STUDIES OF A POSITION EFFECT AND AMINE METABOLISM IN ASPERGILLUS

by

Dionysios-Haralampos Spathas B.Sc.

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Institute of Genetics
University of Glasgow
GLASGOW, SCOTLAND

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To my mother

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SUMMARY

The purpose of these studies was mainly the investigation of a variegated position effect affecting the conidiation system of Aspergillus nidulans.

Genetical and biochemical investigations were undertaken to explore the nature of the variegated process and therefore gain information about the mechanism(s) underlying the relationship of chromatin structure and gene expression.

The main lines towards which these studies were finally orientated include (a) studies of the brlA12 by means of modifiers isolated as such or found in stock strains; such studies aim to trace other features of these mutants and to correlate them to their modifying action; (b) studies of the brlA12 by means of environmental effects on its variegation, caused by chemicals administered in the growth medium; such studies would produce evidence about the biochemical nature of the variegation; (c) search for biochemical defects either in brlA12 itself or its modifiers in combination with the other approaches.

The main points which emerged from the above studies can be summarised here as follows:

(1) brlA12 grows normally on any nitrogen or carbon sources tested. Its variegation is shifted towards wild type appearance (increased conidiation) by salts, methylamine, and to a minor extent, by a number of other compounds, low pH and temperature.

(2) A number of mutants isolated as such or found in the stock strains act as suppressors or enhancers of the variegation (increasing or reducing the conidiation further, respectively).

(3) One class of the suppressors, represented by rev-2 is unable to utilize galactose as carbon source; no clear pattern correlating galactose utilization and brlA1 2 variegation has emerged however. A connection of galactose utilization and polyamine metabolism may exist (as in yeasts) since iproniazid, an amine oxidase inhibitor, repairs to some extent growth of these suppressors on galactose.

(4) The suppressors which are defective on galactose are extra sensitive to molybdate, but this pattern is followed only by modifiers isolated as such; gam and some gal mutants which are all defective on galactose and enhance the brlA1 2 mutant are molybdate resistant.

(5) Molybdate resistant strains (isolated as such or found to be modifiers of brlA1 2)act as enhancers with the exception of molA which has no effect on the variegation; this mutant however may have a different basis of resistance.

(6) rev-2 has low viability rate and high spontaneous mutation frequency to selenate resistance.

(7) rev-2 has subnormal growth on putrescine as nitrogen source and it is pigmented pink. The pigmentation is greatly enhanced if nitrate (or nitrite) is present at the same time (glucose as carbon source), in which case conidiation is extremely poor.

(8) brlA1 2 and to a lesser extent all the brl mutants (non-variegated alleles) are also pigmented on putrescine plus nitrate but not on putrescine alone.

(9) Preliminary investigations suggest that the pink pigment, which may be relevant to conidiation processes, may be a prodigiosin-like pigment with putrescine or a compound derived from putrescine as its biosynthetic precursor.

(10) rev-2, rev-5, rev-7 do not alter the conidiation pattern of the non-variegated leaky brlA9; this may indicate that these suppressors affect chromatin structure in general rather than being conidiation specific.

(11) Studies of polyamine metabolism did not reveal any differences in terms of polyamine uptake or putrescine oxidase and transaminase activities. An instance of possibly abnormally high internal putrescine pools for rev-2 after growth on this amine needs further investigation.

Although studies of amine metabolism in this work were orientated to their connection with brlA12, a number of general aspects of polyamine metabolism and regulation in Aspergillus nidulans have been investigated; a number of mutants altered in polyamine metabolism were isolated, two catabolic enzymes for putrescine were characterised, and the uptakes and internal polyamine pools were studied. Also an activation of glutamate dehydrogenase by polyamines was examined. The main points which arise from these studies can be summarised here as follows:

(1) spsA1 is a spermidine-spermine sensitive mutant which, in conjunction with the putrescine auxotroph will allow the latter to grow on low spermidine as an alternative to low putrescine. This mutant has altered spermidine and spermine internal pools as a result of

still unknown defects, which may account for its sensitivity to these polyamines. The rate of polyamine uptake in this mutant is 30-50% higher than that of the wild type and the Km for spermidine may be different in the mutant.

(2) Mutants unable to utilize putrescine as nitrogen source have been produced by mutagenesis of the wild type and selection on putrescine medium; some, but not all of these mutants were found to be sensitive to the amine oxidase inhibitor isoniazid which therefore appears to mimic putrescine and compete with it for degrading enzyme(s) which are absent from the sensitive strains.

(3) Chlorate, an analogue of nitrate, shuts down putrescine metabolism as it does for other organic nitrogen sources such as amino acids. The *nirA*^{c}1 constitutive mutant for nitrate-nitrite reductases is unable to grow normally on putrescine and also on certain amino acids; these two observations suggest a link between putrescine and nitrate metabolism.

(4) *tamA*^{r}50 and *areA*^{r}550 do not grow on putrescine as nitrogen source which indicates that putrescine utilization is subject to ammonium repression.

(5) Uptake and enzyme studies showed that the transport of polyamines and one of the two enzymes studied are indeed under ammonium repression.

(6) Internal polyamine pools were estimated after various growth conditions; concentrations of spermidine are approximately 3 times higher than those of spermine and 10 times higher than those of putrescine, after growth on nitrate or nitrogen starvation.

(7) A putrescine oxidase and a putrescine transaminase were found to

degrade putrescine; the first enzyme is under ammonium repression, the other under carbon catabolite repression. Both, however, appear to be of minor importance for the degradation of putrescine since their levels in various mutants do not correlate with ability to grow on this nitrogen source. The existence of a third enzyme is therefore most likely.

(8) An activation of the NADP linked glutamate dehydrogenase by polyamines or magnesium ions was detected, enabling this enzyme to utilize tris (hydroxylmethyl) aminomethane as a poor substrate instead of glutamate.

ABBREVIATIONS

α-ABA	α-aminobutyric acid
α-A-i-BA	α-amino-iso-butyric acid
γ-ABA	γ-aminobutyric acid
EDTA	Ethylenediaminetetra-acetic acid
FAD	Flavin-adenine dinucleotide
MGBG	Methyl glyoxal bis-(guanyl hydrazone)dihydrochloride monohydrate
M.S.E.	Master Strain E
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
NAD(P)-GDH	NAD(P)-linked glutamate dehydrogenase
NO_3^-	(sodium nitrate)
NO_2^-	(sodium) nitrite
NH_4^+	Ammonium (tartrate)
NTG	N-methyl-N-nitro-N-nitrosoguanidine
PMSF	Phenylmethylsulfonylfluoride
PU	Putrescine
pyr.PO_4	pyridoxal-5'-phosphate
SAM	S-Adenosylmethionine
SPD	Spermidine
SP	Spermine
T.L.C.	Thin Layer Chromatography
tris	tris(hydroxylmethyl)aminomethane
UDP-galactose	Uridine-diphosphogalactose

SECTION 1

INTRODUCTION

1.1. General remarks

The work described in this thesis started as an attempt to investigate the case of a variegated position effect in Aspergillus nidulans (Clutterbuck, 1970). Such phenomena have been described in a number of organisms, predominantly in Drosophila species, and they have been invariably found associated with changes in chromosome structure (Baker, 1968).

As an extension of these studies the metabolism and regulation of polyamines in this fungus were also investigated.

This introduction will cover only the general aspects of the above mentioned topics and those directly relevant to the work done. For further information on the details of the subjects there exist excellent reviews such as those by Lewis (1950), Baker (1958) and Spofford (1976) on position effects, those by Cohen (1971), Bachrach (1973) and Kapeller-Adler (1970) on polyamines, and those by Lewin (1974), Elgin and Weintraub (1975) and JeiLi and Eckhardt (1977) on chromosome structure.

1.2. Chromosome structure and function

The fact that the normal expression of a gene may be dependent upon its position on the chromosome has been established for more than fifty years (Sturtevant, 1925). However, and despite the considerable work done since then, the existing evidence has still to be incorporated into a consistent theory. The main obstacle in understanding such phenomena is still our limited knowledge of the molecular structure of the chromosome. This structure involves the complex of the DNA molecule with nuclear proteins and other minor

components to give the aggregate which is known as chromatin. Protein is a major constituent of chromatin and it may approach, in certain tissues and organisms, as much as twice the DNA content of the cell; the well studied calf thymus chromatin according to Bonner et al. (1968) has a chemical composition of 1.00 DNA to 1.14 histone to 0.33 nonhistone protein to 0.007 RNA (content relatively to DNA).

Histones are basic low molecular weight proteins and they have been found in all eucaryotic cells, and possibly, in small quantities, in some procaryotes (Leaver and Cruft, 1966). In some instances such as spermatogenesis in fish another class of basic nuclear proteins, protamines, progressively replaces the histone protein so that finally there is no histone left in the mature sperm of these organisms. Histones are typically classified into five classes according to their relative contents of lysine and arginine, the two main basic amino acids present. The nonhistone proteins are less well defined and they are mainly acidic proteins which bind with other chromosomal proteins and the DNA itself.

It was inevitable that histone and nonhistone proteins because of their big share in the chromosome would attract special attention in the search for mechanisms which should regulate the genetic material and the selective processes needed for cell differentiation and development. Indeed a huge amount of information has been gathered concerning the interactions of histones, nonhistones and DNA and their implications in the expression of the genetic material (see reviews by Phillips, 1971; De Lange and Smith, 1971; Hnilica, 1972).

All the available evidence suggests that (a) histones may

regulate the replication and transcription of DNA by influencing the access to it of the appropriate regulatory molecules, (b) histones are structural elements important to the integrity of the chromosomes and they may protect them from various types of destruction, (c) non-histone proteins and other molecules present in chromatin interact with the DNA-histone complex and may affect its nature and confer specificity to the functions of histones, which by themselves are non-specific in their interactions with DNA.

The transcriptional capacity of chromatin is very much dependent on these interactions as well as on histone modifications. A few examples of these modifications which indeed result in transcriptional changes will be mentioned here. It is known for some time that proteins can have their terminal amino acid residues acetylated. Histones were found to be subjected to more extensive acetylation with the arginine rich ones being the best acceptors of labile acetyl groups (Desai and Foley, 1970). Histone phosphorylations, methylations, thiolations and ribosylations have also been demonstrated to occur in vivo and the importance of these reactions and the enzymes which mediate them is evident. Acetylations and phosphorylations have been correlated with increased activity in transcription while methylations and thiolations appear to correlate with condensation of chromatin and loss of RNA synthetic activity (Allfrey, 1971). The presence of hydroxyproline in lysine-reach histones (Srivastava, 1970) also suggests that hydroxylation of proline might be another modification mechanism.

In addition to these processes the significance of interactions of both histones and DNA with other minor components of the chromosome should not be ignored. The acidic nature of DNA implies

that cations will bind to it to neutralise the electrostatic repulsion of the phosphate groups thus stabilising its structure. Na^{+}, Li^{+}, Cs^{+}, Ca^{++}, Mg^{++}, basic amino acids such as arginine, lysine, tetralysine and polyamines all bind to the DNA molecule *in vitro* and most probably *in vivo* as well. They compete with histones or protamines for binding sites. Polyamines in particular are the most active with a preference for adenine + thymine rich polynucleotides; these substances will be discussed later in detail. Tryptophan and other indole derivatives, methionine, alanine, acridine dyes and polypeptide antibiotics all have stabilising effect on DNA and at least some of them are known to affect its template activity as well.

The importance of DNA modification mechanisms, such as methylation of adenine or cytosine as it occurs in bacteria has been discussed by Holliday and Pugh (1975) in relation to the control of gene activity during development. They suggest the presence of specific modification enzymes which during development interact and modify specific DNA sequences thus programming genes as active or inactive after a specific number of cell divisions.

All the above evidence makes clear the importance of the chromatin structure in gene expression. However, although the constituents of chromatin and their importance are fairly well documented the nature of the molecular interactions by which they accomplish their diverse functions is not yet known.

Electron microscopy shows that chromatin is distributed into two easily distinguishable forms; dense clumps of compacted fibril and diffuse regions of loosely extended filaments (Hay and Revel, 1963).

The condensed areas probably because of their higher DNA concentration stain more heavily with feulgen stain - heterochromatin - as opposed to the diffuse regions - euchromatin. Euchromatin passes through a cycle of condensation and dispersion in successive divisions of the cells which suggests that it is the dispersed phase which is transcriptionally active and contains the conventional genes which code for proteins. Heterochromatin is further divided into (a) constitutive, which is thought to represent inactive chromosome regions with highly repetitive DNA sequences; these areas remain always condensed during interphase (b) facultative, which is similar in DNA sequence to that of the euchromatin but which remains for most of the time transcriptionally inactive (e.g. X chromosome of female mammals).

Both heterochromatin and euchromatin are known to contain histone and nonhistone proteins. Cytological methods (Berlowitz, 1965) suggested that the histone content may be higher in heterochromatin thus accounting for its condensed state, however quantitative and qualitative analyses did not support this idea (Comings, 1967). Instead it was found that the nonhistone protein content is considerably higher in euchromatin (Loewus, 1968). This suggests that non-histone proteins may change the configuration of the DNA-histone complex making DNA more accessible to RNA polymerase. On the other hand Musich et al. (1977) associate certain non-histone proteins with the heterochromatic organization of chromatin.

A significant step in the elucidation of the chromosome structure comes from rather recent evidence from a number of workers who suggest that chromatin is composed of a flexible chain of spherical particles named nucleosomes connected by DNA filaments (Kornberg, 1974;

Oudet et al., 1975; Noll, 1976). A length of DNA comprising about 200 nucleotides is present in each nucleosome with most of the DNA (140 base pairs) apparently wrapped around a core of two molecules of each of the histones F2A1, F3, F2A2 and F2B, arranged in the form of an octamer. The basic structure of each nucleosome could probably be the same in all eucaryotes as suggested by the finding of the same DNA length (always 140 base pairs) in a variety of higher organisms and fungi. However, a shorter nucleosome repeat has been found in fungi (154 base pairs as opposed to 198 base pairs in higher organisms; Noll, 1976; Morris, 1976). This must reflect a shorter interval between the nucleosomes in fungi.

Nucleosomes have been detected in two main fractions, either as regularly repeated "beads on a string" or as closely packed aggregates. The latter suggests that nucleosomes may have a superstructure of their own and the aggregates have been tentatively associated with the type of structure seen in the condensed heterochromatin (Comings and Okada, 1976). This might be the case, however no definite proof has been obtained yet.

Evidence for a nucleosome superstructure comes also from recent studies of human mitotic chromosomes (Bak et al., 1977). These authors propose in fact three further levels of coiling to account for the degree of chromatin condensation in the mitotic chromosome: (a) the nucleosomal string which allows for a sevenfold chromatin condensation is coiled in a solanoid form which gives a further sixfold chromatin condensation; (b) the solanoid form is coiled again to a supersolanoid structure, called unit fiber, which still increases condensation by a

factor of 40; (c) the unit fiber is postulated to be coiled again with a resultant further fivefold condensation to give the final structure of the human mitotic chromosome whose total chromatin condensation is of the order of 10000 (7X6X40X5). Whether the same pattern of folding is followed by all eucaryotes still remains to be seen. The progress in this area is fast and it is hoped that soon significant results will provide definite clues of the chromosome structure and its implications.

1.3. Position effects

1.3.1. The nature of variegated position effects

Position effects in general are phenomena in which the normal expression of a gene has changed as a result of its removal from its normal position on the chromosome. When these rearrangements involve wholly euchromatic regions of the chromosomes, a gene adjacent to the translocation point may exhibit a stable change of its phenotype. If however the rearrangements involve heterochromatic areas, the affected gene exhibits a variegated phenotype usually consisting of a mixture of wild type and mutant appearance. Cytological analyses of variegated position effects have shown that the gene affected has generally been brought close to a heterochromatic region on the chromosome. Since "sticking" of euchromatic pieces to unbroken heterochromatic ends seems to be improbable (Swanson, 1957) a second break within a heterochromatic region seems to be necessary for a variegated position effect to be observed.

In contrast to stable type position effects which are confined to single phenotypic expressions, the variegated ones have a

"spreading effect" such that a number of genes can simultaneously exhibit variegation with more extreme phenotype for the ones closest to the heterochromatic area (Baker, 1958).

The variegated phenotype may be shifted towards the wild type appearance (suppression) or towards the mutant type appearance (enhancement) by modifying factors such as temperature (Gowen and Gay, 1933b, 1934), presence or absence of extra Y chromosomes (Gowen and Gay, 1933a, 1934; Schultz, 1936), even other rearrangements present in the same nucleus (Schultz, 1941a).

A complete reversion of the position effect results if by a new translocation the affected gene is brought back to a euchromatic region (Hinton, 1950). This demonstrates that the affected gene has not been actually altered in any permanent way and it is the proximity of heterochromatin which upsets its normal function. Unfortunately in view of our limited knowledge of the chromosome structure, the influence of heterochromatin on the expression of the variegated genes can still only be expressed in terms of "spreading effects" or heterochromatization.

It is hoped, however, that further studies of variegated position effects may in fact contribute to our understanding of the complexities of chromosome structure which they reflect.

1.3.2. The brlA12 variegated position effect in A. nidulans

1.3.2.1. Genetics and features

In a search for aconidial mutants in Aspergillus nidulans Clutterbuck (1969) was able to find two types of such mutants

corresponding to two loci both on the VIII chromosome but not closely linked. Mutants at the abacus locus (abaA) develop on the secondary sterigmata rod-like structures with swellings at intervals, instead of the normal conidial clumps. It seems therefore that this locus controls the change in the budding pattern from sterigmata to conidial chains.

The second locus is the bristle locus (brlA), so called because mutants lacking its normal activity fail to develop any differentiated structures such as vesicles, sterigmata and conidia. Instead, the conidiophore continues to elongate more than twenty times the normal length remaining as a stiff bristly hypha. As a result no conidia are formed and the strains are propagated either by subcultures of mycelium or sexually by the formation of cleistothecia. Leaky bristle mutants were also isolated with a more or less normally developed vesicle and followed by forked bristles which occasionally resembled sterigmata, depending on the degree of leakiness.

The brlA12 allele of this locus is unique since it exhibits a mosaic phenotype, consisting of a very small number of normal (wild type) conidial heads and a majority of bristle-like undifferentiated conidiophores. Subcultures using cleistothecia or any one of the two extreme structures (conidia or hyphae) always give colonies identical to the parental type.

The case was recognised as a possible variegated position effect and the fact that the genetical analysis of the strain revealed a translocation involving the bristle locus, seems to strengthen this hypothesis (Clutterbuck, 1970). The translocation is a non-reciprocal

one with the broken part of the VIII chromosome, bearing the bristle locus next to the breaking point, being attached to the apparently unbroken end of the III chromosome. Whether the sticky point is the unbroken end of the III chromosome, or whether it is a damaged but heterochromatic end so that it is not genetically detectable is not clear. Perkins (1974, 1977) has reported a number of similar "pseudoterminal" translocations in Neurospora suggesting that such translocations may be common in fungi.

At the start of this work the presence of heterochromatin in Aspergillus was only speculative and in any case the chances of seeing it in the minute chromosomes of Aspergillus were not great. However, very recently the presence of heterochromatin in Aspergillus nidulans (Morris, personal communication) as well as the debated (Leighton et al., 1971) presence of histones in fungi have been confirmed (Felden, Saunders, Morris, 1976) in support of the evidence that fungi are true eucaryotes, showing similar degrees of genetic complexity to higher eucaryotic organisms. Investigating the brlA12 mutant, Clutterbuck (1970 and personal communications) found that the variegation is subject to a variety of modifying factors which either enhance or suppress the mutant phenotype. Plates inoculated with the mutant and left at room temperature after initial incubation at 37°C conidiate better (i.e. they form more normal conidial heads) than those left steadily at 37°C. Conidiation is also induced by lowering the pH of the media. Addition of salts in general to the minimal or complete media have the best effect in suppressing inactivation of the bristle locus and improving conidiation. Salts which at the same time lower the pH, like NaH_2PO_4 for example are the most effective, increasing conidiation dramatically, although well below wild type levels.

Mutagenic treatment produced strains with increased conidiation and genetic analysis showed that the reactivation of the bristle locus was due to suppressors rather than retranslocation of the bristle gene. (However the original terminology as revertants will also be used here.) The mutants were classified in three classes according to the degree of reversion; class 'A' includes the best suppressors with almost wild type appearance; class 'B' includes those of intermediate conidiation, and class 'C' includes mild suppressors, with only slightly more conidia than brlA12. Representatives of the above classes were respectively rev-5 also called drkB because of its darker than normal conidial colour, rev-2 also known as galG because of its inability to utilise galactose as carbon source, and rev-7. These mutants which have been extensively used in tests described in this work, are recessive and map on chromosomes II, VIII and VII respectively.

An interesting aspect of the brlA12 variegation was found when Clutterbuck (personal communication) crossed this strain with certain strains already existing in the Glasgow collection of Aspergillus nidulans strains. He noticed that some markers in these strains were able to shift the conidiation of brlA12 to either direction. In particular gamA, gamB, gamC, galC, pacC and subpalB7 act as enhancers while molB acts as a suppressor. Other mutants including pal , pac molA and gal mutants do not affect brlA12.

1.3.2.2. Approaches to further study

The present work started as an attempt to evaluate the observations made so far, extend them further and add new evidence through genetical and biochemical investigations, hoping that a pattern would emerge about the mechanism of the inactivation process which

would throw light to the whole question of the relationship of chromatin structure and gene expression. Two main approaches were chosen for further studies: (a) Isolation of nuclei with the objective of comparing brlA12 and modifiers in their nuclear proteins and enzymes, hoping that any detectable differences could be related to differences in the packing of chromatin and hence activation or inactivation of particular chromosome regions. (b) A study of environmental effects on variegation in conjunction with the modifiers. Aspergillus is a haploid eucaryote organism which can be treated as a microorganism and is therefore well suited for such studies. High salt, while it has a remarkable effect on the variegation, is a rather unspecific agent and a search for other more specific factors acting similarly could probably yield further clues to the mechanism of variegation. It was the result of this second approach which suggested that polyamines might play some role in the determination of the brlA12 phenotype. These investigations led to more general studies of the amine metabolism and regulation in Aspergillus.

1.4. Polyamines

1.4.1. Nature and metabolism

The term polyamine mainly refers to spermidine and spermine, but it usually includes their biosynthetic precursor the diamine putrescine as well. Other diamines of related structure and importance in biological material are cadaverine and propanediamine, and these are often called polyamines too.

Polyamines are aliphatic non protein nitrogenous bases of low molecular weight. Their presence in organisms is ubiquitous

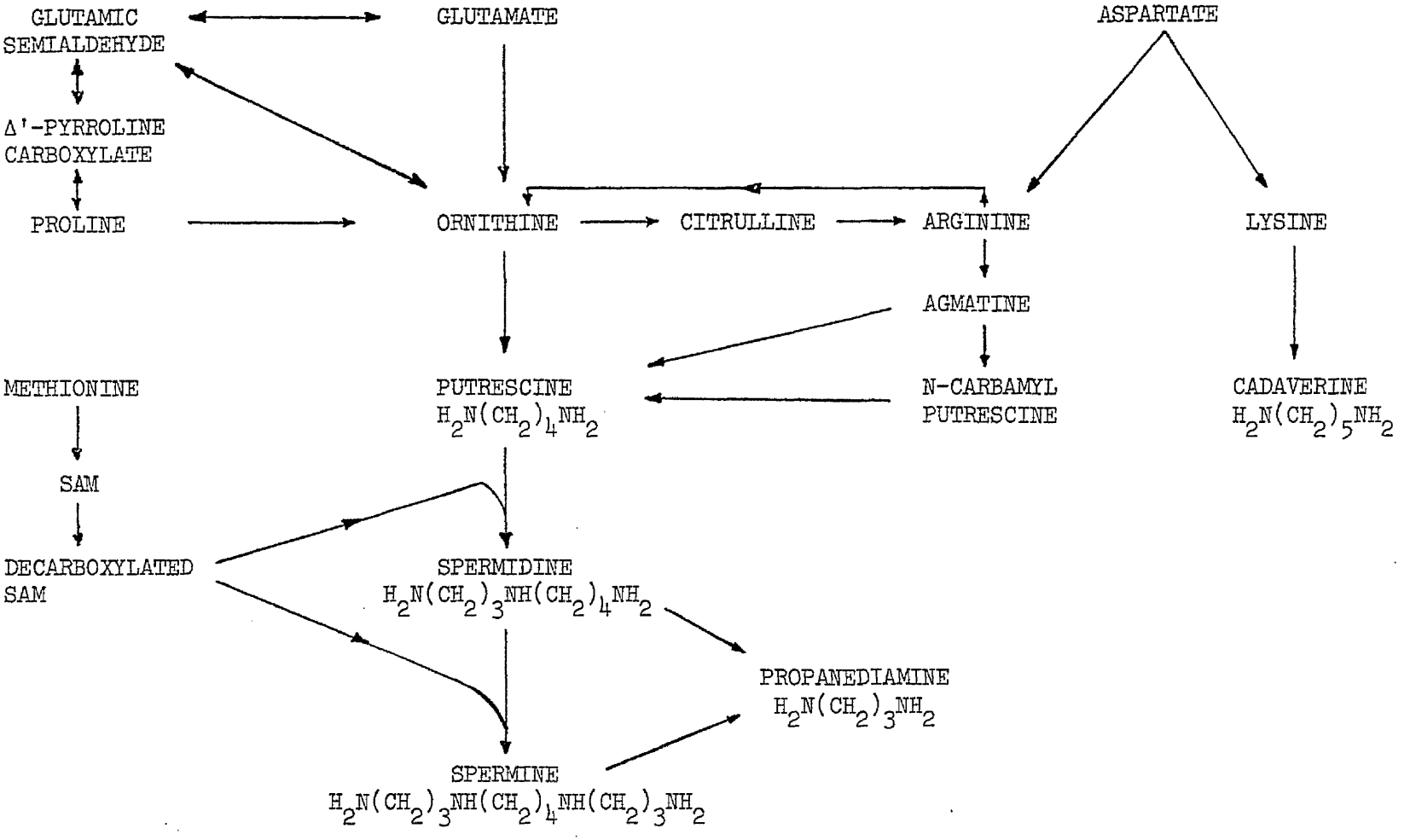


Figure 1. Biosynthesis of polyamines

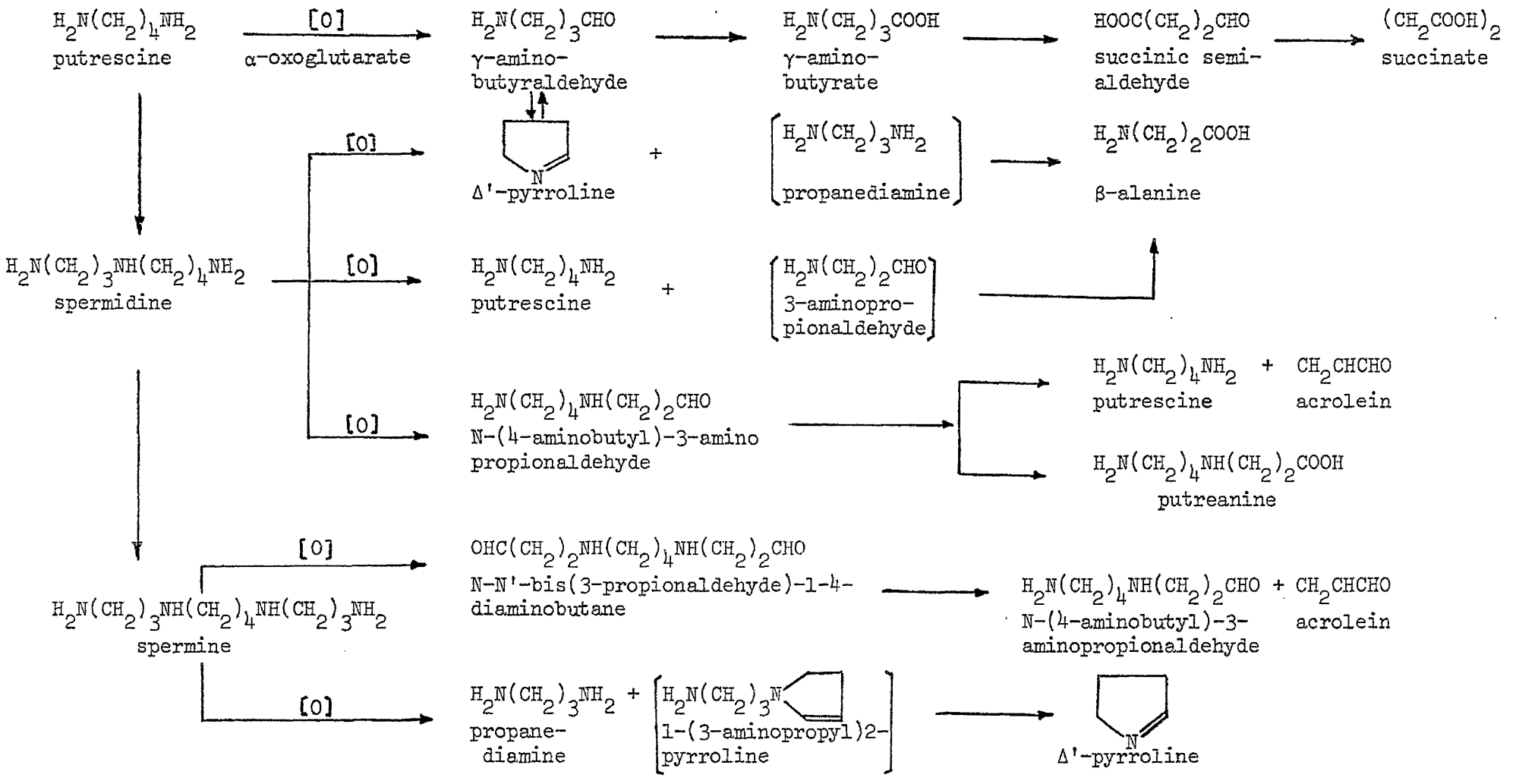


Figure 2. Some catabolic pathways of polyamines

although remarkable quantitative and qualitative differences exist. The main precursors for their synthesis are ornithine and decarboxylated S-adenosylmethionine (SAM) (see fig 1). Putrescine results from decarboxylation of ornithine and can subsequently be converted to spermidine which is the immediate precursor of spermine. Arginine can also give putrescine via agmatine but only in bacteria (Morris and Pardee, 1966). Decarboxylation of lysine gives cadaverine while propanediamine results from oxidation of spermidine or spermine (Smith, 1970).

The biosynthetic pathways are fairly well established, however the mechanisms of polyamine catabolism are less clear mainly due to the complexity of the relevant pathways, the lability of the products and their variety in different organisms. The formation of acetylated (Dubin and Rosenthal, 1960), glutathionyl (Tabor and Tabor, 1971), carbamyl (Roon and Barker, 1972), hydroxylated (Zobari and Tchen, 1971) and other unusual derivatives complicate the situation further. Figure 2 shows some of the most common catabolic pathways and products of the main polyamines. Putrescine (and cadaverine) are normally oxidised by bacteria, fungi and higher organisms to the corresponding amino-aldehydes (Bachrach, 1973) which are spontaneously converted to their heterocyclic amines, that is Δ'-pyrroline for putrescine (and Δ'-piperideine for cadaverine). Transamination of putrescine which occurs in a variety of organisms also leads to the production of γ-aminobutyraldehyde which can be further metabolised to γ-aminobutyrate (Jakoby and Fredericks, 1959) and then via succinate to the citric acid cycle. Acetylation of putrescine followed by monoamine oxidation and deacetylation will also lead to the citric acid cycle in mammalian cells (Seiler and Eichentopf, 1975).

Oxidation of spermidine and spermine will give rise to a

variety of products depending on the particular organism. While the pathways and products in fig 2 are rather well established (see reviews by Cohen, 1971; Bachrach, 1973) it should be mentioned that oxidised polyamines are very labile and their isolation and identification is not easy. The products of oxidation of spermine by Mycobacterium smegmatis have been reported to include spermidine, propanediamine, γ-aminobutyric acid and β-alanine (Bachrach, Persky and Razin, 1960). However the reaction sequences in this system have not been established. Oxidised spermidine can also be converted to putrescine in presence of NAD (Caldarera, 1969).

1.4.2. Function

Polyamines seem to be involved in a variety of metabolic reactions, however this does not provide any conclusive evidence about their function. Their distribution in the cell which should be possibly related to their function is not clear, mainly due to their redistribution in various subcellular particles after homogenisation. They are very soluble in aqueous media and because of their polycationic nature they bind strongly to polyanions such as phospholipids and nucleic acids. They have also been found by many workers associated with ribosomes, cell membranes and nuclei. Although precautions have been taken in such experiments to avoid redistribution, the isolation procedures are not reliable enough to conclude that the in vitro distribution represents that in vivo.

Research in polyamines has associated them with a variety of biological effects most of them related to their polycationic

nature. However the fact that they are present in all cells and in large amounts as well as the existence of mutants with specific requirements for polyamines such as those of Aspergillus nidulans (Sneath, 1955) and Haemophilus parainfluenzae (Herbst and Snell, 1949) suggest that these substances must have some essential function rather than just being a non-specific form of intracellular cation. The main topics in the physiology of the cell with which polyamines have been related will be discussed briefly.

(A) Cell surfaces and cell division.

The evidence suggests that polyamines at very low concentrations bind to acidic groups in the cell walls and membranes. They thus probably minimise the repulsive effect of these acidic groups and thereby stabilise the surfaces and protect bacterial cells and protoplasts from lysis under stress conditions (low ionic strength, etc.) (Tabor, 1962). Large amounts of Mg^{++} or Ca^{++} have similar effects. Subcellular particles such as mitochondria (Tabor, 1960), ribosomes (Siekevitz and Palade, 1962) and isolated nuclei (Busch et al., 1967) are also stabilised. Indeed spermine is quite useful in nuclear isolation procedures to preserve nuclear and nucleolar integrity (Busch et al., 1967). Leake et al. (1972) studied the effect of various cations including spermine on isolated hen erythrocyte nuclei and they observed swelling of nuclei in absence of cations which was reversible by the addition of 1 mM spermine or higher concentrations of inorganic cations. They also present evidence that changes in the nuclear volume result from changes in the density of chromatin structure.

They conclude that chromatin can be held in a dispersed state by repulsion between negatively charged groups within it; when these groups are neutralised by bound cations the chromatin condenses, a process which *in vivo* could be of physiological importance as far as gene activity is concerned.

Polyamines in addition to stabilizing membranes also seem to affect membrane permeability. Spermine, and to a lesser extent spermidine and cadaverine, inhibit the transport of neutral aliphatic amino acids (Bachrach, 1973). Not necessarily related to the effects of polyamines on membranes are abnormalities in cell division. Davidson and Anderson (1960) reported that intracellular polyamines cause abnormalities in the chromosome spiralization and despiralisation processes. It has also been reported that they cause chromosome condensation, breakage and aberrations and it has been suggested that DNA proteins and polyamines have to reach critical levels before division (Inouye and Pardee, 1970). Sevag and Prabble (1962) reported an antimutagenic effect of polyamines causing diminished response to mutagenic agents. The mechanism is unknown but it should be related to the polyamine's affinity for DNA.

(B) Nucleic acids.

Polyamines being positively charged can bind strongly to such polyanions as DNA and RNA probably neutralizing the highly acidic phosphate groups of the nucleic acids and hence stabilizing their structure as is concluded from denaturation experiments. Two models have been proposed for their binding to the DNA molecule, based on

X-ray diffraction, electron microscopy and other techniques. Liguori et al. (1967) propose that protonated amino groups form hydrogen bonds with phosphate oxygens in the narrow groove of the DNA molecule with bridges between the two strands in a zig-zag form depending on the number of the amino groups (in the amines). Other models (Suwalsky et al., 1969) suggest that all the basic groups of the polyamine form hydrogen bonds with phosphate groups on one strand only of the DNA.

Spermine, and to a lesser extent spermidine, are the polyamines which bind stronger to DNA. Their effect on DNA and RNA polymerases depends on their concentrations being stimulatory at low but inhibitory at high concentrations. The fact that the pH optimum of the enzymes is not affected by the concentrations of spermidine or spermine suggests that these substances act by binding to DNA or RNA rather than with the enzyme itself. The mode of their action on the polymerases is not known, the existing evidence however suggests that they are probably capable of changing the chromatin structure and hence influencing the access of DNA to the polymerases. Their ability to displace histones from chromatin (Schwimmer, 1968) should be mentioned here. There exists a widespread correlation between RNA synthesis and spermidine levels, in systems such as bacteria, chick embryo, rat liver and brain, Drosophila melanogaster, Xenopus laevis and sea urchin (Bachrach, 1973). RNA and spermidine levels increase in parallel in all these systems, suggesting that polyamines regulate RNA synthesis and therefore indirectly promote growth and protein synthesis. Whether spermidine is directly involved is not yet clear mainly because of side effects of spermidine during the experimental procedures (Ezekiel and Brockman, 1968).

(C) Protein synthesis.

It has been already mentioned that polyamines were found associated with ribosomes, and that they can stabilize them, probably by binding directly to RNA phosphate residues or alternatively by attaching free ribosomes to cellular membranes. Khawaja (1971) has explored the second possibility and he indicated that *in vitro* spermine is involved in the binding of free ribosomes to the endoplasmic reticulum membrane. He also found that bound ribosomes are more resistant than free ones to ribonuclease (Khawaja and Raina, 1970). As a consequence, polyamines stimulate protein synthesis *in vitro* in many systems provided that their concentration is not high enough to replace magnesium ions beyond a critical level (Bachrach, 1973). Polyamines also influence stability and structure of tRNA molecules (Ishida and Suecka, 1968). In conclusion the effect of polyamines in stimulation or inhibition of protein synthesis (depending upon their concentration) may be either at the tRNA level, the ribosomal level or (through their interaction with the DNA) at the transcriptional level.

(D) pH and ionic balance.

Potassium ions are known to regulate the cellular ionic balance (Epstein and Schultz, 1965) in *E. coli* as well as the activity of many enzymes (Suelter, 1970) some of them closely related to purine and polyamine biosynthesis. Cohen (1971) transferred a strain of *E. coli* unable to accumulate potassium ions (high levels of which are required for growth) to a high sodium medium. He found that putrescine biosynthesis increased tenfold while spermidine concentration declined

as expected because of the absence of K^+. Furthermore, putrescine had taken the place of spermidine in tRNA molecules suggesting that when K^+ is deficient, putrescine may assume some of the roles of spermidine in nucleic acid structure and function. High putrescine production due to K^+ deficiency has also been reported in plants (Smith and Richards, 1962). In view of this evidence Smith and Sinclair (1967) suggest that accumulation of amines may represent a homeostatic mechanism for maintenance of ionic balance. They also suggest that formation and excretion of amines may serve as an internal compensating mechanism to keep intracellular pH fairly constant. In support of these hypotheses, Munro et al. (1972) have found that in E. coli mono-divalent cations and several sugars added to the medium, resulted in reduction of the cellular putrescine level. This reduction seems to occur by excretion of putrescine to the medium according to the environmental K^+ level. It also appears, however, that the putrescine content of E. coli varies inversely with the osmotic strength of the medium. The same situation applies to mammalian cells (Munro et al., 1975) where the changes of ornithine decarboxylase in relation to the osmolarity of the media were also studied.

(E) Growth processes.

The most clear evidence for the importance of polyamines as growth factors is perhaps the isolation of several specific polyamine requiring auxotrophs in microorganisms such as Haemophilus parainfluenzae (Herbst and Schnell, 1949), Aspergillus nidulans (Sneath, 1955) and the conditionally putrescine deficient mutants of E. coli (Morris and Jorstad, 1970). Stimulation of growth by polyamines occur in a variety of organisms including bacteria (Martin et al., 1952), viruses

(Ferroluzzi-Ames, 1965), plants (Bertossi et al., 1965) and mammalian cells (Ham, 1964) to mention just a few examples. Inhibiting and toxic effects due to the polyamines or their derivatives (oxidised products mainly) are also known. Spermine and spermidine are the most toxic ones for bacteria (Rozansky et al., 1954), viruses (Ames and Dubin, 1960) and mammals including man (Risetti and Mancini, 1954). High concentrations and oxidised products are mainly responsible for the inhibitory effects.

(F) Regulation of enzyme synthesis.

In addition to their effects on the metabolism of nucleic acids, polyamines affect a number of enzymatic activities related or not to their biosynthesis. Ornithine decarboxylase and S-adenosyl methionine decarboxylase are the two key enzymes for the production of putrescine, spermidine and spermine. Extensive studies in various organisms have proved that putrescine represses the production of ornithine decarboxylase activity (Bachrach, 1973), and it is an activator of S-adenosylmethionine decarboxylase (Pegg and Williams-Ashman, 1968). Particularly high ornithine decarboxylase activity seems to be required during rapid growth and the enzyme was demonstrated to have an exceptionally short life-time. This seems to be the case for the SAM decarboxylase as well (Mitchell and Rusch, 1972).

Other enzymes stimulated or activated by polyamines are those related to carbohydrate metabolism. Polyamines were found to stimulate glucose oxidation (Lockwood et al., 1971), phosphoenol-pyruvate carboxylase (Sanwal et al., 1966), glucose-6-phosphate dehydrogenase (Sanwal, 1970), glucosyl transferase (Zimmerman et al., (1962), uridine diphosphogalactose-4 epimerase (Darrow and Creveling,

1964) and NAD glycerophosphate dehydrogenase (Lambros and Bacchi, 1976).

1.4.3. Polyamines in Aspergillus nidulans.

1.4.3.1. A review

The existence and importance of polyamines for Aspergillus nidulans was first realised by the isolation of Sneath's (1955) putrescine auxotroph (puA1), which grows only if supplemented with low amounts of putrescine at the range of 0.006-0.06 mM (Sneath, 1955, present work). Spermidine can replace putrescine but only at 10 times higher concentration. This difference between putrescine and spermidine was attributed by Hope and Stevens (1976) to uptake differences. However the higher accumulation of putrescine counts which Hope and Stevens observed could be explained in terms of faster metabolic processes for putrescine than for spermidine or spermine. Spermine does not help growth of the putrescine auxotroph, at least not up to 10 mM concentrations (see section 8). The biochemical defect of the puA1 mutant according to Stevens (1975) is lack of ornithine decarboxylase activity. In wild type cells there is a rapid increase of this enzyme at the time of onset of growth (i.e. between 5 to 8 hrs after incubation of conidia at 37°C). The wild-type enzyme seems to have a short half-life in A. nidulans as in other organisms, since there is a rapid loss of activity after the first twenty hours of growth, which according to Stevens suggests that putrescine is required only for germination but not for subsequent hyphal growth. The rate of the putrescine uptake for the auxotroph seems to be 3 times higher than that for the wild type after 5 hrs of incubation, but it eventually falls down to wild type levels after 7 hrs.

Changes in SAM decarboxylase are very similar to those in ornithine decarboxylase suggesting that these two decarboxylases maintain the supply of spermidine which appears to be necessary for the conidia at an early stage in germination (Stevens et al., 1976). The rates of conversion of ornithine to polyamines and of polyamine accumulation during this stage were studied by Winther and Stevens (1976). The fact that lack of ornithine decarboxylase results in complete auxotrophy in A. nidulans suggests that the alternative pathway for biosynthesis of putrescine from arginine via agmatine does not operate in fungi as it does in bacteria. Putrescine in A. nidulans, according to Stevens et al. (1976) inhibits ornithine decarboxylase and activates SAM decarboxylase. 1-4-Diaminobutanone acts as an analogue of putrescine in these respects and it also causes a slight reduction of the intracellular concentrations of spermidine and spermine (Stevens et al., 1977). Bushell and Bull (1974) studied the levels of polyamine, magnesium and RNA in steady state cultures of A. nidulans, and they found a constant ratio of the combined polyamine and magnesium levels to RNA in such cultures. Genetical aspects of ornithine catabolism via putrescine formation and its subsequent degradation through the citrate cycle are summarised by Arst (1977).

1.4.3.2. Approaches to further study.

Stevens and his group have mainly studied the biochemical aspects of ornithine decarboxylase activity in A. nidulans and the putrescine auxotroph was a useful tool in these studies. Some preliminary but rather inconclusive observations were also made for the uptake of polyamines, and their internal concentrations in A. nidulans.

The studies in the present work were orientated to a number of aspects, the investigation of which was thought to be important for understanding the metabolism and regulation of polyamines and their possible influence on the variegation of the bl*Al2* phenotype. These aspects included:

(a) Search for mutants altered in amine metabolism

(b) Studies of the uptake systems for polyamines

(c) Estimation of internal polyamine pools under various conditions

(d) Catabolic enzyme studies.

The results of these approaches will be presented and discussed in the relevant sections.

SECTION 2

MATERIALS AND METHODS

2.1. Introduction

The main emphasis will be given to techniques and methods applied in this work. Details about the general techniques and procedures involved in genetical work with *Aspergillus nidulans* can be found in the works of Pontecorvo *et al.* (1953) and Clutterbuck (1974). Techniques related to the isolation of nuclei and related studies will be dealt with in the relevant section (see section 4).

2.2. Strains

The nomenclature follows Clutterbuck's (1973) system. For instance, for the symbol *spsA1* : *sps* indicates spermidine sensitivity, *spsA* indicates the locus, *spsA1* is a specific mutant. In the symbol *spd-6* the hyphen indicates that the locus has not been identified. Strains which may contain unidentified mutations, are indicated by phenotypic symbols, e.g. TAMPU (a derivative of the *tamA*^{r}*50* mutant).

Markers, such as auxotrophies, when irrelevant to the results presented, were usually omitted from multimarked strains for the sake of clarity.

Most of the strains used in these studies belong to the Glasgow collection (Clutterbuck, 1969). Other strains isolated or kindly supplied for the purpose of this work are the following. The whole series of bristle mutants and suppressors from Dr. Clutterbuck's collection including *brlA12* (variegated position effect allele), *brlA9* (leaky allele), *brlA1* (non-leaky allele), *brlA42* (temperature sensitive allele). *abaA1* (abacus like sterigmata) comes from the same collection. Strains from Dr. Kinghorn's collection include the fully ammonium repressed (*tamA*^{r}*50* and *areA*^{r}*550*), the partially repressed (*tamA*^{r}*119*)

and the derepressed (*tamA*^{d}1 and *areA*^{d}520) alleles of the *tamA* gene and of the *areA* gene (Kinghorn and Pateman, 1975a; Pateman and Kinghorn, 1975, 1976, 1977). The fully repressed alleles grow satisfactorily only on ammonium as nitrogen source. *gdhA1*, *gdhA2*, *gdhA3*, *gdhA8*, *gdhA9* are alleles of the structural gene for NADP-L-glutamate dehydrogenase (Kinghorn and Pateman, 1975b). *gdhB1* is a mutant lacking NAD-L-glutamate dehydrogenase activity (Pateman and Kinghorn, 1976). *mauA2* and *mauB4* are mutants lacking monoamine (methylamine) oxidase activity (Page and Cove, 1972) and they were supplied by Dr. M.M. Page. *trypA69* is a tryptophan requiring strain (Clutterbuck, 1974) supplied by Dr. C.F. Roberts. *molA33*, a molybdate resistant strain, was supplied by Dr. D.J. Cove.

Strains isolated during this work include: *sltA1*, a salt sensitive strain on chromosome VI (Spathas, 1977), *osm-20*, a probable osmotic mutant with impaired growth on high salt or glucose. *spsA1* is a spermine, spermidine and methylglyoxalbis sensitive strain which also, in combination with the *puA2* strain, allows the growth of the latter on low concentrations of spermidine as an alternative to putrescine. It maps on chromosome III. *spd-6* is the provisional name given to a representative of a class of mutants not sensitive to spermidine or spermine but similar in all other respects to *spsA1*. *punA11* is a mutant distinguished by its inability to grow on putrescine as nitrogen source. It maps on chromosome II. Other mutants of similar type but not mapped were given also the provisional symbol *pun* followed by the isolation number (pun-7, -8, -9, -10, -22, etc). TAMPU indicates a mutant derivative of the *tamA*^{r}50 strain, which allows the latter to grow on putrescine as nitrogen source.

Enhancers of the brlA12 variegation were given the provisional symbol enh followed by their isolation number. Suppressors bear the provisional symbol sup. Dr. Clutterbuck's suppressors mentioned in this work are rev-5, rev-2 and rev-7 (see section 1).

Other strains used from the Glasgow collection (Clutterbuck, 1974) are: biA1, normally used as the wild type; meaA8 and meaB6 are methylammonium resistant, ammonium derepressed mutants; cnxB11 lacks the molybdate cofactor for nitrate reductase; niaD and niiA are structural gene mutants for nitrate and nitrite reductase respectively; nirA⁻1 is a non-inducible mutant for the nitrate reductase regulatory gene nirA; nirA^{c}1 is a constitutive mutant for nitrate nitrite reductases, it is semidominant and it maps in the same gene as the nirA⁻1 mutation (Pateman and Kinghorn, 1975). galA, B, C, D, E, F, G are mutants not utilizing galactose as carbon source. gamA, B, C are galactose non-utilizing molybdate resistant strains. pal and pac mutants lack alkaline and acidic phosphatases respectively. puA2, isolated by C. Herman (personal communication) is a translocation-free allele of the putrescine auxotroph puA1 (Sneath, 1955).

2.3. Media and supplements

Analytical grade chemicals were used where possible without further purifications. Supplements were kept as sterile concentrated aqueous solutions at 4°C and they were normally added to the media immediately before use. Freshly made solutions were used for uptake and enzyme assays. ^{14}C-compounds (putrescine, spermidine, spermine, α-oxoglutarate) were obtained from the Radiochemical Centre, Amersham.

2.3.1. Media

Liquid media where mentioned are made in the same way as the relevant solid media but without adding agar.

(a) Solid minimal medium

D-glucose	10 g	
Sodium nitrate ($NaNO_3$)	6 g	1 N Czapek-Dox solution
Magnesium sulphate ($MgSO_4$)	0.52 g	
Potassium chloride (KCl)	0.52 g	
Potassium dihydrogen orthophosphate (KH_2PO_4)	1.52 g	
Agar No. 3 Oxoid	12 g	
Trace elements solution	1 ml	

Made up to 1 lt with distilled water. pH adjusted at 6.5, before autoclaving for 15 minutes at 10 lbs.

(b) Solid nitrogen-less medium

This is the same as solid minimal medium but with no sodium nitrate and with Difco agar which is more refined than Oxoid No. 3.

(c) Solid carbon-less medium (Base medium)

Same as solid minimal medium but without D-glucose.

(d) Solid complete medium

It is solid minimal medium to which has been added:

Peptone	2 g
yeast extract	1 g
casein hydrolysate solution	5 ml
vitamin solution	1 ml

pH is adjusted at 6.0-6.2 before addition of the vitamin solution.

Autoclave as for minimal medium.

Trace elements solution

Sodium borate ($Na_2B_4O_7.10H_2O$)	40 mg
copper sulphate ($CuSO_4.5H_2O$)	400 mg
ferric orthophosphate ($FePO_4.4H_2O$)	800 mg
manganese sulphate ($MnSO_4.4H_2O$)	800 mg
sodium molybdate ($NaMoO_4.2H_2O$)	800 mg
zinc sulphate ($ZnSO_4.7H_2O$)	8 g

Made up to 1 litre with distilled water.

Vitamin solution

para-aminobenzoic acid (PABA)	40 mg
aneurin-HCl	50 mg
biotin	1 mg
inositol	400 mg
nicotinic acid	100 mg
calcium D-pantothenate	200 mg
riboflavin	100 mg
pyridoxine	50 mg

Made up to 1 litre with distilled water.

Casein hydrolysate solution

30% solution of casein amino acids (Difco) in distilled water.

Agar

Usually media were made up with No. 3 Oxoid agar, or in special cases with Difco agar. 2.5% agar in distilled water was used for cleaning up cleistothecia from adhered conidia and mycelium.

2.3.2. Supplements

Supplement	Stock solution	Added amount 100 ml medium	final concentration
(a) Vitamins			
biotin	40 µg/ml	0.1 ml	0.04 µg/ml
nicotinic acid	2 mg/ml	0.1 ml	2 µg/ml
pyridoxine HCl	1 mg/ml	0.1 ml	1 µg/ml
riboflavin	200 µg/ml	0.1 ml	0.2 µg/ml
para-amino-benzoic acid	10 mM	0.1 ml	10 µM
(b) Toxic agents			
acriflavin	0.5%	1 ml	0.005%
p-fluorophenyl-alanine	1%	2.5 ml	0.025%
D-serine	1 M	0.5 ml	5 mM
aspartate hydroxamate	16 mM	2 ml	0.32 mM
methylammonium	-	6.75 g	1 M
thiourea	1 M	1 ml	10 mM
chlorate	-	1.2 g	100 mM
bromate	1 M		100 mM
sodium azide	100 mM	1 ml	1 mM
sodium selenate	-	-	1 mM

(c) Others

sodium thiosulphate	0.2 M	1 ml	2 mM
sodium deoxycholate	16%	0.5 ml	0.08%
putrescine	-	-	0.06 mM
spermidine	-	-	0.06 mM

(d) Nitrogen sources

1 M stock solutions were used and normally they were added to give a final concentration of 10 mM with respect to nitrogen in the medium. Concentrations are specified in the text.

(e) Carbon sources

Galactose and lactose were added to a final concentration of 0.5%. Sodium acetate to a final concentration of 1.25%. Other carbon sources were added as specified in the text.

2.4. Genetic techniques

2.4.1. Cultures

Strains were kept in tubes of complete medium supplemented with any extra substances required. Diploid strains were kept in minimal medium. For uptake, enzymatic assays, auxonography tests, and other experiments which require a large number of conidia, 2-4 weeks old cultures were used. Spores were collected by adding Tween-80 diluted in distilled sterile water and scraping off with a loop clumps of conidia which were further shaken with a bench-top mixer. The suspension was filtered through cotton filter tubes and conidia were washed free from the Tween by centrifuging and resuspending them in

distilled sterile water. Cultures of strains are normally stored on silica gels in the conidial form as described by Ogata (1962).

2.4.2. Isolation of mutants

(a) Nitrous acid treatment (Siddiqi, 1962).

1 ml of a heavy conidial suspension is added to 8 ml of acetate buffer 0.1 M (6 parts acetic acid plus 4 parts of sodium acetate), pH 4.4 incubated at 37°C. To start the treatment 1 ml of 0.2 M sodium nitrite is added which at the acidic pH will act as the mutagenic agent in the form of nitrous acid. The mutagenesis is stopped after 10-15 minutes incubation (10-3% survival) by diluting 1:10 with phosphate buffer 0.066 M (3 parts NaH_2PO_4 plus 7 parts Na_2HPO_4), pH 7.1, which will raise the pH. Conidia are centrifuged down and resuspended in distilled water. After determination of the survival rate, selective plating follows for the isolation of mutants.

(b) Ultraviolet treatment.

A conidial suspension in distilled water, is placed in a glass petri dish with a stirrer. The plate goes under an ultraviolet lamp preheated for 30 minutes and 35 cm distance from it. The stirrer is then switched on at low speed. Exposure for approximately 10 minutes normally gives a 5-10% survival. The petri dish is covered after treatment with the lid and left in dark for at least two hours to avoid light repair. Then conidia are ready for use. The disadvantage of this method is a rather high freqnency of chromosomal translocations.

(c) N-methyl-N-nitro-N-nitrosoguanidine (NTG) treatment (Adelberg et al., 1965)

A large number of conidia are incubated for 30 minutes at 37°C with 10 ml of 200 mM tris maleate buffer pH 7-7.5 in which 2.5 mg of NTG have been dissolved. After the incubation the suspension is centrifuged and the conidial precipitate is resuspended in distilled water. Conidia are centrifuged and resuspended for another two or three times to dilute out NTG as much as possible.

Conidia from above treatments, after the determination of viability, were usually plated on suitable selective media in petri dishes and isolation of mutants was done by velvet replication. In the case of the attempts for osmotic mutants a filtration concentration technique was applied in some cases allowing the retention of conidia which would not grow on high salt and high glucose (Woodward et al., 1954, see section 4).

2.4.3. Formation of heterokaryons

The usual procedure was to streak the two different strains at about half centimetre apart on complete medium plates. After 2-3 days of growth very small pieces of mixed hyphae, as judged by appropriate colour markers were cut out with as little medium as possible and they were plated on to minimal medium. After 4-5 days incubation at 37°C heterokaryotic mycelium is formed recognised by its fast growth and the balanced conidial colour markers. An alternative procedure is to establish the heterokaryon on the surface of liquid complete medium, wash it with distilled water and distribute it into solid minimal medium for further growth and conidiation. This second procedure seems to select strongly for diploids (Roberts, 1964).

2.4.4. Production of diploids

Selection for diploid nuclei in Aspergillus is possible after Roper's (1952) work on this subject. To obtain diploids, heterokaryons are first established between strains with different auxotrophs and dilute suspensions of conidia from heterokaryons are poured into molten minimal medium and plated out. On incubation only diploid colonies will emerge due to the selection medium.

2.4.5. Haploidization

Clumps of conidia from the diploid strains were point inoculated (9 inocula per plate) on to complete medium supplemented with riboflavin and p-fluorophenylalanine to induce the production of haploid sectors. Acridine yellow and benlate can also substitute for p-fluorophenylalanine. After 5-7 days of incubation, the haploid sectors produced were picked up, purified when necessary and analysed for requirements. Haploidization is the last step of the parasexual cycle in Aspergillus nidulans and is mainly used for assigning unknown genes to chromosomes. For this purpose a diploid is formed between the unmapped mutant and a marker strain, so called because it is marked with at least one known locus on each chromosome. The recovery of no recombinants between the unknown locus and a marker locus indicates the chromosome on which both these markers map. The master strain mainly used in this work was

	I	II	III	IV	V	VI	VII	VIII
M.S.E:	yA2	w-3	galA1	pyroA4	facA3	sB3	nicB8	riboB2

2.4.6. Crosses

Any strain in Aspergillus nidulans can be crossed with any

other as a result of its homothallic nature, to give parental or hybrid cleistothecia. Crosses are normally made on thick minimal medium plates, sometimes enriched with extra glucose which favours cleistothecia formation. Conidia of the two strains differing in spore colour and auxotrophic markers are mixed together in the centre of the plate with a spot of complete medium to initiate growth. The plates are left incubated for 24 hours and then sealed with cellulose to create anaerobic conditions which favour cleistothecia formation. After another 15 days of incubation at 37°C, the matured cleistothecia were isolated and cleaned from conidia and other debris by rolling them with a dissecting needle on the surface of hard agar (2.5%). Hybrid cleistothecia are normally larger than parental ones. Cleaned cleistothecia were washed in distilled water and kept at 4°C. For analysis suitable amounts of ascospore suspension were plated onto complete medium to establish the hybrid nature of the cleistothecium, first by the balanced segregation of conidial colour markers and then by subsequent analysis of the segregants for requirements. The latter was usually done by making "master plates" which were then replicated by a 26-pin replicator on selective media.

2.4.7. Phosphatases in gel electrophoresis

Mycelium for detection of phosphatases was grown for 40 hr on complete medium thin layer cultures (20 ml of solid complete medium with 4 ml of liquid complete medium on top inoculated with 10^5 spores) and it was extracted in the cold with a glass homogenizer. Extracts were appropriately diluted to contain the same amount of protein before applying on the gels. The method for phosphatase detection is basically that of Dorn (1965). 12% starch in a mixture of

tris (0.076M final concentration) and citric acid (0.005M final concentration) at pH 8.7 was used to make starch gels in trays (23 cm long x 12 cm wide x 0.6 cm thick). Extracts of cells were applied on the gel and electrophoresed for 15 hr approximately with a current of 5-6 mA per cm^2. The tank buffer was a mixture of 0.3M boric acid (final concentration) and 0.05M sodium hydroxide (final concentration) at pH 7.95. After electrophoresis, the gel was normally sliced into two, and stained for acid and alkaline phosphatases with 90 ml of 0.5M acetate buffer pH 4.8 containing 60 mg of sodium a-naphthyl phosphate and 600 mg of fast garnet G.B.C. salt.

2.4.8. Internal polyamine pools

Cells were grown in liquid shaken cultures at 30°C for 17 hr with conical 1 l Erlenmeyer flasks containing 200 ml nitrogen-less minimal medium supplemented with 10 mM sodium nitrate as the nitrogen source (inoculum approximately 10^6 conidia/ml). After this initial period of growth cells were harvested on a millipore filter, washed with nitrogen-less medium and 1 g of the mycelium mat (pressed weight) was transferred for 3 hours treatment in shaken cultures again at 30°C with fresh medium as specified in the text. Cells were harvested and washed again as above, and immediately freeze-dried overnight. The method of extraction and polyamine estimation was a modification of the one described by Dion and Herbst (1970). 50 mg of mycelium (dry weight) were homogenised in a cold mortar with 4 ml of 0.2 N ice-cold perchloric acid. The homogenate was centrifuged and 0.2 ml of the resulting supernatant was dansylated by the addition of 0.4 ml dansyl chloride (25 mg per ml of acetone, fresh solution), followed immediately by 50 mg $Na_2CO_3.10H_2O$. Dansylation was allowed

to proceed for 16 hours in the dark at room temperature. Excess dansyl chloride was then converted to dansyl proline by reaction with 0.1 ml of proline (100 mg per ml of water) for 30 minutes. The dansylated derivatives were extracted with 0.5 ml benzene followed by vigorous shaking. 10 µl of the benzene extract were applied to activated (1 hour at 110°C) silica gel G thin layer chromatography plates prepared by shaking 30 g of silica gel G and 60 ml of distilled water and applying the slurry to precleaned glass plates (20 x 20 cm) with a Brinkmann-Desaga variable thickness applicator set at 200 µ.

The separation of the dansyl polyamines was effected by development in ethylacetate/cyclohexane (2:3 v/v) unless otherwise stated. The separated polyamine spots of the extract were identified under ultra-violet light, by comparison with commercial polyamines applied on the same plate. Spots were marked with a needle and carefully scraped off the plate. The dansylated polyamines were then extracted from the gel with 3 ml of ethylacetate-cyclohexane (2:3 v/v). The gel was centrifuged down and the supernatant was used for fluorimetric scanning at 345 mµ excitation and 485 mµ emission. Both commercial and Aspergillus polyamines gave peaks at the above mentioned wavelengths which are slightly lower than those given by Dion and Herbst (365 mµ - 512 mµ), probably depending on the purity of the dansyl chloride and other technical factors. Plates were immediately used after development for quantitative assays.

2.4.9. Polyamine transport assays

2.4.9.1. Preparation of cells

Conidial suspensions (approximately 10^6 conidia/ml final concentration) were added to 1 l Ehrlenmeyer siliconised conical flasks containing 200 ml of nitrogen-less or carbon and nitrogen-less sterile minimal medium. Nitrogen and carbon sources and other supplements were added at the time of inoculation as described in the text. Flasks were incubated at 30°C in a Gallenkamp orbital incubator for 17 hours. The mycelium was then harvested on a large millipore filter and washed with nitrogen-(carbon)-less medium preheated at 30°C. Then it was resuspended to new flasks with treatment medium for a further 3 hours of incubation. Cells were harvested again, washed with distilled water, pressed in paper towels, weighed and 1.5 g pressed wet weight from each flask was divided into three equal parts of 0.5 g each and immediately resuspended in 3 uptake flasks, one for the putrescine, one for the spermidine and one for the spermine uptake.

2.4.9.2. Uptake assays

250 ml Erlenmeyer conical flasks with 50 ml of uptake medium were used, incubated in a shaking waterbath at 25°C. The medium normally used was nitrogen-less minimal medium, growth in which results in high polyamine uptake. All three polyamines were added at a final concentration of 3 mM which is probably close to the saturation point. Radioactive polyamines were added to give approximately 40,000 counts per minute per ml of medium. 5 ml aliquots of the cell suspensions were taken at 1 minute intervals over a 6 minute period, filtered and washed free from medium. Cells had to be washed with 25-30 ml of 0.05 N HCl because polyamines tend to stick on the cell walls. The actual active uptake was not affected by the washing (see section 10).

The resultant cell pads were weighed and suspended in 5 ml Bray's scintillation fluid and the radioactivity counted with a Beckman Liquid scintillation counter. The rate of uptake was linear for the first 5 minutes for all three polyamines. The uptake capacity of the cells is expressed as nmoles of substrate taken up per mg wet weight of cells. The wet weight of cells as normally harvested is approximately 8 times their oven dried weight.

2.4.9.3. Accumulation of ^{14}C-polyamines

Cells after nitrogen free treatment were incubated for 30 minutes with ^{14}C polyamines (no cold polyamines were added). Then the whole mass of cells was collected, washed with 0.05 N HCl and suspended in scintillation fluid. The medium and the washings were also counted for radioactivity. The counts incorporated into the cells represent the actual uptake of the polyamines. To determine the fate of the counts after they were transported into the cells, an exact replica of the above experiment was done at the same time and the cells after harvesting and washing were immediately freeze-dried overnight. They were then extracted with 0.2 N perchloric acid and the dansylated extracts were applied and developed on thin layer chromatography plates as described in section 2.4.6. The separated polyamines were then scanned for radioactivity with a thin layer scanner RTLS-1A (Panax Equipment Ltd., Redhill, Surrey with 95% argon and 2% propane as the scanning gas) and quantitative measurements were done by extracting the dansylated polyamines in scintillation fluid.

2.4.10. Enzyme assays

2.4.10.1. Growth of mycelium for enzyme assays

1 litre Ehrlenmeyer flasks containing 200 ml of nitrogen-carbon-less minimal medium were inoculated with conidial suspension (approximately 10^6 conidia/ml final concentration) prepared as described in section 2.4.1. Nitrogen and carbon sources were added as specified in the text. After 17 hr initial growth at 30°C on a shaker incubator, the mycelium was harvested, washed with nitrogen-less medium, and resuspended in new medium for a treatment period of 3 hr. Cells were harvested again, washed with distilled water and pressed dry. 1.5 g pressed weight mycelium, kept at 4°C was immediately homogenised and used for all assays.

2.4.10.2. Preparation of cell free extracts

1.5 g of freshly harvested mycelium was ground in a cold mortar for 1.5-2 minutes with traces of cold, acid washed sand and 6 ml of cold buffer. Phenylmethylsulfonylfluoride (PMSF), a protease inhibitor, was also added at 1-2 mM during homogenization, when tested for enzyme protection (section 11, 12). The slurry was centrifuged for 30 minutes at 27000 g at 4°C and the supernatant used for assays and protein determination. In the case of the glutamate dehydrogenase assays the supernatant had to be treated further with protamine to precipitate nucleic acids which caused turbidity. For this purpose, protamine was dissolved in distilled water immediately before use and it was added to a final concentration of 0.11% (Veronese et al., 1974). After 20 minutes at 4°C, the precipitate was removed by centrifugation at 27000 g for 30 minutes. The clear supernatant was used for assays and protein determinations. It should be mentioned here that the success of the protamine step depends upon many variables (Sanwal, 1966) and pilot runs were necessary to establish the best conditions.

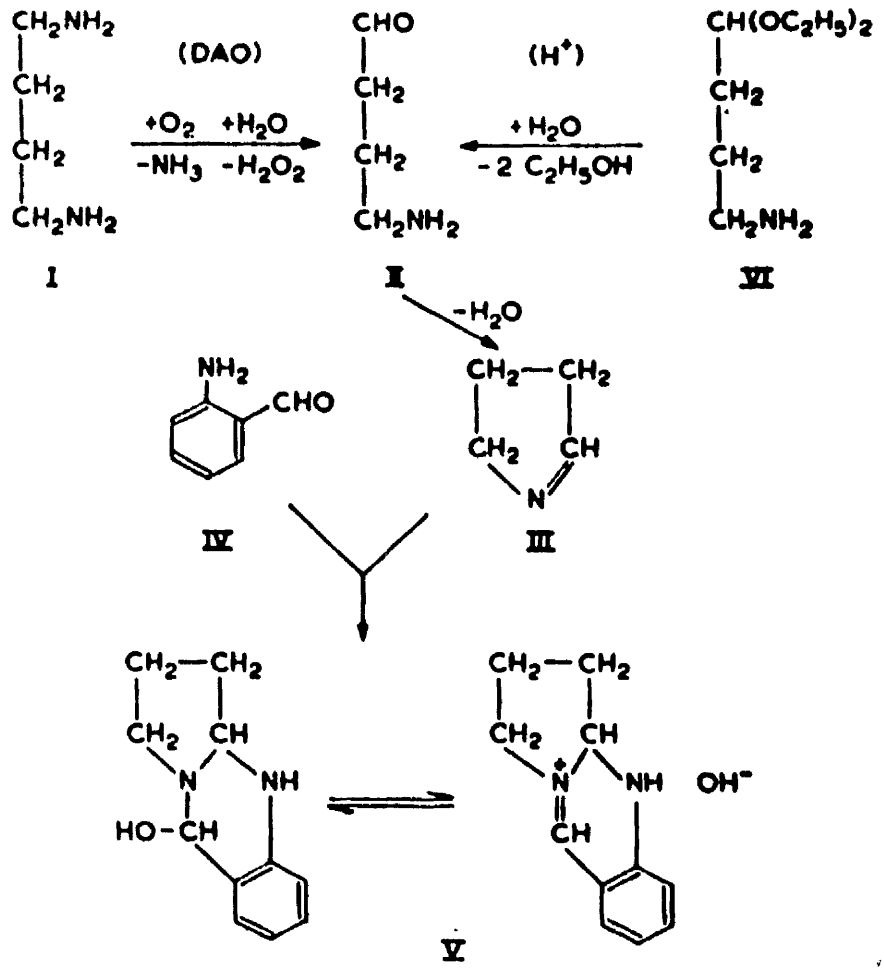


Figure 3. Assay of putrescine oxidase via Quinazolinium production. I, putrescine, II, γ-aminobutyraldehyde, III, Δ^1-pyrroline, IV, o-aminobenzaldehyde, V, trimethylene dihydroquinazolinium, VI, γ-aminobutyraldehyde diethyl acetal.

2.4.10.3. Putrescine oxidase activity

2.4.10.3.1. Assay

Amine oxidases are enzymes which oxidatively deaminate amines (mono-, di- and polyamines) with the formation of one molecule each of aldehyde, ammonia and hydrogen peroxide (Kapeller-Adler, 1970):

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2$$

In the case of putrescine the aldehyde produced would be the γ-amino-butyraldehyde which spontaneously converts to its more stable cyclic form Δ^1-pyrroline (see fig 2 and 3). This compound in the presence of o-amino-benzaldehyde condenses to form a coloured product, 2,3-tri-methylene-1,2-dihydroquinazolinium hydroxide with a strong absorption maximum at 435 nm. The complete reactions are illustrated in figure 3 and they provide a useful spectrophotometric assay for the determination of the putrescine oxidised (Holmstedt and Tham, 1959, 1961). The extinction coefficient of the coloured product was found to be 1.86 x 10^3 $M^{-1}cm^{-1}$ and therefore the oxidase activity can be expressed as nmoles of putrescine oxidised per minute per mg of protein in the extract assayed. The assay procedure as it was modified for use with Aspergillus nidulans was as follows:

Reaction mixture:

0.6 ml of tris buffer pH 8.8, 1.0 ml of 5 mM o-aminobenzal-dehyde in tris buffer pH 8.8 (final concentration 2.5 mM), 0.2 ml of 250 mM putrescine-HCl in tris buffer pH 8.8 (final concentration 25 mM), 0.2 ml of extract in tris buffer pH 8.8.

All reagents were freshly made and brought up to 37°C before extract was added. The extract was added last to initiate the reaction which proceeded for 25 minutes at 37°C in a shaken water bath.

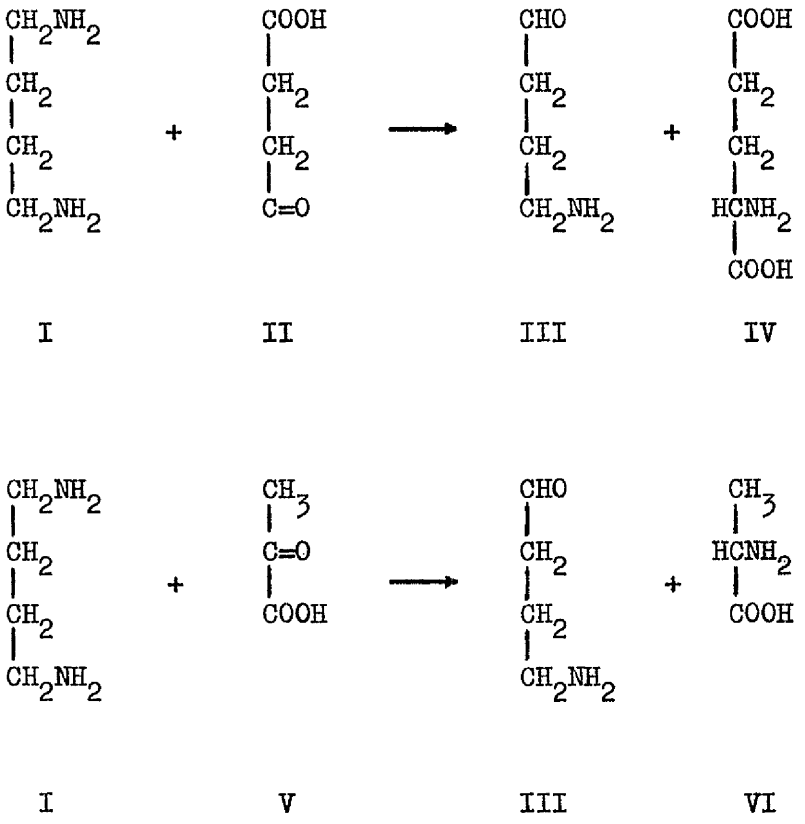


Figure 4. Putrescine transamination in Aspergillus nidulans. I, putrescine, II, a-oxoglutarate, III, γ-aminobutyraldehyde, IV, glutamate, V, pyruvate, VI, alanine.

It was stopped by adding 0.1 ml of 40% trichloroacetic acid (1.9% final concentration) and the precipitating protein was centrifuged down. The clear yellowish supernatant was read at 435 nm against a putrescine-less blank treated as above. The change in absorbance was used to determine the amount of enzyme activity.

2.4.10.3.2. Requirement for oxygen

To demonstrate that the activity observed was due to an oxidase, the reaction was carried out under anaerobic conditions in a Thunberg tube. Before mixing the extract with the other reagents the tube was evacuated with the help of a water-pump, flushed with a stream of nitrogen and re-evacuated. After half an hour incubation at 37°C, 0.1 ml of 40% trichloroacetic acid was added, and after centrifugation the absorbance at 435 nm was read. Three different samples and blanks were made and activities were compared with those found from the same extract under aerobic conditions.

2.4.10.4. Putrescine transaminase activity

2.4.10.4.1. Assay

Kim and Tchen (1962) demonstrated a putrescine-α-oxoglutarate transaminase in E. coli which converts putrescine to γ-aminobutyraldehyde. It was found during the putrescine oxidase assays in Aspergillus nidulans that increased activity was obtained when α-oxoglutarate or sodium pyruvate were present. This suggests that a transaminase activity is operating in Aspergillus most probably according to the reactions shown in figure 4. The transaminase activity was assayed again via the quinazolinium produced from the condensation of Δ'-pyrroline and o-aminobenzaldehyde. The final reaction mixture was as follows.

Reaction mixture:

0.4 ml of carbonate-bicarbonate buffer pH 9.8, 1 ml of 5 mM o-aminobenzaldehyde in same buffer (final concentration 2.5 mM), 0.2 ml of 250 mM putrescine-HCl in same buffer (final concentration 25 mM), 0.2 ml of 180 mM α-oxoglutarate in same buffer (final concentration 18 mM), 0.2 ml of extract in carbonate-bicarbonate buffer pH 9.8. Extract was added last to initiate the reaction.

All reagents were freshly made and brought up to 37°C before the extract was added. The reaction proceeded for 25 minutes at 37°C in a shaken water bath. It was stopped by adding 0.1 ml of 40% trichloroacetic acid (1.9% final concentration) and the precipitating protein was centrifuged down. The clear yellowish supernatant was read at 435 nm against a α-oxoglutarate-less blank treated as above. The change in absorbance was used to determine the amount of enzyme activity.

2.4.10.4.2. Tracing of products

The presumed products of the transaminase reaction with α-oxoglutarate are γ-aminobutyraldehyde and glutamate. The aldehyde was actually used for detecting the transaminase activity. The production of glutamate was followed by two methods. The reaction was carried out in the presence of ^{14}C α-oxoglutarate (18 mM α-oxoglutarate present as well) and the glutamate produced was detected on a chromatogram by means of its radioactivity and its staining with ninhydrin. The chromatogram was obtained after low voltage vertical ionophoresis of the extract and controls (Evered, 1959). The tank buffer pH 1.9 was a mixture of 50 ml acetic acid and 25 ml of 25% formic

acid and the extracts were run for 17 hr at 200V. Chromatograms were dried and stained with ninhydrin, or in the case of the radioactive ones, they were cut into 1 cm sections (3 cm x 1 cm) and counted for radioactivity. A histogram was constructed to show the position of the counts.

2.4.10.5. An activation of glutamate dehydrogenase by polyamines in tris buffer

Glutamate dehydrogenase activities were measured in cell free extracts with glutamate, polyamines or magnesium ions added in the assay reaction mixture. It was initially thought that polyamines could be used in some extent as poor substrates for the glutamate dehydrogenase. The fact however that magnesium had a similar effect suggested that it was rather an activation process than polyamine utilization. Activity was present only in tris buffer which indicates that tris (an amine) might act as a poor substrate for glutamate dehydrogenase in the presence of cations such as polyamines or magnesium. The assays were based on the reduction of NADP (change of absorbance at 340 nm), which has an extinction coefficient of $6.22\ M^{-1}cm^{-1}$. The assay mixture was:

775 µl tris buffer pH 8.2, 100 µl of 150 mM glutamate or amine in tris buffer (15 mM final concentration), 25 µl of 40 mM NADP (1 mM final concentration) and 100 µl of extract. Magnesium sulphate was added to a final concentration of 25 mM when used.

The change in absorbance at 340 nm was read in 1 cm cuvettes for 3 minutes against a glutamate or amine-less blank. The extract had to be incubated at $4^{\circ}C$ with 0.11% protamine sulphate for

20 minutes before use, in order to precipitate nucleic acids, which otherwise cause turbidity by their binding to polyamines, especially spermidine and spermine.

2.4.10.6. γ-aminobutyraldehyde dehydrogenase

In Pseudomonas fluorescens an NAD linked enzyme was isolated which converts γ-aminobutyraldehyde to γ-aminobutyrate (figure 2) (Jakoby and Fredericks, 1959). Attempts were made to detect a similar enzyme in Aspergillus nidulans. Since γ-aminobutyraldehyde is not commercially available, γ-aminobutyraldehyde diethyl acetal was hydrolysed in acid for 3 hours to produce it (Fig 3) (Holmstedt, Larsson and Tham, 1961). Ethanol is also produced during the acid hydrolysis. The hydrolysed product adjusted at pH 7 was used as the substrate at a concentration of 15 mM. 1 mM of NAD or NADP were used as the cofactors and phosphate, tris or carbonate buffers were used. Cells grown with nitrate or ammonium or nitrogen and(or) carbon starved were assayed for activity to exclude the possibility of nitrogen metabolite or carbon catabolite repression. In no case was appreciable activity detected.

2.4.10.7. Protein determination

All protein determinations were carried out by the procedure of Lowry et al. (1951). In 0.2 ml of diluted cell free extract, 1 ml of copper alkaline reagent freshly mixed (100 parts of 2% sodium carbonate in 0.1M NaOH, 1 part of 2% sodium tartrate, 1 part of 1% copper sulphate) were added. After 10 min 0.1 ml of Folin's reagent (Folin and Ciocalteu's phenol reagent diluted 1:1 with water) was added and colour was left to develop at room temperature for 30 minutes.

Absorbance was read at 700 nm, with bovine serum albumin as reference standards. Protein concentration in most extracts was in the range of 2-4 mg/ml.

SECTION 3

STUDIES OF THE *bz1Al2* VARIEGATED POSITION EFFECT

In section 1 the background information concerning the variegated mutant brlA12 has been given and also the approaches to its further investigation were outlined.

For the sake of clarity these approaches and their rational will be summarised here again.

(1) Clearly the best way to study a position effect would be by direct investigations on the chromatin organization. Both nucleoprotein and DNA are subjected to a rather wide variety of structural changes which mostly reflect changes in the transcriptional capacity of DNA. Quantitative and qualitative histone content analyses and studies of nuclear modification enzymes (e.g. phosphorylases, methylases, acetylases, etc) would be the most obvious aspects to look at. A prerequisite of such studies, however, would be isolation of nuclei substantially unaltered and free from extranuclear material.

(2) Modifiers of the variegation are a valuable tool for the determination of any significant changes in nuclear contents in the case of direct studies as above and also for indirect studies. The latter will include search for additional characteristics in the modifiers other than their action on brlA12 and possible correlation of these characteristics to the modifying action.

(3) A third approach would include environmental effects on the variegation of brlA12. This approach has not been previously undertaken in studies of position effects, simply because the organisms where these phenomena have been mainly described are not suitable for such studies. Aspergillus nidulans however is a eucaryote organism which can be easily plated in high numbers in desirable media. In particular, for

the brlA12 mutant it is already known that high salt will shift the variegation towards the wild type appearance. This effect suggests that a search for other more specific substances which cause similar responses to that of salt might prove worthwhile. In addition, administration of analogues of essential metabolites might cause similar effects to high salt and thus provide a clue to the biochemical processes involved in the brlA12 variegation.

The results of the above lines of investigations will be presented in the following three sections.

SECTION 4

ISOLATION OF NUCLEI AND RELATED STUDIES

4.1. Introduction

Whatever "genetical change" produced the brlA12 phenotype, and in view of the evidence presented by Clutterbuck (1970) in favour of a variegated position effect, this change would clearly have to do with the structure of chromatin at the end of chromosome III. This area should be heterochromatic and therefore affect the normal transcription of the bristle locus, possibly by making it not accessible to the transcribing molecules.

On this hypothesis only a local change in chromatin packing distinguishes the brlA12 mutant from the wild type. Such a small difference would obviously be difficult to detect by biochemical or physical means.

However there is a possibility that one mode of action of modifier mutants would involve, for instance, general changes in chromosome packing which would be biochemically detectable in isolated chromatin. While there is at present no evidence as to whether the available modifier mutants do produce such general changes, this seemed a sufficiently interesting possibility to justify the attempt to isolate nuclei and hence chromatin.

As an alternative approach to the isolation of nuclei from intact mycelium the possibility of using protoplasts derived from osmotic mutants (cf. Neurospora, Emerson and Emerson, 1958; Hamilton and Calvet, 1964) was considered.

4.2. Isolation of nuclei

4.2.1. Methods

(a) Growth of mycelium

It was considered important to use mycelium from logarithmic growth on rich medium to minimise the dangers from autolytic enzymes. Some growth studies have been done to find suitable growth systems, combining high yield and exponential growth. Finally 2 litre Ehrlenmeyer flasks were used throughout, containing 150 ml of liquid complete medium and inoculated with 5×10^5 conidia/ml. Flasks were incubated at $37^{\circ}C$ on a shaker incubator for 16 hr. Under these conditions growth was exponential with a doubling time of 2 hr and a yield of approximately 400 mg dry weight of mycelium per flask. Batches of five flasks were usually used for each experiment.

Mycelium was harvested after incubation on a nylon net and washed with a chilled solution of sucrose - EDTA-tris at pH 6.5 (0.5M sucrose, 1 mM EDTA, 0.01M Tris, Dwivedi et al., 1969).

All subsequent operations were carried out at $4^{\circ}C$ unless otherwise stated.

(b) Disruption of cells

(1) Homogenization in mortars

Harvested mycelium was pressed dry, chopped into small pieces and ground with acid washed sand until a smooth paste was obtained. Five volumes of sucrose EDTA solution at pH 6.5 were added gradually. The paste was repeatedly filtered through layers of cheese cloth to get rid of unbroken hyphae and other cell debris. Extra sucrose-EDTA solution was added during filtration. Finally the filtrate was passed

through a sintered glass filter (porosity grade 2,40-50 μ). This last filtrate was then centrifuged at 2900 g for 30 minutes and the DNA in the pellet was estimated. As a second step the pellet from the first centrifugation was carefully layered over half its volume of 1.65M sucrose and centrifuged for 1 hr at 2200 g in a swing-out head. DNA estimations were carried out again in the second pellet which, according to the same procedure in Neurospora (Dwivedi et al., 1969), should be a purified nuclear preparation.

(2) Homogenization in blenders

Harvested mycelium was suspended in sucrose-EDTA-tris pH 6.5. The ATO-MIX was used for homogenization of the suspension in the glass vessel, normally for 3 minutes full speed. The slurry was filtered and treated as in the previous case.

(3) Disruption by ethylene glycol

This method involved extension of observation by Bates and Wilson (1974) in Neurospora where osmotic lysis of cells occurs at high concentrations of ethylene glycol. Harvested mycelium was suspended in a 1 litre Ehrlenmeyer flask containing 150 ml of medium (2 volumes of distilled water, 1 volume of Czapek-Dox solution) and incubated on a shaker at 37°C. One extra volume of ethylene glycol was added to the medium to a final concentration of 4 M added at a rate of 9 ml every 15 minutes. Gradual increase of the glycol concentration was found to be necessary, otherwise hyphae would plasmolyse irreversibly at concentrations higher than 1.6M. The flask was left incubated at 37°C for another 2½ hours after the last portion of glycol was added. Then mycelium was quickly harvested on a nylon net and immediately

suspended in a cold solution containing 0.25M sucrose, 1 mM EDTA, 0.01M tris and 5 mM $CaCl_2$. After vigorous shaking, filtration through a sintered glass filter (porosity 2, 40-50 μ) followed. Extra sucrose was added to bring the concentration at 0.5M in the filtrate and stabilise the nuclei. The filtrate was centrifuged at 2900 g for 30 minutes and the DNA in the pellet was estimated.

(c) Nuclear isolation medium

The choice of the isolation medium is probably important. Only sucrose-EDTA solutions with or without $CaCl_2$ were used here according to the methods of nuclear isolation in Neurospora (Dwivedi et al., 1969; Leighton et al., 1971; Hsiang and Cole, 1973).

(d) Recognition of nuclei

Acridine orange (0.05 mg/ml) was used for staining the nuclei and monitoring them under a fluorescent microscope (Clutterbuck and Roper, 1966). No fixation was used. DNA after proper staining is green while RNA is orange. The method was also useful to check for bacterial contamination and the purity of the preparations. A phase-contrast microscope was also used for recognition of nuclei. Effectiveness of homogenization procedures was estimated with a light microscope. The DNA in the pellets was estimated quantitatively by the diphenylamine method (Burton, 1956) as modified by Giles and Myers (1965). DNA estimations were then converted to the corresponding number of nuclei according to estimations by Bainbridge (1971).

4.2.2. Results

Liquid cultures under exponential growth were used throughout,

to ensure healthy growth although this prerequisite minimised the yields of mycelium.

The main difficulty in nuclear isolation procedures with fungi is the presence of a cell wall which makes the utilisation of vigorous methods of cell breakage a necessity. In these investigations the use of a blender (ATO-MIX) was finally adopted instead of the mortars initially used.

Preliminary observations by Clutterbuck (personal communication) suggest that Aspergillus nuclei are fragile; it was thought however that because of their small size (1-2 μ) they would not be appreciably damaged by the blender although this possibility cannot be excluded. The main difficulty of this method however was increase of temperature during homogenization. Although all operations were carried out in the cold room ($4^{o}C$) it was found rather difficult to keep the temperature of the homogenate lower than 5-$10^{o}C$. The addition of ice caused other problems due to dilution of the sucrose concentration and of the final homogenate.

The yields with the blender were slightly better than those obtained by mortar homogenization, however they did not exceed 9 μg DNA per flask in the first pellet; assuming that this preparation was essentially free from broken or unreleased nuclei this would correspond to approximately 10^{8} nuclei per flask which roughly represents a 2% recovery of the total DNA present. This recovery is about ten times lower than those obtained in workable isolation procedures. One possibly interesting method of cell rupture was investigated although it did not prove any more successful than other systems; this involved

extension of observations by Bates and Wilson (1974) in Neurospora who produced osmotic lysis of cells in high concentrations of ethylene glycol. Aspergillus mycelium transferred to successively high concentrations of ethylene glycol and then after equilibration to a relatively low osmotic strength nuclear isolation medium, did burst and yield a proportion of free intact nuclei which observed under the phase contrast or fluorescent microscopes appeared to be swollen with a volume about 3 times the normal size. Recovery of nuclei in terms of DNA was approximately 1%.

4.3. Isolation of osmotic mutants

As an alternative to the direct nuclear isolation approaches, isolation of osmotic mutants was considered. Osmotic mutations are not uncommon in Neurospora (Perkins, 1959; Livingston Mays, 1969a) and they have been extensively used in this organism for production of protoplasts (Hamilton and Calvet, 1964; Emerson and Emerson, 1958, etc.) as well as for the production of the cell wall deficient slime variant of Neurospora crassa (Emerson, 1963).

4.3.1. Methods

Methods like those employed in Neurospora crassa were used to start with (Livingston-Mays, 1969) where osmotic mutants have been isolated by their inability to grow on media supplemented with 4% NaCl. Preliminary experiments with Aspergillus nidulans however suggested that it can withstand much higher concentrations of salt than Neurospora; therefore higher concentrations had to be used for isolation of osmotic mutants although 4% NaCl was also used hoping that good osmotic mutants should be sensitive at this low concentration as well. The basic

method consisted of mild NTG mutagenesis of the wild type followed by plating in minimal medium supplemented with vitamins and 0.025% deoxycholate. After 3-4 days incubation at 37°C the plates were velvet replicated on minimal medium supplemented with vitamins and 4% or 8% of NaCl. The colonies which did not grow after 1-2 days further incubation were isolated, purified and retested on high salt or high glucose. This method was found to select for salt sensitive mutants rather than true osmotic ones which should be sensitive on glucose as well. One representative of this salt-sensitive class (sltA1) was explored further.

To select strongly for real osmotic mutants a modification of Woodward's technique (Woodward et al., 1954) had to be applied for further experiments. Mutagenised conidia (survival 30-35%) were used to inoculate 250 ml of liquid minimal medium supplemented with 1 M NaCl. The inoculum was 10^8 - 10^9 conidia per flask. Cultures were incubated on a shaker at 37°C for a total of 42 hours. After initial growth of 18 hours the germinated spores were harvested with a millipore filter and discarded. The remaining ungerminated conidia, passing through the millipore filter with the medium, were concentrated by centrifugation and resuspended in new culture medium of the same composition. They were incubated again for another 24 hours and then the ungerminated ones were collected as before. The survival rate was estimated again (5-6% now) and conidia were plated on solid minimal media, and replicated after three days growth on media supplemented with extra salt, and media with extra glucose. Colonies which grew feebly on the last two plates were isolated, purified and retested.

4.3.2. Results

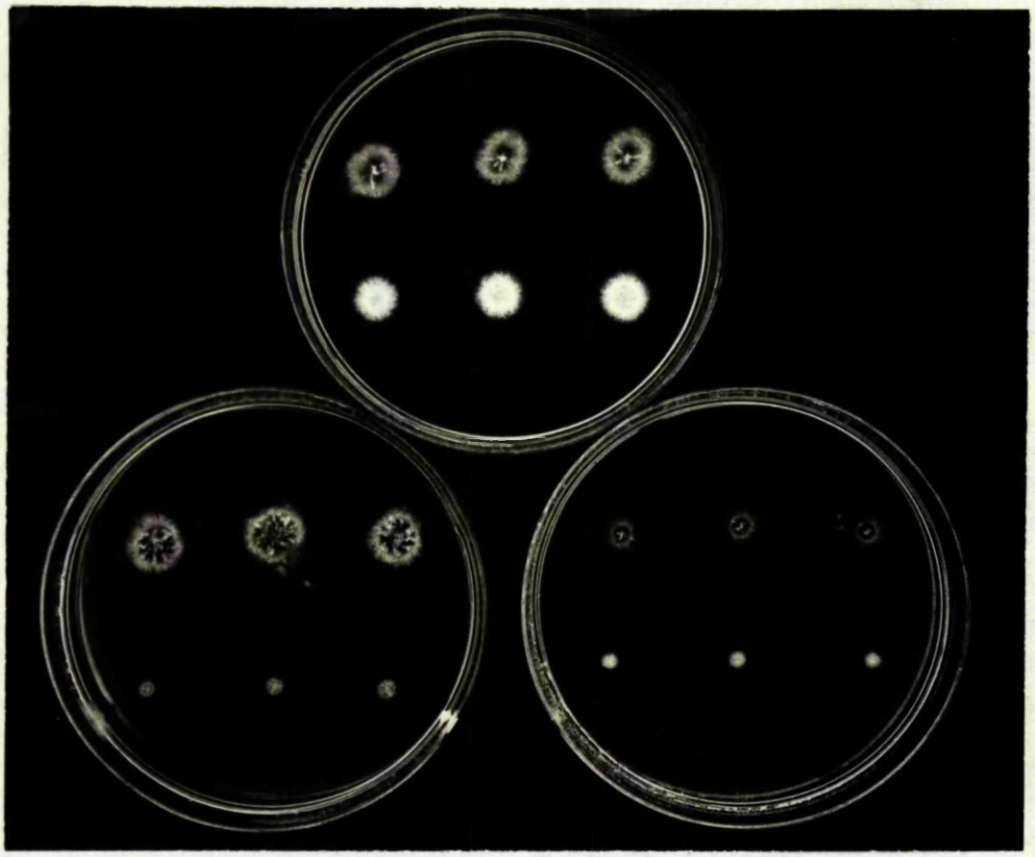


Plate 1. Growth responses of wild type and sltA1 in 10 mM nitrate (top) , 10 mM nitrate + 0.1 M ammonium tartrate (left) and 10 mM nitrate + 1 M sodium chloride (right)

Strains in three replicates : top : biA1

bottom : pabaA1 yA2;sltA1

Salt or sugar	molarity	biAl	sltAl
sodium chloride	0.7-1.8	+	-
potassium chloride	0.5-1	+	-
magnesium chloride	0.5-1	+	-
calcium chloride	1	-	-
ammonium chloride	0.5-1.5	+	-
ammonium sulphate	0.3-0.6	+	-
ammonium tartrate	0.1-1	+	-
sodium tartrate	0.1-1	+	-
sodium nitrate	1	+	-
potassium orthophosphate	1	+	-
sodium orthophosphate	1-1.5	+	-
sodium pyrophosphate	0.1	+	-
" "	0.5	-	-
glucose	0.5-2.5	+	+
sucrose	1	+	+

Table 1. Comparison of biAl and sltAl on media of high osmolarity (CM + salt or sugar).

(+) denotes normal or almost normal growth.

(-) denotes very defective growth or almost no growth.

4.3.2.1. sltA1 - isolation and features

One isolate distinguished by its feeble growth on minimal medium supplemented with 4% NaCl was obtained from the wild type after treatment with N.T.G. (survival 12%). On backcrossing to a normal strain (pabaA1 yA2;pyroA4), this isolate was shown to contain two mutations. The first called sltA, was responsible for failure to grow on media with high salt concentrations, the second conferred inability of the strain to utilize a number of carbon sources other than glucose (unpublished results). Here certain aspects of the sltA gene only will be considered. Tests were performed on a pabaA1 yA2;sltA1 single mutant derivative from the above cross.

(a) Growth on nitrogen sources

The mutant was tested on -N solid minimal medium supplemented with the following substances, each one as the sole nitrogen source: 10 mM sodium nitrate, 10 mM sodium nitrite, 10 mM NH_4^+ (5 mM ammonium tartrate), 5 mM urea, 10 mM alanine, 10 mM arginine, 10 mM asparagine, 10 mM sodium glutamate, 10 mM glutamine, 10 mM proline. No difference between the mutant and the wild type was observed.

(b) Growth on carbon sources

Growth similar to that of the wild type was observed with the following carbon sources, added to base minimal medium: 1% glucose, 1% fructose, 1% sucrose, 0.5% galactose, 0.5% lactose, 1% sorbitol, 1% mannitol, 1% mannose, 1% ribose, 1% arabinose, 1% xylose, 1% glycerol, 1% inositol.

Sodium acetate can be utilised as carbon source up to 50 mM concentration, but at higher concentrations it acts as a growth-restricting salt.

(c) Growth on media of high osmolarity

Table 1 presents the effect of high osmolarity on the wild type and the sltA mutant. Tartrate salts were the most effective, restricting the mutant's growth almost to point inoculum at 0.1-0.2M concentrations (Plate 1). Comparison of the mutant with a gdhA strain which does not grow at high concentrations of ammonium ions added in minimal medium (Kinghorn and Pateman, 1973) show that the sensitivity of the sltA1 is not related to ammonium. This can also be concluded from table 1. Furthermore the mutant has normal internal ammonium pools and tests for ammonium derepression give wild type results (Spathas, 1977). Urea at concentrations of 5-500 mM has no effect. Sugars such as glucose or sucrose do not affect the growth of sltA1 and therefore it is a salt sensitive strain rather than an osmotic one (Livingston Mays, 1969).

In Neurospora, strains carrying the osmotic mutant gene form protoplasts at high hemicellulase and salt concentrations (Emerson and Emerson, 1958). Similar attempts in Aspergillus using the sltA1 strain did not produce any protoplasts. On the contrary 3% hemicellulase reversed to some extent the salt effect restoring growth and conidiation.

sltA1 was crossed to brlA12; no effect of the former to the conidiation of the latter was detectable in the double mutants.

Chromosome	gene marker	sltA$^-$	sltA$^+$
I	y$^+$	3	0
	y	1	7
II	w$^+$	4	7
	w	19	8
III	gal$^+$	4	8
	gal$^-$	19	7
IV	pyro$^+$	5	6
	pyro$^-$	18	9
V	fac$^+$	2	1
	fac$^-$	21	14
VI	s$^+$	23	0
	s$^-$	0	15
VII	nic$^+$	10	2
	nic$^-$	13	13
VIII	ribo$^+$	5	6
	ribo$^-$	18	9

Table 2. Haploidization analysis of sltA1/M.S.E. diploid

	Markers	Recombinants sltA$^-$	Recombinants sltA$^+$	Parentals sltA$^-$	Parentals sltA$^+$	Recombination fraction	Significance of linkage (x_1^2)
CROSS I	sB	14	39	20	27	53%	$P > 0.5$
	lacA	19	39	15	27	58%	$P > 0.1$
	nicC	18	38	16	28	56%	$P > 0.2$
	sbA	8	40	26	26	48%	$P > 0.5$
CROSS II	tamA					>25%*	-
CROSS III	argA	3	3	19	25	12%±4.4	$P < 0.001$

*Data of J.R. Kinghorn (personal communication)

Crosses: I $\frac{yA2\ +}{+\ biA1}$ $\frac{nicC10\ lacA1\ SB3\ sbA3\ +}{+\ +\ +\ +\ sltA1}$

II $\frac{pabaA1\ yA2\ +}{+\ +\ biA1}$ $\frac{tamA^r50\ +}{+\ sltA1}$

III $\frac{pabaA1\ yA2\ +}{+\ +\ biA1}$ $\frac{argA1\ +}{+\ sltA1}$

Table 3. Crosses and recombination fraction of sltA1 with other markers on chromosome VI.

(d) Genetic mapping

sltA1 is recessive in the diploid with Master Strain E. Haploidisation of this diploid with p-fluorophenylalanine located the mutant on chromosome VI (Table 2). sltA1 was then crossed to various markers of this chromosome to locate its exact position. Table 3 presents the recombination fraction between sltA1 and the markers with which it was crossed. The only close linkage found was that with the arginine marker (12% recombination fraction), and since no close linkage of sltA1 with tamA was observed (> 25% recombination fraction, J.R. Kinghorn, personal communication) it is concluded that sltA1 maps at the end of a newly mapped group of mutants on chromosome VI:

$$- - \text{-pacC-} \overset{15}{-} \text{-uaX-} \overset{33}{-} \text{-molA-} \overset{28}{-} \text{-tamA-} \overset{18}{-} \text{-argA-} \overset{12}{-} \text{-sltA-} - -$$

4.3.2.2. Isolation of mutants with the filtration concentration technique

A number of mutants were isolated by selecting for defective growth on 1 M NaCl and also 1 M glucose added to the minimal medium. Three mutants with somewhat impaired growth on both of these media were finally selected, but none of these mutants was sufficiently distinctive to justify further examination. One of them however, provisionally called osm-20, was crossed to brlA12. The double mutant produced forked bristles, resembling leaky bristle appearance. No effect on the rate of conidiation was detectable.

4.4. Discussion

Attempts have been made to isolate nuclei from Aspergillus

nidulans with the primary aim of approaching the brlA12 variegated position effect through studies of nuclear enzymes and proteins.

In all methods studied yields of nuclei have been low, and this is mainly attributed to (a) rather low yields of mycelium to start with, (b) homogenization problems such as rising temperature and fragility of nuclei.

After these experiments were abandoned, Gealt et al. (1975) overcame these problems by freezing appreciable amounts of mycelium (60-120 g) into liquid nitrogen and then homogenizing them in sucrose-piperazine buffer, with a blender. A diploid strain of Aspergillus nidulans was used for this purpose and the yield of nuclei (in terms of percentage of DNA recovered) in their preparations was 15-20%.

A last approach to the nuclear isolation was made by selecting for osmotic mutants.

Isolation of such mutants in Aspergillus in connection with the present work seemed attractive for at least two reasons:

(a) to facilitate the isolation of nuclei via protoplast formation as in Neurospora (Hamilton and Calvet, 1964).
(b) the brlA12 mutant is strongly affected by salt and this response might be modified in osmotic mutants.

Selection for osmotic mutants in Aspergillus produced mainly salt sensitive mutants which were not substantially if at all affected by high concentrations of glucose. At least 25000 colonies were examined in total and about 30 colonies were finally selected and tested further. The filtration concentration technique did not prove

substantially better than a direct selection. A rather extreme type of these mutants, sltA1, was further investigated and proved to be a general salt sensitive mutant, which behaves normally on standard media. It was mapped on chromosome VI, and it is a useful marker for mapping purposes. Its morphological defects were not examined, but it is possibly a cell wall mutant. It does not affect the brlA12 morphology and it does not produce protoplasts under conditions in which they are produced by the osmotic strains of Neurospora.

osm-20, a mutant isolated by the filtration-concentration technique with a rather impaired growth on high salt or high glucose, was also crossed to brlA12 and it produced a leaky bristle phenotype. This contrasts with the action of suppressors of brlA12 (section 5) which increase the frequency of conidiating heads but do not appear to alter the structure of the "bristles". The nature of this mutant however is not yet clear.

In conclusion the nuclear isolation approach did not look hopeful for any further direct or indirect investigations. Indeed, even if a successful isolation procedure was devised, there is no guarantee that biochemical or cytological differences in nuclear enzymes or chromatin structure between brlA12 and its modifiers would be detected. Subsequent studies were therefore orientated to the behaviour of brlA12 and its modifiers in relation to various environmental effects, to gain more evidence on the nature of the variegation process.

SECTION 5

MODIFIERS OF brlAl2

5.1. Introduction

Mutagenesis or spontaneous mutations of the brlAl2 strain led to the isolation of two distinct types of mutants in relation to the original variegated phenotype; (a) those which caused increase of the normal conidial heads, called in general suppressors and (b) those which reduced the number of normal conidial heads further, called in general enhancers. The first set of about 28 suppressors was isolated by Clutterbuck (1970) in an attempt to obtain back mutants with normal morphology, resulting from the retranslocation of the brl locus to a new location which would not inactivate it any more. Genetic analysis however revealed that the restoration of the more or less wild type appearance in these isolates was due to new mutations suppressing the inactivation of the bristle locus rather than to a second translocation of the affected gene. Modifiers of this type were therefore suppressors rather than revertants, and they were able to restore in lesser or greater extent normal morphology.

During the present work, a number of enhancers and new suppressors were isolated with the objective of tracing and investigating any further features they had in relation to their modifying action on brlAl2.

5.2. Isolation procedures

Clutterbuck's suppressors were isolated from complete medium plates after mutagenesis of the biAl;brlAl2 strain (ultra violet treatment).

About a total of 300 more suppressors were isolated during this project either after nitrous acid treatment (survival 4.5%) or

ultra violet irradiation (4.3% survival). The original green strain was used (biA1;brlA12) for this purpose or a yellow recombinant (pabaA1 yA2;brlA12) mainly to avoid green contaminants which are more common. The same strains were used for the isolation of about 30 enhancers by ultra violet treatment (4.3% survival), the best of which are capable of inactivating the bristle locus completely, i.e. they show no conidiation even in the presence of salt. It was observed, as it would be expected, that the majority of double mutants (suppressors or enhancers in conjunction with brlA12) isolated from salt-free medium plates conidiated better when salt was added. Isolation of good suppressors was therefore achieved by plating on complete medium without salt, while addition of salt was essential for isolation of good enhancers. All isolates were purified on CM and retested for the appropriate requirements to detect contaminants or additional auxotrophies.

5.3. Classification and main features

5.3.1. Suppressors

The first and main classification criterion was the degree of reversion towards wild type morphology. Comparisons with the original strain were made on minimal and complete media in absence and presence of 0.71M NaH_2PO_4 in terms of empirical estimations of the rate of conidiation observed under a binocular microscope. Variations in mycelial growth rate and morphology were also considered. Three main categories were observed, which is in agreement with similar observations by Clutterbuck (1970) although some variation may exist in each class. Class A includes the best suppressors which restore brlA12 to wild type appearance on the media tested. Representative of this class is

Clutterbuck's isolate rev-5 on chromosome II. 45 out of the 300 new isolates belonged to this class. Ten of them were streaked adjacent to the rev-5 strain: no lines of bristles emerged along the junction. These, if present, would have indicated complementation of recessive suppressors (Clutterbuck, 1970).

Class B includes suppressors morphologically intermediate to those of class A and class C (see below). Representative of this class will be Clutterbuck's isolate rev-2 which maps on chromosome VIII. 50 of the 300 new isolates belonged to this class, and 35 of them, like rev-2, cannot utilise galactose as a carbon source. 29 of the galactose non-utilisers were tested for complementation with rev-2 in the same way as class A suppressors, and only one of them gave a line of bristles at the junction. This probably indicates the presence of a new galactose non-utilising suppressor locus, which however was not mapped because of difficulties in haploidization.

Class C includes rather poor suppressors, which only slightly increase the conidiation of brlA1 2. Representative of this class is Clutterbuck's isolate rev-7 on chromosome VII. They are the most common product of mutagenic treatment, but their poor nature did not justify any further work with them.

From all three classes the most interesting one was considered to be the one represented by the galactose non-utilizing suppressors. This defect in their physiology was thought to provide the most interesting biochemical clue to the mode of action of suppressors. Further studies were therefore concentrated on this class. In this connection the behaviour of the existing gal and gam mutants of Aspergillus nidulans was also investigated.

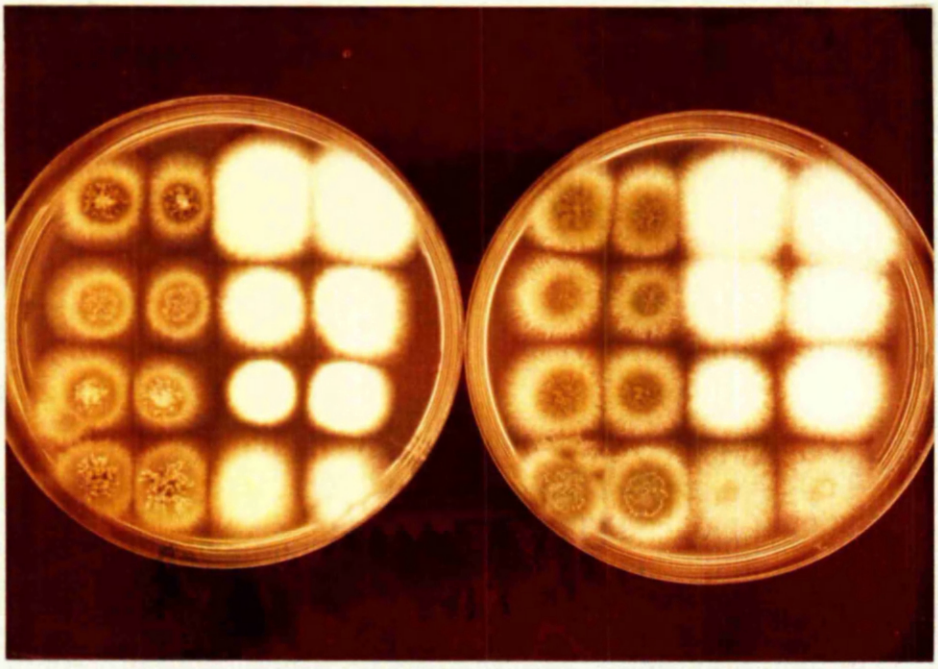


Plate 2. Growth and conidiation of brlA12 and modifiers on CM (left) and CM + 0.7lM NaH_2PO_4 (right) strains in duplicate from top to bottom, left to right:

rev-5;brlA12	brlA12;enh-1
rev-2 brlA12	brlA12;enh-15
rev-7;brlA12	brlA12;enh-24
biA1	brlA12

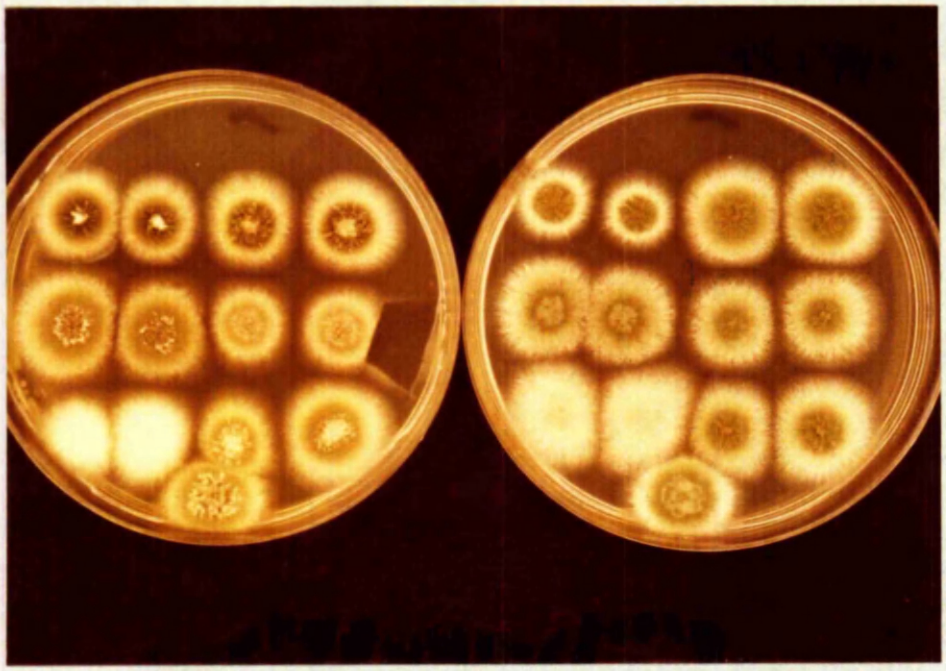


Plate 3. Growth and conidiation of *brlA1 2* and modifiers on CM (left) and CM + 0.7 1M NaH_2PO_4 (right). Strains in duplicate from top to bottom, left to right:

rev-5 puA2	*rev-5;brlA1 2*
rev-2	*rev-2 brlA1 2*
brlA1 2	*rev-7;brlA1 2*

biA1

		Growth on galactose				
		-	±	+	++	Total number tested
suppressors	class A	1	1	35	9	46
	class B	35	3	10	1	49
	class C	3	26	51	51	131
enhancers	class A	1	2	6	2	11
	class B	3	7	2	0	12

Table 4. Growth responses of brlAl2 modifiers on base medium + 0.55% galactose (55 mM). Controls show the following growth on galactose : galAl (-) ; galC7 (±) ; biAl and brlAl2 (+) ; molB33 (++).

5.3.2. Enhancers

Further inactivation of the bristle locus by enhancers results in additional reduction of the normal conidial heads in brlA12 to the extent of complete absence of conidiation even in the presence of salt. About 30 of these mutants were isolated. Only preliminary tests were carried out with enhancers, mainly for two reasons: (a) it is difficult to distinguish enhancement of the bristle phenotype from other unrelated deleterious effects on conidiation. As an example, enhanced bristle mutants could be distinguished from "fluffy" mutants by the stiff and thick-walled conidiophores, but only on close examination. (b) The total absence of conidia causes difficulties in the manipulation of these strains, especially for biochemical work.

The enhancers were classified into two classes; class A in conjunction with brlA12 produces no conidia even in the presence of salt, class B produces relatively few conidia. Plates 2 and 3 present the growth and conidiation of the suppressors and enhancers in relation to the brlA12 and the wild type, on complete media with and without salt.

5.4. Further tests

5.4.1. Galactose utilization

Most of the suppressors and enhancers were classified for utilization of galactose as the sole carbon source. Table 4 summarises these classifications. It can be seen that no clear pattern emerges from these tests, although the majority of the class B suppressors seems to be defective on galactose, while a significant proportion of classes A and C, appear to grow faster on galactose than the biA1 and brlA12 controls.

Some of the observed variation may be explained in terms of inaccurate classification. Also the possibility of some modifiers bearing at the same time galactose utilization abnormalities irrelevant to the variegation cannot be excluded, since most of the mutants have not been genetically analysed further. However the fact that the suppression action of some modifiers is coupled with defects in galactose metabolism (e.g. rev-2) and also the fact that some galactose non-utilizing strains like galC7 (Clutterbuck, personal communication), cause enhancement of the brlA12 mutant phenotype suggest a consistent if unknown connection between these phenomena.

It can be seen from table 4 that some of the suppressors as well as some of the enhancers grow faster on galactose than the wild type. In this respect they behave like molB33 which also slightly suppresses the variegation of brlA12. molA however, another molybdate resistant strain, has no effect on the variegation or on galactose utilization (Arst and Cove, 1970; A.J. Clutterbuck, personal communication). Although these single observations already look rather inconclusive, it was decided to classify all the modifiers for molybdate resistance. All suppressors defective on galactose (that is gal^- or $gal^{\pm}$) with one or two doubtful cases were found to be more sensitive than the wild type on 33 mM molybdate medium (10 mM NH_4^+ as nitrogen source). All the suppressors which utilize galactose well (that is gal^+ or gal^{++}) were no more sensitive than the wild type. Similarly the galactose defective enhancers were extra sensitive to molybdate, while the galactose utilizers were similar to the wild type in sensitivity, with only one exception which was resistant. To complete the picture, the nature of other strains such as gamA, B and C

Genotype	Growth on D.galactose as carbon source	Growth on 33 mM $MoO_4^=$ (10 mM NH_4^+ as N.source)	Effect on brlA12 variegation
biA1	+	-	none
brlA12	+	-	-
brlA12;sup.(45 mutants)	+	-	suppression
brlA12;sup.(10 mutants)	++	-	suppression
brlA12;sup.(40 mutants)	-	--	suppression
brlA12;sup.(4 mutants)	±	--	suppression
brlA12;enh.(8 mutants)	+	-	enhancement
brlA12;enh.(2 mutants)	++	-	enhancement
brlA12;enh.(4 mutants)	-	--	enhancement
brlA12;enh.(9 mutants)	±	--	enhancement
brlA12;enh-11	+	+	enhancement
molA33;brlA12	+	+	none
molB35;brlA12	++	+	suppression
brlA12;galA1	-	-	slight enhancement
brlA12;galC7	±	+	enhancement
brlA12;gamA55	±	-	slight enhancement
brlA12;gamB65	±	+	enhancement
brlA12;gamC66	±	+	enhancement
pacC5	+	+	enhancement
palA,B	+	-	none
palcA,B	+	-	none
suB2;palB7	+	+	enhancement

Table 5. Growth responses of strains affecting the brlA12 phenotype.

(galactose non-utilization, molybdate resistance) and pacC5 (acid phosphataseless) which affect the brlA12 phenotype (Clutterbuck, unpublished) should be considered. The observations are summarised in table 5.

It can be seen that the ability of the modifiers to utilize galactose does not follow a sufficiently consistent pattern to suggest any close correlation with the modifying action of these strains. All the molybdate resistant mutants however act as enhancers of brlA12. The only exception is molA33 which has no effect on the variegation. This mutant, however, may have a basis for molybdate resistance different from the others and possibly related to nitrate metabolism (Arst et al., 1970).

In Aspergillus nidulans seven different loci are known related to defects of galactose metabolism (galA, B, C, D, E, F, G) (Clutterbuck, 1974). From those only galA, galD, galE, are known to have defects in the Leloir pathway which converts galactose to glucose (Roberts, 1970), and they all lead to "total" mutations. The defects of the rest of the loci are not known or they have not been investigated. In particular nothing is known about galG which was isolated as a suppressor (rev-2) of brlA12 (Clutterbuck, 1970; unpublished). Studies by Lindberg on Ophiostoma multiannulatum (1970a,b) suggest that an alternative minor route of galactose utilization, other than the Leloir pathway which converts galactose to glucose, could be via galactonic acid and the pentose phosphate pathway. Evidence for this includes stimulatory effects of pentoses such as D-xylose and D-arabinose on the utilization of D-galactose. This effect was tested in Aspergillus using rev-2 and also other galactose non-utilizing mutants, which

	Base medium supplemented with:						
	55 mM galactose	55 mM galactose + 0.9 mM xylose	55 mM galactose + 0.9 mM arabinose	66 mM arabinose	55 mM inositol	57 mM shikimic acid	46 mM glucosamine
galA1	0	5	9	15	14	8	5
galB3	5	18	9	0	21	15	8
galC7	1	12	17	17	15	11	6
galD5	2	6	7	14	15	14	6
galE9	3	10	15	18	20	15	7
galF2	1	10	12	12	10	6	5
galG (rev-2)	3	10	12	12	15	14	2
gamA55	4	11	18	17	17	11	10
gamB65	3	12	15	15	15	14	6
gamC66	3	13	7	16	16	14	8
molB35	25	25	25	20	22	13	15
biA1	10	20	20	20	20	15	11

Table 6. Relative growth rates of galactose defective strains on pentoses and related hexoses (mm of colony diameter).

influence the brlA12 phenotype. Growth on pentoses and relevant hexoses was also tested in an attempt to correlate defects of the pentose phosphate pathway with galactose non-utilization and effect on brlA12. Table 6 summarizes some of these results. It can be seen that addition of 0.0135% (0.89 mM) xylose or arabinose to the galactose medium stimulated growth of all galactose defective strains including rev-2 and gam mutants. No other major effects were observed except the known inability of galB to grow on arabinose. rev-2 was rather normal apart from its possibly limited growth on glucosamine but this might be attributed to the relatively slow growth of this strain even on glucose. A last aspect to be explored in connection with the galactose utilization and the molybdate resistance was related to the acid phosphatase activity. It is known that lack of acid phosphatases is correlated with molybdate resistance (Arst and Cove, 1970) and also with enhancement of the brlA12 variegation (Clutterbuck, unpublished). Preliminary observations by Clutterbuck suggested that possibly another fast moving acid phosphatase band is reduced in suppressors and increased in enhancers. A number of modifiers (double mutants with brlA12) including rev-2, rev-5, rev-7, enh-15, enh-24, galC7, etc., were therefore repeatedly tested with appropriate controls on starch electrophoresis gels. The extra band however was not always obtained and its correlation with the various strains tested was doubtful.

5.4.2. Spontaneous mutation rate of rev-2

Isolation of suppressors or enhancers of the brlA12 is not difficult after mild mutagenic treatments. In fact spontaneous enhancers or suppressors are not unusual and purification of the strain

		Viability				Spontaneous mutant frequency on 1 mM selenate			
Strains	No.	conidia plated	colonies found	viability	mean viability	viable conidia plated	resistant mutants	mutant frequency per 10^6 conidia	mean* mutant frequency/10^6 conidia
	1	205	198	96.6%		19.93×10^6	21	1.05	
	2	257	282	109.7%		20.37×10^6	35	1.71	
biA1	3	117	132	112.8%	105.80%	30.95×10^6	47	1.51	1.401 ± 0.137
	4	300	316	105.3%		12.25×10^6	14	1.14	
	5	-	-	-		-	-	-	
	1	64	69	107.8%		17.39×10^6	28	1.61	
	2	-	-	-		-	-	-	
biA1;brlA12	3	52	45	86.5%	86.80%	20.29×10^6	72	3.54	2.155 ± 0.229
	4	37	19	51.4%		19.44×10^6	44	2.26	
	5	41	45	102.3%		24.06×10^6	31	1.28	
	1	288	194	67.4%		14.99×10^6	41	2.73	
	2	296	188	63.5%		13.22×10^6	26	1.96	
biA1;rev-2 brlA12	3	155	101	65.2%	68.86%	14.50×10^6	79	5.44	3.988 ± 0.271
	4	266	209	78.6%		14.08×10^6	47	3.33	
	5	355	247	69.6%		13.92×10^6	89	6.39	

*(total mutants obtained/total number of conidia; error in viability and survival platings estimated assuming Poisson distribution).

Table 7. Viability and spontaneous mutant frequency of biA1, brlA12 and rev-2 brlA12

was found rather necessary from time to time to avoid the accumulation of spontaneous modifiers during subculture. rev-2 also has the tendency to become "impure" during successive subcultures. This can possibly be seen in plate 5 presented for other purposes. Fluffiness occurs rather often and in fact it was noticed that it could be either inhibited or suppressed in media supplemented with ammonium or high salt. These observations suggested that at least rev-2 might have a higher mutation frequency than the wild type or brlA12. To check for this hypothesis comparisons were made among conidia of the biA1, brlA12 and rev-2 brlA12 strains, using as a criterion of the spontaneous mutation rate of each strain the occurrence of selenate resistant mutants (Jansen, 1972). Conidia of the above-mentioned strains were plated on CM in very dilute suspensions to give single colonies. Five normal looking single colonies from each strain were then taken to inoculate respectively five tubes of CM + phosphate for each strain. After 2 weeks incubation at 37°C, conidial suspensions were made from each tube and the viability of conidia was repeatedly estimated by comparisons of haemocytometer counts and platings on supplemented minimal medium (table 7). rev-2 gave repeatedly approximately 70% viability compared to the wild type.

Conidia after appropriate dilutions from each suspension were incorporated into minimal medium supplemented with 1 mM sodium selenate. After growth at 37°C, the resistant colonies were counted in each plate and the mutant frequency was calculated after adjusting for viability (table 7). Conidia from cultures of wild type grown in the presence or absence of phosphate salt gave similar results as far as viability and mutant frequency are concerned. The mean mutant frequency of each strain was compared to the other two to determine whether the

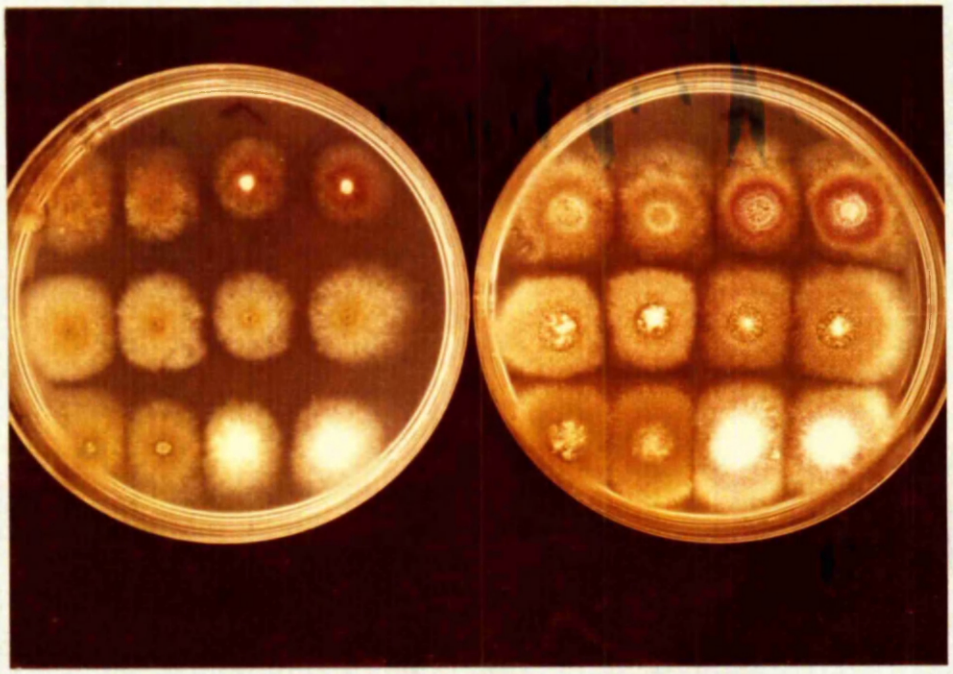


Plate 4. Growth and conidiation of brlA1 2 and modifiers on - N + 10 mM putrescine (left) and on - N + 10 mM putrescine + 10 mM nitrate (right). Strains in duplicate from top to bottom, left to right:

rev-2	brlA1 2 rev-2
puA1 rev-5	brlA1 2;rev-5
biA1	brlA1 2

values obtained are statistically different. It was assumed that (a) the experiments are homogeneous, (b) the Poisson distribution defines the error in estimating the total viable cells plated and the mutants surviving selection, (c) for the numbers given in the experiment the Poisson distribution is not different from normal distribution. A "t" test based on these assumptions demonstrates that: rev-2 brlA12 gives statistically higher mutant frequency than biA1 ($t_{\infty} = 8.51$, $P << 10^{-6}$) or brlA12 ($t_{\infty} = 5.16$, $P \sim 10^{-6}$); the difference in mutant frequency between biA1 and brlA12 is at the borders of significance ($t_{\infty} = 2.82$, $P = 0.0048$). rev-2 may therefore have lower genetic stability than brlA12 or the wild type.

5.4.3. Polyamines and rev-2

Polyamines are known among other things as antimutagenic agents (Johnson and Bach, 1966) apparently because of their affinity for DNA. It would be interesting to examine the behaviour of brlA12 and its modifiers, especially of rev-2 in the presence of these substances. brlA12 rev-2 was inoculated on a plate of minimal medium supplemented with 10 mM putrescine. After 2 days growth at 37°C the strain had taken a pink rather unhealthy appearance with its conidiation restricted to a limited number of immature conidia at the centre of the colony (Plate 4). brlA12;rev-5 and brlA12;rev-7 were also affected (pink with reduced conidiation) but to lesser extent. The wild type was normal with occasional traces of pigmentation only. Rather surprisingly, brlA12 was also pink without any other change in its phenotype. rev-5 and rev-7 by themselves were not pigmented, but rev-2 was. It appears therefore that high putrescine in the minimal medium (nitrate as nitrogen source) affects brlA12 itself as well as

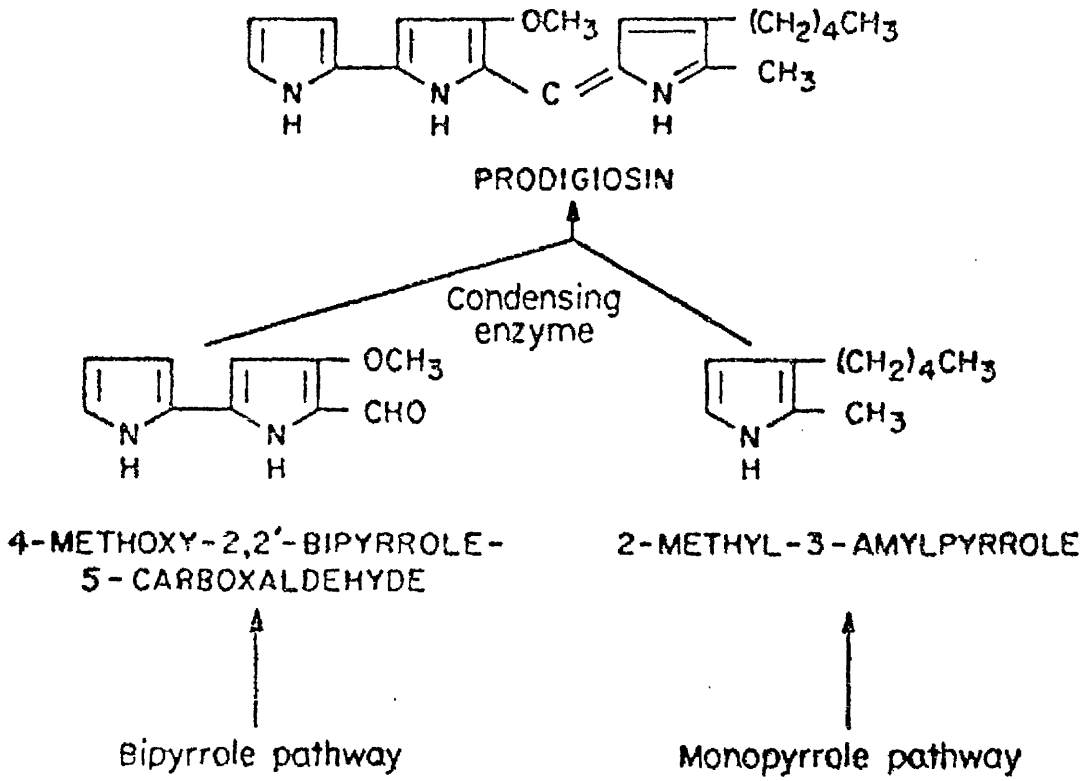


Figure 5. Terminal biosynthetic pathway for prodigiosin in Serratia marcescens

one class of its revertants. The pigment did not spread at all into the medium and it stained most of the hyphae. Although conidiation of brlA12 rev-2 was reduced in its presence no increase of bristles was noticed on this medium. On nitrogen-less minimal medium supplemented with 10 mM putrescine only rev-2 and brlA12 rev-2 were pink (Plate 4). In fact brlA12 rev-2 showed the most marked difference on this medium where putrescine was the sole nitrogen source present, with limited growth, pink pigmentation, no conidiation, and with a bristly spot right at the centre of the colony which might indicate an early and temporary failure of rev-2 to suppress brlA12. rev-2 by itself was also pink with unhealthy growth and conidiation. Putrescine was utilized by rev-5, brlA12 and the wild type as a fairly good nitrogen source, although germination of conidial inocula is delayed on this as compared to other nitrogen sources. The heavy pigmentation of brlA12 rev-2 and its sick appearance on high putrescine were suggestive of possible anomalies of this strain related to amine metabolism. In fact Bachrach (1962) has reported in Serratia marcescens, the association of a pink pigment, identified as prodigiosin (Fig 5), with high spermidine oxidase activity. Prodigiosin is the characteristic pigment of Serratia marcescens but its physiological role and biosynthesis are not known (Williams and Hern, 1967). A simple hypothesis at this stage was that rev-2 might have a higher rate of amine oxidase activity than normal with resultant heavy pigmentation and unhealthy appearance. To test for such a possibility in a rather indirect way, the rev-2 brlA12 mutant was inoculated on minimal medium supplemented with putrescine and also iproniazid or isoniazid which are known amine oxidase inhibitors (Kapeller-Adler, 1970). It was found that both iproniazid and isoniazid inhibited production of the

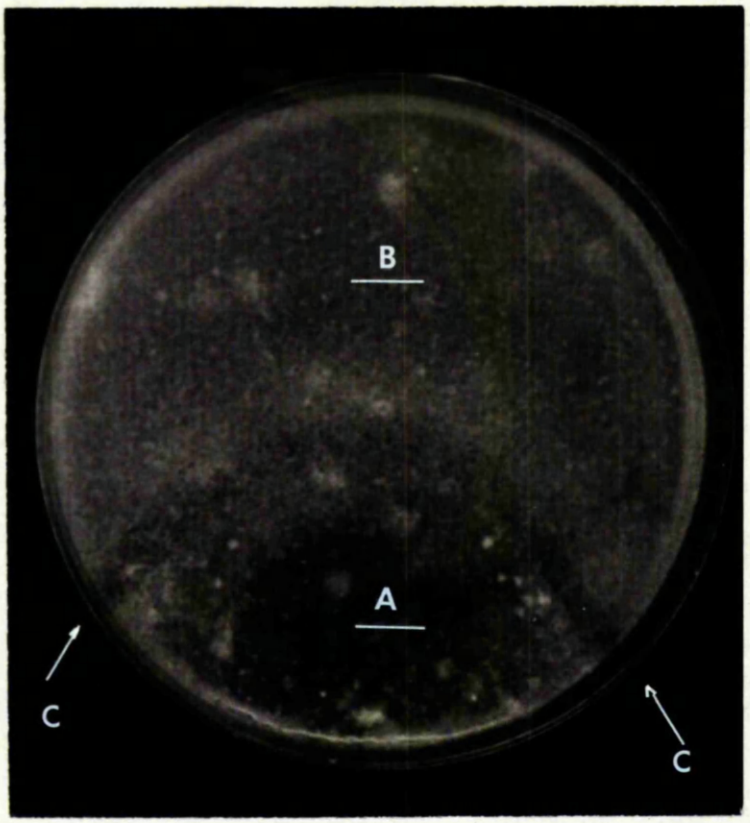


Plate 5. Response of rev-2 brlA12 on BM + galactose in presence of iproniazid phosphate.

A : inhibition area

B : background growth

C : ring of improved growth and conidiation

pigment with the latter being the more effective.

In order to see whether there is any connection between putrescine metabolism and the inability of rev-2 to grow on galactose medium, conidia of the rev-2 brlA12 mutant were plated into galactose medium with some iproniazid added at the edge of the plate. After some days of growth a ring of better than background growth and conidiation for rev-2 appeared in this plate (Plate 5). All the galactose non-utilizing suppressors were similarly tested on galactose medium supplemented with iproniazid and they all clearly behaved like rev-2. These rather positive responses led to further studies of the behaviour and nature of the pink pigment in view of its connection with the brlA12 mutant and its modifiers.

(1) Plate tests.

Heavy pigmentation for brlA12 and rev-2 was first observed on minimal medium (nitrate + glucose present) when supplemented with high putrescine. On this same medium, brlA1, brlA2, brlA9 and brlA42 (non-variegated bristle mutants - probably point mutations) were inoculated and they all formed pink pigment although to a lesser extent than brlA12. It therefore appears that two types of mutation related to conidiation processes lead to the same effect (see discussion). All bristle mutants form the pigment which by subsequent comparisons was found to be the same to that produced by rev-2. Further plate tests showed that the optimum medium for the production of the pink pigment had to contain glucose, nitrate or nitrite and putrescine. Lack of any of the three or inability of the strains to utilize them results in no pigmentation. rev-2 cnxB11 (a nitrate non-utilizing

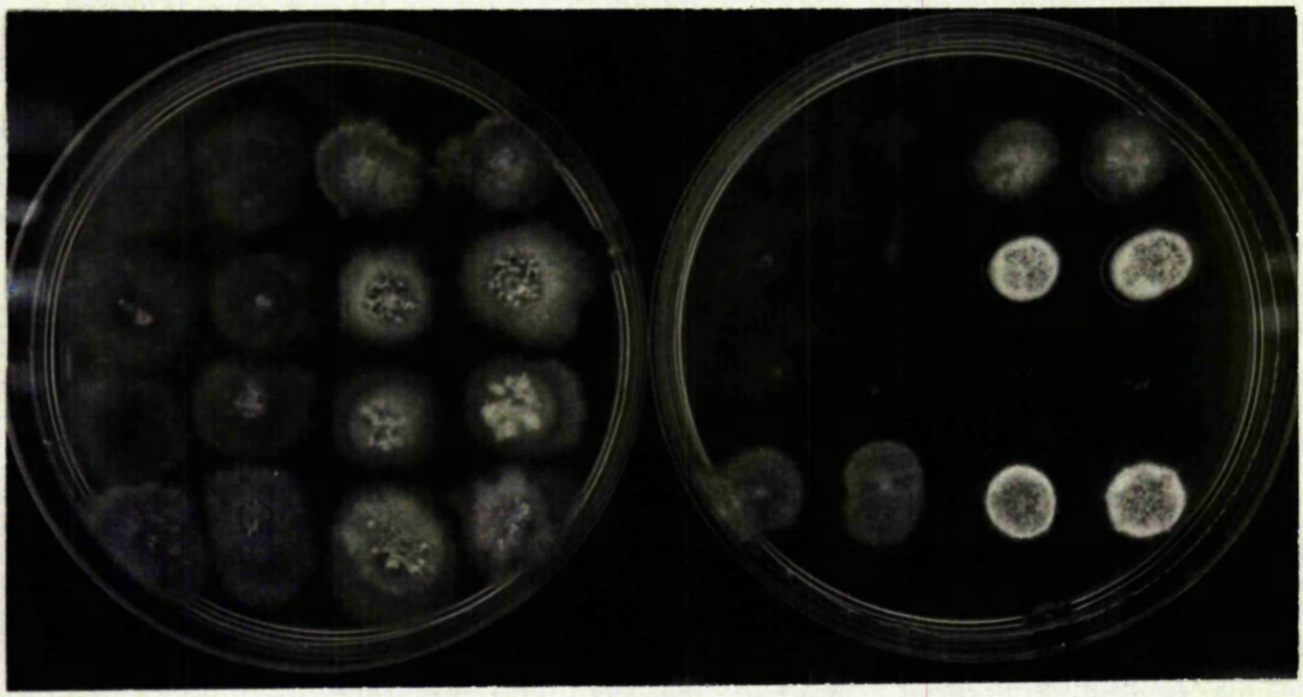


Plate 6. Growth of wild type and mutants on 10 mM nitrate + 10 mM putrescine in absence (left) and presence (right) of 5 mM isoniazid. Strains in duplicate, from top to bottom, left to right:

cnxB11	rev-2
niaD17	brlA12
niiA4	punA11
$nirA^{c}1$	biA1

strain) therefore will not produce the pigment with nitrate but it will do so with nitrite. rev-2 niiA$^-$ or rev-2 nirA$^-$ recombinants which cannot utilize nitrate or nitrite do not produce the pigment. Ethanol or glycerol but not galactose can substitute for glucose but much less pigmentation is produced. Spermidine but not spermine, instead of putrescine, will produce slight pigmentation only. Nitrate or nitrite are the only nitrogen sources which will allow formation of the pigment. Ammonium, or amino acids are ineffective, and in fact ammonium (or methylammonium) if present at a higher concentration than the nitrate will repress the formation of the pigment.

Pigment formation is also repressed by high salt in the medium (0.7 M NaH_2PO_4 or KCl). Plates inoculated with more than 10^4 conidia of rev-2 or bristle strains do not produce the pigment which possibly indicates participation of trace elements in its synthesis. The pigment is not produced in liquid cultures by rev-2 or brlA1^2.

Further tests with iproniazid and isoniazid showed that both prevent or inhibit pigmentation at concentrations around 5 mM, with isoniazid being the most effective. Another observation was that isoniazid and iproniazid at that concentration inhibited almost completely the utilization of putrescine as nitrogen source (Plate 6) which might explain the absence of pigment in the first case. On the other hand at the low concentration of 1 mM both iproniazid and isoniazid could substitute for putrescine and produce pigment in presence of nitrate and glucose. This may mean that, in addition to acting as inhibitors of amine oxidases in competition with putrescine (Kapeller-Adler, 1970), these compounds may mimic putrescine in other respects. This hypothesis together with the significance of amino oxidases for

Pigments in acetone		Pink pigment wavelength (nm)	colour	Prodigiosin (present work) wavelength (nm)	colour	Prodigiosin (Williams et al. 1956; Hubbard and Rimington, 1950) wavelength (nm)	colour
acid	maxima	460*, 305	yellow	535*, 368 280/288, 256/260	pink-red	535/540* 270/275	pink-red
	minima	240		415/420, 332/336 245/252, 276		420/425, 330/325 245/260	
alkaline	maxima	330/340	yellow-orange	470/472 * 532/534 256	yellow-orange	470 270/276	yellow-orange
	minima	310		376/384, 516/520 248		380, 250/260	
neutral	maxima	540/544*, 305 260/264	pink	536 * 256/260	pink	535*, 510 270	pink
	minima	468, 230, 244		512, 376, 248		515, 375, 250	

*main peaks

Table 8. Spectral properties of prodigiosin and pink pigment from A. nidulans.

(Where wavelengths are given as e.g. 535/540 this indicates variation between experiments.)

the utilization of putrescine as nitrogen source will be discussed later in view of further evidence (sections 8, 11, 14).

(2) Characterization of the pink pigment.

Solubility tests showed that the pigment was insoluble in water, petroleum ether, cyclohexane and benzene. It was soluble in methanol, ethanol, acetone, chloroform, toluene and ethylacetate, and therefore is not related to asperthecin, the cleistothecial pigment (Howard and Raistrick, 1955). For its production, brlA12 rev-2 was grown on thin layer cultures of minimal medium supplemented with 10 mM putrescine (approximately 200 conidia in 5 ml of liquid medium layered over 20 ml of solid medium). Similar plates without any putrescine (no production of pigment) were used as controls. After 4-5 days incubation at 37°C the mycelia in the form of a mat were harvested, washed with distilled water and extracted with acetone in a mortar. The homogenate was shaken for 2 hours at room temperature in the dark, then centrifuged. The sedimented debris was washed twice by resuspension in acetone, shaking for 30 minutes and centrifuging. The washings were combined with the supernatant from the original centrifugation and the solution was filtered. It was then dried in a rotary evaporator and dissolved in acetone, which took a deep purple colour. For comparisons with prodigiosin, Serratia marcescens was grown on complete medium plates and the prodigiosin produced was extracted as above. The visible and ultraviolet spectra of the two pigments were compared at neutral, acidic and alkaline pH (Table 8).

To purify the pink pigment further, columns of Sephadex LH-20 were used with a mixture of chloroform:heptane:methanol (10:10:1 vol respectively) being the eluant. Five well separated fractions

were eluted, coloured, dark violet, yellow-orange, yellow, light pink and dark pink. None of these were present in the controls. Prodigiosin also seems to consist of at least four fractions (Williams et al., 1956) although with the present chromatography method only two were recovered (violet and pink).

The dark violet fraction is eluted first and rapidly in both cases and it might represent the dimer of the compound (Harned, 1954). The abundance of fractions may be attributed to the formation of various salts of the main compound, by exchange with organic acids or metals present in the crude extracts. In fact a wide variety of prodigiosin-like pigments exist in organisms including Pseudomonas species and Actinomycetes in addition to Serratia (Gerber, 1975). These pigments differ in having extensive modifications of the basic tripyrrylmethane structure (prodiginine). The pink pigment found in Aspergillus might be one of these although only systematic analysis could prove that.

The main similarities and differences of the two pigments will be summarised here for an overall picture:

(a) The biosynthetic pathways which lead to the formation of both pigments are unknown; both however seem to be somehow connected with polyamines (putrescine for Aspergillus, spermidine for Serratia). If they are of similar nature then their most probable and rather distant precursor could be Δ^1-pyrroline, the cyclic oxidation product of putrescine and spermidine (see fig 2 and 5). In fact Δ^1-pyrroline was one of the compounds used for the synthesis of prodigiosin in vitro (Rapoport and Holden, 1961).

(b) In both cases the formation of the pigment is inhibited or

repressed by high salt in the medium (Silverman and Munoz, 1973). In addition it was found that Aspergillus pigment was also repressed by iproniazid, isoniazid, ammonium and methylammonium. Low, non-toxic concentrations of o-aminobenzaldehyde added to the medium prevented the formation of the pigment probably by trapping any Δ^1-pyrroline available.

(c) Both pigments are soluble in organic solvents only. A difference here is that one of them but not the other is soluble in benzene and petroleum ether, from the solvents tested.

(d) Both pigments are pink at neutral pH and yellow-orange at alkaline pH. In acidic pH however prodigiosin is red while the other is yellow. This difference may account for altered spectra although there is some correspondence between the main peaks of the same colour (Table 8).

(e) Prodigiosin formation requires iron (Silverman and Munoz, 1973) which affects its biosynthesis on media with high bacterial populations. A similar effect has been observed with the other pigment, although the specific requirement has not been identified.

(f) The evidence from Aspergillus nidulans suggests that enzymes or products involved in the metabolism of glucose and nitrate-nitrite as well as putrescine are essential for the formation of the pigment.

By transferring colonies grown for 24, 36 and 48 hours to new media, it was found that the appearance of the pigment is not related to the age of the medium. There is also a clear connection of the pigment with conidiation processes; the pigment is produced by all bristle mutants and when produced by other strains it inhibits their conidiation. Its appearance in solid media cultures after about

24 hrs of growth at 37°C almost coincides with the beginning of conidiation. Its absence from liquid cultures may also suggest dependence on conidiation. The connection of rev-2 with conidiation will be discussed later.

5.4.4. Hydroxylamine sensitivity.

Hydroxylamine (NH_2OH) is a toxic compound for Aspergillus nidulans at concentrations of 1 mM and above (Drainas, 1978). The reasons for its toxicity are not known. It is a powerful mutagenic agent (Budowsky et al., 1972) reacting with cytosine and its nucleotides and nucleosides to give base pair transitions (CG → AT). It is also known to induce chromosome breaks (Freese and Freese, 1964) and to inhibit amino acid decarboxylases and oxidases (Clark, 1963). It was found to increase the conidiation of brlA12 but only in presence of high salt in the medium (section 5) and it was therefore tested with the modifiers at the 1 mM level for effect on conidiation or toxicity in minimal medium. No change in the conidiation pattern occurred. As far as toxicity is concerned enhancers and suppressors were heterogeneous in their sensitivity to hydroxylamine, although hypersensitivity was more common among the enhancers. The situation was reversed when putrescine was used instead of nitrate as the sole nitrogen source.

5.4.5. Salt sensitivity.

In connection with the studies related to the isolation of osmotic mutants (section 3) all suppressors and enhancers were tested at 0.75 M and 1.5 M of NaH_2PO_4 and also NaCl added to complete medium. None were found to be sensitive at any of the above-mentioned two concentrations.

5.4.6. Rate of enhancement in *brlA12;enh-37*

In the course of mapping experiments one of the galactose defective enhancers, *brlA12 enh-37*, in heterokaryons with M.S.E. gave the impression of better conidiation as if the inactivation of the bristle locus was suppressed. To test for this, heterokaryons of *pabaA1 yA2;brlA12* + M.S.E. and *pabaA1 yA2;brlA12;enh-37* + M.S.E. were cultured under identical conditions (thickness of medium, incubation time, etc.) and equal surfaces of medium with heterokaryotic conidia were suspended in Tween. 1.6×10^4 conidia from each suspension after washing out the Tween, were plated on minimal medium supplemented only with p-aminobenzoic acid to select for conidia other than those of M.S.E. After incubation 65 colonies emerged from the heterokaryon with the original *brlA12* and only 5 from the heterokaryon with the enhanced *brlA12*. M.S.E. therefore does not appear to affect the enhancer tested, the check however provides a quantitative comparison of the change in the phenotype of the two strains.

5.4.7. Effect of *rev-2* on *brlA9*

brlA9 (Clutterbuck, 1969) is a non-variegated but leaky bristle aconidial mutant with a rudimentary vesicle having elongated forked bristles. It is assumed to be a point mutation. It responds to high salt by reducing the length of its bristles to resemble sterigma-like structures. No conidia are formed however. *rev-2* was crossed with *brlA9* to see whether it would have any effect on its phenotype. The double mutants were examined for conidiation and for other morphological changes under a binocular microscope. No conidia were formed. Some of the segregants gave the impression of shorter

and more branched bristles in presence of rev-2 than those of the original brlA9, however this was a minor effect and it might be due to other modifying factors, existing in the cross. rev-5 and rev-7 when crossed also with brlA9 also did not influence the latter (Clutterbuck, 1970 and personal communication).

Discussion

About 300 mutants suppressing the inactivation of the bristle locus in brlA12 were isolated. Another set of 30 mutants was isolated enhancing the brlA12 inactivation. No genetical analysis was carried out beyond attempts to map two or three of the modifiers which met with haploidization difficulties.

Complementation tests and classification according to the degree of modification indicate three different groups of suppressors, corresponding to the classes observed by Clutterbuck (1970) and represented by rev-5 (chromosome II, good suppressors), rev-2 (chromosome VIII, intermediate) and rev-7 (chromosome VII, inefficient suppressors). The possibility of more loci is not excluded. The most interesting class appears to be the one represented by rev-2 which mostly includes suppressors with a simultaneous defect in galactose utilization. Some of the enhancers however are galactose non-utilizers as well. Attempts to correlate molybdate resistance and acid phosphatase activity (Arst and Cove, 1970) with modifications of the brlA12 phenotype, did not produce any consistent pattern of behaviour, although some connection, not obvious at the moment, may exist. Mutants which affect the bristle morphology are known (Clutterbuck, unpublished) like molB (suppressor), and gamA, B, C, (enhancers), galC7 (enhancer), pacC (enhancer) but again their mode

of action cannot be predicted in relation to their other features since strains similar for one feature may give positive, negative or normal responses in other tests. Only the knowledge of the precise biochemical defects of these strains might give information about which ones are relevant to the brlA12 case and therefore provide clues about the nature of the variegation.

A new possible candidate for investigation emerged when putrescine was found to cause heavy pigmentation and unhealthy growth of rev-2. Polyamines are known to bind with nucleic acids and therefore could qualify for some role. They are also considered to be antimutagenic agents and rev-2 in addition to its poor growth on putrescine, was found to have low viability and high spontaneous mutation rate, which suggest genetic instability. Furthermore all the galactose non-utilizing suppressors grew better to some extent on this carbon source when iproniazid, an amine oxidase inhibitor was present at the same time. It should be mentioned here that polyamines such as spermidine and spermine activate UDP-galactose-4-epimerase in yeasts (Darrow and Creveling, 1964) the enzyme which converts UDP-galactose to UDP-glucose. If a similar activating mechanism operates in Aspergillus nidulans as well, then, in view of the evidence above, the inability of rev-2 to utilize galactose might be explained in terms of alterations in polyamine content in this mutant.

brlA12 and to a lesser extent all the bristle strains also produce the same pink pigment as rev-2. It appears therefore that polyamines, assuming at the moment that they are the precursors of the pigment, might be involved in conidiation processes in general. The absence of pigment in liquid cultures may also support this hypothesis.

However rev-2 although it induces conidiation in brlA12 does not affect the non-variegated aconidial brlA9. Therefore rev-2, like rev-5 (= drkB5, Clutterbuck, 1970) acts specifically on the variegated position effect mutant. It might therefore be expected to affect other variegated position effect mutants, although, unfortunately no other such mutants are available for test. If this is the case however the brlA12 mutation can be regarded merely as a detector for rev-2 and no specific connection of rev-2 with conidiation is implied. In this case, the occurrence of the pink pigment in both rev-2 and bristle mutants is a curious coincidence.

Some evidence has been presented to correlate the pigment with prodigiosin, an antibiotic produced by Serratia marcescens, and associated with high spermidine oxidase activity. Although similarities exist the two pigments are different, but possibly related in structure in view of the abundance and variety of prodigiosin-like pigments. In Aspergillus nidulans, presence in the medium and utilization of nitrate or nitrite by the strains which produce the pigment seems to be essential for its formation, in addition to the participation of putrescine and glucose. Its production is inhibited by ammonium, amine oxidase inhibitors and high salt which is known to suppress the brlA12 variegation. The pigment itself when formed inhibits conidiation, although partial recovery will occur after long incubation.

SECTION 6

TESTS WITH brlAl2

MM + salt	pH	Extra conidiation in brlA12 (scale 0-10)
none	7	0
1 M NaCl or KCl	7	8
0.1 M NaCl or KCl	7	2
0.7 M NaH_2PO_4	6	10
0.1 M NaH_2PO_4	6	3
0.7 M $NaNO_3$	8	4
0.7 M $(NH_4)_2SO_4$	6	6
0.7 M CH_3COONa	9	0
0.6 M $(CHOH.COONH_4)_2$	8	5
0.5 M $[CH(OH)COONa]_2$	8	0
0.2 M $CaCl_2$	6	0
0.7 M glucose	8	3

Table 9. Responses of <u>brlA12</u> conidiation on various salts.

6.1. Introduction

When this work started, salt was the only agent able to cause a major effect on the variegation of brlA12. Unfortunately, it is not specific enough to provide any information about its mode of action. Its effect however suggested that other substances more specific in their action, may exist, which could provide further information. An attempt was made to this direction and an extensive number of chemicals, inhibitors and other analogues were used. brlA12 was mainly studied although some modifiers were also looked at. Media used were as specified in each particular case. While a number of compounds did affect conidiation of brlA12, the effects were not dramatic. 0.7 M NaH_2PO_4 converts about 20% of bristles to conidial heads a rate obtained only by methylamine addition (see below).

6.2. Plate tests

6.2.1. Salt effect

Extending Clutterbuck's observations, brlA12was inoculated on minimal medium supplemented with a variety of salts (glucose was also tried) at non-toxic concentrations, to see their influence on the induction of conidiation (Table 9). The pH of the medium was not specially adjusted, and it was estimated by paper testers after growth. Phosphate salt at an optimum concentration of 0.71 M (approximately 10%) was the most effective and this salt was used in all further tests when required. The low concentration of 0.1 M which is not enough to affect brlA12 substantially was used occasionally to buffer the medium for relevant tests.

6.2.2. pH effect

brlA1 2 responds to pH changes (Clutterbuck, 1970) as it also can be seen from Table 9. Low pH increases wild type activity although its effect is rather small compared to that of the salt.

6.2.3. Temperature

Plates left at room temperature, after initial growth at 37°C show improved conidiation by comparison to plates constantly at 37°C for the same time.

6.2.4. Carbon sources

brlA1 2 grows similar to the wild type with no effect on its conidiation on the following carbon sources added to base medium. Acidic compounds were neutralized before addition. The concentrations used were : 1% D-glucose, 1% galactose, 1% ethanol, 1.4% acetate, 0.55% D-galactose, and 100 mM of the remaining compounds : D-mannose, D-mannitol, D-sorbitol, i-inositol, D-arabinose, D-xylose, L-ascorbate, pyruvate, malate, lactate, fumarate, succinate, tartrate.

6.2.5. Nitrogen sources

Nitrogen-less minimal medium was used with nitrogen sources added at the concentration of 10 mM: urea, uric acid, methylamine, putrescine, γ-aminobutyrate, acetamide, glycine, L-alanine, L-arginine, L-lycine, L-proline, L-asparagine, L-glutamine, L-glutamic acid, L-aspartic acid, L-methionine, L-histidine, L-serine, L-tyrosine, L-phenylalanine, L-tryptophan, L-ornithine, nitrate, nitrite and ammonium.

brlA12 grows like the wild type on all the nitrogen sources tested. None was found to affect its conidiation.

6.2.6. Analogues and toxic agents

These tests were the extension of a first experiment where ethionine, an analogue of methionine, was tested for an effect on the variegation of brlA12. According to Holliday and Pugh (1975), methylated DNA may increase heterochromatization. If therefore methionine, the main methyl donor, is replaced by ethionine, the latter should be expected to increase conidiation. Although the result of this test was not the expected one (see below), a number of other analogues of natural compounds were tested for an effect on brlA12.

Normally for this purpose, brlA12 conidia were incorporated into solid minimal medium and the analogue was added in a well at the edge of the plate, so that a range of concentrations would develop, the highest ones, around the well usually being toxic. A second set of plates was also made as above but with 0.7 M phosphate salt added in the medium. Plates with the natural compounds were occasionally used as controls. The chemicals tried were: D-alanine, DL-alanine hydroxamate, N-acetyl-L-alanine, α-N-benzoyl-L-alanine, L-alanine methyl ester, 3,4,dihydro-DL-proline, 4-hydroxy-L-proline, L-azetidine-2 carboxylic acid, L-thiazolidine-4-carboxylic acid (thioproline), D-threonine, D-histidine, D-valine, D-phenylalanine, p-fluorophenyl-alanine, D-tyrosine, 3-amino-L-tyrosine, D-tryptophan, methyl-DL-tryptophan, 3-2-aminoethyl-L-cysteine, D-lysine, D-serine, D-ornithine, L-methionine sulphoxide, DL-ethionine, D-isoleucine, β-fluoropyruvic acid, methylamine, aminopterin, 1-4-diaminobutanone hydroxylamine, 5-bromodeoxyuridine, thiodisuccinic acid, selenate, methyl-urea,

ethyl-urea, thiourea, isoniazid, iproniazid phosphate. Only those which had a detectable effect on the conidiation of brlA12 will be discussed here.

(a) L-methionine analogues: L-methionine is the donor of methyl groups whose importance in DNA modification mechanisms and gene activity has been particularly emphasized by Holliday and Pugh (1975). It also participates in the synthesis of vitally important polyamines. When replaced by its analogue, DL-ethionine, the latter in the form of S-adenosyl ethionine traps available adenine and prevents synthesis of ATP with a resulting decline in m-RNA and protein synthesis. This effect is reversed by administration of ATP or adenine (Harper, 197). Ethionine according to the hypothesis of Holliday and Pugh, was expected to cause an increase in the conidiation of brlA12. In fact it had no effect on conidiation in media without phosphate in the presence or absence of adenine. When phosphate was present a reduction of conidiation, irreversible by adenine was apparent.

It was found however that a similar reduction of conidiation by ethionine occurred in the wild type as well; this reduction could be minimized in both cases, if the plates were incubated with their lids removed. This test suggests that the effect of ethionine was probably due to some volatile compound produced from ethionine, which inhibited conidiation in general. Decarboxylation of ethionine in Coprinus lagopus (R.H. Wilson, 1970 and personal communication) produces 3-ethylthiopropylamine, a volatile compound with inhibitory effects.

L-methionine added in control plates in presence of phosphate

salt had a very slight positive or negative effect in the conidiation of brlA12, depending on the concentration.

(b) L-alanine analogues: In presence of phosphate salt, D-alanine causes further reduction of conidiation in brlA12. This effect and similar ones with other analogues, cannot be regarded as necessarily related to the mechanism of the variegation unless enhancement of the bristle morphology is observed at the same time as the reduction of the conidiation. Observation of such a result is however difficult unless a really big effect occurs, which is not usually the case.

In contrast to D-alanine, DL-alanine hydroxamate under the same conditions suppresses brlA12, increasing its conidiation. L-alanine used as control had no effect. The pH of the plates was unchanged at pH 6-7.

(c) L-proline analogues: No effect on conidiation was observed on media without phosphate salt. In its presence thioproline and to a lesser extent α-azetidine-2-carboxylic acid, induced conidiation. A well conidiating ring separated a zone of better conidiation and growth (next to the analogue) from that with the normally expected conidiation (distant from the analogue). The hydroxy- and dehydro-proline had no positive effect, in fact they might have caused slight reduction of conidiation. L-proline used as control slightly improved conidiation in the presence of phosphate only.

(d) methylamine: Concentrations of 10-100 mM of this monoamine which behaves as an ammonia analogue (Pateman and Kinghorn, 1976) were added in minimal medium without phosphate salt (pH 8.6). Conidiation was induced at relatively good levels with an optimum at concentrations

of 50-70 mM. At this rather low concentration methylamine was much more effective than equimolar concentrations of phosphate salt, however less effective than high phosphate (0.7 M). Unfortunately comparisons at the high concentration were not possible because of the toxic effect of this analogue when used above 100 mM. Methylamine was less effective in changing the conidiation of brlA12 when nitrite, ammonium, arginine or putrescine were used as nitrogen sources instead of nitrate. Ammonium used at similar concentrations in any medium, also had no effect.

(e) aminopterin: This compound is known to affect variegated position effects (Schultz, 1956), probably because it is an analogue of folic acid which participates in purine and pyrimidine synthesis (Woolley, 1963). When tested with brlA12 it caused a slight improvement of conidiation in the presence of phosphate. p-aminobenzoic acid (PABA) is a precursor of folic acid: starvation of a PABA requiring brlA12 strain for this compound did not produce any special effects.

(f) 1-4-diaminobutanone: This may be regarded as a putrescine analogue ($H_2N(CH_2)_2O(CH_2)_2NH_2$) which affects polyamine synthesis via its inhibitory and stimulatory effects on ornithine and S-adenosyl-methionine decarboxylases (Stevens, 1977a,b). When added to plates with conidia of brlA12 in the presence of phosphate salt it caused reduction of conidiation.

(g) hydroxylamine: It is a strong mutagenic agent in bacteriophage. It is toxic to Aspergillus at concentrations above 1 mM. Its effects on the modifiers of brlA12 have already been mentioned (section 5). On the brlA12 itself in the presence of phosphate salt only, it caused a good increase of conidiation and growth. The pH of the medium is not affected by the addition of hydroxylamine.

6.2.7. Other agents

Addition of amines (histamine, tryptamine, putrescine, spermidine, cadaverine, spermine), butyryl cyclic monophosphoric acid (C-AMP), purines, pyrimidines and their nucleosides did not affect the conidiation in presence or absence of phosphate salt. Ethanol and trypsin stimulated conidiation only in the presence of phosphate. Arginine also improved growth and conidiation in the presence of phosphate (at pH 6.5).

6.3. Summary and Discussion

The present state of knowledge concerning brlA12 may be summarized as follows:

brlA12 grows normally on all nitrogen or carbon sources tested.

A number of chemical agents, mostly analogues of compounds important in the physiology of the fungus were tested in an attempt to influence the rate of its conidiation and therefore provide hints of their modifying action.

Those with negative action, that is further reduction of the conidiation, include mainly, D-alanine, DL-ethionine and 1-4-diamino-butanone. Their effect however might be unrelated to the position effect since no obvious enhancement of the bristly morphology accompanied the reduction of conidiation. However as already mentioned observations of this type are not easy because of the relatively large number of bristles compared to conidial heads, even in the presence of salt.

Those with positive action, that is increase of the number of conidial heads include, methylamine and salts at relatively low pH. Other agents also with positive action but depending on the simultaneous presence of salt in the medium include arginine, hydroxylamine, DL-alanine hydroxamate, thioproline, ethanol and trypsin.

Clearly no one of these compounds with the possible exception of methylamine is capable of a major effect similar to that manifested by the addition of salt. Moreover, their diverse nature makes interpretation difficult. This is indeed not an unusual situation with variegated position effects (Baker, 1968) where the investigator has to determine which of the information obtained is the most relevant. Methylamine might be of some importance in this case most probably by its property of being an ammonium analogue. However it is not clear why it should therefore affect variegation.

The effect of salt might be expected at least because of its property <u>in vitro</u> to dissociate DNA and nuclear proteins. High external osmolarity will probably increase the internal osmolarity as well, with effects on chromosome organization.

SECTION 7

STUDIES OF AMINE METABOLISM IN *Aspergillus nidulans*

In section 1 the importance of polyamines was described and some of their functional aspects were emphasized in view of their probable connection with the brlA12 variegated position effect, as based on the evidence presented in the previous sections. For convenience this evidence will be summarized here.

(1) Polyamines, like histones, are of basic nature and therefore have high affinity for polyanions such as nucleic acids, to which they bind strongly. In fact polyamines may compete with histones for binding sites on the chromosome (Schwimmer, 1968). Hence they qualify as possible candidates affecting position effect phenomena.

(2) The polyamine content of various organisms and cell cultures is inversely related to the osmolarity of the growth medium (Munro et al., 1972, 1975). Similarly, inactivation of the bristle locus is inversely related to the osmolarity of the medium.

(3) Polyamines by their stabilizing effect on DNA act as antimutagenic agents. brlA12 rather easily gives spontaneous suppressors or enhancers and in fact, rev-2, one of its suppressors, has a statistically significant high spontaneous mutation frequency compared to the wild type or brlA12.

(4) Uridine diphosphogalactose epimerase in yeasts is activated by polyamines. rev-2 is a galactose non-utilizing suppressor of brlA12 which is repaired to some extent on this medium by iproniazid, an amine oxidase inhibitor.

(5) Cultures of brlA12 and also of rev-2 are heavily pigmented pink when grown on minimal medium supplemented with putrescine. Spermidine

is less effective. Serratia marcescens produces a pink pigment, identified as prodigiosin and associated with high spermidine oxidase activity.

(6) Comparisons of prodigiosin with the pink pigment of Aspergillus nidulans indicate that they are different but possibly of similar nature (prodigiosin-like pigments).

(7) rev-2 is not able to grow on putrescine as the sole nitrogen source to the same extent as the wild type. This may be due to failure of putrescine catabolism or alternatively to growth inhibition by putrescine.

These are the main points which are suggestive of some role for polyamines in the regulation of the brlA12 variegation; it was therefore decided to pursue further investigations in this direction. Unfortunately very little work has been done on polyamine metabolism in Aspergillus nidulans (Kinghorn and Pateman, 1977) and therefore elucidation of at least the main aspects of their catabolism and regulation in this organism was necessary before any conclusions could be drawn about their action on brlA12. The genetical and biochemical investigations undertaken to this direction involve the three main polyamines (putrescine, spermidine and spermine) and they will be discussed in the following sections.

SECTION 8

MUTATIONS AFFECTING AMINE METABOLISM IN *Aspergillus nidulans*

8.1. Introduction

Investigations of the metabolism of polyamines are facilitated by genetical and biochemical comparisons between the wild type and appropriate mutants. When this work started the only known mutation of *Aspergillus nidulans* connected with polyamines was the putrescine auxotroph *puA1* (Sneath, 1955), which in fact demonstrates the importance of polyamines for the organism rather than providing any information about their metabolism (Stevens, 1975, section 1). Isolation of mutants was therefore considered necessary and the search was directed along three main lines (a) the isolation of spermidine or spermine auxotrophs and also related mutants which would allow the *puA1* to grow on these polyamines as alternatives to putrescine; such mutants might be obtainable if in fact the main (or only) function of putrescine is as a precursor of spermidine and spermine. These mutants should be informative about the uptake system(s) for all three polyamines and also about functions other than their main interactions (putrescine → spermidine → spermine), (b) a search for mutants which could not grow on putrescine as nitrogen source either because of defective uptake or lack of catabolic enzymes; such mutants should produce evidence about the biochemical pathways involved in polyamine catabolism and possibly explain the defective growth of *rev-2* on putrescine, (c) a search for mutants already existing in the stocks which could be additionally defective in amine metabolism as a result of defects in regulatory loci; aspects of polyamine regulation in respect to other regulatory systems could be explored by studies with such mutants.

8.2. spsA1 a spermidine-spermine sensitive mutant of Aspergillus nidulans

Repeated mutagenic treatments of the wild type were made in a search for mutants which would require spermidine or spermine for growth. The detection method was based on velvet replication to unsupplemented medium of colonies grown on media supplemented with spermidine or spermine. No such auxotrophs were found. Mutagenesis of the puA2 strain however (nitrous acid, 45% survival) produced a series of derivative strains which, unlike the original (section 1), could also grow on 0.06 mM spermidine as an alternative to 0.06 mM putrescine. The new mutants still could not utilize spermidine as nitrogen or carbon source, and further tests separated them into two classes:

(a) One class, less frequent than the other is represented by spd-6 which exhibits the above mentioned characteristics. The altered locus has not been identified mainly because of the difficulties arising from the dependence of the mutant phenotype on the putrescine auxotroph. Later however, it was found that the spd-6 mutant was, unlike the wild type, sensitive to methylglyoxalbis(guanylhydrazone) (MGBG), an analogue of polyamines (Williams-Ashman and Schenone, 1972; Birgitta-Brown et al., 1975). This new property would now allow identification of the mutant independently of the putrescine marker.

(b) The second, more frequent, class of mutant in addition to being able to accept low concentrations of spermidine in place of putrescine, was also inhibited by high concentrations of spermidine or spermine (5-10 mM) in minimal medium. Inhibition was also produced by the polyamine analogue MGBG as in the case of spd-6. This sensitivity

chromosome	gene marker	spsA$^-$	spsA$^+$
I	y^+	0	2
	y	6	17
II	w^+	6	19
	w	6	16
III	gal^+	12	0
	gal^-	0	35
IV	$pyro^+$	4	14
	$pyro^-$	8	21
V	fac^+	6	22
	fac^-	6	13
VI	s^+	7	24
	s^-	5	11
VII	nic^+	3	14
	nic^-	9	21
VIII	$ribo^+$	6	16
	$ribo^-$	6	19

Table 10 : Haploidization analysis of spsA1/M.S.E. diploid.

cross	Markers	Recombinants		Parentals		Recombination fraction	Significance of linkage (X_1^2)
		spsA$^-$	spsA$^+$	spsA$^-$	spsA$^+$		
I	galA	13	25	45	7	42.2%	P > 0.1
	argA	0	32	58	0	33.5%	P > 0.001
	methH	14	17	32	27	34.4%	P > 0.001
	ivoA	7	17	53	13	26.6% ± 4.6	P < 0.001
	sC	6	16	52	16	24.4% ± 4.5	P < 0.001
II	sorB	20	27	74	72	24.3% ± 3.1	P < 0.001
	cnxH	18	16	76	83	17.6% ± 2.7	P < 0.001
III	cnxH	5	10	27	54	15.6% ± 3.7	P < 0.001
IV	sorB galE*	9	39	64	77	25% ± 3.1	P < 0.001
V	brlA12	3	9	39	47	12.2% ± 3.3	P < 0.001

* See text.

I $\frac{+\ biA1}{yA2\ +}$ $\frac{+}{wA3}$ $\frac{spsA1\ +\ +\ +\ +\ +}{+\ sC7\ ivoA1\ methH2\ argA1\ galA1}$

II $\frac{pabaA1\ yA2\ +}{+\ +\ biA1}$ $\frac{spsA1\ +\ +}{+\ cnxH4\ sorB11}$

III $\frac{+\ biA1}{yA2\ +}$ $\frac{spsA1\ +}{+\ cnxH4}$ $\frac{+}{pyroA4}$

IV $\frac{pabaA1\ yA2\ +}{+\ +\ biA1}$ $\frac{+\ spsA1\ sorB11}{galE9\ +\ +}$

V $\frac{+\ +\ biA1}{pabaA1\ yA2\ +}$ $\frac{puA2}{+}$ $\frac{+\ spsA1}{brlA12\ +}$

Table 11. Crosses and recombination fraction of spsA with other markers of chromosome III.

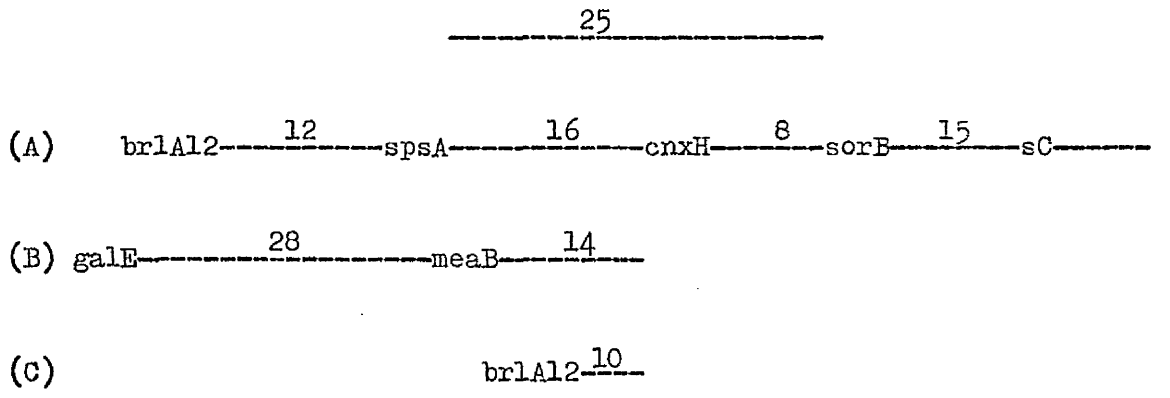


Fig. 6 Markers at the end of chromosome III

(A), present data (see text)

(B), Käfer, 1977

(C), Clutterbuck, 1970.

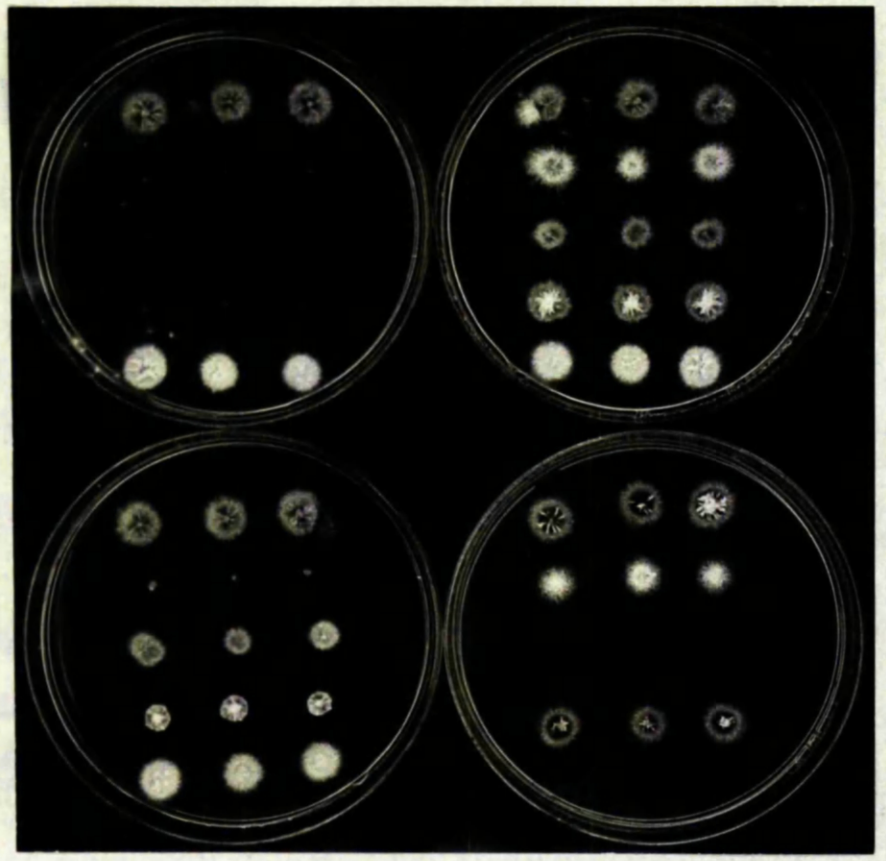


Plate 7 : Growth responses of spsA1, spd-6 and controls on 10 mM nitrate (top left), 10 mM nitrate + 0.06 mM putrescine (top right), 10 mM nitrate + 0.06 mM spermidine (bottom left) and 10 mM nitrate + 10 mM spermidine (bottom right).

Strains in 3 replicas from top to bottom:

biA1

biA1;puA2

biA1;puA2;spsA1

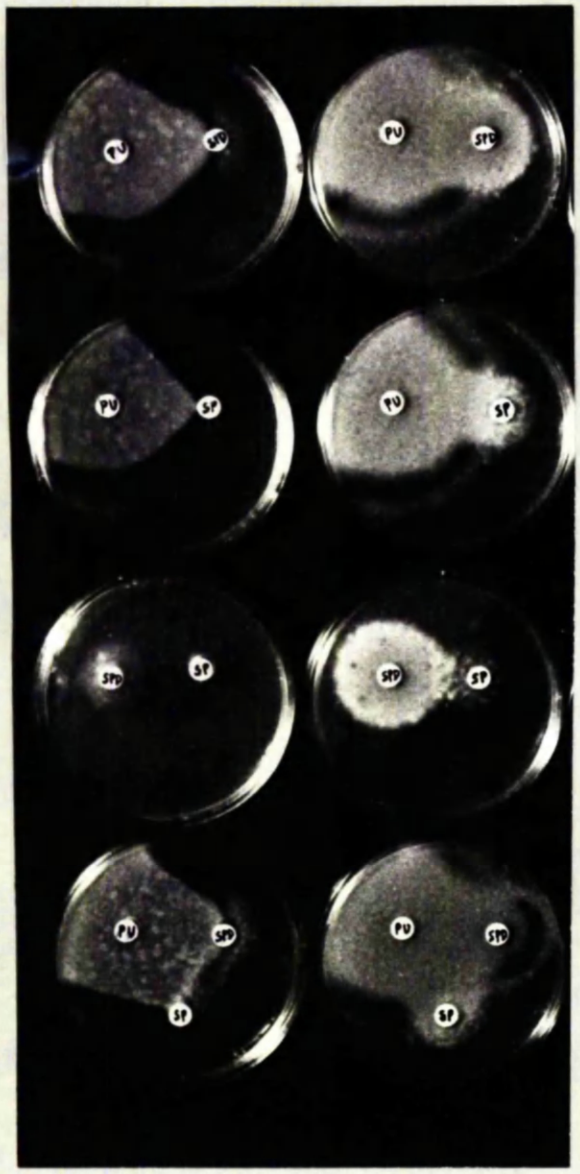
biA1;puA2;spd-6

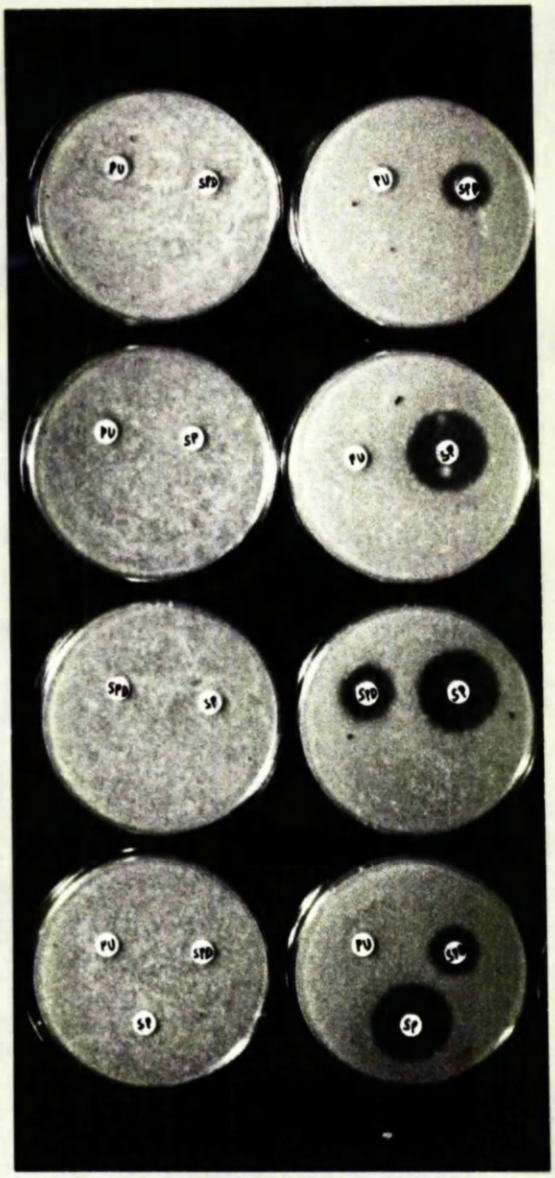
biA1;spsA1

was not reversed by ammonia or any other nitrogen source added at the 10 mM level. 20 segregants requiring putrescine and spermidine sensitive were recovered from various crosses where the double mutant was one of the parents. All of them could be supplemented with low concentrations of spermidine instead of putrescine, which suggests that unless two closely linked genes are involved, the two properties of the new mutants are due to a single gene. The gene by itself (i.e. in pu^+ recombinants) still confers sensitivity to high concentrations of spermidine or spermine and therefore it was given the symbol spsA. spsA1 is recessive in the diploid with master strain E (M.S.E.). Haploidization analysis of this diploid located the mutant on the chromosome III (Table 10). Further mapping followed to locate its position on this chromosome. Table 11 presents the recombination fraction of spsA1 and the markers with which it was crossed. Close linkage was found with cnxH4 and brlA12 only (see also Fig 6).

brlA12 according to Clutterbuck (1970) must be located approximately 10 units on the left of cnxH on this chromosome, while the present data place it about 12 units on the left of spsA (Fig 6). Both these distances, however, are probably distorted because of the translocation.

galE is probably the only marker not in agreement with the evidence that brlA12 is at the end of chromosome III. Crosses involving the galE and meaB markers are not presented because interactions with unknown markers made unequivocal classification difficult. Plate 7 illustrates the main growth properties of spsA1 and spd6 in





biAl;puA2 biAl;puA2;spsAl biAl biAl;spsAl

Plate 8a: Auxanographic responses of biAl;puA2 and biAl;puA2;spsAl to polyamines.

Polyamine concentrations in wells:

(PU) : 100 µl of 6 mM putrescine
(SPD): 100 µl of 6 mM spermidine
(SP) : 100 µl of 6 mM spermine

Plate 8b: Inhibition of biAl and biAl;spsAl by polyamines.

Polyamine concentrations in wells:

(PU) : 25 µl of 6 mM putrescine
(SPD): 100 µl of 100 mM spermidine
(SP) : 100 µl of 100 mM spermine

(dark areas at the borders of growth in plate 8a are due to dark brown pigmentation).

medium		mutants				
Supplements other than biotin	Nitrogen source	biA1	puA2	puA2;spsA1	puA2;spd-6	spsA1
0.06 mM PU	10-70 mM NO_3^- or 10 mM NO_2^- or 5-100 mM urea, or 10 mM alanine, or 10 mM glutamate, or 10 mM asparagine or 10 mM proline or 10 mM arginine	+	+	+	+	+
0.06 mM SPD	same as above	+	-	+	+	+
0.06 mM PU	10 mM NH_4^+ or 10 mM methylamine	+	+	+	+	+
0.06 mM SPD	same as above	+	-	-	-	+
0.06 mM PU	70 mM NO_3^- + 1-10 mM NH_4^+	+	+	+	+	+
0.06 mM SPD	same as above	+	-	-	-	+
0.06 mM PU	70 mM NH_4^+ + 1-10 mM NO_3^-	+	+	+	+	+
0.06 mM SPD	same as above	+	-	-	-	+
0.06 mM PU	10 mM NO_3^- + 10 mM NH_4^+	+	+	+	+	+
0.06 mM SPD	same as above	+	-	+	+	+
0.06 mM PU + 0.7 M NaH_2PO_4 (or 1M KCl)	10 mM NO_3^-	+	+	+	+	+
0.06 mM SPD + 0.7 M NaH_2PO_4 (or 1M KCl)	10 mM NO_3^-	+	-	-	-	+

Table 12 (1st part) Growth of spsA1, spd-6 and related mutants on various media.

medium		mutants				
Supplements other than biotin	Nitrogen source	biA1	puA2	puA2;spsA1	puA2;spd-6	spsA1
-	10 mM PU	+	+	+	+	+
-	10 mM SPD	-	-	-	-	-
-	10 mM SP	-	-	-	-	-
-	10 mM MGBG	-	-	-	-	-
6-50 mM SPD	10 mM NO_3^- or 10 mM NH_4^+	+	+	-	+	-
10 mM SP	10 mM NO_3^- or 10 mM NH_4^+	+	-	-	-	-
1-10 mM MGBG	10 mM NO_3^-	+	-	-	-	-
0.06 mM PU + 1-5 mM MGBG	10 mM NO_3^-	+	-	-	-	-
10 mM MGBG	10 mM PU	+	+	-	-	-
0.05-0.5 mM MGBG	10 mM NO_3^-	+	-	-	-	+
10 mM SPD + 0.7M NaH_2PO_4 (or 1M KCl)	10 mM NO_3^-	+	+	+	+	+
6 mM SPD + 0.7M NaH_2PO_4	10 mM NO_3^-	+	±	±		+
6 mM SPD 1 M KCl	10 mM NO_3^-	+	+	+	+	+
0.06 mM PU + 0.06-10 mM SPD + 0.7M NaH_2PO_4	10 mM NO_3^-	+	+	+	+	+

Table 12 (2nd part) Growth of spsA1, spd-6 and related mutants on various media.

comparison with other strains. Plate 8 illustrates the same properties for spsA1 in the form of auxanographic tests. The response of puA2;spsA1 on spermine (a sparing effect) is discussed along with additional evidence below. The growth conditions of the strains were investigated further to establish all their properties and possibly obtain information about their biochemical defects. Table 12 presents most of the tests done and these results will be summarised here for convenience:

(1) spsA1 grows like biA1 on the nitrogen sources tested. Both strains can utilize putrescine but not spermidine or spermine as the sole nitrogen source.

(2) spsA1 does not require any polyamines for growth and it can be easily distinguished from the wild type by its sensitivity to high concentrations of spermidine, spermine or MGBG.

(3) This sensitivity is reversed by high salt concentrations, eg 0.7M NaH_2PO_4 or 1M KCl with phosphate being the more effective. Ammonium added at 10 mM does not protect from toxicity.

(4) The double mutant with the putrescine auxotroph (puA2;spsA1) behaves similarly to spsA1 in all above tests when supplemented with 0.06 mM putrescine.

(5) Unlike the putrescine auxotroph, the double mutant will also grow on 0.06 mM spermidine, unless ammonium or methylamine or high salt concentrations are present.

(6) High nitrate (70 mM) or high urea (100 mM) as the sole nitrogen sources do not impair the growth of the double mutant when supplemented with putrescine or spermidine. Combinations of, high ammonium (70 mM)

with or without nitrate, or high nitrate (70 mM) with low ammonium (1-10 mM) restrict the growth of the double mutant when supplemented with spermidine (but not when putrescine is used).

(7) Low nitrate (10 mM) or low ammonium (10 mM) do not affect growth of the double mutant supplemented either with putrescine or spermidine.

(8) Spermine cannot substitute for putrescine or spermidine at least not at concentrations up to 10 mM. The response of puA2;spsA1 on spermine in the auxanography test (Plate 8a) appears therefore to be a sparing effect, detected only if putrescine or spermidine are present at the same time with spermine. This fits with the theory that in the absence of spermine, sufficient putrescine or spermidine needs to be supplied for conversion to spermine. However, while spermine cannot be converted back to spermidine and therefore will not, on its own, support growth of the puA1 mutant, it can reduce the requirement for the simpler polyamines.

A rather similar case of a sparing effect has been reported by Pontecorvo et al. (1953) with an arginine auxotroph which could also be supplemented with proline but not lysine; when, however, both proline and lysine were present, an additional response to the latter was observed.

(9) puA2;spsA1 and puA2;spd-6 behave similarly as far as their growth is concerned at low levels of putrescine or spermidine. puA2;spd-6 is not sensitive to high spermidine or spermine. It is sensitive however on MGBG and by this test should be distinguished from the wild type in the absence of the putrescine marker.

(10) MGBG (1-5 mM) by competing with putrescine (0.06 mM) does not allow the growth of putrescine auxotrophs.

(11) In brlA12;spsA1 recombinants, spsA1 had no obvious effect on the rate of conidiation of brlA12 in the presence or absence of non-toxic concentrations of spermidine.

A very simple hypothesis which could easily explain the features of spsA1 is that it is an uptake mutant which can take up spermidine more efficiently than the wild type. Low concentrations allow growth while high concentrations are toxic. Ammonium may regulate the uptake as is the case for some other uptake systems in Aspergillus nidulans (Dunn and Pateman, 1972; Pateman et al., 1974). High salt may decrease the internal polyamine pools, reversing spermidine toxicity at high concentrations and not allowing entry of sufficient spermidine. Putrescine is not affected by ammonium or high salt either because of higher pools, or differences in regulation and metabolism.

Assuming that spsA1 is an uptake mutant, it would provide the means for testing for any effect of spermidine on the brlA12 variegation. A negative result can be interpreted either as showing that spsA1 does not allow access of spermidine to the nucleus or that variations of spermidine levels so produced, do not influence heterochromatization.

All the above possibilities will be discussed later on in the light of uptake and enzyme assays and internal polyamine pool estimations.

The relationship of spsA1 to spd-6 is not yet known. The latter may represent a leaky allele of spsA1, however it still grows at concentrations of spermidine up to 50 mM, despite being as sensitive to MGBG as spsA1. This may reflect mutation at another locus resulting in a different spectrum of polyamine sensitivites.

8.3. Isolation of mutants unable to utilize putrescine as the sole nitrogen source

Spermidine or spermine cannot be utilized by Aspergillus nidulans as nitrogen or carbon sources. Putrescine also cannot be utilized as a carbon source, but wild type grows fairly well on it when used as the sole nitrogen source. There is a small delay however in germination, compared to other nitrogen sources. Although amines are rather unusual compounds to be treated as nitrogen sources the fact that putrescine can be utilized as such, and also the defective growth of rev-2 on the same (Plate 4, section 5) were tempting enough to look for mutants which would not grow on putrescine either because of a defect in the uptake system or as a result of defects in catabolic enzyme activities. Unfortunately no toxic analogues of polyamines for the wild type are available to select for resistance (Apirion, 1962) and therefore direct selection had to be used by plating mutagenised conidia (u.v. treatment, survival 6%) on MM and replicating onto medium containing 10 mM putrescine as sole nitrogen source. biA1 and yA2;pabaA1;pyroA4 were the mutagenised strains. A number of mutants which grew poorly on putrescine as nitrogen source were isolated and purified. A common feature of most of them was that they were leaky to some extent which probably

Chromosome	gene marker	punA$^+$	punA$^-$
I	bi$^+$	26	19
	bi$^-$	8	0
II	w$^+$	0	19
	w	34	0
III	gal$^+$	26	9
	gal$^-$	8	10
IV	pyro$^+$	7	14
	pyro$^-$	27	5
V	fac$^+$	31	14
	fac$^-$	3	5
VI	s$^+$	31	10
	s$^-$	3	9
VII	nic$^+$	2	16
	nic$^-$	32	3
VIII	ribo$^+$	26	14
	ribo$^-$	8	5

Table 13 : Haploidization analysis of punA11/M.S.E. diploid

Cross	Marker	Recombinants		Parentals		Recombination fraction	Significance of linkage (x^2_1)
		punA$^-$	punA$^+$	punA$^-$	punA$^+$		
	acrA	27	22	19	21	55%	P > 0.3
	wA	19	18	27	25	41.5%	P > 0.1
	riboE	19	23	27	20	47%	P > 0.5
I	thiA	17	27	29	16	39.3%	P > 0.9
	ileA	18	27	28	16	50.5%	P > 0.9
	cnxE	14	21	32	22	39.3%	P > 0.02
	ygA	3	12	43	31	16.8% ± 3.9	P < 0.001
II	puA	7	22	47	18	30.8% ± 4.7	P < 0.001
III	trypA	5	3	69	114	4.1% ± 1.4	P < 0.001

Crosses: I

$$\frac{\text{biA1}}{+} \qquad \frac{+ \quad + \quad + \quad + \quad + \quad + \quad \text{punA11} \quad +}{\text{acrA3 wA4 riboE6 thiA4 ileA1 cnxE14} \quad + \quad \text{ygA6}}$$

II

$$\frac{+}{\text{luA1}} \quad \frac{+}{\text{yA2}} \quad \frac{\text{biA1}}{+} \qquad \frac{+}{\text{puA2}} \quad \frac{\text{punA11}}{+} \qquad \frac{+}{\text{mauA2}}$$

III

$$\frac{+}{\text{pabaA1}} \quad \frac{+}{\text{yA2}} \quad \frac{\text{biA1}}{+} \qquad \frac{+}{\text{trypA69}} \quad \frac{\text{punA11}}{+}$$

Table 14 : Crosses and recombination fraction of punA11 with other markers of chromosome II.

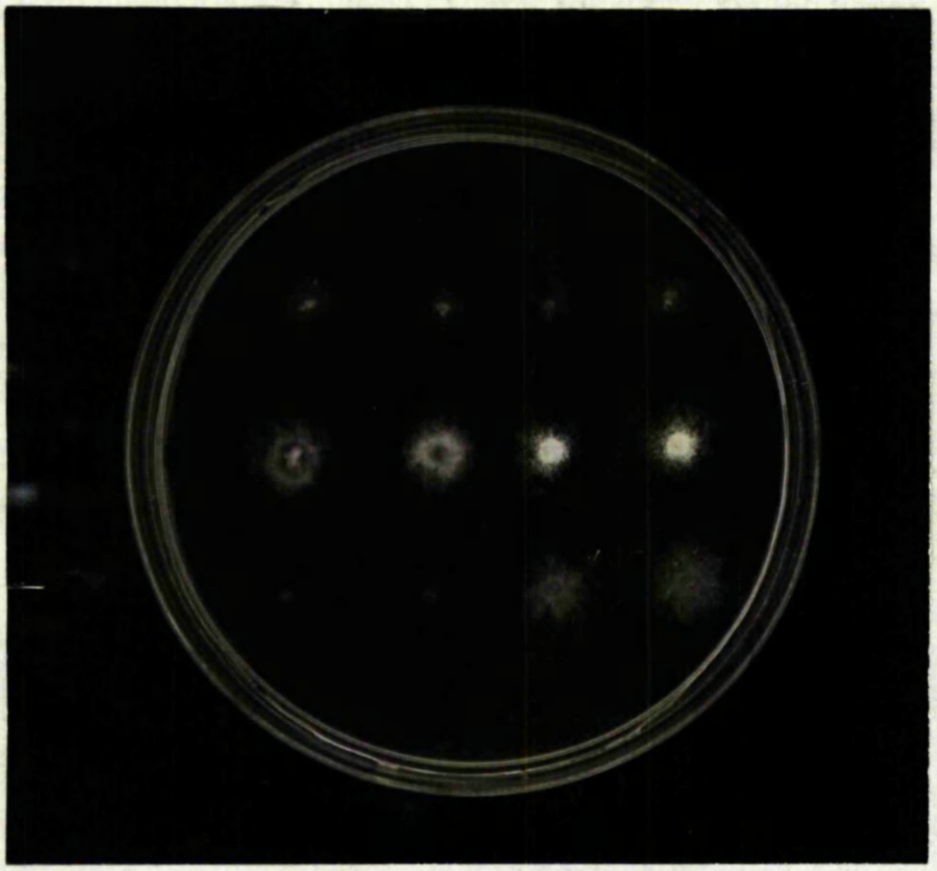


Plate 9 : Growth tests of tamA and areA strains on putrescine as the sole nitrogen source (two days incubation at 37°C). Strains in duplicate top to bottom and left to right .

tamA^{r}50	areA^{r}550
tamA^{d}1	areA^{d}520
TAMPU	biA1

(Note that TAMPU has grown no more than tamA^{r}50 at this stage ; cf plate 10).

suggests the presence of more than one enzyme contributing to putrescine utilization. The whole series of mutants was given the symbol *pun* (putrescine as nitrogen source) and the best isolate *punA11* was mapped on chromosome II (Table 13). *punA11* was recessive in the diploid with M.S.E. Further crossing located the mutant close to the *trypA* marker on this chromosome (Table 14), between the *trypA* and *ygA* markers. Complementation tests in heterokaryons between *pun*$^-$ mutants were difficult because of the leaky growth of these strains; at the moment it seems likely that at least two loci are involved since *pun-8* complements partially with *pun-9*, *punA11* and *pun-22*.

The growth properties of the most interesting mutants were screened and they will be presented in conjunction with the properties of mutants discussed in the following subsection.

8.4. Regulatory loci affecting putrescine utilization as nitrogen source

tamA^r*50* and *areA*^{r}*550* are the completely ammonium repressed alleles of the *tamA* and *areA* genes respectively (Pateman and Kinghorn, 1975, 1976), the products of which are thought to regulate a number of enzyme activities subjected to ammonium repression. The *tamA*^{r}*50* and *areA*^{r}*550* alleles are altered in such a way that they do not allow the expression of these enzymes and therefore they can grow only on ammonium as the nitrogen source. It was found that strains carrying either of these alleles of two distinctly different regulatory loci, are also unable to make effective use of putrescine as nitrogen source (Plate 9); the partially repressed *tamA*^{r}*119* and the ammonium derepressed *tamA*^{d}*1* and *areA*^{d}*310* can utilize putrescine similarly to

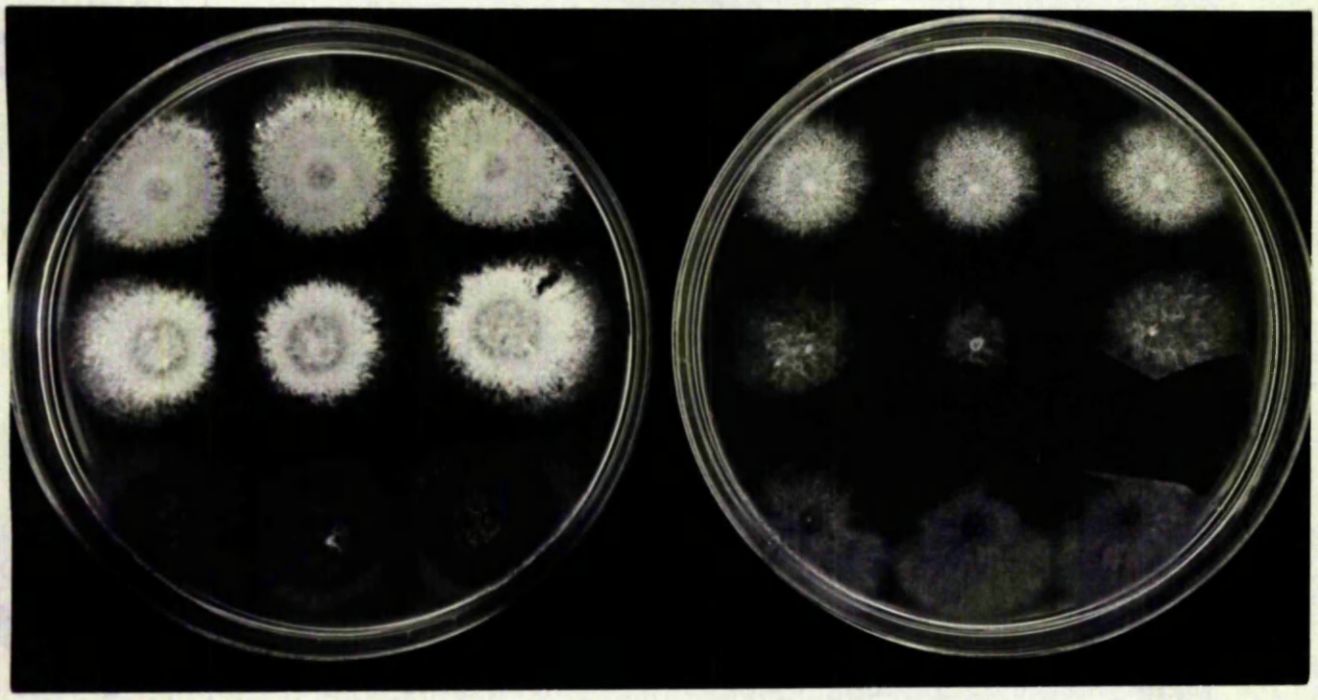


Plate 10 : Growth response of TAMPU, tamA^{r}50 and biA1 on 10 mM ammonium (left) and 10 mM putrescine (right) (6 days incubation at 37°C) strains in 3 replicates top to bottom.

yA2;TAMPU

yA2;tamA^{r}50

biA1

(Note: strains carrying the yellow spore marker (yA2) appear lighter in the photograph).

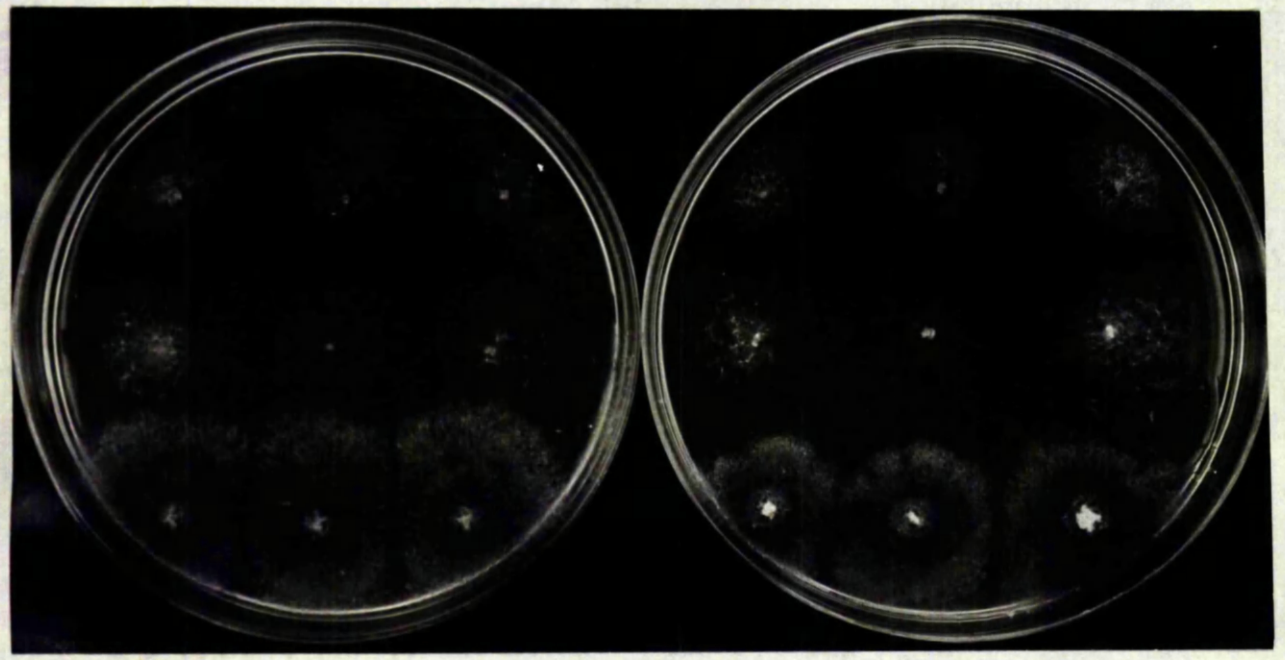


Plate 11 : Growth response of TAMPU, *tamA*^{r}50 and *biA1* on 10 mM glutamate (left) and 10 mM alanine (right).
(6 days incubation at 37°C).
Strains in 3 replicates top to bottom:

yA2;TAMPU

yA2;*tamA*^{r}50

biA1

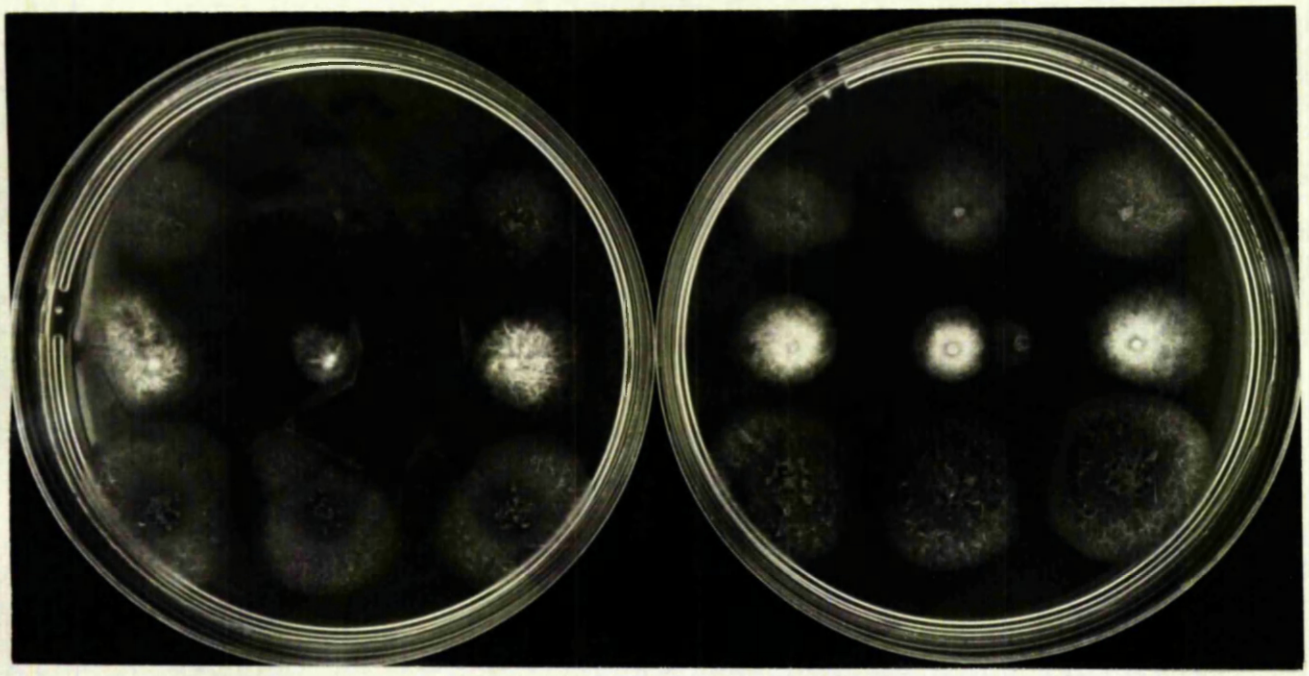


Plate 12 : Growth response of TAMPU, *tamA*^{r}50 and *biA1* on 3 mM nitrite (left) and 3 mM nitrate (right).
(6 days incubation at 37°C).
Strains in 3 replicates top to bottom.

yA2;TAMPU

yA2;*tamA*^{r}50

biA1

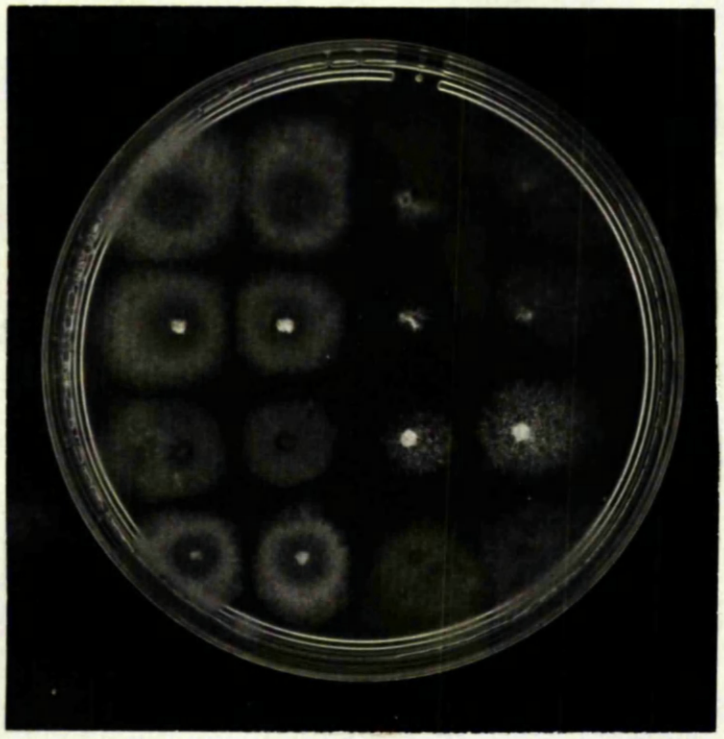


Plate 13 . Growth response of *nirA*^{c}1 and other strains on putrescine as the sole nitrogen source . Strains in duplicate , from left to right , top to bottom :

cnxB11	*nirA*^{c}1
niaD17	*nirA*^{c}1 + *gdhB1*
nirA4	
niiA4	*biA1*

the wild type. TAMPU represents a strain which resulted from mutagenesis of tamA^{r}50 and was selected for specific growth on putrescine. Plate 10 shows that it grows much better than the original strain on putrescine. It is however very delayed in its germination (compare plate 9 to plate 10) and this is probably explained by the absence of active uptake for putrescine in this strain (see section 10). It is as bad as tamA^{r}50 on alanine or glutamate (Plate 11) and it is even worse than tamA^{r}50 on nitrate or nitrite as nitrogen sources (Plate 12). TAMPU therefore, specifically allows utilization of putrescine only and does not resemble tamA^{r}50 back-mutants which restore growth on any nitrogen source. Back-mutants would probably result by mutations affecting the tamA^{r}50 product, presumably a regulatory protein; for TAMPU however it is more likely that this protein is the same as the one produced by the original mutant (tamA^{r}50), and the new mutation may be in a regulatory region close to the gene coding for putrescine utilization. Such a mutation would allow expression of the putrescine utilization system in the presence of the tamA^{r}50 repressing protein, which however would be still capable of repressing other systems.

The poor growth of TAMPU on nitrate or nitrite, apparently worse from tamA^{r}50's suggested a possible interaction between nitrate and putrescine utilization. To test for this possibility, strains with abnormalities in the utilization of nitrate and nitrite were tested on putrescine as the sole nitrogen source. Interestingly it was found that cnxB11, niaD17, nirA4 and especially niiA4 grew better than the wild type on putrescine, while nirA^{c} the constitutive mutant for nitrate reductase did not grow (Plate 13). The nirA^{c} allele in

Growth of mutants on various nitrogen sources relative to biA1	minimal medium (70 mM NO_3^-)	complete medium	−N + 10 mM NO_3^-	−N + 10 mM NO_2^-	−N + 1 mM NH_4^+	−N + 10 mM putrescine	−N + 10 mM γ-aminobutyrate	−N + 10 mM propanediamine	−N + 10 mM cadaverine	−N + 10 mM tryptamine	−N + 10 mM methylamine	−N + 10 mM L-glutamine	−N + 10 mM L-glutamate	−N + 10 mM L-ornithine	−N + 10 mM L-asparagine	−N + 10 mM glycine	−N + 10 mM L-tyrosine	−N + 10 mM L-lysine	−N + 10 mM L-arginine	−N + 10 mM L-proline	−N + 10 mM L-alanine	−N + 10 mM urea	−N + 10 mM L-methionine
(standard) biA1	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
pun-7	5	5	5	3	5	2	5	3	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5
pun-8	6	6	6	5	6	2	6	6	5	2	5	6	6	6	6	6	6	1	6	6	6	6	5
pun-9	5	5	5	4	4	2	5	3	4	5	6	4	4	5	5	5	5	6	5	5	5	5	4
pun-10	5	6	5	5	6	2	5	2	1	5	5	4	5	6	6	6	5	6	6	6	6	6	5
punA11	6	6	6	6	6	1	6	6	4	2	5	6	4	6	6	6	5	1	6	6	3	6	4
pun-22	3	6	6	6	6	3	6	5	4	4	5	6	6	5	6	6	5	5	6	6	6	6	4
Growth of wild type (biA1) on the above nitrogen sources relative to its growth on ammonium																							
biA1	6	6	6	5	6	5	6	4	2	3	4	6	5	5	6	5	3	2	6	5	5	6	4

Table 15 : Growth of pun⁻ mutants on various nitrogen sources

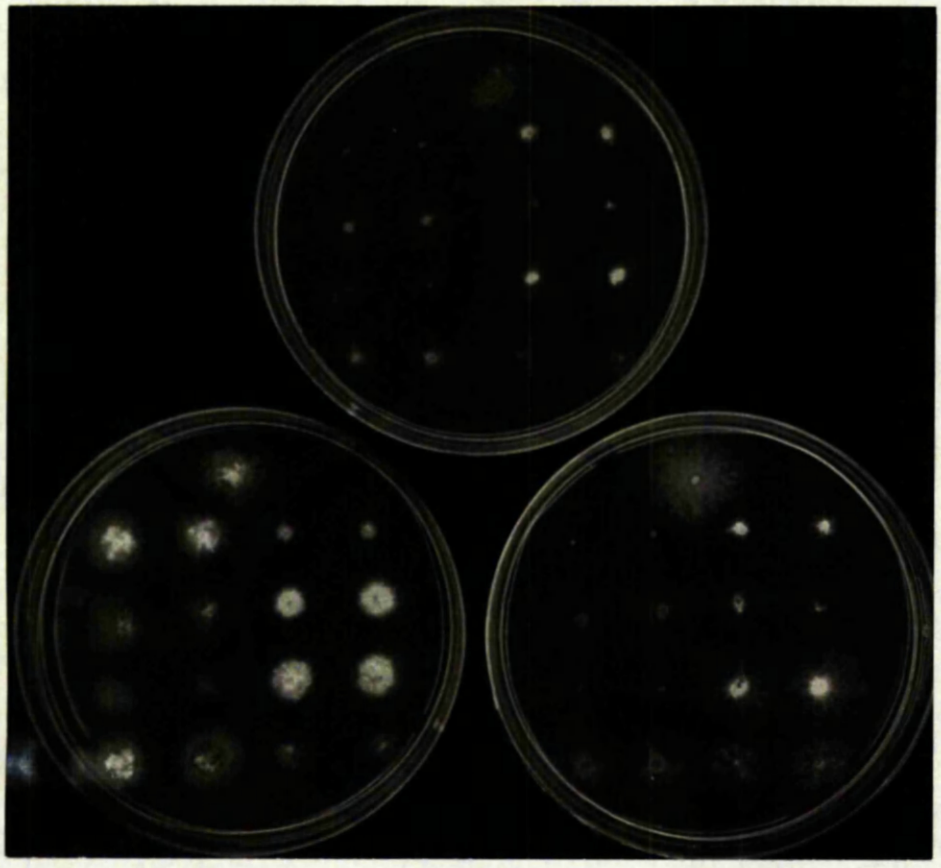


Plate 14 : Growth of *pun*$^-$ and *nirA*^{c}*1* mutants on 10 mM putrescine (top) ,10 mM putrescine+0.1 M Na_2HPO_4 (left) and 10 mM putrescine+0.2 M NaCl (right). Stains in duplicate ,left to right , top to bottom.

biA1

punA11	*pun-7*
pun-22	*pun-8*
nirA^c*1*	*pun-9*
nirA^c*1* +	*pun-10*

the *nirA*^{c}/*nirA*$^{+}$ heterozygous diploid was dominant or semidominant for putrescine non-utilization as it is for constitutivity of nitrate and nitrite reductases (Pateman and Cove, 1967). It therefore appears that putrescine catabolism is in some way linked with nitrate assimilation in *Aspergillus nidulans*. The mutants described here, together with those mentioned in the last subsection were further tested to obtain more information.

8.5. Growth properties of mutants defective on putrescine as the sole nitrogen source

Table 15 presents the growth rates of the most interesting *pun*$^{-}$ mutants on various nitrogen sources, in relation to the growth of the wild type taken as the optimum growth on each particular nitrogen source. The relative growth of the wild type on all tests is also presented. *pun-7* and *pun-9* are defective on putrescine and also on its oxidation product, propanediamine; *pun-8* and *punA11* are defective on putrescine and tryptamine; *pun-10* on putrescine and cadaverine; *pun-22* on putrescine. It therefore appears that all the mutants can utilize more or less normally amino acids and inorganic nitrogen as a nitrogen source, and they are specifically defective on putrescine and possibly other amines as well; it should be noted however that growth on propanediamine, cadaverine and tryptamine is rather poor for the wild type as well (see last row, table 15). Plate 14 presents the growth of *pun*$^{-}$ strains and also of *nirA*^{c} on 10 mM putrescine. It also shows that addition of 0.1M Na_2HPO_4 (but not of 0.2M NaCl) enables *pun-8*, *pun-9*, *punA11* and *nirA*^{c} (but not *pun-7*, *pun-10*, *pun-22*) to grow again on putrescine as nitrogen source.

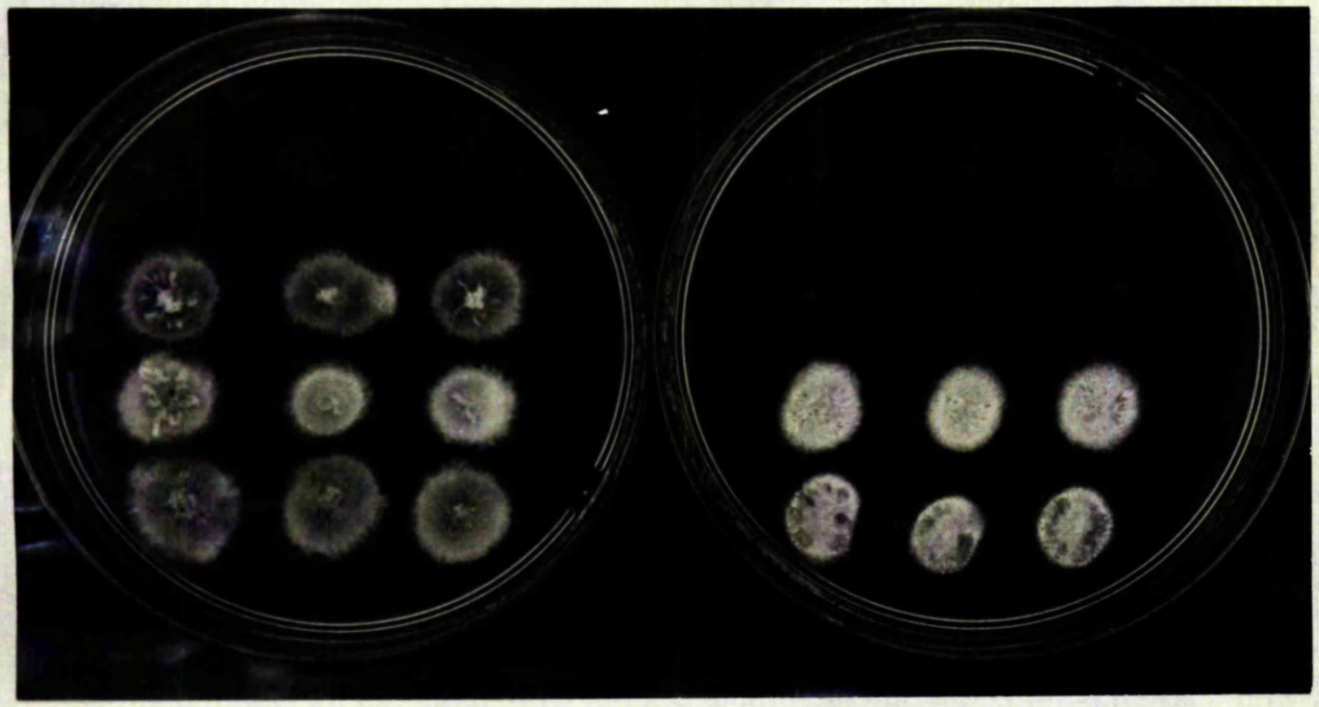


Plate 15 : Growth of punA11 and other strains on 10 mM nitrate (left) and 10 mM nitrate + 5 mM isoniazid (right).

Strains in 3 replicates top to bottom:

cnxB11

punA11

spsA1

biA1

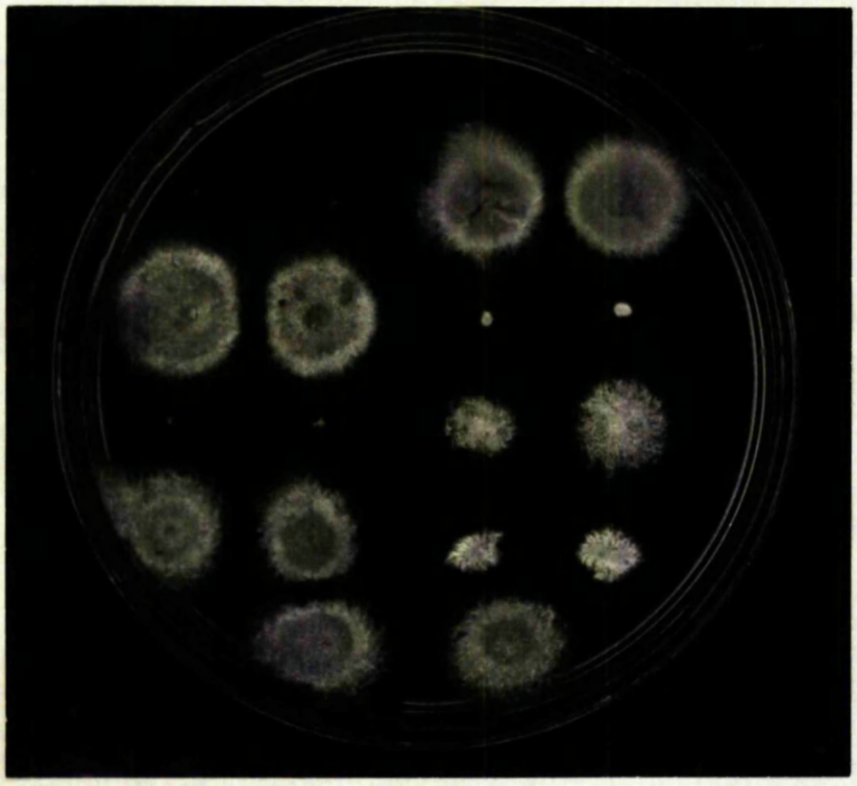


Plate 16 : Growth of pun⁻ strains on 10 mM nitrate + 5 mM isoniazid . Strains in two replicates , left to right , top to bottom :

punA11	pun-7
pun-22	pun-8
punA11	pun-9
biA1	pun-10

biA1

Strains	NO_3^-	NH_4^+	putrescine	γ-ABA	α-ABA	α-A-i-BA	L-alanine	L-leucine	L-isoleucine	glycine	L-serine	L-threonine	L-lysine	L-methionine	L-glutamate
biA1	6	6	5	6	5	4	4	4	4	5	5	4	3	3	5
spsA1	6	6	5	6	5	5	4	5	5	5	5	5	3	3	5
rev-2	5	6	2.5	6	5	3	4	3	3	4	4	3	3	2.5	3
punA11	6	6	0	6	1	2	2	2	2	5	4	2	0	2	3
$tamA^r50$	3	6	2	3	1	1	1	1	1	1	1	1	1	1	1
$tamA^d1$	6	6	5	6	5	5	5	5	5	5	5	5	2	3	5
cnxB11	0	6	4	6	3	2	3	2.5	3	4	3	4	2	1.5	4
niaD17	0	6	6	6	3	2	3	2.5	3	5	3	3	2	1.5	4
nirA4	0	6	6	6	6	5	5	5	5	5	5	5	3	4	5
niiA4	0	6	6	6	6	6	6	6	6	6	6	6	3.5	5	5
$nirA^c1$	5	6	1	6	1	0	0	0	1	2	1.5	1.5	1	1	1
$nirA^c1/+$	5	6	2	6	3	1	1	1	1	3	1.5	1.5	1.5	1.5	2

Table 16: Growth of <u>$nirA^c1$</u> and other strains on various nitrogen sources

The phosphate but not the chloride salt brings the pH of the medium up to 8-9. This effect may be due to some putrescine degrading enzymes which operate at high pH but not at relatively low pH (6-7). The situation might be similar (if not connected) to that of the galactose utilization (Roberts, 1970) where all of the galactose non-utilizing mutants can grow at high pH. Alternatively alterations in amine metabolism may upset the intracellular pH (section 1) of some strains with a detectable effect only when these substances are supplied as nutrients in high quantities. It has been mentioned in section 4 that isoniazid, an amine oxidase inhibitor, almost completely inhibited the utilization of putrescine as nitrogen source (Plate 6). This could be explained either by attributing a major role for amine oxidases in the utilization of putrescine as nitrogen source, or by assuming that isoniazid affects the utilization of putrescine by competing with it as the substrate for other degrading enzymes. The second hypothesis is more likely because enzyme assays (section 10) showed that strains which do not grow on putrescine still possess oxidase activity (punA11 for example). In further support for the second alternative it was found that punA11 unlike the wild type is sensitive to isoniazid at concentrations of 1-5 mM (Plates 6 and 15). It may be that the enzyme which breaks down putrescine in the wild type, can also break down the isoniazid which otherwise would be toxic. punA11 lacking this enzyme does not grow on putrescine and it is sensitive to isoniazid with any nitrogen source tested. These two properties segregate together in crosses and the latter now provides a clearcut test to distinguish the mutant.

nirA^{c}1 is not sensitive to isoniazid (Plate 6) and therefore

its inability to grow on putrescine may have a different basis. Also pun-7, pun-9 and pun-22 are insensitive (Plate 16). pun-8 and punA11 are completely inhibited while pun-10 is of intermediate phenotype. rev-2 (Plate 6) and also $tamA^{r}50$ and $areA^{r}550$ are not sensitive. Iproniazid (another amine oxidase inhibitor - see section 4) is not toxic for the wild type or any of the above mentioned mutants. At high concentrations of isoniazid (10 mM) the wild type is also very impaired but still distinguishable from the sensitive mutants. Its poor growth is possibly due to blockage of the pathways which will convert putrescine to substances indispensable for growth.

Isoniazid and the related compounds, isonicotinic acid and nicotinic acid cannot be utilized as nitrogen sources by the wild type. Further tests explored growth of $nirA^{c}1$ on various nitrogen sources (Table 16). punA11 and other strains were also tested, and their growth compared to the relative growth of the wild type (6 points for best growth). All nitrogen sources were added at the 10 mM level in nitrogen-less minimal medium. Rather unexpectedly it was found that $nirA^{c}1$ has subnormal growth on a number of nitrogen sources; this contrasts with observations by Cove (1970, 1976) implying that $nirA^{c}1$ grows similarly to wild-type strains on most nitrogen sources tested. The disagreement may be due either to the use of more refined agar in the present work or to acquisition of unknown modifiers by $nirA^{c}1$, through crosses or subculturing.

punA11 is also poor on some of these nitrogen sources. It therefore appears that both mutants have wide-ranging effects on nitrogen source utilization.

All strains defective on putrescine, including rev-2, grow well on γ-aminobutyrate which results from the oxidation of putrescine (Fig 2).

A last observation concerning the utilization of putrescine as nitrogen source is that chlorate, an analogue of nitrate which is known to inhibit utilization of amino acids as nitrogen source (Cove, 1976), also prevents putrescine utilization.

It was hoped that enzyme and uptake assays could possibly clarify the metabolic defects of the mutants and in general the various aspects of putrescine metabolism.

8.6. Discussion

A number of mutants altered in the metabolism of polyamines were found and their properties were studied. The most interesting of them include (a) a spermidine and spermine sensitive strain (spsA1) which also in combination with the putrescine auxotroph allows the latter to grow equally well at low concentrations of spermidine or putrescine. The general features of the mutant fit in with an altered uptake hypothesis, and this aspect will be discussed later on (section 10), (b) mutants unable to utilize putrescine effectively as nitrogen source. These were either directly isolated as such (pun$^-$) or were among mutants in the stocks with pleiotropic defects in nitrogen utilization (tamA^{r}50, areA^{r}550, nirA^{c}1). rev-2 is also defective on putrescine. One of the pun$^-$ mutants (punA11) maps on the second chromosome, but probably more loci are involved in other mutants. This is indicated by preliminary heterokaryotic complementation tests and possibly by the sensitivity of some but not all of

these mutants, to concentrations of isoniazid where the wild type is resistant. nirA^{c}1, rev-2, tamA^{r}50, areA^{r}550 and nir^{c}A are not sensitive on isoniazid. Inability of the fully ammonium repressed alleles of the tamA and areA genes to grow on putrescine indicates that possibly the enzymes necessary for its utilization as nitrogen source are under ammonium control.

The connection of the nitrate assimilation mutants with the putrescine utilization is not very clear; cnxB11, niaD17, niiA4, nirA4, which do not grow either on nitrate or nitrite or both, grow better than the wild type on putrescine. nirA^{c}1, the constitutive mutant for nitrate and nitrite reductases, which maps at the regulatory locus of these two enzymes, does not grow on putrescine and a number of amino acids tested. A simple hypothesis would be that the nirA product, an inducer necessary for the expression of the nitrate and nitrite reductases structural genes, may at the same time act as a repressor of putrescine and amino acid catabolism. Such a mechanism would prevent catabolism of amino acids if nitrate is available. This would be at least partially in agreement with Cove's model (Cove, 1976) where both the nirA product and the nitrate reductase are implicated in mediating a shut-down of amino acid catabolism, in presence of nitrate or its toxic analogue, chlorate. In support of the above model, potassium chlorate (100 mM) was also found capable of inhibiting utilization of putrescine as nitrogen source.

Some biochemical aspects of polyamine utilization by the wild type and the mutants now available will be considered in the following sections.

SECTION 9

INTERNAL POLYAMINE POOLS

9.1. Introduction

One possible explanation for the anomalies observed in certain mutants like brlA12, rev-2, spsA1, in relation to amine metabolism would be altered internal polyamine concentrations as a result of defects in biosynthetic or catabolic pathways. It has been suggested (see section 1) that in view of the participation of polyamines in a wide spectrum of activities inside the cells, their concentration may be of critical importance.

Estimations of the size of the pools and of their response to external factors could possibly provide clues about biochemical processes and defects which would be difficult to detect otherwise.

The size of each particular pool should also be informative about the relative importance of each polyamine in the physiology of the wild type. Studies by Stevens et al. (1977) and Bushell and Bull (1974) indicate that spermidine is the predominant polyamine in Aspergillus nidulans. A last aspect would include evidence about the presence of spermine in Aspergillus nidulans, a feature of filamentous fungi which has recently been questioned (Nickerson et al., 1977).

9.2. Pool estimations

Commercial samples of putrescine, spermidine and spermine at various concentrations were dansylated, extracted with benzene and run on T.L.C. plates (see section 2 for details). The fluorescent spots on the gel were extracted and assayed to obtain a standard

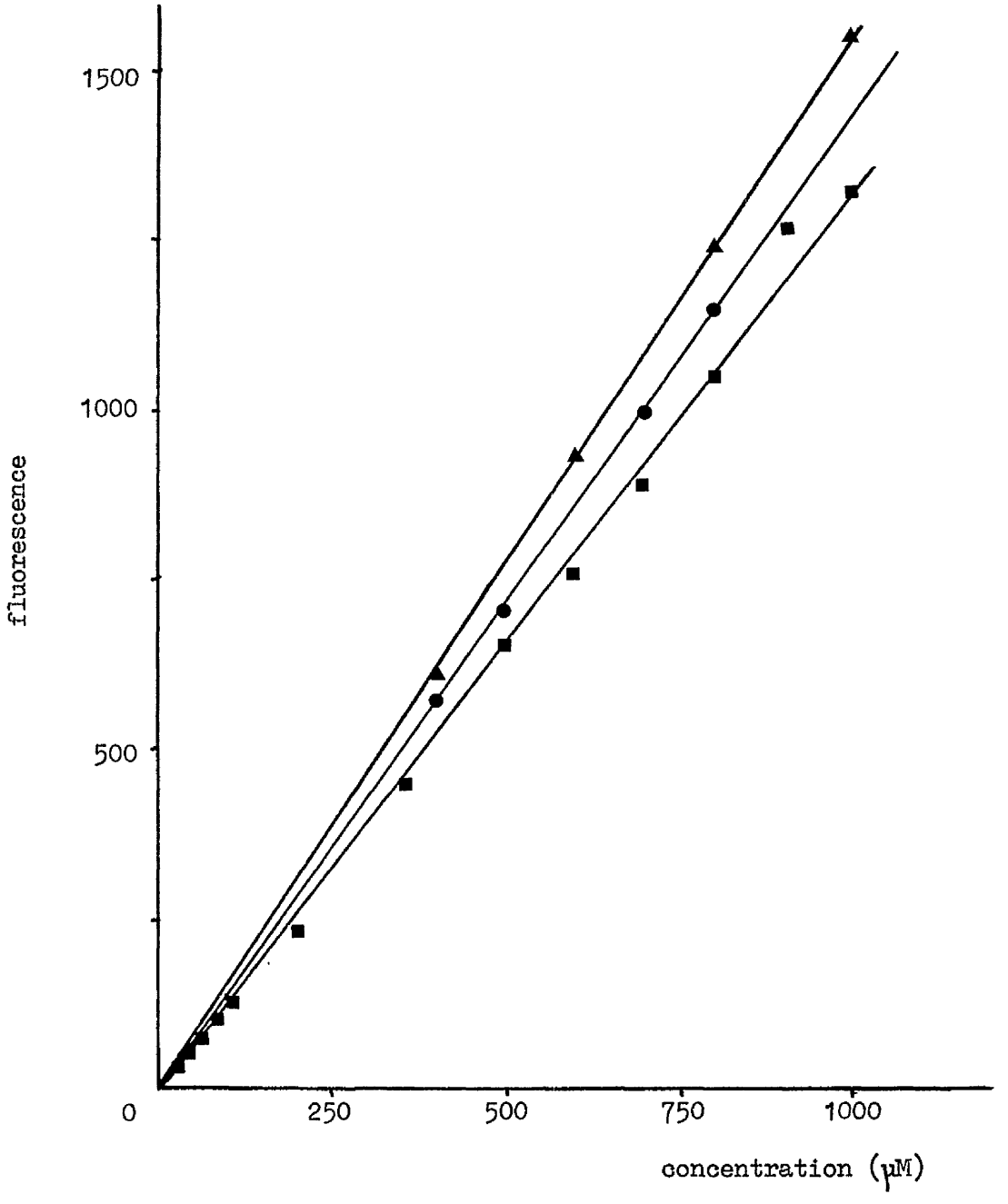


Figure 7 . Standard curve of fluorescence intensities and concentration of dansyl derivatives of putrescine (■) , spermidine (▲) and spermine (●) .

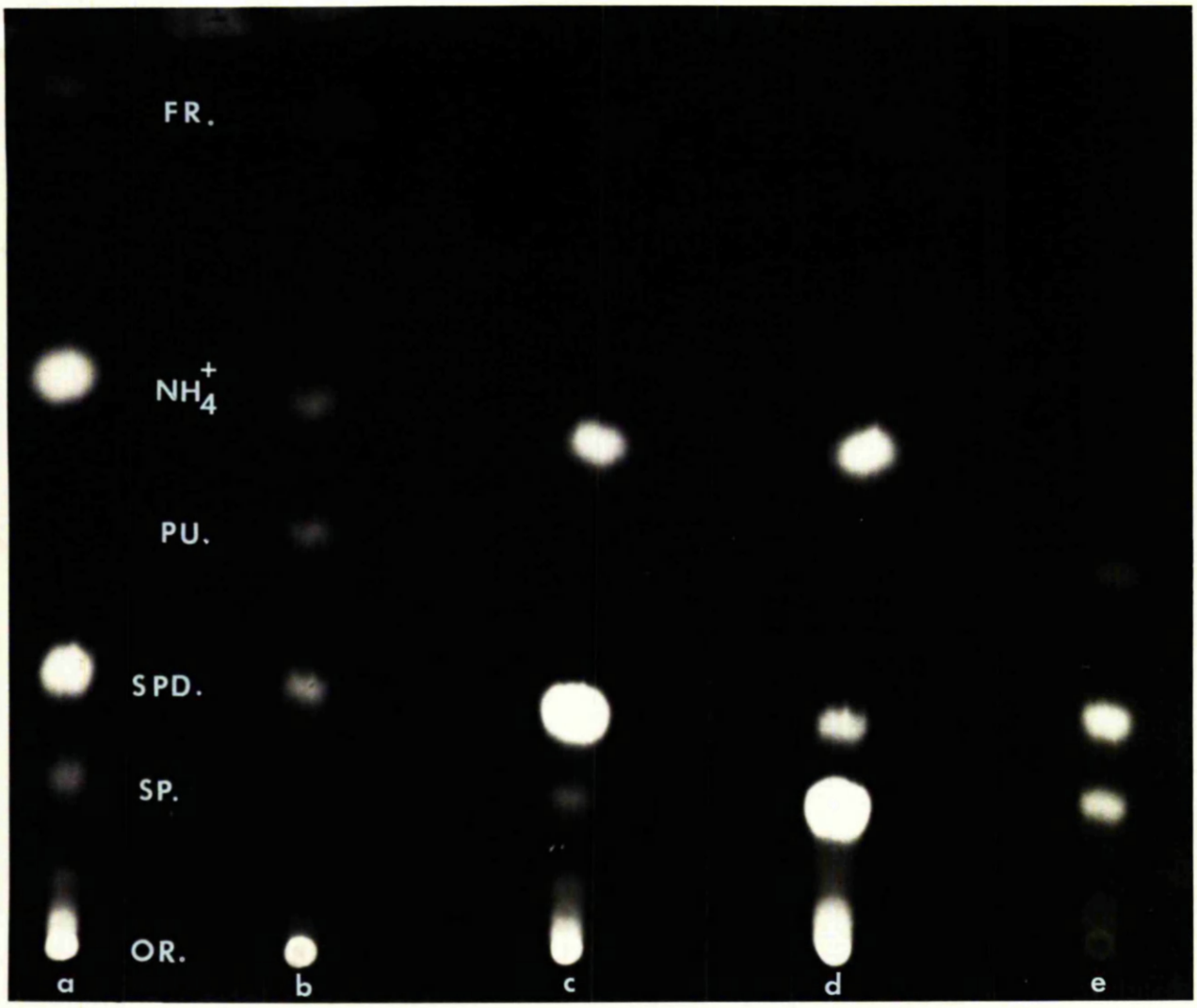


Plate 17 : Typical separation of dansylated polyamines on TLC plates (solvent, ethylacetate-cyclohexane 2:3, v/v).

a : cell free extract pretreated : 3 hrs nitrogen free growth

b : -"- -"- -"- -"- : 3 hrs 5 mM NO_3^- + 5 mM PU

c : -"- -"- -"- -"- : 3 hrs 5 mM NO_3^- + 5 mM SPD

d : -"- -"- -"- -"- : 3 hrs 5 mM NO_3^- + 5 mM SP

e : commercial samples of putrescine (PU), spermidine (SPD), and spermine (SP).

OR. = origin, FR. = front.

	treatment (3 hrs)	acid washed cells (0.05 N HCl)	mutant	Internal Polyamine Pools (µmoles/g dry weight) putrescine	spermidine	spermine
(1)	nitrogen free	NO	biA1	0.70	7.00	2.00
			brlA12	1.00	8.00	1.80
			rev-2	0.70	5.00	1.90
			spsA1	0.60	4.50	1.20
(2)	5 mM nitrate	NO	biA1	0.70,0.57,0.62	8.50,6.50,8.50	2.50,3.50,2.40
			brlA12	-	-	-
			rev-2	0.77	7.20	2.00
			spsA1	0.57,0.55	7.00,7.50	1.60,2.00
(3)	5 mM nitrate + 5 mM putrescine	NO	biA1	40.00	14.00,14.00	2.70,2.50
			brlA12	40.00	14.00	2.50
			rev-2	110.00	15.00	2.50
			spsA1	50.00	13.00	2.50
(4)	5 mM nitrate + 5 mM spermidine	NO	biA1	2.00,0.90	52.50	4.30,4.00
			spsA1	2.10,1.00	80.00	4.50,4.00
(5)	5 mM nitrate + 5 mM spermine	NO	biA1	5.50,0.80	9.00,8.00	72.50,90.00
			spsA1	3.00,1.30	10.00,9.00	150.00,180.00
(6)	5 mM nitrate + 5 mM putrescine	YES	biA1	6.60,6.90,9.80,7.70	9.60,9.50,10.50,7.90	1.04,1.08,1.40,1.00
			rev-2	27.60,7.50,10.20,60.00,9.00	9.40,7.90,12.80,10.80	1.06,1.08,0.86,0.93
(7)	5 mM nitrate + 5 mM spermidine	YES	biA1	0.40,0.68,0.96,0.76	24.40,31.40,35.88,30.18	2.20,2.24,2.60,2.28
			spsA1	1.40,1.20,0.86,0.96,0.57	56,00,58.80,88.40,53.20,55.00	2.72,2.80,3.00,2.90,2.77
(8)	5 mM nitrate + 0.06 mM spermidine	YES	biA1	0.88,1.00	12.80,13.60	2.24,2.08
			spsA1	0.88,0.76	16.00,11.20	1.84,1.84

Table 17 : Internal Polyamine Pools in wild type and mutants after various growth conditions.

samples of mycelium	pressed weight (mg) A	dry weight (mg) B	ratio A/B	mean ratio
1	253	68	3.720	
2	248	75	3.306	
3	215	58	3.706	3.55
4	225	62	3.629	
5	221	61	3.622	
6	195	58	3.362	

Table 18 : Weights of hard pressed (A) and dried (B) mycelium after 20 hrs of growth in liquid cultures.

curve relating polyamine concentrations and fluorescent intensities. The linear relationship shown in Fig 7 was reproducible, however variations in fluorescence intensity from plate to plate necessitate the presence of dansyl polyamine standards on each plate. The spermine and spermidine curves were very similar to that obtained for putrescine (Fig 7). Cells of wild type and mutants were grown in liquid cultures with minimal medium supplemented with 10 mM nitrate as the nitrogen source. After the initial growth of 17 hrs, cells were harvested and transferred to treatment medium, as specified, for another 3 hrs. After extraction, dansylation and thin layer chromatography, the bands corresponding to the main polyamines (Plate 17) were assayed for fluorescence intensity which was subsequently converted to μmoles of polyamines present per gram dry weight (Table 17). Each figure represents a single independent experiment. For the last three treatments, cells have been washed with 0.05 N HCl after harvesting which at this concentration will wash out the adhered polyamines without damaging the cells (see section 10, uptake experiments).

In order to estimate the molar concentration of polyamines in the cells it was necessary to estimate the volume of internal water. Cells, after 20 hours of growth in liquid cultures, were pressed dry until no trace of water was left on the paper towels. The weights were taken and the cells were subsequently freeze-dried to obtain the dry weight (Table 18). It appears that 3.55 g pressed weight correspond to approximately 1 g dry weight; assuming that the pressed mycelium is free from external water then the internal water

for every g of dry weight (3.55 g pressed weight) is approximately 2.55 g. This factor was used for the conversion of μmoles/g dry weight to mM of internal polyamines.

The main conclusions which can be drawn from Table 17 will be summarised here for convenience:

(1) In wild type cells, under normal growth conditions (i.e. low or no external polyamines added, Table 1, treatments 1,2,8), spermidine concentrations (7.8 μmoles/g dry weight = 3 mM) are approximately 3 times higher than those of spermine (2.8 μmoles/g dry weight = 1 mM) and about 10 times higher than those of putrescine (0.63 μmoles/g dry weight = 0.24 mM).

(2) Cells grown at high concentrations of polyamines, when washed with 0.05 N HCl give lower pools than those given by cells washed with distilled water (compare treatments 3-4 to 6-7). The values obtained by acid washed cells are considered to represent the actual pool size since evidence in section 10 (uptake experiments), suggests that polyamines adhering to the cell walls are not washed out with distilled water but are removed by 0.05 N HCl.

(3) High concentrations of polyamines in the medium caused increased internal pools for the particular polyamine added. In wild type cells putrescine has increased more than tenfold (3 mM as opposed to 0.24 mM normally), while spermidine increased fourfold (12.6 mM as opposed to 3 mM normally). Spermine concentrations (treatment 5) also rise to high levels, possibly even higher than those of spermidine. It appears therefore that cells may concentrate at least spermidine and spermine at concentrations much higher than those originally supplied in the medium.

(4) Only the internal pool of the particular polyamine added in the medium changes significantly; the other polyamines retain their standard concentrations.

(5) For rev-2, after growth on high nitrate and putrescine (i.e. conditions which will result in defective growth on plate tests - section 5), the internal putrescine concentrations have been found either normal (3 out of 6 independent experiments) or approximately 4-8 times higher than those of the wild type, under the same growth conditions. The cause of this inconsistency is not known at the moment and further estimations are required to determine whether a significant difference exists in this particular case (see also Discussion).

(6) spsA1, after growth on high spermidine or spermine consistently gives an almost twofold increase in the internal concentrations of these polyamines compared to the wild type. This difference may account for spsA1's sensitivity to high spermidine or spermine in solid media since at least for the case of spermidine, the internal concentration of this polyamine in spsA1 rises to 22 mM as opposed to 12 mM for the wild type under the same conditions. Spermine concentrations may be even higher than those for spermidine as judged by treatment 5 (Table 17).

9.3. Presence of spermine in Aspergillus nidulans

Conflicting reports exist about the presence of spermine in fungi. Bushell and Bull (1974) and Stevens et al. (1977) do not question its presence in Aspergillus nidulans. On the other hand

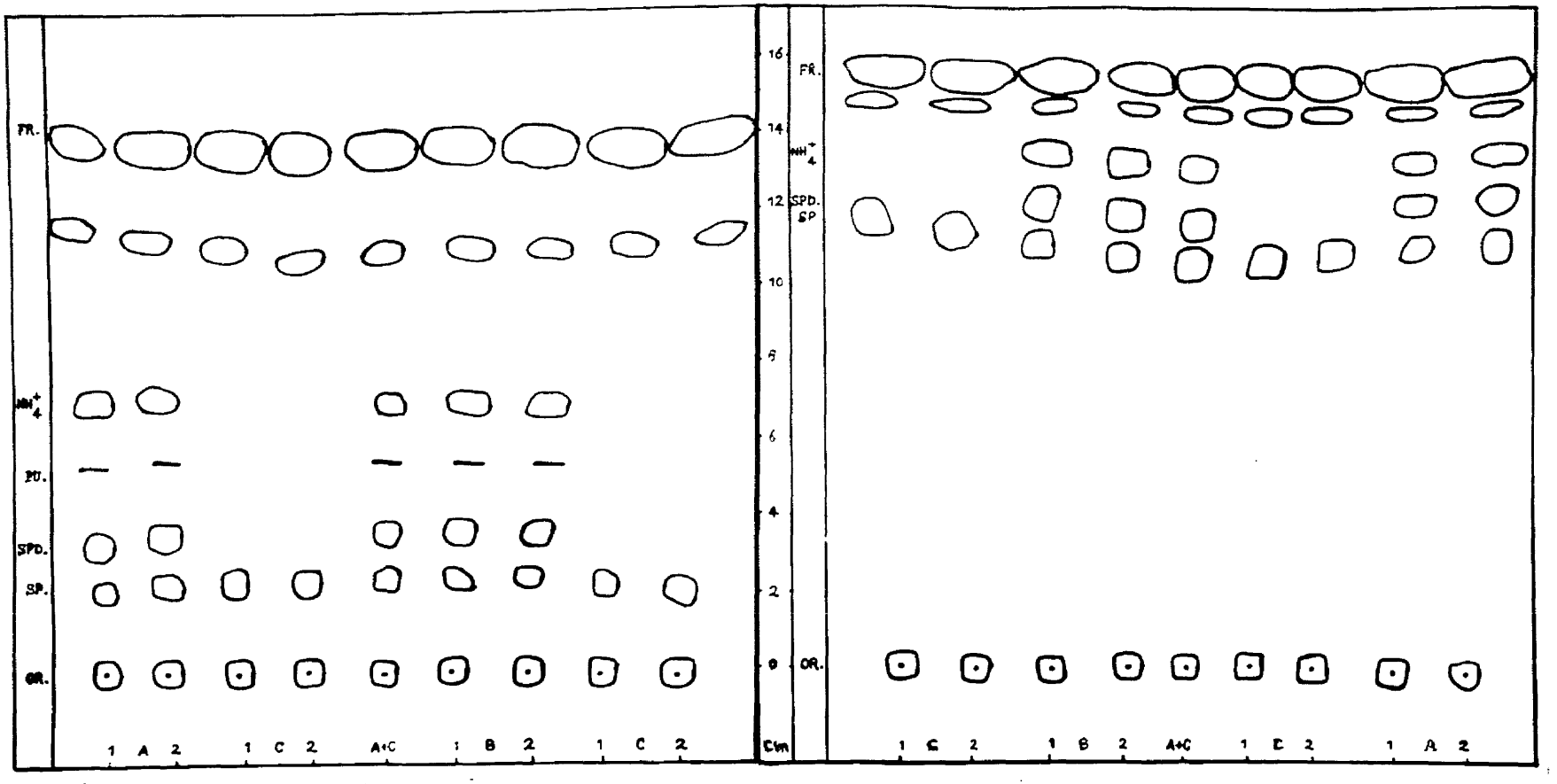


Plate 18 : Separation of Aspergillus nidulans polyamines on TLC plates.

18a: solvent, ethylacetate/cyclohexane (2/3 v:v). 18b: solvent, ethylacetate/cyclohexane (3/2) v:v)

samples: A (1,2), cell free extract No. 1

B (1,2), cell free extract No. 2

C (1,2), commercial spermine

A+C, mixture of A and C.

Nickerson et al. (1977) suggested recently that in Neurospora crassa and other fungi an unknown compound with an Rf value very close to that of spermine could be easily mistaken for it under the standard procedures. Development of T.L.C. plates with extracts in a mixture of ethylacetate-cyclohexane (2:3 v/v) causes the unknown compound to migrate similarly to authentic spermine; however if the ethylacetate-cyclohexane ratio is altered (3:2 v/v) a distinct separation of spermine and the unknown compound is achieved. To see whether this is the case with Aspergillus nidulans as well, in view of its similarities with Neurospora crassa, the same procedures were applied. The same extracts after nitrogen free treatment (two independent extractions) were applied in two replicas on T.L.C. plates, with controls and developed in ethylacetate-cyclohexane (2:3 v/v and also 3:2 v/v). All dansylated spots were marked after development and they are presented in Plate 18. Although the Rf values are quite different from the one solvent to the other no extra spot appeared in the extract samples and the correspondence of commercial spermine to that of Aspergillus was good in both cases.

Clearly this test cannot provide conclusive evidence about the existence of spermine. It however favours such a hypothesis.

9.4. Discussion

The purpose of these investigations was mainly to compare quantitatively the internal polyamine pools of various mutants to those of the wild type.

In particular, rev-2 and spsA1 are the mutants for which

alterations in the size of the pools under the appropriate growth conditions could possibly explain the behaviour of these strains on the relevant plate tests.

spsA1 is sensitive at concentrations of spermidine or spermine above 5 mM and the internal pools indicate that under these conditions spsA1 accumulates almost twice as much spermidine or spermine as the wild type. This difference which will bring the internal concentration of spermidine up to approximately 22 mM in the spsA1 as opposed to approximately 12 mM in the wild type, may therefore be responsible for the sensitivity of spsA1 to high spermidine. The situation with spermine appears to be similar if not more extreme.

Whether rev-2 is different in putrescine pools from the wild type (after growth on putrescine) is not yet clear. A difference if existed, could possibly explain the defective growth of this strain on solid media supplemented with putrescine and it could also strengthen the evidence relating the brlA12 variegation to amine metabolism. At the moment the inconsistencies detected in the estimations of the pools are rather difficult to explain; they have been observed only in this particular case while all other estimations gave consistently reproducible results. It could be that the concentration of putrescine used (or something produced from putrescine) represents a critical value, responsible for a threshold effect in which a drastic shift of metabolism may occur, leading to greatly increased putrescine accumulation. This may correlate with the apparent toxicity of putrescine to rev-2 in plate tests.

A second possibility could be that cultures of rev-2 give rise to heterogeneous mycelium as a result of the high mutation frequency of this mutant; it seems unlikely however that in this case such extreme variations would be seen.

Further estimations at various concentrations of putrescine are required to explore these possibilities.

SECTION 10

TRANSPORT OF POLYAMINES

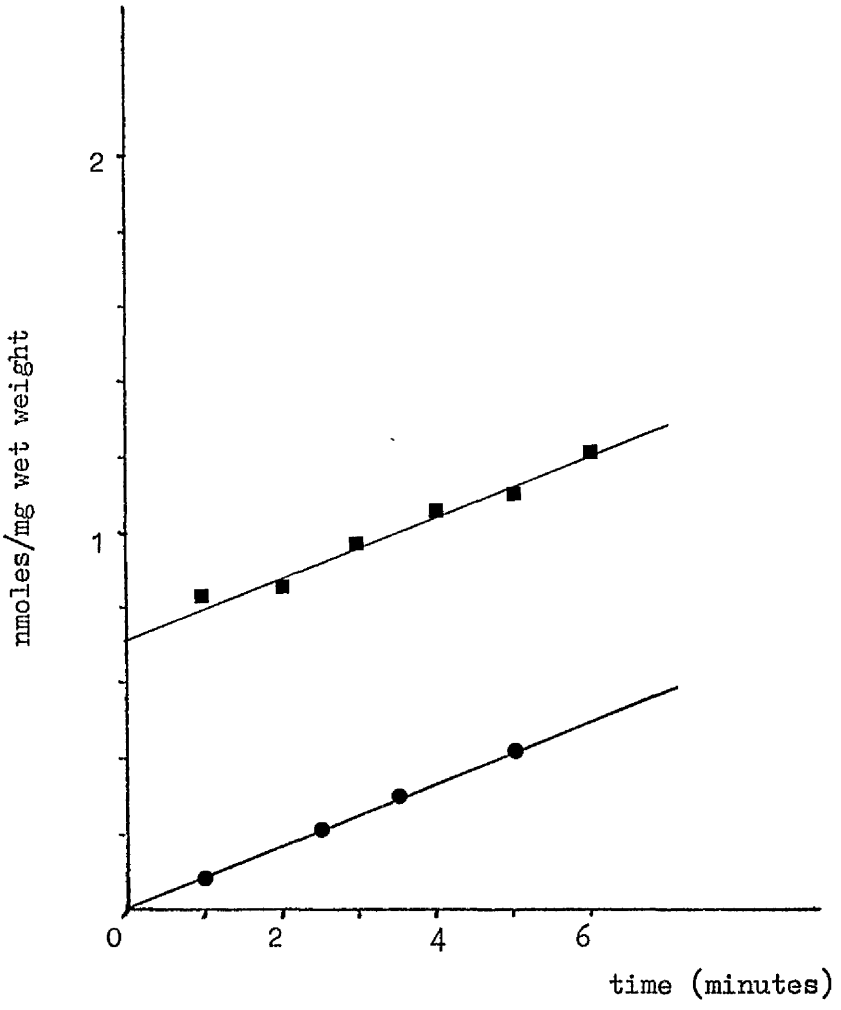


Figure 8 . Uptake of ^{14}C-putrescine(0.2 mM cold putrescine present) by wild type cells (biA1) .

■:Cells washed with distilled water

●:Same cells washed with 0.05 N HCl

Treatment : Nitrogen-free, 1% Glucose

10.1. Introduction

The uptakes of putrescine, spermidine and spermine were estimated in Aspergillus nidulans using ^{14}C labelled polyamines (see section 2 for details of method). Nitrogen-starved cells were normally used for this purpose, since such cells were found to transport all three polyamines most efficiently. Cells were initially grown in minimal medium, with nitrate as nitrogen source, or ammonium for those mutants unable to utilize nitrate. Wild type controls, grown on nitrate or ammonium, did not differ in their uptake activities. The characteristics of the uptakes and their regulation were studied in the wild type and certain other strains. Complications in the experimental procedure were similar to those found by Tabor and Tabor (1966) in Escherichia coli. These amines and particularly spermine, have the tendency to adsorb to cell surfaces, millipore filters and glassware and suitable washing procedures had to be devised to permit reliable measurement of the active uptake. A concentration of 0.05N HCl was found to be strong enough to clean up the cells from adhered polyamines without affecting the transported material. This is evident from figure 8, by comparisons between water and acid washed cells; it can be seen that a high level of "uptake" is found in water-washed samples even at the shortest incubation time; however this instantaneously absorbed material can be removed with acid-washing without damaging the active time-dependent uptake.

All polyamines are presented for uptake at the 3 mM level unless otherwise specified, and the results are expressed as nmoles of polyamine taken up per mg of wet weight in relation to time. Since

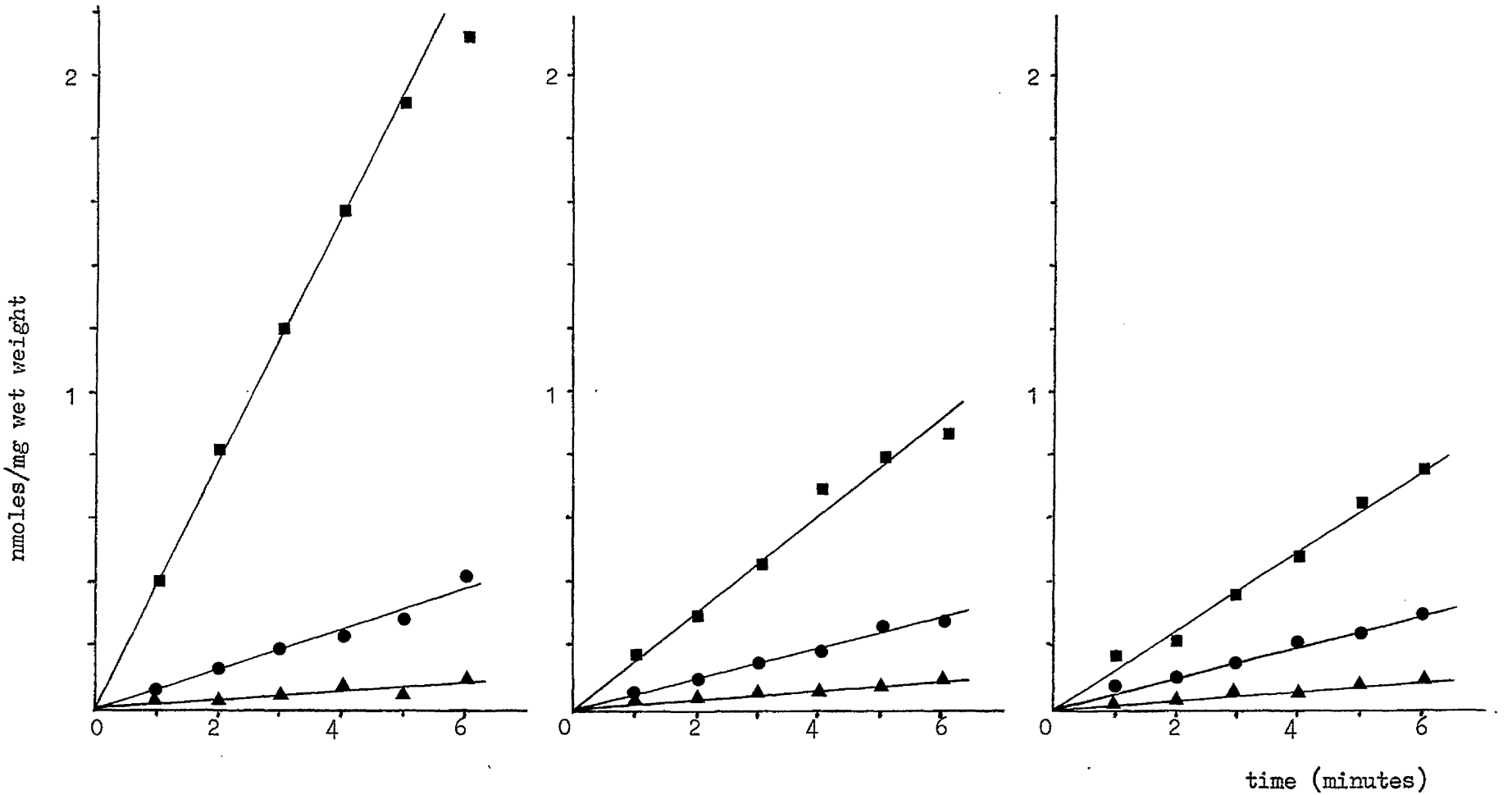


Figure 9. Uptakes of (left to right) , putrescine , spermidine and spermine in wild type cells (biA1).

■:Nitrogen free treatment , ●:Nitrogen free treatment with cycloheximide (10 mg/ml)

▲:Nitrogen free treatment with 1 mM azide in uptake flasks .

	Uptake (nmoles/min/mg wet weight)		
mutant	putrescine	spermidine	spermine
biA1	0.388 ± 0.010	0.161 ± 0.007	0.118 ± 0.006
spsA1	0.525 ± 0.050	0.196 ± 0.011	0.152 ± 0.020

Table 19 . Polyamine uptakes by biA1 and spsA1 from 3 mM solutions.

this mycelium is not blotted, unlike that used in polyamine pools estimations (see section 9), a factor of approximately 8 is required to convert this wet weight to oven dried weight (see also section 2).

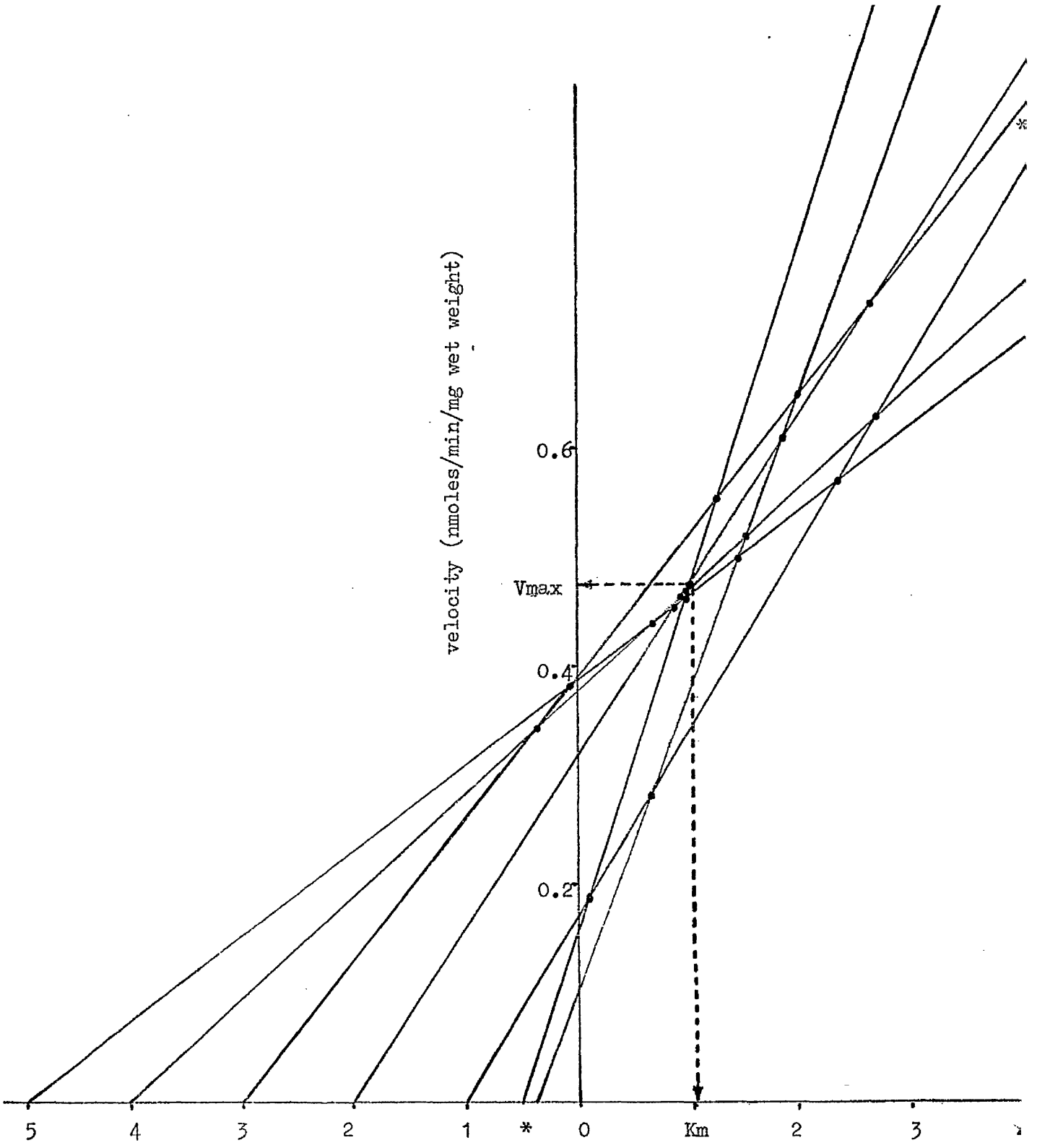
10.2. Characteristics of the active uptake

(a) Polyamine uptake by wild type and spsA1

The uptakes into wild type cells were studied using cultures grown for 17 hrs and then given a 3 hrs nitrogen free treatment. The kinetics of the transport systems are linear for the first five minutes after the addition of cells to the uptake medium (Fig 9) and this time was adhered to throughout the uptake experiments. The uptake decreased after the first five minutes, under the particular experimental conditions used, but this effect was not investigated.

Table 19 presents the uptakes for biA1 and spsA1 at the 3 mM level. The standard errors have been calculated and a "t" test was done to see whether the two strains are statistically different in their uptakes. It was found that they differ significantly for putrescine uptake ($t_9 = 2.85$, $P < 0.02$) and spermidine uptake ($t_9 = 2.79$, $P < 0.05$), but not for spermine uptake ($t_9 = 2.01$, $P > 0.05$). This result was further investigated by considering the effect of polyamine concentration on their transport, over the range of 0.35 - 5 mM.

The Michaelis constants (Km) were determined by the direct linear plot (Eisenthal and Cornish-Bowden, 1974) which is more advantageous than the Lineweaver-Burk reciprocal plot in cases of



Substrate concentration (10^{-3}M putrescine)

Figure 10 . Determination of a putrescine uptake Km by the direct linear plot; the median of the points which intersect the lines drawn for each particular s and v represents the Km and Vmax values;the asterisks indicate off-scale intersections (see also text).

Strain	Experiment No.	Uptake kinetics putrescine Km	putrescine Vmax	spermidine Km	spermidine Vmax	spermine Km	spermine Vmax
biA1	1	1.00	0.470	4.00	0.370	1.30	0.148
	2	1.25	0.470	4.00	0.376	0.70	0.098
	3	1.40	0.525	1.70	0.173	1.10	0.154
spsA1	1	1.25	0.515	2.50	0.392	1.00	0.132
	2	1.35	0.725	2.25	0.360	1.30	0.290
	3	-	-	2.40	0.340	2.30	0.300

Table 20. Putrescine, spermidine and spermine kinetics for biA1 and spsA1. (Km value in mM, Vmax values in nmoles/min/mg wet weight.)

uncertainty about the main kinetic parameters (Atkins and Nimmo, 1975). An example of estimating the Km by this method is presented in figure 10; for each observation (s, v) the points - s and v have been marked on the relevant axes and the line through these two points was drawn and extended to the right side of the V axis. This is done for each observation, and the intersections of these lines then provide estimations of Km and Vmax. The coordinates of the median of these points is accepted as the best estimate of Km and Vmax that satisfy the Michaelis-Menten equation. The total number of intersections is $\frac{1}{2}n(n-1)$ with n being the number of lines (= observations). Some off-scale intersections will occur in cases of poor experimental precision.

Three independent experiments were performed for the estimation of each Km, which revealed rather considerable variation among them, most probably due to the fact that more than one culture had to be used for the same experiment. The values obtained are represented in Table 20.

A possibly real difference may exist between wild type and spsA1 for the spermidine Km which, if true, may mean higher affinity of spsA1 for spermidine than that of the wild type. However the reliability of this conclusion is put into doubt by one inconsistently low Km value for the wild type.

Km values for putrescine and spermine do not appear to show any differences between the two strains but it is just possible that there is a higher Vmax for spermine uptake by spsA1.

^{14}C-putrescine Uptake

polyamine present	activity (nmoles/min/mg wet weight)
3 mM putrescine	0.394
3 mM putrescine 3 mM spermidine	0.330
3 mM putrescine 3 mM spermine	0.440

^{14}C-spermidine Uptake

polyamine present	activity (nmoles/min/mg wet weight)
3 mM spermidine	0.164
3 mM spermidine 3 mM putrescine	0.122
3 mM spermidine 3 mM spermine	0.102

^{14}C-spermine Uptake

polyamine present	activity (nmoles/min/mg wet weight)
3 mM spermine	0.130
3 mM spermine 3 mM putrescine	0.124
3 mM spermine 3 mM spermidine	0.140

Table 21. Uptake competition experiment for putrescine, spermidine and spermine in wild type (biA1) cells. Nitrogen free conditions.

(b) Competition data

The specificity of the uptake system for each polyamine was investigated in relation to the other two (Table 21). No significant competition exists among putrescine, spermidine and spermine. This is in agreement with observations from auxanography tests (Plate 8).

(c) Energy requirement of uptakes

Sodium azide is a respiratory inhibitor and it prevents the uptake when added to the medium at the concentration of 1 mM (Fig 9). This suggests (but does not prove) that energy is in some way required in the transport process.

(d) Effect of cycloheximide on polyamine transport

Wild type cells after the initial 17 hrs growth period were transferred for 3 hrs to nitrogen free medium supplemented with cycloheximide (10 µg/ml). The activity was substantially less than that found after a nitrogen free treatment without cycloheximide (Fig 9). This suggests that protein synthesis is necessary for the development of a high polyamine transport capacity.

(e) Accumulation of ^{14}C polyamines in the cells

Comparison of counts taken up into the cells, and those left in the medium after 30 minutes of uptake gave a 480 fold concentration for the putrescine uptake, an 80 fold concentration for the spermidine uptake and a 1.7 fold concentration for the spermine uptake inside the cells. These figures refer to total counts transported into the cells. In order to see whether the polyamines

	Uptakes(nmoles/min/mg wet weight)		
treatment* (3 hrs)	putrescine	spermidine	spermine
nitrogen free	0.390	0.168	0.106
carbon and nitrogen free	0.133	0.055	0.038
5 mM alanine	0.216	0.071	0.076
2.5 mM urea	0.225	0.076	0.083
5 mM nitrate	0.100	0.060	0.045
5 mM ammonium	0.053	0.026	0.066
10 mM ammonium	0.040	0.023	0.050

* 1% glucose present unless otherwise indicated

Table 22 . Uptakes of putrescine , spermidine and spermine by wild type cells (biA1) after various treatment conditions. (Initial growth on nitrogen-less minimal medium supplemented with 10 mM nitrate).

taken up were further metabolised the cells were extracted and the radioactivity in the separated polyamine spots on T.L.C. plates was measured. For the putrescine uptake 27% of the recovered counts were in the putrescine spot while 34% were on the spot corresponding to spermidine; this clearly indicates the conversion of putrescine to spermidine. For the spermidine and spermine uptakes most of the counts recovered stayed at the chromatographic origin, probably indicating that these polyamines are bound to, or converted to low mobility compounds. In all three cases a fair proportion of the counts were not recovered as discrete spots on the chromatography plate.

10.3. Regulatory aspects of polyamine transport

10.3.1. Polyamine transport in wild type cells grown under various conditions

The transport of putrescine, spermidine and spermine was studied in wild type cells grown under various conditions for 3 hrs after the initial period of growth (17 hrs with 1% glucose and 10 mM nitrate), in an attempt to elucidate its regulatory patterns (Table 22). The highest activities were obtained after the nitrogen starvation treatment. Under these conditions the rate of uptake of putrescine is approximately $2\frac{1}{2}$ times faster than the rate of uptake of spermidine, which is $1\frac{1}{2}$ times faster than that of spermine. Alanine or urea or carbon and nitrogen free conditions result in reduced uptakes for all three polyamines. The most significant reduction occurs after treatment with nitrate or ammonium, which suggests that the system is ammonium repressible. The effect of nitrate may be either explained by its conversion to ammonium or by its direct action in shutting down nitrogen catabolism as proposed by Cove (1976).

mutants	treatment*	uptakes (nmoles/min/mg wet weight) putrescine	spermidine	spermine
biA1	N.F	0.388	0.161	0.118
	5 mM NH_4^+	0.053	0.026	0.066
brlA12	N.F	0.380	0.168	0.110
rev-2	N.F	0.400	0.175	0.115
nirA^{c}1	N.F	0.400	0.140	0.110
	5 mM NH_4^+	0.066	0.047	0.063
puA2	N.F	0.420	0.160	0.122
	5 mM NH_4^+	0.070	0.036	0.052
puA2;spsA1	N.F	0.550	0.204	0.138
puA2;spd-6	N.F	0.478	0.200	0.160
biA1;spsA1	N.F	0.525	0.196	0.152
pun-8	N.F	0.365	0.125	N.T
pun-9	N.F	0.380	0.140	N.T
pun-10	N.F	0.375	N.T	N.T
punA11	N.F	0.400	0.175	0.120
pun-22	N.F	0.400	0.140	N.T
tamA^{r}50	N.F	0.052	0.033	0.033
	5 mM NH_4^+	0.066	0.044	0.056
TAMPU	N.F	0.026	0.016	N.T
areA^{r}550	N.F	0.025	0.016	0.040
	5 mM NH_4^+	0.068	0.126	0.090
areA^{d}520	N.F	0.333	0.160	0.150
	5 mM NH_4^+	0.206	0.126	0.130
meaA8	N.F	0.380	0.173	N.T
	5 mM NH_4^+	0.375	0.176	N.T
gdhA1	N.F	0.375	0.150	N.T
	5 mM NH_4^+	0.120	0.072	N.T

*1% glucose as carbon source

Table 23. Polyamine uptakes by various mutants from 3 mM solutions. (N.F: Nitrogen free, N.T: not tested).

Genotype	% of derepression		
	putrescine	spermidine	spermine
biA1	13.4	15.3	55
$areA^{d}520$	61.8	78.7	86.6
meaA8	98.6	101	not tested
gdhA1	32	48	not tested

Table 24. Derepression of polyamine transport by certain ammonium derepressed mutants (growth on ammonium).

10.3.2. Nitrogen metabolism and polyamine transport

Table 23 presents the uptakes of polyamines by various mutants after nitrogen starvation and/or growth on ammonium. *punA11*, *pun-8*, *pun-9*, *pun-10*, *pun-22*, *rev-2* and *nirA*^{c}*1* which all have defective growth on putrescine (section 7), have wild type uptakes under nitrogen free conditions for all three polyamines. *brlA12* and *puA2* are also normal. The repressed alleles of the *tamA* and *areA* genes have very low uptakes as it should be expected if the transport is under ammonium control. Rather surprisingly TAMPU (selected from *tamA*^{r}*50* for growth on putrescine, see section 8), has very low uptake of putrescine and spermidine as well (spermine was not tested). This result, together with the observed delayed germination of this strain, suggest that putrescine is taken up passively and can be metabolised further by catabolic enzyme(s) which are probably not functioning in the original *tamA*^{r}*50*.

The ammonium derepressed mutants *areA*^{d}*520*, *gdhA1* and *meaA8* have normal uptakes after nitrogen starvation. After growth on ammonium, *meaA8* is fully derepressed for polyamine transport whereas *areA*^{d}*520* and *gdhA1* are partially derepressed.

The degree of ammonium derepression in the above strains for all three uptakes is expressed as a percentage in Table 24. The percent of derepression is calculated as the activity after ammonium treatment divided by the activity observed after nitrogen starvation for each particular strain, multiplied by 100.

10.4. Discussion

The results presented in this section suggest that polyamines are transported into wild type cells of *Aspergillus nidulans* by an active system which concentrates at least putrescine and spermidine against a concentration gradient. For quantitative estimations it is essential that the cells are first freed from polyamines adhering to the cell walls.

Evidence is presented that the polyamine transport systems are regulated by ammonium although it is not clear if this is due to inhibition of already synthesized systems or repression of the synthesis of such systems. As would be expected, the fully ammonium repressed mutants, *tamA*^{r}50 and *areA*^{r}550 have low uptake rates for all three polyamines. Nitrate also has a repressing effect on the uptakes acting either as such (Cove, 1976) or by its conversion to ammonium.

Best uptake activities for all three uptakes were detected after nitrogen starvation (3 hrs) with glucose as carbon source. Activities were also present after growth on urea or alanine which indicates that uptake will occur without induction by polyamines.

No mutants with altered uptakes were found among those with defective growth on putrescine; *rev-2* and *brlA12* were also normal.

spsA1, the spermidine-spermine sensitive mutant, appears to have higher uptake, at least for putrescine and spermidine, than the wild type. This might be the result of higher affinity for spermidine as indicated in kinetic experiments.

The uptakes however in the mutant, do not exceed those of the wild type by more than 30-50% and it is not clear at the moment whether this difference alone could explain the sensitivity of this strain on polyamines. As it has already been shown in section 9, spsA1 has increased levels of internal polyamine pools which could be the consequence of an increased uptake, or anomalies in the biosynthetic and/or catabolic steps for these polyamines.

Some indication of modification of polyamines after uptake by wild type is given by the experiment using ^{14}C labelled materials (section 10.2.(e)) in which much of the spermidine and spermine products remain at the chromatographic origin.

SECTION 11

PUTRESCINE OXIDASE ACTIVITY IN *Aspergillus nidulans*

11.1. Introduction

An obvious candidate for the degradation of putrescine in Aspergillus nidulans is putrescine oxidase which would convert it to the corresponding aldehyde (γ-aminobutyraldehyde) with release of ammonia and hydrogen peroxide at the same time:

$$H_2N(CH_4)NH_2 + O_2 + H_2O \longrightarrow H_2N(CH_4)_3CHO + NH_3 + H_2O_2$$

An amine oxidase activity has been detected (Yamada et al., 1965) in a number of fungi including Aspergillus species. This enzyme, according to the authors, can utilize a number of monoamines and diamines as substrates. On the other hand, Page (1971) was unable to detect any monoamine oxidase activity with diamines as substrates in Aspergillus nidulans.

In the present work a modification of the method by Holmstedt et al. (1959, 1961) was used to detect putrescine oxidase in Aspergillus nidulans. This assay method is based on trapping the oxidation product, Δ^1-pyrolline with o-aminobenzaldehyde to form quinazolinium, which can then be easily detected spectrophotometrically (see section 1 for details). Activities are expressed in nmoles of putrescine consumed per minute per mg of protein. Cells, for assaying, were normally grown for an initial period of 17 hrs in minimal medium (10 mM nitrate as nitrogen source) and then transferred to medium supplemented with 5 mM putrescine (sole nitrogen source) for another 3 hrs.

11.2. Characteristics of the enzyme

(a) pH and temperature

Phosphate and tris buffers adjusted with HCl or NaOH where

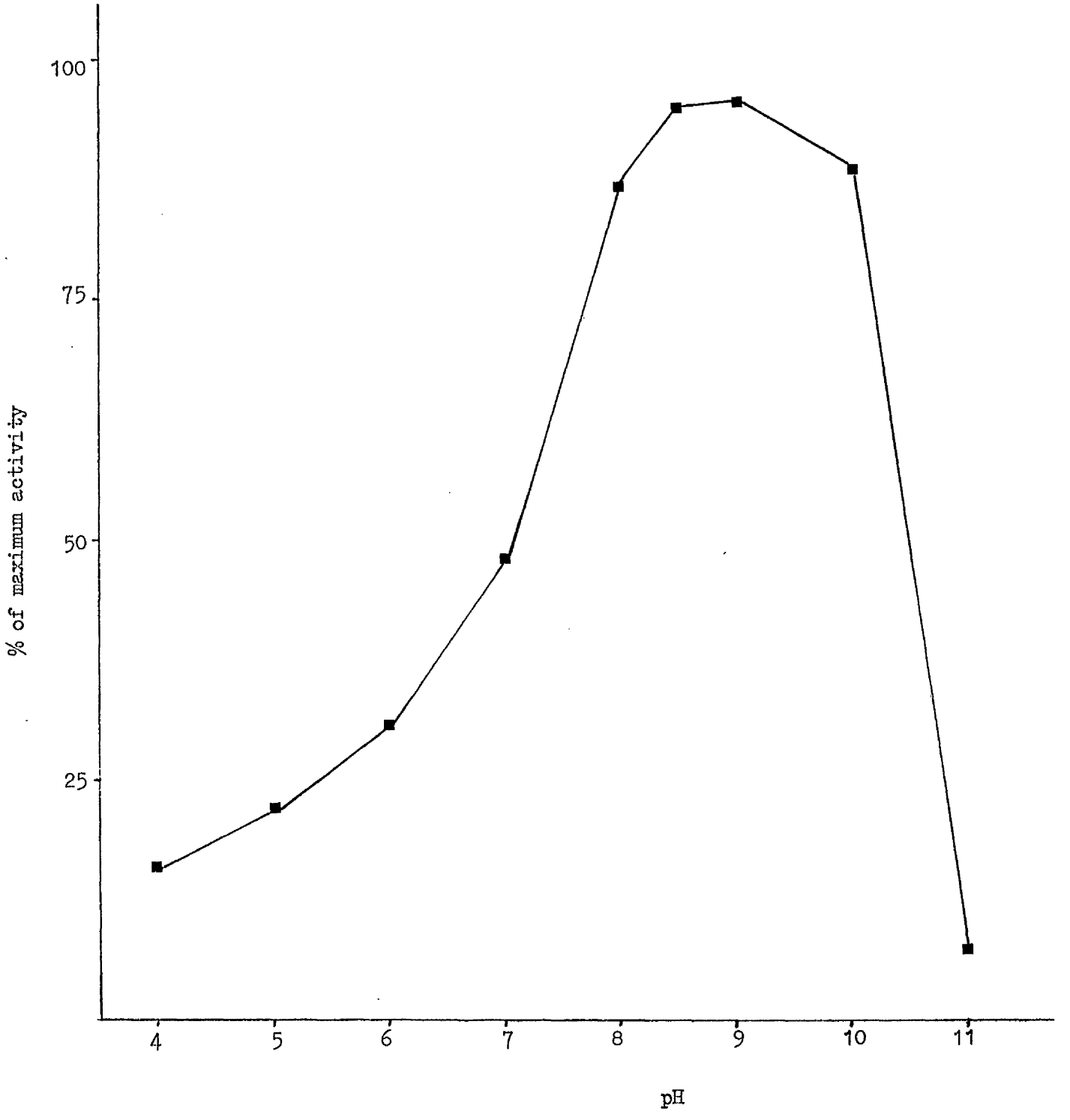


Figure 11 . pH dependence of putrescine oxidase activity .

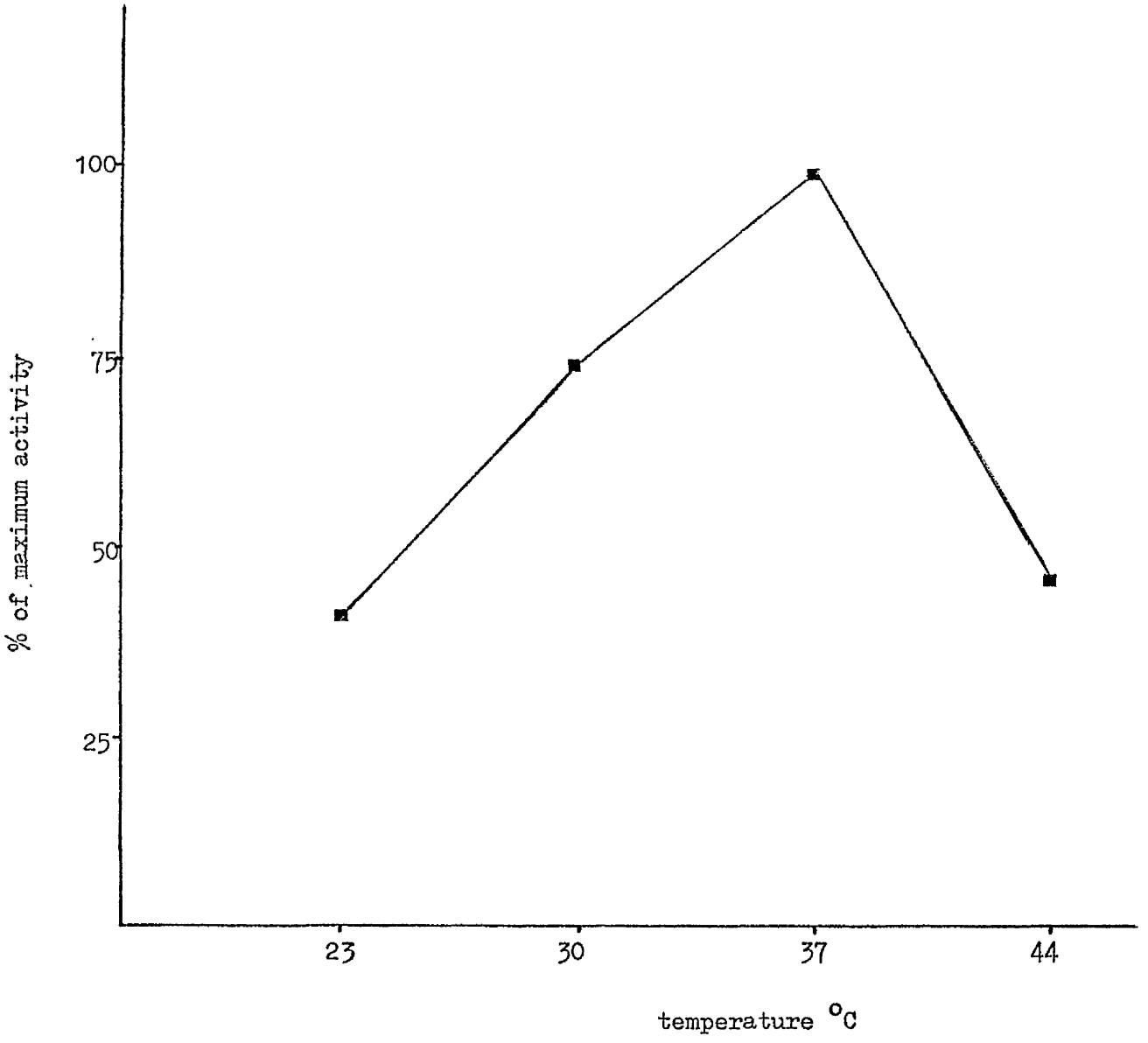


Figure 12 . Temrerature dependence of putrescine oxidase activity (activity measured over a 25 minutes period) .

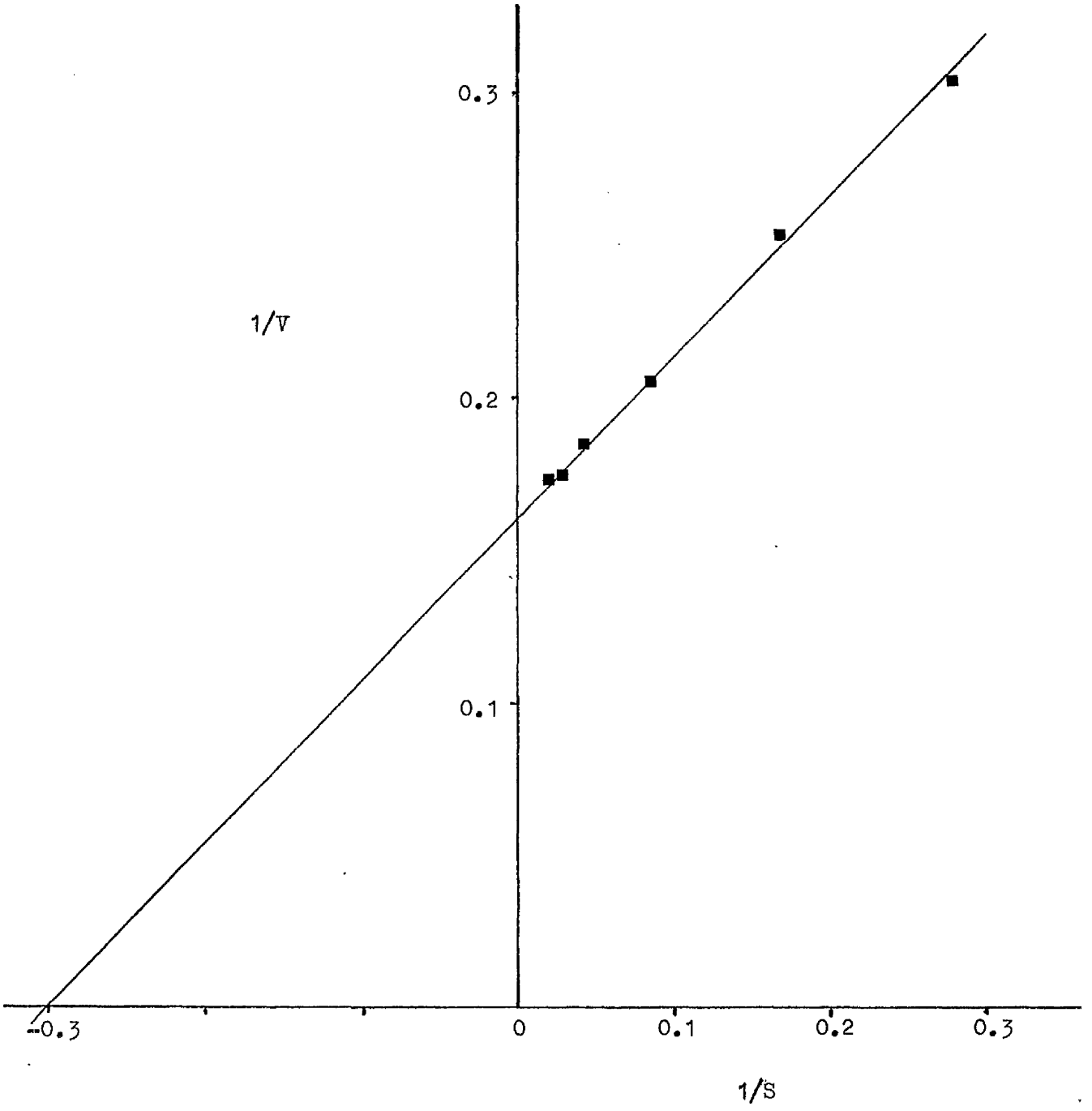


Figure 13 .Influence of putrescine concentration on putrescine oxidase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM putrescine, 1% glucose

V : nmoles/min/mg protein , S : 10^{-3}M putrescine

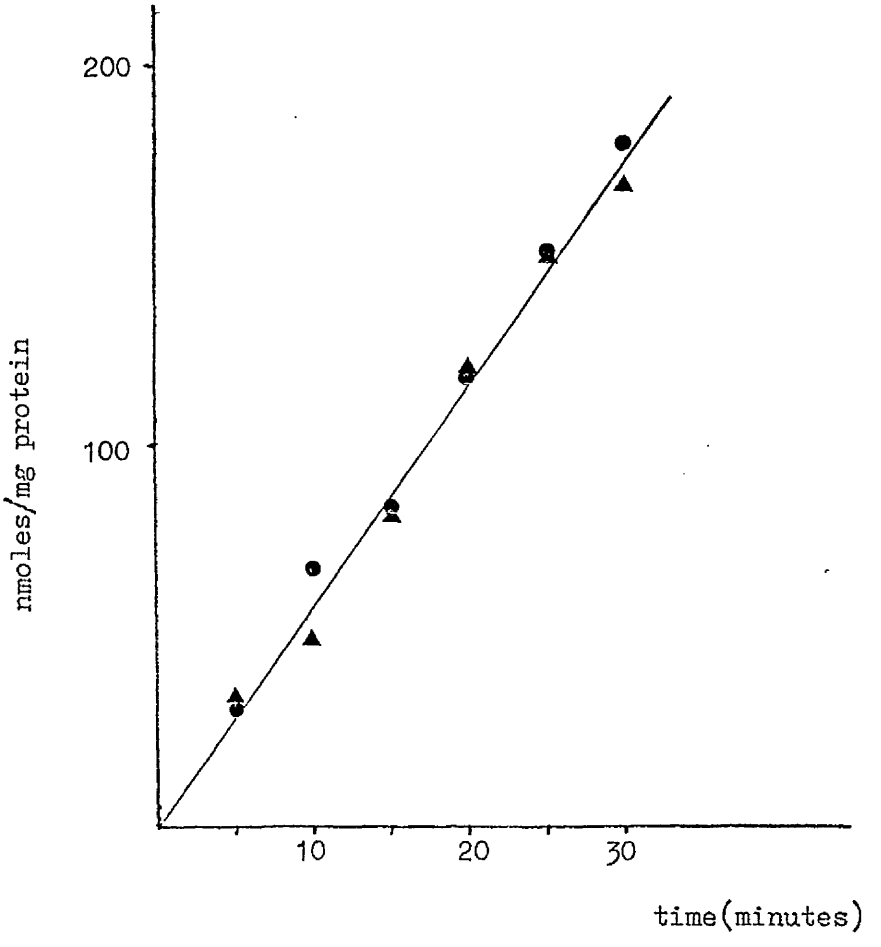


Figure 14 . Time course of putrescine oxidase activity in wild type cells(biA1) , in presence(▲) and absence(●) of PMSF .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM putrescine, 1% glucose

necessary were used to obtain the data for the pH graph (Fig 11). Maximum activity was obtained about pH 8.5 - 9 and therefore all subsequent experiments were performed at pH 8.8 with Gomori's tris buffer at 37°C (Fig 12).

(b) Kinetics

The effect of the substrate concentration on the rate of the putrescine oxidase activity was investigated over the range of 3.6 - 48 mM final concentration of putrescine in the reaction mixture. A Lineweaver-Burk double reciprocal plot (Lineweaver and Burk, 1934) of the data was constructed (Fig 13) and this shows that the enzyme has a Michaelis Constant (Km) of 3.3 mM and a maximum velocity (Vmax) of 6 nmoles/min/mg of protein, which corresponds to approximately 50 mM substrate concentration.

Activity is linear under the standard assay conditions (see section 2) for the first 25 minutes (Fig 14) and this time was adhered to throughout the assays. Phenylmethylsulfonylfluoride (PMSF), a protease inhibitor (Fahrney and Gold, 1963) was tested to see if it prevented enzyme decay but it did not affect activity (Fig 14).

(c) Prosthetic groups

Flavin adenine dinucleotide (FAD, final concentration 5 μM), pyridoxal-5'-phosphate (final concentration 0.2 mM), cupric chloride (final concentration 0.2 mM) added in the assay reaction mixture gave 104%, 106% and 56% respectively of the activity produced in their absence. This however does not exclude the possibility that the purified enzyme may require one of the above mentioned groups.

	replicates	activity in nmoles/min/mg of protein	% of activity
aerobic	1	4.951	
conditions	2	5.048	100
	3	4.951	
anaerobic	1	0.388	
conditions	2	0.000	3.23
	3	0.097	

Table 25. Putrescine oxidase activity in the wild type (biA1) under aerobic and anaerobic conditions.

treatment (3 hrs)		activity nmoles/min/mg protein
carbon source	nitrogen source	
1% glucose	none	1.769
"	5 mM pu	5.820
"	5 mM Spd	2.000
"	5 mM Sp	0.930
"	5 mM NO_3^-	0.088
"	5 mM NH_4^+	0.416
"	5 mM ala	1.047
"	2.5 mM urea	0.571
"	5 mM pu + 5 mM NO_3^-	1.021
"	5 mM pu + 5 mM NH_4^+	0.340
none	none	2.600
"	5 mM pu	8.725
"	5 mM NO_3^-	2.810
"	5 mM NH_4^+	2.340
"	5 mM ala	1.520
"	2.5 mM urea	1.850
"	5 mM pu+ 5 mM NO_3^-	9.770
"	5 mM pu + 5 mM NH_4^+	7.500

Table 26. Putrescine oxidase activity after various nitrogen and carbon conditions. (Initial growth on nitrogen-less minimal medium supplemented with 10 mM nitrate.)

(d) Activity under anaerobic conditions

Putrescine oxidase activity was determined in the same extract (3 replicates) under aerobic and anaerobic conditions (see methods, section 2). Only 3.23% of the normal activity was left present under anaerobic conditions (Table 25), probably due to traces of oxygen still present in the Thunberg tube.

(e) Substrate specificity

Only substrates whose oxidation product is or yields Δ^1-pyrroline could be tested as substrates with the assay system used. Spermidine or spermine gave only 3% of the activity obtained with putrescine which suggests that these two polyamines are not oxidised to give Δ^1-pyrroline in Aspergillus nidulans, at least not under the conditions investigated here.

11.3. Regulatory aspects of putrescine oxidase

11.3.1. Activity in wild type cells pretreated with various conditions

Wild type cells after the initial period of growth (17 hrs with 1% glucose and 10 mM nitrate) were transferred for another 3 hrs to various treatment media to investigate the effect of carbon and nitrogen sources in the rate of activity (Table 26). The main conclusions drawn from these results are:

(a) Putrescine oxidase activity is at its highest after treatment with putrescine in presence or absence of glucose, i.e. putrescine appears to have some inducing effect.

Mutants	Activity nmoles/min/mg protein
biA1	6.0
brlA12	6.7
rev-2	4.9
puA2	4.9
spsA1	5.8
punA11	6.6
pun-7	3.7
pun-8	4.4
pun-9	5.8
pun-10	5.7
pun-22	3.1
nirA1	6.0
$nirA^{c}1$	5.0
niiA4	7.6
$areA^{r}550$	0.7
$areA^{d}520$	6.1
$tamA^{r}50$	1.0
$tamA^{r}119$	4.1
TAMPU	0.7
mauA2	4.1
mauB4	6.5

Table 27. Putrescine oxidase activity in various mutants (treatment : 3 hrs 1% glucose + 5 mM putrescine).

(b) Activity is repressed by nitrate or ammonium only in presence of glucose.

(c) Glucose or a metabolite produced from glucose, may have a repressing effect on the enzyme activity.

(d) In general the enzyme does not appear to be very active (in comparison, for instance with the NADP L-glutamate dehydrogenase (Kinghorn and Pateman, 1973)), although the activities obtained are 10 times higher than those reported for Aspergillus niger with putrescine as substrate (Yamada et al., 1965).

11.3.2. Mutations affecting putrescine oxidase activity

Table 27 presents the mutants tested and the activities obtained. The only mutants which clearly have very low activity are $tamA^{r}50$, TAMPU, $areA^{r}550$ ("ammonium repressed" mutants, see section 2). The pun^{-} mutants tested, rev-2 and $nirA^{c}$ which are all defective in growth on putrescine as nitrogen source, have more or less normal putrescine oxidase activity. The conclusion is therefore that this enzyme may not be responsible for utilization of putrescine as nitrogen source; this may also be indicated by the rather low activities obtained. mauA and mauB mutants which grow on putrescine but lack monoamine oxidase activity are normal as far as putrescine oxidase is concerned.

11.4. Discussion

A putrescine oxidase activity was detected in Aspergillus nidulans. It operates at pH 8-9 and the Km for putrescine is 3.3 mM. Spermidine and spermine are probably not substrates for this enzyme,

at least not so as to produce Δ^1-pyrroline as an oxidation product. The maximal formation of enzyme was observed after growth with putrescine which suggests some kind of induction. Other sources of nitrogen such as urea, alanine, nitrate or ammonium resulted in minimal enzyme production. Furthermore nitrate or ammonium repressed the activity in presence of glucose. The system was not repressed by ammonium or nitrate when activities were measured after carbon starvation. The situation may be similar to that of glutamate uptake (Pateman et al., 1974) or the acetamidase (Hynes, 1970). Both these systems are ammonium repressible in presence of glucose, but become insensitive to ammonium repression when their substrates are used as carbon sources. Putrescine however in this case cannot be utilized as carbon source by Aspergillus nidulans. In addition, glucose or some derivative appears to exercise carbon catabolite repression since activities after growth with putrescine were higher in the absence of glucose than in its presence.

As expected for an ammonium repressible enzyme, its activity is minimal in the tamA^{r}50 and areA^{r}550 strains (the fully ammonium repressed alleles of the tamA and areA regulatory genes). However TAMPU, although able to grow on putrescine, is not derepressed for oxidase activity.

On the other hand all the other mutants with impaired growth on putrescine such as rev-2, nirA^{c}1 and pun$^-$ mutants have about normal enzyme activity. It should be mentioned however that nirA^{c}1 and some, but not all, of the pun$^-$ mutants are repaired on plate tests for growth on putrescine at pH 9 which is also the pH optimum for this enzyme.

The general outcome of the above observations is that the oxidase probably does not play a major role in the degradation of putrescine except perhaps during growth at high pH.

The situation must therefore be more complex for diamines (putrescine) as opposed to monoamines which are degraded by the monoamine (methylamine) oxidase (Page, 1971).

The mutants, mauA2 and mauB4, which lack the latter and do not grow on methylamine as nitrogen source (Page and Cove, 1972) still grow on putrescine. They also have normal putrescine oxidase activity.

SECTION 12

PUTRESCINE TRANSAMINASE ACTIVITY IN *Aspergillus nidulans*

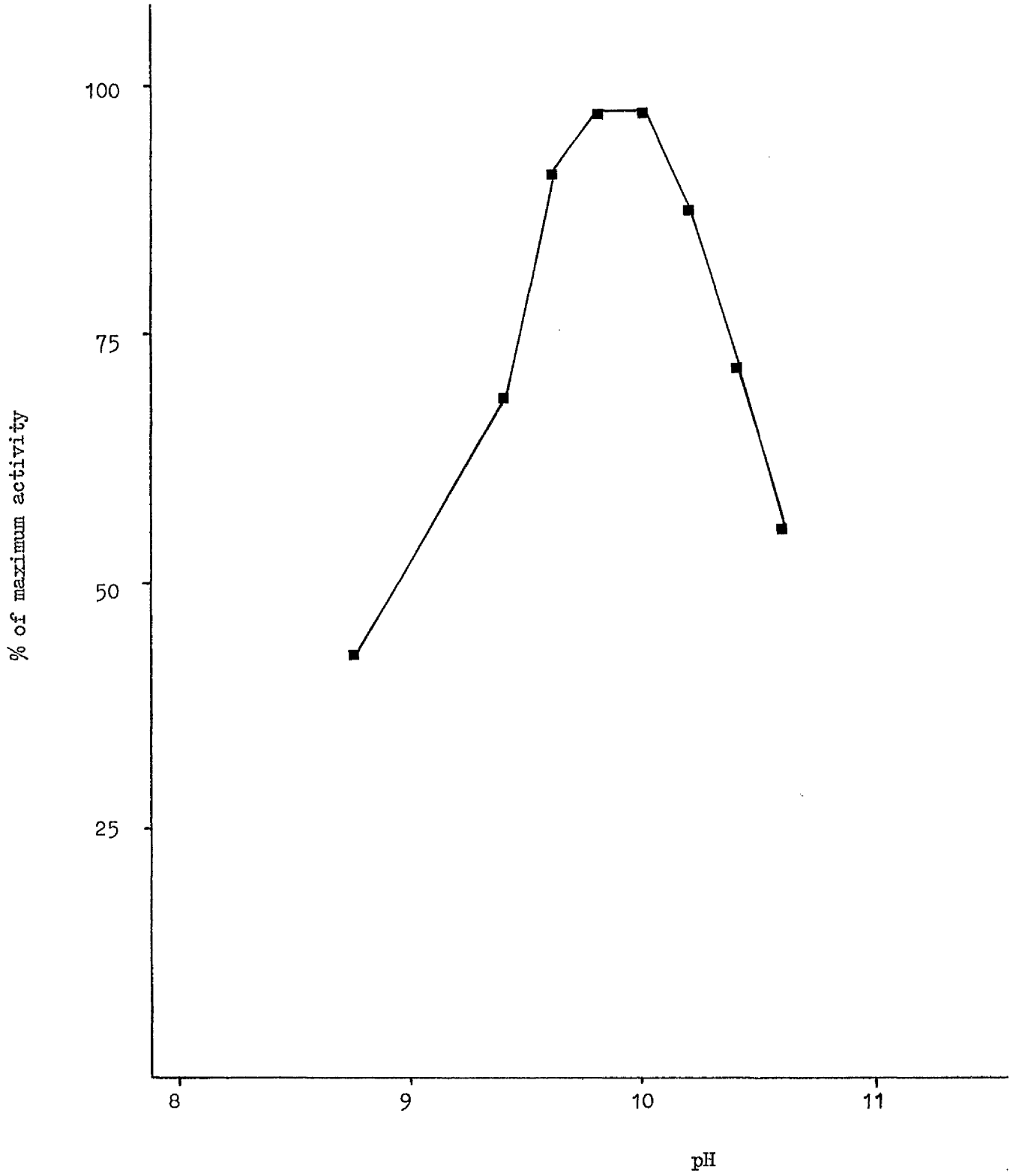


Figure 15 . pH dependence of putrescine transaminase activity

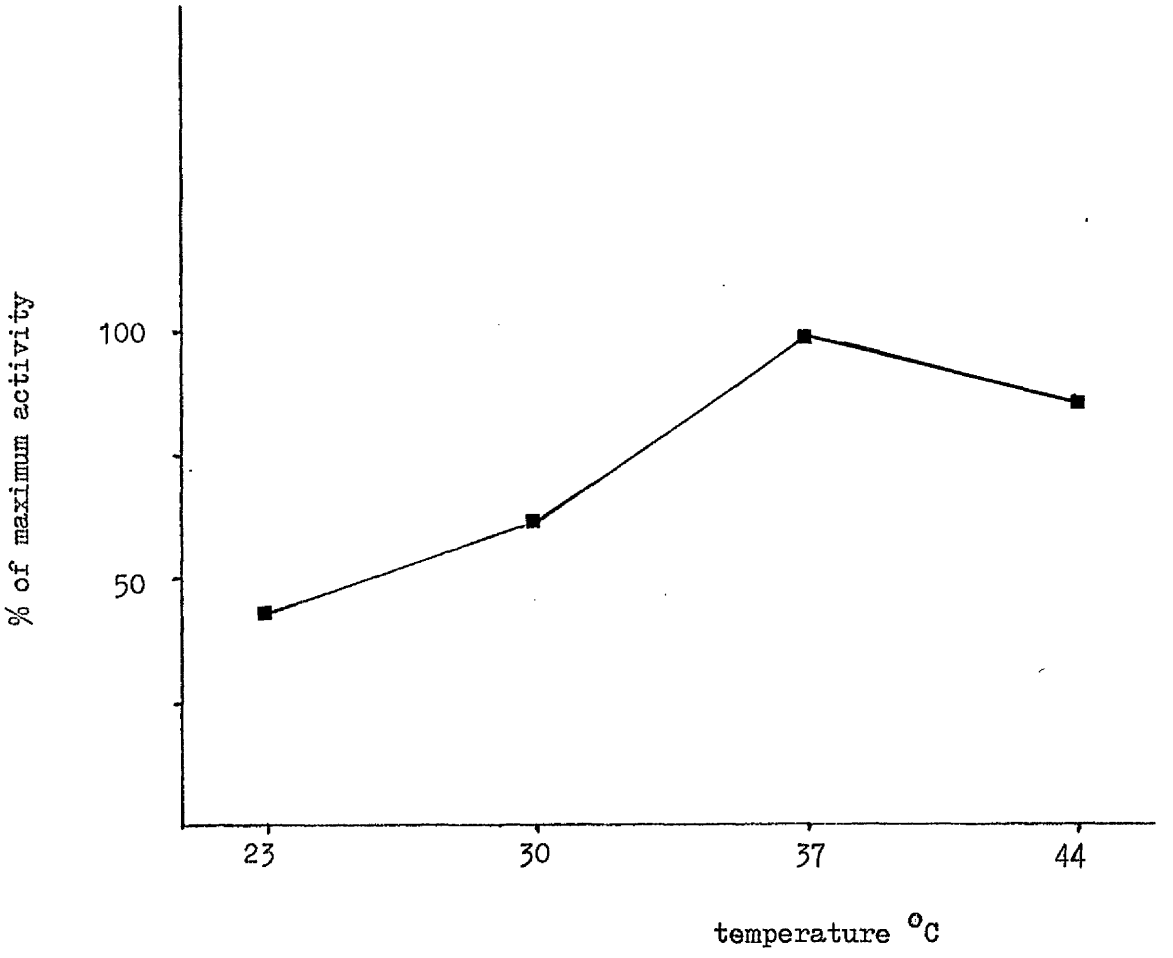


Figure 16 . Temperature dependence of putrescine transaminase activity .

12.1. Introduction

A putrescine transaminase activity has been reported in Escherichia coli (Kim and Tchen, 1962), converting putrescine to γ-aminobutyraldehyde. It was noticed in extracts of Aspergillus nidulans that if α-oxoglutarate or pyruvate was added in the putrescine oxidase assay, extra quinazolinium product could be detected. This suggested the presence of a putrescine transaminase in this organism as well, and an assay was developed for its estimation based on the production of Δ^1-pyrroline (Section 2). Cells were initially grown for 17 hrs in minimal medium (10 mM nitrate as nitrogen source), then transferred to medium supplemented with putrescine for another 3 hrs.

12.2. Characteristics of the enzyme

(a) pH and temperature

Tris and carbonate-bicarbonate buffers were used for the pH graph (Fig 15). Maximum activity was obtained about pH 9.7 - 10 and therefore all subsequent experiments were performed at pH 9.8 with Delory and King's carbonate-bicarbonate buffer at the optimum temperature of 37°C (Fig 16).

(b) Kinetics

The effect of the substrate concentration (putrescine) and also of the co-substrates (α-oxoglutarate or pyruvate) on the rate of putrescine oxidase activity was investigated over a range of 2.5 - 50 mM for putrescine and 3.6 - 48 mM for α-oxoglutarate or pyruvate. Lineweaver-Burk double reciprocal plots of the data were constructed

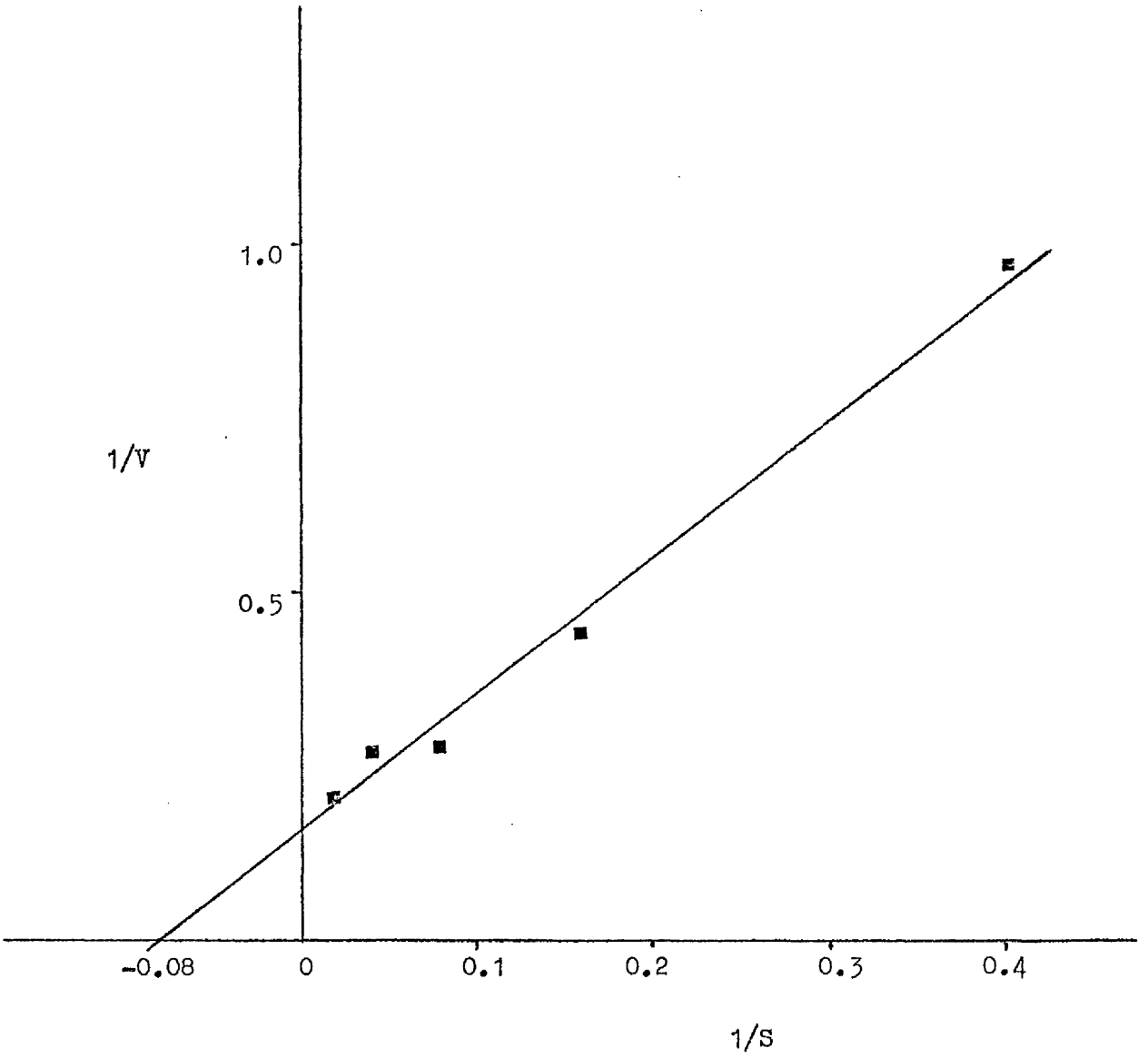


Figure 17 . Influence of putrescine concentration on putrescine transaminase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM putrescine, 1% glucose

V : nmoles/min/mg protein, S : 10^{-3}M putrescine

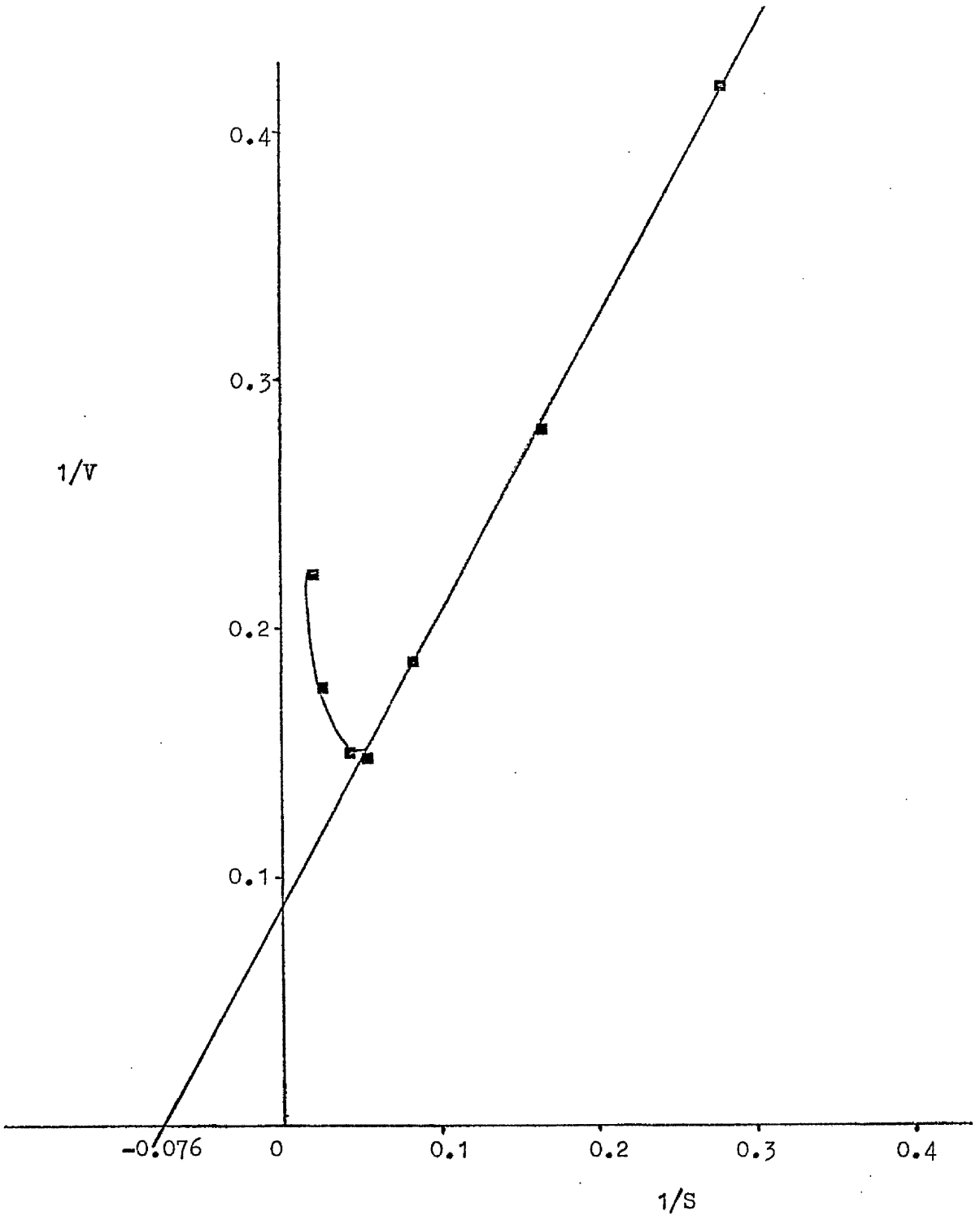


Figure 18 . Influence of α-oxoglutarate concentration on putrescine transaminase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM putrescine, 1% glucose

V : nmoles/min/mg protein, S : 10^{-3}M α-oxoglutarate

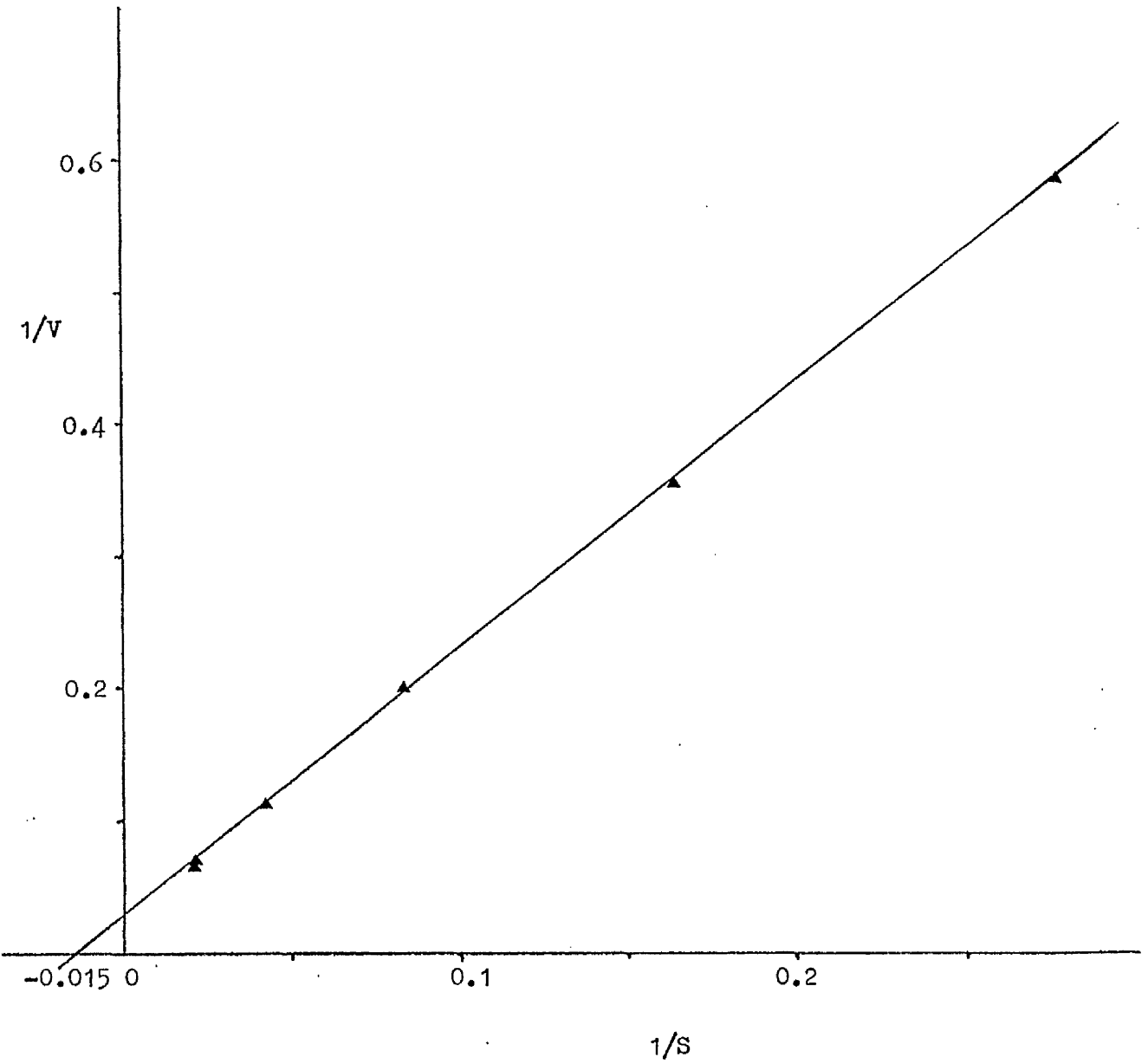


Figure 19 . Influence of pyruvate concentration on putrescine transaminase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM putrescine, 1% glucose

V : nmoles/min/mg protein, S : 10^{-3}M sodium pyruvate

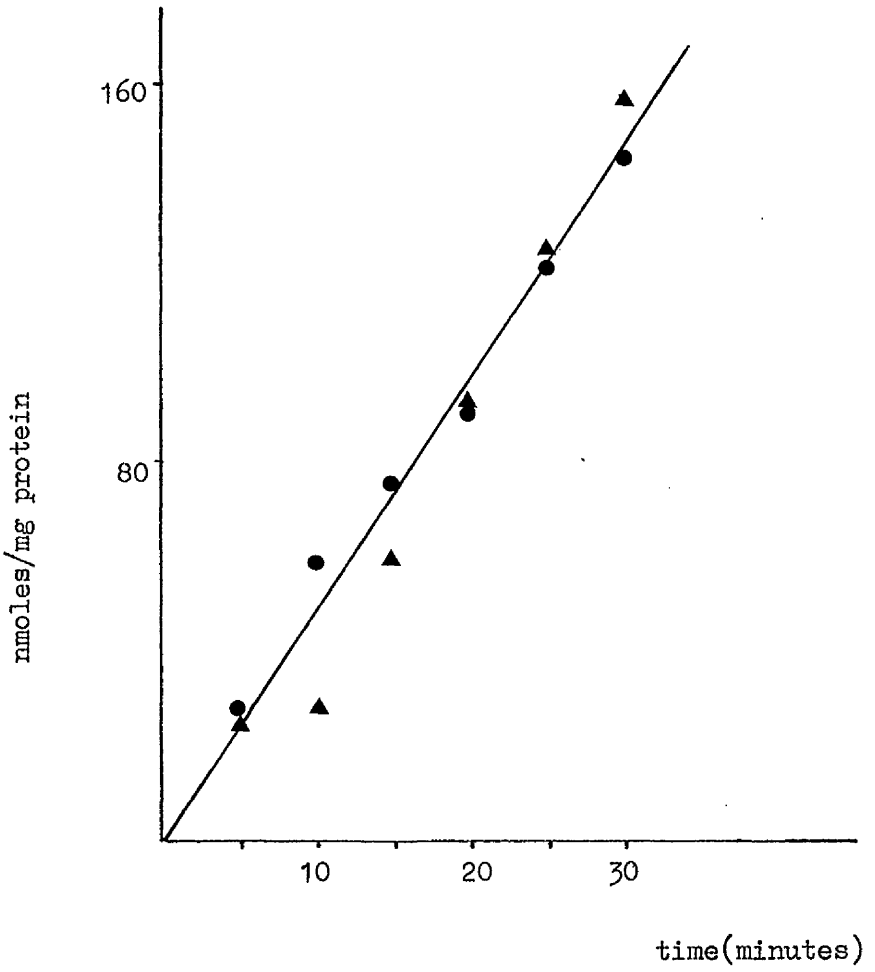


Figure 20 . Time course of putrescine transaminase activity in wild type cells (biA1) , in presence(▲) and absence(●) of PMSF .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM putrescine, 1% glucose

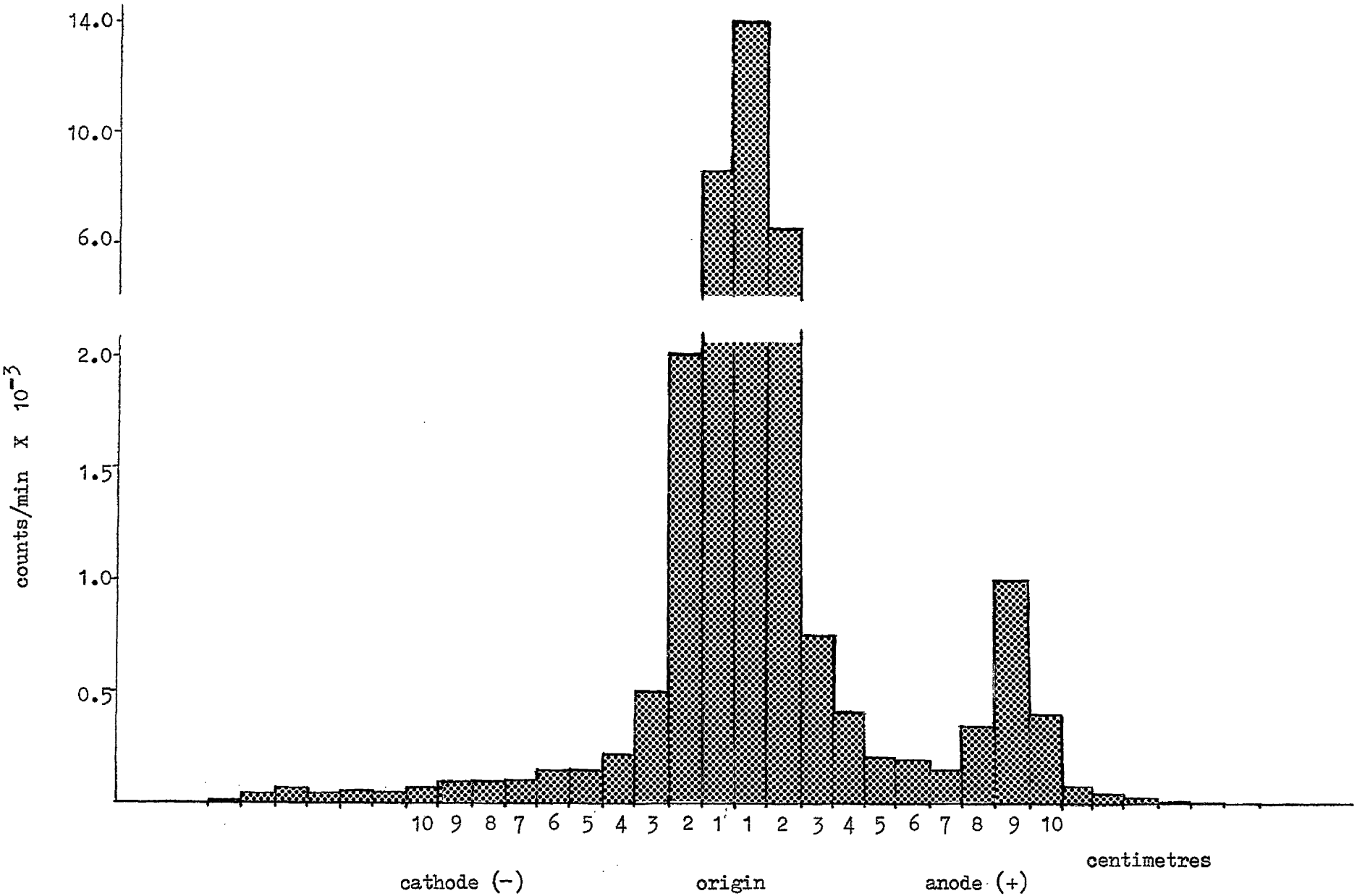


Figure 21 . Histogram of ^{14}C-glutamate(peak 8-10) , synthesized from ^{14}C-oxoglutarate(origin) via putrescine transaminase activity of wild type extracts .

(Fig 17, 18, 19) and these show that the enzyme has a Michaelis Constant (Km) of 12.5 mM for putrescine, 13.1 mM for α-oxoglutarate and 66 mM for pyruvate. Concentrations of α-oxoglutarate above 15 mM are inhibitory to the enzyme.

Activity is linear under the standard assay conditions (Section 2) at least for the first 25 minutes (Fig 20) and this time was adhered to throughout the assays. PMSF, the protease inhibitor, did not affect activity substantially (Fig 20).

(c) Prosthetic groups

Flavin adenine dinucleotide (FAD, final concentration 5 μM) pyridoxal-5'-phosphate (final concentrations 12 and 200 μM), cupric chloride (final concentration 0.2 mM) added in the assay reaction mixture gave 96%, 92% and 43% respectively of the activity produced in their absence.

(d) Detection of glutamate

Transamination of putrescine will produce γ-aminobutyraldehyde and L-glutamate or L-alanine depending on the co-substrate (Section 2, Fig 4). The aldehyde has been used for the quantitative estimation of the enzyme. Glutamate was also traced on a chromatogram (a) as a small consistent radioactive peak produced from radioactive α-oxo-glutarate added in the standard putrescine transaminase assay mixture (Fig 21). This peak corresponded to the Rf values given by control L-glutamate stained with ninhydrin. Radioactive α-oxoglutarate when included in the assay mixture in the absence of putrescine as the

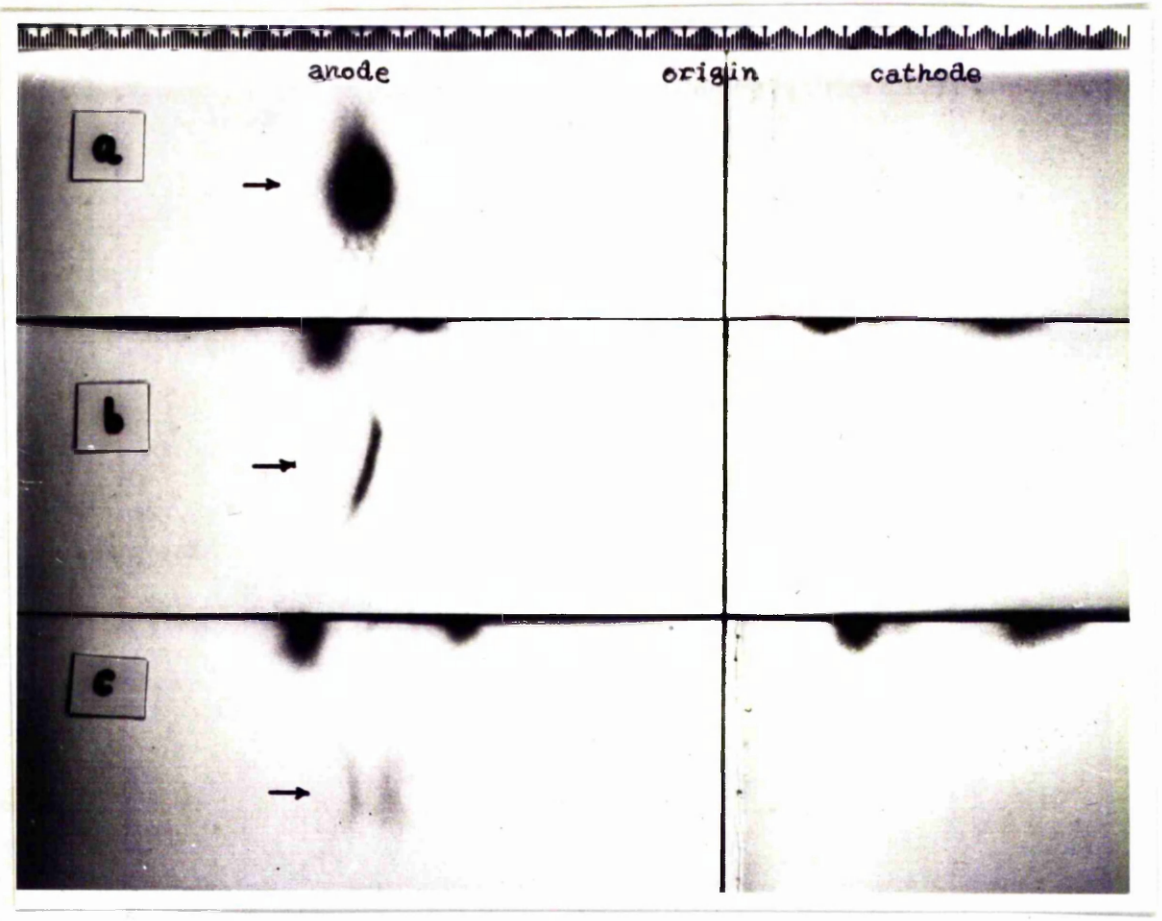


Plate 19. Detection of glutamate production via putrescine transaminase activity (staining with ninhydrin).

a: commercial L-glutamate

b: glutamate in reaction mixture after the transaminase assay (putrescine and α-oxoglutarate present)

c: glutamate in control reaction mixture after the transaminase assay (no putrescine added, α-oxoglutarate present).

(Spots at the edges of chromatograms are indicator dyes).

Treatment (3 hrs)		activity nmoles/min/mg protein
carbon source	nitrogen source	
1% glucose	none	1.010
"	5 mM pu	4.746
"	5 mM spd	2.166
"	5 mM sp	1.121
"	5 mM NO_3^-	3.893
"	5 mM NH_4^+	5.208
"	5 mM ala	1.523
"	2.5 mM urea	1.047
"	5 mM pu + 5 mM NO_3^-	4.010
"	5 mM pu + 5 mM NH_4^+	5.210
none	none	9.505
"	5 mM pu	10.380
"	5 mM NO_3^-	11.300
"	5 mM NH_4^+	8.503
"	5 mM ala	5.200
"	2.5 mM urea	10.700
"	5 mM pu + 5 mM NO_3^-	9.420
"	5 mM pu + 5 mM NH_4^+	9.100

Table 28. Putrescine transaminase activity after various treatment conditions. (Initial growth on nitrogen-less minimal medium supplemented with 10 mM nitrate.)

co-substrate, gave only the one (α-oxoglutarate) peak which remained at the origin; (b) by staining with ninhydrin (Plate 19): commercial L-glutamate used as a control migrated at the same distance with the glutamate produced via transaminase activity. When putrescine, the co-substrate was omitted from the transaminase assay, traces only of glutamate were detected with ninhydrin (Plate 19,c), most probably due to glutamate already synthesised in vivo. Glutamate in all above cases had moved about 8-10 cm to the anode (+) with the ionophoresis system used (see Section 2 for method).

(e) Substrate specificity

In addition to putrescine, only spermidine or spermine which may produce Δ^1-pyrroline could be tested as substrates under this particular assay system. Spermidine and spermine gave 20% and 16% respectively of the activity produced by putrescine.

12.3. Regulatory aspects of putrescine transaminase

12.3.1. Activity of wild type cells grown under various conditions

Wild type cells after the initial period of growth (17 hrs with 1% glucose and 10 mM nitrate) were transferred for another 3 hrs to treatment media to investigate the effect of carbon and nitrogen sources in the rate of activity (Table 28).

It can be concluded that

(a) putrescine transaminase activity is present under all conditions and it is not subjected to ammonium repression

genotype	activity nmoles/min/mg protein
biA1	5.00
brlA12	5.90
rev-2	4.37
puA2	4.94
spsA1	6.90
punA11	4.89
pun-7	4.32
pun-8	3.69
pun-9	5.20
pun-10	3.54
pun-22	7.50
nirA1	7.40
$nirA^{c}1$	5.50
niiA4	6.38
$areA^{r}550$	0.97
$areA^{d}520$	6.70
$tamA^{r}50$	1.60
$tamA^{r}119$	5.90
TAMPU	1.57
mauA2	3.15
mauB4	5.18

Table 29. Putrescine transaminase activity in various strains. treatment (3 hrs): 1% glucose + 5 mM putrescine).

(b) glucose or a product of glucose exercises carbon catabolite repression on the enzyme.

12.3.2. Mutations affecting putrescine transaminase activity

Table 29 presents the various mutants tested and their activities after growth on nitrate and then transfer to nitrogen-less medium supplemented with 5 mM putrescine (3 hrs treatment). Although the enzyme activity in the wild type is apparently not ammonium repressible, it appears that the only mutants with low putrescine transaminase activity are *tamA*^{r}50, TAMPU and *areA*^{r}550.

12.4. Discussion

A putrescine transaminase activity was detected in *Aspergillus nidulans* utilizing either α-oxoglutarate or pyruvate as co-substrates. The enzyme does not require induction and it is not ammonium repressible. It is however apparently under carbon catabolite repression, which seems odd since putrescine cannot be utilized as carbon source by *Aspergillus nidulans*. It might therefore be that putrescine is not the natural substrate of the enzyme, a hypothesis also supported by the fact that the enzyme's Km for putrescine is rather high (12.5 mM).

Activities are in general rather low and the *tamA*^{r}50, TAMPU and *areA*^{r}550 are the only defective mutants found. This may indicate that the *tamA* and *areA* genes are also involved in the regulation of systems other than those repressed by ammonium (carbon repression for example).

pun$^-$ mutants, *nirA*^{c}*1* and *rev-2* have apparently normal activities; this suggests that the transaminase, like the oxidase (Section 11) does not play a major role in the utilization of putrescine as nitrogen source.

SECTION 13

ACTIVATION BY POLYAMINES OF NADP-LINKED GLUTAMATE DEHYDROGENASE IN TRIS BUFFER

13.1. Introduction

The previous two sections indicate that putrescine degradation is achieved by at least two enzymes, neither of which however seems to be responsible for its utilization as nitrogen source. The γ-aminobutyraldehyde produced by the above mentioned enzymes exists probably in equilibrium with its cyclic form Δ^1-pyrroline. The fate of Δ^1-pyrroline is not clear in Aspergillus nidulans. It is probably involved in the production under certain conditions of a prodigiosin-like pigment (Section 5). On the other hand γ-aminobutyraldehyde can be converted to γ-aminobutyrate and further to succinate (Arst, 1976, 1977). The conversion of the aldehyde to the corresponding butyric acid is catalysed in Pseudomonas by an NAD-linked dehydrogenase (Jakoby and Fredericks, 1959); attempts to assay a similar NAD or NADP dependent enzyme in Aspergillus using hydrolysed γ-aminobutyraldehyde diethyl acetal as substrate (see also section 2) did not reveal the presence of such an enzyme. When, however, putrescine, the immediate precursor of the actual substrate was incubated with NADP, an apparent NADP-linked activity was detected with the formation of an NADPH absorbance peak at 340 nm. Other polyamines were equally effective. The evidence to be presented in this section suggests that the observed activity is due to the NADP-glutamate dehydrogenase (Kinghorn and Pateman, 1973) which in presence of polyamines or magnesium can utilize the tris buffer (an amine) as a poor substrate.

13.2. Characteristics of the activity

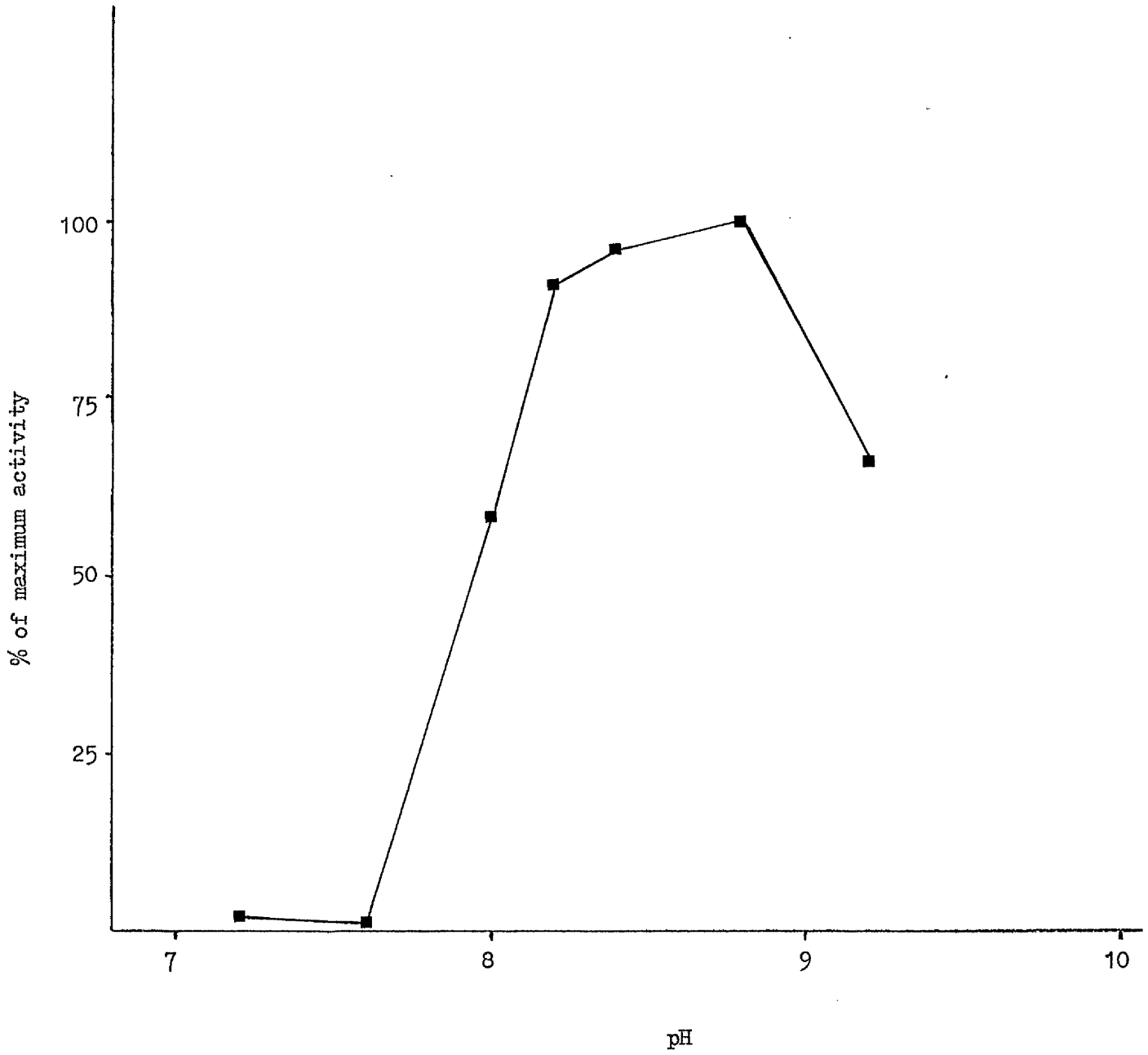


Figure 22 . pH dependence of activation by putrescine of NADP linked glutamate dehydrogenase .

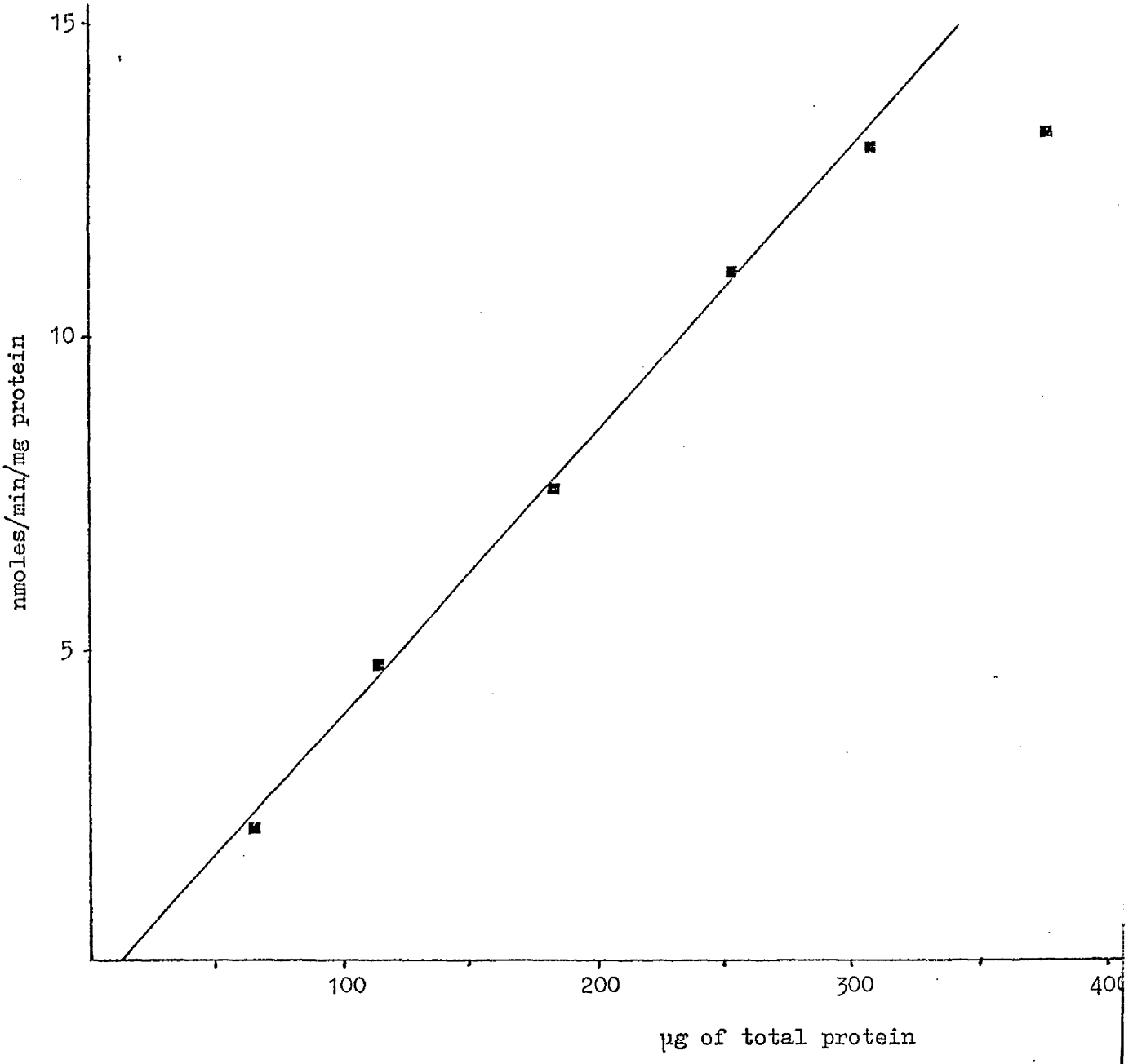


Figure 23 . Dependence of NADP linked glutamate dehydrogenase activity on enzyme concentration (total protein).

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM putrescine+5 mM ammonium , 1% glucose

"substrate" (15 mM final concentration)	activity (nmoles/min/mg protein)
propanediamine	18-21
putrescine	25-28
spermidine	36-38
spermine	29-31
cadaverine	21
MGBG	0
methylamine	0
histamine	0
glutamine	0
γ-aminobutyrate	0
L-glutamate	165
L-alanine	0
L-ornithine	0
L-arginine	0
L-proline	0
L-glycine	0
L-lysine	0

Table 30. Reduction of NADP-linked activities in presence of amines or amino acids. Growth conditions: 17 hrs with 1% glucose and 10 mM NO_3^-, then 3 hrs 1% glucose and 5 mM putrescine.

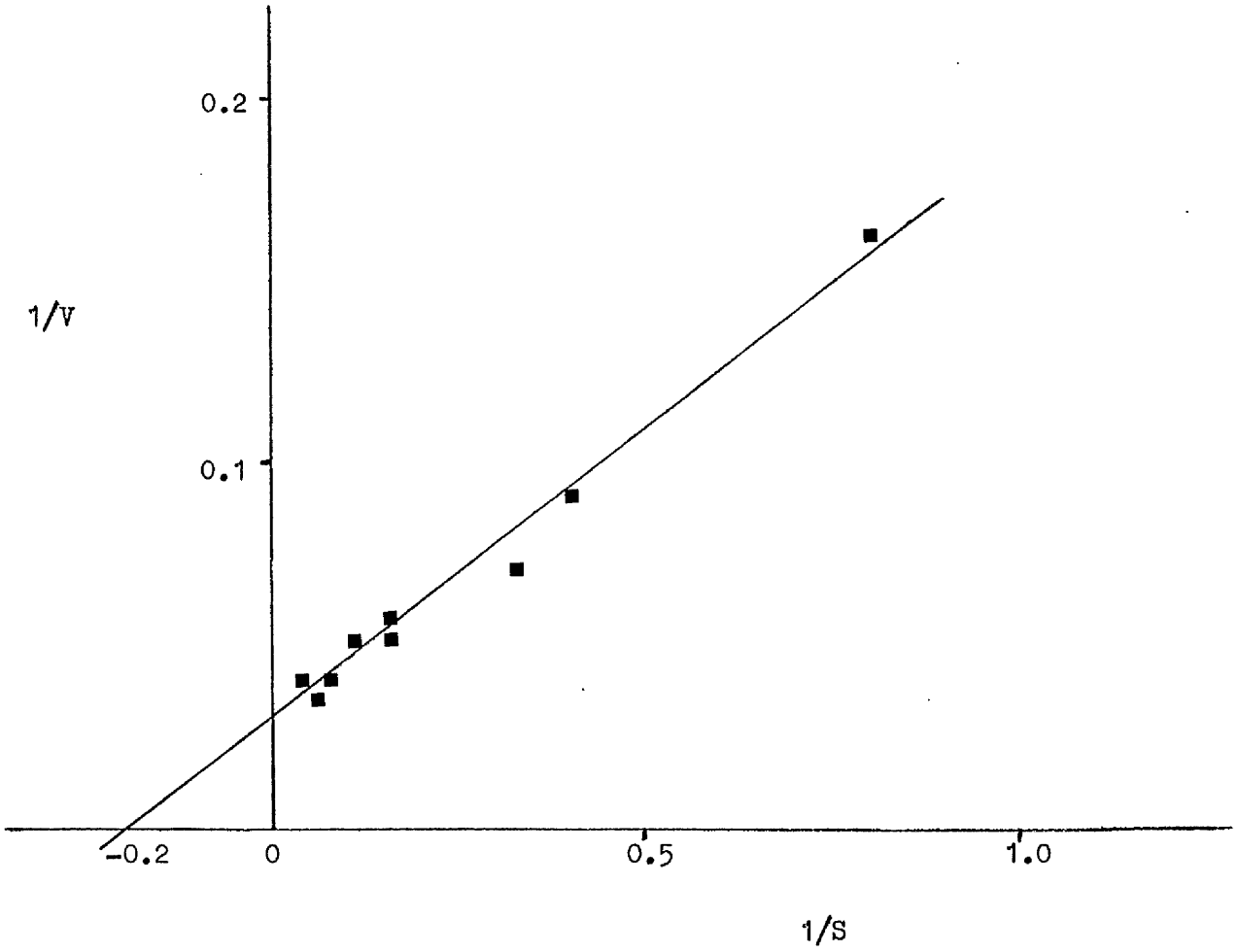


Figure 24 . The effect of putrescine concentration on NADP linked glutamate dehydrogenase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM nitrate , 1% glucose

V : nmoles/min/mg protein , S : 10^{-3} M putrescine

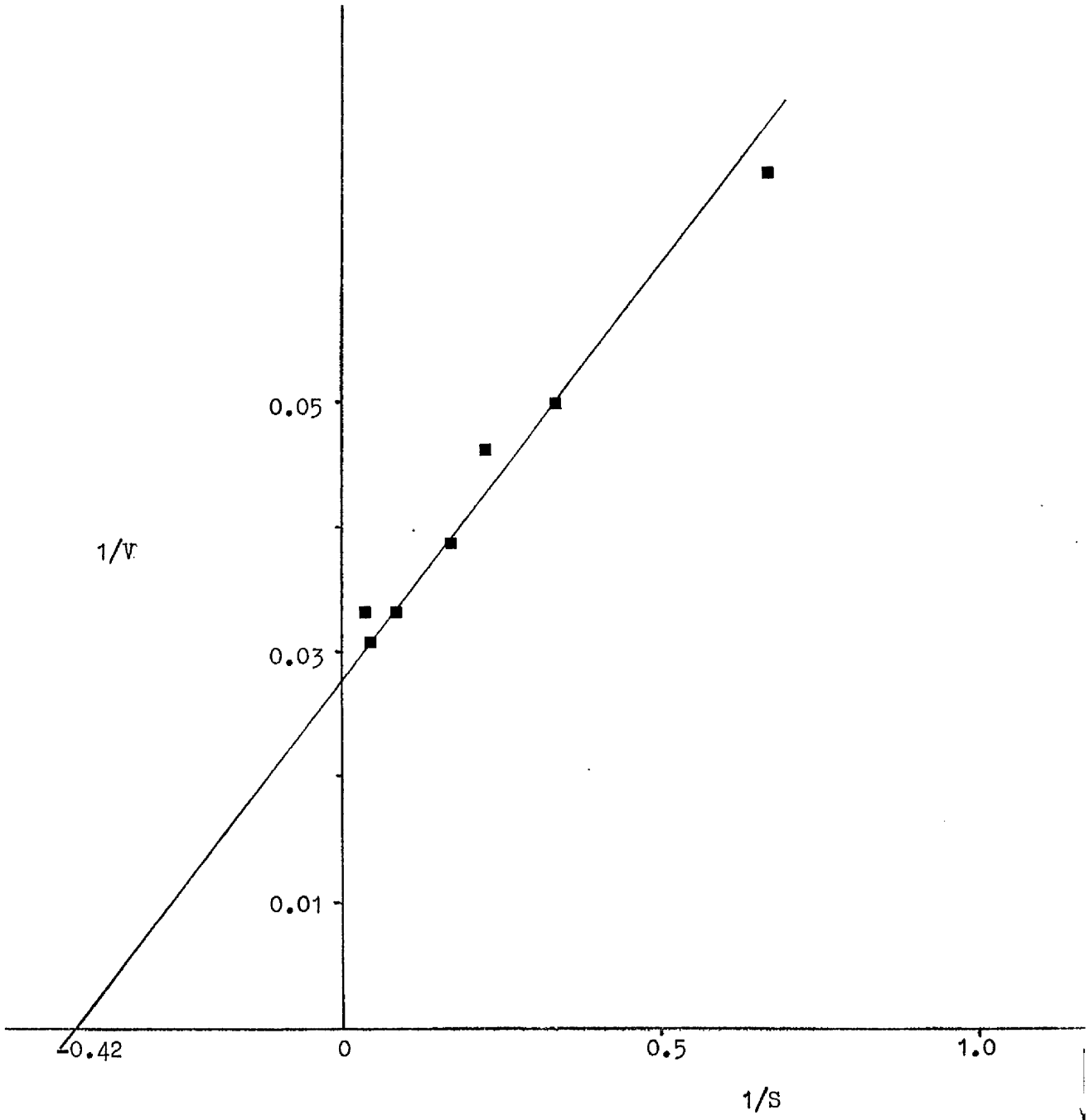


Figure 25 . The effect of spermidine concentration on NADP linked glutamate dehydrogenase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM nitrate , 1% glucose

V : nmoles/min/mg protein , S : 10^{-3} M spermidine

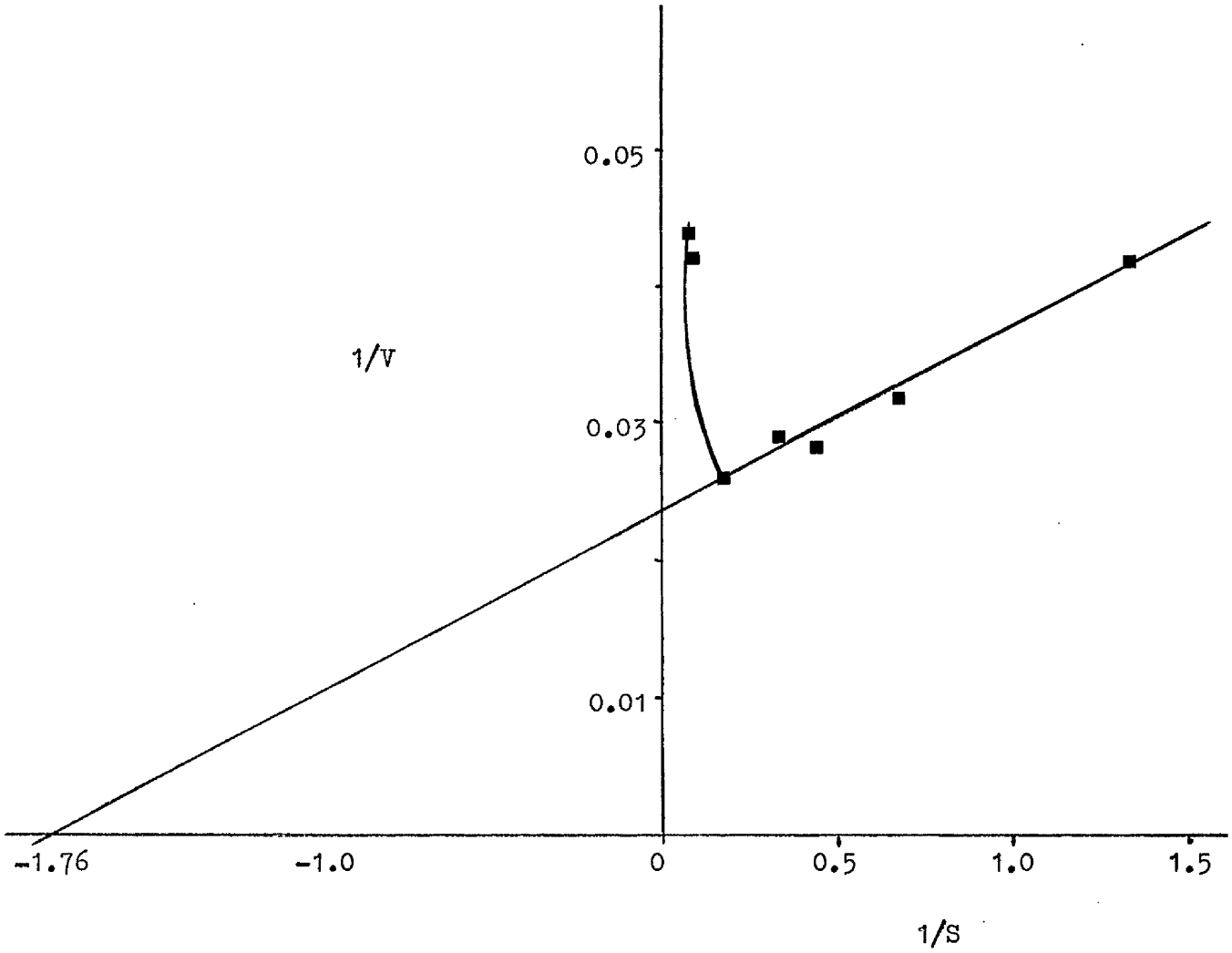


Figure 26 . The effect of spermine concentration on NADP linked glutamate dehydrogenase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM nitrate , 1% glucose

V : nmoles/min/mg protein , S : 10^{-3} M spermine

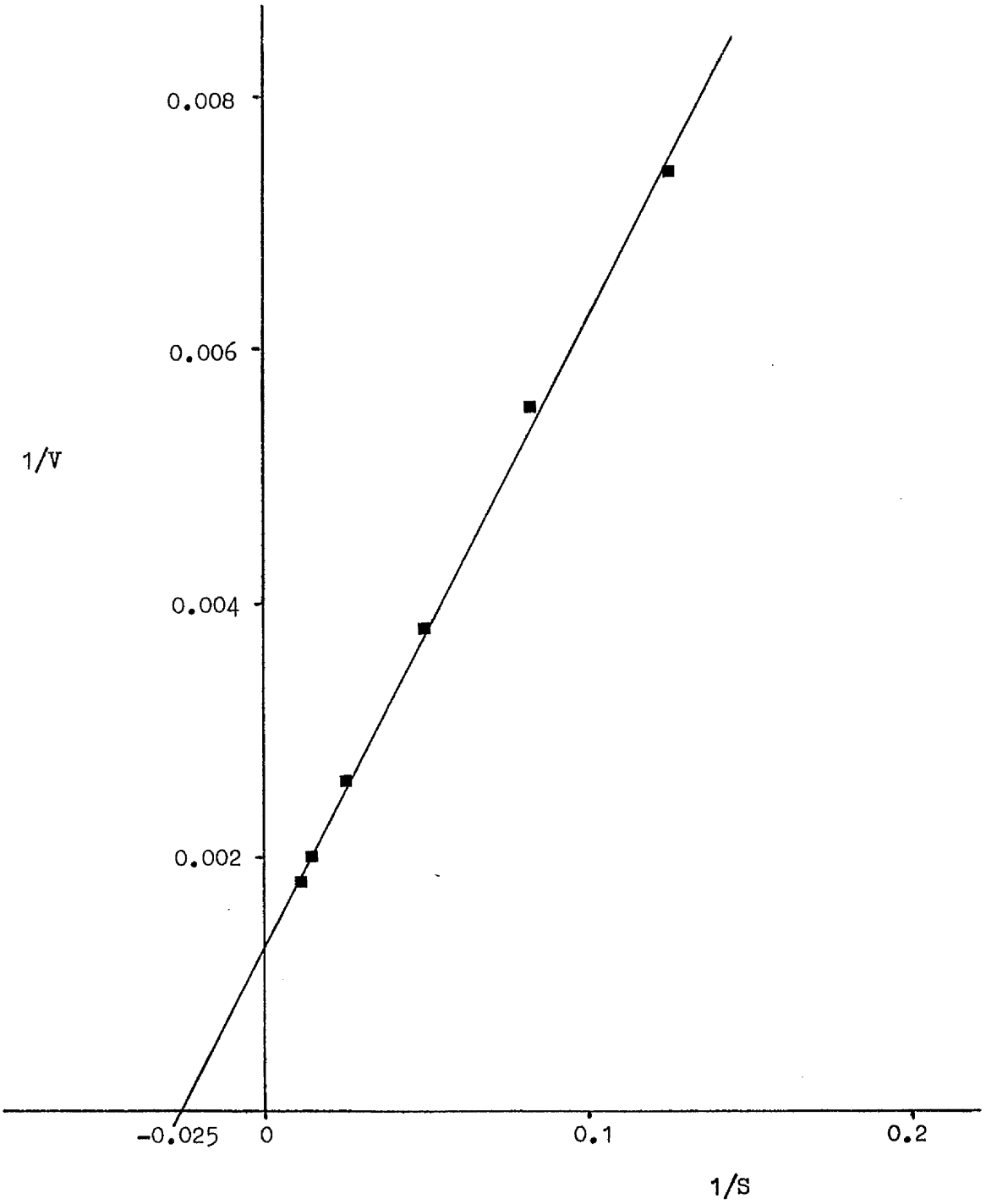


Figure 27 . Influence of glutamate concentration on NADP linked glutamate dehydrogenase activity .

Growth conditions : 10 mM nitrate , 1% glucose

Treatment (3 hrs) : 5 mM nitrate , 1% glucose

V : nmoles/min/mg protein, S : 10^{-3}M glutamate

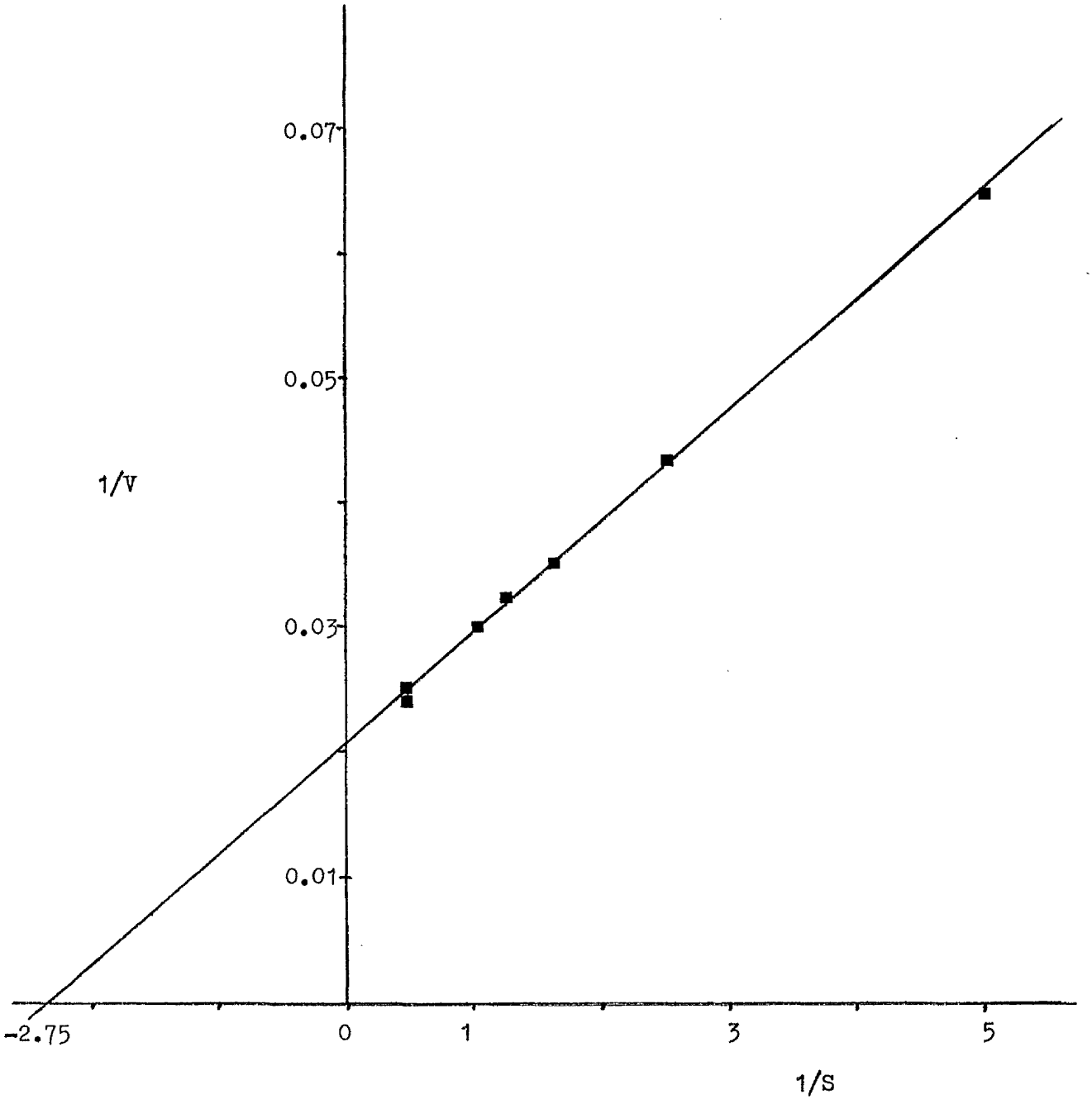


Figure 28 . Influence of NADP concentration on NADP linked glutamate dehydrogenase activity . Growth conditions : 10 mM nitrate , 1% glucose Treatment (3 hrs) : 5 mM nitrate , 1% glucose V : nmoles/min/mg protein , S : 10^{-3}M NADP .

(a) pH and temperature

Maximum activity was detected at pH 8.2-8.8 with Gomori's tris buffer at 37°C (Fig 22) with putrescine present at a concentration of 15 mM. Later it was found that no activity could be detected with Clark and Lubs's borate buffer at pH 8.2 or with Sorensen's phosphate at pH 8.2.

(b) Activity and enzyme concentration

With 15 mM putrescine present activity was linear for concentrations of total protein less than 300 µg in the reaction mixture (Fig 23).

(c) "Substrate" specificity

Since the activity was initially observed in presence of putrescine it was thought that this polyamine should be the substrate. A number of other amines and amino acids were therefore tested as "substrates" (Table 30). Activity was detected only with polyamines and L-glutamate. The Km for putrescine is 5 mM, for spermidine 2.38 mM, for spermine 0.57 mM, for L-glutamate 40 mM, for NADP 0.36 mM (Fig 24, 25, 26, 27, 28 respectively). Other amino acids or monoamines were ineffective. Maximum activity was obtained with L-glutamate (approximately 8 times that observed with polyamines) and it is apparently due to the presence of the NADP-GDH enzyme catalysing the reaction:

$$\text{L-glutamate} + \text{NADP} \rightleftharpoons \alpha\text{-oxoglutarate} + \text{NADPH} + NH_3$$

To test the hypothesis that both activities observed were due

growth conditions (liquid nitrogen-less medium) initial growth (17 hours)	treatment* (3 hours)	activity (nmoles/min/mg protein) (a) 15 mM putrescine	(b) 15 mM L-glutamate	ratio b/a
1% glucose, 10 mM NO_3^-	nitrogen free	20	223	11
" "	5 mM NO_3^-	44	296	7
" "	5 mM NH_4^+	28	287	10
" "	5 mM ala	15	121	8
" "	5 mM glut	25	147	6
" "	5 mM pu	16	217	14
" "	5 mM pu + 5 mM NO_3^-	50	310	6
" "	5 mM pu + 5 mM NH_4^+	34	216	6
1% glucose, 10 mM NO_3^-	none	37	274	7
1% glucose, 10 mM NH_4^+	none	12	159	13
1% glucose, 10 mM glut.	none	9	103	11
0.075% glucose, 100 mM glut.	none	0	4	-

*1% glucose present

Table 31. Wild type NADP-linked activities in presence of putrescine or L-glutamate.

Mutants	Activity (nmoles/min/mg protein) 15 mM putrescine	15 mM L-glutamate
biA1	39	280
gdhA1	0	0
gdhA2	0	0
gdhA3	0	0
gdhA8	0	0
gdhA9	0	0

Table 32. NADP-linked activities of gdhA mutants in presence of putrescine or L-glutamate.

Growth conditions: 17 hrs with nitrogen-less minimal medium supplemented with 10 mM nitrate and 2.5 mM L-alanine.

to the same enzyme, the following points were investigated:

(a) Determination of glutamate and "polyamine" activity under various conditions which would allow comparison of the ratio between the two activities. The NADP-glutamate dehydrogenase activity is known to vary considerably with the conditions of growth (Kinghorn and Pateman, 1973) and if the "polyamine" activity was also due to this enzyme it should vary similarly.

(b) Determination of both activities in gdhA mutants which are altered in the structural gene coding for the NADP-linked glutamate dehydrogenase would be most informative. These mutants have no NADP-GDH activity (Kinghorn and Pateman, 1975).

Tables 31 and 32 present the results of these studies. The ratio of the activities observed with L-glutamate to those observed with putrescine under the same conditions is approximately 9 with less consistency in very high or very low values which may be explained in terms of NADP dependence or limits of detection respectively. With NAD instead of NADP no "polyamine" activity was observed under the same conditions or conditions which would allow formation of high NAD-glutamate dehydrogenase activity (i.e. growth on 100 mM glutamate in absence of glucose; Kinghorn and Pateman, 1974).

Five gdhA alleles were tested and all were found to lack both activities. These results therefore favour the hypothesis that the NADP-linked glutamate dehydrogenase is the enzyme responsible for both activities. This would not be too surprising because the GDH

molecule is known to exist in various polymeric states upon which its activity towards the substrate depends (Vessel, 1972). In Neurospora the enzyme has both L-glutamate and L-alanine dehydrogenase activities (Burk and Pateman, 1962). However the main evidence so far against the hypothesis of polyamines acting as poor substrates is that activity is detectable only with tris buffer; this is not very unusual for some enzymes and in fact in this particular case it was found that borate at pH 8.2, greatly reduces glutamate dehydrogenase activity as well (L-glutamate as substrate).

An alternative hypothesis is that polyamines may activate the NADP-GDH enzyme rather than act as substrates. In support, 25 mM Mg^{++} (SO_4^{--}) in tris buffer only, in presence of NADP and extract, produced the same amount of activity given by polyamines. All the gdhA alleles tested lacked any NADP-linked activity in presence of tris plus glutamate, or tris plus polyamines, or tris plus magnesium ions.

13.3. Discussion

The most plausible explanation of the results presented is that polyamines or magnesium ions can activate the NADP-linked glutamate dehydrogenase to utilize tris, an amine, as a poor substrate.

The most thoroughly investigated NADP-L-glutamic dehydrogenase is the crystalline enzyme from beef liver. The bovine GDH molecule is an allosteric protein with various polymeric states, ranging from molecular weight of 250,000 to 1,000,000, which are regulated by a variety of compounds (allosteric effectors) such as cations, co-enzymes,

nucleotides and hormones (Vesell, 1972). The activity towards the substrate depends upon the polymeric state of the molecule; the lower molecular weight form exhibits most activity towards alanine, whereas the higher molecular weight polymer is more active with glutamate than alanine.

The Neurospora crassa enzyme shows both glutamic and alanine dehydrogenase activities and it has a molecular weight of about 200,000.

The postulated activation of the NADP-GDH by polyamines in Aspergillus is not very surprising since a number of other enzymes are also known to be activated by these polycations in fungi and other organisms (see section 1). However this appears to be the first report that tris may serve as a substrate for NADP-GDH under the specified conditions. The physiological importance of this observation is not easy to judge. Most probably tris may be just one of a number of compounds which can act as a substrate for this important enzyme under certain conditions.

SECTION 14

GENERAL DISCUSSION

14.1. Polyamine metabolism

Polyamines have been recently the subject of extensive investigations in a variety of organisms. This interest arises from their involvement in a wide spectrum of activities inside the cells, their ubiquitous presence in organisms in large amounts and especially their ability to bind to nucleic acids.

The studies in this work although mainly orientated to the possible connection between polyamines and *brlA1 2*, were extended further in an attempt to study general aspects of amine metabolism in *A. nidulans*.

A number of mutants with a variety of properties have been isolated to facilitate genetical and biochemical investigations.

Putrescine, spermidine and possibly spermine are transported into the cells by an active uptake which is regulated by ammonium; since no significant competition between the three polyamines was observed in the uptake assays, more than one system may be involved.

Once taken up, putrescine is converted to spermidine which is the precursor of spermine. The *puA2* auxotroph which is unable to convert ornithine to putrescine requires the latter at small amounts in the medium. It can also be supplemented by high concentrations of spermidine but not spermine. To investigate whether this difference is the result of a lower uptake capacity for spermidine, the double mutant *puA2;spsA1* was isolated by its ability to grow at low concentrations of spermidine as an alternative to putrescine.

In the wild type spermidine uptake is approximately 2½ times lower than that of putrescine, however spsA1 had only 30-50% higher uptake than the wild type for spermidine and putrescine. This difference may sound rather small to account for all the properties of spsA1; this would imply either that putrescine has other functions in addition to its conversion to spermidine or that the latter is converted or bound to something which reduces its availability. These conversions might be reduced in spsA1. It is known that in E. coli, depending on the conditions of growth, spermidine is converted to glutathionyl-spermidine, a compound of unknown function (Tabor and Tabor, 1970, 1971).

The predominant polyamine in A. nidulans is spermidine at intracellular concentrations of approximately 3 mM as opposed to 0.2 mM for putrescine and 1 mM for spermine. Spermine on its own does not support growth of the puA2 or puA2;spsA1 mutants but for the case of puA2;spsA1 it exhibits a sparing effect, that is, while spermine cannot be converted to the other two polyamines it reduces the requirement of the latter for spermine synthesis when either putrescine or spermidine are present at the same time.

A second property of spsA1 is its sensitivity to high concentrations of spermidine or spermine. This at the moment appears to be the result of high internal pools for these two polyamines, as a consequence of increased uptake and/or blocks in presently unknown catabolic/biosynthetic pathways. At the moment the function of these pathways in the wild type is not known, although some evidence suggests that these polyamines may be converted to non-toxic derivatives. spsA1 should be examined for such ability.

Putrescine can obviously be catabolised since it is the only one of the three which can support growth when used as the sole nitrogen source. So far two enzymes have been found and assayed. Both convert putrescine to γ-aminobutyraldehyde with simultaneous production of ammonia in the case of the oxidase, and glutamate or alanine in the case of the transaminase. The oxidase is mainly under ammonium control while the transaminase is under carbon catabolite repression.

Both enzymes however seem at the moment rather unlikely to be responsible for utilization of putrescine as nitrogen source for a number of reasons: (a) mutants have been isolated (e.g. punAll), which are incapable of growing on putrescine as nitrogen source although they still possess both putrescine oxidase and transaminase activities. (b) Other mutants (e.g. TAMPU) are able to grow on putrescine although they have very low levels of the above enzyme. (c) Both oxidase and transaminase have rather low specific activities. The transaminase, and to a lesser extent the oxidase, are subject to carbon catabolite repression, although putrescine cannot be utilized as carbon source, and in addition the transaminase has a high Km for putrescine: all these points may indicate that putrescine may not be the natural substrate for these enzymes. (d) In view of the above evidence the existence of a third enzyme seems very probable; such a hypothesis is also supported by the property of some putrescine non-utilizing strains which, unlike the wild type, are sensitive to isoniazid. Since these strains still possess oxidase and transaminase activities, it might therefore be that the enzyme which they lack

can also break down isoniazid which would otherwise be toxic. Isoniazid is an amine oxidase inhibitor and in view of the above observations it may mimic putrescine as the substrate of degrading its enzymes; this property now can be explored for an easy and effective isolation of mutants unable to utilize putrescine as nitrogen source.

A last aspect of putrescine metabolism is its connection with nitrate metabolism which might be significant for the case of brl mutants and rev-2 as discussed later. The structure of putrescine is such that it could be mistaken for an amino acid, therefore it is not surprising that its metabolism is affected by mechanisms regulating amino acid metabolism. This is indicated by studies with mutants such as nirA^{c}1, tamA^{r}50 and areA^{r}550, which do not grow on putrescine. These are all mutants with alterations in the regulation of amino acid metabolism. nirA^{c}1 is a constitutive mutant for nitrate and nitrite reductases; the product of the nirA locus in conjunction with nitrate reductase is believed to mediate a shut-down of amino acid metabolism in presence of nitrate. nirA^{c}1 is unable to utilize putrescine and also a number of amino acids as nitrogen sources. Chlorate mimicing the action of nitrate, also causes this shut-down resulting in the subsequent starvation of the cells for nitrogen again in the presence of either amino acids or putrescine. tamA^{r}50 and areA^{r}550 in which all the ammonium repressible systems (amino acid uptakes and utilization) do not function, were also found to be repressed for the transport and utilization of putrescine.

These are the main points which emerged during the present studies. It is obvious that further investigations are required to

complete the elucidation of polyamine metabolism. It is hoped that such studies will uncover the whole spectrum of polyamine activities in fungi and may provide more definite evidence about their importance in such organisms.

14.2. The brlA12 variegated position effect

The purpose of this work was mainly the investigation of a variegated position effect concerning the conidial system of A. nidulans.

As it has already been discussed, aspects of polyamine metabolism have been investigated in an attempt to strengthen early evidence (see section 7 for a summary) suggesting that polyamines may participate in the brlA12 variegation. This evidence is rather indirect since the rate of conidiation in brlA12 does not change on addition of putrescine, spermidine or spermine to the growth medium. To exclude the possibility that this negative effect might be due to permeability barriers, at least for spermidine and spermine, the uptakes of all three polyamines were investigated in the wild type and mutants. No differences in the uptake capacity of wild type and brlA12 or rev-2 were found; also spsA1 which has 30-50% higher uptake than the wild type for putrescine and spermidine did not change the phenotype of brlA12 in the double mutants.

One of the connections of brlA12 and its modifiers to polyamines is the pink pigmentation observed in brlA12 and also rev-2 on media supplemented with nitrate and putrescine (less pigment is formed with spermidine). This pigment, however, exists in all brl strains tested which, together with other evidence, suggests that it

might be related to conidiation processes. Metabolically the pigment can be postulated to be a catabolic product of putrescine, however considering the evidence that putrescine is not catabolised in presence of nitrate, it might be an unknown biosynthetic product with nitrate or nitrate metabolism derivatives also being involved in its biosynthesis. Since it is found in the brl strains which are defective at early stages of conidiation it might accumulate in such strains while in the wild type whatever is produced from putrescine may be used in the later steps of the conidiation process (aba strains, defective at the late stages of conidiation, do not produce the pigment.) If this is the case however, the reasons why rev-2 is also pink in the presence of putrescine are not clear. It is probable that this mutant has abnormally high internal putrescine pools and the pigment might represent an attempt of this strain to reduce the level of putrescine by converting it to pigmentation which, however, might be deleterious to conidiation.

The putrescine oxidase and transaminase activities do not seem likely to be involved in the above processes since they are related to putrescine catabolism and in any case no differences in these activities were detected between the wild type and brlA12 or rev-2.

Studies of the brlA12 variegation in terms other than polyamine metabolism should also be considered since although polyamines at the moment appeared as the most promising candidate for an effect on the variegation, they may be only one component of the system.

Methylamine, like salt, had a remarkable effect on the variegation and although it is an amine, it might have a completely different mode of action which should be investigated.

Although a great many modifiers have been isolated, their contribution to these investigations so far is less than that expected. Enhancers might be the result of deleterious effects on conidiation probably not related to the variegation; suppressors, however, should be more informative. Some evidence suggests that the isolated suppressors may affect chromatin structure in general rather than conidiation specifically. A locus with similar effects on heterochromatization has been identified in Drosophila (Spofford, 1967, 1976) suppressing the variegation of the w^m gene of that organism. With Aspergillus however the situation is more advantageous since the study of other phenotypic effects exhibited by the suppressors is possible. Indeed a number of biochemical defects were found, some of them obviously correlated to the mode of variegation, their study however did not lead to a consistent pattern. In the case of galactose utilization, iproniazid, the amine oxidase inhibitor, improved to some extent, the utilization of galactose by gal$^-$ suppressors. The situation may be related to that found in yeasts where UDP-galactose epimerase, one of the enzymes involved in the conversion of galactose to glucose, is activated by polyamines. It is therefore possible that the connecting factor in the modification of the variegation and galactose utilization is provided by polyamines or perhaps other cations.

Possibly there exist a variety of ways to influence and alter the conidiation of brlA1^2 which make it difficult to envisage a general mode of modifying action. This is also shown by the number of other loci which are capable of shifting the variegation in either direction. While the suppressors available do not affect other brl

mutants, it is not known whether they will act on variegated position effects in general, since brlA12 is the only mutant of this type.

It is hoped that further studies on the above lines of investigation, together with the recent progress on the molecular structure of the chromosomes will yield evidence enough to clarify the brlA12 variegation and possibly position effects in general.

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