Figure 3). If this were the case then mutations resulting in the loss of γ-aminobutyric acid transaminase (gat A) or succinic semialdehyde dehydrogenase (ssu A) should lead to the inability to utilize putrescine as a nitrogen source.

Putrescine utilization, like that of most other nitrogen sources, is probably ammonium repressible in A. nidulans. An ammonium repressed mutant that does not grow on putrescine still possesses diamine oxidase activity (N. Spathas, unpublished observations) suggesting either that this enzyme is not involved in putrescine catabolism or that other ammonium repressible enzymes are required for putrescine catabolism. It is also possible that some putrescine non-utilizers may perform the initial steps in oligoamine catabolism leading to an accumulation of toxic intermediates which halt growth.

(iii) Control Mechanisms in Oligoamine Biosynthesis

Ornithine decarboxylase catalyzes the first step in the oligoamine biosynthetic pathway and under most growth conditions is probably the rate limiting step. The activity of this enzyme is very low in quiescent tissues but is much higher in rapidly proliferating cells and other conditions involving an increase in protein synthesis (see Stevens, 1970; Tabor and Tabor, 1976). In fact fluctuations in ornithine decarboxylase are among the most rapid changes to occur during the resumption of rapid growth (Jänne et al, 1978a). Both ornithine decarboxylase and S-adenosyl methionine decarboxylase activities have extremely short half-lives and pronounced inducibility (Raina and Jänne, 1975; Jänne et al, 1976). This rapid turnover may be part of the mechanism bringing about an increase in activity during rapid growth. Schimke and Doyle (1970) demonstrated

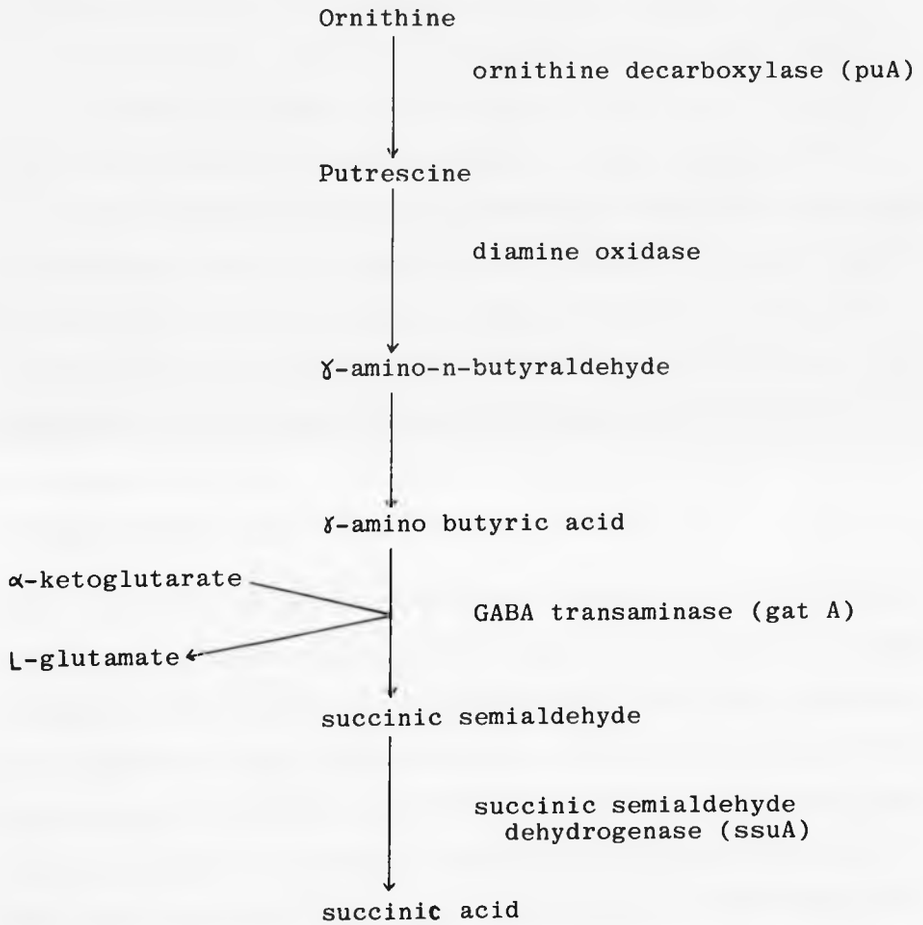

Figure 3 Possible route for the catabolism of ornithine and oligoamines (after Arst, 1977) see text for further details.

than an increase in total protein synthesis will first affect those enzymes turning over most rapidly.

Eukaryotic ornithine decarboxylase activities are not controlled by guanine nucleotides or other low molecular weight effectors (except putrescine) as is the enzyme from E. coli (Hölltä et al, 1972, 1974). Rather, in higher eukaryotes and fungi, putrescine has a central role in controlling oligoamine metabolism as shown in Figure 4; putrescine is an activator of SAM decarboxylase, an inhibitor of ornithine decarboxylase, a substrate for spermidine synthesis and an inhibitor of spermine synthase (Williams-Ashman, 1972; Kallio et al, 1977). Oligoamines inhibit the induction of ornithine decarboxylase in mammalian cells at extremely low concentrations and at higher concentrations induce the formation of a non-competitive, protein inhibitor of ornithine decarboxylase termed an "antizyme" (Heller et al, 1978; Fong et al, 1976).

There is some good evidence that ornithine decarboxylase activity can be controlled at the post-transcriptional level. The activity of this enzyme increases in both nucleated and enucleated mouse L cells when stimulated by the addition of fresh medium (McCormick, 1977). Apparently, conformational changes or covalent modification of ornithine decarboxylase gives rise to two forms of the enzyme in Physarum polycephalum (Mitchell and Carter, 1977) and mouse fibroblasts (Clark and Fuller, 1976) which differ in their affinities for pyridoxal phosphate and may affect their in vivo decarboxylating activities.

The long half-lives of the oligoamines are consistent with the view that they are not involved in any "fine control" of growth rates. This would appear to be in conflict with the very short half-lives and close regulation of ornithine decarboxylase and SAM decarboxylase. This conflict could be resolved in two ways: 1) simply because the turnover of

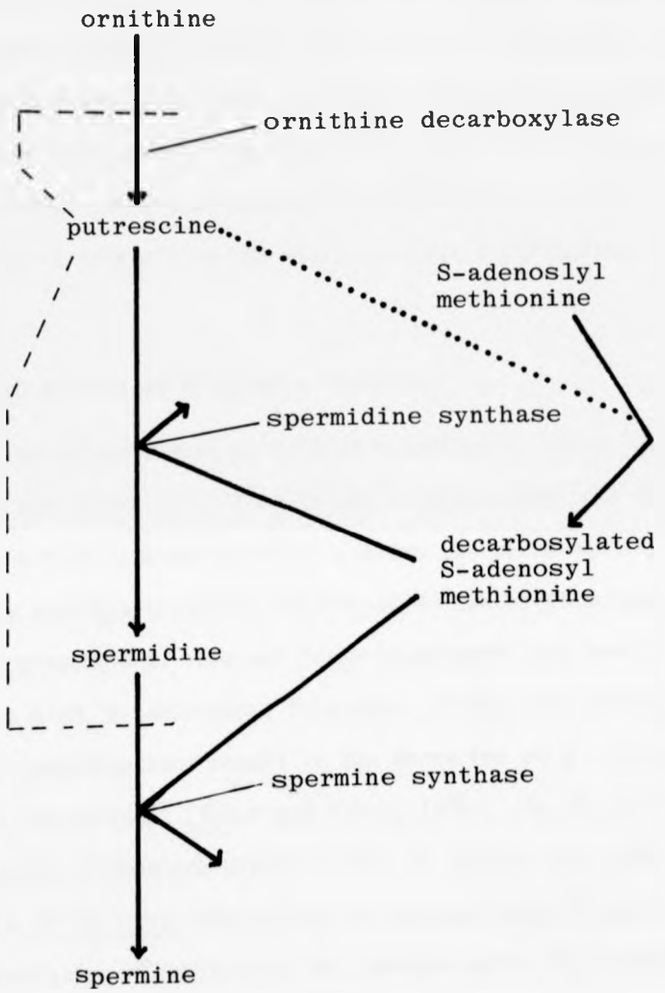

Figure 4 The central role of putrescine in oligoamine metabolism

See text for further discussion

inhibition _ _ _ _ _ _ _ _

stimulation .....

oligoamines is slow, it is extremely important to avoid overproduction, especially as spermidine and spermine can be toxic to fungi (Razin et al, 1958; Sakurada and Matsumura, 1964; Brown and Woodcock, 1973), and 2) though the total oligoamine levels turn over slowly the bulk of the amines are passively bound to organelles, ribosomes and membranes, and a smaller, soluble pool of oligoamines concerned with growth control may turn over much more rapidly. This last hypothesis is difficult to test because of the difficulty in determining the intra-cellular distribution of oligoamines.

(iv) In vitro Studies of Oligoamine Functions

Oligoamines can affect a wide variety of reactions in vitro (Jänne et al, 1978a; Tabor and Tabor 1972, 1976) though in many cases this is only a non-specific effect as other divalent cations can serve equally well. Virtually any reaction involving nucleic acids can be stimulated or inhibited by oligoamines. However these experiments are hard to interpret as amines can bind to substrates, templates, primers and products and in large enough concentrations result in the formation of an oligoamine-nucleic acid precipitates (Tabor and Tabor, 1976). As the concentration of free, soluble oligoamines within a cell is unknown this complicates extrapolation of in vitro observations to proposed physiological functions. Even the association of spermidine and spermine with ribosomes which is found in cell extracts has not been proven to occur in vivo. Oligoamines have a clear role in the packaging of DNA in bacteriophage T_4 (Ames and Dubin, 1960), herpes virus (Gibson and Roizman, 1971) and λ phage (Kaiser et al, 1975). Either spermidine or the DNA-unwinding protein are required for elongation of a primed single strand template of ØX 174 (Geider and Kornberg, 1974). Replication of the unprimed ØX 174 template apparently requires both factors. The known antagonism

of spermidine to unwinding or denaturation of the DNA duplex appears in conflict with these observations. These two agents may act at different substages of the initiation reaction. There is a small stimulation by spermidine of DNA polymerase activity in vitro (Chiu and Sung, 1972; Geider and Kornberg, 1974).

Nelson et al, (1978) found that spermidine produces only a small, non-specific increase of RNA polymerase activity in vitro. The extent of stimulation varies with the nature of the type of polymerase and template used (Jänne et al, 1975). However as the rate of chain elongation in these systems is very low at about 1 nucleotide per second (Jänne et al, 1975) which is less than 5% of the rate occurring in vivo and the nature of the RNA produced is uncertain,these experiments do not come close enough to duplicating in vivo conditions.

It is quite clear that oligoamines may have a vital role in protein synthesis (Tabor and Tabor, 1976; Algranati and Goldemberg, 1977a). A wide variety of effects have been reported. Igarashi et al, (1978) found that the increase in polyphenylalanine synthesis by oligoamines occurs at the level of aminoacyl-tRNA binding rather than peptide bond formation and translocation. Other studies have also demonstrated that oligoamines increase the initiation of polypeptide synthesis (Atkins et al, 1975) particularly the formation of the "initiation complex" (Algranati and Goldemberg, 1977b).

The presence of spermine does not markedly affect the efficiency of translation of poly A^+ RNA by a wheat germ cell free system when measured by $[^{14}C]$-leucine incorporation into acid insoluble precipitates (Raj and Pitha, 1977). However when the specific production of antiviral activity by interferon was assayed an absolute requirement for spermine was found (Raj and Pitha, 1977; Thang et al, 1975). The amount of

spermine required for interferon production is 80 to 100uM. Concentrations greater than this prevent interferon glycoprotein production. The effect on interferon may be unique to a small subclass of messengers with the template activity of the majority of the mRNA species not affected by spermine. Alternatively a critical level of oligoamines may be required for correct initiations, terminations and/or codon reading. Atkins et al, (1975) have shown that the patterns of proteins synthesized in vitro directed by a viral template in the presence of spermine, more closely resemble the pattern occurring in vivo than did the pattern produced in vitro in the absence of oligoamines. Furthermore, high concentrations of oligoamines can stabilize nucleotide-nucleotide interactions to the extent of causing miscoding (Friedman and Weinstein, 1964). Thus for experiments investigating the stimulation of protein synthesis by oligoamines, it is essential to establish that "meaningful" translation occurs. This has been done by Raymondjean et al, (1977) who found a nine to fourteen-fold stimuation by spermidine or spermine haemoglobin synthesis in a reticulocyte cell free protein synthesizing system.

Putrescine starved auxotrophs of E. coli have an increased amount of defective 30S subunits which are inactive in protein synthesis and a decreased affinity for 50S subunits (Echandi and Algranati, 1975). These defective particles disappear with the return to normal oligoamine levels following putrescine addition. The association of oligoamines with ribosomal RNA may be necessary for ribosome biogenesis. Other researchers have found that oligoamines promote ribosomal subunit association (Cohen and Lichtenstein, 1960; Garcia-Patrone et al, 1975).

The stimulation of reactions by oligoamines may not proceed exclusively by stimulation of the rate of the reaction itself but by altering the specificity of the reaction or eliminating competing, inhibiting reactions.

Thus while Evans and Deutscher (1976) found that oligoamines were not required for rabbit liver tRNA nucleotidyl transferase, the rate of reaction could be stimulated three-fold by increasing the specificity of the enzyme for tRNA substrates and inhibiting the reverse reaction. Ribosomes can inhibit rat liver isoleucine-tRNA formation in the presence of Mg^{++} and this inhibition is prevented by oligoamines (Igarashi et al, 1978). Oligoamines also alter the specificity of ribonucleases as well as stimulating the overall rate of reaction (Frank et al, 1975). Spermine specifically blocks the non-covalent bonding of IF3 and 30S ribosomal subunits in E. coli which would otherwise lead to the formation of inactive subunits (Pon et al, 1976; Cooperman et al, 1977).

The recent observation that low concentrations of spermidine and spermine (0.2-0.5mM) catalyze the polymerization of muscle actin (Oriol, 1978) suggests some novel possibilities for oligoamine functions. Muscle actin is similar to cytoplasmic actin (Landon et al, 1977) which is essential for cell division. Sanger (1975a,b) has demonstrated that actin is localized in chromosomal spindle fibres and its localization changes during cell division. If spermidine and spermine are present in the cytoplasm (McCormick, 1977) the dissolution of the nuclear membrane in mitosis could produce rapid changes in oligoamine concentrations during the cell cycle, which may in turn affect the polymerization of actin.

(v) Putrescine Auxotrophs

The existence of organisms requiring putrescine for growth is evidence that oligoamines have essential functions in cellular metabolism. Putrescine requiring mutants have been described for E. coli (Maas, 1972), N. crassa (McDougall et al, 1977; Deters et al, 1974), S. cerevisiae (Whitney and Morris, 1978) and A. nidulans (Sneath, 1955). Research,

utilizing putrescine auxotrophs of E. coli has had some success in determining the in vivo functions of the naturally occurring oligoamines. These studies have led to the conclusion that these organic cations participate directly in protein synthesis and production of the small ribosomal subunits (Algranati and Goldemberg, 1977a). Oligoamine starved E. coli grow very slowly with greatly reduced rates of macromolecular synthesis. Addition of putrescine to starved bacteria results in a rapid increase in protein synthesis followed by increases in RNA and DNA synthesis (Young and Srinivasan, 1972). Furthermore, oligoamine starved auxotrophs are unable to support the growth of RNA phage f_2. Putrescine stimulates phage growth by increasing the rate of translation of the parental RNA (Young and Srinivasan, 1974). Cell-free studies, free of the complications arising from the coupling of transcription and translation in E. coli, confirmed the defect in polypeptide synthesis in putrescine starved cultures (Echandi and Algranati, 1975). The defect is related to a deficiency in the 30s ribosomal subunit (Goldemberg and Algranati, 1977; Echandi and Algranati, 1975). Thus oligoamines may have a physiological role in ribosome assembly and/or biogenesis. Oligoamines also increase the formation of initiation complexes in polypeptide synthesis (Igarashi et al, 1978; Algranati and Goldemberg, 1977b).

Mutant strains of S. cerevisiae, defective in ornithine decarboxylase, SAM decarboxylase and possibly propylamine transferase have been isolated (Cohn et al, 1978, Whitney and Morris, 1978). Whitney and Morris (1978) reported that the growth rate of S. cerevisiae was only depressed two-fold following a twenty-fold reduction in oligoamine pool levels. Oligoamine limited cultures showed an increase in autolysis and continuing decline in growth rate. The mutants deficient in SAM decarboxylase isolated by Cohn et al, (1978) grew at only one-sixth the rate of wild

type cells but growth was completely restored by 10^{-6}M spermidine or 10^{-5}M spermine. Significantly, diploids homozygous for this mutation completely failed to sporulate in the absence of spermidine or spermine. The oligoamine requirement for sporulation is somewhat surprising as SAM decarboxylase activity falls off rapidly during nutrient starvation induced sporulation (Shapiro and Ferro, 1977). Possibly the cells are not metabolically competent to sporulate when grown without oligoamines. The fact that the cells can grow with spermine and spermidine levels decreased by one hundred to one thousand-fold, could indicate that these oligoamines are not absolutely essential for many normal growth processes or that the cell maintains levels vastly greater than are absolutely essential. At low oligoamine concentrations, there should be less non-specific binding and those functions requiring oligoamines would be expected to have a high affinity for the limited spermine and spermidine molecules available.

The putrescine auxotroph, puA_1, isolated by Sneath (1955), requires 6μM putrescine or 60μM spermidine for optimal growth. Spermine supports growth, but to a much lesser extent (Hope and Stevens, 1976). The rapid uptake of putrescine as compared to spermidine probably accounts for the ability of putrescine to support growth at a lower concentration (Hope and Stevens, 1976). A mutant derived from an A. nidulans putrescine auxotroph, that has an increased ability to grow on spermidine, designated $spsA_1$, is also more susceptible to the toxic effects of spermidine and spermine , which occurs at higher concentrations (N Spathas and J Clutterbuck, unpublished observations).

Putrescine auxotrophs of A. nidulans, when inoculated on to solid media lacking putrescine, grow slowly with a restricted morphology. The hyphae appear short, highly swollen and contorted (J A Hope, unpublished

observations). Putrescine starved N. crassa have a similar abnormal morphology (Davis et al, 1970). This form of growth is quite different from that occurring during nitrogen starvation. When a nitrate non-utilizer is grown on a media with nitrate as the only nitrogen source and nitrogen starvation results, a characteristic sparse but spreading morphology occurs. Nitrate non-utilizing, putrescine-requiring double auxotrophs, when grown on nitrate media lacking putrescine, show the sparse, spreading morphology typical of nitrogen starvation rather than the restricted morphology of putrescine starved cultures. Thus sectors of sparse but rapid growth ("spidery growth"), occurring naturally or after mutagenesis, in a putrescine auxotroph grown on restricted putrescine levels, are likely to be double auxotrophs: nitrogen source non-utilizers as well as putrescine-requiring. This allows a means of selecting nitrate (or other nitrogen source) non-utilizers in a puA background by selecting those growth sectors showing "spidery growth" (Herman and Clutterbuck, 1966).

The "spidery growth" occurs in that sector of the colony with a faster radial growth rate (the sparse but spreading morphology of the double auxotroph) even though the rate of dry weight increase is unchanged. Other researchers have found that the radial growth rates are not solely dependent on the rate of increase of the mycelial mass. Trinci (1971) observed that two particular strains of A. nidulans with identical specific growth rates in submerged culture had different radial growth rates on solid media due to differences in the widths of the "peripheral growth zones". Putrescine starvation alone could result in a narrow peripheral growth zone, possibly as a result of excessive formation of branches and/or septa. In support of this model it has been observed that in A. nidulans, osmotic shock and cycloheximide inhibit elongation

and induce chitin incorporation and eventually branching along the length of the hyphae (Katz and Rosenberger, 1971). The double auxotroph (putrescine-requiring, nitrate non-utilizing) would revert to a "normal" nitrogen starvation morphology by restricting branch formation and/or forming fewer septa. Putrescine starvation should lead to a departure from the normal relationship between the average number of branches per unit hyphal length and the growth rate (Katz et al, 1972).

(vi) Inhibitors of Oligoamine Biosynthesis

In order to determine the physiological importance of oligoamines, it would be useful to selectively deplete the cell of these compounds and ascertain the resulting metabolic defects. As isolation of oligoamine auxotrophs is difficult for animal cells, much attention has been placed on the development of specific inhibitors of oligoamine biosynthesis (Stevens, 1978; Jänne et al, 1978a). In certain systems α-Methylornithine, α-hydrazino-ßamino valeric acid, 1,3 diaminopropane and 1,2 diaminoethane inhibit the activity or synthesis of ornithine decarboxylase while methylglyoxal bis(guanylhydrazone) (MGBG) and 1,1'-(methylethanediylidine-dinitrilo)-bis-(3-aminoguanidine) (MBAG) inhibit SAM decarboxylase activity. Useful inhibitors of spermidine or spermine synthase have not yet been developed.

In a variety of experimental systems the prevention of the accumulation of oligoamines before cell replication by specific inhibitors of oligoamine biosynthesis results in the inhibition of cell proliferation (see Jänne et al, 1978b). In some cases the primary block resulting from oligoamine depletion appeared to involve DNA synthesis. However all these systems require long periods of exposure to the inhibitors so that the eventual manifestations of oligoamine starvation are likely to be

indirect or possibly even artifactual. There is also a potential danger of using analogues of oligoamines as inhibitors as the analogues themselves may sustitute for some functions served by the naturally occurring oligoamines. Metabolic inhibitors may also have unexpected side effects as shown by the ability of methylglyoxal (bisguanylhydrazone) to inhibit RNA polymerase activity *in vitro* (Nelson et al, 1978).

The conclusion arising from the use of inhibitors, i.e. that DNA synthesis is most severely restricted during oligoamine starvation, is somewhat at odds with the results obtained from the studies of auxotrophs in which protein synthesis is preferentially affected. In part this may be a difference between the types of organisms used in these experiments; microorganisms for the isolation of auxotrophs and cultured mammalian cells for most of the experiments using inhibitors. The essential function(s) performed by oligoamines may be different for the two types of organisms. Alternatively, a similar range of essential functions may be performed by oligoamines in all cells but that the functions which are most susceptible to a drop in oligoamine levels are different depending on the particular cell type and growth conditions.

RESEARCH AIMS

Investigations of oligoamine metabolism in a large number of plant, animal and bacterial species have thus far been unable to definitively assign essential biochemical functions to putrescine, spermidine or spermine. It appeared likely that the use of auxotrophic mutants to achieve oligoamine depletion would be an advantageous system to study. In particular the putrescine requirement for germination in *A. nidulans* presented a relatively simple system for experimentation. This thesis presents a study of the biochemical events occurring during the germination

of the conidia of A. nidulans, with an emphasis on oligoamine and macromolecular synthesis. A knowledge of the events required for germination would help establish which oligoamine-stimulated processes are required for germination. Examination of the biochemical changes occurring during oligoamine starvation and subsequent supplementation was also undertaken in order to investigate the in vivo functions of the oligoamines.

MATERIALS AND METHODS

A Materials

(i) Chemicals

Cycloheximide, dithiothreitol, phenylmethylsulphonyl fluoride, ATP, UTP, GTP, CTP, adenine, albumin (bovine serum), deoxyribose, pancreatic ribonuclease (Bovine type 1A), polyadenylic acid (Type I), putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride were all obtained from Sigma London Chemical Company Limited, Kingston upon Thames.

α-Amanitin was from Boehringer Corporation (London) Limited.

Nutrient agar and malt extract agar were obtained from Oxoid.

All radioactive compounds were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

All other reagent, solvents and other chemicals used were of the highest commerical grade available and obtained from BDH Chemicals Limited, Poole, Dorset.

(ii) Organisms

The prototroph of Aspergillus nidulans used for these experiments was strain BWB 272 which is a biotin prototrophic recombinant derived from the Glasgow strain W_3b_1. The putrescine auxotroph A. nidulans (puA_1) is a biotin prototrophic recombinant obtained from Sneath's 1955 original isolate (b_1, W_3, puA_1). Temperature sensitive nuclear replication cycle mutants (12, 48 and 316) and the strain from which they were isolated (46 NXW) were obtained from E Orr and R F Rosenberger. The phenotypes, in addition to the temperature sensitive mutations, are: 12 - adl5, cnx B, y; 48 - adl5, cnx, y; 316 - adl5, cnx, w; 46 NXW - adl5, cnx, w. For explanations of the gene symbols see Clutterbuck (1973).

B Growth Conditions

(i) Growth on solid media

Wild type conidia were routinely maintained on nutrient agar (2.8% w/v) and glucose (1% w/v). The nuclear replication cycle mutants were grown on nutrient agar media supplemented with adenine (0,2 mg/ml) and biotin (0.001 mg/ml). The putrescine auxotroph was grown on malt extract agar (5% w/v) and glucose (1% w/v) supplemented with putrescine dihydrochloride (0.05 mg/ml). For large scale production of conidia the fungi were grown in Roux bottles containing 125ml of the appropriate agar media.

All media and agars were sterilized by autoclaving for fifteen minutes at 15 lb/square inch. Sugars were sterilized separately by autoclaving 50% solutions.

Conidia from the stock cultures were transferred under sterile conditions, inoculated and incubated at $37^{o}C$ for five to eight days. After the growth period conidia were removed from the cultures by washing with a solution of dilute detergent (Tween-80, 0.1% v/v) and filtered through muslin. It was necessary to wash the conidia of the putrescine auxotroph by centrifugation and resuspension in fresh sterile Tween-80 (0.1% v/v) five or six times to remove any putrescine which may have been carried over from the supplemented malt extract agar media. Conidial densities were determined by counting at least 200 conidia in an improved Neubauer haemocytometer.

(ii) Growth on Liquid Media

All biochemical experiments were carried out on conidia during germination and growth in liquid media. Conidia were grown at a density of 0.1 - 2×10^{7} spores/ml. The composition of the minimal liquid media in one litre distilled water was: 1.0g $NaNO_3$, 1.6g Na_2SO_4, 0.5g KCl, 0.56g K_2HPO_4,

0.93g KH_2PO_4, 0.25g $MgSO_4 \cdot 7H_2O$, 0.01g $ZnSO_4 \cdot 7H_2O$, 0.005g $CuSO_4 \cdot 5H_2O$, 0.01g $FeSO_4 \cdot 7H_2O$ and 5% w/v glucose. The nuclear replication cycle mutants were grown in nutrient broth (2% w/v) supplemented with adenine (0.2mg/ml) and biotin (0.001mg/ml). Conidia were grown in an orbital incubator at 160 revolutions per minute. The wild type and putrescine auxotroph were grown at 37°C and the temperature sensitive nuclear replication cycle mutants at the restrictive temperature (43°C) in nutrient broth liquid media. Unless otherwise stated all times given are those after inoculation of the conidia in to liquid media.

Conidia and mycelia were harvested on 47mm diameter membrane filters, pore size 0.45μm, washed with 50mls distilled water or appropriate buffer, and extracted immediately or stored at -12°C. For determination of the extent of conidial germination at least 200 spores were examined microscopically. Germination was defined as when a visible protuberance was greater than one-half the spore diameter.

C Analysis

(i) Nucleic Acid Estimation

Extraction

Conidia and mycelia were disrupted by sonication for 3 minutes in 3% (v/v) $HClO_4$ maintained at 0-4°C in an ice bath. After centrifugation at 1200g X 10 minutes the residue was re-extracted with 3% (v/v) $HClO_4$ to remove contaminating bases and nucleotides. RNA and DNA were then extracted from the residue in 3% $HClO_4$ at 90°C for 20 minutes, cooled, centrifuged and supernatent removed. The residue was re-extracted at 90° as above and the combined supernatants used for the assay of ribose and deoxyribose to determine the original amounts present of RNA and DNA respectively.

RNA estimation (adapted from the method of Schneider, 1957)

1ml of freshly made up orcinol reagent (1% orcinol, 0.5% Ferric chloride in concentrated HCl) was added to 1ml of the hot $HClO_4$ extract and incubated at 100°C for 20 minutes with marbles on top of the tubes to stop evaporative losses. The samples were allowed to cool and the extinctions read at 600nm. The standard used was D-ribose (0 - 20µg/ml).

DNA estimation (method of Burton, 1956 as modified by Hames et al, 1972)

To each 0.4ml of sample (in 3% $HClO_4$) was added 0.1ml of a $HClO_4$/trichloroacetic acid mixture, 0.5ml diphenylamine solution (recrystallized, 4g/100ml of acetic acid) and 0.025 ml/aqueous acetaldehyde (1:500 v/v). The solutions were mixed and left for 20 to 24 hours in the dark at 20°C to 25°C. The difference in the optical densities at 595nm and 700nm was determined for each sample and compared to a standard curve using deoxyribose (0 - 20µg/ml).

(ii) Protein Estimation

Modified method of Lowry et al, 1951

The following stock solutions were made up on the day required: 0.05g $CuSO_4$-$5H_2O$ (AR) in 10ml sodium-potassium-tartrate (1%) added to 100ml 10% Na_2CO_3 (anhydrous) with 0.5N NaOH (A) and Folin Ciocalteu reagent diluted 1 $\longrightarrow$ 11 with water just before use (B). Then 1.0ml of the protein solution to be determined was mixed with 1.0ml of solution (A) and allowed to stand for exactly 10 minutes. 3.0ml of the solution (B) was then added as rapidly and as uniformly as possible to each tube, followed by immediate thorough mixing. After 30 minutes the optical density at 650nm was determined. A calibration curve was made with bovine serum albumin (0 - 100µg/ml).

Figure 5

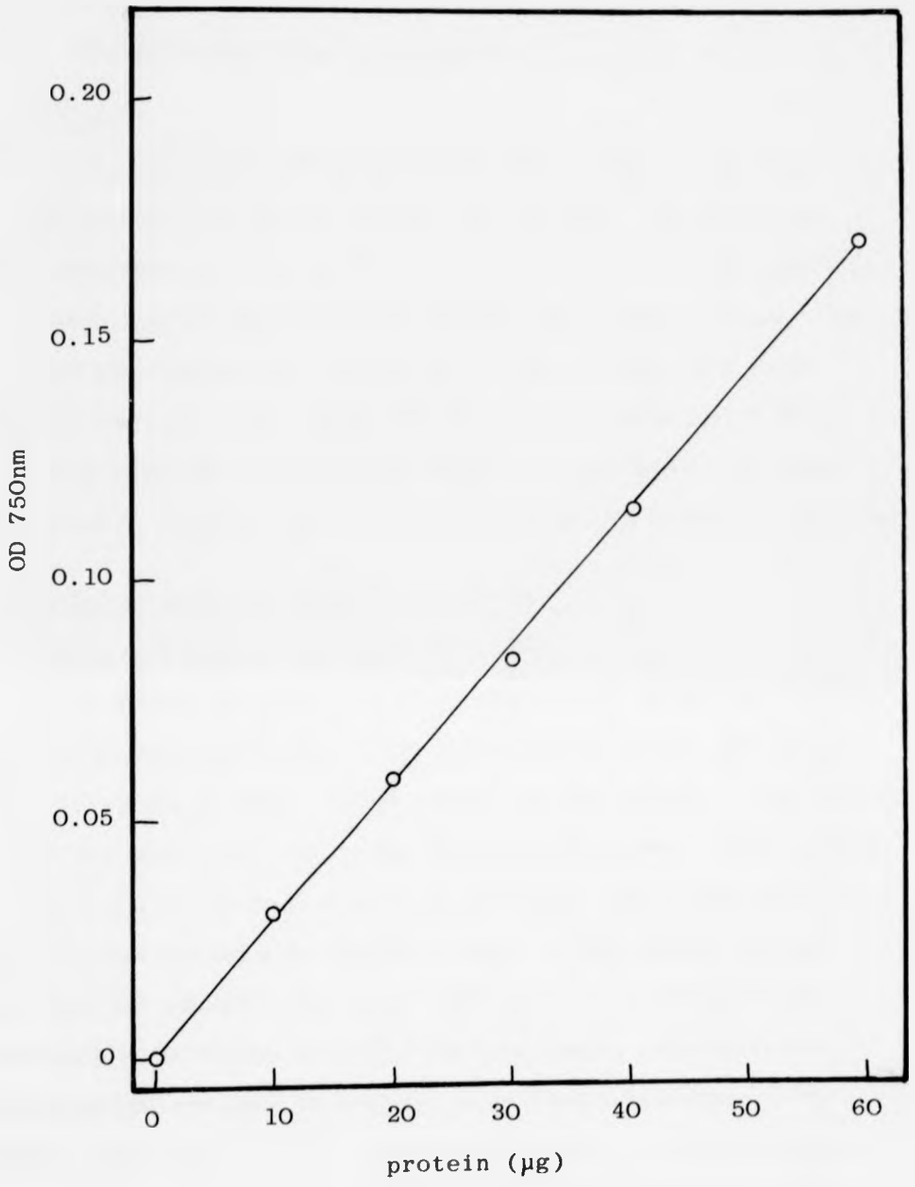

Figure 5 Standard curves for Protein Estimation

Protein was estimated by the method of Lowry et al (1951) using Bovine Serum Albumin as the standard.

(iii) Oligoamine and Ornithine Estimation

Extraction

The spores, harvested on Millipore filters were washed off in 5-15mls 2% $HClO_4$ and sonicated for one minute in an ice bath. The extract was then centrifuged at 500g for five minutes (setting 3 in bench centrifuge). The precipitate was re-extracted in $HClO_4$ and centrifuged as above. The combined supernatants were applied to a column of Dowex-50 W resin (200-400 mesh, 8% cross-linked) 1 X 8cm and washed with 20ml 0.5M HCl. The oligoamines and ornithine were eluted with 15ml 6M HCl and rotary evaporated to dryness. The residue was dissolved in 0.02M HCl (0.2-0.5ml).

Oligoamine estimation by Paper Electrophoresis

a) Citric Acid Method (Raina and Cohen 1966)

Strips of Whatman 3MM paper, 3 X 28cm, were lightly marked 8cm from one end as the origin and wetted in the citrate buffer pH 3.6 (21g citric acid, 3.2g NaOH, 1L H_2O). 5-40µl extract was then spotted in the central 2cm of the paper strip, run in the electrophoresis tank in 0.1M citrate buffer pH 3.6 for 60 to 90 minutes at 240 volts. The strips, dried at 80°C were then stained with ninhydrin reagent (100mg cadmium acetate, 10ml H_2O, 5ml glacial acetic acid, 100ml acetone, 1g ninhydrin) and developed for 60 minutes at 110°C. For quantitative colourimetric determinations the spots were eluted in 3mls extraction reagent (H_2O: glacial acetic acid: ethanol; 10:50:40, with 0.02% w/v cadmium acetate). After 30 minutes, with shaking at intervals, the solutions were read at 505nm. Standards were run from 10-200 µmoles per strip.

b) Sulphosalicylic Acid Method (Raina, 1963)

This method is very like the citric acid method described above with the substitution of 65mM 5-sulphosalicylic acid, pH 3.2 for wetting the paper and as running buffer. Electrophoresis was for 60 to 90 minutes

at 300 volts. This method was superior for the separation of diamines.

Ornithine Estimation by Paper Electrophoresis (Evered, 1959)

This method is again very like the citric acid method with the substitution of citric acid by 0.05M borate buffer (500ml 0.2M boric acid plus 440ml 0.2 N NaOH to pH 10.2, make to 2 l with H_2O). Electrophoresis was for 16 hours at 120 volts, staining as above.

Oligoamine Estimation by Thin Layer Methods

a) Method of Tobari and Tchen (1971)

Silica gel plates were prepared by mixing 30g silica gel G+ with 55ml H_2O and pouring layers of 200µl with a variable thickness applicator (clean the glass plates thoroughly rinsing finally with ethanol, then chloroform). The extract, 5-40µl, was then spotted on the plates and developed in a tank with methanol:conc. ammonia (7:3 v/v) as solvent. Standards were run at 10-200 nmoles per spot. Oligoamines ran with the following R_f values: 1,4-diaminobutanone 0.56, hydroxyputrescine 0.25, putrescine 0.17, spermidine 0.08, spermine 0.04. The ninhydrin spray was the reagent used for electrophoresis as described previously.

b) Dansylation Methods

Method of Dion and Herbst (1970)

0.1ml sample was put in a small vial with 50mg $Na_2CO_3 \cdot 10\ H_2O$ and dansylated with 0.2ml dansylchloride (30mg/ml acetone), mixed, stoppered and left in darkness at 25°C for 16 to 20 hours. The unreacted dansyl-Cl was then removed by adding 50µl proline (100mg/ml) for 30 minutes and extracting the Dans-amides in 0.2ml benzene with vigorous shaking. Silica gel plates were prepared as above and activated by drying at 115°C for one hour before use. 5 to 50µl of the benzene extracts were spotted on and the plates developed in ethyl acetate:cyclohexane (2:3 v/v) for 60 minutes in darkness. The plates were then dried at 130°C for 10 minutes, the

spots marked under U V light and scraped off. The Dans-amides were extracted in 3mls benzene:triethylamine (95:5) and the fluorescence determined. Excitation was at 345 X 4nm and emission at 480 X 9nm. Standards were run from 1-50 nmoles, see Figure 6. The dansylated polyamines ran with R_f values: putrescine 0.32, spermidine 0.21, spermine 0.12.

Method of Dreyfuss et al, (1973)

The extract was dansylated as above but after the overnight dansylation the oligoamines were extracted into ether by partition between 1ml 0.5N NaOH and 2ml ether. After shaking and centrifuging the lower, aqueous phase was discarded. The ether was evaporated to dryness and the residue dissolved in 0.2ml chloroform. 5-50µl was applied to a TLC alumina plate (30g alumina+ 45ml H_2O prepared as silica plates) which were developed in dioxane:glacial acetic acid : chloroform (2:1:97) for 60 minutes, visualised in U V, scraped off and eluted in 3ml ethanol. Excitation was at 335nm and emission at 515nm.

R_f values: putrescine 0.28, spermidine 0.40, spermine 0.51

(iv) Magnesium (Alcock and MacIntyre, 1966)

Magnesium was estimated by atomic absorption spectroscopy. The mycelial or conidial sample for which the magnesium content was to be determined was first washed with 0.1mM EDTA (pH 6.5) to remove any magnesium passively bound to the fungal walls. The mycelia or conidia were then disrupted by sonication in doubly distilled water for 6 minutes. After centrifugation at 10,000g for 10 minutes the clear supernatent was used for the estimation of magnesium by atomic absorption spectroscopy at 285.2nm wavelength. Concentrated samples were diluted to less than 0.025mM magnesium where there is a linear response between magnesium concentration and light absorption, see Figure 7.

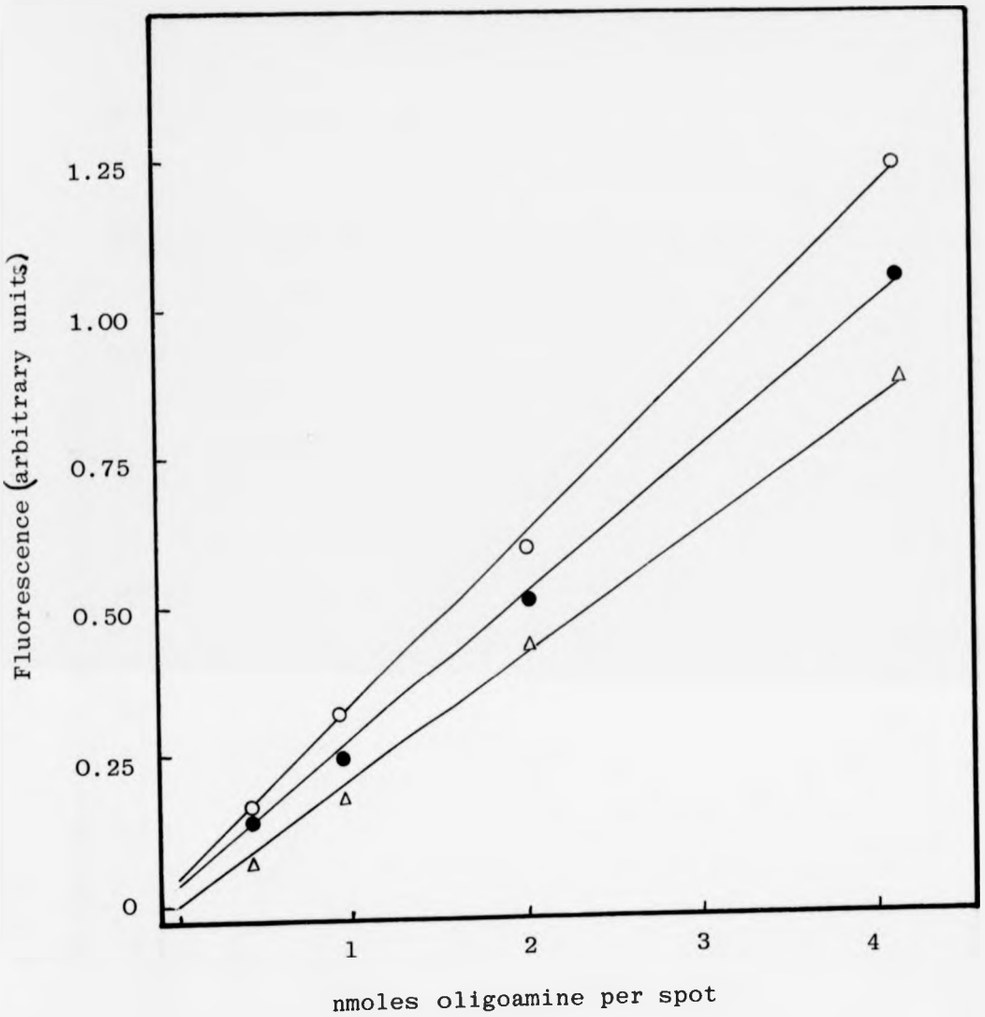

Figure 6 Typical standard curves for the determination of oligoamines by dansylation and thin layer chromatography

Spermine (○), spermidine (●) and putrescine (△) were determined as described in Metarials and Methods.

Figure 7

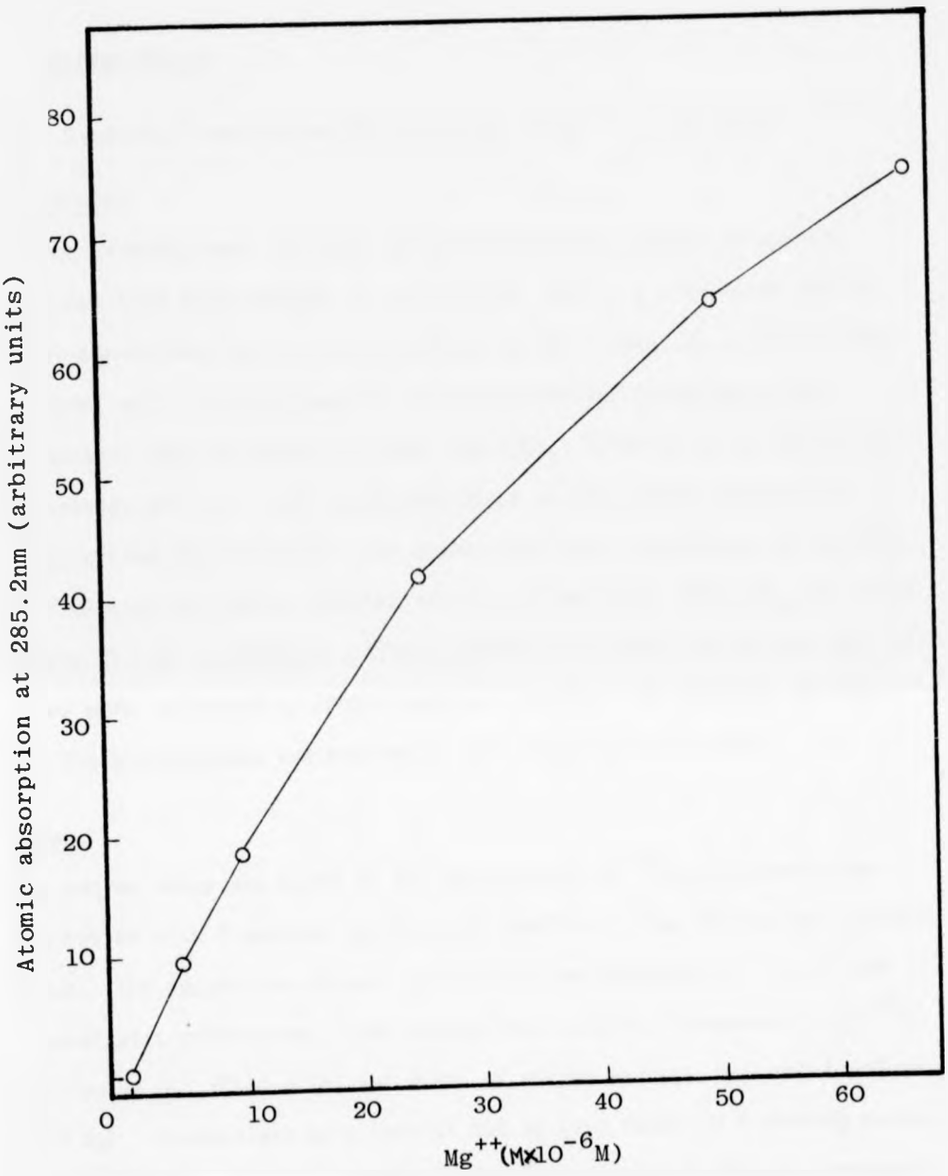

Figure 7 Standard curve for the determination of magnesium concentration

The estimation of magnesium was made by atomic absorption (285.2nm wavelength, 0.09nm slit width) as described in Materials and Methods.

D Enzyme Assays

(i) S-adenosyl Methionine Decarboxylase EC 4.1.1.50

Extraction

Enzyme extracts were prepared by grinding frozen conidia or mycelia with one-half their weight of acid-washed sand in a pre-cooled mortar or by sonication for 3 minutes followed by 10 strokes in a potter homogenizer, with 2 to 5 volumes of extraction buffer (10mM potassium phosphate, 2mM 1,4-dithiothreitol, 1mM $MgCl_2$, 0.1mM EDTA, 0.1mM pyridoxal phosphate, pH 7.6). All subsequent steps in the enzyme extraction were carried out at 0-4^{o}C. The suspensions were centrifuged at 15,000g X 15 minutes to remove cellular debris. 430mg/ml of $(NH_4)_2SO_4$ was added slowly to the supernatent. After a futher 10 minutes on ice the precipitates were collected by centrifugation (12,000g X 15 minutes) at 4^{o}C and the precipitates redissolved in 1ml of extraction buffer.

Assay

The enzyme assay was based on the measurement of $^{14}CO_2$ released after incubation with S-adenosyl-methionine labelled in the methionine carboxyl group. The incubation mixture was 0.1 M sodium phosphate pH 7.4, 0.2mM S-adenosyl-L-methionine, 1.0mM putrescine, 0.025uC: S-adenosyl-L-[1-^{14}C] methionine (45 mCi/m mole) and 0.1ml of enzyme extract in a total volume of 0.4ml. Incubations were carried out in test tubes in a shaking waterbath at 37^{o}C. Small vials (12mm diameter) containing 0.05ml of 2-methoxyethanol/ethanolamine (2:1 v/v) were suspended by thread near the top of the test tubes which were sealed with self-sealing caps. After 30 minutes, 0.1ml of 50% (w/v) trichloroacetic acid was injected into the test tubes and they were incubated for a further 60 minutes to completely trap the liberated CO_2. The small vials were then removed, 2.5mls scintillation mixture added and the samples assayed for radioactivity in a liquid

scintillation analyser. For measurement of protein by the method of Lowry et al (1951) the extracts were dialysed against water to remove pyridoxal phosphate and dithiothreitol.

(ii) RNA Polymerase EC 2.7.7.6

Extraction of RNA polymerase

The extraction and assay procedure or RNA polymerase were similar to that employed by Young and Whitely (1975). After harvesting and freezing the spores or mycelia they were homogenized in 50mM Tris-HCl, 5mM $MgCl_2$, 0.1mM EDTA, 25% (v/v) glycerol, 1mM dithiothreitol, 1.7mM phenylmethylsulphanyl fluoride, pH 7.5 (designated Buffer A) containing 0.02M$(NH_4)_2SO_4$ and sonicated in an MSE sonicater in an ice bath for 12 X 15-second bursts at 12μ amplitude. This suspension was then rehomogenized for 20 strokes and debris and unbroken cells removed by centrifugation at 12,000g X 20 minutes. This and all subsequent steps were performed at 0-4^{o}C. The pellet was re-extracted by repeating the homogenization and sonication procedure just described. The combined supernatants, ("crude extract") were then slowly stirred while protamine sulphate (10mg/ml in Buffer A) was slowly added to give a final concentration of 1mg/ml. After stirring for a further 15 minutes the preparation was sedimented at 23.000g X 20 minutes. The supernatant was discarded and the surface of the pellet washed with buffer A. The washings were discarded. The pellet was homogenized in buffer A containing 0.12M-$(NH_4)_2SO_4$, sedimented at 23,000g X 30 minutes and the supernatant kept. The pellet was re-extracted with buffer A containing 0.12M $(NH_4)_2SO_4$ and the two supernatants combined ("protamine eluate").

Chromatography of RNA polymerase

The "protamine eluate" was diluted with buffer A to give a final concentration of 0.02M $(NH_4)_2SO_4$, and loaded on to a DEAE-cellulose column

(2.5 X 8cm), suitably precycled and equilibrated with buffer A at a flow rate of 2ml/min. The column was then washed with two column volumes of buffer A containing 0.02M $(NH_4)_2SO_4$. The total RNA polymerase activity was then eluted with buffer A containing 0.6M $(NH_4)_2SO_4$. The eluate was passed through a Sephadex G50 column (2.5 X 15cm) to remove the $(NH_4)_2SO_4$ and Mg^{++}. This solution was then loaded on to a phospho-cellulose column (1 X 8cm) at a rate of 0.4ml/min and washed with 3 column volumes of buffer containing 50mM Tris-HCl-0.1mM EDTA-25% (v/v) glycerol-1mMDTT-1.7mM PMSF, pH 7.5 (Buffer B). Proteins were eluted with a linear gradient of 0.02M to 0.45M $(NH_4)_2SO_4$ dissolved in buffer B (120ml total volume). Fractions of 1.2ml were collected, alternate fractions being collected in tubes containing 50µl of solution containing 1mg bovine serum albumin and 10mg $MgCl_2$ and used for the assay for RNA polymerase. The unsupplemented fractions were used for measurement of conductivity and E_{280}.

RNA polymerase assay

The reaction mixture contained 2.5mM GTP, CTP and ATP, 4mM $MgCl_2$, 12,5mM Na_2HPO_4, 0.07M Tris-HCl, pH 7.5, 300µg/ml denatured salmon sperm DNA, 2uCi ^{3}H-UTP and 20µl enzyme extract in a final volume of 0.08ml. After 30 minute incubation at 37°, 50µl samples of the reaction mixture were spotted on numbered 2.5cm circles of Whatman No. 1 filter paper, dried and washed in batches three times with cold 5% trichloroacetic acid - 0.5% $Na_4P_2O_7$, twice with cold ethanol and twice in acetone. 5ml of solutions per filter paper were used for each washing. The filter papers were air-dried and radioactivity determined as described earlier in the Methods.

(iii) Ornithine Transaminase EC 2.6.1.13

Extraction of ornithine transaminase

The mycelia were disrupted by sonication for 3 minutes in buffer (0.1 M-

Tris-HCl, 0.1mM pyridoxal phosphate, 0.5mM EDTA, pH 7.6) placed in an ice bath to maintain a low temperature during extraction. After centrifugation at 30,000g X 15 minutes the supernatent was carefully removed and used for the enzyme assay.

Assay of ornithine transaminase

This assay was basically the same as that described by Stevens et al, (1976). 1.4ml of enzyme extract plus distilled water was added to 0.15ml L-ornithine (0.25M, pH 7.6), 0.15ml 2-oxoglutarate (0.25M, pH 7.6) and 0.3ml Tris-HCl (0.2M, pH 7.6) and incubated for 30 minutes at 37^{o}C. After 25 mintues 0.1ml O-aminobenzaldehyde solution (54mg dissolved in 1.08ml ethanol plus 1.92ml distilled water) was added to each tube. The reaction was stopped after 30 minutes by the additon of 0.5ml 20% trichloro-acetic acid. Precipitated proteins were removed by centrifugation at 1,000g X 10 minutes and the extinction at 443nm was determined. Controls were carried out by incubating the enzyme extracts in the absence of oxoglutarate, which was added after the reaction had been stopped. The amount of product produced was calculated from the extinction coefficient for the condensation product between O-aminobenzaldehyde and Δ^{1} pyrroline-5-carboxylic acid (2.71 litre$\cdot$mole$^{-1}\cdot$cm^{-1}).

E Oxygen Uptake

Oxygen consumption was determined in a Rank oxygen electrode. Calibration was carried out using oxygen-free-nitrogen to determine the zero O_2 content point, the upper level determined with fully oxygenated buffer which is assumed to contain 240μM O_2. 3ml samples of the growing conidia or mycelia were placed in the measuring chamber maintained at 37°C and the rate of oxygen uptake determined over the following 5 to 15 minutes. The initial slope was used for calculating the rates of oxygen consumption.

F Intracellular volume

This method was based on that of Slayman and Tatum (1964). The intracellular volume of germinating conidia was determined by estimating the volume from which inulin was excluded in a conidia suspension. /^{3}H/-Inulin (2.5 Ci/mmole, 500μCi/mg, average molecular weight 5,000 daltons) was added at a final concentration of 0.1μCi/ml along with 2-20 X 10^{9} conidia, harvested at various stages of germination, to a small volumetric flask and distilled water added to bring the total volume to exactly 2, 5, 10 or 25mls, depending on the sample. The radioactivity of the filtered supernatant was determined and compared with the radioactivity in a control sample not containing any spores. The concentration of labelled inulin is higher in samples containing conidia or mycelia as the inulin is not able to pass through cell membranes and so becomes excluded from the intracellular space of the conidia. Thus the intracellular volume of the conidial suspension equals the total volume of the flask minus the volume which is available to the inulin molecules. The total conidial volumes as determined were normally between 1% and 5% of the total volume of the flask. All determinations were made in triplicate.

G Rates of Macromolecular Synthesis

Conidial suspensions (5ml at a density of 10^7 conidia/ml) were incubated with a) 1ml 1.2 X 10^{-4}M DL-leucine containing 0.1 μCi [^{14}C]-leucine for the measurement of incorporation into protein, or b) 1ml 6 X 10^{-6}M adenine containing 1.0 μCi [^{3}H]-adenine for incorporation into nucleic acids. Incubation was for 10 minutes at 37°C and stopped by the addition of 0.7ml cold trichloroacetic acid (50% w/v). After disruption of the conidia in an MSE ultrasonic disruptor the insoluble material was collected on Whatman GF/C glass fibre filters and washed in 40ml ice-cold 5% trichloroacetic acid followed by 5ml cold acetone. To measure separately the rates of RNA and DNA synthesis, precipitates were incubated with 0.5 M NaOH for 2 hours at 37°C to hydrolyze the RNA, and the DNA repricipitated with trichloroacetic acid (5% w/v final concentration) and collected on GF/C filters. After air-drying the filters, radioactivity was determined. The concentrations of leucine and adenine used for incorporation studies were sufficiently high to ensure that incorporation was linear over the time course and was proportional to the number of conidia present.

H RNA Isolation

Extraction of RNA

Approximately 1g wet weight of harvested conidia was disrupted in a Mickle disruptor with 3.5mls buffer (0.2 M ammonium carbonate, 0.002 M-EDTA, 0.2% SDS pH 9.0, bentonite 100µg/ml, 3.mls redistilled phenol containing 0.01%w/v 8-hyroxyquinoline and 2.8g glass beads (size 50uM) and shaken for 6 minutes at 4°C. After centrifugation at 100g X 10 minutes the aqueous layer was carefully removed and re-extracted with an equal volume of phenol containing 8-hydroxyquinoline (1%w/w). The mixture was again shaken at 4°C for 3 minutes and the aqueous layer separated by centrifugation. The combined aqueous layers were made 2% with respect to potassium acetate and 2 volumes of ethanol added. The RNA was allowed to precipitate for at least 4 hours at -12°C. Precipitates were collected by centrifugation at 12,000g X 10 minutes and dissolved in a small volume of 0.2% SDS.

Oligo (dT) cellulose chromatography

The separation of poly A containing RNA,carried out by the method of Darnbrough and Ford (1976):

The oligo (dT) was hydrated in 0.1 M NaOH and packed in a 2ml plastic syringe. In between experiments the column was stored in 0.1 M NaOH. Before each sample was applied the column was equilibrated with 25mls of binding buffer (0.4 M NaCl, 1mMEDTA, 10mM Tris-HCl (pH 7.5), 0.1% SDS) The RNA sample, dissolved in binding buffer, was passed through the column followed by 1ml of binding buffer. The eluate was reapplied to the column, followed by 8 - 30 ml binding buffer to remove any non-specifically bound RNA. The bound RNA, presumably containing poly A tracts, was eluted with 4 mls of elution buffer (1mM EDTA, 10mM Tris-HCl-

(pH 7.5), 0.1% SDS). The amount of RNA and radioactivity in each sample was determined. The binding capacity of the oligo (dT) cellulose column is between 2 and 3 optical density units of poly A. When necessary to precipitate bound RNA, fractions were made 0.4M with respect to NaCl before addition of 2 volumes of ethanol.

Sucrose gradient analysis

Separation of RNA species was made on the basis of velocity sedementation in a linear sucrose gradient, 7 $\longrightarrow$ 30% w/v sucrose, in NETS buffer (100mM NaCl, 1% SDS, 10mM EDTA 20mM Tris-HCl pH 7.5). 0.5ml of samples containing 2.5 OD units were layered on 5ml gradients in 6ml tubes and centrifuged, 60,000 rpm for 3 hours at 16^{o}C. For larger scale separations 1-2ml of sample containing up to 25 OD units was layered on 34 ml gradients in 38ml tubes and centrifuged, 25,000rpm for 16 hours at 16^{o}C. 50% (w/v) sucrose was pumped into the bottom of the tube displacing the contents through a flow through cell (0.5cm) and continuously monitored at 260nm. When necessary, fractions were collected in a Gilson Fraction Collector.

Polyacrylamide gel electrophoresis

Gel electrophoresis was performed on 3% and 10% polyacrilamide gels (5% N', N'-methylenebis-acrilamide cross-linked) in a buffer containing 0.04M Tris, 0.033M sodium acetate, 0.001M EDTA and 0.2% SDS, pH 7.2. 10% gels were pre-run for 3 hours at 6mA/gel. 50µl samples having approximately 4.0 E_{260} units dissolved in 5% sucrose were applied to the 3% or 10% gels and electrophoresed for 2½ hours at 5mA/gel. The gels were scanned at 260nm either in a Pye Unicam SP 1800 or with a Gilford Model 2410 linear transport system.

For measurement of radioactivity the gels were sliced into 1 or 2mm slices. The slices were heated at 60^{o}C in 0.5ml 10% piperidine overnight in scintillation vials. 12ml scintillation cocktail (31.5g of

5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3, 4-diazole, 500ml of methanol, 3 litres of toluene) was added to each vial and the radioactivity was measured in a Philips Scintillation Analyser. Measurements were corrected to dpm by the channels - ratio method.

I Polysome Isolation

Extraction

Mycelia or conidia were harvested on Millipore filters and disrupted in one of three ways i) grinding with 2 volumes sand plus 0.1ml buffer (0.03 M T iethylamine-HCl, 0.01 M KCl, 0.01 M $MgCl_2$, pH 7.5) for 60 seconds followed by addition of 3-4 mls buffer and further grinding for 30 seconds, all at 4^oC (Mirkes, 1974), ii) grinding in liquid N_2 for 2 minutes followed by grinding with 2 volumes of sand and 3-4 mls buffer for 30 seconds and iii) disrupting in a Mickle Cell Disrupter for 3 minutes in 2-7 mls buffer with glass beads (0.4g/ml buffer). In all cases the extracts were then centrifuged at 15,000g X 10 minutes at 4^oC.

Sucrose gradient analysis

Volumes of the resulting supernatant containing 4 to 15 OD units (E_{260}) were then layered on a linear sucrose gradient (10 - 40% wt/wt sucrose) in 38 ml tubes and spun for 3 hours at 25,000 rpm at 4^oC. 50% sucrose (wt/wt) was pumped into the bottom of the gradients displacing the contents through a 0.5cm flow through cell, continuously monitored at E_{260}. When necessary a Gilson Fraction Collector was used to collect 1ml samples. Addition of the detergent Triton X-100 to the breaking buffer did not increase the recovery of polysomes from extracts. The addition of cycloheximide (200µg/ml) 5 minutes prior to harvesting ensured that ribosomes did not "run off" by completing the translation of a mRNA during harvesting and extraction.

Radioactivity of Ribosomal Nascent chains

200uCi of L-[^{3}H] leucine (500 Ci/mmole) was added rapidly to 200mls of spores and 50ml portions rapidly removed at short intervals over 3 minutes and poured on to frozen aqueous cycloheximide (500µg/ml). The conidia were then harvested on membrane filters, washed with cold polysome

isolation buffer containing cycloheximide (200µg/ml) and disrupted in a Mickle Cell Disruptor. The suspensions were then centrifuged 15,000g X 10 minutes to remove debris and portions of the supernatent (S-15) spun at 150,000g X 3 hours. This centrifugation step separated labelled nascent polypeptides (ribosome bound, in the pellet) from labelled completed protein molecules released into the supernatent (S-150).

Portions of the S-15 and S-150 supernatents were heated for 20 minutes at 90^{o}C in 10% w/w trichloroacetic acid to hydrolyze aminoacyl-tRNA molecules and cooled. The precipitates were collected on membrane filters, washed with cold 10% w/w tricholoracetic acid and diethyl ether, air dried and assayed for radioactivity by scintiallation spectroscopy.

The experimental data were plotted and interpreted according to Scornik, (1974).

J Polypeptide Size Distribution

Labelling and extraction of proteins

To 50mls of conidial suspension (2 X 10^7/ml), 50µCi ^{3}H-Leucine was added for a 20 minute "pulse" at 37°C followed by a "chase" of 10 minutes with leucine (1mM final concentration). The conidia were rapidly harvested and disrupted by shaking with glass beads in buffer (100mM KCl, 10mM Tris (pH 7.4), 1mM Mg acetate). The suspension was then centrifuged at 12,000g X 20 minutes, the supernatent dialyzed against 3 changes of distilled water and lyophylized. The freeze-dried precipitate was then redissolved in buffer (0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue dye). Before electrophoresis the samples were heated in a boiling water bath for 1.5 minutes to completely denature the protein chains.

Polyacrylamide gel electrophoresis of proteins

Electrophoresis was carried out by the method of Laemmli (1970) utilizing gels containing 3% acrylamide, 0.3% bis-acrylamide (stacking gel) and 15% acrylamide, 0.75% bis-acrylamide (separating gel). In later experiments the bis-acrylamide was replaced by diallyltartardiamide on a 1:1 molar basis to produce a gel which can be solublized in 2% periodic acid (Anker, 1970).

The final buffer concentrations in the separation gel were as follows: 0.375 M Tris-HCl (pH 8.8), 0.1% SDS. The gels were polymerized chemically by the addition of 0.025% (v/v) tetramethylethylenediamine and ammonium persulphate. Gels were prepared in glass tubes 8cm X 0.6cm or in a slab gel apparatus.

The stacking gels of 3% acrylamide contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS and were polymerized chemically in the same way as for the separating gel.

The electrode buffer contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Electrophoresis was for 4 hours at 5mA/gel.

K Determination of Radioactivity

The scintillation cocktail used for the determination of radioactivity was 31.5g butyl-PBD, 500ml methanol, 3000ml toluene and 1500ml Triton X-100. This scintillation cocktail is able to solubilize up to two mls of aqueous sample with only a minor decrease in counting efficiency.

Radioactivity in sucrose containing samples from gradients was determined by counting 0.2ml sample plus 1.6ml H_2O with 10ml scintillation cocktail. Alternatively 100µg/tube carrier RNA was added, precipitated with an equal volume of 10% TCA and collected on glass fibre filter paper.

Corrections to dpm were made by the channels-ratio method except for those measuring tritium in sucrose gradients and gels which were corrected to dpm by the external standards method.

RESULTS

A Conidial Germination in "Wild Type" Aspergillus nidulans

The first objective of this project was to map out some of the events of germination in the "wild type" strain Aspergillus nidulans BWB 272 to provide a basis for the examination of perturbations in the normal course of events brought about by inhibitors, by nutrient depletion in auxotrophs and in temperature-sensitive mutants. Interest was particularly directed towards studying the details of the mechanisms by which active metabolism and a high rate of macromolecular synthesis is established in germinating conidia. The experiments presented here utilized aqueous harvested conidia in which some of the earliest events may have already occurred. This did not present any difficulties in studying the quantitative and qualitative aspects of macromolecular and oligoamine synthesis in germination as they are not immediate changes resulting from hydration.

Morphological and Physiological Changes in Germination

One of the earliest visible events in germination is the swelling of the conidia due to both passive and "active" uptake of water and sperical growth, which precedes germ-tube emergence. This is reflected in an increased intracellular volume between 0 and 4 hours after inoculation, before germ-tube emergence, as shown in Figure 8. The volume of the dormant conidia, 0.29×10^{-13} litre per conidia, is equivalent to that expected from a sphere of 3.8µm diameter while that of the 6 hour swollen conidia, 0.91×10^{-13} litre, would result from a sphere 5.7µm in diameter. Border and Trinci (1970) found that during the initial stages of germination the diameter of the conidia of A. nidulans increased from about 3µm to 5µm, which is consistent with the intracellular volumes

Figure 8

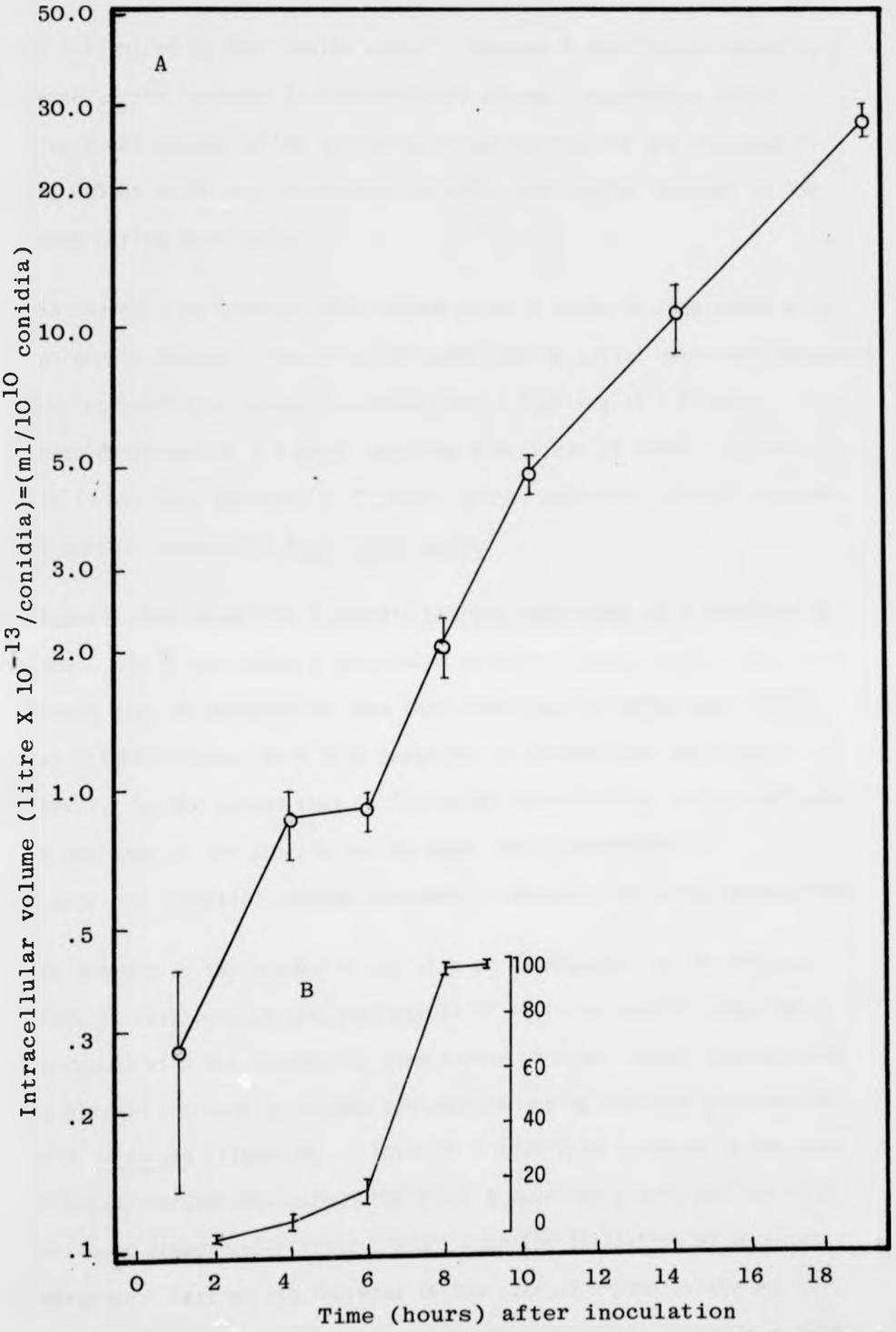

Figure 8 Intracellular volume and % germ-tube emergence in wild type conidia

A Intracellular volume

B The % conidia bearing germ-tubes

Determinations were made in triplicate, with standard deviations indicated, as described in Methods

as determined by the "inulin space". Between 4 and 6 hours there is a pause in the increase in intracellular volume. Bainbridge (1971) also found pauses in the synthesis of macromolecules and increase in dry weight which may be association with organization changes in the spore during germination.

The increase in intracellular volume after 6 hours is consistent with two growth phases. From 6 to 10 hours, during active germ-tube emergence, the intracellular volume increased with a doubling at 1.6 hours, which decreased to 3.5 hours doubling time after 10 hours. Trinci (1971) has also observed multiphasic growth patterns in batch cultures of another ascomycete Geotrichum lactis.

Figure 8 also shows the % conidia bearing germ-tubes as a function of time. 80% of the conidia germinated between 6 and 8 hours. This is a slower rate of germination than that described by Bainbridge (1971), but slightly faster than that described by Rosenberger and Kessel (1967). To the extent that an increased intracellular volume reflects an increase in the $\frac{\text{mass}}{\text{cell}}$/it can be seen that germination is a very proliferative process presumably requiring an active metabolism.

One measure of the metabolic activity of an organism is the rate at which it respires, or consumes oxygen if it is an aerobic organism. In common with the process of germination of other fungal spores there is a rapid increase in oxygen consumption during conidial germination in A. nidulans (Figure 9). There is a four-fold increase in the rate of oxygen consumption within the first 4 hours of growth but the rate increases dramatically after 6 hours with the initiation of germ-tube emergence. Part of the increase in the rate of oxygen uptake may be due to the increase in the number of mitochondria which occurs in this organism during hydration and germ-tube emergence (Florance et al,

Figure 9

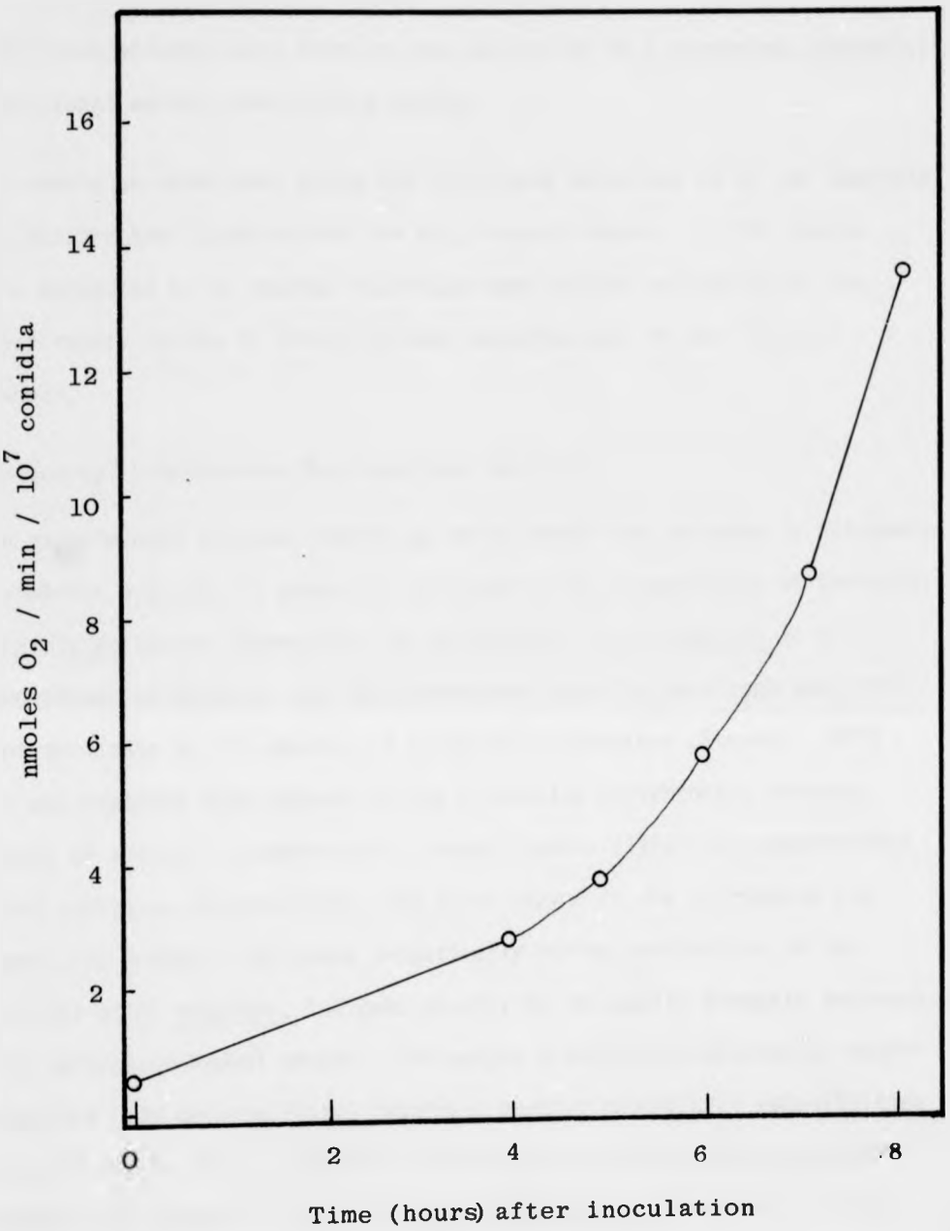

Figure 9 The Rate of Oxygen consumption during germination in wild type

The uptake of O_2 was determined as described in Methods.

1972) but probably also involves the activation of a preserved, potentially functional aerobic respiratory system.

It should be noted that using the techniques described it is not possible to measure the oxygen uptake for dry, dormant spores. As the spores are harvested by an aqueous technique some initial activation of the respiratory system is likely to have occurred even in the "0 hour" sample.

S-Adenosyl-L-Methionine Decarboxylase Activity

In experimental systems undergoing rapid growth the increase in oligoamine synthetic activity is generally followed by the accumulation of spermine, spermidine and/or putrescine. As germination in A. nidulans is a proliferative process, and the putrescine-requiring auxotroph puA_1 will not germinate in the absence of exogenous oligoamines (Stevens, 1975) it was expected that enzymes of the oligoamine biosynthetic pathway would be active in germination. Indeed Stevens (1975) has demonstrated that ornithine decarboxylase, the first enzyme in the oligoamine biosynthetic pathway, increases dramatically during germination of the conidia of A. nidulans, followed shortly by an equally dramatic decrease during subsequent hyphal growth. The enzyme S-adenosyl-L-methionine decarboxylase (SAM decarboxylase) catalyzes another potentially rate-limiting step in spermidine and spermine biosynthesis so it was of interest to examine the changes in its activity accompanying germination. As this enzyme had not been measured in any filamentous fungi it was necessary to establish basic characteristics of the enzyme. The extraction buffer used was similar to that employed for the extraction of ornithine decarboxylase from this organism, and in fact, both enzymes could be measured in the same 25,000g supernatants, suggesting cytoplasmic localizations.

The experimental results presented in Figure 10 show that the enzyme activity is proportional to the amount of protein added, that the assay is linear for at least 30 minutes, has a temperature optimum, under these conditions, near 37°C and a pH optima symmetrical about pH7.4. These properties are characteristic of an enzyme catalyzed process rather than a non-enzymic decarboxylation of the labelled S-adenosylmethionine (SAM) present in the assay mixture.

The SAM decarboxylase activities of a number of eukaryotic organisms, except plants, are strongly stimulated by putrescine whereas those from bacterial sources require Mg^{++}. The enzyme activity from A nidulans was also found to be markedly stimulated by putrescine (Figure 11). One-half of the maximum stimulation occurs between 0.08 and 0.12mM putrescine, depending on the particular cell extract examined. In some experiments with crude extracts putrescine concentrations greater than 1.0mM could be slightly inhibitory. Ornithine, spermidine and spermine, even up to 10mM, are not stimulatory. Thus the SAM decarboxylase activity in A. nidulans is similar to that found in yeast (Pösö et al, 1975) and animal tissues (Raina and Hannonen, 1970).

The apparent K_m of the enzyme SAM decarboxylase for S-adenosylmethionine was found to be dependent on the presence of putrescine (Figure 12). Thus the addition of 1.0mM putrescine not only stimulates the activity by over twenty-fold it also decreases the K_m from 0.48mM to 0.16mM. In the presence of 3.1mM putrescine the K_m is decreased even further to 0.063mM. Putrescine decreases the K_m for S-adenosylmethionine in S. cerevisiae (Pösö et al, 1975) which is consistent with the results presented here for A. nidulans. It would appear that the putrescine stimulated increase in the rate of decarboxylation can only partially be accounted for by the increased affinity for the substrate and may

Figure 10

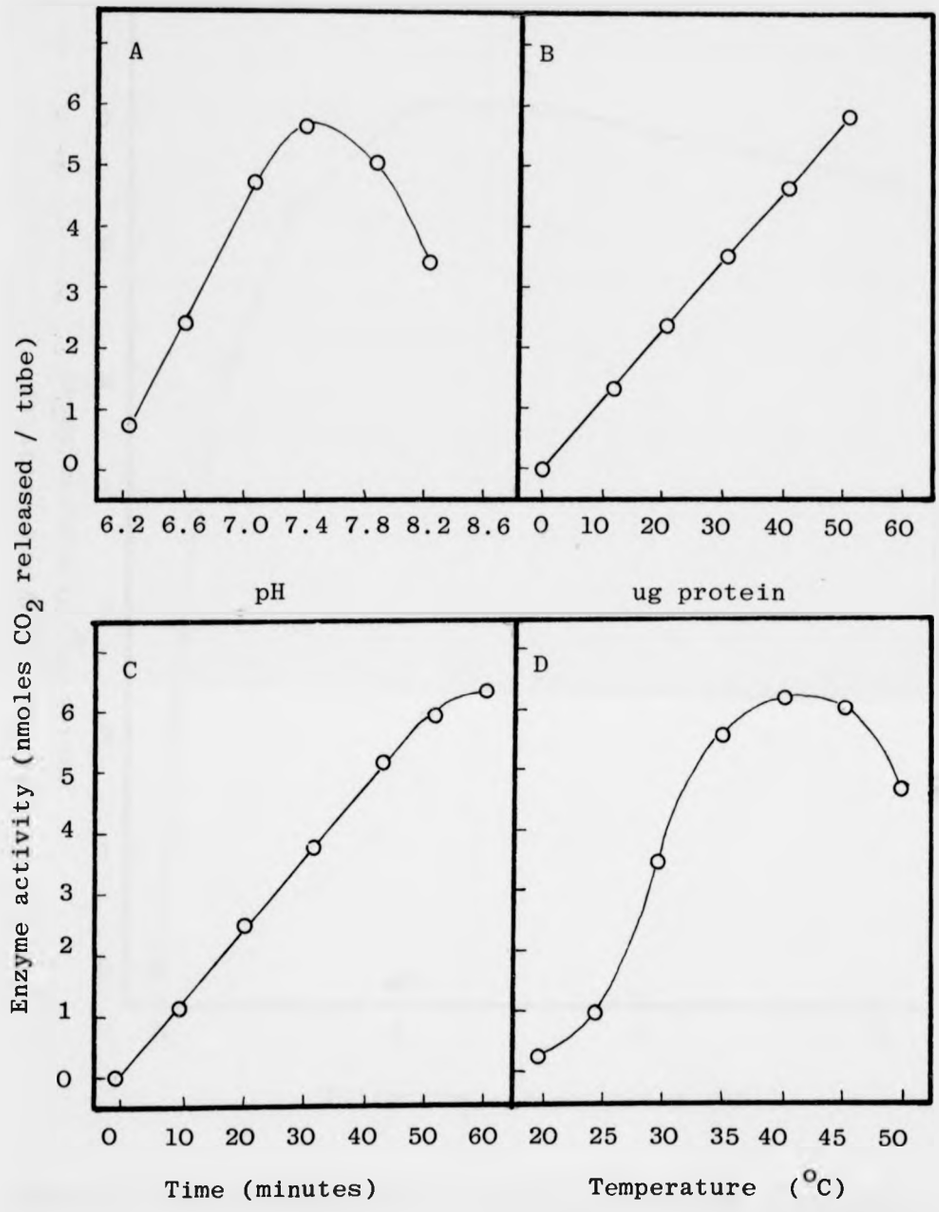

Figure 10 Properties of the S-adenosylmethionine decarboxylase activity extracted from the germinating (8 hr) conidia of A. nidulans (wild type)

Enzyme activity was measured as described in materials and methods with the exception of the sodium phosphate buffer in A, the time of assay in C and the temperature in D. Crude extract from 8 hr conidia was added in the range of 0 to 60μg protein per assay in B.

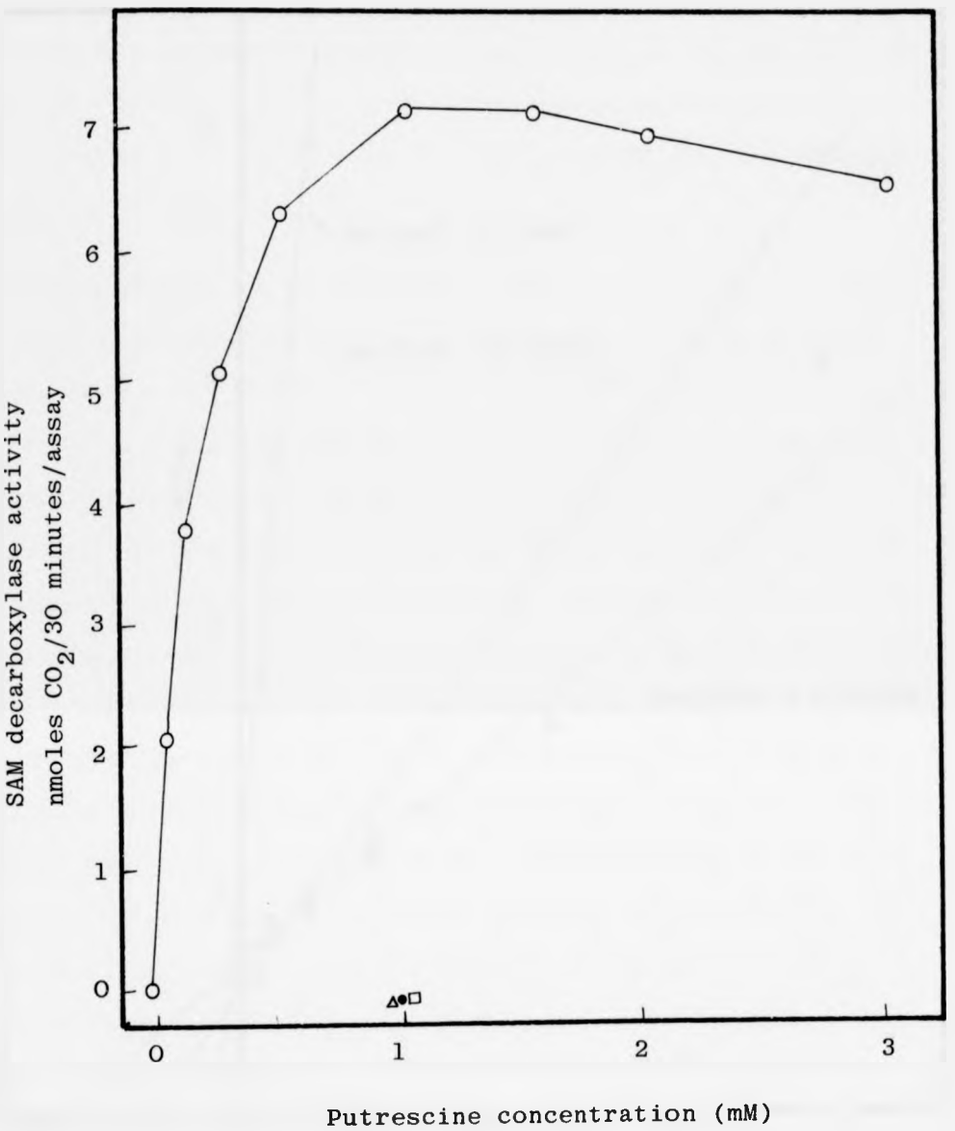

Figure 11 Putrescine stimulation of S-adenosyl-methionine decarboxylase activity in A. nidulans (wild type)

S-adenosylmethionine decarboxylase activity was measured as described in Materials and Methods, except that varying levels of putrescine were added to the assay mixture to give the final concentrations as shown. Spermine (Δ), spermidine (●) and ornithine (□) were not stimulatory at a concentration of 1mM.

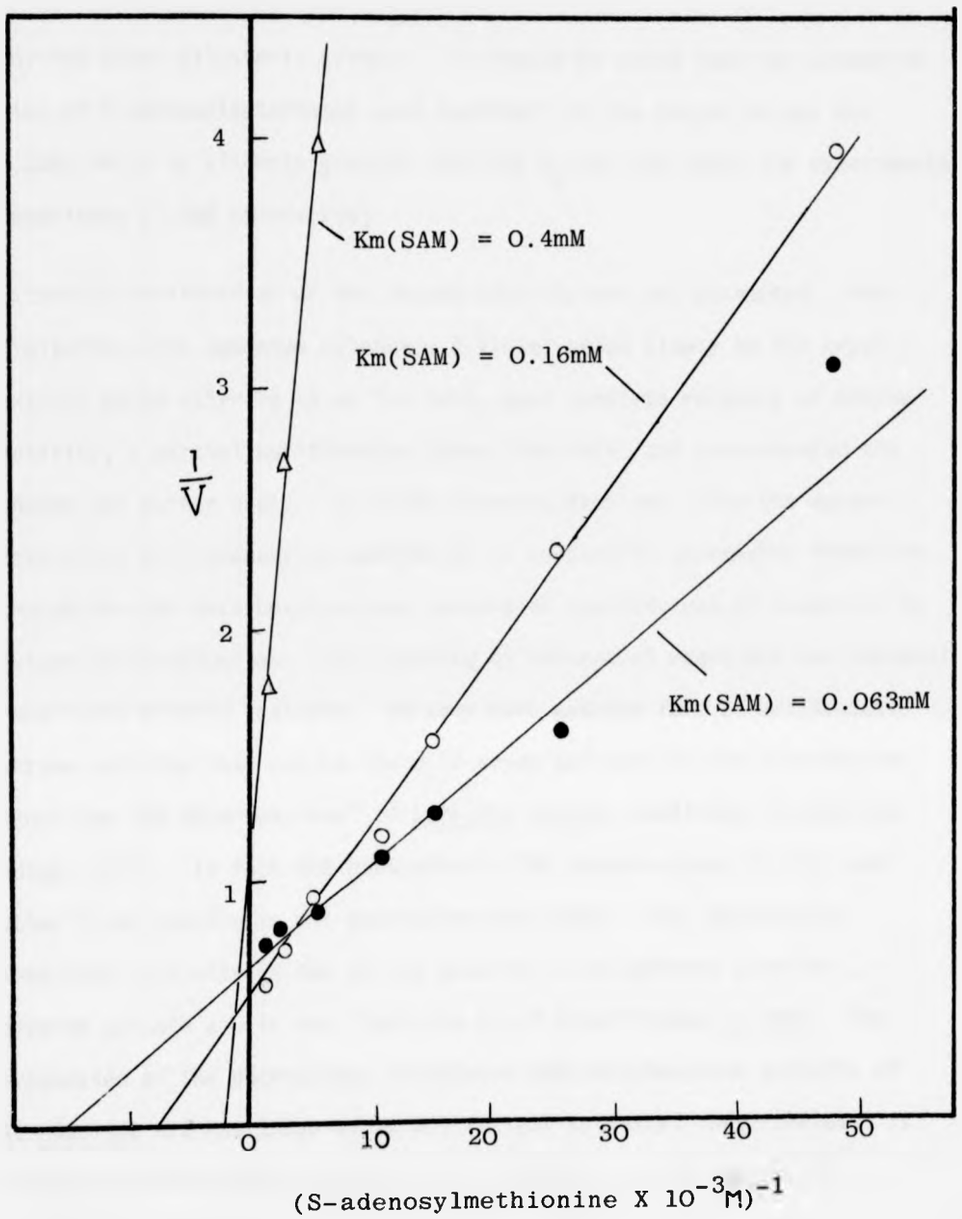

Figure 12 The effect of putrescine on the Km for S-adenosylmethionine of SAM decarboxylase (wild type)

A kinetic analysis of SAM decarboxylase was carried out in the absence of putrescine (Δ), with 1.0mM putrescine (○), 3.1mM putrescine (●) in the assay mixture

involve other allosteric effects. It should be noted that the concentration of S-adenosylmethionine used routinely in the enzyme assays was 0.2mM, which is slightly greater than the K_m for SAM under the experimental conditions (1.0mM putrescine).

Extensive purification of the enzyme activity was not attempted. Precipitation with ammonium sulphate, 0.41g/ml added slowly to the crude extract while stirring in an ice bath, gave complete recovery of enzyme activity, a partial purification (about two-fold) and concentrated the enzyme for easier assay. In crude extracts from rat liver the decarboxylation of S-adenosyl-L-methionine is coupled to spermidine formation, though the SAM decarboxylase and spermidine synthase can be separated by extensive purification. The coupling of sequential reactions can certainly complicate kinetic analysis. An even more extreme form of artifactual enzyme activity that can be found in crude extracts is the "putrescine-sensitive SAM decarboxylase" of Lathyrus sativus seedlings (Suresh and Adiga, 1977). In fact the biosynthetic SAM decarboxylase in this and other plant species is not putrescine activated. The "putrescine-sensitive" activity is due to the generation of hydrogen peroxide by diamine oxidase and is not likely to be of significance in vivo. The properties of the putrescine - stimulated SAM decarboxylase activity of A. nidulans are not those of an artifactual activity. Nevertheless, it illustrates the need for caution when analysing enzyme properties in crude extracts.

There is a rapid increase in the activity of SAM decarboxylase during germination for the first 10 hours, followed by an equally rapid decrease (Figure 13). The time course for this enzyme is thus very similar to that of ornithine decarboxylase in which the maximum activity occurs slightly earlier at 8 hours (Stevens, 1975). The most rapid increase in

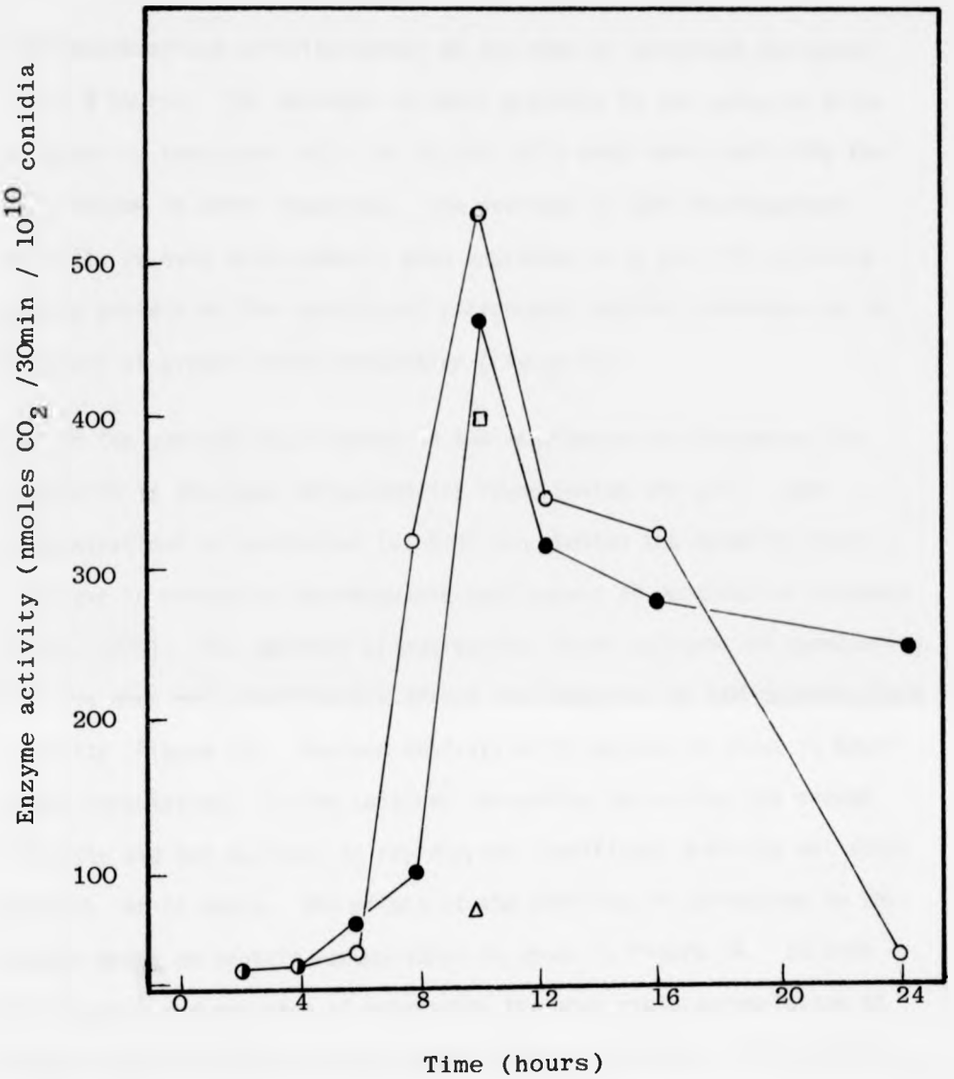

Figure 13 The effect of putrescine on the development SAM decarboxylase activity during germination (wild type)

The levels of enzyme activity were determined as described in Materials and Methods in extracts from conidia germinated in the absence (○) or presence (●) of 0.56mM putrescine, 1.6mM spermidine (△) and 1.1mM spermine (□).

SAM decarboxylase activity occurs at the time of germ-tube emergence (6 to 9 hours). The decrease in total activity in the cultures after 10 hours is consistent with the reports of a very short half-life for this enzyme in other organisms. The decrease in SAM decarboxylase activity is even more dramatic when expressed as a specific activity per mg protein as the quantity of extractable protein increases up to 24 hours of growth after inoculation (Figure 14).

One of the controlling elements in the regulation of oligoamine biosynthesis is the level of putrescine found inside the cell. Low concentrations of putrescine (0.56mM) can abolish the normally rapid increase in ornithine decarboxylase that occurs at germination (Stevens et al, 1976). The addition of putrescine in the cultures of germinating conidia does not significantly affect the induction of SAM decarboxylase activity (Figure 13). Maximum activity still occurs at about 10 hours after inoculation. In the cultures containing putrescine, the enzyme activity did not decrease as rapidly, and significant activity was still present at 24 hours. The effect of the addition of putrescine to the growth media on protein accumulation is shown in Figure 14. In both the absence and presence of putrescine the most rapid accumulation of protein occurs between 6 and 16 hours after inoculation. The levelling off of the quantity of protein after 16 hours suggests that nutrients become limiting and a "stationary phase" results under the conditions of growth utilized here (i.e. shaken batch cultures at high spore densities).

The addition of spermidine (1.6mM) to the growth media produced a dramatic decrease in the activity of SAM decarboxylase found in 10 hour germinated conidia (Figure 13). Thus this enzyme is sensitive to the intracellular oligoamine concentration, though in a rather different way than that by which ornithine decarboxylase is regulated. Spermine (1.1mM) did not

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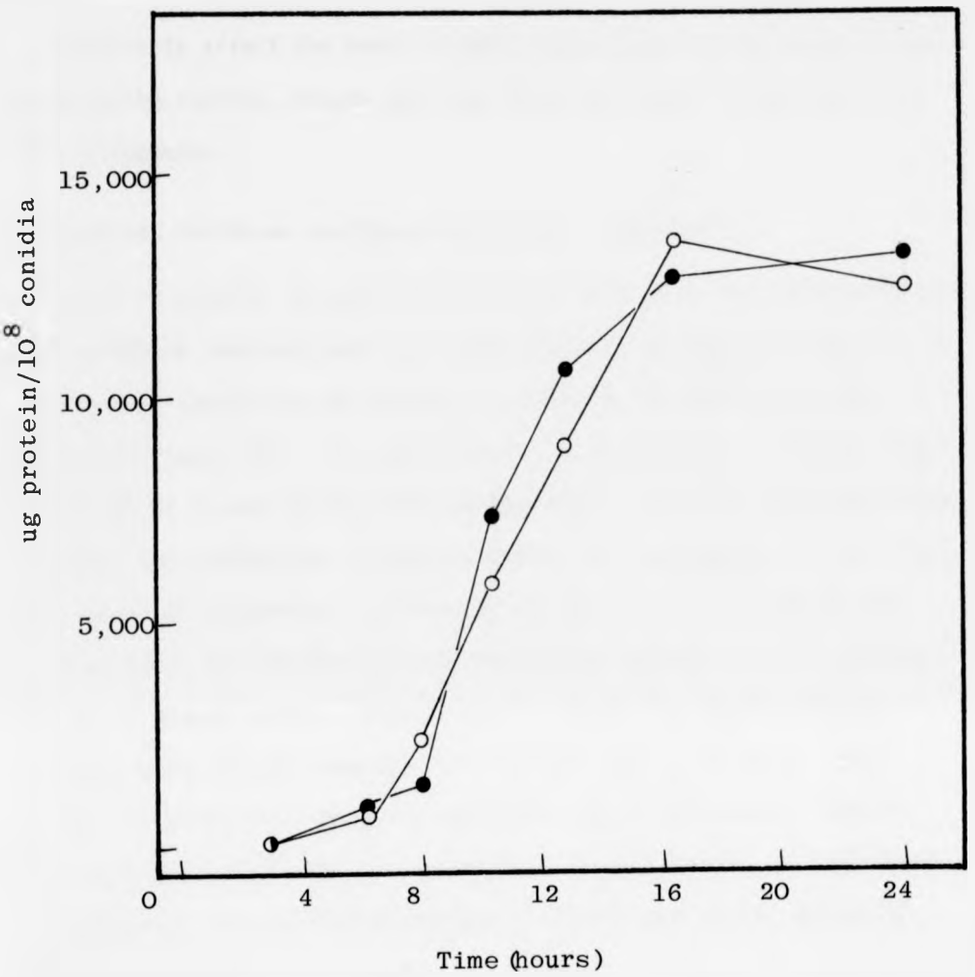
Figure 14



wild type

Figure 14 The quantity of protein present in the crude extracts of λconidia grown in the absence (O) or presence (●) of 0.56mM putrescine was determined as described in Materials and Methods. Protein was estimated by the method of Lowry et al (1951).

significantly affect the level of SAM decarboxylase found in the 10 hour germinating conidia, though this may simply reflect a lesser uptake of this oligoamine.

Oligoamine, Ornithine and Magnesium Levels in Germination

In order to clarify the physiological significance of the SAM decarboxylase and ornithine decarboxylase activities measured in vitro, the amounts of putrescine, spermidine and spermine present during germination were measured (Figure 15). The concentration of oligoamines increases dramatically prior to and during germ-tube emergence. As with yeast and animal tissues, the predominant oligoamine present in A. nidulans is spermidine. The amount of spermidine increases by the greatest amount during the first 8 hours so that the ratio of spermidine:spermine is 7-9:1 during the early growth period. However after 8 hours the spermine levels increase more rapidly reducing this ratio to 3:1 by 16 hours. The levels of putrescine remain low thoughout the entire period. This is consistent with the view that putrescine serves primarily as a precursor to spermidine and spermine biosynthesis, rather than having essential physiological roles of its own.

Where sufficient material was available the oligoamines were estimated by paper electrophoretic separation and staining with ninhydrin. The identifications of the oligoamines were confirmed by thin layer chromatography of dansylated extracts under the two different running conditions. All three methods also gave similar values for the amounts of the various oligoamines present in germinating conidia. Confirmation of the identity of spermine was important as an earlier report (Nickerson et al, 1977) suggested that filamentous fungi lacked this specific oligoamine. The presence of spermine in a number of species of filamentous fungi has been demonstrated by Hart et al (1978).

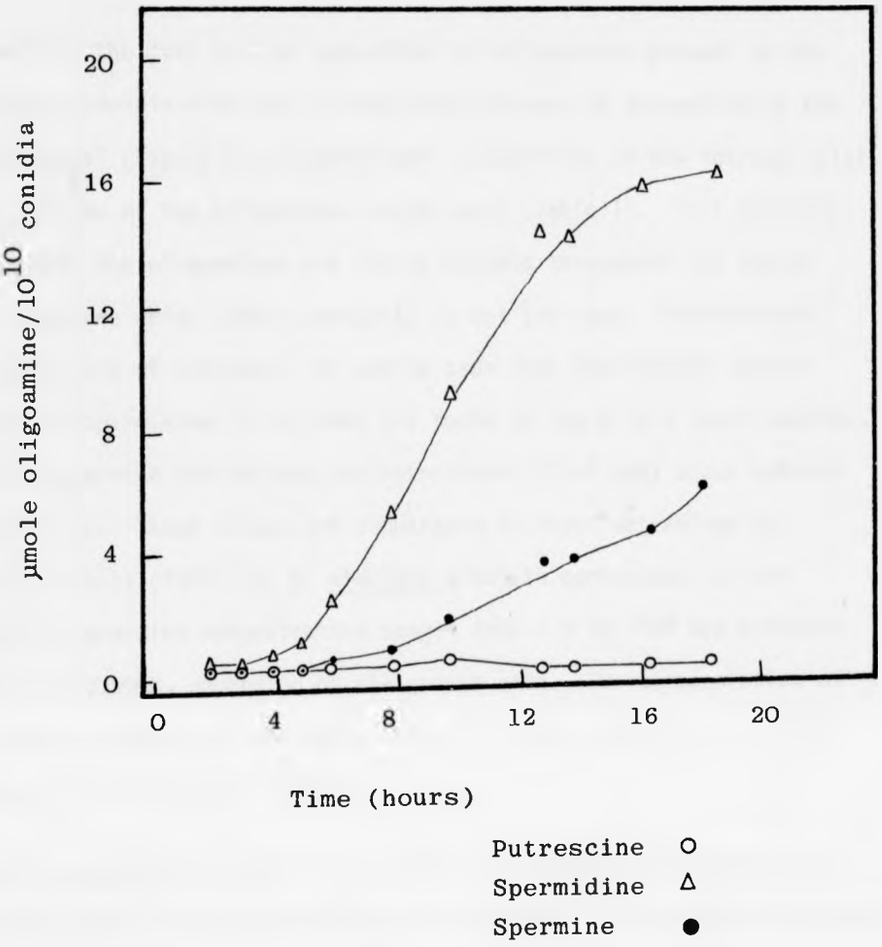


Figure 15 Oligoamine Accumulation during the germination of *A. nidulans* wild type conidia

The amounts of putrescine (○), spermidine (△) and spermine (●) were determined by thin layer chromatography of dansylated extracts as described in Materials and Methods

By combining the data for the quantities of oligoamines present in the germinating conidia with the intracellular volumes as determined by the "inulin space" (Figure 8) an approximate calculation of the intracellular concentrations of the oligoamines can be made (Table 1). This analysis assumes that the oligoamines are freely soluble throughout the intracellular volume which almost certainly is not the case. Nevertheless, the results are of interest. It can be seen that the highest concentrations of spermidine (2.6-2.7mM) are found in the 6 to 8 hours samples, while for spermine the maximum concentrations (0.3-0.4mM) occur between 8 to 14 hours. These values are comparable to those determined by Bushell and Bull (1974) for A. nidulans grown in continuous culture where the spermidine concentration ranges from 2.9 to 15mM and spermine from 0.4 to 0.59mM, depending on the growth rate. The concentration of putrescine is highest in the early stages of germination, being around 0.2mM, and then decreases rapidly.

A possible controlling factor in regulating oligoamine biosynthesis is the concentration of ornithine within the conidia. The amount of ornithine present during conidial germination is shown in Figure 16. Ornithine is barely detectable in the dormant spores and increases dramatically (50-fold) during germination. The amount of ornithine at 4 hours, 0.2μmole/10^{10} conidia, is equivalent to an intracellular concentration of only 0.2mM. As this is less than one quarter of the amount of oligoamines present at this time it would seem that continuous ornithine synthesis and turnover is necessary to meet the increasing requirements of oligoamine biosynthesis. Thus a limitation in the ornithine supply in dormant conidia could be one factor maintaining a low level of oligoamine synthesis. The accumulation of large amounts of ornithine, arginine and citrulline is an early event in the germination of conidia of N. crassa (Schmit and Brody, 1975).

Intracellular concentration of Oligoamines in wild type

µmole/10^{10} conidia and concentration (mM)						
	Spermine		Spermidine		Putrescine	
Time (hours)	µmole	mM	µmole	mM	µmole	mM
1	0.05	0.17	0.35	1.21	0.1	0.3
4	0.08	0.09	0.59	0.65	0.15	0.2
6	0.26	0.27	2.52	2.63	0.15	0.15
8	0.73	0.36	5.39	2.68	0.35	0.2
10	1.41	0.29	9.22	1.90	0.4	0.1
14	3.61	0.36	14.19	1.42	0.2	0.02
19	5.03	0.14	16.25	0.46	0.2	0.01

The amounts of putrescine, spermidine, and spermine were determined by thin layer chromatography of dansylated extracts, and the intracellular concentrations of the oligoamines assumes that the amines are freely distributed within the intracellular volume.

Figure 16

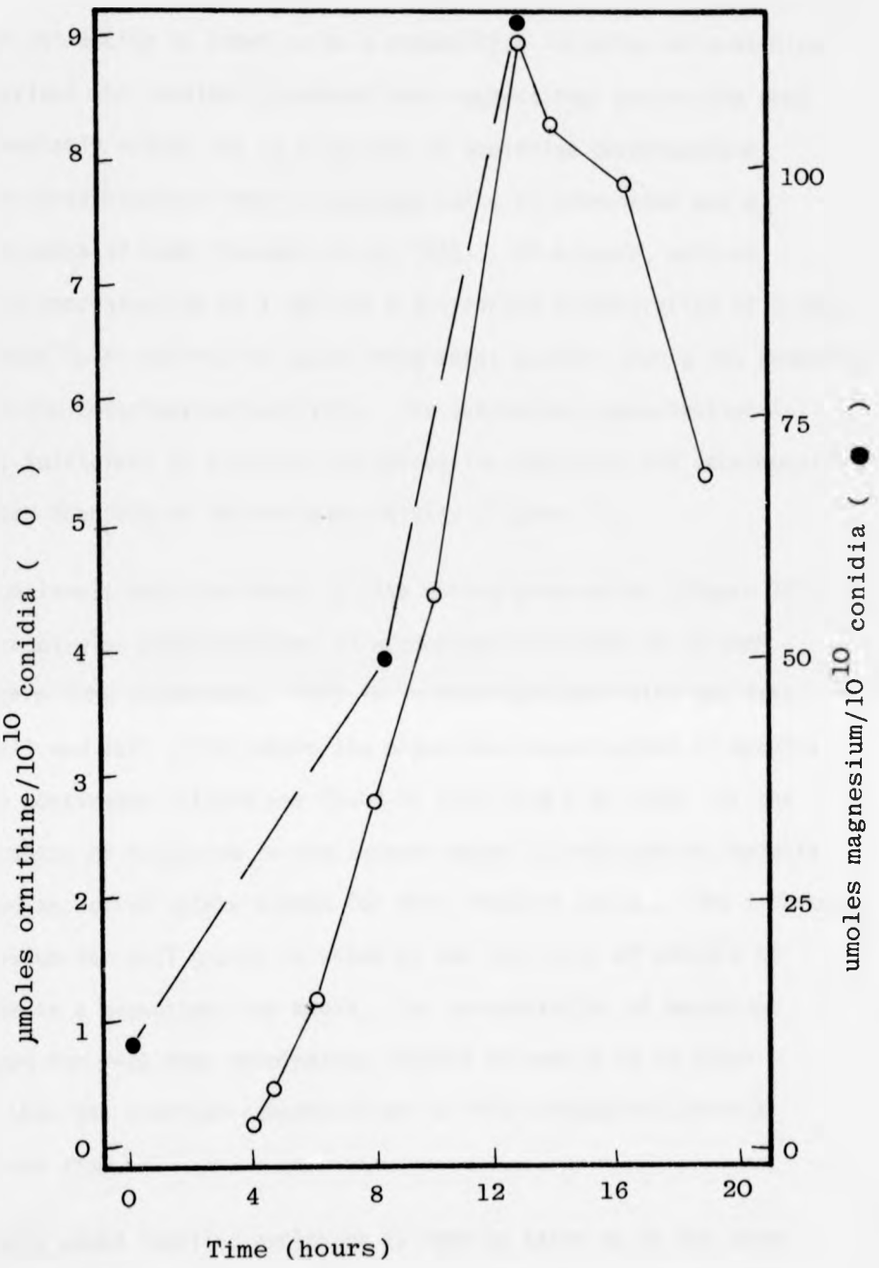


Figure 16 Ornithine and Magnesium accumulation during germination of A. nidulans conidia (wild type)

The amounts of ornithine (O) and magnesium (●) were determined as described in Materials and Methods.

Although putrescine is known to be a competitive inhibitor of ornithine decarboxylase the results presented here suggest that putrescine does not appreciably affect the *in vivo* rate of ornithine decarboxylase. Ornithine decarboxylase from *A. nidulans* has a K_i putrescine and K_m ornithine each of 60μM (Stevens et al, 1976). At 8 hours, with an ornithine concentration of 1.4mM and a putrescine concentration of 0.2mM, the intracellular putrescine could bring about no more than a 15% reduction in ornithine decarboxylase activity. The putrescine concentration is, however, sufficient to activate the putrescine-dependent SAM decarboxylase to greater than 65% of the maximum activity (Figure 11).

Magnesium levels are also found to rise during germination (Figure 16). The intracellular concentrations of magnesium would thus be 20-25mM during germ-tube appearance. This is in good agreement with the data of Bushell and Bull (1974) where the magnesium concentration of mycelia grown in continuous culture was found to vary from 6 to 37mM. As the concentration of magnesium in the aqueous media is only 1mM the mycelia must have an active uptake system for this divalent cation. The importance of magnesium for cell growth is shown by the inability of conidia to germinate in a magnesium-free media. The concentration of magnesium determined for 8-12 hour germinating conidia is some 8 to 10 times greater than the combined concentrations of the oligoamines present at the same time.

Exogenously added labelled ornithine is readily taken up by the germinating conidia and is used for the synthesis of putrescine, spermidine and spermine. It is possible to calculate the rate at which ornithine is decarboxylated *in vivo* (Figure 17) by measuring the amount of radioactivity found in the oligoamines as a function of time, assuming that they are not appreciably metabolized to other compounds during this

Figure 17

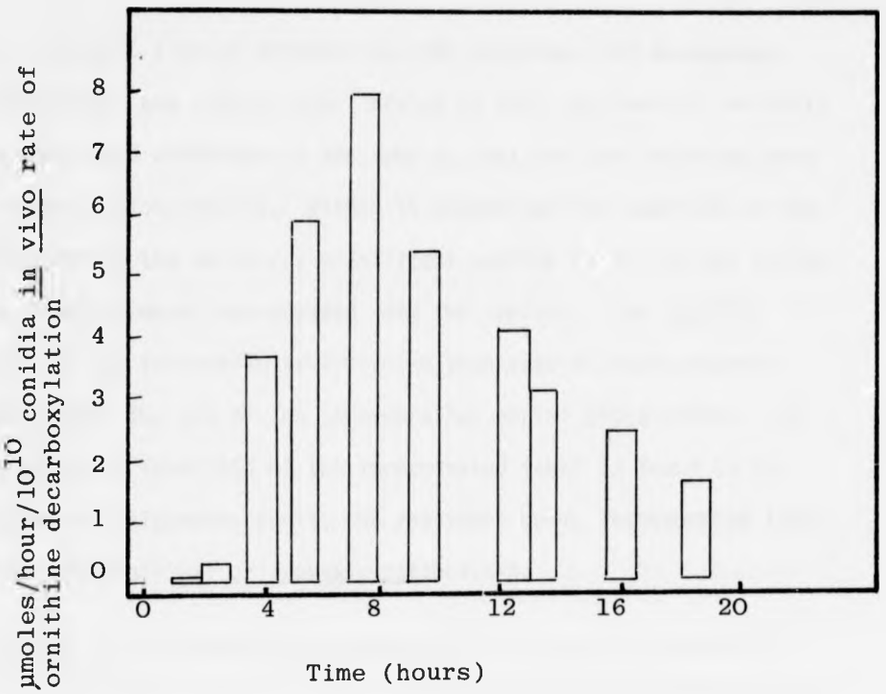


Figure 17 The rate of conversion of ornithine into oligoamines during germination (wild type)

20uCi ^{3}H-ornithine (5.0 Cu/m mole) was added to the submerged cultures 1.1 X 10^{9} conidia in 225mls, at various times and harvested one hour later. Ornithine and oligoamines were extracted, separated by paper electrophoresis and the amounts and radioactivity of each compound determined as described in materials and methods. The in vivo rate of ornithine decarbosylation was calculated from the specific activity of the intracellular ornithine and the sum of the ^{3}H-ornithine incorporated into putrescine, spermidine and spermine, assuming that the specific activity of the intracellular ornithine remains approximately constant during the one hour incorporation period.

period. It must also be assumed that the exogenous and endogenous ornithine pools are rapidly equilibrated so that the specific activity of the extracted ornithine is the same as that for the ornithine used in oligoamine biosynthesis. Within 15 minutes of the addition of the ^{3}H-ornithine to the culture, a significant portion (3-5%) of the radioactive label becomes incorporated into the conidia. The specific activity of the intracellular ornithine then remains approximately constant until the end of the incorporation period (60 minutes). In most samples at least 50% of the incorporated label is found in the ornithine and oligoamine pools, the remainder being incorporated into protein (via arginine) or probably catabolized.

The *in vivo* rate of ornithine decarboxylation (Figure 17) shows a pattern remarkably similar to the rise and fall of ornithine decarboxylase activity *in vitro* (Stevens, 1975). The decarboxylation of ornithine is low or undetectable until 4 hours and is maximal at 7 to 8 hours. The rate of decarboxylation *in vivo* then falls off, but not as rapidly as does the rate of enzyme activity detectable *in vitro*. One puzzling aspect of the ornithine decarboxylase activity is that extensive dialysis is necessary to remove some inhibitory compound present in the extracts (Stevens et al, 1976). The results presented here suggest that the "inhibitor" is not a significant controlling factor *in vivo*.

A comparison of *in vivo* and *in vitro* methods for measuring the decarboxylation of ornithine is shown in Table 2. While all three methods agree roughly in the pattern of decarboxylation in germinating conidia, it can be seen that the *in vivo* methods give a rate some five to ten times greater than that found by measuring the enzyme activity in cell free extracts. This discrepancy would suggest either that the conditions for measuring the enzyme activity *in vitro* are not fully optimised or that

A comparison of *in vivo* and *in vitro* methods for measuring the decarboxylation of ornithine in wild type strain

A The *in vivo* rate of ornithine decarboxylation as determined by the incorporation of ^{3}H-ornithine into oligoamines (see Figure 17)

Time period of incorporation	μmole/hour/10^{10} conidia
1-2 hours	0.08
3.5-4.5 hours	3.7
5-6 hours	5.9
7-8 hours	7.95
9-10 hours	5.4
13-14 hours	3.1
18-19 hours	1.55

B The *in vivo* rate of ornithine decarboxylation as determined by the actual increases in the total intracellular amount of oligoamines (from figure 15)

Time Period	μmole/hour/10^{10} conidia
2-4 hours	0.12
4-6 hours	1.08
6-8 hours	1.72
8-10 hours	2.27
10-14 hours	1.74
14-16.5 hours	1.03
16.5-19 hours	0.35

C The *in vitro* measurements of ornithine decarboxylase activity (Stevens et al, 1976)

Time	μmole/hour/10^{10} conidia
4 hours	0.004
6 hours	0.032
8 hours	0.21
10 hours	0.42
14 hours	0.16
16 hours	0.09
20 hours	0.04

the enzyme is not completely extracted from the conidia. The in vivo rate as determined by the incorporation of ^{3}H-ornithine (method A) is some three times greater than is necessary to account for the actual accumulation of oligoamines in vivo (method B). This higher rate could be due to the occurrence of turnover of oligoamines in the conidia or a lack of equilibration of the exogenous and endogenous pools of ornithine. Over 90% of the intracellular arginine and ornithine in N. crassa growing in minimal media is present in specific vesicles which exchange slowly with the cytoplasmic pools (Subramanian et al, 1973) but it is not known if this also occurs in A. nidulans. Artifacts due to the possible separation of the intracellular ornithine into two pools are not likely to be large in these experiments. The specific activity of putrescine, which is rapidly turning over, is very close to that of ornithine which would not be expected if the exogenous ornithine was preferentially used for oligoamine biosynthesis.

Macromolecular Synthesis During Germination

The previous results have amply demonstrated that oligoamine biosynthesis occurs rapidly during germination of the conidia of A. nidulans and thus could have a role in controlling other metabolic events in germination. As numerous reports have established various correlations between oligoamine metabolism and macromolecular biosynthesis (see Tabor and Tabor, 1976) it was of interest to see if such a correlation exists also within the germinating conidia of A. nidulans. In particular the rates of RNA and protein synthesis were investigated as well as the types of macromolecules produced.

Figure 19 shows the rates of protein and RNA synthesis during germination as determined by the incorporation of labelled precursors into a cold trichloroacetic acid insoluble precipitate. The rates of synthesis of

Figure 18

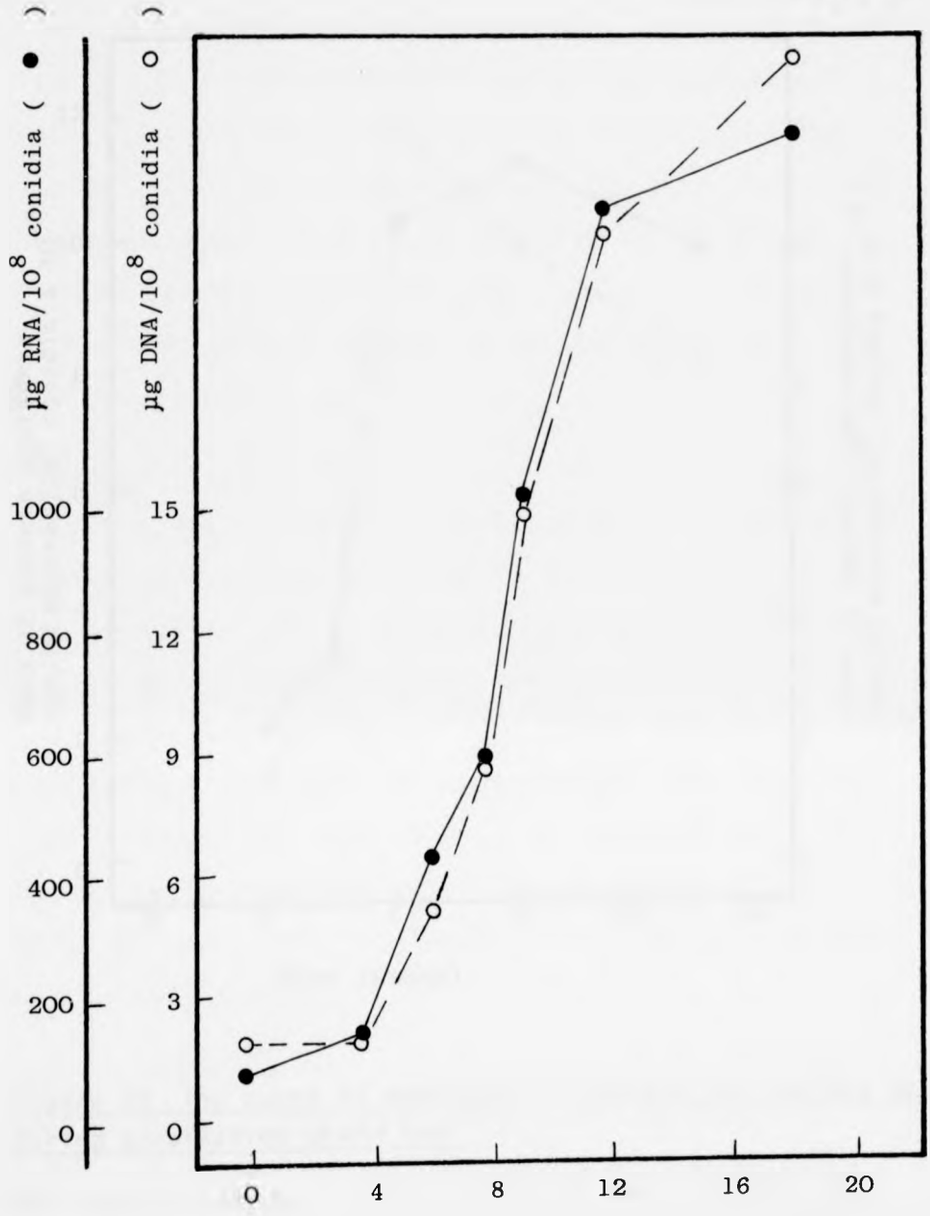


Figure 18 Nucleic acid accumulation during germination (wild type)

RNA (●) and DNA (○) were extracted and estimated as described in Materials and Methods

Figure 19

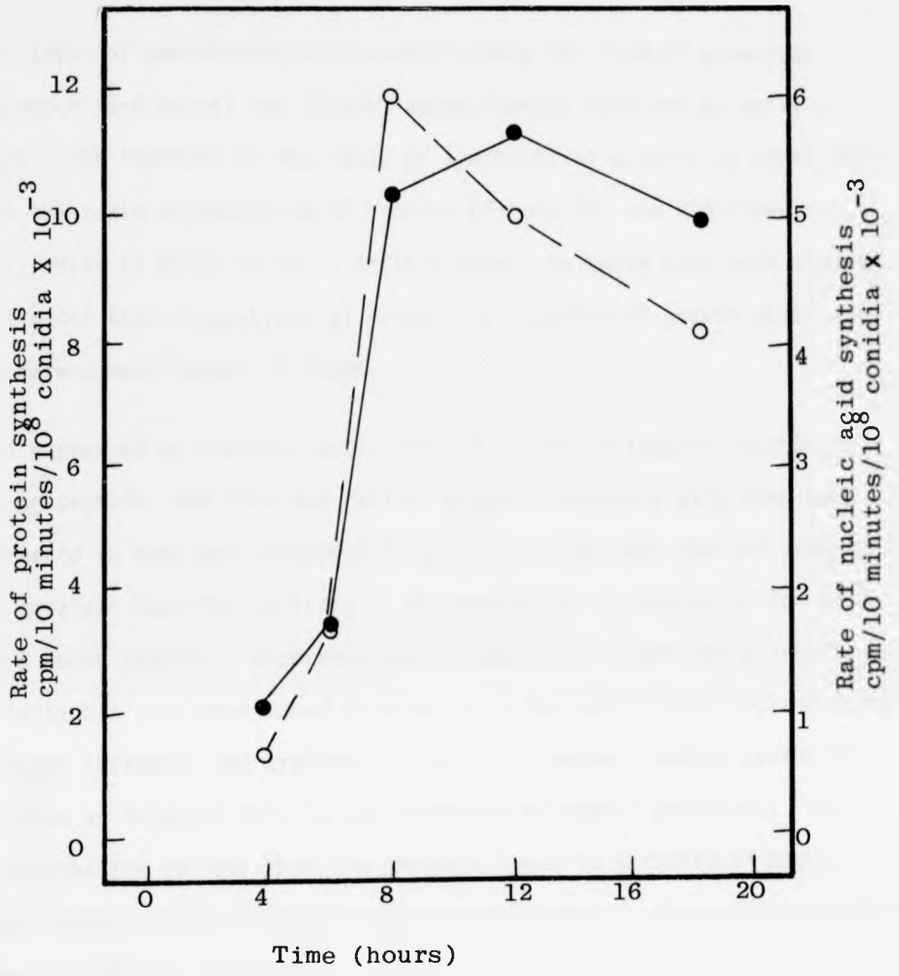


Figure 19 The rates of synthesis of protein and nucleic acid during germination of wild type

The rates of synthesis of protein (O) and RNA (●) were determined by measuring the incorporation of ^{3}H-leucine and ^{14}C-adenine, respectively into cold trichloroacetic acid insoluable material as described in Materials and Methods.

both types of macromolecules increase during the time of germ-tube emergence (6-8 hours) and remain approximately constant up until 18 hours. The increase in the rates of synthesis of protein is consistent with the rapid accumulation of protein (Figure 14) and RNA (Stevens, 1975) which is known to occur at this time. It would also seem clear that under these conditions of growth the exponential growth phase does not extend much beyond 12 hours.

When expressed as specific activities, i.e. cpm ^{3}H-leucine incorporated per ug protein, the rise and fali of protein synthesis with germ-tube emergence is even more apparent (Figure 20). For both RNA and protein the greatest specific activity of macromolecular synthesis is found in the 8 hours samples. A correlation of peak protein and RNA synthetic activity has also been found to occur with the germinating sporangiospores of Mucor (Orlowski and Sypherd, 1978b). Accelerated protein synthesis may have an integral role in the formation of hyphal germ-tubes. It is interesting to note that the greatest specific activity of macromolecular synthesis occurs at the time at which the oligoamine concentrations are highest (Table 1).

Another sensitive way to measure the protein synthetic activity of a system is to determine the number of ribosomes actively translating natural messengers. Messenger RNA species present in the cytoplasm may have many ribosomes attached, engaged in protein synthesis. The number of ribosomes which can actively translate a single messenger RNA molecule is quite large, depending partly on the size of the message as well as the availability of ribosomes. The extraction of intact polyribosome complexes (polysomes) requires delicate methods for disrupting the cells as the mRNA linking ribosomes within the polysome complex is very sensitive to mechanical shear or ribonuclease activity, Another

Figure 20

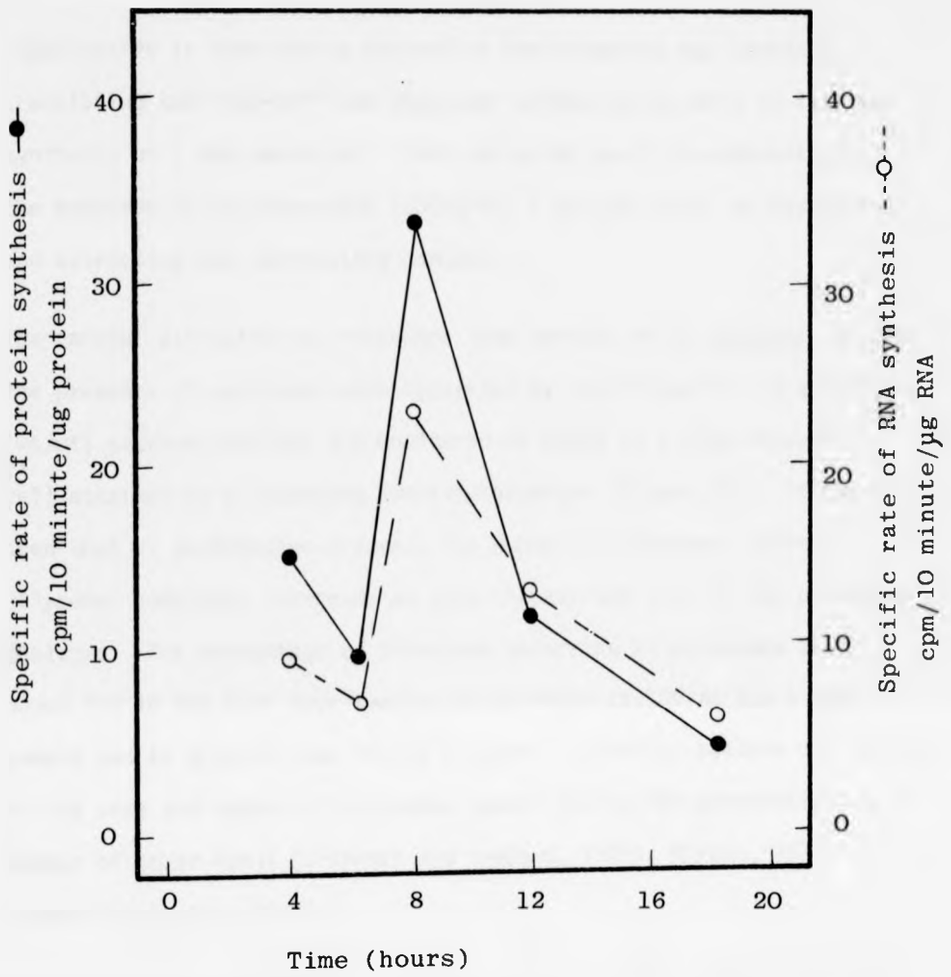


Figure 20 The specific rates of protein and nucleic acid synthesis during germination of wild type

The specific rates of synthesis were determined by the rates of incorporation (figure 18) and the amounts of protein and RNA determined as described in Materials and Methods.

complication is that during extraction the ribosomes may continue translating and "run-off" the messenger without being able to initiate synthesis on a new messenger. This can quite easily be prevented by the addition of cycloheximide (200µg/ml) 5 minutes prior to harvesting and extracting the germinating conidia.

The careful extraction of ribosomes from conidia of A. nidulans reveals the presence of polysomes when separated by centrifugation in a 10-40% (wt/wt) sucrose gradient and monitored at 260nm in a flow-through cell attached to a recording spectrophotometer (Figure 21). It can be seen that as germination proceeds, the number of ribosomes found in polysomal complexes increases as does the average size of the polysomes isolated. The percentage of ribosomes occurring as polysomes is only about 15% in the zero hour sample, which rises to 70% in the 4 hour sample and to greater than 90% by 8 hours. A similar pattern of increase in the size and number of polysomes occurs during the germination of a number of other fungi (Orlowski and Sypherd, 1978b; Mirkes, 1974; Leaver and Lovett, 1974).

The existence of polysomes in dormant spores would be evidence for the existence of preformed messengers which could direct protein synthesis during the early stages of germination. However the occurrence of polysomes in the zero hours sample (Figure 21,A) does not prove that polysomes are also present in the dormant conidia. This is because the conidia are harvested by an aqueous technique that may hydrate the conidia and allow some of the earliest events of germination to occur before the ribosomes are extracted. Indeed Mirkes (1974) found that dry harvested conidia of N. crassa had less than 3% of its ribosomes in polysomes compared to 25% found in the wet harvested spores. In both Blastocladiella emersonii and N. crassa over 50% of the ribosomes

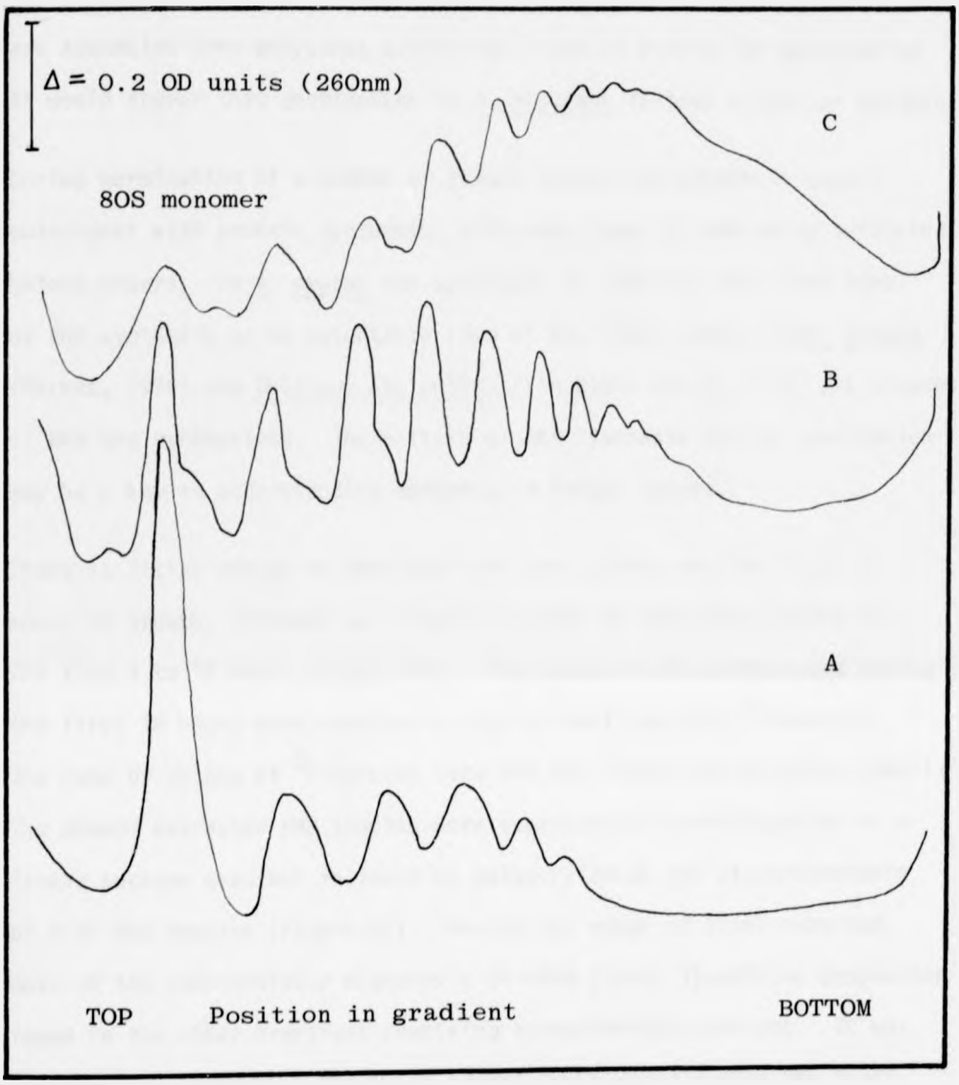


Figure 21 Ribosome distribution in conidia during germination of wild type

Conidia were harvested at 0 hours (A), 4 hours (B) or 8 hours (C) and the polysomes extracted by grinding with sand, fractionated by centrifugation, the contents displaced by 50% (wt/wt) sucrose and continuously monitored at 260nm as described in Materials and Methods.

are assembled into polysomes within the first 20 minutes of germination It would appear that germination in A. nidulans follows a similar pattern.

During germination of a number of fungal spores RNA synthesis occurs coincident with protein synthesis, with some types of RNA being initiated before others. In A. oryzae the synthesis of rRNA was the first type of RNA synthesis to be detectable (Ono et al, 1966), while in N. crassa (Mirkes, 1974) and Rhizopus stolonifer (Van Etten et al, 1976) all classes of RNA are synthesized. The pattern of RNA synthesis during germination may be a key to understanding dormancy in fungal spores.

There is little change in the total RNA per conidia for the first 4 hours of growth, followed by a rapid increase in the total amount of RNA from 4 to 16 hours (Figure 18). The types of RNA synthesized during the first 16 hours were examined by pulse-labelling with ^{3}H-adenine. The rate of uptake of ^{3}H-adenine into RNA was linear during pulse-labelling. The phenol extracted RNA samples were separated by centrifugation in a linear sucrose gradient followed by polyacrylamide gel electrophoresis of 4-5S RNA species (Figure 22). During the range of times examined, most of the radioactivity migrates with rRNA (Table 3) with the proportion found in the other fractions remaining approximately constant. It was not possible to measure the types of RNA synthesized during the first 3 hours of germination due to the lack of ^{3}H-adenine uptake and incorporation. The radioactivity in the fraction labelled "remainder" would be expected to include some DNA, precursors of rRNA and messenger RNA species lacking polyadenylated tracts.

The results presented in Table 3 also show that the conidia of A. nidulans contain polyadenylated RNA species. Messenger RNA species containing poly(A) tracts have been found in a wide variety of eukaryotes, including fungi (see Van Etten et al, 1976).

Figure 22

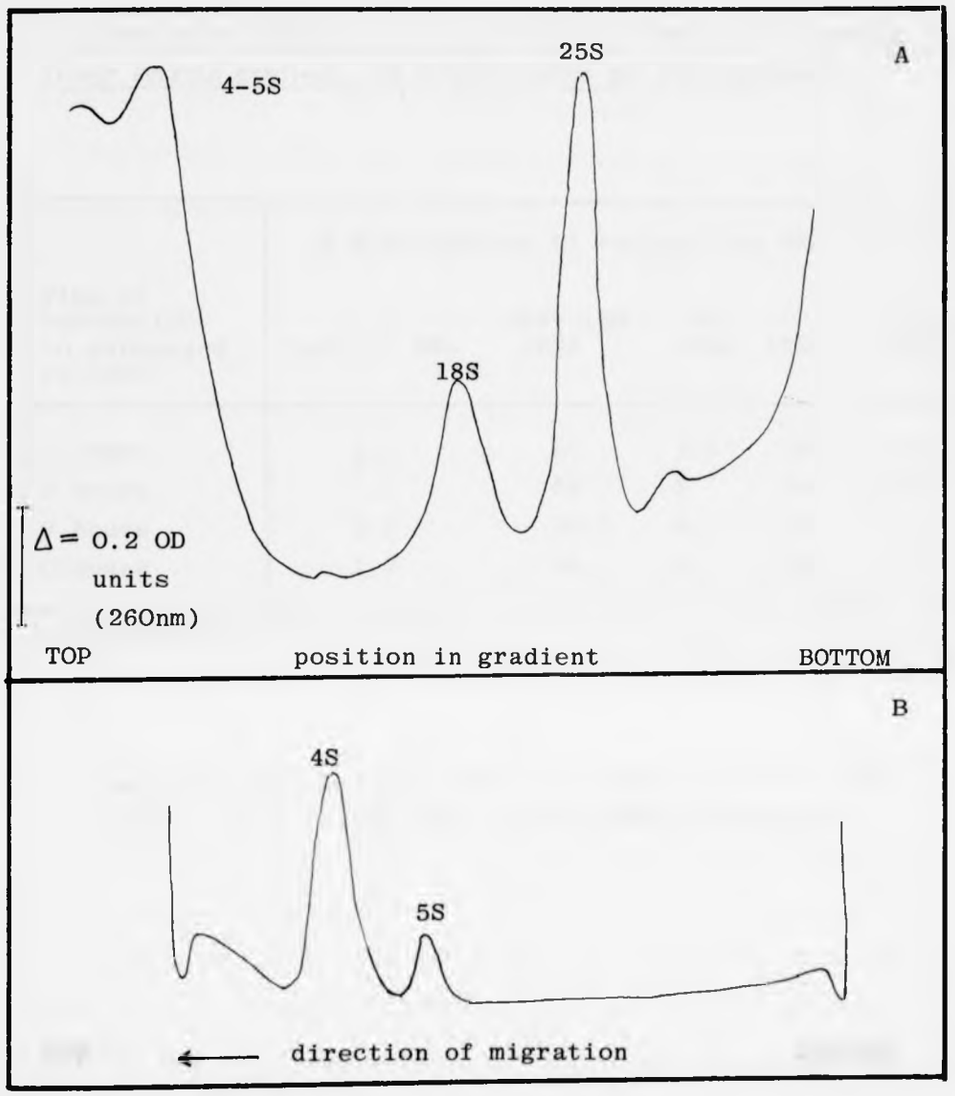


Figure 22 Typical separation of RNA from germinating conidia of A. nidulans wild type

RNA was extracted from conidia and the poly (A) containing RNA separated by oligo (dT) cellulose chromatography. The poly (A) lacking RNA was then separated by centrifugation in a 7-30% (w/v) linear sucrose gradient, monitored at 260mn and fractions collected A The fractions containing the 4-5S RNA were then concentrated by precipitation with ethanol and separated using 15% polyacrylamide gels,B.

Table 3

Distribution of radioactivity in pulse-labelled RNA after separation by linear sucrose gradients and polyacrylamide gel electrophoresis

Time of incubation in submerged culture†	% Distribution of radioactive RNA *				
	poly A^+ RNA	25S+ 18S rRNA	5S rRNA	tRNA	remainder
3 hours	2.1	64	3.8	19	11
6 hours	1.3	58	3	24	12
9 hours	1.5	70.5	4	17	7
12 hours	1.2	68	2	19	11

† wild type strain

* "remainder" refers to radioactivity not binding to an oligo (dT) cellulose column or coincident with the rRNA and tRNA peaks

RNA Polymerase activities

A partial purification of RNA polymerase from germinating conidia has been achieved (Table 4) and RNA polymerase activities separated into three peaks on phosphocellulose (Figure 23). Although the three peaks were not sufficiently purified to distinguish on polyacrilamide gels some differences in their properties were found which suggests they are distinct enzymes. All three activities were shown to be DNA dependent, sensitive to actinomycin D and inhibited by pyrophosphate (Table 5), but they show different Mn^{++} optima and the third peak showed a slight sensitivity to α-amanitin. The α-amanitin sensitivity is similar to the pattern observed with other fungal RNA polymerases (Young and Whiteley, 1975; Pong and Loomis, 1973; Gong and Van Etten, 1972).

The total RNA polymerase activity increases very rapidly at the onset of germination and continues to increase during apical growth of the hyphae (Table 6). The specific activity of the enzyme however only increases in the first 6 hours; thus after 6 hours the increase is only in parallel with the general increase in protein synthesis which is occurring. All three peaks of RNA polymerase activity could be detected between 4 and 17 hours. If a high conidial density (greater than 2.5×10^7/ml) was used to obtain 14 and 17 hours mycelial extracts then only one RNA polymerase peak was detected on the phosphocellulose columns. Under these conditions growth may be restricted by 14 and 17 hours through depletion of nutrient or oxygen. In the fungus Histoplasma capsulatum only one RNA polymerase was detectable during mycelial growth whereas three can be detected during the yeast growth phase (Boguslawski et al, 1974). This is due to an RNA polymerase inhibitor present in the mycelial phase. It is possible that a similar inhibitor might account for only one RNA polymerase being detectable at the higher mycelial densities.

Purification of RNA polymerase from extracts of 10 hour germinating conidia (wild type)

Fraction	Recovery[1]	Purification[2]
Crude extract	100%	1
Protamine sulphate	39%	2.3
DEAE-cellulose	63%	5.4
Sephadex G50	65%	6.1
Phosphocellulose Peak I		25
" Peak II	23%	27
" Peak III		31

1. Recoveries are expressed as a % of the total activity in the crude extract
2. The purifications are relative to that of the crude extract which has a specific activity of 316000cpm/30min/100mg

Figure 23a

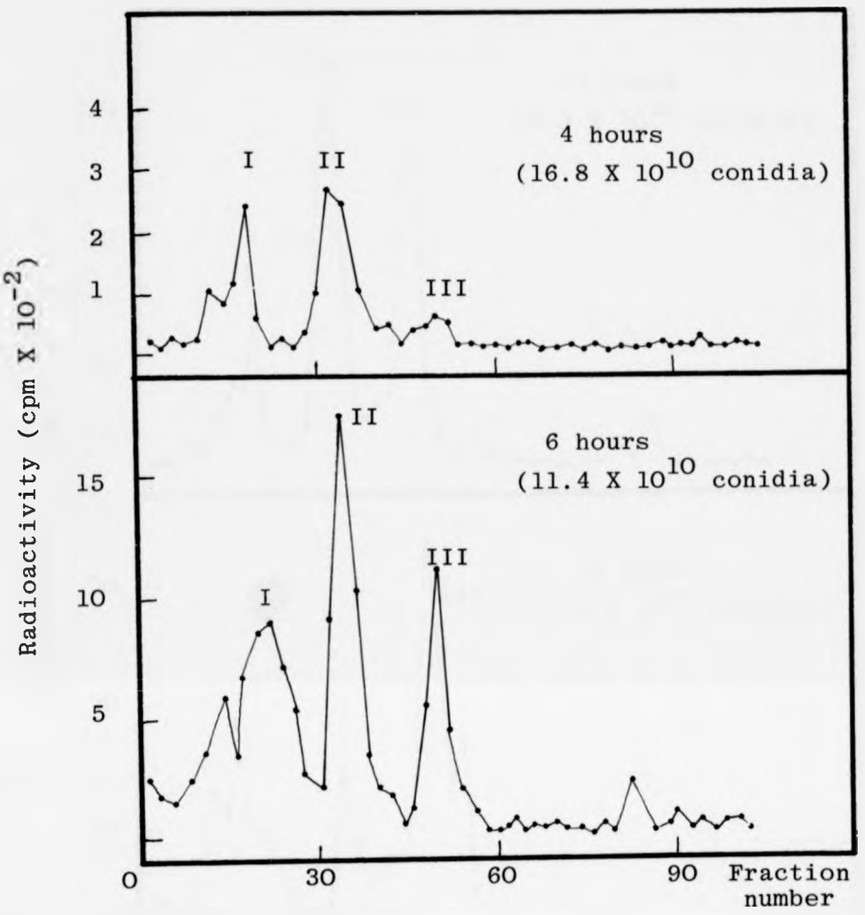


Figure 23a Separation of RNA polymerase activities from germinating conidia of Aspergillus nidulans by phosphocellulose chromatography (wild type)

RNA polymerase activity was extracted from conidia grown for the times indicated, purified and separated as described in Materials and Methods.

Figure 23b

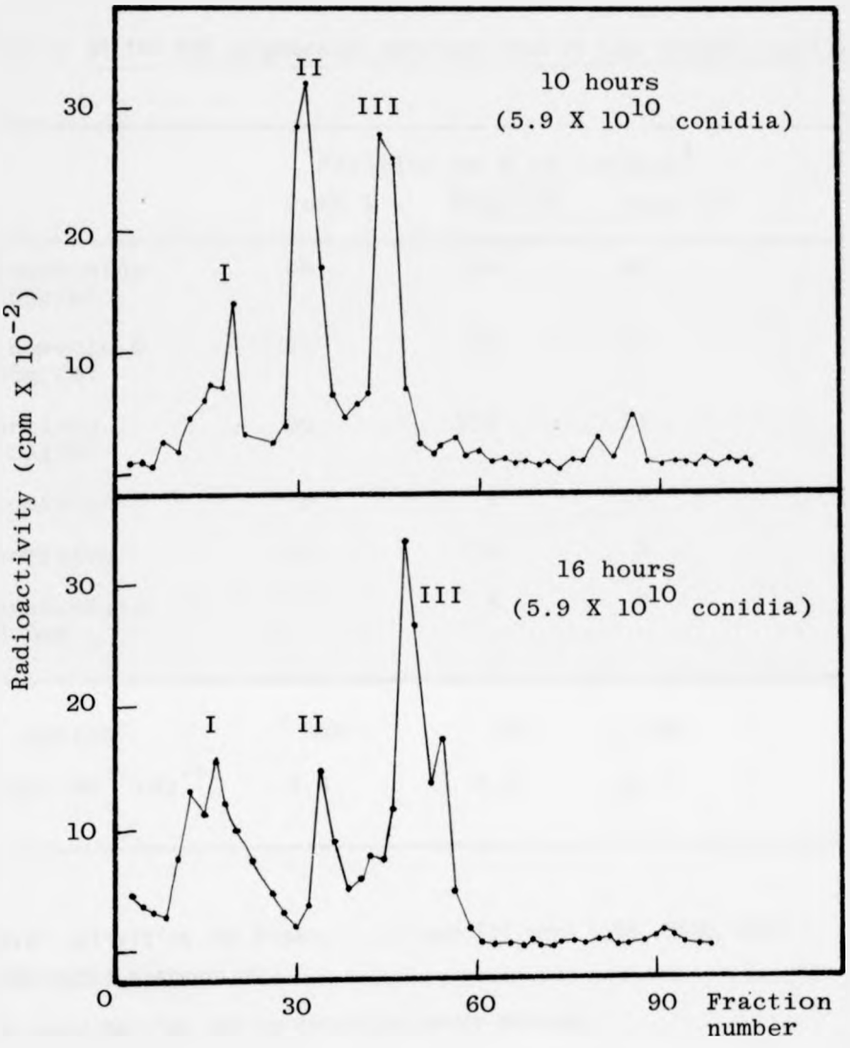


Figure 23b Separation of RNA polymerase activities from germinating conidia of Aspergillus nidulans by phosphocellulose chromatography (wild type)

RNA polymerase activity was extracted from conidia grown for the time indicated, purified and separated as described in Materials and Methods.

Table 5

Properties of the RNA polymerases obtained from 10 hour conidia (wild type)

	Activity as % of control[1]		
	Peak I	Peak II	Peak III
Cycloheximide 125µg/ml	98	99	98
Actinomycin D 125µg/ml	22	26	43
∝-Amanitin 2.5µg/ml	96	101	83
DNA omitted	5	1	8
ATP omitted	12	19	3
Pyrophosphate 12.5mM	7	8	6
Mn^{++} optima	3mM	2mM	5mM
Optimal Mn^{++}/Mg^{++}	2.4	3.9	3.5

[1]Control activities for Peaks, I, II and III were 1358, 3640, 2621 cpm/30minutes respectively.

Assays were carried out as described under Methods.

Total RNA polymerase activity during conidial germination of wild type

Time	Total activity cpm/30min/10^{10} conidia	specific activity cpm/30min/100mg protein
4 hours	64,000	1.07×10^5
6 hours	343,000	3.40×10^5
10 hours	1,050,000	3.16×10^5
17 hours	7,820,000	3.38×10^5

Activity was measured as described under Methods

Discussion on Germination in Wild Type A. nidulans

Biochemical evidence increasingly supports the concept that lower eukaryotes such as Aspergillus resemble higher eukaryotes rather than prokaryotes in their mode of gene expression. The relative distribution of the naturally occurring oligoamines and enzymatic processes of oligoamine biosynthesis in A. nidulans are very similar to those found in higher animal eukaryotes. The S-adenosylmethionine decarboxylase activity in extracts of A. nidulans is stimulated by the addition of putrescine and has other properties, kinetic and regulatory, similar to those of the animal and yeast cell enzymes. The genetic material of A. nidulans is complexed with histones in a nucleosome structure (Morris, 1976c) and transcription involves a multiplicity of RNA polymerase activities (Figure 23). As A. nidulans is easy to handle experimentally and has been the subject of extensive genetic analysis it has certain advantages as a model system in which to study the functions of oligoamines in cellular metabolism and other aspects of the molecular basis of gene control in eukaryotes.

The S-adenosylmethionine decarboxylase activities of A. nidulans and yeast are both stimulated over fifty-fold by the addition of putrescine. Hart et al, (1978) found a large variation in the extent of stimulation of enzyme activity by putrescine ranging from four-fold to 350-fold increases. The measurement of the extent of enzyme stimulation in crude extracts is subject to a variety of influences. Extensive dialysis is required to remove oligoamines passively bound to proteins in the cell extract and other enzymes may be present catalyzing competing or inhibiting reactions.

Nevertheless it is clearly the case that the stimulation of SAM

decarboxylase activities by putrescine is of a significantly greater magnitude in fungi than in animal cells where only two-fold to ten-fold increases are commonly observed.

The pattern of oligoamines present within A. nidulans is typical of that found in higher animal eukaryotes in which spermidine is normally the predominant oligoamine, spermine is also present in relatively large amounts and the putrescine levels remain low. This is consistent with the suggestion of Pösö et al, (1975) that in general, organisms capable of spermine synthesis possess putrescine activated S-adenosylmethionine decarboxylase activities. The enzymology and oligoamine content of P. polycephalum, D. discoideum and B. emersonii are quite different from other fungi and appear more similar to bacteria. The reason for this is unknown, though it may reflect the wide phylogenetic separation of these organisms from most fungi. The high putrescine content may reflect a role in osmotic protection of membrane stabilization quite distinct from the role of oligoamines in macromolecular synthesis.

Investigations of the process of germination in a variety of fungal species have shown that a great many events take place prior to germ-tube emergence. In A. nidulans there is a marked swelling of the conidial spores and an increased respiratory metabolism detectable in the early stages of germination. The quantities of protein, RNA, oligoamines and ornithine increase several fold prior to germ-tube emergence and apical growth. The dramatic increases in oligoamine synthesis and accumulation in germination are similar to the events occurring in other systems during proliferative growth. Thus the study of germination would appear to have parallels to embryonic and tumour cell growth and tissue regeneration as the cells all grow at rapid rates, presumably with optimal utilization of the components of macromolecular synthesis. These systems are all characterized by the rapid accumulation of oligoamines (Jänne et al, 1978;

Stevens and Winther, 1978) suggesting that the molecular basis of rapid growth has certain universal features.

The increases in the rates of ornithine decarboxylase (Stevens, 1975) and S-adenosylmethionine decarboxylase activities during germination are approximately one hundred-fold while the increase in the amount of oligoamines is somewhat less at about twenty-fold. However an excess capacity of oligoamine biosynthesizing enzymes does not necessarily occur. The calculated rates of *in vivo* ornithine decarboxylation are three to five times as great as the enzyme activities measured *in vitro*. Results from the incorporation of ^{3}H-ornithine into putrescine, spermidine and spermine are consistent with only one pathway of oligoamine biosynthesis occurring within the cell and with a relatively slow turnover of oligoamines. The rise and fall of ornithine decarboxylation *in vivo* parallels the measured enzyme activities, suggesting that the pattern of changing activity in oligoamine biosynthetic enzymes is not simply an artifact of the extraction process.

Increases in ornithine decarboxylase activity occur during the cell cycle, often preceding and following the period of DNA synthesis or S phase (see Stevens and Winther, 1978). The partially synchronous germination of *A. nidulans* conidia can also be viewed as a cell cycle process with the dormant conidia present in the G_1 phase and moving through S phase 6 to 8 hours after inoculation. The peak in ornithine decarboxylase at this time may be associated with cell cycle changes in cellular metabolism rather than specific processes unique to germination. The synchrony of nuclear division does not extend much after the second nuclear division so subsequent peaks in ornithine decarboxylase activity would be diffuse and so less prominant. Possibly more synchronized cultures of germinating conidia would have multiple peaks of ornithine decarboxylase activity. It seems that most studies of germination have neglected to consider

parallels to cyclical changes normally occurring within the cell and more such comparisons would help to identify unique events of the germination process.

The massive increase in the intracellular concentration of ornithine during germination is probably necessary to support the increase in oligoamine biosynthesis at this time. If the ornithine supply were not continually resupplied then the reserves would be depleted within a few hours. The biosynthesis of ornithine could be an important control point in regulating the production of spermine, spermidine and putrescine. Such a control could not operate during the later stages of mycelial growth when the necessity for oligoamine biosynthesis is less. The affinity of ornithine decarboxylase for ornithine is considerably greater than is found for the other ornithine metabolizing enzymes (see Stevens and Winther, 1978) ensuring the continuation of oligoamine biosynthesis even during restricted ornithine levels. The availability of ornithine may also be affected by the subcellular localization of this amino acid as has been found in N. crassa (Bowman and Davis, 1977). There is no evidence for any such sequestering of ornithine in cytoplasmic vescicles in A. nidulans and the results from monitoring ^{3}H-ornithine incorporation into oligoamines are consistent with only a single pool of functional or available ornithine being present in the cell.

The ratio of oligoamines to RNA or protein amounts increases with germination as oligoamine biosynthesis is more rapid than nucleic acid biosynthesis. The highest ratio is reached between 6 and 12 hours when the growth rate is fastest. A high oligoamine:RNA ratio is also found in A. nidulans grown at a fast rate in continuous culture (Bushell and Bull, 1974). At the maximum intracellular concentrations the sum total of the oligoamine nitrogens is sufficient to neutralize 15% to 20% of the nucleic acid phosphates. Although the precise intracellular distributions are unknown,

binding studies suggest that oligoamines may be a significant counter ion for nucleic acids (see Stevens and Winther, 1978).

RNA accumulates most rapidly in the germinating conidia of A. nidulans between 6 and 14 hours after the initiation of germination. The proportion of ^{3}H-adenine incorporated into the various classes of RNA does not change markedly between 3 and 19 hours. Because of the low rate of RNA synthesis during the first few hours of germination the distribution of incorporated radioactivity was not determined. During the first hour of germination in spores of B. theobromae (Knight and Van Etten, 1976) Peronospora tabacina (Hollomon, 1970) and Aspergillus oryzae (Ono et al, 1966) the sequential initiation of RNA synthesis has been observed. It remains possible that in the first hour of germination of the conidia of A. nidulans initiation of synthesis of the various classes of RNA begins sequentially. However as the amounts of RNA synthesized at this time are very small it is uncertain if this is of regulatory significance. The synthesis of one limiting specific class of RNA may not be the key to the initiation of germination as evidence suggests that dormant spores may contain all of the components necessary for protein synthesis (see Lovett, 1976).

The polyadenylated RNA found in A. nidulans has some of the properties associated with mRNA species, including a heterogeneous size distribution (7-20S) and a rapid rate of turnover. Polyadenylated RNA has been found in a number of fungal species and in at least one case has been shown to support portein synthesis in vitro (Wenzler and Brambl, 1978). The proportion of poly (A) containing RNA synthesized remains approximately constant in germinating conidia of A. nidulans. This is in marked contrast to germination of Botryodiplodia theobromae where the majority of RNA synthesized is presumptive mRNA (Knight and Van Etten, 1976).

The increases in incorporation of ^{3}H-adenine into RNA are matched by increases in RNA polymerase activity. In common with many other fungi (Van Etten et al, 1976; Young and Whiteley, 1975; Pong and Loomis 1973; Gornicki et al 1974) A. nidulans has multiple RNA polymerases. It is difficult to assign functions to these various polymerases for a number of reasons. In higher eukaryotes three classes of polymerases are recognized (Chambon, 1974) with class A insensitive to ∝-amanitin, class B sensitive to low concentration of ∝-amanitin (0.001-0.01µg/ml) and class C sensitive to high concentrations of ∝-amanitin (10-100µg/ml). There is good evidence that RNA polymerase class B is largely reponsible for hnRNA and mRNA synthesis (Chambon 1974). In fungi, however, there is no polymerase activity as sensitive to ∝-amanitin as RNA polymerase B. Levels of 1-33µg/ml ∝-amanitin are required for inhibition (Young and Whiteley, 1975; Pong and loomis, 1973; Gornicki et al 1974; Tellez de Inon et al 1974; Gong and Van Etten 1972) which makes them closer to RNA polymerase C in character. Also it has not been possible to definitely locate the fungal RNA polymerases in specific regions of the nucleus as has been shown with higher eukaryotes (Chambon 1974), although there is an enrichment of ∝-amanitin resistant RNA polymerase activity in nucleolar fractions obtained from Physarum polycephalum (Grant 1972; Hildebrandt and Sauer 1973). A method has recently been described for the isolation of nuclei of A. nidulans (Gealt et al, 1976) and thus isolation of nucleoli may be possible in future.

Bainbridge (1971) found that an increased amount of RNA was detectable within 30 minutes of the initiation of germination while the amount of protein did not significantly increase until 150 minutes. If RNA synthesis were to precede protein synthesis during the initiation of germination then the dormant spore must contain preformed RNA polymerases. Three RNA polymerase activities are detectable at an early stage of germ-

ination in A. nidulans and are maintained at approximately constant proportions during germ-tube emergence and the early exponential growth phase. The total extractable RNA polymerase activity increases dramatically prior to germ-tube emergence, suggesting that synthesis of the RNA polymerase protein molecules is an important event contributing to the increased rate of macromolecular synthesis in germination.

The process of germination in the conidia of A. nidulans and a number of other fungal species involves two distinct stages. The dry, dormant spores can maintain viability for long periods of time by reducing their metabolic rates to the barest minimum, sufficient to maintain vital repair and maintenance mechanisms. To this end the conidia require only a basal level of machinery for macromolecular synthesis. However once dormancy has been broken and germination initiated it is to the selective advantage of the spore to produce a germ-tube and grow rapidly as long as environmental conditions remain suitable. This can best be achieved by having the machinery of macromolecular synthesis already present in the conidia in an inactive state which can readily be "switched on". Thus the earliest stages of germination must involve the activation of pre-existing systems while the later stages require the synthesis of new material for protein and nucleic acid synthesis. It can be calculated that the number of ribosomes increases three-fold prior to germ-tube formation in the germinating conidia of A. nidulans. The mechanism(s) for breaking dormancy, which could result from the sequestering of mRNA or some other component of protein synthesis, self-inhibitors, or simply nutrient depletion, are probably independent of the mechanisms which increase all of the components of macromolecular synthesis prior to and during germ-tube emergence. The later mechanisms are concerned with the overall control of growth rate and are not unique to the germination process.

Germination is an advantageous system in which to study the biochemical machinery associated with a rapid growth rate. The specific activities of nucleic acid and protein synthesis are greatest during germ-tube emergence (Figure 20). This may simply be due to the fact that in mycelial fungi only the hyphal tip contributes to growth and in freshly germinated spores the hyphal tip constitutes a greater percentage of the total fungal mass than it does at any other time. Additionally in batch cultures grown at relatively high spore densities at 37^{o}C, "exponential growth" becomes limited soon after germination and does not extend much beyond 16 hours. The increase in oligoamines in germinating conidia may reflect the need for a high oligoamine/RNA ratio during fast growth as occurs in A. nidulans growing rapidly in continuous culture (Bushell and Bull, 1974) rather than a germination specific event. As the peak rate of oligoamine biosynthesis coincides with the maximum rates of macromolecular synthesis as well as being near the time of germ-tube emergence it is not possible to distinguish between the two hypothesis on the basis of these experiments alone. Possibly growth at a lower temperature (25^{o}C) may help to lengthen the period of unrestricted growth and might result in different patterns of oligoamine accumulation. Alternatively the normal pattern of germination may be perturbed by metabolic inhibitors and in nutrient deprived auxotrophic mutants.

B Perturbations in the Pattern of Germination brought about by Inhibitors, by Nutrient Depletion in Auxotrophs and in Temperature-Sensitive Mutants

Having gained an understanding of some of the biochemical processes occurring during germination in A. nidulans, it becomes possible to analyse the contribution each makes towards the completion of germ-tube emergence. Germination is a complex process involving changes in most all aspects of cellular metabolism. A large number of enzyme activities increase in germination though not all of these are essential to the process of germ-tube emergence. For example, nicotinamide adenine dinucleotide (phosphate) glycohydrolase, which increases in activity in germinating conidia of N. crassa has been shown to be dispensible as mutants lacking this enzyme are still able to germinate normally (Nelson et al, 1975). The inability of many nutrient depleted auxotrophic mutants to produce germ-tubes suggests that the genetic defects do occur in functions essential for germination. Thus mutations at the puA locus in A. nidulans prevent germination in the absence of putrescine, suggesting that oligo-amines have an important role to play in this process. The role of essential non-nutritional biochemical functions, such as nuclear replication, in germination can also be studied with the aid of termperature-sensitive mutants.

(i) Inhibitors

The discovery of a number of compounds which can specifically affect a limited number of metabolic processes has led to a deeper understanding of control mechanisms, developmental processes and the interrelatedness of cellular metabolic pathways. However metabolic inhibitors may have unpredictable side effects in certain systems but not in others. It cannot always be assumed that an inhibitor will be specific for the

same *in vivo* function in every organism in which it is used. Additionally, the inability of an inhibitor to affect some function *in vivo* may simply be due to the lack of uptake.

Inhibition of Protein Synthesis

The germination of fungal spores has been found to require protein synthesis in every species tested (see Van Etten et al, 1976). This has been determined largely on the basis of the ability of cycloheximide to inhibit the germination process. Cycloheximide is a commonly used inhibitor of protein synthesis which interferes with the normal functioning of the ribosomes (Siegal and Sisler, 1964). Even so it is not without its reported side effects. Fiala and Davis (1965) found evidence that the synthesis and methylation of rRNA was also affected in *N. crassa*. In *A. nidulans* Cybis and Weglenski (1972) were able to inhibit the induction of arginase by low levels of cycloheximide (5µg/ml). However larger quantities are required to inhibit total protein synthesis for any length of time. 5µg/ml cycloheximide delays germination by about 2 hours (Figure 24). This rise in the activity of SAM decarboxylase is delayed by a similar amount of time. Stevens et al, (1976) found that the peak of ornithine decarboxylase was also delayed 2 hours by the addition of cycloheximide to the germination media. Thus this organism is capable of overcoming the block presented by the antibiotic. The fact that germination and oligoamine biosynthesis are delayed by similar times is evidence for a correlation between the two events. It is also clear that some protein synthesis is necessary for the complete germination of the conidia of *A. nidulans*.

Figure 24

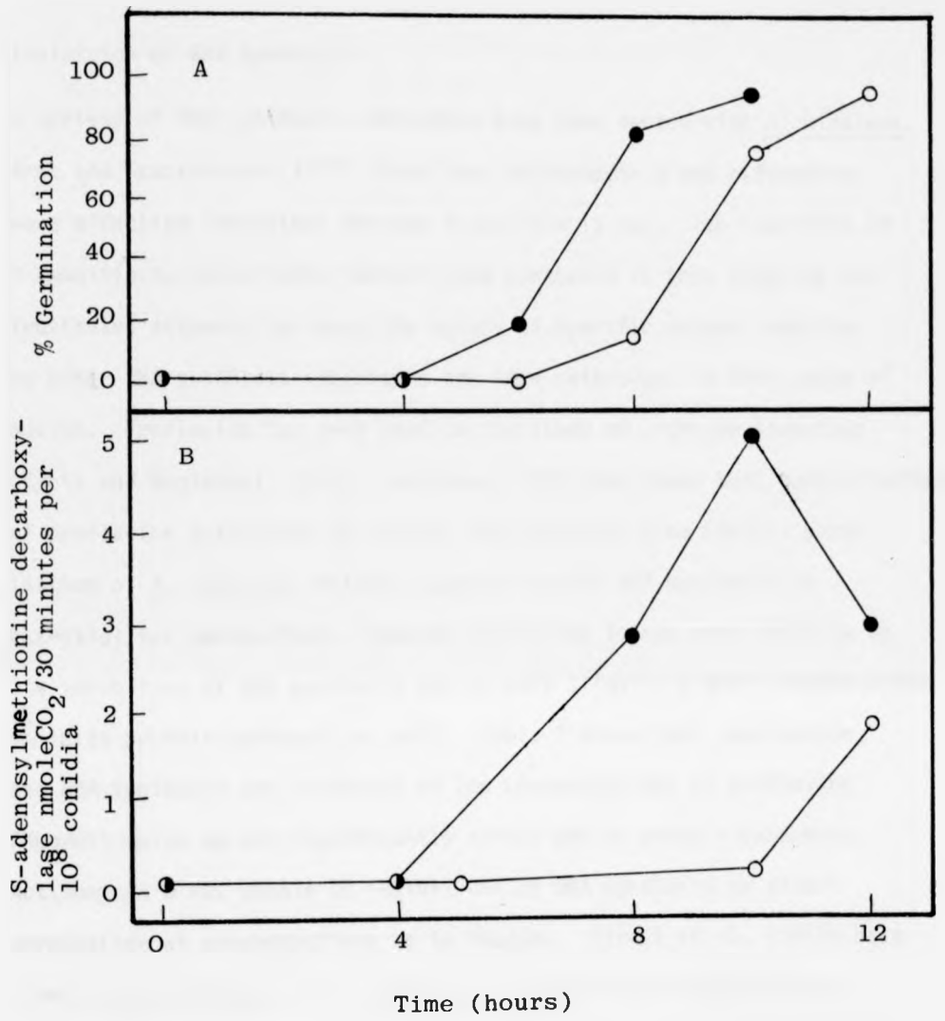


Figure 24 Effect of cycloheximide on germination and development of S-adenosylmethionine decarboxylase activity of wild type

A. The percentage of conidia bearing germ-tubes and B. S-adenosylmethionine decarboxylase activity were measured in the presence (○) and absence (●) of cycloheximide as described in Materials and Methods. Cycloheximide (5µg/ml) was added at the time of inoculation.

Inhibition of RNA Synthesis

A variety of RNA synthesis inhibitors have been tested with A. nidulans. Arst and Scazzocchio (1972) found that actinomycin D and rifampicin were effective inhibitors whereas ∝-amanitin is not. The inability of ∝-amanitin to selectively inhibit mRNA synthesis in this organism has frustrated attempts to study the nature of specific enzyme induction as other RNA synthesis inhibitors are less selective in their mode of action. Proflavine has been used in the study of arginase induction (Cybis and Weglenski, 1972). Hollomon (1970) has shown that concentrations of proflavine sufficient to inhibit RNA synthesis also inhibit germination of A. nidulans conidia, suggesting that RNA synthesis is essential for germination. However proflavine is not very specific in its inhibition of RNA synthesis and at only slightly higher concentrations inhibits protein synthesis as well. Table 7 shows that germination and RNA synthesis are inhibited at low concentrations of proflavine (3µg/ml) which do not significantly affect DNA or protein synthesis. Actinomycin D was unable to inhibit RNA or DNA synthesis or affect germination at concentrations up to 30µg/ml. Firtel et al, (1973) have shown that actinomycin D is unable to inhibit mRNA synthesis in D. discoideum even at very high concentrations. It can be concluded from the results presented in Table 7 and from other research (Hollomon, 1970) that conidial germination in A. nidulans requires some RNA synthesis.

Inhibition of DNA Synthesis

The accumulation of DNA during germination occurs in a number of fungal species. As the increase in this nucleic acid occurs prior to or at about the same time as germ-tube emergence it is unclear as to whether DNA was essential for germination. Many researchers have utilized various inhibitors of DNA synthesis to see if the blockage of DNA synthesis prevents germination. Van Etten et al (1976) tried a number of DNA

Table 7

The effects of proflavine and actinomycin D on Germination and macromolecular synthesis (wild type)

Addition to Growth Media	% Germination	Rates of synthesis as a percentage of control	
		RNA	DNA
Control	95	100	100
Proflavine			
3 µg/ml	30	43	98
10 µg/ml	10	20	71
30 µg/ml	1	11	12
Actinomycin D			
10 µg/ml	95	104	112
30 µg/ml	90	92	101

The inhibitors were added to the growth media at 4 hours and germ-tube emergence determined at 8 hours. RNA and DNA synthetic rates were determined at 6 hours by the incorporation of ^{3}H-adenine and ^{14}C-leucine, respectively, as described in Materials and Methods. The control values for RNA and DNA synthesis were 14,300 cpm and 1,820 cpm respectively.

synthesis inhibitors on germination in R. stolonifer and Botryodiplodia theobromae including hydroxyurea, mitomycin C, 6-fluorodeoxyuridine, adenine arabinoside and cytosine arabinoside but only nalidixic acid and 5-fluorouracil affects the rate of extent of spore germination. However these experiments did not determine the specificity of inhibition of DNA synthesis or if the inhibitor was taken up from the media, though it was tentatively concluded that DNA synthesis is not essential for germ-tube emergence.

The percentage of conidia showing visible germ-tubes and the percent increase in DNA during germination in the presence of hydroxyurea, nalidixic acid and 5-fluorouracil are shown in Table 8. In general it can be seen that for all three inhibitors over a wide range of concentrations there is a decrease in germination coincident with the decrease in the amount of DNA synthesis. Conidial germination is extremely sensitive to 5-fluorouracil but as well as inhibiting DNA synthesis this compound can cause errors in transcription and translation and thus affect the fidelity of protein synthesis (Bellisario et al, 1976).

Hydroxyurea inhibits DNA synthesis by interfering with the synthesis of deoxyribonucleotides and at higher concentrations interferes with RNA synthesis. Relatively high concentrations are required to inhibit germination (Table 8) which is accompanyed by a parallel decrease in the extent of DNA accumulation. Nalidixic acid is effective at lower concentrations and is also able to preferentially inhibit DNA synthesis. Thus at 10µg/ml 26% of the conidia can produce germ-tubes in the absence of detectable DNA synthesis and accumulation. This is in contrast to the parallel inhibition of DNA synthesis and germination which is observed with 5-fluorouracil and hydroxyurea. The effect of these latter two inhibitors is probably explained by the hypothesis that the germination

The inhibition of DNA synthesis and germination by hydroxyurea, nalidixic acid and 5-fluorouracil

Experiment I Inhibitor	% Germination	% increase of DNA ($\Delta T/To \times 100\%$)
Control	30	166
Hydroxyurea		
30 µg/ml	21	32
100 "	19	14
300 "	29	2
1000 "	13	16
Nalidixic acid		
3 µg/ml	24	13
10 "	26	0
30 "	23	4
100 "	13	0
300 "	7	0
Experiment II		
Control	90	190
Hydroxyurea		
30 µg/ml	62	188
100 "	59	130
300 "	48	122
1000 "	15	52
3000 "	1	0
5-Fluorouracil		
1 ng/ml	90	185
3 "	69	129
10 "	30	57
30 "	3	12
100 "	1	3

Germ-tube emergence and DNA were measured 7 hours (Exp I) or 8 hours (Exp II) after inoculation into media containing the inhibitors.
(Wild type strain)

process is sensitive to "side effects" of the inhibitors which are manifested at concentrations below those sufficient to inhibit DNA synthesis.

Although the most sensitive means of studying the mode of action of of inhibitors is to measure the effects on the incorporation of labelled precursors, such an approach was not possible here. Some of the inhibitors gave some extremely anomolous results, in some cases suggesting that they brought about an increase in the rate of DNA synthesis when clearly the accumulation of DNA had ceased. For example 100μg/ml hydroxyurea increases the rate of labelled adenine incorporation into DNA by over 50% though the actual accumulation of DNA is somewhat reduced from the control values (Table 8). This somewhat contradictory result could be explained by assuming that hydroxyurea changes the pool size of the nucleotide precursors. Without a knowledge of the exact intracellular precursor pool sizes it is not possible to compare the data for the incorporation of radioactivity in different samples, unless it can be assumed that the pool sizes are comparable.

Inhibition of Oligoamine Biosynthesis

A number of inhibitors of oligoamine biosynthesis are now known (see Stevens, 1978). Mostly these are inhibitors of ornithine decarboxylase or SAM decarboxylase as these are the rate-controlling enzymes of the biosynthetic pathway. One of the aims in the development of such inhibitors has been to use them to bring about a depletion of oligoamines *in vivo* as a means of elucidating the roles played by these compounds. Methylglyoxal bis (guanylhydrazone), or MGBG, is known to be a potent inhibitor of putrescine-stimulated S-adenosylmethionine decarboxylase activities in animal tissues (Williams-Ashman and Schenone, 1972) and is effective also in *A. nidulans* (Table 9). When added to cell free extracts this

The effect of methylglyoxal bis(guanylhydrazone) on SAM decarboxylase activity *in vitro* A, germination and oligoamine metabolism *in vivo* B.

A

Inhibitor concentration	SAM decarboxylase activity (% control)
0 µM	100
0.1 "	101.5
1.0 "	79
10.0 "	28.6
100.0 "	16
1000.0 "	11.8

Enzyme activity was measured as described in Materials and Methods

B

inhibitor concentration	% germination at 7 hours*	µmole/10^{10} conidia			*in vivo* enzyme activity µmoles/hour/10^{10} conidia	
		putrescine	spermidine	spermine	Ornithine decarboxylation	SAM decarboxylation
0	87	0.2	10.3	1.67	5.40	5.70
0.1 mM	84	0.89	10.5	1.37	7.29	5.19
1.0 mM	59	1.13	7.5	0.98	4.64	2.05

Methylglyoxal bis(guanylhydrazone) was added at 8.45 hours, ^{3}H-ornithine at 9 hours and the conidia harvested 10 hours after inoculation. *In vivo* enzyme activity was estimated on the basis of ^{3}H-ornithine incorporation into oligoamines as described in Materials and Methods, using the wild type strain.

* The % germination was determined separately, with the inhibitor added at the time of inoculation.

inhibitor brings about a 50% reduction in enzyme activity at a low concentration (5μM). The SAM decarboxylase activity from S. cerevisiae is fifty times more sensitive to inhibition (Pösö et al, 1975) by MGBG.

Methylglyoxal bis(guanylhydrazone) is also effective at inhibiting oligoamine biosynthesis in vivo and to a lesser extent germination. Although SAM decarboxylase is sensitive to the inhibitor at low concentrations in vitro much higher concentrations are required to observe an effect in vivo. At 0.1mM MGBG, germination and the levels of spermidine and spermine are not significantly different from the control values but the putrescine concentration is raised over four-fold (Table 9). This can probably be explained by the 9% reduction in the rate at which putrescine is metabolized to spermidine and spermine (i.e. the "in vivo rate of SAM decarboxylation"). The addition of 1.0mM MGBG reduces the rate of spermidine and spermine synthesis by 64% and causes an even greater increase in the level of putrescine. These observations on the effects of MGBG on oligoamine synthesis in vivo are consistent with the mode of action being an inhibition of SAM decarboxylase activity. However even at the highest concentrations tested germination is only slightly inhibited. The intact cells do not appear to be sufficiently sensitive to MGBG to make it useful for preparing oligoamine-depleted cells as the higher concentrations required may have more general toxic effects. The increase in putrescine brought about by MGBG may compensate for the decrease in spermidine and spermine that occurs during inhibition. Furthermore, MGBG itself may be capable of carrying out various roles normally performed by spermidine and spermine, and so frustrate any attempts to study the consequences of oligoamine depletion.

1,4-Diaminobutanone is a powerful competitive inhibitor of ornithine decarboxylase activity in A. nidulans (Stevens et al, 1977) with a

Figure 25

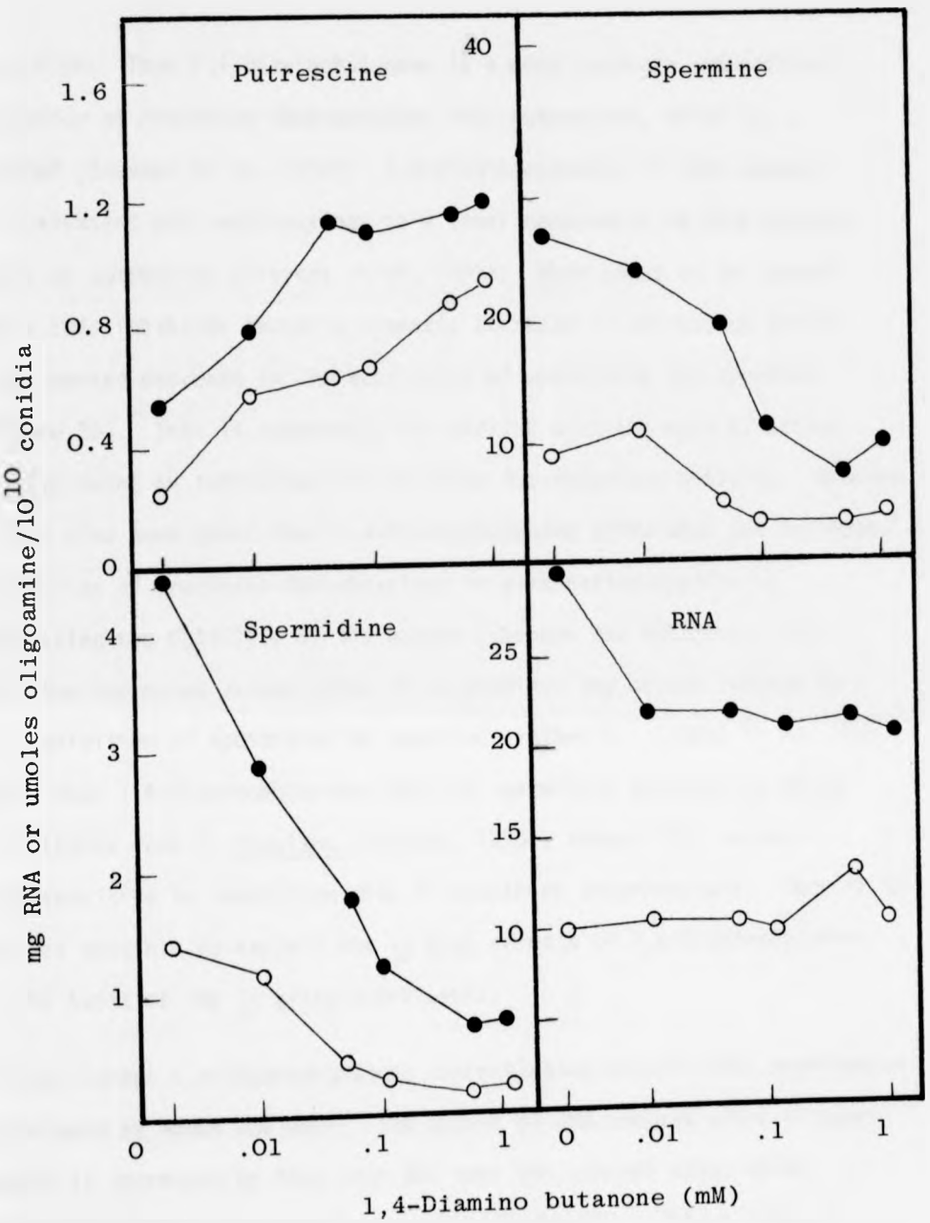


Figure 25 Effect of 1,4 Diamino butanone on accumulation of oligoamines and RNA

Inhibitor added at the time of inoculation and the amounts of RNA and oligoamines determined at 8 (○) and 12 (●) hours as described in Materials and Methods, using the wild type strain.

K_i=0.91μM. Thus 1,4-Diaminobutanone is a much stronger competitive inhibitor of ornithine decarboxylase than putrescine, which has a K_i=60μM (Stevens et al, 1976). 1,4-Diaminobutanone is also capable of activating SAM decarboxylase to a level comparable to that brought about by putrescine (Stevens et al, 1977). When added to the growth media this inhibitor causes a dramatic increase in putrescine levels and a marked decrease in the quantities of spermidine and spermine (Figure 25). This is apparently in conflict with the mode of action *in vivo* being an inhibition of ornithine decarboxylase activity. However it has also been shown that 1,4-Diaminobutanone stimulates the increased production of ornithine decarboxylase in germinating conidia by increasing the half-life of the enzyme (Stevens and McKinnon, 1977). Thus the decreased accumulation of oligoamines may be due instead to the inhibition of spermidine or spermine synthesis. Indeed it has been found that 1,4-Diaminobutanone inhibits spermidine synthase *in vitro* in extracts from *A. nidulans* (Stevens, 1978), though this enzyme is less sensitive to inhibition than is ornithine decarboxylase. Thus it is not yet possible to explain the *in vivo* effects of 1,4-Diaminobutanone on the basis of the *in vitro* experiments.

At the highest 1,4-Diaminobutanone concentration tested (1mM) germination is delayed by about one hour. The amount of RNA present after 12 hours growth is decreased by less than 30% over the control value while spermidine levels are down by over 80% (Figure 25). Thus a constant ratio of spermidine to RNA is not maintained under these conditions. However it is also possible that the functions normally fulfilled by spermidine and spermine are compensated for by the increased putrescine concentrations present during inhibition, or even by 1,4-Diaminobutanone. This is always a possibility where close structural analogues of the

oligoamines are used as inhibitors. 1,4-Diaminobutanone presents the additional problem of being unstable in solution and polymerizes at high concentrations, and so did not seem a suitable tool for investigating the essential cellular roles of the oligoamines.

(ii) Germination in Nuclear Replication Cycle Mutants

A correlation between the accumulation of oligoamines and the onset of macromolecular synthesis is apparent in the germinating conidia of A. nidulans (Figures 15 and 18). However as a great many other events also occur during germination, an extension of these observations is needed in order to establish functional relationships. By examining variations in the germination process it may be possible to more precisely define the various biochemical events which are intrinsically associated with and necessary for germination. As DNA synthesis and nuclear division are features of germ-tube emergence in A. nidulans this appeared to be a potentially important area to investigate. A series of termperature-sensitive mutants were examined which are able to germinate at the restrictive temperature (42°C) but contain only a single nucleus (Orr and Rosenberger, 1976a). The defects in these mutants have not been defined at a molecular level. The mutant strains 12 and 316 have defects appearing in the early stages of the cell cycle while the nuclei of strain 48 contain condensed chromosomes and are unable to complete mitosis, the final stage of the cell cycle (Orr and Rosenberger, 1976b).

The accumulation of spermidine, RNA and DNA in the nuclear replication cycle mutants and the parent strain from which they are derived (NXW) are shown in Figure 26. It is clear from the data for NXW that there is a parallel accumulation of oligoamines and nucleic acids in balanced

Figure 26

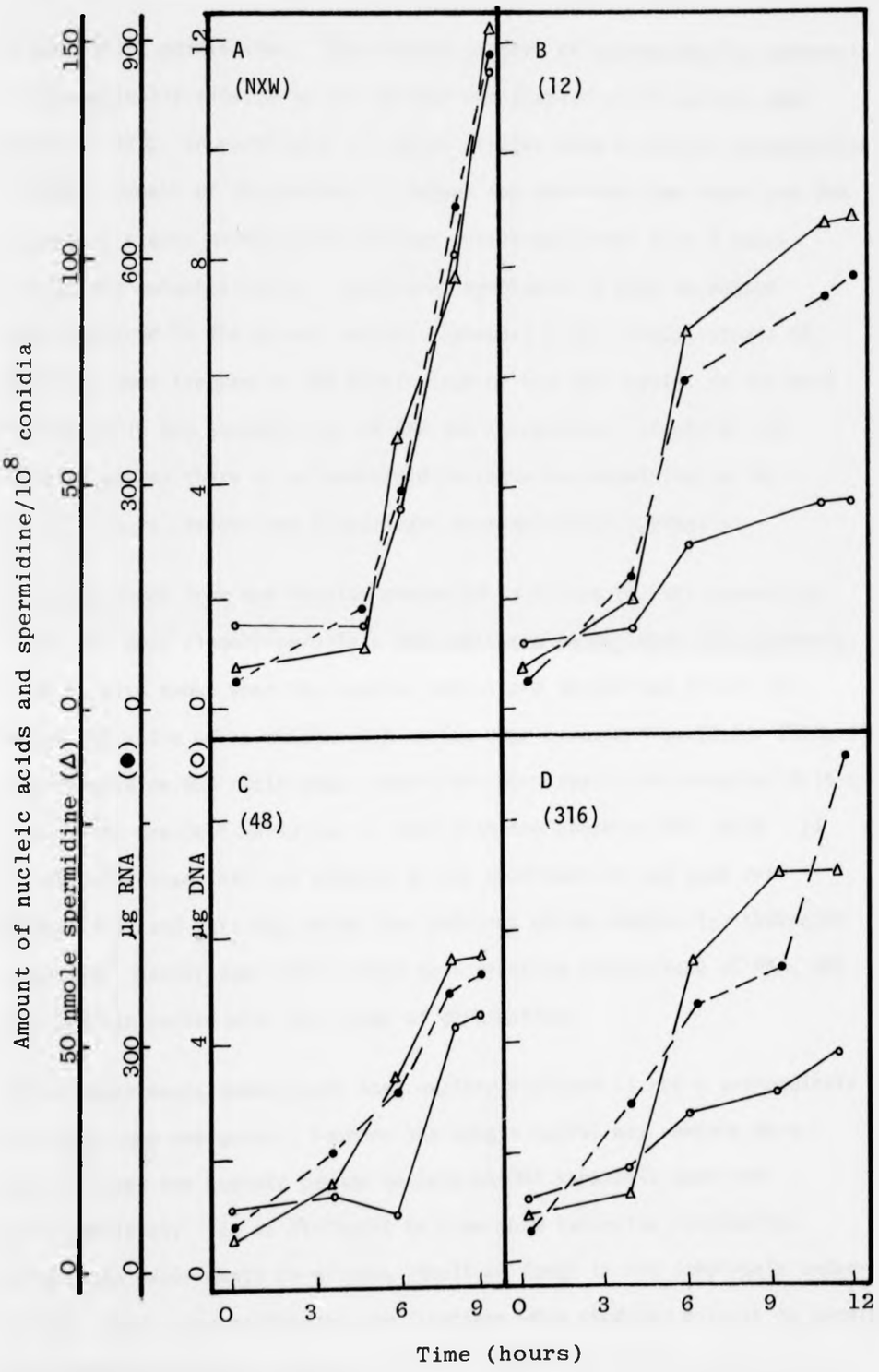


Figure 26 Accumulation of Spermidine and nucleic acids in nuclear replication cycle mutants grown at 42°C.

growth after germination. This normal pattern of macromolecular synthesis is dramatically altered in the nuclear replication cycle mutants when grown at 42°C. In particular all three strains show a reduced accumulation of DNA. Growth of the mutants is normal for the first few hours and RNA synthesis starts normally but becomes restricted after 7 to 8 hours in all the mutant strains. Spermidine synthesis is also decreased when compared to the parent strain. Somewhat surprisingly, strain 48, which becomes trapped in the final stage of the cell cycle, is the most inhibited in the accumulation of RNA and spermidine. In all of the strains tested there is a three-fold increase in spermidine in the first 4 hours, before any significant macromolecular synthesis.

It seems clear from the results presented in Figure 26 that spermidine synthesis more closely parallels RNA synthesis rather than DNA synthesis. This is also shown when the precise ratios are determined (Table 10). While the ratio of spermidine:DNA varies over a range from 12.5 - 25.8μmole/mg the spermidine:RNA ratio stays within the more restricted range of 0.14 - 0.18. The greatest variation is seen with the spermine:DNA ratio. It is also the case that the mutants do not germinate at the same rate (Table 10), and this may affect the patterns of macromolecular synthesis expected. Bainbridge (1971) found that relative proportions of RNA, DNA and protein varied with the stage of germination.

These experiments demonstrate that nuclear division is not a prerequisite for germ-tube emergence. However the single nuclei may contain more than 4 times the haploid genome content as DNA synthesis does not stop completely. It is difficult to draw more extensive conclusions from these experiments as mitosis, itself in fungi is not completely understood. There are additional complications when studying mitosis in germination. For example, Orr and Rosenberger (1976b) found that blocking mitosis in growing hyphae affects RNA synthesis more than it does in germinating

Table 10

Germination (A) and oligoamine:macromolecule ratios (B) in nuclear replication cycle mutants grown at 42°C.

A

Strain	% Germination at various time			
	6 hours	8 hours	9 hours	12 hours
N X W	51	95	95	-
12	89	95	95	-
48	25	84	95	-
316	10	-	20	55

B

Strain	μmole oligoamine/mg macromolecule			
	Spermidine / protein	Spermidine / RNA	Spermidine / DNA	Spermine / DNA
N X W	0.15	0.16	12.5	4.9
12	0.12	0.15	25.8	14.7
48	0.16	0.18	15.1	8.3
316	0.21	0.14	18.1	18.8

Spermidine, spermine, RNA and DNA were determined after 9 hours growth in nutrient broth at 42°C as described in Materials and Methods.

conidia. Thus the mechanisms which interlock nuclear replication with RNA and protein synthesis may be different in conidia.

(iii) Germination in the Putrescine Auxotroph puA_1

One of the approaches in the study of oligoamines is to bring about variations in the patterns of macromolecular synthesis and observe the changes in the intracellular concentrations of putrescine, spermidine and spermine (see Section ii). A more direct approach would be to selectively deplete or increase the amounts of the oligoamines and observe the consequent effects on cellular metabolism. The use of inhibitors of oligoamine synthesis (Section i) has yielded some pertinent information but it is difficult to avoid artifacts arising from the use of inhibitors. These difficulties do not arise with the use of oligoamine-requiring mutants, which are known in bacteria (Maas, 1972), yeast (Cohn et al, 1978), N. crassa (Deters et al, 1974) and A. nidulans (Sneath, 1955).

The putrescine requirement of the A. nidulans strain puA_1 is due to a deficiency in ornithine decarboxylase at a time when there is a rapid increase in this enzyme in the prototrophic strain (Stevens, 1975). Some ornithine decarboxylase activity is detectable in the mutant though at an insufficient level to allow detectable oligoamine biosynthesis. This is also confirmed by measurement of the intracellular oligoamine levels. The combined levels of the oligoamines decreases with time in the unsupplemented auxotroph. The total amount of oligoamines present in the germinating conidia of the auxotroph can always be accounted for by the amount of exogenous oligoamine added, if any, and the amounts already present in the dormant conidia. When supplemented with putrescine, germination in the auxotroph occurs at the normal rate (Table 11). Remarkably low concentrations of putrescine will allow complete germination of the conidia. The conidia must first, however, be washed by

Table 11

Germination of the conidia of the putrescine auxotroph puA_1 in the presence of oligoamines

Oligoamine	% Germination at 9 hours
None	4
Putrescine	
3nM	3
30nM	39
300nM	86
3µM	95
30µM	95
300µM	95
Spermidine	
3µM	15
300µM	71
Spermine	
3µM	7
300µM	62

Oligoamines were added to the growth media at the time of inoculation and the % conidia bearing germ-tubes 9 hours later determined as described in Materials and Methods.

repeated centrifugation and resuspension in fresh sterile 0.1% tween-80 to remove contaminating oligoamines which may be carried over from the solid media which of necessity contains putrescine to allow conidiation. If this step is not carried out only a partial putrescine requirement for germination and growth is observed.

Spermidine and spermine will support some germination in the auxotroph but only when at about one hundred times the concentration of putrescine. It is difficult to eliminate the possibility that a low level of contaminating putrescine in the spermidine and spermine samples may be responsible for allowing some germination, though this does not seem to be the case. The oligoamine distribution of conidia grown in liquid media containing 0.3mM putrescine, spermidine or spermine is shown in Table 12. Putrescine supports the greatest amount of growth, as judged by RNA accumulation. The intracellular putrescine concentration is greatly depressed when the auxotroph is supplemented with spermine and to a lesser extent with growth on spermidine. It is also noticeable that with growth on spermine the intracellular spermine/spermidine ratio is reversed from the normal pattern of spermidine being the predominant oligoamine.

The accumulation of protein, RNA and DNA in the putrescine auxotroph grown in the presence or absence of putrescine is shown in Figure 27. In the absence of exogenously supplied putrescine there is only a slight increase in the amount of protein and RNA. DNA doubles in amount, prior to germ-tube emergence suggesting that the primary effect on germination exerted by oligoamine deficiency is not simply a limitation of DNA synthesis. All types of macromolecular synthesis are affected. This is very different from the observation that DNA synthesis is preferentially inhibited during oligoamine depletion in lymphocytes (Fillingame et al, 1975), rat liver and a number of other animal tissues (Jänne et al, 1978a).

Intracellular oligoamine distribution of the putrescine auxotroph supplemented with putrescine, spermine or spermidine

	Supplement to the Growth Media			
	Putrescine	spermidine	spermine	none
nmole oligoamine per 10^8 conidia				
putrescine	30	7.5	0.5	1
spermidine	141	52	31.5	4
spermine	93	29.5	66	32
µg RNA per 10^8 conidia	165	103	104.5	99

Conidia were grown in media containing putrescine, spermidine or spermine at a final concentration of 0.3mM, harvested after 8 hours growth and oligoamines and RNA estimated as described in Materials and Methods

Figure 27

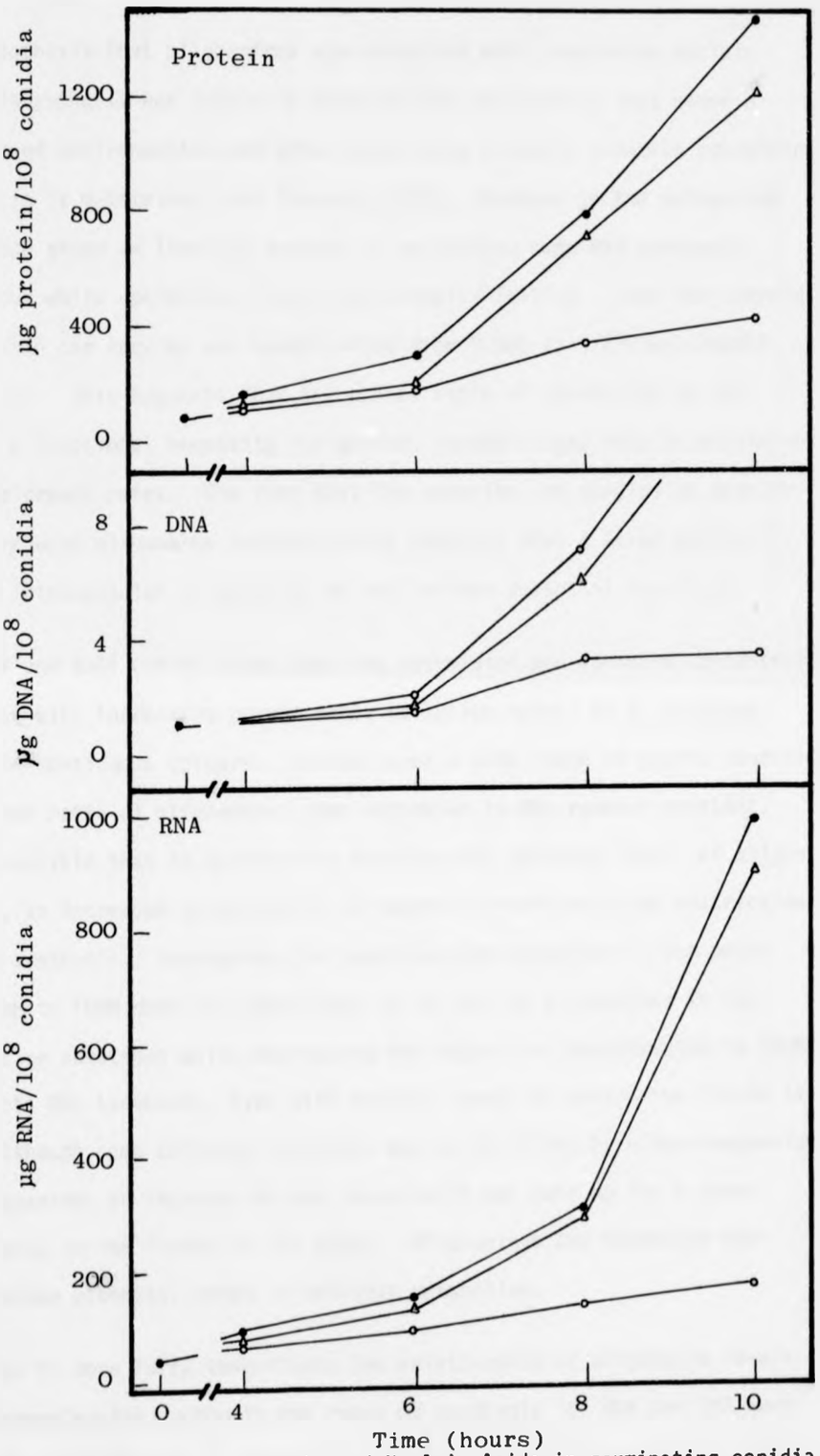


Figure 27 Accumulation of Protein and Nucleic Acids in germinating conidia of the putrescine auxotroph - putrescine was added at the time of inoculation to a final concentration of 300µM (●), 3µM (Δ) or 0µM (○).

The hypothesis that oligoamines are concerned with regulating nucleic acid biosynthesis was initially based on the observation that under a variety of environmental and other conditions a nearly constant spermidine: RNA ratio is maintained (see Stevens, 1970). However in the putrescine auxotroph grown on limiting amounts of putrescine some RNA synthesis can occur while spermidine levels are actually falling. Thus the spermidine: RNA ratio can vary by one hundred-fold from 0.003 to 0.28 μmole/mgRNA (Table 13). This suggests that the normal ratio of spermidine to RNA is not a functional necessity for growth, though it may help in attaining maximum growth rates. The fact that the organism can survive on drastically reduced oligoamine concentrations suggests that a large portion of the intracellular oligoamines do not perform essential functions.

Bushell and Bull (1974) found that the spermidine and spermine concentrations increase with increasing growth rates (dilution rates) in A. nidulans grown in continuous culture. However over a wide range of growth conditions the molar ratio of oligoamines plus magnesium to RNA remains constant. It is possible that in germinating conidia with depleted levels of oligoamines, an increased accumulation of magnesium could ease any restrictions on RNA synthesis. Increasing the magnesium concentration in the media from 1mM to 10mM does not substitute for a lack of oligoamines in the putrescine auxotroph while decreasing the magnesium concentration to 10μM restricts RNA synthesis, even with optimal levels of putrescine (Table 14). Thus although some cellular functions may be fulfilled by either magnesium or oligoamines an increase in one cation will not make up for a gross deficiency in the levels of the other. Oligoamines and magnesium must have unique essential roles in cellular metabolism.

In order to more fully investigate the relationship of oligoamine levels and macromolecular synthesis the rates of synthesis for RNA and DNA were

The ratio of spermidine to RNA (μmole/mgRNA) for the putrescine auxotroph *A. nidulans* puA_1

Putrescine Concentration	Culture Age 0 hr	8 hr	24 hr
0.0	0.073	0.004	0.003
0.003 μM	0.073	0.028	0.015
0.03 μM	0.073	0.268	0.090
0.3 μM	0.073	0.240	0.281
Wild type in minimal media	0.091	0.186	0.203

RNA and spermidine were measured as described in Materials and Methods.

Effect of exogenous putrescine and magnesium concentrations on RNA accumulation in the putrescine auxotroph.

µg RNA/10^8 conidia				
	putrescine concentration in growth media			
A Culture Age	0µM	3µM	30µM	300µM
0 hour	45	45	45	45
8 hours	91	143	154	143
12 hours	173	321	348	451
B mM Mg^{++} in growth media				
0 mM	132	122	-	134
0.01 "	-	221	267	298
1.0 "	173	321	348	451
10.0 "	160	330	-	420

The magnesium concentration of the growth media in experiment A was 1mM (standard minimal media). The conidia were harvested at 12 hours for experiment B employing a range of magnesium concentrations. The estimation of RNA was carried out as described in Materials and Methods.

determined by measuring the incorporation of labelled precursors (Figure 28). The initial experiments utilizing a very low concentration of labelled adenine (0.1μM) gave results suggesting that nucleic acid synthesis continued at the control rate during oligoamine depletion and that both of the rates decrease after 4 hours growth. This is not consistent with the actual accumulation of RNA and DNA (Figure 27). When the rate of nucleic acid synthesis is determined with higher concentrations of adenine (100μM) the results show that nucleic acid synthesis during oligoamine depletion is normal until 6 hours when the rates begin to fall behind the control values. A more complete time course of nucleic acid synthesis (Figure 29) confirms that oligoamine depletion results in a limitation of nucleic acid biosynthesis between 4 and 6 hours after inoculation. This is the time at which the rapid synthesis and accumulation of oligoamines begins in the prototroph (Figure 15). The inability of the putrescine starved auxotroph to increase the rate of synthesis after 6 hours growth could account for the inability of the conidia to form germ-tubes, as germination requires RNA synthesis (Table 7). When the specific rates of nucleic acid synthesis are compared (Figure 30) it can be seen that the increase in RNA and protein synthesis at 6 hours in the putrescine supplemented conidia of the auxotroph does not occur in the putrescine starved cultures. This is consistent with the view that oligoamines are required to optimize the ability of the intracellular machinery to synthesize macromolecules. As an increase in the specific rates of synthesis normally takes place in germination the oligoamine depleted conidia are unable to maintain a high rate of macromolecular synthesis to allow germination to proceed.

The difference between the rates of nucleic acid synthesis determined with 0.1μM and 100μM ^{3}H-adenine (Figure 28) is probably explained by

Figure 28

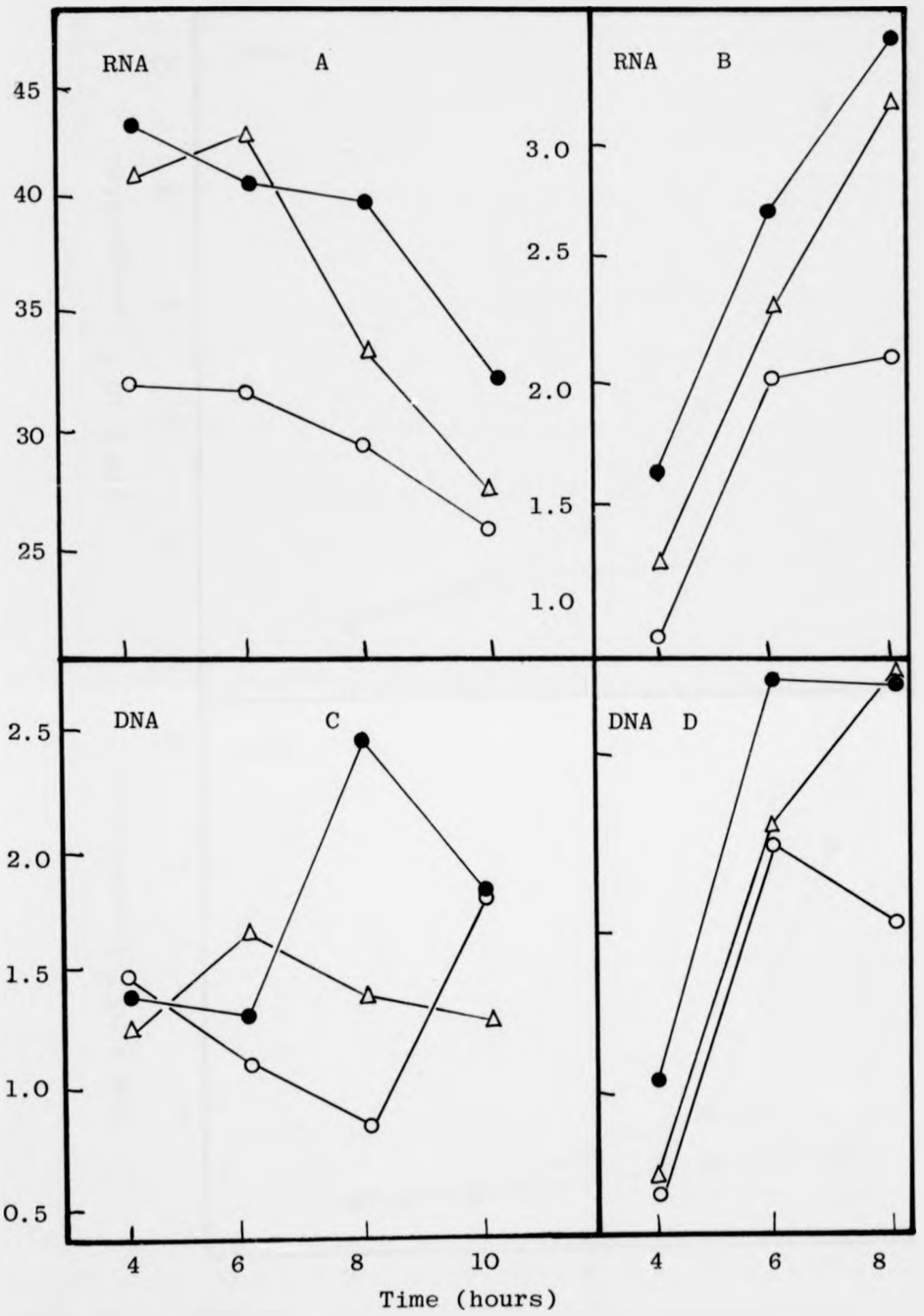


Figure 28 Influence of adenine concentration on precursor incorporation into nucleic acids. Wild type conidia were grown for the times shown in the presence of 300μM putrescine (●), 3μM putrescine (Δ) or in the absence of putrescine (O). Nucleic acids were labelled with ^{3}H-adenine (5μCi) in 20mls media containing 6 X 10^{6} conidia for 15 minutes along with unlabelled adenine to a final concentration of 0.1mM (B and D) or 0.1μM (A and C)

Figure 29

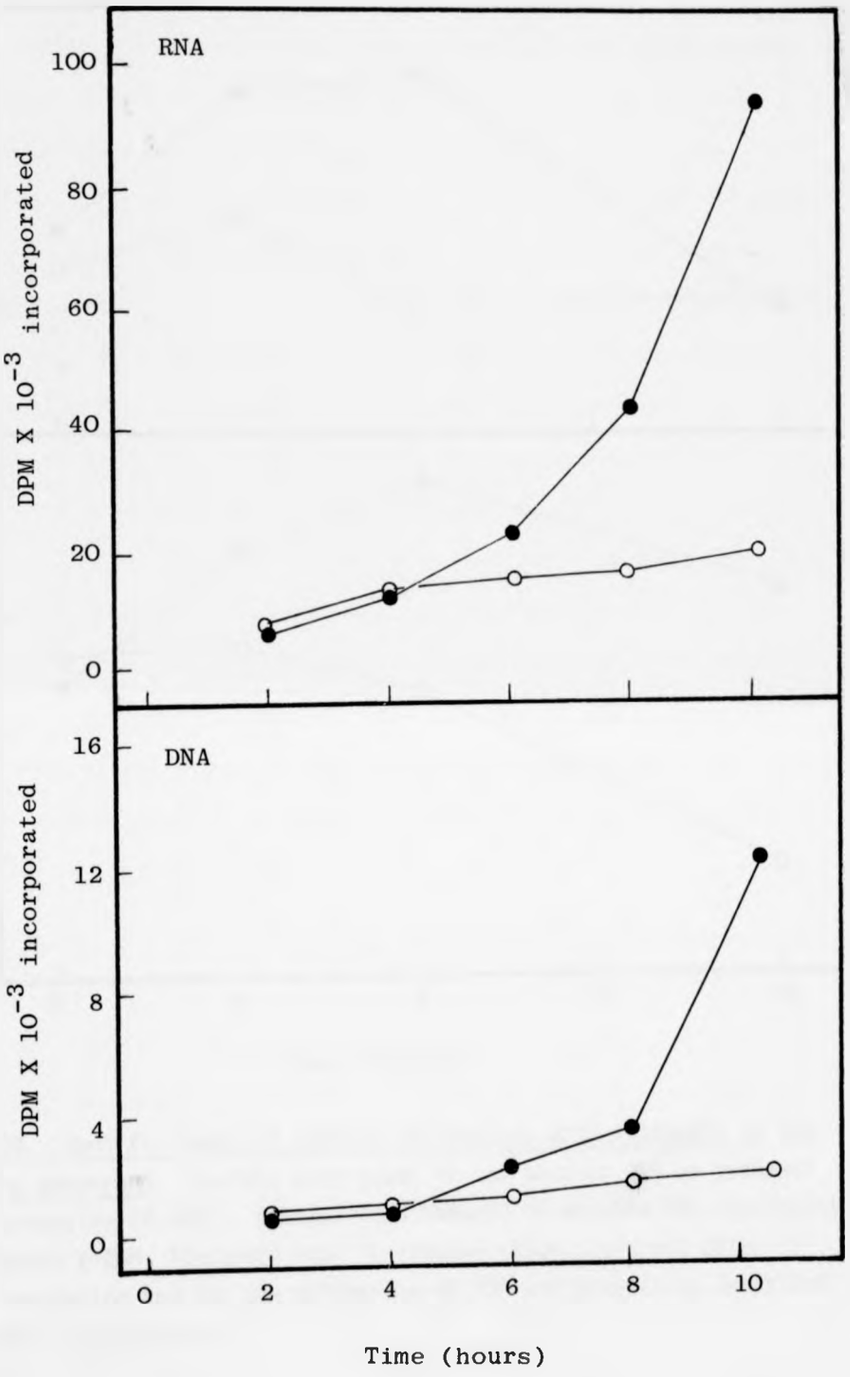


Figure 29 Nucleic acid synthesis in the putrescine auxotroph puA1

The incorporation of ^{3}H-adenine (100μM, 5μCi/ml) into RNA and DNA in the putrescine auxotroph in the presence (●) or absence (○) of putrescine (0.3mM) was determined as described in Materials and Methods.

Figure 30

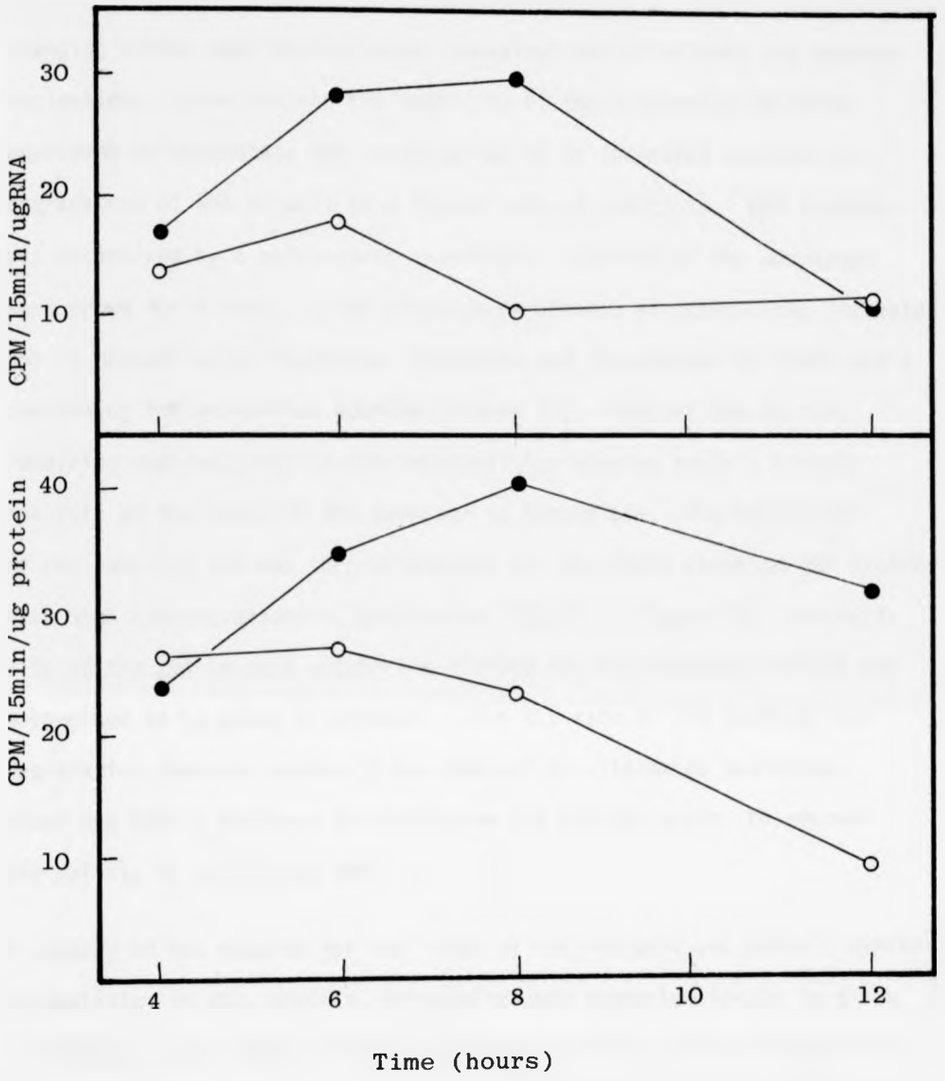


Figure 30 Specific rates of protein and nucleic acid synthesis in the putrescine auxotroph. Conidia were grown in the absence (O) or presence (●) of putrescine (0.7mM). Samples were removed to measure the incorporation of ^{3}H-adenine (10μM, 10nCi/ml) and ^{14}C-leucine (10μM, 2nCi/ml) after 15 minutes incubation and for the estimation of RNA and protein as described in Materials and Methods.

changing uptake and intracellular concentrations of adenine and adenine nucleotides. Nevertheless the inability of the oligoamine depleted auxotroph to accumulate RNA could be due to an increased turnover or degradation of RNA as well as a lesser rate of synthesis. RNA turnover was determined by a pulse-chase experiment. Conidia of the auxotroph were grown for 8 hours in the presence or absence of putrescine, incubated for 15 minutes with ^{3}H-adenine, harvested and resuspended in fresh media containing 1mM unlabelled adenine (Figure 31). However due to the remaining radioactivity in the intracellular adenine pools a kinetic analysis of the rates of RNA turnover is impossible. The half-life of the labelled RNA was then determined by completely stopping RNA synthesis with high concentrations of proflavine (40µg/ml) (Figure 32). The half-life of the RNA in both putrescine starved and supplemented conidia was determined to be about 66 minutes. Thus the rate of RNA turnover and degradation does not appear to be affected by oligoamine depletion. After one hour's exposure to proflavine the conidia begin to recover the ability to synthesize RNA.

A summary of the results for the rates of nucleic acid and protein synthesis, accumulation of RNA, protein, oligoamines and magnesium levels is given in Table 15. The rate of protein synthesis appears to be restricted by the greatest amount during oligoamine starvation or supplementation at low levels where the rate actually decreases between 8 and 12 hours. There is a reduced accumulation of magnesium during oligoamine depletion suggesting that the uptake of magnesium requires an active metabolism. The ratio of magnesium to RNA is not significantly affected during putrescine starvation.

As protein synthesis is one of the processes most severely affected during oligoamine starvation this was further investigated by determining

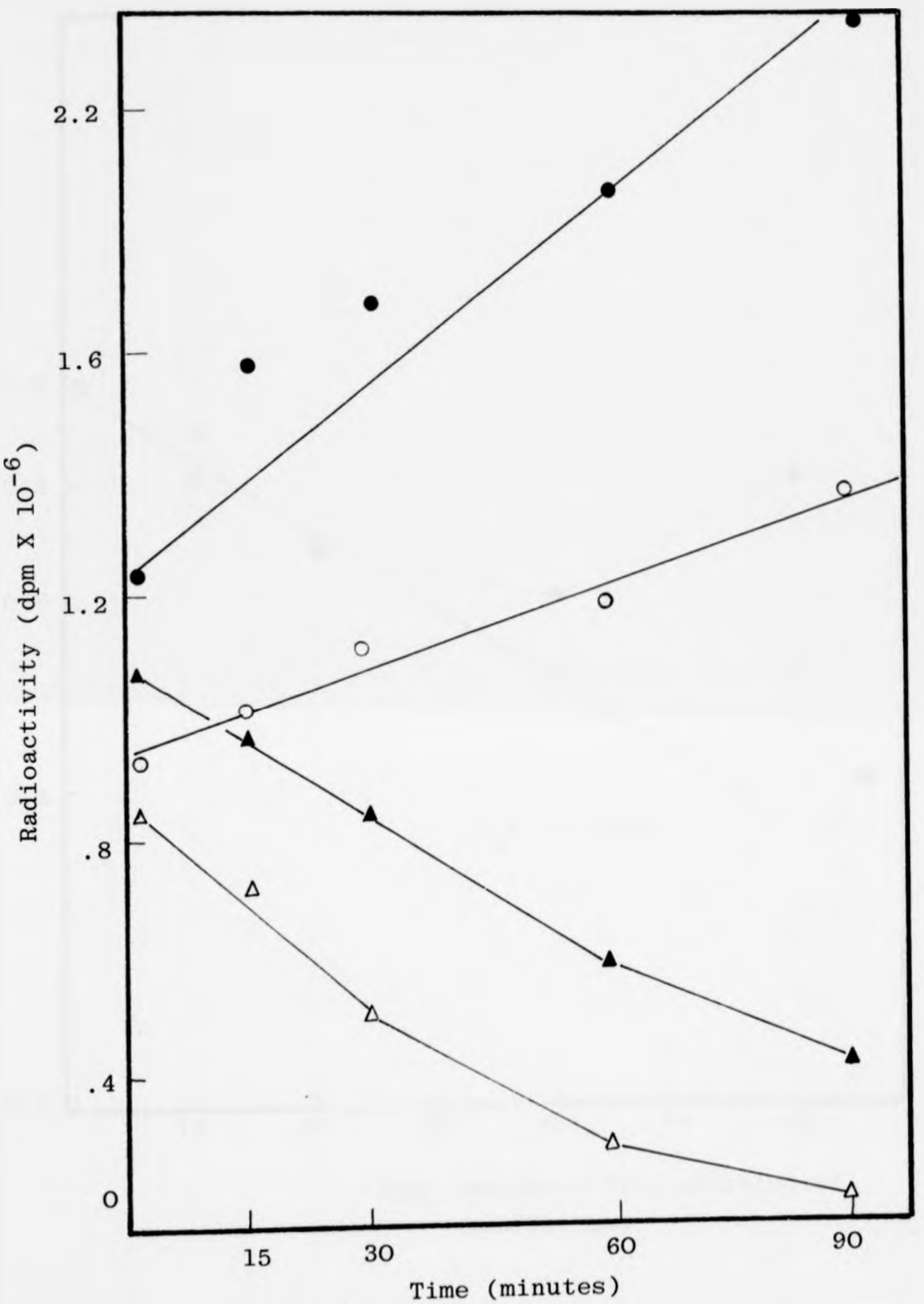


Figure 31 Turnover of RNA in the wild type strain

Conidia were grown in the absence (open figures) or presence of 0.7mM putrescine (closed figures) and incubated with ^{3}H-adenine for 15 minutes. The conidia were harvested, washed, resuspended in media containing 10mM unlabelled adenine and the radioactivity in the acid insoluble (○) and soluble (△) fractions determined at intervals.

Figure 32

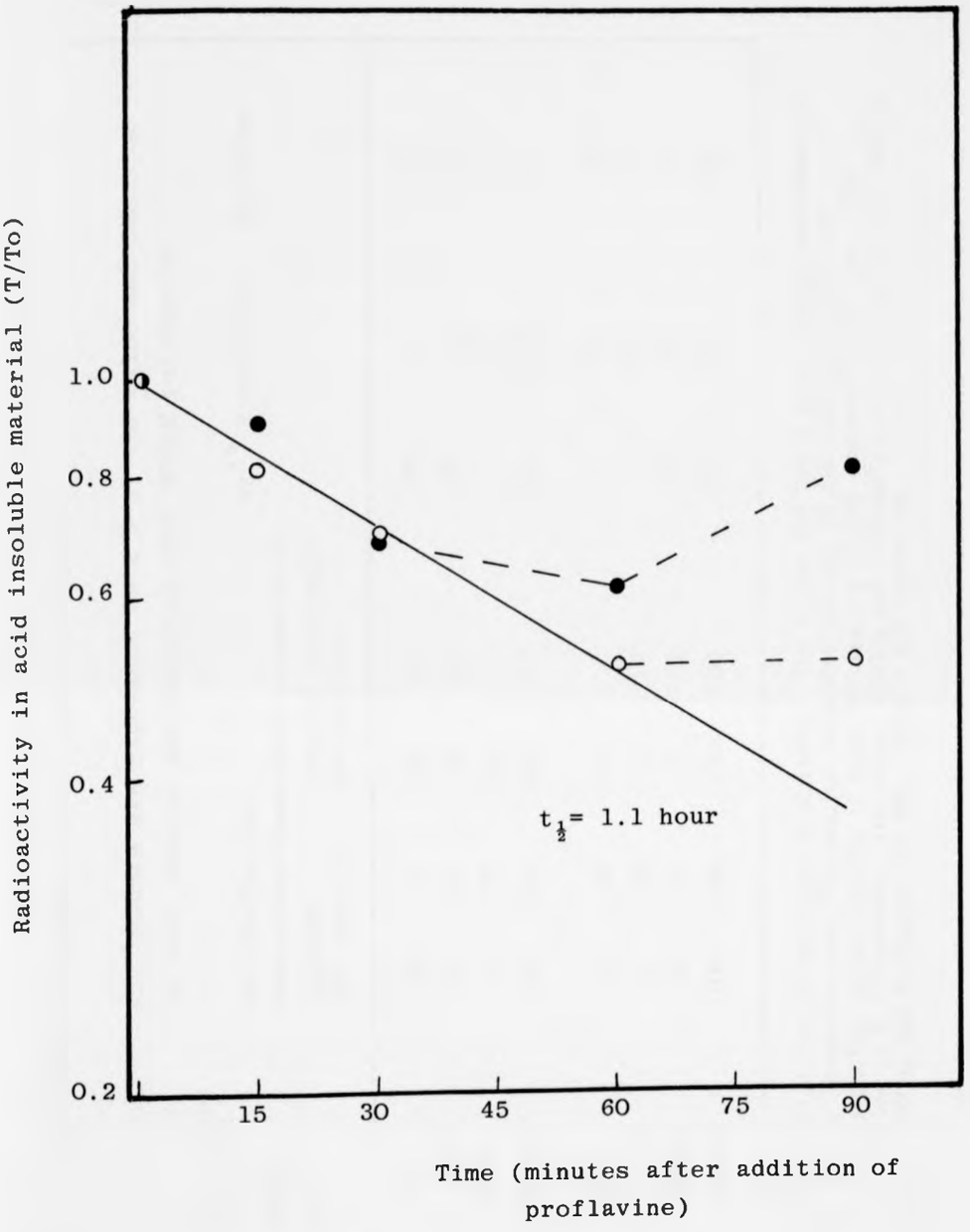


Figure 32 Half-life of total RNA

Wild type conidia were grown in the absence (○) or presence (●) of 0.7mM putrescine for 8 hours and exposed to ^{3}H-adenine for 15 minutes after which time proflavine (40µg/ml) was added and radioactivity remaining in the acid insoluble material determined at intervals.

Oligoamine content and macromolecular synthesis in the putrescine auxotroph

Growth Time	Putrescine addition to growth media	All amounts are expressed as a % of the value for the 8 or 12 hour samples supplemented with 300nM putrescine						
		Macromolecules				Mg^{++}	Spermidine	Spermine
		Content		Rate of synthesis				
		RNA	protein	RNA	protein			
8 hours	none	58	52	32	30	48	10	16
	3nM	66	51	48	30	50	15	23
	30nM	67	73	67	67	91	27	81
	300nM	100	100	100	100	100	100	100
12 hours	none	29	36	29	9	31	19	8
	3nM	31	40	31	10	38	17	12
	30nM	37	81	57	23	63	30	23
	300nM	100	100	100	100	100	100	100

All estimations were carried out as described in Methods. The values for the 8 hour conidia supplemented with 300nM putrescine for ^{3}H-adenine and ^{14}C-leucine incorporation are 18,600 cpm and 53,800 cpm respectively. The corresponding values for the 12 hour conidia are 22,600 cpm and 75,830 cpm. The contents for 8 hour samples (4 X 10^{8} conidia) are 988µg, 1340µg and 1.98µmole respectively and for the 12 hour samples 2,280µg, 2,300µg and 5.07µmole for RNA, protein and magnesium.

Table 15

Types of RNA synthesized during putrescine starvation of *puA*$_1$

Growth Time	18-25S rRNA	4-5S rRNA	poly (A)$^+$ RNA	Number of ribosomes/ conidia X 10^{-5}
Plus putrescine (0.3mM)				
6 hours	61	18.4	1.70	3.2
12 hours	59.5	15.9	1.45	11.8
Minus putrescine				
6 hours	52	33	1.12	2.9
12 hours	32	57	1.31	3.3

RNA was labelled for 15 minutes with ^{3}H-adenine (0.1μCi/ml, 100mCi/mmole) extracted and separated by oligo (dT) cellulose chromatography and centrifugation in a sucrose gradient as described in materials and methods. The estimation for the number of ribosomes assumes that all of the extracted rRNA is present in ribosomes.

the percentage of ribosomes present in polysomal complexes active in protein synthesis. The polysome profiles (Figure 33) demonstrate that the number of ribosomes present as polysomes is much reduced in the oligoamine depleted cultures. Furthermore the number and size of polysomes decreases between 6 and 12 hours in the putrescine starved cultures, reflecting the decrease in the rate of protein synthesis that also occurs over this period (Table 15)

The types of RNA synthesized during putrescine starvation and supplementation are shown in Table 15b. There is a very marked decrease in the proportion of rRNA synthesized when conidia are grown in the absence of putrescine. This is consistent with the previous work of Sinha (1978). The proportion of poly (A) containing RNA was not significantly changed under these conditions. If it is assumed that the rRNA present was assembled into ribosomes a rough calculation of the number of ribosomes can be made (Table 15b). This shows that the number of ribosomes is severely restricted by oligoamine depletion, and is an important factor in the reduction of protein synthesis in the auxotroph.

The biosynthesis of all cellular constituents requires energy and in aerobic organisms this necessitates a functional mitochondrial system. As germination is normally accompanied by a massive increase in mitochondrial activity, it appeared possible that a defect in mitochondria brought about by oligoamine depletion could indirectly affect the rates of nucleic acid and macromolecular synthesis. The rate of oxygen consumption in the putrescine supplemented auxotroph increases dramatically during germ-tube emergence (Figure 34). This massive increase in oxidative metabolism is absent in the putrescine starved conidia where only a slow, gradual increase in oxygen uptake is detectable. The reduced rate of oxygen uptake in the unsupplemented auxotroph can be explained as being

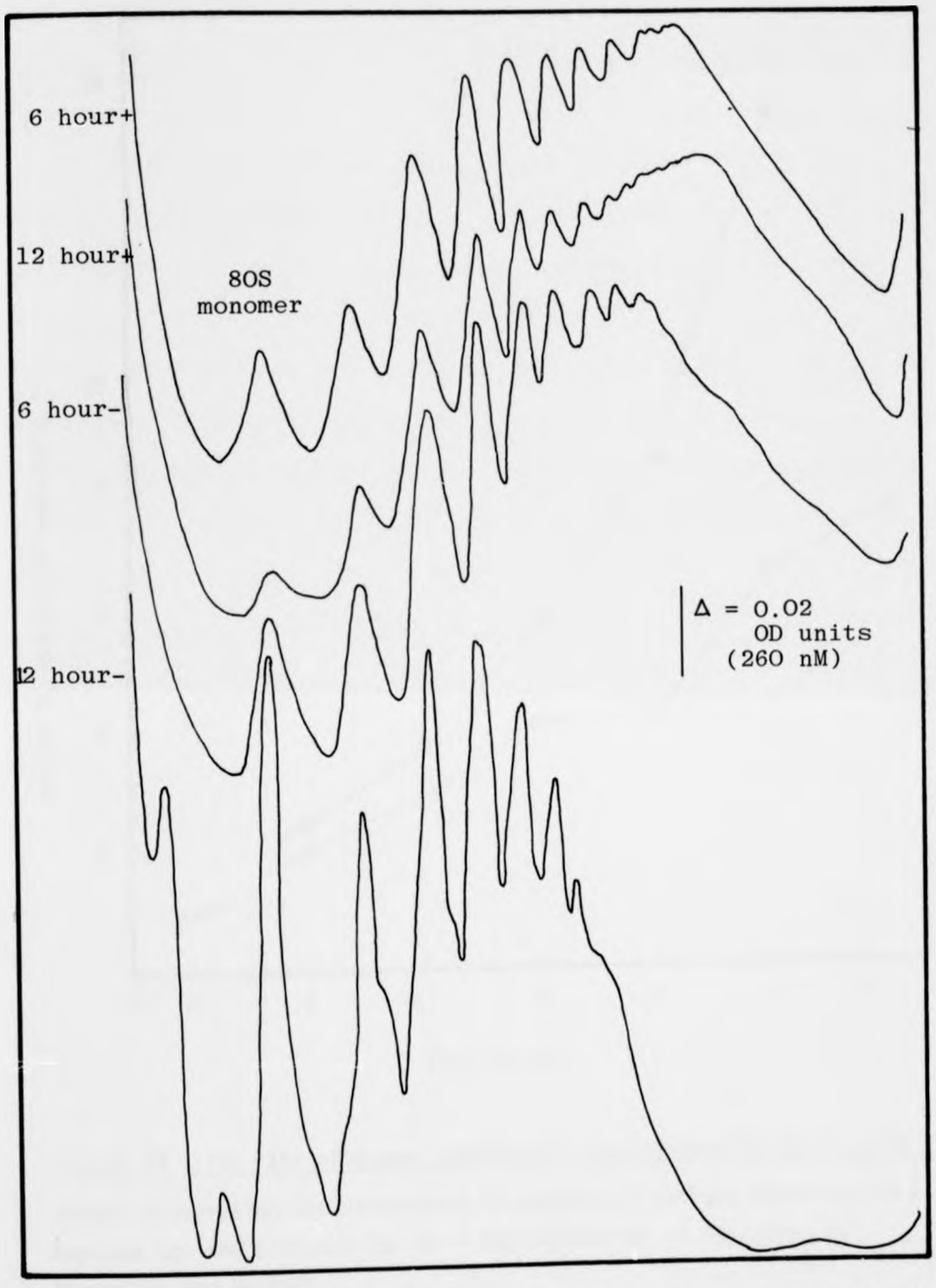


Figure 33 Polysome profile from conidia grown in the absence or presence of putrescine (*puA*$_1$)

Polysomes were extracted from conidia grown for 6 or 12 hours in the absence (-) or presence (+) of 0.3mM putrescine and separated by centrifugation as described in Methods.

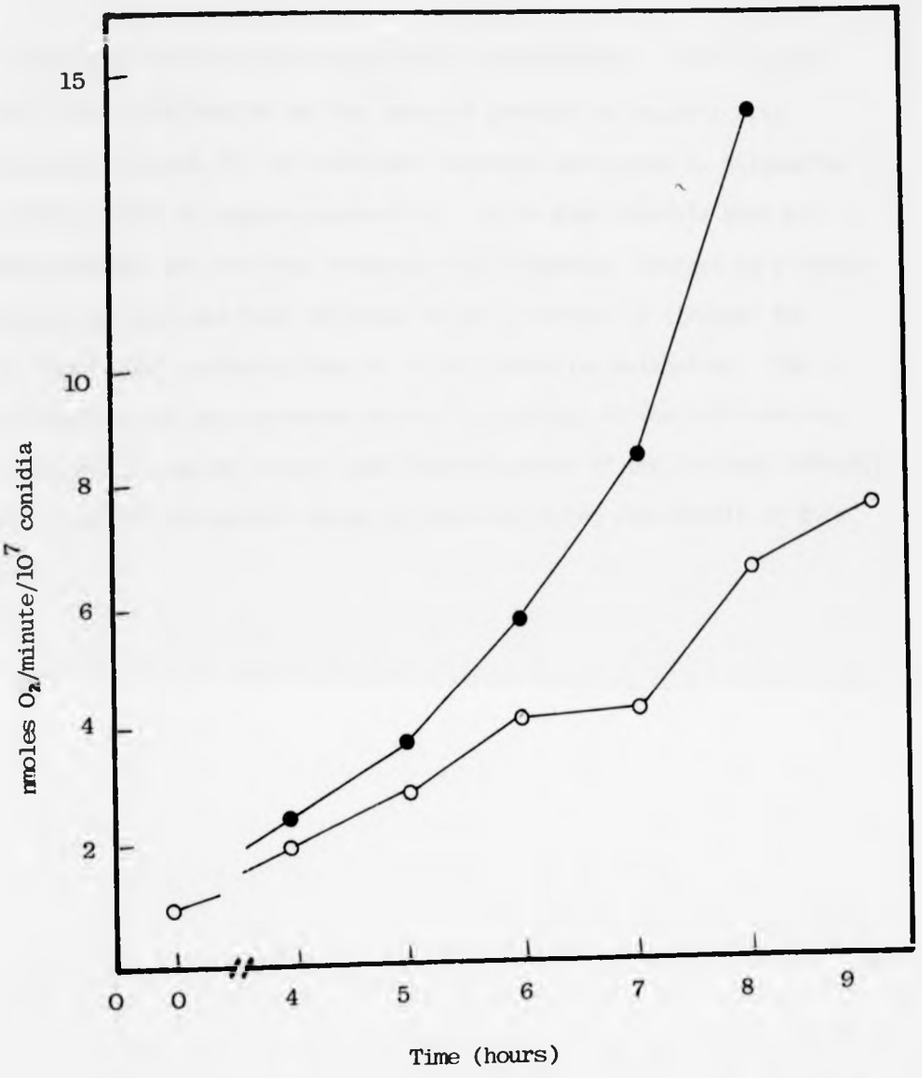


Figure 34 The rate of oxygen consumption during germination of puA_1

Oxygen consumption was determined in samples of conidia grown in the absence (○) and presence (●) of 0.3mM putrescine as described in Materials and Methods.

a secondary effect of the inhibition of germination. This seems the most likely explanation as the rates of protein and nucleic acid synthesis (Figure 27) are much more severely restricted by oligoamine depletion than is oxygen consumption. It is also possible that all these effects are indirect responses to biochemical changes on a rather different level and that the coupling of processes is stronger for macromolecular synthesis than it is for oxidative metabolism. The interactions of the different metabolic pathways in the cell are of a sufficiently complex nature that identification of the "primary effect(s)" of oligoamine starvation cannot be made utilizing experiments of this type.

Discussion on the Effects of Inhibitors and Nutrient Depletion of Auxotrophic Mutants on Germination

The use of metabolic inhibitors such as cycloheximide and proflavine has established that some synthesis of both RNA and protein is required for germ-tube emergence of the conidia of A. nidulans. A requirement for macromolecular synthesis is found in germination of a wide number of fungal species. The results are less conclusive in demonstrating the necessity of DNA synthesis prior to and during germ-tube emergence. It is quite clear that the early transcriptional and translational events of germination occur before the initiation of DNA synthesis. Although DNA synthesis is not required for these early events, it may be important during the later stages of germination. DNA replication increases the number of rRNA and tRNA genes within the conidia, which may be important factors governing the level of ribosome production and protein synthesis. DNA synthesis and subsequent increase in the available genome quantity could well be necessary for the increasing rates of macromolecular synthesis occurring at the time of germ-tube emergence.

DNA synthesis and nuclear replication may not be required for conidial germination but are essential for subsequent growth to continue. Conidia of A. nidulans mutant strains defective in mitosis are able to germinate but subsequent extension of germ-tubes is severely restricted. The early stages of germination proceed normally in these mutants when grown at the restrictive temperature but by 6 to 8 hours after inoculation the defect in nuclear replication results in marked decreases in RNA and protein levels as well as in DNA synthesis. This is further evidence that the increases in macromolecular synthesis during germ-tube emergence are dependent on an amplified genome. The quantity of DNA is probably not the only factor limiting growth. Some of the nuclear replication cycle mutants support a several-fold increase in DNA levels though still contained within a

single nucleus. As this results in a restriction of growth it would appear that the distribution of DNA in nuclei located in separate areas of the cytoplasm is essential for efficient utilization of the genome.

Starvation of the putrescine auxotrophic mutant puA_1 results in an inhibition of conidial germination. If conidia of the putrescine auxotroph are germinated in the absence of putrescine there is a gradual decline in the total quantities of oligoamine present within the cell, though most of the spermidine and putrescine is metabolized to form spermine. Furthermore when the auxotroph is supplemented with oligoamines the intracellular quantities are always found to be less than the sum of the exogenous supply plus the amount already present within the spore. This confirms that the loss of ornithine decarboxylase prevents oligoamine accumulation.

Spermine and spermidine are only partially able to substitute for the putrescine requirement for germination and growth of strain puA_1. Growth of A. nidulans puA_1 when supplemented with putrescine occurs at the same rate as growth of the wild type strain, and the kinetics of germination are similar. As putrescine completely compensates for the loss of ornithine decarboxylase activity in the mutant strain it is probably safe to assume that the other biochemical events occurring during germination of the wild type strain occur also in the supplemented auxotroph. This certainly seems to be the case for those processes which have been examined in both strains as shown by comparing Figure 21 with 33, 20 with 30, and 11 with 34.

The very close correlation between oligoamine and RNA amounts found in the wild type strain is no longer found when the auxotroph puA_1 is grown on limiting supplies of putrescine. The fact that the conidia will germinate with less than 10% of the normal oligoamine content suggest that the fungus normally maintains putrescine, spermidine and spermine at levels in excess of those required to fulfil essential functions. The higher oligoamine

concentrations may optimize growth conditions by non-essential or non-specific functions which can be filled by other divalent cations during oligoamine deprivation. There is, however, an absolute requirement for oligoamines for germination. As DNA synthesis does not appear to be essential for germ-tube emergence the inhibition of germination by oligoamine starvation can not be due to the lack of DNA synthesis alone. As RNA and protein synthesis are both required for germination, limitation of the synthesis of either macromolecule could prevent germ-tube emergence.

The conidia of the putrescine-requiring auxotroph are able to germinate when supplemented with very low levels of putrescine (Table 11). Thus if they are supplied with excess putrescine for the first 6 hours of growth and then harvested, washed free of unincorporated putrescine and resuspended in media lacking oligoamines germination proceeds normally (Stevens, 1975), although a partial restriction in growth is apparent by 24 hours (J A Hope, unpublished observations). This can now be explained by the ability of the conidia to rapidly store sufficient oligoamines to enable germination and growth. Once germination has been completed subsequent oligoamine requirements are small and can be met by the reserves present in the germinated conidia. The "transient requirement" for oligoamines for germination only is thus not because hyphal growth after germ-tube emergence is independent of the intracellular concentration. Instead, it is most likely a reflection of the fact that "exponential" or log phase growth exists for only a short time in batch cultures of A. nidulans grown at the densities and temperatures used in these experiments. A decrease in the growth rate of A. nidulans occurs when the organism concentration exceeds 7 mg dry weight/ml (Trinci, 1969) probably due to the accumulation of toxic products in the media. At spore densities of around 2×10^6 conidia/ml, 7mg/ml is reached after 25 hours growth at $37^{o}C$ and is reached much earlier at spore densities of up to 2×10^7 conidia/ml such as used in many of the experiments

reported here. The increase in the rate of growth is greatest between 6 and 10 hours and subsequent requirements are small. It is thus difficult to starve the mycelia for oligoamines after germination. This may account for the observation of Arst and Scazzocchio (1972) that putrescine starvation of puA_1 did not affect incorporation into protein or nucleic acid synthesis. An additional aspect of these experiments is that the ratio of incorporation into RNA and protein was determined, and thus would be unable to detect any effect on both types of macromolecular synthesis.

Bushell and Bull (1974) found that the oligoamine:RNA ratio increases at faster growth rates of A. nidulans in continuous culture but that the oligoamine and magnesium:RNA ratio remains constant. The functional significance of this is that to a certain extent magnesium and oligoamines are interchangeable for a number of reactions in vitro, and this may occur in vivo as well. Magnesium concentrations increase during germination of conidia and germ-tube emergence is completely dependent on the presence of this divalent cation. Magnesium is known to be a co-factor for a number of intracellular reactions, including nucleic acid and protein synthesis. The oligoamine and magnesium:RNA ratio also falls during growth of the auxotroph on limiting putrescine. Increased supplies of magnesium can not compensate for oligoamine depletion, nor will increased oligoamine levels allow germination in the absence of magnesium. Although some intracellular functions may be filled by either magnesium or oligoamines the cell also has absolute requirements for both types of cations separately. Unfortunately due to the long time required for oligoamine starvation to have significant physiological effects it is not possible to be more precise about the roles of the oligoamines in cell metabolism by such starvation experiments alone.

C Oligoamine Supplementation of the Putrescine Auxotroph puA_1

In the examination of the biochemical details of germination discussed in earlier sections it became apparent that a large number of events occur simultaneously, confusing any functional correlations that might be made. Similarly, during depletion of oligoamines in the putrescine starved cultures of strain puA_1 there is a slow run-down in a number of metabolic processes including RNA and protein synthesis and oxidative metabolism. Because of the difficulties in interpreting the slow, gerneral run-down of metabolism during putrescine starvation, it is difficult to elucidate the primary effects of oligoamines on cellular metabolism. Starvation for oligoamines is a gradual process probably because they turn over very slowly in A. nidulans and are normally maintained at levels greater than that required for the essential functions they perform. However a large, rapid change in oligoamine levels may be brought about by growing the conidia in the absence of putrescine for 12 hours, by which time they have depleted their endogenous supply, and then administering putrescine or another oligoamine. This brings about a rapid increase in the germination of the conidia, even after 24 hours of starvation (Figure 35). Germination proceeds only very slowly in the absence of putrescine. The conidia are not, however, dormant and a decrease in the ability to germinate is apparent after 24 hours starvation.

Protein and Nucleic Acid Biosynthesis

The increase in the rates of protein and nucleic acid biosynthesis is a major event in the germination of the conidia of A. nidulans (Figure 19). As germination follows rapidly after the addition of putrescine to oligoamine starved cultures, the effect of putrescine on macromolecular synthesis was also investigated. The incorporation of ^{3}H-adenine and ^{14}C-leucine

Figure 35

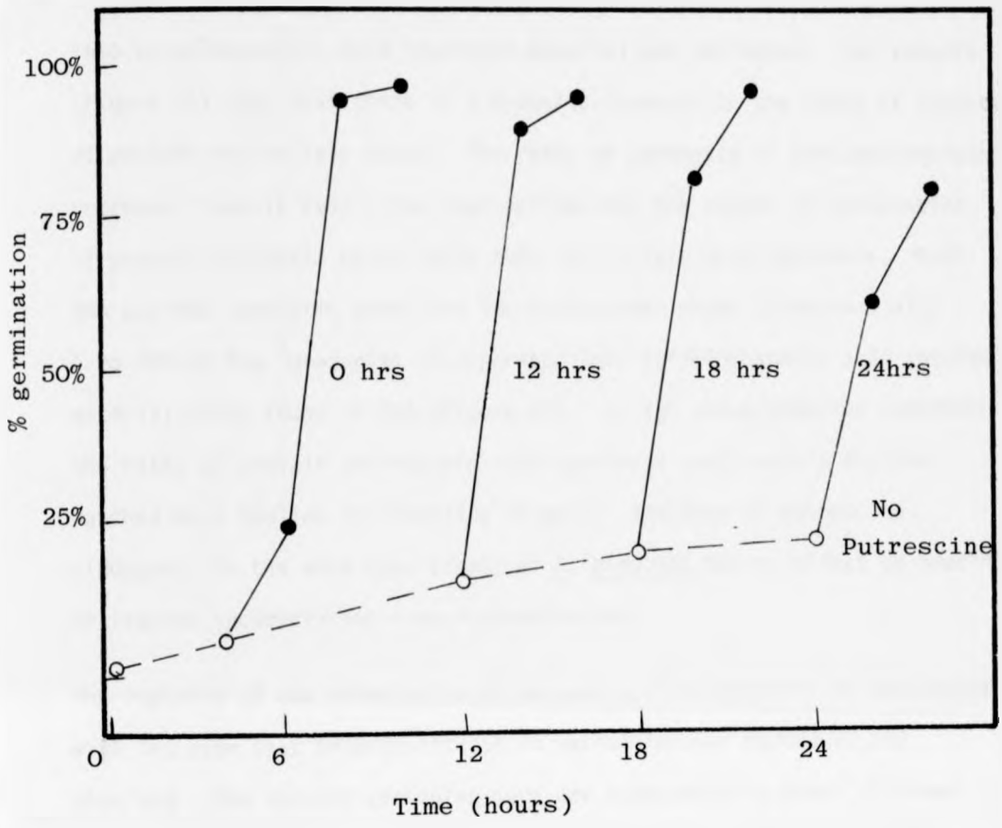


Figure 35 Germination of conidia of the putrescine auxotroph

Putrescine (0.7mM) was added to cultures of the auxotroph at the times indicated and the percentage germination determined. The dashed line indicates the extent of germination in the absence of putrescine (O).

into trichloroacetic acid insoluble material was monitored. The results (Figure 36) show that there is a dramatic increase in the rates of synthesis of protein and nucleic acids. The rates of synthesis of both macromolecules increase linearly over a two hour period with the extent of stimulation of protein synthesis being twice that for nucleic acid synthesis. Both RNA and DNA synthetic rates are increased under these conditions with 6 to 12% of the ^{3}H-adenine incorporated into trichloroacetic acid insoluble material being found in DNA (Figure 37). In the unsupplemented auxotroph the rates of protein and nucleic acid synthesis continuously decline, matched by a decline in viability as well. Addition of putrescine (100µg/ml) to the wild type strain of A. nidulans has no effect on adenine or leucine incorporation into macromolecules.

The rapidity of the stimulation of macromolecular synthesis is consistent with the view that primary effects on macromolecular synthesis are observed. The results presented here are comparable to those obtained with an oligoamine auxotroph of E. coli (Young and Srinivasan, 1972) where an immediate increase in protein content is detectable upon addition of putrescine to putrescine depleted cultures, while RNA and DNA do not increase until one hour later. In this latter study the accumulation of radioactiviaty was measured over an 8 hour period following a single addition of radioactive precursor molecules. In the experiments reported here (e.g. Figure 36), however, short period incorporations of 10 minutes were utilized which give a much more sensitive measurement of the instantaneous rates of synthesis. Both studies are consistent in observing a preferential stimulation of protein synthesis.

Before the stimulation of macromolecular synthesis by putrescine can be studied in greater depth is is important to establish that the rate of incorporation of labelled precursors into acid insoluble material reflects the in vivo rates of macromolecular synthesis. The greatest

Figure 36

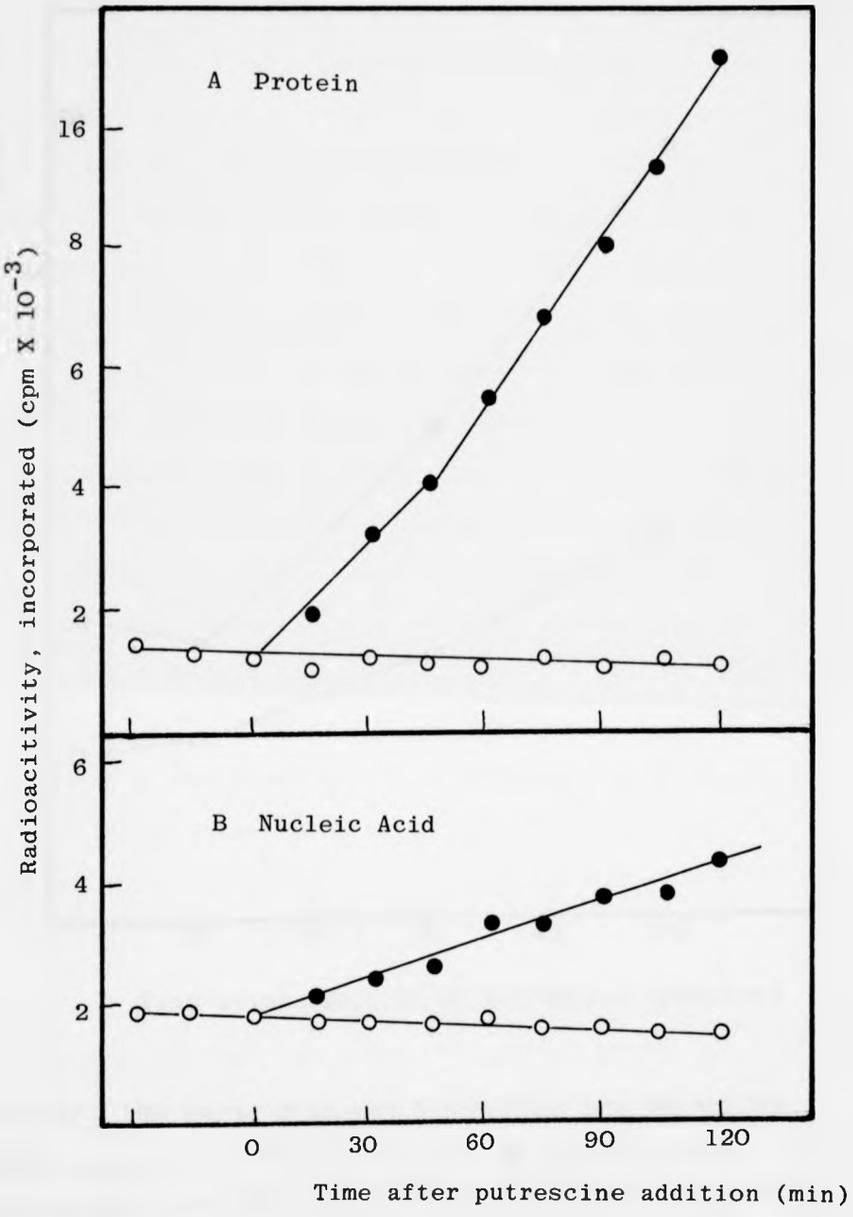


Figure 36 Time course of putrescine stimulation of macromolecular synthesis in puA_1

The incorporation of ^{14}C-leucine (A) and ^{3}H-adenine (B) was measured at intervals in the absence of putrescine (O) and after the additon of putrescine at 0.7mM (●) to one part of the culture after 12 hours growth in the absence of putrescine.

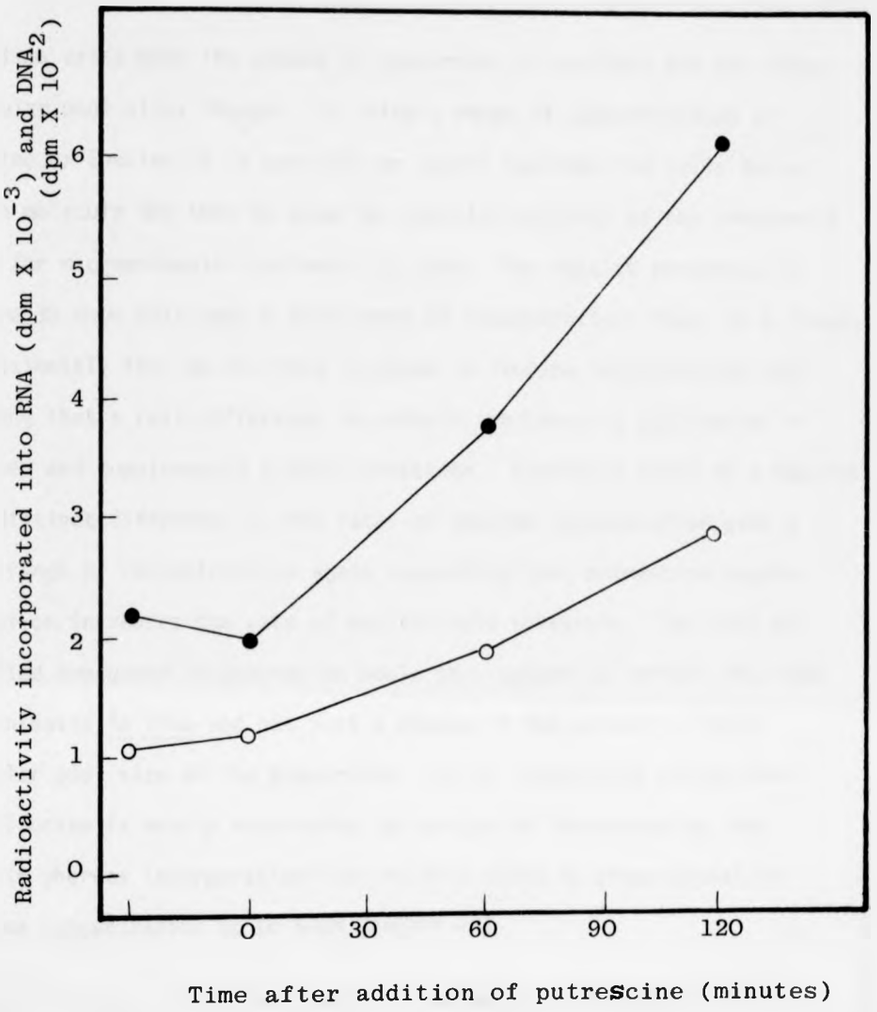


Figure 37 Time course of adenine incorporation into RNA and DNA

The incorporation of ^{14}C-adenine into RNA (●) and DNA (○) was distinguished on the basis of solubility in 0.5M NaOH as described in Materials and Methods. Putrescine (0.05mg/ml) was added 12 hours after inoculation with conidia of strain puA_1.

problems arise when the uptake of precursors is variable and the intracellular pool sizes change. By using a range of concentrations of adenine or leucine it is possible to nearly saturate the cells for a given molecule and thus to know the specific activity of the precursors used for macromolecular synthesis in vivo. The results presented in Figure 38 show that over a wide range of concentrations there is a large, approximately five to six-fold increase in leucine incorporation suggesting that a real difference in protein synthesis is detectable in starved and supplemented growth conditions. Similarly there is a smaller but distinct difference in the rates of adenine incorporation over a wide range of concentrations again suggesting that putrescine supplementation increases the rate of nucleic acid synthesis. The rate of labelled precursor incorporation would thus appear to reflect the rate of synthesis in vivo and not just a change in the uptake or intracellular pool size of the precursors. It is interesting to note that 20μM leucine is nearly saturating for precursor incorporation into protein whereas incorporation into nucleic acids is proportional to adenine concentration up to 60μM, (Figure 39).

The specificity of the stimulation of macromolecular synthesis by oligoamines is shown in Figure 40. Putrescine brings about the greatest stimulation of both protein and nucleic acid biosynthesis but spermidine and spermine are active in this respect as well. Cadaverine, 1,3-diaminopropane, ornithine and arginine have little effect on macromolecular synthesis. The differential abilities of the oligoamines to stimulate macromolecular synthesis correlate well with the observation that putrescine is taken up from the media forty times as rapidly as spermidine or spermine and that spermine is taken up more rapidly than spermidine (Hope and Stevens, 1976). When grown with putrescine at 10μg/ml for one hour nearly 2%

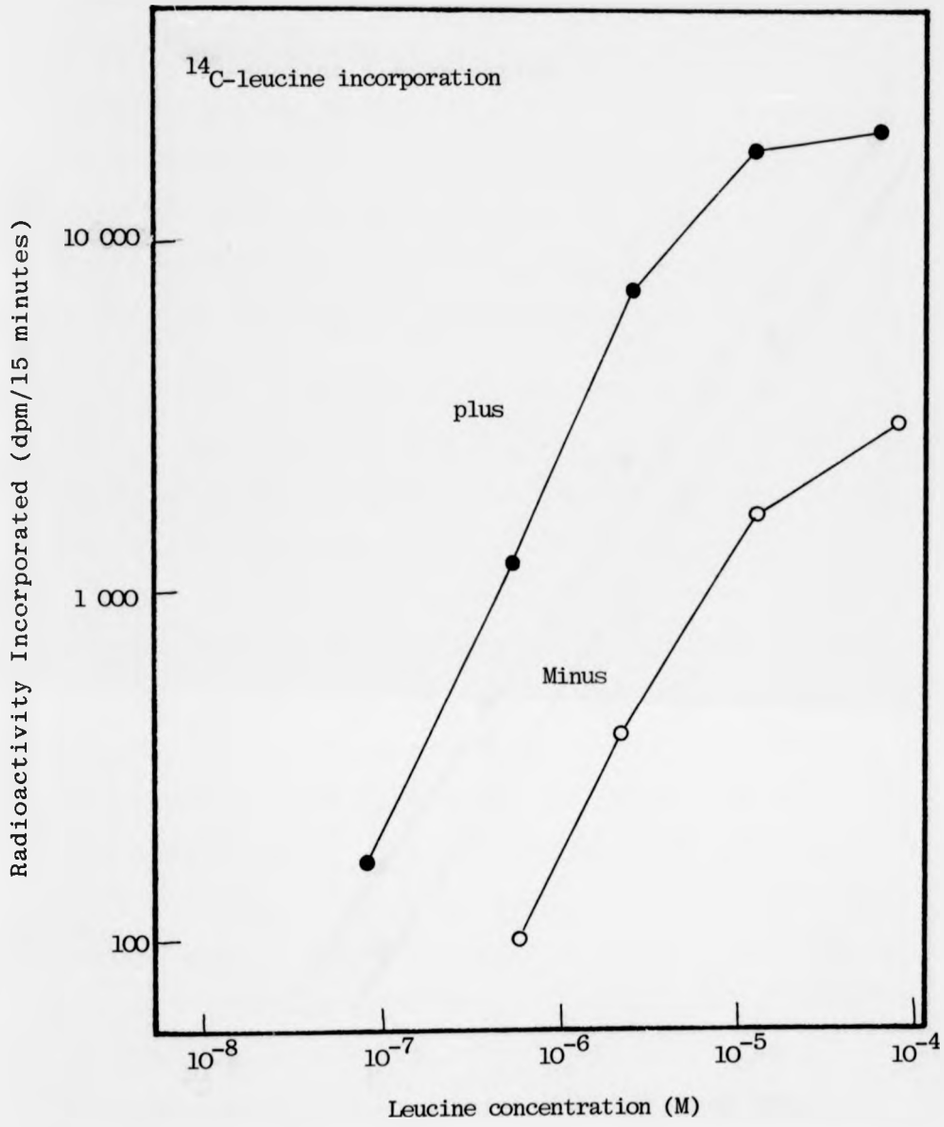


Figure 38 Effect of leucine concentration on the incorporation of precursor into protein in puA$_1$

Conidia were grown for 12 hours in the absence of putrescine (O), with putrescine added to one part of the culture for a further hour(●). ^{14}C-leucine (167μCi/mmole) was added to 5 mls conidial suspension at at range of concentrations and after 15 minutes incubation the acid insoluble counts were estimated as described in Methods.

Figure 39

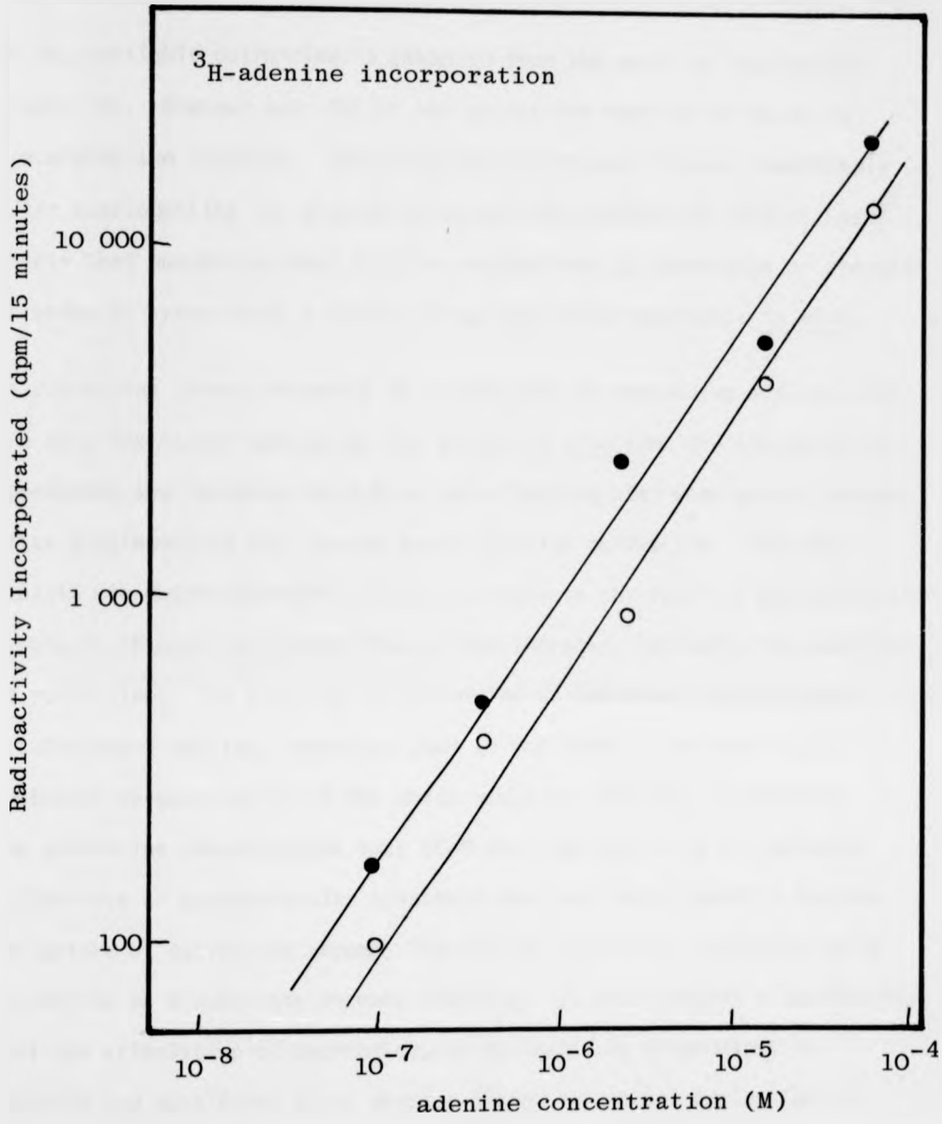


Figure 39 Effect of adenine concentration on the incorporation of precursor into nucleic acids in puA$_1$

Conidia were grown for 12 hours in the absence of putrescine (O), with putrescine added to one part of the culture for a further hour (●). ^{3}H-adenine (17mCi/mmole) was added to 5mls conidial suspension at a range of concentrations and after 15 minutes incubation the acid insoluble counts were estimated as described in Methods.

of the available putrescine is taken up from the media by the conidia (Table 16). However over 98% of the putrescine taken up is found in spermidine and spermine. Thus oligoamine synthesis resumes immediately after supplementing the starved auxotroph with putrescine, and it seems likely that putrescine must first be metabolized to spermidine or spermine in order to bring about a change in macromolecular synthesis *in vivo*.

If putrescine serves primarily as a precursor to spermidine and spermine and only the latter two amines are active *in vivo* then the synthesis of spermidine and spermine could be a rate-limiting step when growth resumes after supplementing the starved auxotroph with putrescine. This may explain the observation of a linear increase in the rates of macromolecular synthesis (Figure 36) rather than a step increase, following the addition of putrescine. The addition of spermidine or spermine directly does not produce a massive, immediate jump in the rates of macromolecular synthesis because uptake of the amine would be limiting. Increasing the putrescine concentration over 60μM does not result in an increase in the rate of macromolecular synthesis one hour later possibly because the uptake of putrescine becomes limiting or spermidine synthesis using putrescine as a substrate becomes limiting. It also remains a possibility that the stimulation of macromolecular synthesis by oligoamines is indirect and must first bring about a change in another process which may be the rate-limiting step.

One approach to investigate which oligoamines are active in stimulating macromolecular synthesis *in vivo* is to obtain mutants with altered uptake of oligoamines. If a mutant could be found with an uptake of spermidine equivalent to that of putrescine it would be a simple matter to see which allowed greater macromolecular synthesis. A mutant that is very sensitive to low concentrations of spermidine and spermine, designated $spsA_1$, has been

Table 16

Metabolic Fate of Incorporated Putrescine in puA$_1$

Location	
Total Radioactivity	% Total Radioactivity
Incorporated (taken up from media)	1.8
unincorporated	98.2
Incorporated Radioactivity	% Incorporated Radioactivity
Oligoamines	92
Other acid soluble material	2
Acid insoluble Material	6
Oligoamines	% Radioactivity in various Oligoamines
Putrescine	1.3
Spermidine	73.9
Spermine	24.8

Conidia (0.5×10^7ml) were grown in the absence of putrescine for 12 hours. Putrescine incorporation was for one hour (0.05μCi/ml, 0.6mM) after which time the conidia were rapidly harvested, washed thoroughly and the oligoamines extracted and separated by paper electrophoresis as described in Materials and Methods.

identified, (N Spathas and J Clutterbuck, unpublished observations). The double mutant puA_2,$spsA_1$ will grow on lower concentrations of spermidine than can support growth and conidiation of the single auxotroph puA_2. Though these observations are compatible with an increased uptake of spermidine and spermine by $spsA_1$ direct measurements of the uptake of oligoamines are not consistent with this simple hypothesis, (N Spathas, unpublished observations).

Starvation of this mutant ($spsA_1$, puA_2,bi_1) for 12 hours followed by the addition of putrescine, spermidine or spermine resulted in an increase in leucine incorporation (Figure 41) almost identical to that found with the putrescine auxotroph puA_1 (figure 40). Spermidine and spermine are slightly better able to stimulate protein synthesis in $spsA_1$ but only at one hundred times the concentration of putrescine required to obtain an equal stimulation of macromolecular synthesis. Particularly noticeable is the inhibition of protein synthesis at the highest concentrations of spermine. Spermine can be toxic not only to A. nidulans but also to a variety of other fungi (Razin et al, 1958; Sakurado and Matsumura, 1964). Overall the stimulation of protein synthesis by putrescine in $spsA_1$,puA_2 is greater than that in puA_1 and the former may also be a good organism for further study of oligoamine functions. The puA_1 and puA_2 mutations do, however, appear to have very similar properties with regards to the stimulation of macromolecular synthesis by oligoamines.

The rapidity of the stimulation of the rates of protein and nucleic acid biosynthesis by oligoamines is consistent with a primary role in macromolecular synthesis. As the stimulation of protein synthesis is more marked than nucleic acid synthesis the latter could simply be a secondary

Figure 40

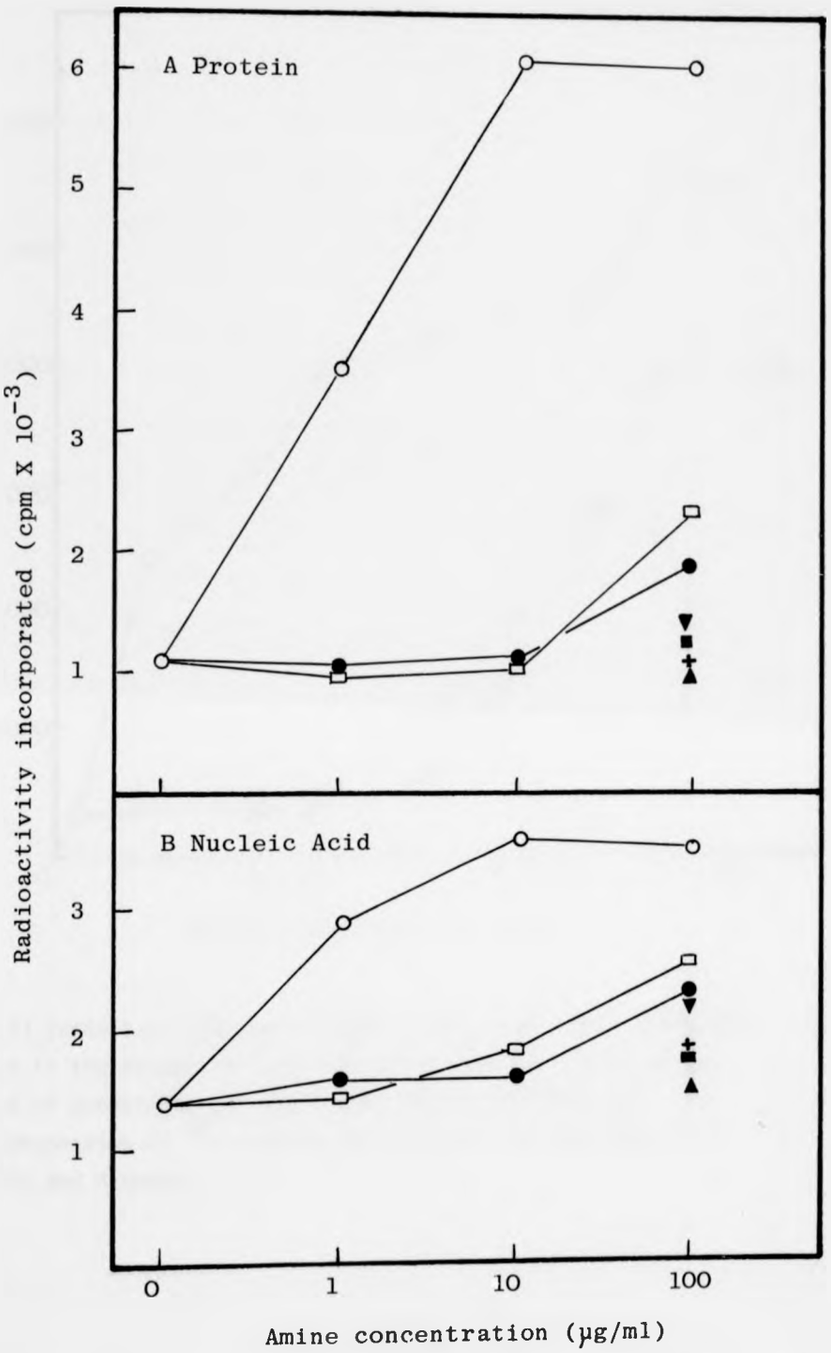


Figure 40 Stimulation of macromolecular synthesis by amines and amino acids - The incorporation of ^{14}C-leucine A and ^{3}H-adenine B was measured as described in Methods, one hour after the addition of putrescine (○), spermidine (●), spermine (□), 1,3 diaminopropane (▼), cadaverine (■), arginine (+) or ornithine (▲) at the concentrations indicated using strain puA_1.

Figure 41

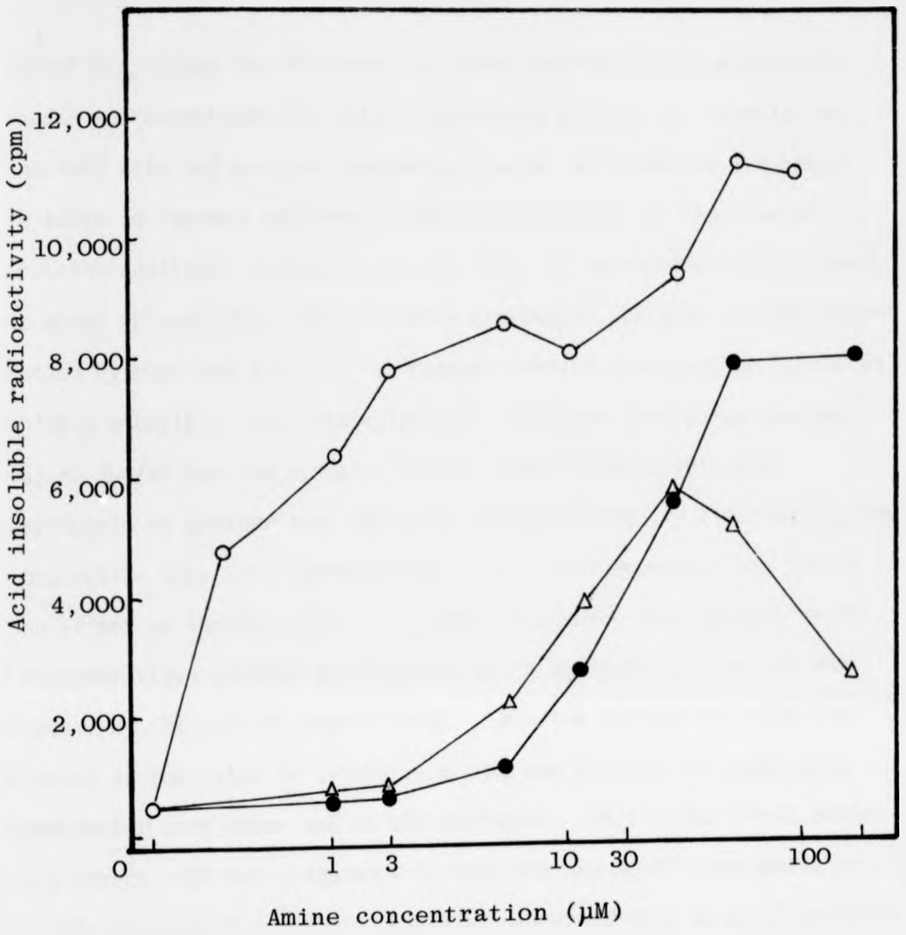


Figure 41 Conidia of the double mutant (puA_2 $spsA_1$) were grown for 12 hours in the absence of putrescine and then for 1 hour in the presence of putrescine (O) spermidine (●) or spermine (Δ). The incorporation of ^{14}C-leucine was monitored as described in Materials and Methods.

response to a change in the former but other explanations are possible. In order to investigate the interrelationship between the stimulation of nucleic acid and protein synthesis, a series of metabolic inhibitors were added to starved cultures of the auxotroph puA_1 at the time of putrescine addition (12 hours) and the rates of macromolecular synthesis determined (Figure 42). Cycloheximide at 20µg/ml inhibits leucine incorporation by over 55% but does not depress adenine incoporation into acid insoluble material. The transcriptional inhibitor proflavine (Waring, 1966) at 3µg/ml has the opposite effect, inhibiting nucleic acid biosynthesis by greater than 60% while not significantly altering leucine incorporation into acid insoluble material. 5-Fluorouracil has little or no effect on incorporation of leucine or adenine into protein or RNA at concentrations (1µg/ml and 50µg/ml) which decrease the rate of DNA synthesis by 48% and 63% respectively. Thus the putrescine-stimulated increases in the rates of synthesis of RNA and protein are apparently independent of each other and of DNA synthesis. This is consistent either with a direct role for oligoamine in both RNA and protein synthesis or with RNA and protein synthesis responding independently to an oligoamine affected process of another sort, such as energy metabolism or availability of precursors and co-factors. However these experiments do appear to rule out the possibility that the sole primary effect of oligoamines on cellular metabolism is a stimulation of DNA synthesis as has been suggested for other systems (Wiegland and Pegg, 1978; Morris et al, 1977; Pösö and Jänne 1976; Sunkara et al, 1977). Oligoamines may have a primary role in DNA synthesis in A. nidulans but this would not account for the oligoamine induced changes in RNA and protein synthesis or for the oligoamine requirement during conidial germination.

A summary of the results for the oligoamine and macromolecular contents

Figure 42

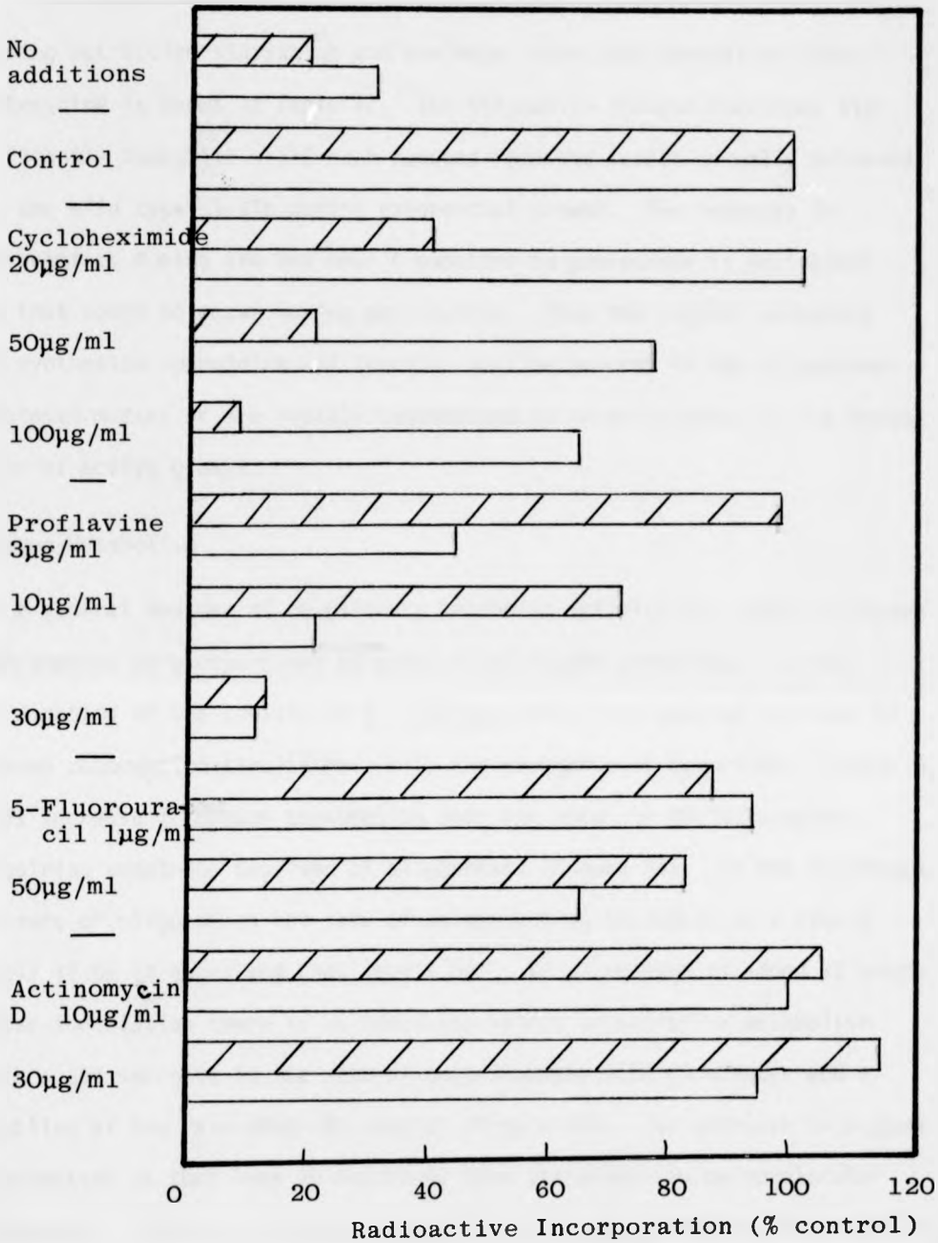


Figure 42. The effects of metabolic inhibitors on macromolecular synthesis

The incorporation of ^{3}H-adenine and ^{14}C-leucine was measured one hour after the addition of inhibitors. Putrescine (0.7mM) was added to all samples except for 'no additions'. Control values for adenine and leucine incorporation were 2040 and 6130 cpm respectively, using strain puA$_1$.

during putrescine starvation and one hour after supplementation with putrescine is shown in Table 17. The oligoamine content increases significantly though it is still much reduced from the levels normally achieved by the wild type strain during exponential growth. The increase in oligoamines during the one hour exposure to putrescine is equivalent to that found to occur during germination. Thus the enzymes necessary to synthesize spermidine and spermine must be present in the oligoamine-depleted mutant or are rapidly synthesized as an early event in the resumption of active growth.

Oxygen Metabolism

As a general measure of respiratory metabolic activity the rates of oxygen consumption were determined by means of an oxygen electrode. In the germination of the conidia of A. nidulans there is a massive increase in oxygen consumption simultaneous with the emergence of germ-tubes (Figure 9). This increase in oxygen consumption does not occur in the putrescine-requiring auxotroph deprived of oligoamines (Figure 34). In the continued absence of oligoamines the rate of oxygen uptake increases only slowly until 12 to 14 hours and then levels off. If putrescine is added 12 hours after inoculation there is an immediate effect on oxidative metabolism with a 35% increase in the rate of oxygen uptake with 15 minutes and a doubling of the rate after 90 minutes (Figure 43). The increase in oxygen consumption is thus less in magnitude than the effect on macromolecular synthesis. The rate of oxygen consumption is proportional to the amount of protein found in the cells. It would appear that the oxidative metabolism of the cell is dependent on the growth rate and/increased oxygen uptake demonstrates that metabolic activity of the cells is a rapid response to the addition of putrescine to oligoamine-depleted cultures and probably affects many aspects of cell metabolism. Though it is not possible to

Table 17

Macromolecular synthesis and accumulation following the addition of putrescine to starved cultures of the auxotroph puA_1.

	Addition to media after 12 hrs growth in the absence of putrescine.	
	none	putrescine
Macromolecular Content ($\mu g/10^8$ conidia)		
protein	490	610
RNA	184	282
DNA	3.91	5.15
Oligoamine Content (nmole/ 10^8 conidia)		
Spermidine	7.2	18
Spermine	8.1	9
Macromolecular Rates of synthesis (cpm/ 15 minutes)		
Protein	1,930	9,810
RNA	1,160	3,500
DNA	130	400

All extimations were carried out as described in Methods.

Figure 43

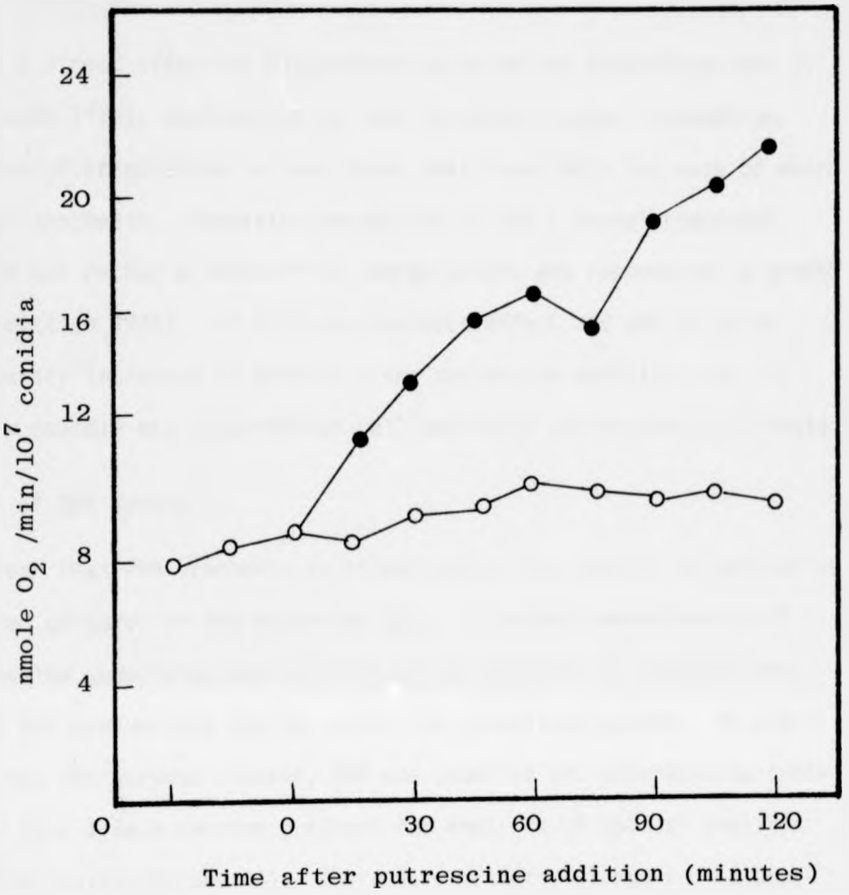


Figure 43 The stimulation of oxygen consumption by the addition of putrescine to starved cultures of the auxotroph puA_1

0.3mM Putrescine (●) was added to part of the culture after 12 hours growth in the absence of putrescine and oxygen consumption determined as described in Materials and Methods.

rule out a direct effect of oligoamines on oxidative metabolism, this is not the most likely explanation for the increased oxygen consumption. The extent of stimulation is less thant that found with the rate of macromolecular synthesis. Oxidative metabolism is not a normal regulator of growth but rather a "mirror" as energy levels are responsive to growth rates (Gottlieb,1976). If it is an indirect effect and yet is quite significantly increased 15 minutes after putrescine addition, then it shows how rapidly the interrelated cell machinery can respond to stimuli.

Patterns of RNA Synthesis

It is clear that RNA synthesis is stimulated by the additon of putrescine to starved cultures of the auxotroph puA_1. A better understanding of the mechanism underlying this process may be obtained by studying the types of RNA synthesized during putrescine stimulated growth. In order to separate the various classes, RNA was labelled and separated by centrifugation in a linear sucrose gradient for analysis of optical densities and radioactivity (Figure 44). There is a dramatic change in the proportions of the various RNA classes synthesized brought about by the addition of putrescine. The proportion of small RNA (4-5S) clearly accounts for the majority of the RNA synthesized during putescine starvation, while the addition of putrescine reverses this pattern and large rRNA (18-28S) accounts for the majority of the RNA synthesized. The transition from one pattern to the other occurs within 30 minutes. During this time there is little change in the rate of tRNA synthesis while the rate of rRNA synthesis increases approximately four-fold. The material at the bottom of the gradient, heavier than 25S rRNA, probably represents the precursor of rRNA. The amount of DNA present in these samples is less than 5% wt/wt of the RNA.

Figure 44a

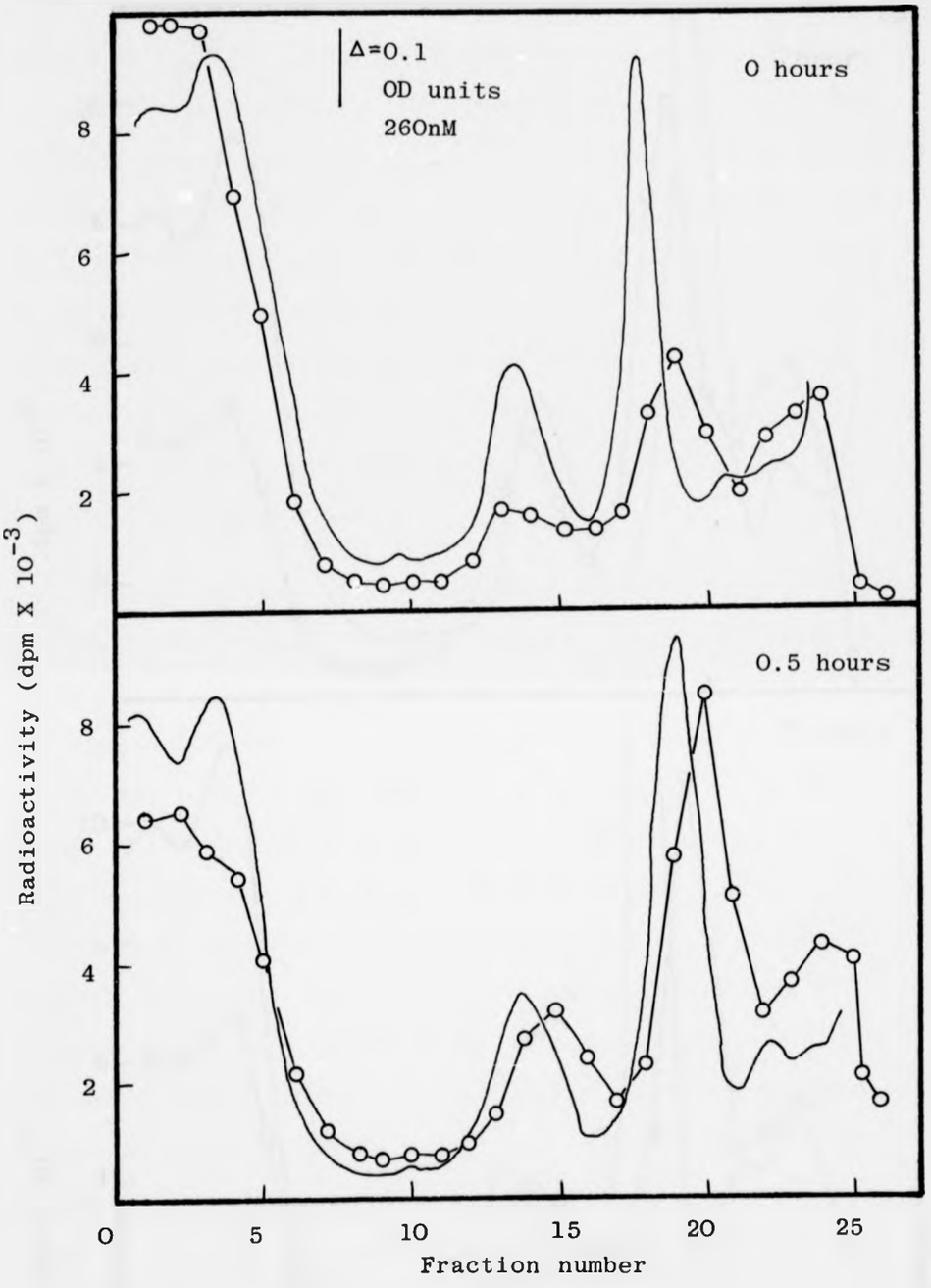
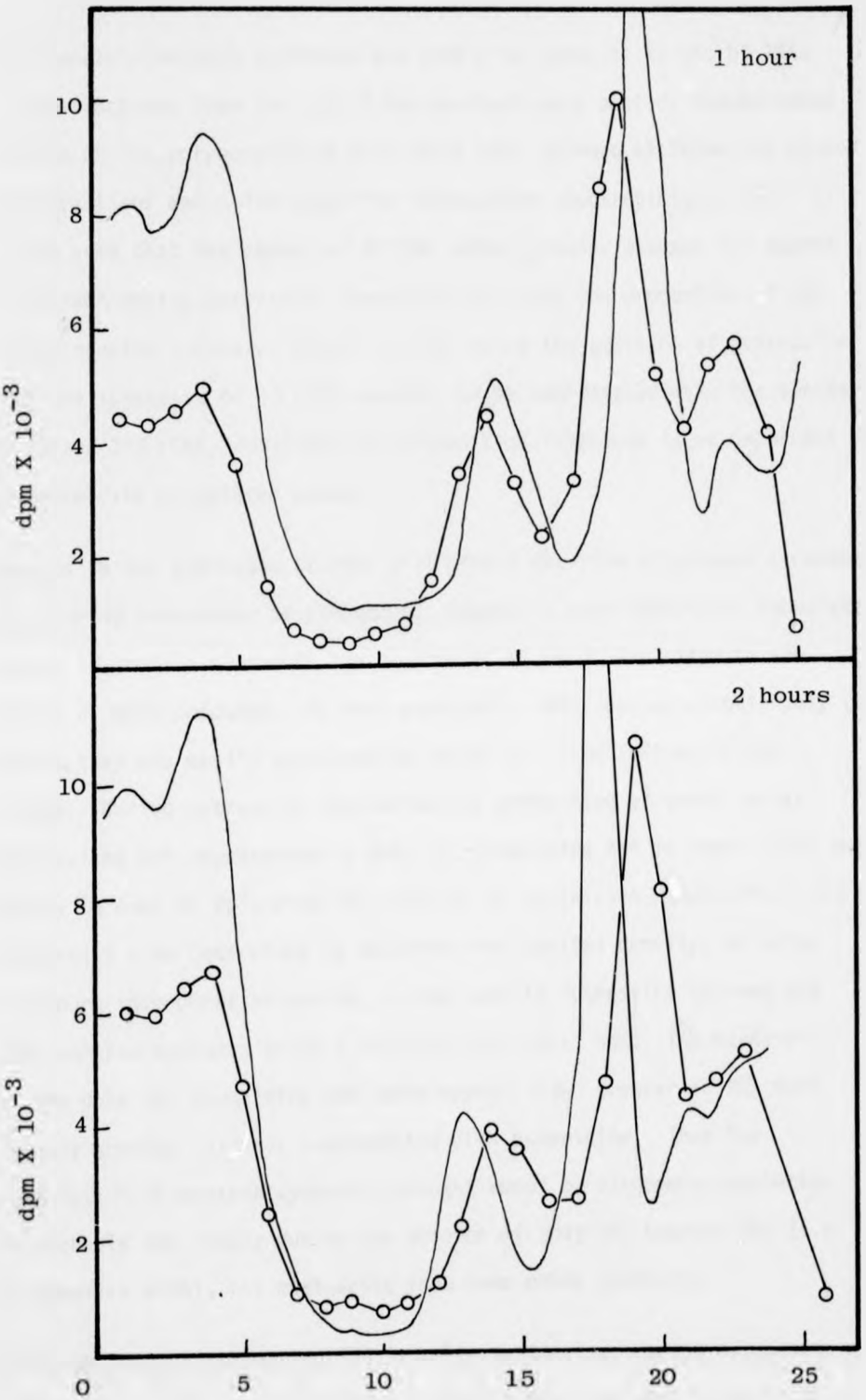


Figure 44 Separation of RNA by sucrose gradient centrifugation

Conidia were grown for 12 hours in the absence of putrescine, then supplemented with putrescine (0.7mM) and samples taken at the times indicated and labelled with ^{3}H-adenine (0.1 µCi/ml, 100mCi/mmole). RNA was extracted and separated as described in Methods, using strain puA$_1$.

Figure 44b



Unfortunately sucrose gradients are unable to separate 4S and 5S RNAs so the fractions from the top of the gradient were pooled, concentrated and run on 10% polyacrylamide gels which were scanned at 260nm and sliced into fractions and solubilized for radioactive counting(Figure 45). It can be seen that the amount of 4S RNA (tRNA) greatly exceeds the amount of 5S rRNA during putrescine starvation but that the proportion of the latter species increases (Table 19) following the addition of putrescine. Thus the synthesis of 5S rRNA appears to be coordinated with the synthesis of 18 and 25S rRNA. Additionally, synthesis of ribosomes is an important event in putrescine stimulated growth.

Changes in the synthesis of rRNA will affect the rate of protein synthesis by altering the number of ribosomes. However a more direct and immediate change in protein synthesis can be brought about by variation in the amount of mRNA produced. As most eukaryotic mRNA species contain poly (A) tracts, they are easily separated by use of an oligo (dT)-cellulose column. During putrescine starvation the proportion of total newly synthesized RNA represented by poly (A)-containing RNA is about 1.5%, which rises to over 2% following the addition of putrescine, (Table 18). The proportion when determined by measuring the optical density, or after two hours incorporation period, is the same in putrescine starved and supplemented cultures being 1.1-1.2% of the total RNA. The turnover of the poly (A) containing RNA would appear to be greater in the more rapidly growing cultures supplemented with putrescine. Thus the restriction of protein synthesis brought about by oligoamine depletion is probably not simply due to the absence of poly (A) bearing RNA (i.e. presumptive mRNA), but must arise from some other factor(s).

Although poly(A) containing RNA is being synthesized during putrescine starvation it may be qualitatively differerent from that produced in

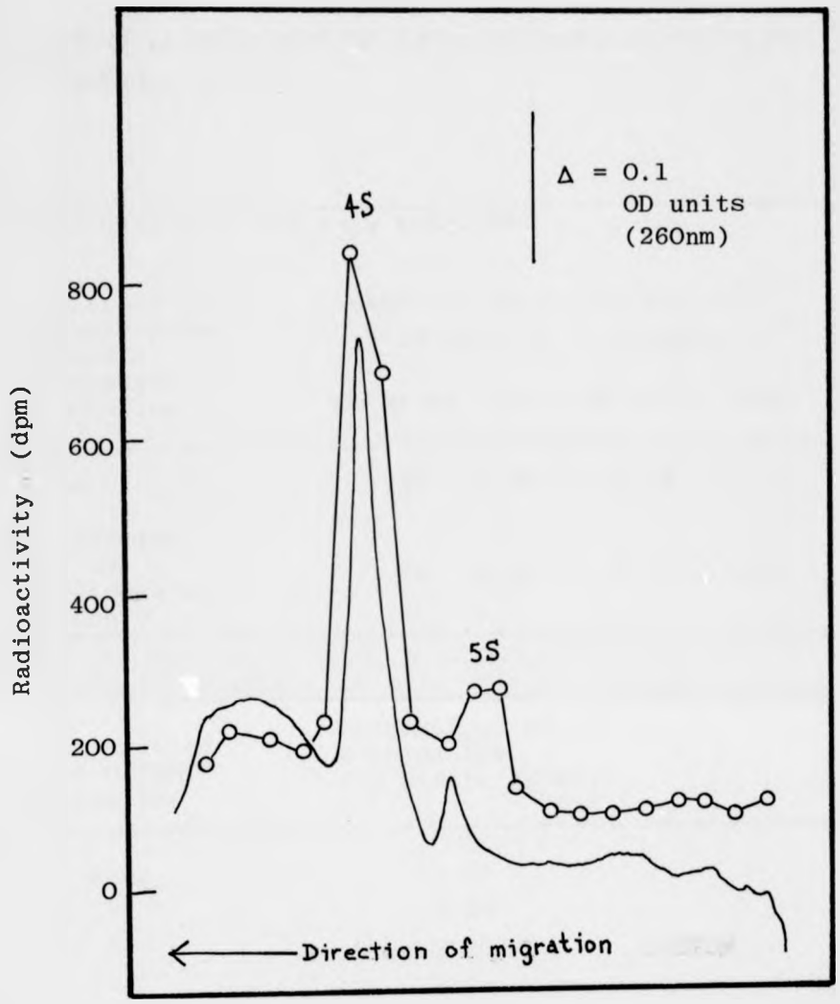


Figure 45 Typical separation of 4S and 5S RNA from puA1

Conidia were grown for 12 hours in the absence of putrescine then for a further 0.5 hours with putrescine (0.7mM). Extraction of RNA, separation by SDS polyacrylamide gel electrophoresis (15% acrylamide) and determination of radioactivity (O) were carried out as described in Materials and Methods.

Synthesis of polyadenylated RNA during putrescine starvation and supplementation of puA_1.

A

Polyadenylated RNA as % total RNA				
Additions to culture after 12 hours putrescine starvation	Length of labelling period 15 minutes		2 hours	
	OD units	dpm	OD units	dpm
None	1.21	1.36	1.18	1.11
Putrescine (O.7mM) supplemented (1 hour)	1.36	2.16	1.30	1.49

B

Time supplemented with O.7mM putrescine	Polyadenylated RNA as % total RNA (15 minute label)
O hour	1.36
O.5 "	2.36
1 "	2.16
2 "	3.31

Table 18 RNA was labelled, separated by oligo (dT) cellulose chromatography and the optical density (260nm) and radioactivity determined as described in Methods.

Patterns of RNA synthesis during putrescine starvation of puA_1.

Time after putrescine addition (hours)	Amounts as % total RNA				
	4S tRNA	5S rRNA	18-25S rRNA	poly(A)$^+$ RNA	remainder
0	57	1.9	32	1.31	8
0.5	35	2	49	1.73	12
1	27	2.5	53	1.6	16
2	19	3	51	1.65	25

* "remainder" refers to radioactivity not binding to an oligo (dT) cellulose column or coincident with the rRNA and tRNA peaks.

Conidia were grown for 12 hours in the absence of putrescine and then supplemented with putrescine for the times indicated. The amounts of radioactivity present in the phenol extract of RNA from 5×10^8 conidia incubated with ^{3}H-adenine (2μCi, 1μM) were 29,780, 58,100, 66,400 and 73,300 dpm for conidia grwon with putrescine for 0, 0.5, 1 and 2 hours respectively.

putrescine supplemented cultures. If oligoamines are necessary for faithful transcription then non-functional mRNA may be produced and due to premature termination may be shortened in length. Sucrose gradient analysis (Figures 46 and 47) reveals a similar spectrum of molecular weights for the poly $(A)^+$RNA extracted from both putrescine starved and supplemented cultures, with an average size of between 5S and 18S. A small amount of rRNA binds non-specifically to the affinity column (Figure 46) but can be removed by a more extensive washing of the column before eluting the poly $(A)^+$RNA (Figure 47). In order to determine the accuracy and translatability of the transcripts an in vitro protein synthesizing system capable of producing functional products is required. However gross defects in the RNA produced can probably be ruled out on the basis of the experiments reported here.

A summary of the patterns of RNA synthesized during oligoamine depletion and subsequent additon of putrescine is shown in Table 19. The dramatic shift in the relative proportions of rRNA and tRNA is evident 15 minutes after the addition of putrescine. The total amount of tRNA synthesized remains approximately constant while the synthesis of rRNA increases markedly. The amount of the presumptive precursor of rRNA, present in large amounts during oligoamine depletion, increases further during the putrescine stimulated growth. This may indicate that the resumption of pre-rRNA synthesis is so rapid that processing and ribosome assembly becomes rate-limiting, possibly for a lack of ribosomal proteins. Echandi and Algranati (1975) proposed that oligoamines have a role in ribosome assembly in E. coli. Analysis by sucrose gradient centrifugation shows that 30S ribosomal subunits isolated from oligoamine-depleted bacteria have a high proportion of defective particles which bind only weakly to the 50S subunits.

Figure 46

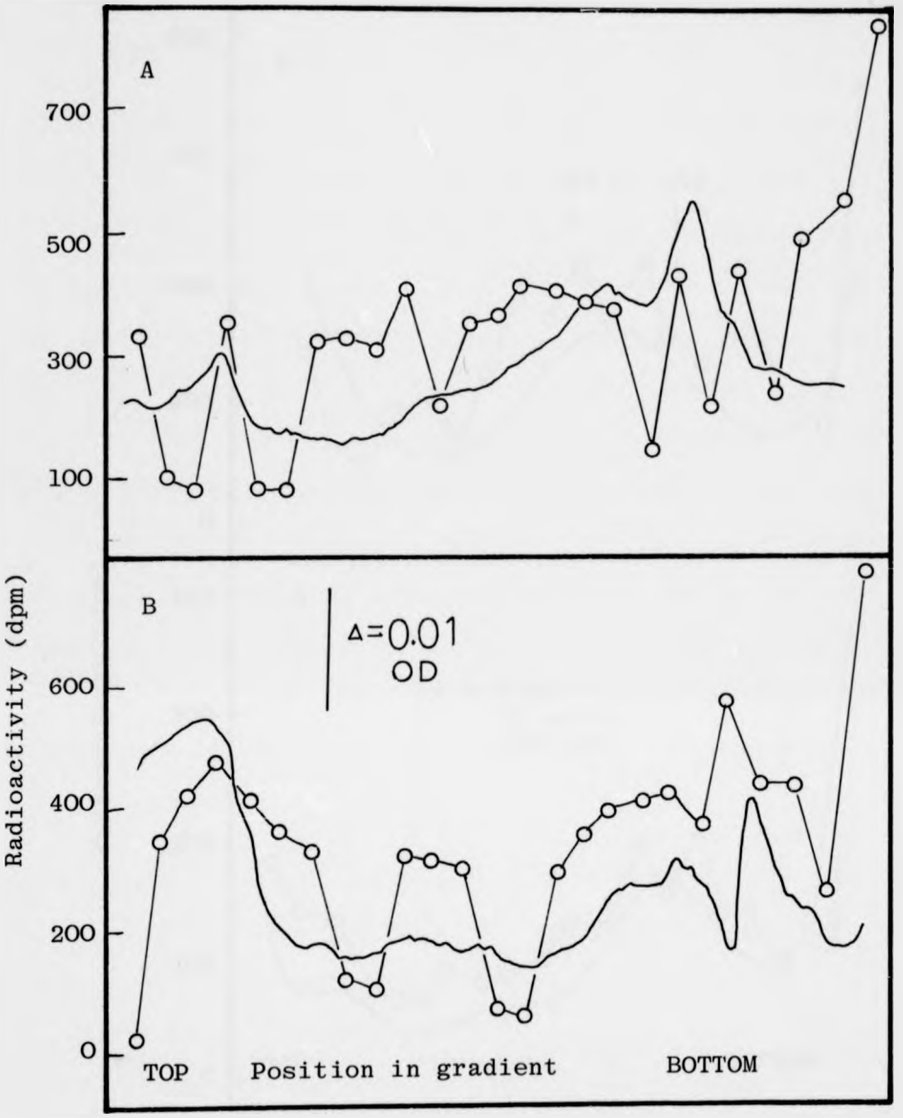


Figure 46 Sucrose gradient analysis of polyadenylated RNA of puA1

RNA was extracted from conidia grown in the absence of putrescine(B) and supplemented with 0.7mM putrescine for one hour(A)and separated by centrifugation in a linear sucrose gradient as described in Methods. The RNA bound to the oligo (dT) cellulose column was washed with 8mls binding buffer before elution of the poly A RNA.

Figure 47

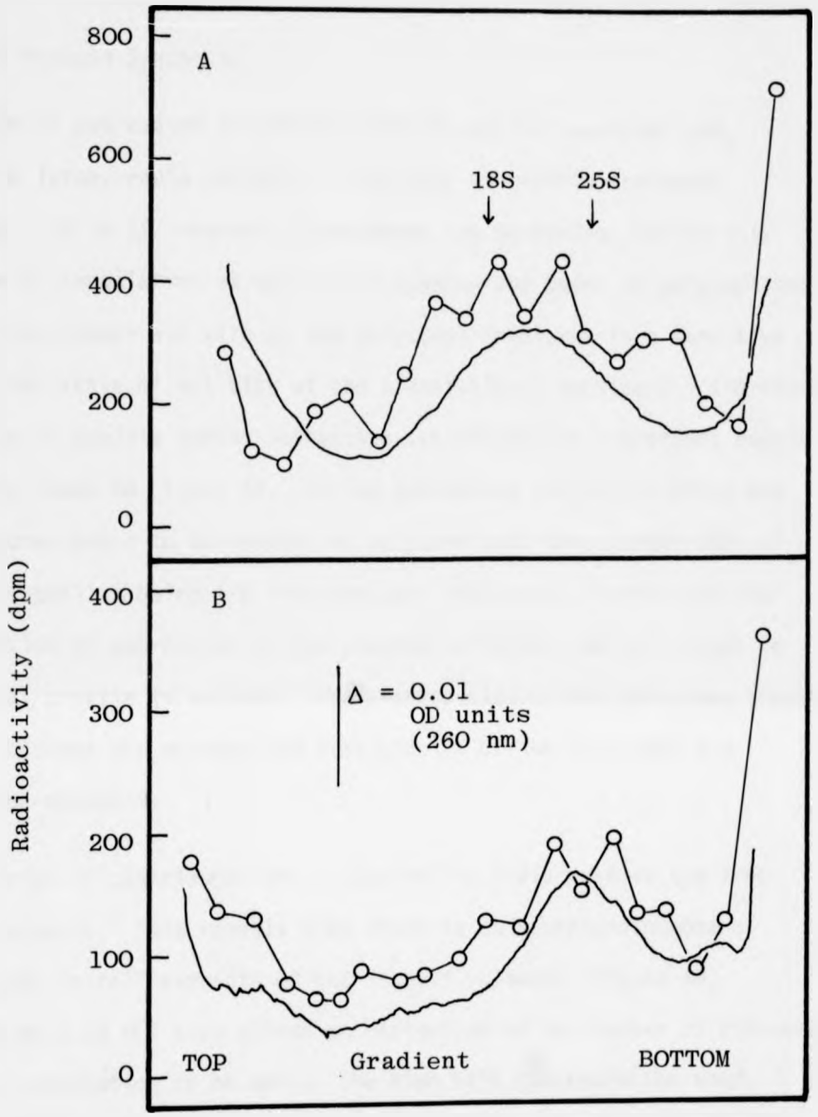


Figure 47 Sucrose gradient analysis of polyadenylated RNA of puA1

RNA was extracted from conidia grown in the absence of putrescine B and supplemented with 0.7mM putrescine for one hour A and separated by centrifugation in a linear sucrose gradient as described in Methods. The RNA bound to the oligo (dT) cellulose column was washed with 30mls binding buffer before elution of the poly A RNA.

Patterns of Protein Synthesis

The addition of putrescine to starved cultures of the auxotroph puA_1 results in a large, rapid increase in the rate of protein synthesis (Figure 36). It is of interest to determine the mechanisms behind this stimulation of translation as well as to examine the types of polypeptides produced. The number and size of the polysomal complexes is a sensitive measure of the state of activity of the translational machinery. The ribosome distribution in conidia during putrescine starvation and subsequent supplementation is shown in Figure 48. During putrescine starvation there are fewer ribosomes bound to messengers as polysomes and the average size of polysomes is smaller, being 4-5 ribosomes per messenger. Within one hour of the addition of putrescine to the starved cultures a marked change in the polysomal profile is evident. The average size of the polysomes increases to 12-15 ribosomes per message and less than 2% of the ribosomes are found as free monomers.

A longer period of centrifugation is required to fully resolve the free ribosomal subunits. This reveals that there is an increased number of subunits found in cell extracts of the starved cultures (Figure 49) The addition of 0.5M KCl also allows an estimation of the number of ribosomes involved in translation to be made. The high salt concentration used in the gradients should have caused any run off 80S ribosomes (i.e. not attached to mRNA) to dissociate into 40S and 60S subunits. Consequently all 80S particles occurring in the sucrose gradients should represent single, actively translating ribosomes as shown with Mucor racemosus (Orlowski and Sypherd, 1978b). The sum of all polysomes plus all 80S ribosomes gives the percentage of cellular ribosomes active in protein synthesis.

The percentage active polysomes and average size of the polysomes isolated

Figure 48

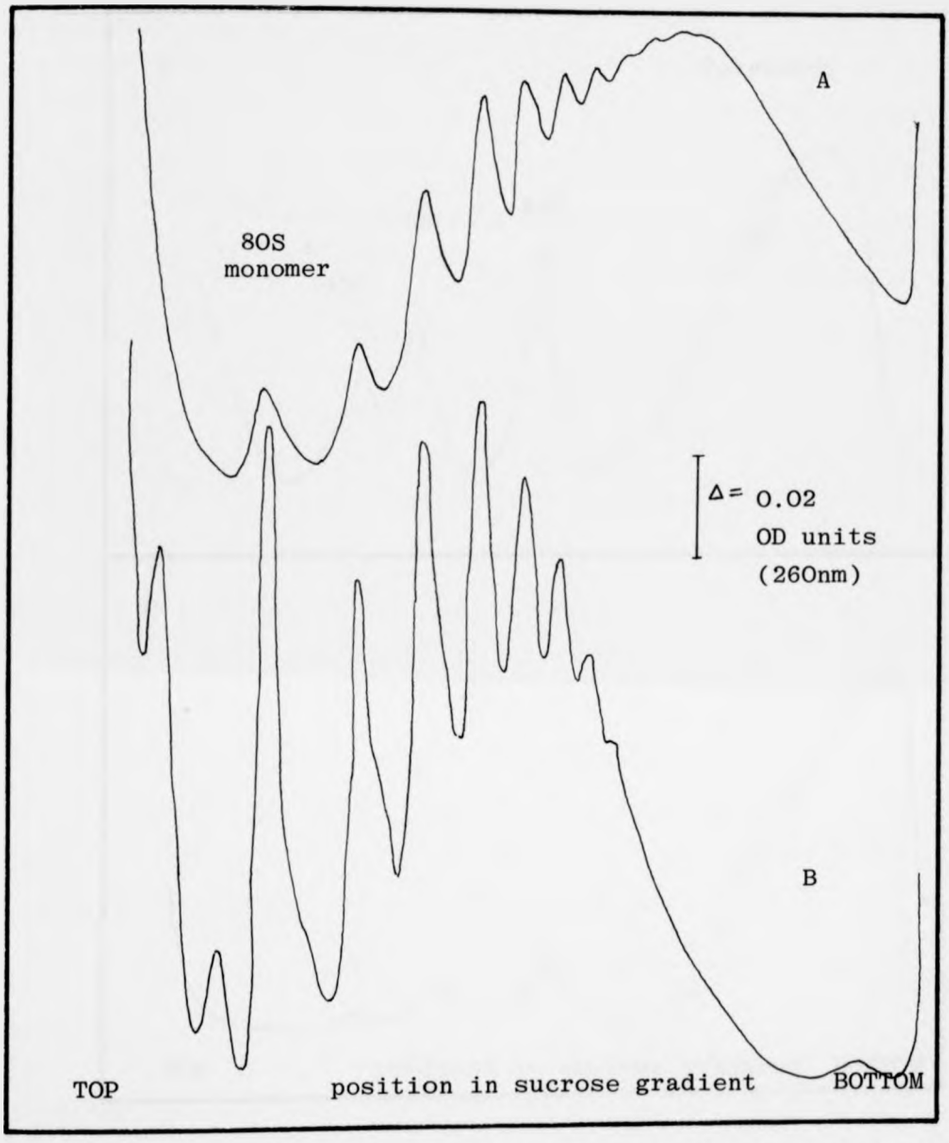


Figure 48 Ribosome distribution in conidia during putrescine starvation of *puA*1

Conidia were inoculated into a media lacking putrescine for 12 hours. One portion of the culture was then incubated in the presence of putrescine (0.3mM) for one hour A, while another was incubated in the continued absence of putrescine B. Extraction and analysis of polysomes was carried out as described in Materials and Methods.

Figure 49

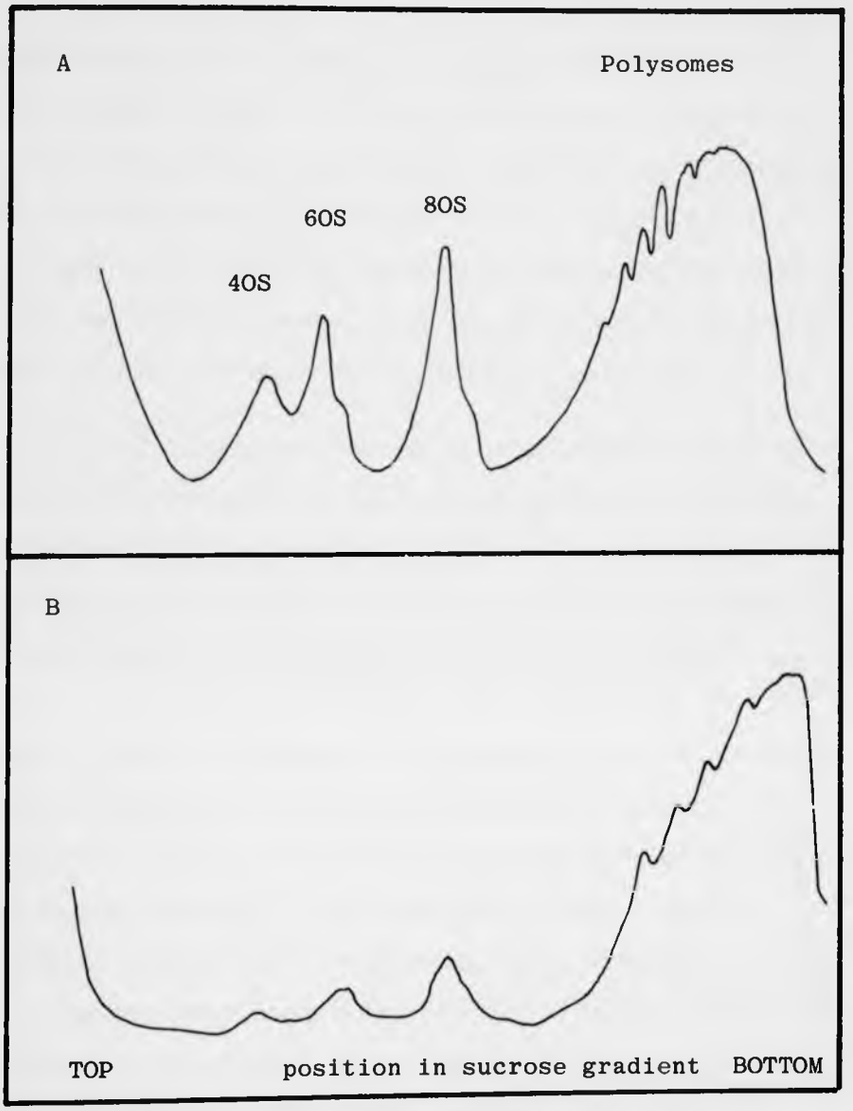


Figure 49 Ribosomes, subunits and polysomes during putrescine starvation and supplementation of pu^{A_1}

Details are as in Figure 48 with the addition of 0.5m KCl to all buffers and a 7 hour centrifugation at 110 000g to fully resolve the ribosomal subunits.

from extracts made at various times after the addition of putrescine to starved cultures is shown in Table 20a. The average size increases rapidly, more than doubling within 30 minutes from 4 to over 10 ribosomes per mRNA. The proportion of the ribosomes found as subunits decreases as well, though even after 12 hours growth in the absence of putrescine some 80% of the ribosomes appear to be engaged in polypeptide biosynthesis, even though the overall rate of protein synthesis is very slow.

As polysomes are quite fragile structures it is possible that the loss of polysomes in the starved auxotroph is due to an increased fragility of the ribosome-mRNA binding interaction. Ribonucleases are not a problem as the cell extracts can be left for 24 hours at $4^{o}C$, at which temperature ribonucleases maintain some activity, and after sucrose gradient centrifugation large polysomes are still present (Table 20b). 20mM EDTA plus 0.5M KCl completely dissociates the polysomal complexes of ribosomes isolated from both putrescine starved and supplemented cultures, as does ribonuclease. The addition of 20mM EDTA alone, while having only a small effect on polysomes of the supplemented cultures, results in nearly complete dissociation of the polysomes of the oligoamine depleted cultures. Thus the latter polysomes would appear to be more dependent on the presence of some divalent cation which is possibly substituting for oligoamines in the ribosome-mRNA binding reaction or that 20mM EDTA is a sufficiently high concentration to chelate the lower quantity of cations found in the starved cultures but not the levels found in supplemented cultures.

Cycloheximide, which inhibits the elongation of polypeptide chains at some stage after the attachment of amino acids on to tRNA (Siegel and Sisler, 1964) can be used to determine at which stage protein synthesis is being affected during oligoamine starvation. Upon addition of

A

Time course of changes in the polysome profile

Time after addtion of putrescine (minutes)	% ribosomes active in protein synthesis	Average number of ribosomes per polysome
0	81	4-5
15	89	8-10
30	91	10-12
45	98	12-15
60	97	12-15

B

Stability of polysomes

% ribosomes occurring as polysomes

Addition to media after putrescine starvation	control	Treatment to polysomes after isolation: 5µg ribonuclease 10 min at 4°C	20mM EDTA	20mM EDTA 0.5M KCl
None	91	1	0	0
Putrescine 0.7mM for 1 hour	85	2	70	0

Conidia of the putrescine auxotroph were grown for 12 hours in the absence of putrescine, after which time putrescine was added to one part of the culture for the times indicated. Polysomes were isolated by centrifugation as described in Methods. The % active ribosomes was taken as the sum of 80S monosomes and polysomes found after addition of 0.5M KCl to all buffers to dissociate inactive 80S ribosomes.

cycloheximide,protein synthesis continues at a very slow rate, with polypeptide chain elongation being the rate limiting step. The addition of cycloheximide (100µg/ml) to starved cultures of A. nidulans puA_1 for one hour before harvesting results in a shift of the average polysome size to larger complexes (Figure 50). This suggests that initiation of new polypeptide chains is normally a rate-limiting step in protein synthesis in the putrescine starved cultures rather than there being a defect in the mRNA or ribosomal structures.

A more direct means of establishing the rate of polypeptide chain elongation is to determine the period of time required for a ribosome to bind to mRNA, complete translation, and release a finished polypeptide. This is referred to as the transit time and can be determined by measuring the kinetics of radioactive amino acid transfer from nascent polypeptide chains to completed proteins (Fan and Penman, 1970; Scornik, 1974). The distribution of radioactivity in nascent and complete polypeptides is shown in Table 21 and Figure 51, for the determination of the transit time in putrescine starved and supplemented cultures. Over very short periods of incubation with ^{3}H-adenine the majority of the radioactivity is found in the pellet of the high speed centrifugation (150,000g) and is due to the newly synthesized nascent chains being associated with polysomal complexes. After further incubation an increasing proportion of the label is found in the supernatant along with the completed polypeptide chains. It can be seen that the transit time is nearly doubled in the putrescine starved cultures, indicating a slower rate of polypeptide chain elongation.

The results presented in this section suggest that the lower rate of protein synthesis during oligoamine starvation is partly explained by the decreased rates of polypeptide chain initiation and elongation. It also remains a possibility that the polypeptide chains synthesized are

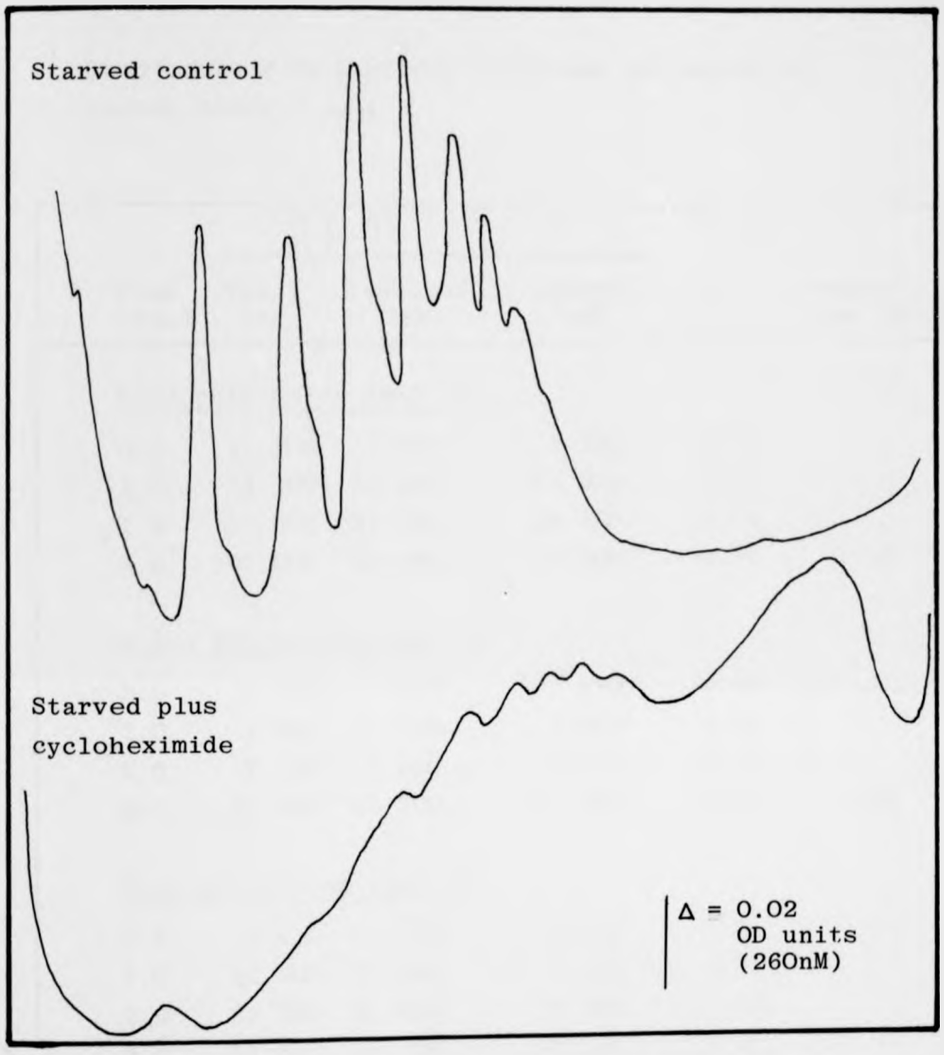


Figure 50 Effect of cycloheximide on polysome profiles of puA$_1$

Conidia were grown for 12 hours in the absence of putrescine. Cycloheximide (100μg/ml) was added to one part of the culture for one hour before harvesting and extraction of polysomes as described in Methods.

Distribution of Radioactivity in released and nascent polypeptide chains of pvA_1

Time (min)	d.p.m. Total (t)	released (r)	nascent (n)	n/t	transit time (min)
Plus putrescine (exp 1)					
0.5	11 200	3 000	8 200	0.73	
1.0	31 100	14 100	17 000	0.55	
1.5	63 800	37 700	26 100	0.41	
2.0	101.000	69 000	32 000	0.32	1.19
Minus putrescine (exp 1)					
0.5	1 420	440	980	0.69	
1.0	4 400	1 500	2 900	0.66	
1.5	7 600	3 300	4 300	0.56	
2.0	27 600	16 200	11 400	0.41	1.60
Plus putrescine (exp 2)					
0.5	4 410	1 720	2 690	0.61	
1.0	16 400	11 100	5 300	0.32	
1.5	35 200	26 800	8 400	0.24	
2.0	71 800	61 700	10 100	0.14	0.83
2.5	67 400	67 300	100	0.00	
Minus putrescine (exp 2)					
0.5	460	170	390	0.63	
1.5	4 420	2 900	1 520	0.34	
2.0	7 550	5 430	2 120	0.28	
2.5	11 200	8 710	2 490	0.22	1.40

The transit time was determined as described in Materials and Methods. Centrifugation at 150000g was used to separate the polyribosome fraction containing nascent polypeptide chains.

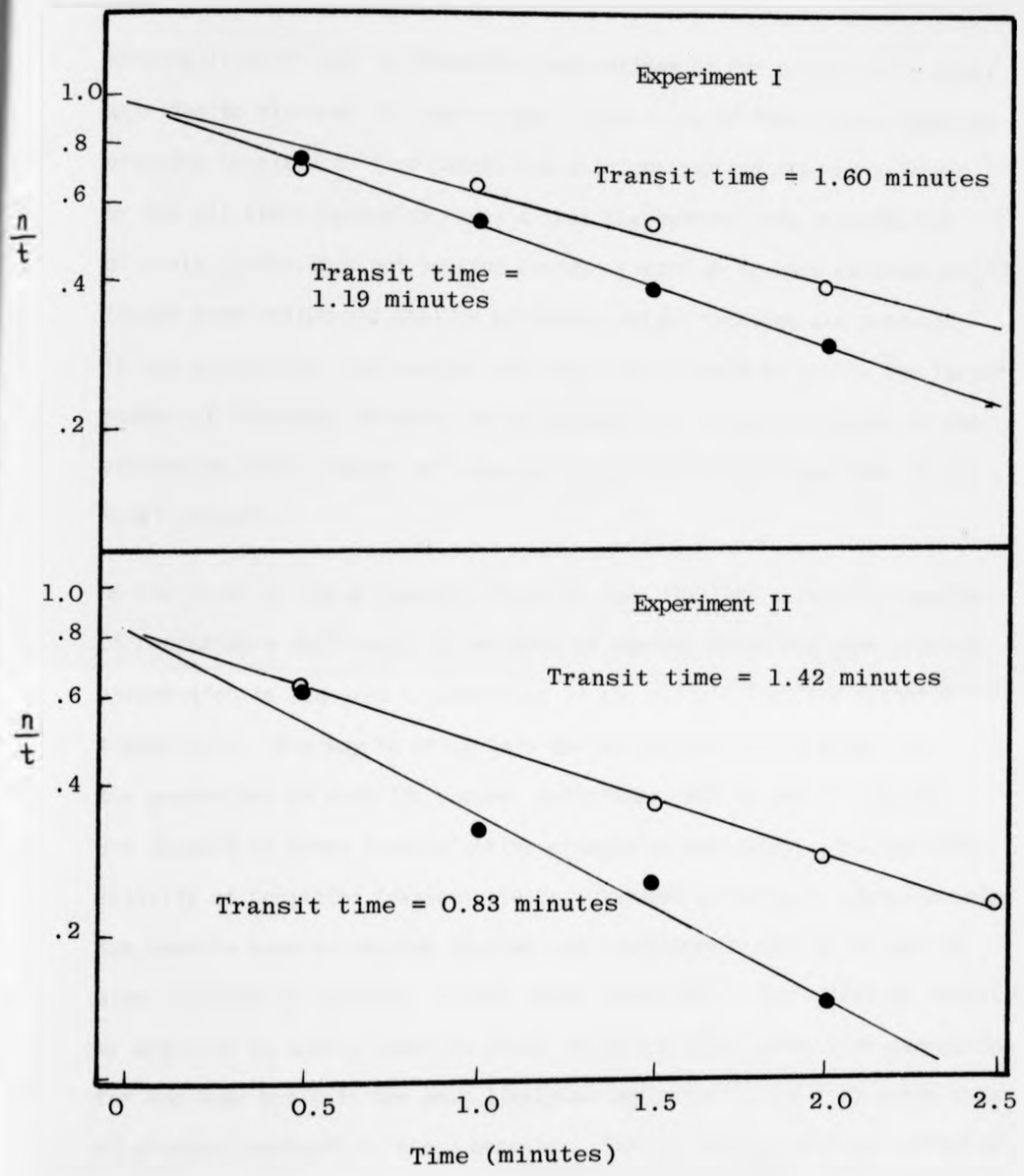


Figure 51 The effect of putrescine on the rate of polypeptide chain elongation
The transit time was determined for cultures starved for 12 hours (O) and when supplemented with 0.7mM putrescine (●) for one hour as described in Methods, using strain $putA_1$.

abnormally short due to premature terminations or are defective in other ways due to mistakes in translation. Separation of total pulse-labelled proteins in extracts from putrescine supplemented and starved cultures by SDS gel electrophoresis reveals that the overall size distribution of newly synthesized polypeptide chains is similar in both cultures, Fig. 52, though more medium and smaller molecular weight proteins are produced in the putrescine supplemented cultures. This could be due to the larger number of ribosomal proteins being synthesized in these cultures as the average molecular weight of ribosomal proteins is less than that of the total protein.

On the basis of these results it can be seen that the mutant is capable of producing a full range of proteins of various sizes but more precise information is required to determine if the polypeptides are faithful transcripts. One way in which this can be achieved is to assay for the production of specific enzymes activities, and to see if enzymes are capable of being induced during oligoamine depletion. The specific activity of ornithine transaminase in uninduced cultures is approximately the same in both putrescine starved and supplemented conditions and is also induced by arginine in both cases (Table 22). The extent of induction by arginine is nearly twice as great in the cultures grown with putrescine for one hour but this can most likely be explained by the more rapid rate of protein synthesis in these mycelia. Thus oligoamine depleted cultures of A. nidulans are fully capable of accurate enzyme production involving transcription and translation, though at a slower rate than in putrescine supplemented cultures.

Figure 52

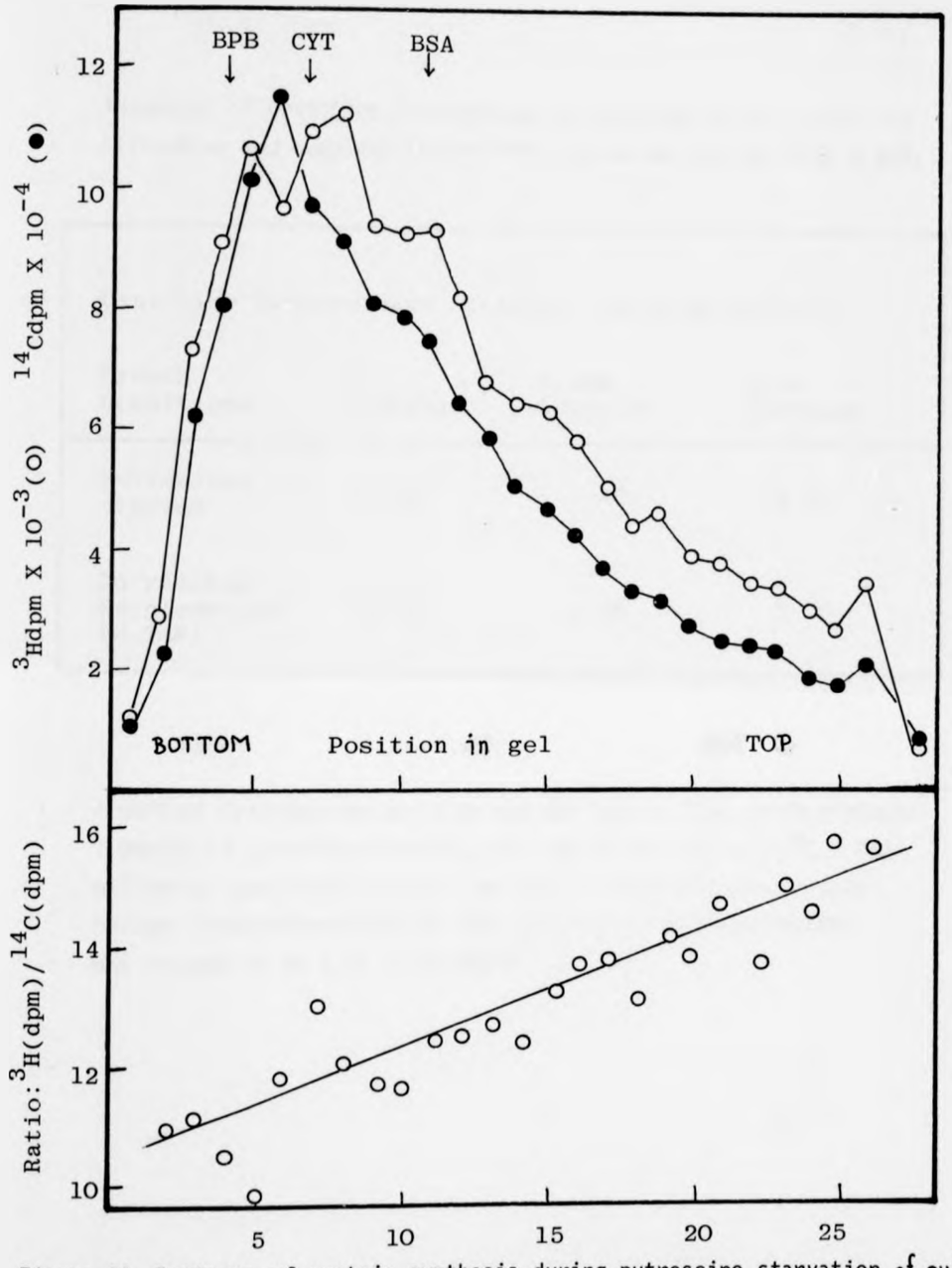


Figure 52 Patterns of protein synthesis during putrescine starvation of *puA*$_1$

Conidia were grown for 12 hours in the absence of putrescine, labelled with ^{3}H-leucine (O) and proteins extracted and separated by SDS gel electrophoresis as described in Methods. Another culture was starved for 12 hours given putrescine (0.7mM) for one hour, labelled with 10μCi ^{14}C-leucine and analyzed as above (●). The molecular weight markers were CYT = cytochrome C and BSA = bovine serum albumin. BPB indicates the position of the bromophenol blue dye.

Induction of ornithine transaminase by arginine during putrescine starvation and supplementation with putrescine for one hour in puA_1

Ornithine Transaminase Activity (units/mg protein)			
Growth Conditions	No Arginine	2.4mM Arginine	Fold Increase
Putrescine starved	0.620	1.44	2.32
Putrescine Supplemented (0.7mM)	0.767	2.88	3.76

A unit of transaminase activity was defined as that which produces 1.0mmole of pyrroline-5-carboxylate per 30 minutes at $37^{o}C$. The millimolar extinction coefficient for the condensation product between O-amino-benzaldehyde and Δ^{1}-pyrrolidine-5-carboxylate was assumed to be 2.71 litre-mmole^{-1} cm^{-1}.

Discussion on Oligoamine Supplementation of the Putrescine Auxotroph puA_1

A detailed study of the events rapidly occurring after administering oligoamines to depleted cultures has not been carried out for many systems. Most experiments with auxotrophic mutants or cells exposed to inhibitors of oligoamine biosynthesis compare normal growth to a "steady-state" of oligoamine deprivation or follow the metabolic processes during starvation. This is not an effective protocol as the oligoamine levels drop slowly and only a general metabolic run-down is observable. It is particularly difficult to distinguish primary effects from secondary or tertiary effects of oligoamine depletion. However a rapid change in oligoamine levels is observable by first starving conidia for putrescine and then providing putrescine or some other oligoamine in large quantities. The cellular response to the addition of putrescine is rapid and complex including qualitative and quantitative changes in the synthesis of RNA and protein, the number of ribosomes, the rate of translation and oxidative metabolism.

Another clue as to the biochemical changes occurring during oligoamine starvation is given by the morphology of the starved mycelia. When grown in the absence of putrescine, the hyphae of the auxotroph puA_1 are short, swollen and excessively branched. This is similar to the morphology of wild type cells after inhibition of cellular protein synthesis by cycloheximide, which drastically alters the normal pattern of apical incorporation of N-acetylglucosamine in A. nidulans (Katz and Rosenberger, 1971). Thus the oligoamine-starved morphology could result from a decreased rate of protein synthesis in the auxotroph.

A summary of the physiological and biochemical changes brought about by the addition of putrescine to cultures starved of oligoamines for 12 hours is given in Table 23. A number of separate aspects of cell

Summary of the observed biochemical changes one hour after the addition of putrescine to starved cultures of the auxotroph puA_1.

1. 60% increase in oxygen consumption
2. 410% increase in the average polysome size
3. 270% increase in the rate of RNA synthesis
4. 200-300% increase in the average polysome size
5. 150% increase in the number of ribosomes as estimated by the amount of rRNA
6. 350-400% increase in the rate of synthesis of polyadenylated RNA
7. 80-100% increase in the rate of polypeptide chain elongation as estimated by the "transit time"
8. Alteration of the proportion of rRNA synthesized from 30-35% to 55-60% of the total RNA synthesized

metabolism are altered by oligoamines, the most dramatic being the eight to ten-fold increase in the rate of protein synthesis after 2 hours and the increased size of polysomes. However this does not establish protein biosynthesis as the primary essential site of action for oligoamines. The fact that a variety of effects are observed could be taken as evidence for oligoamines having many functions in vivo. Alternatively, most, or even all, of the effects presented in Table 23 may be the result of secondary interactions of cell metabolism masking the specific functions of the oligoamines.

The putrescine stimulated increases in the rates of incorporation of adenine and leucine into acid insoluble material appears to reflect an increase in the ability of the existing cellular machinery to synthesize nucleic acids and protein rather than result from a change in uptake or intracellular pools of precursors. The effect is specific for the naturally occurring oligoamines, particularly putrescine, and alters the proportions synthesized of the various types of RNA. Furthermore addition of oligoamines to the wild type strain has no effect on the rates of macromolecular synthesis ruling out any trivial effects of putrescine on precursor incorporation or cell metabolism. The stimulation of nucleic acid synthesis by putrescine can almost entirely be accounted for by the increased rate of rRNA synthesis. It is to be expected that rRNA synthesis would increase with a faster growth rate as there would be an increasing need for ribosomes. Ribosomes are the most expensive item in the protein-synthesizing system and should therefore be used at maximum efficiency (Koch, 1971). However this does not always seem to be the case and a number of slowly growing organisms possess "extra" non-functioning but rapidly mobilized rRNA. In A. nidulans there is a linear increase in ribosomal efficiency with increasing dilution rates up to 65% of the maximum specific growth rate (Bull and Trinci, 1977). Thus a rapid

three-fold increase in ribosomal efficiency can follow from a step up in dilution rates. Similarly the percentage of ribosomes active in protein synthesis and the velocity of ribosome movement along the mRNA are continuously adjusted throughout hyphal germ-tube development and during yeast-to-hyphae morphogenesis in the fungus, Mucor racemosus (Orlowski and Sypherd, 1978a and 1978b). In this organism the increased rate of polypeptide chain elongation is associated with a significant increase in the intracellular concentration of spermidine. Though this can not be taken as evidence in favour of a direct role for oligoamines in protein synthesis it seems clear from the work on A. nidulans reported here, and on Mucor, that an increasing growth rate is accompanied by and possibly dependent upon higher oligoamine concentrations.

If the cellular machinery for protein synthesis was inactive only due to the low levels of oligoamines in starved cultures of the putrescine auxotroph then the stimulation of protein synthesis should be more rapid than is actually observed. The uptake of putrescine and subsequent synthesis of spermidine and spermine could well be rate-limiting steps. This can not be easily tested as the cells take up spermidine and spermine only slowly. If putrescine and spermidine were taken up at the same rate it might be expected that spermidine would bring about the greater stimulation of macromolecular synthesis. However this may not be the case if the localization of oligoamines is crucial. Spermidine synthesized in vivo from putrescine would have a different intracellular localization than spermidine taken up directly from the media, which may become bound non-specifically to a number of structures including cell wall, membranes and organelles. Unfortunately it is not possible to precisely determine the subcellular distribution of putrescine, spermidine or spermine so it is not possible to know if exogenous and endogenous oligoamines have the same subcellular localizations.

The four to five-fold stimulation of leucine incorporation into protein one hour after the addition of putrescine to starved cultures is due to a number of factors. Prominant among these is the two-fold increase in the amount of rRNA (and presumably the number of ribosomes) in the mycelia. This alone probably accounts for nearly half of the observed stimulation. The transit time is decreased in the presence of putrescine, which could increase the rate of protein synthesis if the rate of initiation of new polypeptide chains was not a limiting factor. The increased amount of ribosomal subunits, not active in translation, during putrescine starvation is evidence for initiation of translation being a rate-limiting step. Although the polysome profiles are suggestive of an increase in the rate of initiation caused by oligoamines a more direct measure of this reaction would be desirable.

The rate of protein synthesis *in vivo* could be limited by the availability of mRNA. The proportion of poly (A) containing RNA synthesized does not change markedly during oligoamine depletion so a continued supply of messengers seems to occur. This is confirmed by the presence of polysomes in the starved condition as the maintenance of polyribosome structures is dependent upon the continued synthesis of mRNA, even under conditions of extremely slow polypeptide elongation.

The results presented here can be usefully discussed with respect to experiments with other oligoamine-depleted cells and to *in vitro* experiments of oligoamine functions. Putrescine requiring mutants have been described for various microorganisms, including *E. coli* (Mass, 1972), *N. crassa* (McDougall et al, 1977; Deters et al, 1974), *S. cerevisiae* (Whitney and Morris, 1978) and *A. nidulans* (Sneath, 1955). Consistent with the results presented here for *A. nidulans*, the addition of putrescine to starved bacteria results in a marked increase in the rate of protein synthesis (Young and Srinivasan, 1972). A combination of *in vivo*

and *in vitro* studies has led to the conclusion that oligoamines participate directly in protein synthesis and production of the small ribosomal subunits (Algranatic and Goldemberg, 1977a). Detailed analysis of the requirements for the initiation of polypeptide synthesis on natural eukaryotic mRNAs has also demonstrated the necessity of spermidine for protein synthesis *in vitro* (Anderson et al, 1977) which confirms the general observations of cell metabolism in the auxotrophic microorganisms discussed previously.

Oligoamine-requiring mutants have not been available for mammalian cells so a specific inhibition of the accumulation of oligoamines *in vivo* by chemical inhibitors seems to be almost the only approach to oligoamine depletion. Almost exclusively these are inhibitors of ornithine decarboxylase or S-adenosylmethionine decarboxylase. Experiments utilizing 1,3-diaminopropane (Farwell et al, 1977), ∝-methylornithine (Mamont et al, 1976) and methylglyoxal bis(guanylhydrazone) (Fillingame et al, 1975) suggest that oligaomine depletion with these inhibitors results in the specific inhibiton of DNA synthesis, with virtually no effects on RNA or protein synthesis. This is quite different from what is observed with starvation of auxotrophic mutants and will be discussed further in the next section.

DISCUSSION

The results of the analysis presented here of cell metabolism *in vivo*, including macromolecular synthesis, during oligoamine starvation and supplementation are consistent with the observations of other workers that spermidine and spermine have a role in the initiation of protein synthesis (Algranati and Goldemberg, 1977b and 1977a; Atkins et al, 1975). Oligoamines are capable of stimulating, inhibiting or changing qualitatively a large number of reactions *in vitro*, including aminocyl-tRNA formation, polypeptide synthesis, DNA and RNA polymerases and nucleases (see Tabor and Tabor, 1972 and 1976). A large number of reactions involving nucleic acids are capable of being altered in some way by the presence of oligoamines which may bind strongly to the nucleic acid substrates, products or primers. Since many of the effects of oligoamines observed *in vitro* are sensitive to changes in concentrations of other cations, for which the free intracellular concentrations are not accurately known, it is not a simple process of extrapolating these observations to occurrences in the living organism. Hopefully some of the *in vitro* effects can be confirmed by observation of reactions *in vivo*. A complete analysis of the putrescine requirement of *A. nidulans* will ultimately require analysis of the defect(s) in *in vitro* biosynthetic systems. However the inefficiency of *in vitro* systems for macromolecular synthesis and the likelihood that most of these systems are not dependent upon many components essential for macromolecular synthesis *in vivo* make it essential that the mutant be first analyzed for physiological and biochemical defects *in vivo*.

The changes in ribosome distribution in polysomes of different sizes occurring rapidly after supplying oligoamine-depleted cultures with putrescine suggest that the initiation of protein synthesis is being selectively inhibited, at least to a greater extent than elongation or termination.

It is significant that even after 12 hours of putrescine starvation some of the ribosomes still occur in polysomal complexes. This is quite different from the situation with the yeast strain ts⁻187, a mutant defective in the initiation of polypeptide chains, where all of the polyribosomes decay to monoribosomes within 2 minutes after a shift to the restrictive temperature (Hartwell et al, 1970). The fact that such a dramatic shift does not occur in the A. nidulans puA_1 auxotroph is evidence that the effect of oligoamine depletion is not simply and exclusively an inhibition of the polypeptide initiation reaction but must affect directly or indirectly, a number of other metabolic reactions. Notable among these is the marked decrease in rRNA synthesis during putrescine starvation. This limits the number of ribosomes in the cytoplasm and prevents a massive and wasteful accumulation of subunits and monomers not involved in actively translating messengers. In general the rate of synthesis of rRNA is very sensitive to fluctuations in the growth rate in N. crassa (Alberghina et al, 1975) so this may be an indirect effect of oligoamine starvation in A. nidulans. Identification of the primary essential roles of oligoamines within the cell is complicated by the complex interacting control systems regulating all aspects of cell metabolism. Nevertheless, it is encouraging to note that study of E. coli putrescine auxotrophic mutants has also led to the conclusion that these organic cations participate directly in protein synthesis (Algranati and Goldemberg, 1977b).

The conclusions based upon the study of oligoamine depleted cultures of the A. nidulans putrescine auxotroph puA_1 as discussed here are, at first sight, quite different from those resulting from the inhibition of oligoamine biosynthesis in animal cells using various inhibitors (see Jänne et al, 1978). In the later studies the most common observation is an inhibition of DNA synthesis during oligoamine depletion while RNA and protein synthesis

continue unaffected. Although this may reflect a genuine difference in oligoamine functions there are a number of other ways in which this can be reconciled with the observations that in A. nidulans and E. coli putrescine auxotrophs, protein synthesis appears to be more closely linked with oligoamine content.

(i) Oligoamines may be required for all types of macromolecular synthesis but in animal cells DNA synthesis is the most sensitive to a drop in oligoamine levels while in the microorganisms protein synthesis is most vulnerable. Mammalian cells have some quite different properties than either A. nidulans or E. coli. The typical doubling time of the animal cells is around 24 hours, compared to 0.5-3 hours for the two microorganisms, and mammalian cells contain ten times the amount of DNA (per amount of RNA). The relative contribution of DNA synthesis to growth is thus much greater in animal cells. Microorganisms, with their very fast growth rates, are much more likely to be limited by the rates of ribosome production and protein synthesis (see Bull and Trinci, 1977).

(ii) The majority of the studies with animal cells using inhibitors examine the decline of metabolic precesses during oligoamine depletion. This is less satisfactory than following the first process to pick up as growth resumes with the addition of exogenous oligoamines. Because of the interrelatedness of cell physiological processes slow starvation results in only a general metabolic rundown. It may be that in animal cells a depression of the overall growth rate results in a shutdown of DNA synthesis, though this is only an indirect effect, it could be of a greater magnitude than the "primary effect".

(iii) Oligoamine starvation of A. nidulans, or yeast does not affect the cell physiology until the oligoamine levels are reduced to around 10% or less of the control values. However in the studies of animal cells

very large effects on DNA synthesis occur with only 50% or less reductions in spermidine, and usually no change in spermine levels. The animal cells would appear to be more sensitive to fluctuations in oligoamine levels than the microorganisms. On the other hand this may also be evidence that the metabolic inhibitors used have other toxic effects that result in DNA synthesis inhibition, and do not act via a reduction in spermidine content. The reversal of inhibition, which in some cases can be observed by adding low concentrations of oligoamines to the cells could act by competing with the inhibitors for various subcellular functions, thus preventing the expression of toxic effects.

(iv) Protein synthesis is required for the initiation of DNA synthesis in S. cerevisiae (Hereford and Hartwell, 1973) but once initiated DNA replication can be completed in the absence of protein synthesis. As these proteins are short lived, made only 10 minutes prior to initiation (Williamson, 1973), it is clear that a decrease in protein synthesis may have a dramatic effect on DNA synthesis. Thus oligoamine depletion in higher eukaryotes may result in a decrease in DNA synthesis by limiting the availability of various protein initiation factors or histones and non-histone proteins required for the newly synthesized DNA.

An increase in oligoamine biosynthesis is an early event following the initiation of rapid growth in a wide variety of organisms and is clearly required for germination in A. nidulans and sporulation in S.cerevisiae (Cohn et al, 1978). Though oligoamine depletion resulting from auxotrophy or inhibitors can limit growth and development it is not clear whether or not cells control their rate of growth directly by alterations in the oligoamine content. This seems unlikely for a number of reasons. Oligoamines are observed to turn over slowly, which would not be expected for a regulatory molecule. Also at least 90% reductions in oligoamine levels would be necessary to control growth rates in

A. nidulans or S. cerevisiae, which again is not the range of response suitable for intracellular controlling agents. However oligoamine levels are precisely controlled as shown by the short half-lives of the rate-limiting enzymes of the biosynthetic pathway and as the bulk of the intracellular oligoamines are bound to various cellular constituents the turnover of the soluble, free oligoamines could be very rapid and of regulatory importance.

Although oligoamine biosynthesis is a major event in the germination of conidia of A. nidulans it would not appear to be a controlling factor for dormancy. Starvation of the putrescine auxotroph does not noticeably effect growth for the first 4 or 5 hours after inoculation, during which time the cells dormant state has been broken and active growth initiated. The massive increase in macromolecular synthesis during germ-tube emergence is dependent on an increased supply of spermidine and spermine and so could be factors normally controlling maximum growth rates. However it does not appear likely that oligoamines necessarily have such a role in regulating growth. The morphology typical of oligoamine starvation, short contorted hyphae with excessive branching, is not typically observed in the wild type strain under more natural limitations of growth (e.g. starvation for carbon and nitrogen sources) and would not be of a selective advantage to the organism. A. nidulans and other mycelial fungi are capable of controlling the density of growth as well as the specific and radical growth rates. Such morphological adaptability can not be accomplished by unicellular microorganisms. The filamentous habit permits variation in colony density such that the energy source available in the medium is used to maximum efficiency in extending the colony's diameter allowing it to reach a more favourable environment. The limitation of oligoamines, while not involved in regulation of mycelial growth could have a role in fungal differentiation where hyphal growth occurs in a wider

variety of shapes in conidiosphore and ascus development.

There may not be a simple answer to the question, "What are the intracellular functions of the naturally occurring oligoamines?". Like magnesium, these organic cations are likely to have a wide number of independent functions. As organisms evolved over the ages more and more cellular reactions, particularly those involving nucleic acids, probably came to depend on the oligoamines present within the cells. Indeed such a process is typical of the efficiency of cellular metabolism in utilizing a single molecule in as many ways possible. Of course, identifying the very first essential function of oligoamines in primitive cells is probably impossible and not necessarily of importance to understanding the various functions extent today. Oligoamines are in some ways superior to magnesium as general cations for various metabolic functions. Being organic cations the cell is able to regulate the concentration of oligoamines with a great flexibility by controlling the rates of synthesis, degradation and transport. Only the latter form of control is possible with magnesium, and if it was present in the environment in low cencentrations the cell could not synthesize it, resulting in a limitation of growth. It would appear that the lack of understanding of the details of cell metabolism and macromolecular synthesis in vivo are the primary limitations on understanding the molecular basis of oligoamine functions in the cell. Oligoamine research may be one area which can not progress without a further advance in the general knowledge of the biochemistry of molecular biology of the cell.

There are many areas where further investigation of oligoamine functions may still yield pertinent information. Some emphasis should be given to developing in vitro RNA and protein synthesizing systems from oligoamine-depleted cells. If the defects in protein or nucleic acid synthesis could be repaired

simply by the addition of oligoamines to the *in vitro* systems then this would be powerful evidence in favour of a direct role for oligoamines in macromolecular synthesis. This is preferable to preparing *in vitro* systems from normal cells as it is difficult to remove oligoamines from the extracts without impairing their efficiency in synthesizing macromolecules. Unless the oligoamine levels are drastically reduced a dependency on their presence may not be demonstrated.

It may be necessary to change our conceptual framework for thinking about and studying the biosynthesis of macromolecules to see how oligoamines could be involved in these processes. This includes developing an understanding for dynamics of protein and nucleic acid synthesis rather than view them simply as static tableaux. Thus the potential interactions of oligoamines with rRNA, tRNA, mRNA and probably other factors involved in polypeptide biosynthesis will not be an easy system to unravel. There is, however, reason to attempt this as it would almost certainly help in understanding the mechanisms by which cells optimize their biosynthetic machinery for the most efficient use of resources, and still maintain the flexibility to allow the regulatory control of macromolecular synthesis necessary for the successful fulfilment of the genetic programmes of development and differentiation.

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