PUTRESCINE SYNTHESIS IN
NEUROSPORA CRASSA

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ABSTRACT

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A mutant of Neurospora crassa, designated put-1, requires the polyamine, putrescine, or its immediate product, spermidine, for maximal growth. The mutant grows to a lesser degree on either agmatine, spermine, or cadaverine, but does not respond at all when grown on minimal medium or medium supplemented with ornithine, arginine, or ethylene diamine. This requirement is not removed by growing the mutant in medium having a high osmolarity. The put-1 mutant lacks the ornithine decarboxylase (ODC) activity demonstrated in both wild type 74A and in the arginaseless mutant, aga.

The specific activity of ODC for wild type 74A and aga varies from one batch of cells to another, however, the specific activity is similar from preparation to preparation. Putrescine, which is the product of ODC activity, inhibits the ODC enzyme both in vivo and in vitro. This inhibition is more severe when putrescine is added in vitro, indicating that the mechanism involved is one of feedback inhibition. However, end product repression cannot be ruled out as a possible mechanism of ODC inhibition.
ODC activity is stimulated by the presence of arginine in the medium. In wild type 74A it results in a specific activity three times greater than normal, while in the aga mutant it produces a dramatic increase to almost 50 times greater than the specific activity after growth on minimal medium.

Arginine decarboxylase (ADC) activity is present at very low levels in wild type 74A and in aga, but is not present in the put-1 mutant. The put-1 mutant is able to grow on agmatine, however. Both of these results indicate that unlike other eucaryotes, Neurospora crassa may possess a second pathway to putrescine synthesis.
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INTRODUCTION

Polyamines have been shown to be present in most living organisms and have proven to be required for growth in several procaryotes, and in one eucaryote. Procaryotes, such as *E. coli*, have the ability to produce the polyamine, putrescine, via two pathways utilizing the enzymes ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). In eucaryotes only ODC activity has been demonstrated (Morris and Pardee, 1966).

Some of the *E. coli* mutants which require putrescine have been shown to excrete putrescine when grown in media of high osmolality. Some of these mutants also have the ability to grow in the complete absence of putrescine when the osmolarity of the medium is increased to 0.1M MgCl₂, KCl, or NaCl.

Thus, it remains to be seen whether the polyamines, putrescine and spermidine, are absolutely required for growth by procaryotes or eucaryotes or both. It is also not known what function these substances perform in the cell. Isolation of an absolute putrescine-requiring mutant would greatly accelerate the discovery of their function. This study was undertaken to characterize the put-1 mutant and to determine the pathway or pathways to putrescine synthesis in *Neurospora crassa*. 
Polyamines are non-protein nitrogenous bases which have been found in bacteria, plants, bacteriophages, and animal tissue (Tabor and Tabor, 1964). They include the compounds putrescine, spermidine, and spermine. These three compounds are synthesized by all eucaryotic cells; procaryotic cells can only synthesize putrescine and spermidine. Procaryotes can utilize spermine if present in the medium. A few procaryotes have been found which are unable to synthesize putrescine and these grow slowly or not at all in its absence (Cohen, 1971). In all cases the requirement for putrescine can be replaced by spermidine. No organism or mutants are known which are unable to synthesize spermidine from putrescine.

Fig. 1 shows the structures of three polyamines and some related compounds. Fig. 2 illustrates the synthesis of putrescine. Putrescine (1, 4 diaminobutane) results from the decarboxylation of ornithine by the ornithine decarboxylase enzyme (ODC). In procaryotes the decarboxylation of arginine by arginine decarboxylase (ADC) results in the production of agmatine which can then be converted to putrescine and urea by the enzyme agmatine ureohydrolase.

Spermidine is a product of putrescine and is the next compound in polyamine synthesis (Fig. 3). It results from the combina-
Putrescine (1, 4 Diaminobutane)

\[ \text{H}_2\text{N-}(\text{CH}_2)_4-\text{NH}_2 \]

Spermidine

\[ \text{H}_2\text{N-}(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2 \]

Spermine

\[ \text{H}_2\text{N-}(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2 \]

Cadaverine (1, 5 Diaminopentane)

\[ \text{H}_2\text{N-}(\text{CH}_2)_5-\text{NH}_2 \]

Agmatine

\[ \text{H}_2\text{N-C-CH-(CH}_2)_4-\text{NH}_2 \]

\[ \text{NH} \]

Ethylene diamine

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

Arginine

\[ \text{H}_2\text{N-C-NH-(CH}_2)_3-\text{CH-NH}_2 \]

\[ \text{NH} \]

\[ \text{COOH} \]

Ornithine

\[ \text{H}_2\text{N-(CH}_2)_3-\text{CH-NH}_2 \]

\[ \text{COOH} \]

Fig. 1. Structure of the polyamines and some related compounds.
Fig. 2. Routes leading to the formation of putrescine.
Fig. 3. The biosynthesis of spermidine from putrescine and s-adenosylmethionine.
tion of putrescine and s-adenosylmethionine (SAM) by two enzymes; one decarboxylates SAM and the other transfers the aminopropyl moiety of decarboxylated SAM to putrescine resulting in spermidine. Spermidine is thought to be the essential functional entity since all organisms which require putrescine can synthesize and grow equally well on spermidine.

Spermine, which can only be synthesized by eucaryotes, is a product of spermidine. In eucaryotes there appears to be a single enzyme that possesses the ability to decarboxylate SAM and also transfer the aminopropyl moiety (putrescine). The rate of activity of this enzyme is controlled by the amount of the aminopropyl moiety present. This same enzyme appears to be capable of transferring another aminopropyl moiety to spermidine to form spermine. When spermine becomes oxidized it has an antibacterial effect and is highly toxic. It also has been shown to have a protective effect since it prevents DNA from being degraded by various nucleases (Cohen, 1971).

The polyamines have been implicated in many cellular activities over a wide range of organisms. Much investigation has been carried out to discover whether or not they are essential for growth. Herbst and Snell (1948) discovered that putrescine or related di- and polyamines constituted an essential growth requirement for Haemophilus parainfluenzae. This was the first time that the polyamines were assigned a biological function, even though their occurrence was known to be widespread in nature and their existence had been known for al-
most 300 years.

Subsequently, other bacterial auxotrophs were isolated which required a polyamine for growth. Martin, et al. (1952) isolated a mutant of *Neisseria perflava* which required putrescine for growth. Mager, et al. (1954) isolated a strain of *Pasteurella tularensis* whose growth was stimulated by putrescine. Sneath (1955) isolated a mutant of the eucaryote, *Aspergillus nidulans*, which required 1 µg/ml of putrescine for growth. Maas, et al. (1970) isolated mutant strains of *Escherichia coli* which were putrescine deficient.

*E. coli* has been shown to contain 15 µmoles/gm wet weight of putrescine intracellularly. This is considerably higher than that found in eucaryotes. However, only 1% of this actually appears to be required for growth. 99% of the putrescine can be removed and growth will occur. In such cases the putrescine is replaced by Mg²⁺ (Cohen, 1971).

In the case of the putrescine-deficient *E. coli* mutant of Morris and Jorstad (1970), the intracellular putrescine level was reduced to 1% of normal, however, the wild-type morphology, the levels of protein and RNA, and the stringent amino acid control of RNA synthesis were all maintained at normal levels. The one difference noted between mutant and wild-type was that the doubling time of the mutant was increased approximately 10%.

These mutants of Morris and Jorstad (1970) and of Maas, et al. (1970) were unable to convert arginine to putrescine, while the
presence of arginine in the medium repressed and inhibited ornithine synthesis. Notice (Fig. 4) that *E. coli* has two pathways to putrescine synthesis, one by the degradation of arginine and the other from the synthesis of ornithine.

In *E. coli* it has been shown that there exists two ODC and two ADC enzymes. In each case one is constitutive and one is inducible (Morris and Pardee, 1965).

Induced ODC is not responsible for most of the putrescine found. The formation of this enzyme occurs only under a special nutrient situation in which several amino acids are added to the medium (Sher and Mallette medium without lysine). This enzyme is catabolic in nature and has a pH optimum of 5.3.

Biosynthetic ODC is present under a variety of growth conditions and is responsible for most of the putrescine produced. It has a different heat optimum (stable at 55°C) and works best at a pH of 7.5. Both ODC enzymes require pyridoxal phosphate for activity.

The constitutive biosynthetic ADC requires Mg²⁺ and pyridoxal phosphate and has a pH optimum of 8.4. It was found to be present under various growth conditions. The catabolic ADC enzyme has a pH optimum of 5 and requires the same special growth conditions as seen in the case of the inducible ODC enzyme.

Morris and Pardee (1966) demonstrated the existence of a pathway to putrescine via arginine. They also determined that the conversion of putrescine from ornithine was independent of the con-
Fig. 4. Polyamine synthesis in *E. coli*.
version of ornithine to arginine.

Further proof of the existence of a second pathway to putrescine synthesis was found in E. coli strains which lacked the ability to synthesize ornithine. Such strains should require both putrescine and arginine for growth, since no ornithine can be made via degradation or synthesis. However, such strains only require arginine for growth indicating putrescine production via the second pathway (Morris and Pardee, 1965).

This arginine pathway to putrescine in E. coli is similar to that seen in mycobacteria and in plants. No such pathway from arginine has been found in the other eucaryotes. Either pathway is capable of producing sufficient amounts of putrescine and spermidine for normal growth.

Morris and Pardee (1966) concluded that the most economical pathway to putrescine was from ornithine but when the organism is grown in arginine, putrescine can still be readily formed even though ornithine synthesis is repressed. Also, if the organism is grown in an acidic environment, the inducible ODC and ADC enzymes can be utilized. Cohen (1971) points out that this two pathway system indicates an essentiality of these substances for the organism's growth. It has also made isolation of these mutants difficult.

The mutants of Maas, et al. (1970) were deficient in agmatine ureohydrolase, while the two ADC enzymes showed normal activity. Their mutants are similar to those of Morris and Jorstad (1970). No
E. coli mutants have been isolated which lack ODC activity.

Most of the investigations carried out thus far to determine the functions of the polyamines in the cell have been performed on mutants of the procaryote, E. coli. Since this organism has two pathways for the synthesis of putrescine, researchers have been unable to isolate a strict putrescine mutant.

In Neurospora crassa (Fig. 5) ornithine can be synthesized from glutamic acid or it can be produced by the catabolism of arginine via the enzyme, arginase. The arginase enzyme is found only in eucaryotes.

Ornithine has several possible fates. It can be converted to glutamic semi-aldehyde (GSA) by way of the ornithine transaminase enzyme (OTA). This enzyme is one of arginine catabolism and plays no major biosynthetic role in the synthesis of arginine (Davis, 1968). Exogenous ornithine has been shown to be destined for catabolism by OTA. On the other hand, endogenous ornithine can be converted to citrulline and arginine via the ornithine transcarbamylase enzyme (OTC).

The ornithine pool of arginine-grown strains is entirely from catabolism since ornithine synthesis is feedback inhibited by arginine. Proof of this lies in the fact that the ornithine pools of arginine-grown strains of wild-type and arg-5 are the same even though arg-5 is blocked in ornithine synthesis (Davis, et al. 1970). When the aga mutant, which lacks arginase, is grown on minimal media or media supplemented with
Fig. 5. Ornithine metabolism in *N. crassa*. 
ornithine, the cultures grow normally. However, when grown on arginine, \textit{aga} strains grow much more slowly.

This arginine sensitivity and its effect on polyamine synthesis and the ODC enzyme has been discussed by several authors (Davis, et al. 1970; Hirschfield, et al. 1970; Morris and Jorstad, 1970). Since \textit{aga} cannot make ornithine from arginine and the synthesis of ornithine is feedback inhibited by the presence of arginine, \textit{aga} strains grown on arginine should have a severe putrescine deficiency and impaired growth. This "ornithine starvation" hypothesis would account for the arginine sensitivity of \textit{aga} strains (Davis, et al. 1970). Putrescine can reverse arginine inhibition of an \textit{aga} strain. Spermidine will also fully reverse this inhibitory effect of arginine.

Davis, et al. (1970) tested whether the presence of high concentrations of arginine might itself be inhibitory. They employed an arginine competitor, lysine, for this purpose, and found that except for the highest concentration (1 mM), lysine was not effective in reversing the effect of arginine. Arginine pools of strains grown on 1 mM arginine and either 0.5 mM lysine or 0.5mM putrescine were almost identical (297 and 283 nmol/mg, respectively) and only about 14\% less than strains grown on arginine alone (340 nmol/mg). Since 0.5 mM putrescine has almost maximal effect, and 0.5 mM lysine has none, arginine inhibition is not related directly to arginine pool size.

Several questions arise from the findings of Davis, et al.
(1970). First, why does the aga mutant grow at all on arginine when there is no ornithine pool? Second, is there an ADC enzyme present in Neurospora crassa which is active when "ornithine starvation" occurs? Third, are putrescine and the other polyamines required by Neurospora for growth?

Two hypotheses have been proposed by Davis, et al. (1970) to answer the first question. The first is that possibly a small amount of ornithine is being produced by an incomplete inhibition of the normal biosynthetic route to ornithine; or possibly the OTA reaction is reversible in vivo; or the arginine synthetic pathway is reversible between arginine and ornithine. However, strains of the genotypes arg-5, ota, aga and arg-5, arg-12, aga both grow at about the rate of aga in arginine-supplemented medium. Thus, since these mutations do not accentuate arginine inhibition of aga, the enzymes effected by these mutations probably are not significant in the slow growth of aga on arginine.

The second hypothesis is that putrescine is not absolutely required for growth of Neurospora or that this requirement is manifest only after prolonged growth.

Recent work by Weiss and Davis (1973) has concentrated on the theory of compartmentation of ornithine and arginine pools in Neurospora. Their findings may give an insight into the control of arginine catabolism and putrescine synthesis.

Exogenous arginine enters the cytoplasm and is immediately
utilized for protein synthesis via arginyl-tRNA. The remainder (about one-eighth) is sequestered in a vesicle compartment with the bulk of the arginine pool. Catabolism of this arginine pool does not occur unless there are high concentrations of arginine in the cytoplasm as a result of arginine addition to the medium. Under normal growth conditions this arginine is excluded from protein synthesis except by exchange with cytoplasmic arginine. Cytoplasmic arginine accounts for less than 5% of the total arginine present in the cell. Thus, this vesicle compartment prevents arginine catabolism. Not only is over 90% of the arginine present in this compartment, but also over 90% of the ornithine is found there. Likewise, the biosynthetic enzymes of arginine are found within this vesicle compartment while catabolic enzymes of arginine and ornithine, such as ODC, are cytoplasmic.

Most of the ornithine present in mitochondria is converted to citrulline before it can leave the mitochondrion. One-sixth of the ornithine found in the cell escapes to the cytoplasm from mitochondria with one-half of this being converted to putrescine and the other one-half entering the vesicle compartment (Subramanian, et al. 1973). Citrulline leaves the mitochondrion easily and is converted to arginine. Most of this arginine is converted to protein. The remainder (one-eighth) is sequestered in the vesicle compartment. It was concluded that if arginine is not used within a few seconds after its entry into the cytoplasm, it will enter this compartment.
Weiss and Davis (1973) point out that in most procaryotes, the pool sizes of many endogenous amino acids are often maintained at low levels and catabolic enzymes remain uninduced. However, in eucaryotic cells, such as *Neurospora*, endogenous amino acids frequently accumulate despite the presence of significant levels of catabolic enzymes. They have also investigated putrescine synthesis by ODC and found its activity to be 3 pmol/μg/hr or 0.013 pmol/min in 254 mg of protein. The ODC enzyme was reported to have a high affinity for ornithine with a Km of 0.4 mM.

Weiss (1973) has investigated some of the physical properties of this vesicle compartment which sequesters ornithine and arginine, and proved it to be distinct from mitochondria. Other amino acids besides ornithine and arginine were found in this compartment.

Besides uncovering the metabolic control of putrescine synthesis, much investigation has been carried out to determine the role of the polyamines in the cell and to determine whether they are absolutely required for growth. In several instances putrescine mutants have been isolated in which the putrescine requirement could be overcome or partially replaced by addition of cations or by a change in the osmolarity of the media.

Viotti, et al. (1971) investigated exponentially-grown *Neurospora* mycelia. When grown in medium containing 16 mM Mg++, *Neurospora* mycelia were shown to contain 16 mM spermidine and 0.22 mM spermine. If the Mg++ concentration was lowered, a correspond-
ing increase in polyamine levels was seen, while growth and RNA synthesis declined and several hours later net DNA synthesis declined. Thus, they concluded that polyamines can only partially substitute for Mg$^{++}$ in Neurospora.

Similar findings have been found for E. coli (Hurwitz and Rosano, 1967) and for Pseudomonas (Rosano and Hurwitz, 1969). Smith (1970) has shown in higher plants that starvation by monovalent cations (K$^+$) can be followed by an increase of spermine and spermidine levels.

Morris and Jorstad (1970) found in their putrescine-deficient mutants of E. coli that neither the elimination of 99% of the intracellular putrescine nor a radical change in the relative levels of putrescine and spermidine caused serious physiological defects. In 1973 they isolated a mutant with a more pronounced growth requirement for either putrescine or spermidine. In this case their results indicated that spermidine or a related polyamine was required for growth. Polyamine starvation resulted in an abnormal growth in which the RNA to protein and DNA to cell ratios were higher than expected on the basis of their growth rate. However, the stable RNA appeared normal as judged from size distribution and degree of methylation. Since the cells were in a steady state when measurements were made, the net rate of RNA accumulation and the rate of protein synthesis were shown to be decreasing coordinately.

Their study was carried out to attempt to determine what was limiting growth or more precisely, protein synthesis, in
polyamine-starved cells. The level of ribosomes and tRNA was not found to be limiting. But, it was seen that the rate of protein synthesis per ribosome declined if compared to normal cells. Some possible reasons suggested for this decline in protein synthesis were a change in the amount of mRNA available in the rate of initiation of protein synthesis, or in the rate of polypeptide chain elongation.

If the osmolarity of the medium is increased, E. coli will excrete putrescine (Munro and Sauerbier, 1973). Mutants defective in K⁺ ion transport display a greatly diminished rate of putrescine excretion. They suggest that putrescine excretion and K⁺ uptake may both play a role in the adaptation of E. coli to media of high osmolarity. In contrast external osmolarity has little effect on the cellular spermidine content. No relationship has been demonstrated between K⁺ deprivation and putrescine biosynthesis, however.

Munro and Bell (1973) have suggested that for E. coli polyamines are required for growth and that putrescine may have a specific function during growth in media of low osmolarity. Their study was undertaken to determine whether high levels of intracellular putrescine or other polyamines might be necessary for growth in media of low osmolarity. They found that wild-type strains of E. coli accumulate large amounts of putrescine, but not spermidine, during growth in media of low osmolarity. Also, the growth rate of
cultures grown in low osmolarity media was directly affected by the putrescine present and could not be accounted for or explained by conversion of putrescine to spermidine. It was also found that high concentrations of spermine can completely block any effects of putrescine. They concluded that spermidine may displace putrescine from sites of action within the cell, and that in *E. coli* a normal and rapid rate of growth in media of low osmolarity is specifically dependent on the presence of putrescine.

Other bacteria besides *E. coli* have shown a requirement for polyamines. Spermidine and spermine stimulated the growth of *Pasteurella tularensis* (Traub, et al. 1955), *Lactobacillus casei* (Kihara and Snell, 1957), and *Photobacterium fischeri* (Mager, 1959). Putrescine, spermidine, and spermine are essential growth factors, as previously mentioned, for *Haemophilus parainfluenzae* (Herbst and Snell, 1948), *Aspergillus nidulans* (Sneath, 1955), and *Neisseria perflava* (Mager, 1955). In the last organism polyamine requirements can almost be entirely eliminated by increasing the osmolarity of the media with NaCl, glucose, or sucrose. (Mager, 1955).

Young and Srinivasan (1974) have investigated RNA bacteriophage f2 to determine the function of polyamines in the cell. They demonstrated that the addition of putrescine or spermidine to cells partially depleted of their polyamines initiated a definite sequence of macromolecular events, the first detectable effect being the stimulation of protein synthesis. 60 min later there was seen an increase
in stable RNA and DNA synthesis and an acceleration in the rate of cell division.

This immediate stimulation of protein synthesis suggested that the polyamines are needed in a normal cell for mRNA synthesis and/or for the efficient translation of pre-existing messengers. Because of the close association between transcription and translation in bacteria, it was not known which of the two events or if both events were affected by polyamines. By use of RNA bacteriophage f2, they could dissociate transcription from translation. The presence of polyamines enhances the growth of phage f2. It was found that this effect is due to the direct stimulation of translation of a preformed message. This stimulation is due to an increase in f2 replicase production. More replicase molecules, presumably, are being made per parental RNA in the presence of putrescine than in its absence. It is not known whether this means that there is an increase in the number of translational initiation events or more complete translations of the entire gene. It should be noted that in vitro, polyamines do not stimulate replicase activity. Thus, it is concluded that polyamines act directly in some step of mRNA translation.

Takeda, et al. (1972) have demonstrated that aminoacyl tRNA formation is stimulated by polyamines or by Mg\(^{++}\) with both reactions having the same requirements. The same tRNA is aminoacylated in the presence of either spermine or Mg\(^{++}\). These aminoacyl tRNA's can act as donors of amino acids in polypeptide synthesis.
It has been suggested that stabilization of tRNA conformation is carried out by these cations and that replacements can be made (Igarashi and Takeda, 1970; Ishida and Sueoka, 1968; Lindahl, et al. 1966). Even though the polyamines cannot completely replace Mg$^{++}$ in this reaction, they do produce the same reaction as that occurring in the presence of Mg$^{++}$ alone.

Morris and Hansen (1973) have demonstrated in their putrescine-deficient E. coli mutant a decrease in the rate of polypeptide elongation and mRNA elongation during periods of polyamine starvation. This decrease was shown to be proportional to the decrease in cellular growth rate produced by polyamine starvation. They concluded that, although it was quite unlikely that polyamine levels were involved in the regulation of cell growth, they might be acting as cofactors in the synthesis of RNA or protein, or both.

Munro and Bell (1973) have found that polyamines are required for rapid phospholipid turnover in E. coli growing in media of low osmolarity. Polyamine depletion resulted in reduced phospholipid turnover in media of low osmolarity while in media of high osmolarity, polyamine depletion had no effect on the rate of phospholipid turnover. These turnover rates were shown not to be simply a reflection of the change in growth rates of the cultures or of a shift in the relative amounts of the various phospholipids. It was also found that of the polyamines tested, only spermidine was capable of fully restoring the turnover rate to normal levels.
Many other findings regarding the functions of the polyamines have been discussed by other authors. Polyamines have been shown to bind electrostatically to nucleic acids (Ames and Dubin, 1960; Choi and Carr, 1967; Felsenfeld and Huang, 1961; Razin and Rozansky, 1959; Tabor, H., 1962). They stabilize osmotically fragile forms of bacteria (Harold, 1964; Mager, 1955; Mager, 1959a; Tabor, C.W., 1962). Polyamines lower cell-free protein synthesis (Hershko, et al., 1961; Martin and Ames, 1962). They promote the aggregation of ribosomal subunits (Silman, et al., 1965; Norton, et al., 1968; Moller and Kim, 1965; Cohen and Lichenstein, 1960; Colbourn, et al., 1961). Finally, polyamines have been shown to stimulate DNA-dependent RNA polymerase (So, et al., 1967; Petersen, et al., 1968; Krakow, 1963; Abraham, 1968).
MATERIALS AND METHODS

Organism

The organism used throughout this study was *Neurospora crassa*. The strains used were: 74A (wild-type); aga (arginase-less mutant); arg-5 (arginine-requiring mutant); put-1 (putrescine-requiring mutant). All strains were provided by Dr. K. J. McDougall. The put-1 mutant was recently isolated by Julie Miskimen utilizing the filtration-enrichment technique (Woodward, et al., 1954).

Chemicals

Except where indicated, all chemicals used were of reagent grade purity. DL-ornithine-1-C14 monohydrochloride and DL-arginine-1-C14 monohydrochloride were obtained from Amersham/Searle Corp., Arlington Heights, Ill.; pyridoxal phosphate, L-arginine, L-ornithine, cadaverine, agmatine, spermidine dihydrochloride, spermine, and ethylene diamine dihydrochloride were obtained from Sigma Chemical Co., St. Louis, Mo.; Toluene (spectroquality), methyl cellulose, and EDTA (ethylene diamine tetraacetic acid) were obtained from J. T. Baker Co., Phillipsburg, N. J.; Sephadex, G-25 coarse was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; PPO (2, 5 diphenyloxazole) was obtained from Research Products International Corp., Elk Grove Village, Ill.; Hydroxide of hyamine, 10-X, was
obtained from Packard, LaGrange, Ill.

**Medium**

The basic medium used was Vogel's Medium N (Vogel, 1958). The salt solution was kept as a 50X concentrate. 1.5% agar was added in making conidiation medium. All media were autoclaved for 15 min at 121°C and 15 psi. Medium was supplemented when necessary with 30 mg/100 ml of arginine, 30 mg/100 ml of ornithine, or 20 mg/100 ml of ornithine, or 20 mg/100 ml of putrescine. Other supplements added for growth were added at the concentrations indicated in the tables.

**Growth Conditions**

Conidia were grown in 500 ml Erlenmeyer flasks to which 50 ml of conidiation medium was added or in 3000 ml Erlenmeyer flasks to which 300 ml of conidiation medium was added. Conidia were harvested 7-10 days later by adding 100 ml of sterile water to the flasks and filtering the suspension through sterile cheesecloth to remove hyphae. The inoculum was then added directly to the growth flasks.

Cultures were grown at room temperature. Log and stationary cultures utilized for enzyme assays were grown in 3000 ml Erlenmeyer flasks under vigorous aeration. Stationary cultures were permitted to grow for 24 h unless otherwise indicated. Log phase cultures were 16-18 h old. In some cases the time allowed for growth depended upon the strain used and the conditions specified for the assay.
Growth Studies

Growth studies were carried out either in 25 ml Erlenmeyer flasks to which 10 ml of medium was added or 125 ml Erlenmeyer flasks to which 25 ml of medium was added. Unless otherwise indicated supplements were added at a concentration of 0.1 mg/ml of medium. Innoculation was carried out with one drop of filtered conidia suspension from a sterile 5 ml pipette. Flasks were incubated at 27 C for the desired time. The mycelia were collected with an inoculating loop, pressed dry, and dried in an oven for 4 h at 90 C. Dry weights were measured to the nearest mg.

Osmolality

Osmolality is a measure of solute particle concentration. An osmol is the number of particles \(6.0224 \times 10^{23}\) that will lower the freezing point of a solution 1.858 C, irrespective of whether the particles are ionic or molecular. Osmolality was measured on an Advanced Osmometer, Model 3L, from Advanced Instruments, Inc., Newton Highlands, Mass. Steps for measuring the osmolality were carried out as instructed by the "User's Guide". The instrument was standardized with a 500 m0sm standard and then 2 ml of samples were measured and their osmolality determined.

Enzyme Assays

ODC. Enzyme assays were performed according to the method of Jones, et al., (1972), with slight modifications. Scintillation vials were used as the incubation chambers. The caps were modified by
attachment of a metal pin acting as a hook from which a small square of Whatman No. 1 filter paper was hung. Intramedic tubing was placed through a hole in the cap so that the radioactive substrate and citric acid could be added without exposing the incubation chamber to atmospheric CO$_2$ and without losing any $^{14}$CO$_2$ which had been released through enzymatic activity. The tubing extended below the surface of the incubation media to prevent CO$_2$ addition or loss also.

The enzyme extract was prepared by grinding a fresh mycelial pad of log or stationary phase cultures with mortar and pestle for 5 min in 0.05 M Na/K phosphate buffer (pH 7.4). Following centrifugation at 2200 g for 15 min, the resulting supernatant was passed once through a short (15 ml void vol) G-25 coarse Sephadex column. 1 ml of this extract was added to each sample vial containing 1.5 ml of a buffer with 2 mM EDTA in 0.05 M Na/K phosphate (pH 7.4) with 10% pyridoxal phosphate. Papers moistened with 0.1 ml of hyamine hydroxide were hung from the metal hook and the caps tightly sealed. Vials were pre-incubated for 10 min in a shaking water bath at 30 C. 1 ml of DL-ornithine-1-C14 containing 0.5 uCi/ml (sp. Act. = 29 mCi/mmol) or 1 uCi/ml (Sp Act. = 61 mCi/mmol) was injected into each vial. Controls contained boiled extract. The vials were then placed back in the water bath for 30 min. To stop the reaction 1 ml of 2M citric acid was injected into each vial. All vials were allowed to remain in the bath for an additional 30 min to allow for complete
$^{14}$CO$_2$ absorption. Papers were removed and placed in scintillation vials containing 12 ml of liquid scintillation fluid (3 g PPO, 500 ml methyl cellosolve, 750 ml toluene). Vials were counted on a liquid scintillation counter (Nuclear, Chicago). Total protein was determined by the Lowry method (Lowry, et al., 1951).

**ADC.** Assays for ADC were carried out in the same manner as the ODC assays with a few modifications. DL-arginine-1-C14 monohydrochloride (20 mCi/mmol) was utilized as the substrate. 1 ml of substrate containing 0.5 uCi/ml was added to each vial. Several different buffer systems besides the standard assay buffer were used since Morris and Pardee (1966) had shown in *E. coli* that there were two different ADC enzymes, one requiring EDTA for activity and the other requiring Mg$^{++}$ and pyridoxal phosphate. Buffers containing 2 mM EDTA; varying concentrations of Mg$^{++}$ (0.003M, 0.005M, and 0.008M); 10% pyridoxal phosphate with 0.005M Mg$^{++}$; and the standard buffer with 0.005M Mg$^{++}$ were all tested to determine the presence of any ADC activity.
RESULTS

The put-1 mutant

The minimum amount of putrescine required for maximum growth of the put-1 mutant was determined by measuring the amount of growth after five days in medium containing varying concentrations of putrescine. Fig. 6 indicates that a range of 0.06 to 0.20 mg/ml of putrescine resulted in similar amounts of growth. When putrescine was not present in the medium, there was no visible growth. Putrescine concentrations of less than 0.06 mg/ml resulted in less than the maximum amount of growth.

The put-1 mutant was then tested for growth on medium supplemented with polyamines (0.2 mg/ml) and their derivatives. Fig. 7 illustrates the growth of the put-1 mutant on these compounds and the rate at which the mutant responded. The put-1 mutant grew on putrescine, spermidine, spermine, agmatine, and cadaverine, but it did not grow on ornithine, arginine, or ethylene diamine dihydrochloride.

Growth was evident after one day when either putrescine or spermidine was added to the medium. After four days the amount of growth of put-1 was about the same on either putrescine or spermidine-supplemented medium. The rate of growth of the put-1 mutant
Fig. 6. Growth response of the put-1 mutant to varying concentrations of putrescine. Cultures were harvested after 113 h incubation.
Putrescine (mg/ml)

Dry Weight (mg/25ml)

Putrescine (mg/ml)
Fig. 7. Comparison of the growth of the put-1 mutant on polyamine-supplemented medium (0.2 mg/ml) to growth of wild type 74A on minimal medium and putrescine-supplemented medium (0.2 mg/ml). wild type 74A: on minimal (□— □); on putrescine (■— ■). put-1: on putrescine (○— ○); on spermidine (▲— ▲); on agmatine (○— ○); on spermine (△— △).
was slightly less on spermidine as compared with putrescine-supplemented medium. A comparison of these results with those obtained for wild type 74A grown on minimal medium or putrescine-supplemented medium demonstrates that the growth response of put-1 on putrescine is slower than that of wild type 74A on the same concentration of putrescine (0.2 mg/ml) or on minimal medium. The growth response of wild type 74A on minimal medium is similar to growth on putrescine-supplemented medium, however.

Agmatine-supplemented medium supported a delayed growth response by the put-1 mutant. A dry weight measurement was not possible until the third day. On the fourth day the dry weight was 1.5 mg/25ml but by the fifth day the dry weight reached 12 mg/25ml. One culture continued to grow for nine days resulting in a dry weight of 26 mg/25ml surpassing the growth of put-1 on either putrescine- or spermidine-supplemented medium.

Cultures grown on spermine-supplemented medium showed a delayed response even greater than the delay of agmatine. Growth was evident but not measureable by the fifth day. By the eighth day the culture measured 5.6 mg dry weight per 25 ml. The put-1 mutant never did achieve the amount of growth on spermine that was demonstrated by the mutant when grown on putrescine-, spermidine- or agmatine-supplemented medium.

Several concentrations of cadaverine (0.1, 0.2, 0.5, and 1.0 mg/ml) were tested to determine if put-1 would grow in its presence.
Cultures of the mutant were incubated for 21 days but never grew enough to be harvested. Thus, the dry weight of put-1 grown on cadaverine-supplemented medium was less than 1 mg/25ml even after 21 days.

The put-1 mutant was also tested to see if it would grow on ornithine (0.3 mg/ml), arginine (0.3; 0.5, 1.0, and 2.0 mg/ml), ethylene diamine dihydrochloride (0.2 mg/ml), and minimal medium. In no case was visible growth detected.

Growth at Various Osmolalities. The put-1 mutant was tested in duplicate to see if it would grow on minimal medium in which the osmolality was increased by addition of sucrose, MgCl₂, KCl, NaCl or CaCl₂. Sucrose was tested at eight concentrations resulting in media with osmolalities ranging from 176 mOsm (5.0 mg sucrose per ml) to 475 mOsm (100 mg sucrose per ml). MgCl₂ was tested at seven concentrations resulting in media ranging from 185 mOsm (0.4 mg MgCl₂ per ml) to 241 mOsm (25 mg MgCl₂ per ml or 0.1 M MgCl₂). CaCl₂ was tested at three concentrations resulting in media ranging from 184 mOsm (0.2 mg CaCl₂ per ml) to 194 mOsm (1.0 mg CaCl₂ per ml). NaCl was tested at an osmolality of 220 mOsm (58.5 mg of NaCl per ml or 0.1M NaCl) and KCl was tested at an osmolality of 216 mOsm (74.5 mg KCl per ml or 0.1 M KCl). The minimal medium had an osmolality of 185 mOsm while minimal medium supplemented with 0.2 mg/ml of putrescine had an osmolality of 186 mOsm. No growth was noted in any of these flasks after three weeks except in those flasks to which putrescine was added. Cultures from control flasks containing
0.2 mg/ml of putrescine reached a dry weight of 41 mg/25 ml in 90 h.

**Enzyme Assays.** The wild type strain 74A showed a low but detectable amount of ODC specific activity. The first assays carried out showed that the wild type strain had a specific activity of $0.78 \pm 0.17$ pmol of substrate converted to putrescine per μg of protein per h. When the put-1 mutant was tested at this time, the specific activity of the ODC enzyme was $0.01 \pm 0.01$ pmol/μg of protein/h.

A comparison of ODC specific activities for wild type 74A at various stages of growth on minimal medium showed some variability (Table 1). Assays of cells after 16, 18, and 22 h were not significantly different ($p < 0.05$) from one another, however, assays of cells grown for 36 h showed a specific activity which was significantly lower ($p < 0.01$) than the other specific ODC activities.

Table 2 illustrates the specific activity of the ODC enzyme from the put-1 mutant when grown in minimal medium supplemented with 0.2 mg/ml of putrescine. The specific activity does not vary significantly ($p < 0.05$) when cells were assayed after 24, 48, and 72 h. In all cases the specific activity was $0.01 \pm 0.01$ pmol/μg/h or less.

A measurement of the ODC specific activity at 10, 20, and 30 min (Fig. 8) shows a linear increase when wild type 74A is grown on minimal medium or on medium supplemented with 0.2 mg/ml of putrescine. Putrescine addition resulted in a reduction in ODC specific activity for wild type 74A but these levels clearly indicate enzymatic activity. In contrast to this the put-1 mutant when grown in the presence of 0.2 mg/ml of putrescine showed extremely low levels of enzyme
Table 1. Specific activity of ODC from wild type 74A after growth for various periods of time.

<table>
<thead>
<tr>
<th>Age of Culture (h)</th>
<th>Specific Activity (± S. D.)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>2.38 ± 0.22</td>
</tr>
<tr>
<td>18</td>
<td>2.19 ± 0.11</td>
</tr>
<tr>
<td>22</td>
<td>2.24 ± 0.13</td>
</tr>
<tr>
<td>36</td>
<td>1.66 ± 0.13</td>
</tr>
</tbody>
</table>

<sup>a</sup>Specific activity is expressed as pmol of substrate (DL-ornithine-1-C14) acted upon per μg of protein per h. Values were obtained from at least two experiments each done in triplicate. Analysis of variance indicates that only the 36 h assay is significantly different from the other three assays p<0.01).
Table 2. Specific activity of ODC from the put-1 mutant after growth for various periods of time.

<table>
<thead>
<tr>
<th>Age of Culture (h)</th>
<th>Specific Activity (+ S. D.)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.010 ± 0.010</td>
</tr>
<tr>
<td>48</td>
<td>0.006 ± 0.003</td>
</tr>
<tr>
<td>72</td>
<td>0.008 ± 0.002</td>
</tr>
</tbody>
</table>

<sup>a</sup>Specific activity is expressed as pmol of substrate (DL-ornithine-1-C14) acted upon per µg of protein per h. Values were obtained from at least two experiments done in triplicate. Analysis of variance indicates that there is no significant difference between any of these values (p < 0.05).
Fig. 8. Specific activity of ODC at 10, 20, and 30 min for wild type 74A on minimal medium: assay 1 (○—○); 74A assay 2 (□—□). 74A on medium supplemented with 0.2 mg/ml of putrescine (●—●); put-1 on medium supplemented with 0.2 mg/ml of putrescine (△—△). Specific activity is expressed as pmol of substrate (DL-ornithine-1-C14) acted upon per μg of protein.
activity.

Table 3 shows that the specific activity of the ODC enzyme for wild type 74A grown on minimal medium varied markedly with each assay performed. Specific activities as high as $2.24 \pm 0.13$ pmol/μg/h and as low as $0.16 \pm 0.01$ pmol/μg/h were recorded for 24-h cultures. The put-1 mutant, however, did not demonstrate a similar variability and never exceeded a specific activity of $0.01 \pm 0.01$ pmol/μg/h.

Duplicate assays were performed in which L-ornithine was added as a carrier at final concentrations of 10, 15, 50, 100 and 400 μM. Addition of carrier did not produce an increase in the ODC specific activity indicating that part of the ornithine pool was not being removed when the enzyme extract was passed through the Sephadex column.

A comparison was made of the ODC specific activities of both the wild type 74A and the aga mutant when grown on minimal medium and on medium supplemented with arginine (Table 4). Both strains showed ODC specific activities which were not significantly different from one another ($p < 0.01$) when grown on minimal medium. However, when these two strains were grown on arginine (0.3 mg/ml), each showed a significant increase ($p < 0.01$) in ODC specific activity. The wild type 74A strain showed an ODC specific activity about three times greater than the ODC specific activity on minimal medium. The aga mutant showed an extremely large increase which was almost 50 times greater than the ODC specific activity on minimal medium.
Table 3. Variation in ODC activity in 24-h cultures of wild type 74A.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Specific Activity (± S. D.)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.78 ± 0.17</td>
</tr>
<tr>
<td>2</td>
<td>0.90 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>2.24 ± 0.13</td>
</tr>
<tr>
<td>4</td>
<td>1.30 ± 0.13</td>
</tr>
<tr>
<td>5</td>
<td>0.46 ± 0.07</td>
</tr>
<tr>
<td>6</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific activity is expressed as pmol of substrate (DL-ornithine-1-C14) acted upon per µg of protein per h. Values were obtained from two experiments each done in triplicate. Analysis of variance shows that there is a significant difference between all assays (<i>p</i> < 0.01) except for assays 1 and 2. Assays 1 and 2 show no significant difference (<i>p</i> < 0.05).
Table 4. Comparison of ODC activities of 24-h cultures of wild type 74A and aga when grown on Vogel’s minimal medium or on arginine-supplemented medium.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Strain</th>
<th>Supplement</th>
<th>Specific Activity (+ S. D.)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74A</td>
<td>minimal</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>aga</td>
<td>minimal</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>74A</td>
<td>arginine</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>aga&lt;sup&gt;a&lt;/sup&gt;</td>
<td>arginine</td>
<td>8.12 ± 0.83</td>
</tr>
</tbody>
</table>

<sup>a</sup>These aga cultures were allowed to grow for 28 h to permit them to reach the same stage of growth as wild type 74A and aga when grown on minimal medium (Davis et al, 1970).

<sup>b</sup>Specific activity is expressed as pmol of substrate (DL-ornithine-1-C14) acted upon per μg of protein per h. Values were obtained from two experiments each done in triplicate. Analysis of variance shows that there is a significant difference between all assays (p< 0.01) except for assays 1 and 2. Assays 1 and 2 show no significant difference (p< 0.05).
ODC specific activity was examined in wild type 74A when grown in putrescine-supplemented medium (in vivo) compared to ODC specific activity when grown in minimal medium with putrescine added to the incubation buffer (in vitro). Table 5 shows that both in vivo and in vitro putrescine addition resulted in a reduction in ODC specific activity. However, the reduction was significantly greater for the 0.2 mg/ml putrescine addition in vitro as compared to the in vivo addition.

Since the enzyme extract was normally passed through a Sephadex column prior to incubation, it was probable that some of the putrescine in the in vivo assay was being removed. To determine the effect of the column on the specific activity of ODC, assays were performed in which half of the extract was passed through the column while the other half was not. While the normal wild type 74A ODC specific activity was rather low (0.16 pmol/µg of protein/h), a marked decrease occurred in both cases where putrescine was added (Table 6). The extract from 74A grown on putrescine-supplemented medium, which was passed through the column, showed an ODC specific activity which was approximately one-third the specific activity of an extract taken from cultures grown on minimal medium and passed through the column. However, extracts from the 74A grown in putrescine-supplemented medium which were not passed through the column showed an even greater decrease.

ADC assays were carried out to determine whether wild type
Table 5. Specific activities of ODC from 24-h cultures of wild type 74A after growth on putrescine-supplemented medium (in vivo) compared to growth on Vogel's minimal medium with putrescine addition to the assay buffer (in vitro).

<table>
<thead>
<tr>
<th>Putrescine Concentration (mg/ml)</th>
<th>Specific Activity$^a$ in vivo (Mean ± S. D.)</th>
<th>Specific Activity$^a$ in vitro (Mean ± S. D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.46 ± 0.07</td>
<td>0.46 ± 0.07</td>
</tr>
<tr>
<td>0.2</td>
<td>0.30 ± 0.04</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>1.0</td>
<td>0.02 ± 0.005</td>
<td>0.01 ± 0.003</td>
</tr>
</tbody>
</table>

$^a$Specific activity is expressed as pmol of substrate (DL-ornithine-1-C14) acted upon per μg of protein per h. Values were obtained from two experiments each done in triplicate. Analysis of variance indicates that there is a significant difference between the three in vivo assays ($p < 0.01$). Likewise, a significant difference exists between the three in vitro assays ($p < 0.01$).
Table 6. Specific activity of ODC from 24-h cultures of wild type 74A after growth on medium containing 0.2 mg of putrescine per ml. Comparison of activity when extract was passed through a Sephadex column to activity when extract was not passed through the column.

<table>
<thead>
<tr>
<th>Extract Procedure</th>
<th>Supplement</th>
<th>Specific Activity&lt;sup&gt;a&lt;/sup&gt; (Mean ± S. D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>column</td>
<td>minimal</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>column</td>
<td>putrescine</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>no column</td>
<td>putrescine</td>
<td>-0.02&lt;sup&gt;b&lt;/sup&gt; ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup>Specific activity is expressed as pmol of substrate (DL-ornithine-1-C<sub>14</sub>) acted upon per µg of protein per h. Values were obtained from two experiments each done in triplicate. Analysis of variance indicates that these values are significantly different (p < 0.01).

<sup>b</sup>Values for specific activity were less than the controls.
74A possessed any ADC activity. Several buffer systems were utilized in these assays of cultures grown on minimal medium. Extremely low activities were detected (Table 7) with the highest value being 0.07 pmol/µg/h and the lowest value being 0.01 pmol/µg/h.

Both the put-1 and aga mutants were tested for ADC activity (Table 8). When grown on either arginine-supplemented or minimal medium, put-1 showed specific activities which were less than the controls. One ADC assay of put-1 showed a significant activity (0.24 pmol/µg/h), however, repeated assays did not produce similar results and this high value cannot be explained. The aga mutant showed results similar to wild type 74A with a specific activity of 0.01 pmol/µg/h when grown on minimal medium and 0.06 pmol/µg/h when grown on arginine-supplemented medium.
Table 7. Specific activity of ADC from 24-h cultures of wild type 74A after growth on Vogel’s minimal medium. Comparison of specific activities on various buffers.

| Assay | Buffer | Specific Activity (± S. D.)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.003M MgSO₄</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.005M MgSO₄</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.008M MgSO₄</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>Standard + 0.005M MgSO₄</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>2 mM EDTA</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.005M MgSO₄ + 10% pyridoxal phosphate</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

*Specific activity is expressed as pmol of substrate (DL-arginine-1-C¹⁴) acted upon per µg of protein per h. Values were obtained from two experiments each done in triplicate. Analysis of variance indicates that assay 2 is significantly different from assays 1, 5, 6, and 7 (p < 0.01). Assay 2 is not significantly different from assays 3 and 4 (p < 0.05).*
Table 8. Specific activity of ADC from 24-h cultures of wild type 74A, *aga*, and *put-1* after growth on medium containing various supplements.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Strain</th>
<th>Supplement</th>
<th>Specific Activity (+ S. D.)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74A</td>
<td>minimal</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td><em>put-1</em></td>
<td>putrescine</td>
<td>-0.01 ± 0.003&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td><em>put-1</em></td>
<td>putrescine + arginine</td>
<td>-0.01 ± 0.002&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td><em>aga</em></td>
<td>minimal</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>5</td>
<td><em>aga</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>arginine</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific activity is expressed as pmol of substrate (DL-arginine-1-C14) acted upon per µg of protein per h. Values were obtained from two experiments each done in triplicate. Analysis of variance indicates a significant difference exists between assays 4 and 5 (p < 0.01).

<sup>b</sup> These *aga* cultures were allowed to grow for 28 h to permit them to reach the same stage of growth as wild type 74A and *aga* when grown on minimal medium (Davis et al, 1970).

<sup>c</sup> Values for specific activity were less than the controls.
DISCUSSION

Mutants of other organisms have been isolated which require polyamines for growth (Munro and Bell, 1973a). Herbst and Snell (1948) isolated a putrescine-requiring mutant of *Haemophilus parainfluenzae*. Martin, et al., (1952) isolated a mutant of *Neisseria perflava* which also required putrescine for growth. Other mutants have been isolated whose growth was stimulated by spermidine and spermine (Traub, et al., 1955; Kihara and Snell, 1957; and Mager, 1959). Recently Maas, et al., (1970) and Morris and Jorstad (1970) isolated putrescine-deficient mutants of *E. coli*. However, the only eucaryote in which a putrescine-requiring mutant has been reported is *Aspergillus nidulans* (Sneath, 1955). In this mutant it was demonstrated that maximal growth occurred when the medium was supplemented with 0.001 mg of putrescine per ml.

This paper presents the finding of a polyamine-requiring mutant of the eucaryote, *Neurospora crassa*. This mutant did not grow on Vogel's minimal medium, but responded well to putrescine-supplemented medium or to medium supplemented with the immediate product of putrescine, spermidine. The response of the mutant to putrescine (Fig. 7), however, was somewhat delayed in comparison to the response of wild type 74A to the same concentration of putres-
cine. On the second and third days the dry weights of wild type 74A grown in putrescine-supplemented medium were two times greater than the dry weights of the put-1 mutant grown in the same medium. But, by the fourth day the dry weights of these two strains were very similar.

The put-1 mutant demonstrated an even greater delayed growth response in the presence of agmatine and spermine. In the presence of agmatine, the put-1 mutant did not respond well until the fourth day. However, by the ninth day the growth of the put-1 mutant exceeded all other dry weight measurements. Growth of the put-1 mutant on spermine never reached the level attained when it was grown on putrescine, spermidine or agmatine.

Growth on spermine was possibly due to a reversal of the reaction which results in its synthesis from spermidine. Another possible explanation for growth on spermine is that put-1 can partially carry out the function of spermidine in the cell but it cannot completely substitute for it. Growth on agmatine could be explained by the fact that it is readily converted to putrescine and urea by the enzyme, agmatine ureohydrolase. This enzyme has not been demonstrated in N. crassa. If the enzyme is present, the delayed growth by put-1 on agmatine could be explained as an induction of this enzyme. Another explanation is that the spermidine-synthesizing enzymes are able to utilize the aminopropyl moiety of agmatine when putrescine is not present, but not as efficiently.
The put-1 mutant did not grow on medium supplemented with arginine or ornithine suggesting a lack of ODC and ADC activity. It did not grow in the presence of ethylene diamine or cadaverine. In the case of cadaverine slight growth was seen at the higher concentrations; however, the mutants minimal response to cadaverine indicated an inability to utilize cadaverine as a substitute for putrescine.

Putrescine, cadaverine, and ethylene diamine all possess two amino groups (Fig. 1). They differ in the number of propyl groups between the two amino groups. The fact that neither cadaverine nor ethylene diamine substitute for putrescine suggests that not only is the presence of the two amino groups important for polyamine action in this organism, but possibly the size and configuration of the molecules is important. A second possibility is that the size of the polyamine affects its transport across the membrane. However, agmatine, which is utilized by put-1, is a larger molecule than ethylene diamine and comparable in size to cadaverine. Neither cadaverine nor ethylene diamine are able to support growth by put-1. Spermidine is a larger molecule than either ethylene diamine or cadaverine but it is readily utilized by put-1 suggesting that transport is not the reason for the put-1 mutant's inability to utilize some polyamines.

These results also support the hypothesis that spermidine is the active compound necessary for growth of the organism. Apparently neither cadaverine nor ethylene diamine can be utilized in the absence of putrescine to produce spermidine.
Other putrescine-requiring and putrescine-deficient mutants have the ability to grow in the absence of putrescine if the osmolality of the medium is increased (Morris and Jorstad, 1970; Mager, 1955). The put-1 mutant did not demonstrate such an ability.

Mager (1955) demonstrated in _N. perflava_ that 0.1M NaCl, KCl, or sucrose addition to the medium allowed a putrescine mutant to grow. Addition of these compounds to the medium did not result in growth of the put-1 mutant. When the osmolality of the medium was almost tripled using sucrose, there was still no growth, nor did MgCl₂ or CaCl₂ result in growth. It would be of interest to see if the amount of putrescine required for maximal growth by put-1 is reduced in medium of higher osmolality. Excretion of putrescine by _N. crassa_ (Viotti, et al., 1971) and by _E. coli_ (Munro and Sauerbier, 1973) has been reported when these organisms are grown in media of high osmolality.

As seen in Table 2 and Fig. 8 there is an extremely low ODC specific activity for the put-1 mutant compared to wild type 74A. Since this mutant responded well to the putrescine supplement, this finding was reasonable.

Subramanian, et al., (1973) reported the presence of an ODC enzyme in _N. crassa_ with a specific activity of approximately 3 pmol/μg of protein/h. The highest specific activity obtained in this study for cultures at a similar stage of growth was 2.24 pmol/μg/h which is in agreement with their findings.
It is apparent that ODC is present in *N. crassa* and is the major and possibly the only route to putrescine synthesis in this organism. It is also apparent that repeated assays for this enzyme demonstrated a great deal of variability in the specific activity of the ODC enzyme. The decrease in the specific activity of the ODC enzyme in the 36-h culture is typical for this enzyme and has been reported to occur in mammalian liver cells (Cohen, 1971) and chick embryo cells (Russell and Snyder, 1970).

Variability in the specific activity of the ODC enzyme from assay to assay (Table 3) also is not unusual and is known to occur in some fungal enzymes (Walker and Cooney, 1973). The variability of the ODC enzyme has been reported by others (Jones, et al., 1972 and Russell and Snyder, 1970). The ODC enzyme is stable, it has a short half-life of 11 min (Cohen, 1971), and it is extremely susceptible to freezing and heating (Snyder, et al., 1970). A 75% loss of ODC specific activity was seen in 10-day old chick embryos when the soluble supernatant fraction was frozen prior to assay.

Another possible reason for this variability between assays is that there was not enough ornithine present to saturate the enzyme. The Km of the ODC enzyme was reported to be 400 uM (Subramanian, et al., 1973). Addition of 1 uCi of DL-ornithine-1-C14 (68 mCi/mmol) resulted in a final concentration of approximately 5 uM. Addition of unlabelled L-ornithine at final concentrations of 10, 15, 50, 100, or 400 uM, however, did not result in higher ODC specific activities.
This indicated that the endogenous ornithine pool had not been removed when the cell extract was passed through the Sephadex column, and that sufficient substrate was present for saturation of the enzyme. Some variation may have occurred in the amount of ornithine present, in the cell extract after having passed through the column and could have been responsible for some of the variability in ODC specific activity between assays.

ODC activity is inhibited by the end product, putrescine (Table 5). Addition of 0.2 mg of putrescine per ml in vitro resulted in a reduction of the ODC specific activity for wild type 74A to one-tenth of its specific activity when the culture was grown on minimal medium. Addition of the same amount of putrescine in vivo reduced the ODC specific activity for wild type 74A to two-thirds of its specific activity when the culture was grown on minimal medium. Addition of 1.0 mg of putrescine per ml either in vivo or in vitro almost completely inhibited ODC activity for wild type 74A.

Table 5 suggests that reduction in the amount of ODC specific activity may be due to feedback inhibition. Equivalent putrescine concentrations caused a slight decrease in the ODC specific activity in vivo while producing a large decrease in vitro. Further evidence to support this hypothesis is found in Table 6. Since the enzyme extract was passed through the Sephadex column, much of the putrescine was probably being removed from the extract with enough remaining perhaps bound to the enzyme to inhibit ODC activity. If this same extract
was not passed through the column, there was virtually no ODC activity. This supports the hypothesis that inhibition of ODC activity is due to feedback inhibition, but it does not entirely eliminate the possibility that end product repression also occurs.

When cultures were grown in arginine-supplemented medium, the specific activity of the ODC enzyme was increased. In wild type 74A an arginine-supplemented culture showed an ODC specific activity which was three times greater than the specific activity when cultures were grown on minimal medium. Arginine-supplemented aga cultures showed an increase in ODC specific activity almost 45 times greater than aga cultures grown on minimal medium. In the aga mutant conversion of arginine to ornithine is not known to occur. The aga mutant grows more slowly on arginine-supplemented medium than on minimal medium or ornithine-supplemented medium. Arginine-grown aga strains undergo "ornithine starvation" and have a resulting putrescine deficiency because arginine results in feedback inhibition of the biosynthetic ornithine enzymes. If ornithine cannot be made either by biosynthesis or degradation, it would appear that N. crassa could survive only if putrescine was not a necessary growth factor or there was another path to putrescine synthesis besides ODC action. Since the results presented here show that putrescine is a growth requirement, there are two possible explanations for the ability of the aga strain to grow on arginine. One is that a small amount of ornithine is being produced and rapidly converted to putrescine either by a
reversal of the OTA reaction or a reversal of the arginine-to-ornithine biosynthetic pathway (Davis, 1968). Davis, et al. (1970) also suggested these possibilities but refuted them with evidence from growth studies on several mutants. They showed that the growth of arg-5, ota aga and arg-5, arg-12 on arginine was the same as aga grown on arginine. This suggests that reversal of the OTA reaction, reversal of the arginine-to-ornithine biosynthetic route, or the biosynthesis of ornithine is not producing the ornithine needed for putrescine synthesis.

Another explanation is that there is a second pathway to putrescine synthesis in N. crassa via the ADC pathway. The evidence presented here does not prove this, but it suggests it.

There is ADC activity present at extremely low levels in wild type 74A and in aga, but not in put-1 (Tables 7 and 8). The specific activity for wild type 74A in assay 2 in Table 7 is significantly different from the specific activities for assays 1, 5, 6 and 7 (p < 0.01). Thus, addition of 0.03M MgSO₄ resulted in the only significant ODC specific activity. The ADC enzyme found in E. coli also has been reported to require MgSO₄ for activity (Morris and Pardee, 1966). When arginine was added to the medium, aga cultures also demonstrated significant ADC specific activities (Table 8).

Low ADC activity could be explained by the fact that ADC is probably a cytoplasmic enzyme and that less than 5% of exogenous arginine is found in the cytoplasm. The rest is sequestered in a vesicular compartment (Weiss and Davis, 1973). Thus, much of the
arginine is not available to the enzyme and little catabolism occurs. It may be that ADC production is induced only at very high concentrations of arginine. In the put-1 mutant, however, high concentrations of exogenous arginine did not result in growth. This suggests that ADC activity is not sufficient to supply N. crassa with the necessary amount of putrescine for growth.

The presence of ADC could explain the ability of put-1 to grow on agmatine, as well as the ability of aga to grow on arginine. It could also support the conclusions of Davis, et al. (1970) concerning the "ornithine starvation" hypothesis. However, additional evidence must be obtained before it can be concluded that N. crassa possesses a second pathway to putrescine synthesis.
SUMMARY AND CONCLUSIONS

(1) **Neurospora crassa** requires putrescine or its immediate product, spermidine, for maximal growth. The putrescine mutant (put-1) requires 0.06 to 0.20 mg/ml of putrescine for maximal growth with growth decreasing as the putrescine concentration decreases.

(2) Agmatine supports the growth of put-1 but only after a delay of several days. Spermine supports growth to a lesser extent while cadaverine produced only a very slight growth response.

(3) The put-1 mutant will not grow on minimal medium or medium supplemented with arginine, ornithine, or ethylene diamine.

(4) The put-1 mutant will not grow in minimal medium in which the osmolarity of the medium has been increased unless putrescine is added.

(5) The put-1 mutant lacks the ODC activity seen in wild type 74A and aga.

(6) ODC specific activity shows much variability from one batch of cells to another but not from preparation to preparation.

(7) ODC specific activity decreases in the presence of its end product, putrescine. **In vitro** putrescine produced a much more severe decrease than **in vivo** putrescine.

(8) The presence of arginine in the medium stimulates the production
of ODC particularly in the aga mutant.

(9) The possibility exists that there is a second pathway to putrescine synthesis in N. crassa. Evidence for this lies in the low levels of ADC specific activity, the stimulation of ADC specific activity in the presence of arginine, the growth of put-1 on agmatine, and the growth of aga on arginine.
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