STUDIES ON L-ASPARAGINE METABOLISM IN ASPERGILLUS NIDULANS

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

by Constantine Drainas

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to Virginia

and my parents

CONTENTS

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DECLARATION

I certify that this thesis does not contain any material previously published or written by anyone else except where explicitely stated in the text.

Constantine Drainas B.Sc.

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ABBREVIATIONS

ADP	Adenosino-diphosphate
AH	DL-aspartic acid-β-hydroxamate
asn	L-asparagine
ATP	Adenosino-triphosphate
CPM	counts per minute
GDH	glutamate dehydrogenase
gln	L-glutamine
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dimucleotide phosphate
NADPH	reduced form of NADP

CORRECTION

On tables, figures, or plates for asp20, asn^{-}1, gln^{-}1 read asp$\underline{A}$20, asn^{-}A1, gln^{-}A1.

SUMMARY

A number of mutants (*ahrA*), resistant to the toxic analogue of L-asparagine, DL-aspartic acid-β-hydroxamate, have been isolated and shown to lack asparaginase I activity. The level of aspartic hydroxamate resistance is correlated with asparaginase I production, strongly resistant mutants have no detectable enzyme activity and weakly resistant ones have low enzyme activity. The enzyme activity of various heterozygous diploids containing the mutants indicated that gene expression is under strict dosage effect.

It is proposed that asparaginase I is coded by the *ahrA* structural gene and asparaginase I activity is regulated by ammonia. This ammonia regulation requires protein synthesis and is either inactivation of enzyme activity *in vivo* or repression of enzyme synthesis or both.

A mutant epistatic to *ahrA* and *tamA* (a regulator gene responsible for the expression of a number of ammonia regulated systems), has been isolated. The existance of this mutant, named *aspA20*, and the fact that the *ahrA* mutants and the *tamA50* mutant (completely repressed allele of the *tamA* locus) can utilise L-asparagine as a nitrogen source and as a carbon source respectively, strongly suggests the involvement of more than one enzyme in the utilisation of L-asparagine.

An active transport system for L-asparagine has been

detected in A. nidulans. This transport system is probably under nitrogen metabolite and carbon catabolite control.

It is possible that an L-asparagine synthetase exists in A. nidulans which is coded by the asn⁻A1 gene.

CHAPTER I INTRODUCTION

INTRODUCTION

1. The use of Aspergillus nidulans for scientific research

Aspergillus nidulans is a homothalic fungus, belonging to the class Ascomycetes, subclass Euascomycetes, of the order Plectomycetes (Fincham and Day 1963). Its reproduction includes both asexual and sexual cycles. The asexual cycle results in the formation of uninucleate haploid conidia, while the sexual cycle results in a fruit body, called the perithecium, or more accurately cleistothecium, in which the asci are formed. In these asci the ascospores are enclosed.

Pontecorvo first used A. nidulans for genetical experimentation (Pontecorvo et al., 1949, Pontecorvo and Roper 1952, Pontecorvo et al., 1953). The advantages of A. nidulans for genetical work are: the ease of formation of heterocaryons and diploids, the genetic analysis which can be carried out since the organism has a normal sexual cycle, the excellent conidial colour markers, the easy and cheap culture and manipulation. Thus, powerful microbial techniques can be used in this organism, although it is a eucaryote with well defined organelles such as a nucleus (which includes chromosomes, nucleolus, histones and nuclear membrane), mitochondria, cytoplasmic reticulum etc. An excellent review of A. nidulans genetics is that of J. A. Clutterbuck (1973).

2. Genetic regulation in procaryotic and eucaryotic micro-organisms

The DNA of an organism contains all the information for the synthesis of the protein molecules required for growth and development. However the expression of the information is not uniform; different genes may be active under different conditions in bacteria and in different cell types of eucaryotes.

In many bacterial systems the enzymes that determine a single metabolic pathway are produced from a group of genes that constitute a genetic unit of function called an operon by Jacob and Monod (1961). Epstein and Beckwith (1968) define the operon as a group of adjacent structural genes, showing coordinate expression, and their closely associated controlling sites. Controlling sites are elements which determine the expression of only those genes to which they are attached. The operon is transcribed as a complete unit from the one end to the other. Transcription starts from the promoter sites which are close to the first structural gene of the operon. The structural genes are concerned solely with elaborating proteins required by the cell. This gene expression is controlled by the action of regulator molecules coded for by the regulator genes. The product of the regulator genes interacts at the operator site, affecting the transcription of the structural genes. Gilbert and Muller-Hill (1966) isolated one such regulator molecule which was shown to be a protein.

When the regulator protein prevents gene expression the control is called negative, e.g. in the lactose operon (Jacob

and Monod 1961) in Escherichia coli. Positive control is where the product of the regulator gene is necessary for the expression of the structural genes e.g. the arabinose operon in E. coli (Englesberg et al., 1969) and probably some of the systems of fungi. Many pathways consist of more than one operon. The term regulon has been suggested for systems in which widely separated operons show similar although usually not coordinate expression (Epstein and Beckwith 1968).

Induction is the stimulation of the synthesis of enzymes coded by the structural genes, only in the presence of specific molecules which may be substrates. Induction might be caused either by activation of an inactive precursor form of the enzyme already present in the cell, or by the de novo synthesis of new enzyme molecules. Examples of inducible systems are the lactose operon and the arabinose operon in E. coli. Repression is the specific inhibition of enzyme synthesis. An example of a repressible system is the tryptophan synthetase in Aerobacter aerogenes (Monod and Cohen-Bazire 1953). The term effector has been proposed for the compound which acts as the physiological signal to alter the rate of specific enzyme synthesis by repressing or inducing. Calvo and Fink (1971) proposed that some proteins have a dual role, functioning both as enzymes and effectors.

Although the regulation of gene expression is now relatively well established in bacteria, knowledge of regulation in simple eucaryotes until very recently was limited. In simple eucaryotes the clustering of genes is relatively infrequent

compared to that in bacteria. Examples of these clusters or operons are the histidine-3 region of Neurospora crassa (Ahmed, Case and Giles 1964), the galactose system in yeast (Douglas and Hawthorne 1966), the arom system in N. crassa (Giles, et al., 1967) and the quinic acid system in N. crassa (Rines 1968, Chaleff 1971, Valone et al., 1971).

It is interesting that most of the fungal systems resemble the positive controlled arabinose system rather than the negative controlled lactose system of E. coli. Over the last few years excellent reviews about fungal regulatory systems have been published including those of Gross (1969), Shapiro and Stadtman (1970), Beckwith and Rosson (1974), Metzenberg (1972), Englesberg and Wilcox (1974), Davis (1975), Calhoun and Hatfield (1975) and Pateman and Kinghorn (1976a).

3. Genetic regulatory mechanisms in Aspergillus nidulans

a. Inducible systems

Nitrate reductase is subject to nitrate induction (Cove 1966). A. nidulans cells produce high levels of this enzyme in presence of nitrate and low levels in its absence. The enzymes nitrate reductase and nitrite reductase responsible for nitrate reduction are coded by the structural genes niaD and niiA respectively (Pateman and Cove 1967, Cove and Pateman 1969). These genes are closely linked on linkage group VIII and they are regulated in a positive way by the nirA gene, also mapping on linkage group VIII. Deletions have been isolated which extend from within the niaD structural gene into the neighbouring niiA gene (Cove 1976).

Other examples of inducible systems are the acetamidase and formamidase systems (Hynes 1970, Hynes and Pateman 1970 a & b), the induction of the enzymes of purine degradation pathway by hypoxanthine (Darlington, Scazzocchio and Pateman 1965, Scazzocchio and Darlington 1968, Scazzocchio 1973 a & b).

b. Repressible systems

Nitrogen metabolite repression: Ammonia as a control metabolite of nitrogen metabolism (e.g. as the end product of many deaminating reactions), acts as an effector regulating a number of enzyme and transport systems which are closely connected with nitrogen assimilation. In the presence of ammonia the enzyme

activities or the rate of transport systems are low. These systems include: nitrate reductase (Pateman and Cove 1967), xanthine dehydrogenase (Scazzocchio and Darlington 1968), acetamidase (Hynes and Pateman 1970 a & b), L-glutamate uptake (Kinghorn and Pateman 1972, Pateman, Kinghorn and Dunn 1974), urea uptake (Dunn and Pateman 1972) extracellular protease (Cohen 1972) and histidase (Polkinghorne and Hynes 1974). Consequently ammonia protects against the toxicity of many nitrogen metabolite analogues by repressing the relevant enzyme or uptake system.

Mutants altered in respect with ammonia regulation have been isolated. Such mutants can produce enzyme and/or uptake activity in the presence of ammonia. These ammonia derepressed mutants include the loci meaA (Arst and Cove 1969, Arst and Page 1973), DER-3 a modified meaA (Pateman et al., 1974) xprD (Cohen 1972) and gdhA1 (Kinghorn and Pateman 1973).

Two regulatory loci have been proposed, the products of which play a role in ammonia repression. These are the areA (Arst and Cove 1973) and tamA (Kinghorn and Pateman 1975 c) genes. Completely repressed, partially repressed and derepressed mutants have been isolated in both loci. The relationship between areA and tamA in ammonia repression and their possible functions are reviewed by Pateman and Kinghorn (1976 a).

Carbon catabolite repression: This is the repression of the enzymes involved in carbon metabolism by glucose and certain other readily utilizable carbon sources. Although the mechanism of carbon catabolite repression is relatively well understood in

bacteria (Buettner et al., 1973, Brickman et al., 1973), little is known in eucaryotic cells. In A. nidulans mutations are known in a locus named creA (Arst and Cove 1973, Bailey and Arst 1975) which relieve carbon catabolite repression. $creA^{d}$ mutations allow ethanol to serve as a source of acetate for pyruvate dehydrogenase less strains in the presence of carbon catabolite repressing carbon sources. Page (1971) and Page and Cove (1972) have shown that ethanol is converted to acetate via acetaldehyde in two enzymatic steps and that at least the first, alcohol dehydrogenase, is subject to carbon catabolite repression. Hynes (1970) and Hynes and Pateman (1970 a) have shown that acetamidase, which converts acetamide to acetate, is also subject to carbon catabolite repression.

c. Gene clusters and control regions

In A. nidulans gene clustering is rather rare. The first demonstration of a possible operon was a gene cluster responsible for the proline catabolism described by Arst and MacDonald (1975). Another possible operon is the urea cluster described by Pateman and Kinghorn (1976 b) and Kinghorn and Pateman (1976). Three tightly linked mutants have been isolated in the order url-uX-uru. url is a mutant in a control region and has derepressed urease activity and urea transport in the presence of ammonia. uX is a gene responsible for urease activity and uru gene is responsible for urea transport. Two other genes are required for urease activity, uY and uZ, which are unlinked to each other and with uX.

Mutations in the control region uap-100 (Arst and Scazzocchio 1975) have been isolated, adjacent to a putative structural gene, uapA, coding for the uric acid-xanthine permease. Also a control mutant affecting increased induction of acetamidase synthesis has been isolated by Hynes (1975). An operator type mutant, sBo, closely linked to the sB structural gene for sulphate permease has been isolated by Lukaszkiewuz and Paszewski (1976). This mutation causes hypersensitivity to repression by sulphur amino acids.

d. Transport systems

In A. nidulans the regulation of transport systems has been studied, such as phenylalanine uptake (Sinha 1969) urea uptake (Dunn and Pateman 1972), ammonium and methylammonium uptake (Arst and Page 1973, Pateman et al., 1974) and L-glutamate (Kinghorn and Pateman 1972). Mutants deficient in amino acid transport (aau) have been isolated (Kinghorn and Pateman 1975 b). These mutants are unable to utilize L-glutamate as a sole carbon and nitrogen source.

4. Asparagine metabolism

Asparagine metabolism includes four main areas: a) synthesis from aspartic acid and ammonia, b) deamination by asparaginase, c) transportation into the cell and d) incorporation into proteins (for review see Cooney and Handschumacher 1970). This thesis concerns itself mainly with a), b) and c).

a. L-Asparagine synthetase

L-Asparagine synthetase has been detected in cell extracts of the bacteria Escherichia coli (Cedar and Schwartz 1969 a & b) and Lactobacillus arabinosus (Ravel et al., 1962), as well as in cell extracts of higher organisms such as in chicken embryo liver (Arfin 1967) and in Novikoff Hepatoma tumor cells transplanted into the peritoneal cavity of rats (Patterson and Orr 1968).

b. L-Asparaginase

L-Asparaginase or L-asparagine amidohydrolase is an amidase (E. C. 3.5.1.1.) normally decomposing L-asparagine. The products of this catalytic activity are L-aspartate and ammonia. Over the last ten years asparaginase has been the subject of more than two hundred papers and excellent reviews (Cooney and Handschumacher 1970, Capizzi et al., 1970, Wriston and Yellin 1973).

Sources of isolation: The first report about ferments which released a volatile base (NH_4^+) from L-asparagine were made by Lang in 1904 (for review article see Capizzi et al., 1970). As early as 1909, Dox detected asparaginase in preparations from yeast and moulds. Since then asparaginase has been detected and isolated in the extracts of cells of many organisms such as in vertebrates (Furth and Freidmann 1910), in guinea pig serum (Clementi 1922), in plants (Grover and Chibnal 1972), in gram-negative bacteria (Altenbern and Housewright 1954), in human and animal sera (Lee and Bridges 1968), in gram-negative and positive bacteria, yeast and filamentous fungi (Imada et al., 1973).

Two L-asparaginases have been found in E. coli, which differ in a number of properties such as solubility, chromatophoretic behaviour, pH optima and affinity for L-asparagine. These two L-asparaginases were called EC-1 and EC-2 (Mashburn and Wriston 1964) or I and II (Schwartz et al., 1966). L-Asparaginase I has low activity, low affinity for L-asparagine (Campbell et al., 1967) and locates within the spheroplasts in the cytoplasm, while asparaginase II has high activity, high affinity for asparagine and locates near the cell surface in the periplasmic region, between the bacterial plasma membrane and the cell wall (Cedar and Schwartz 1967). L-Asparaginase II is formed under anaerobic conditions helped by high concentrations of amino acids and with low production in presence of sugars (Cedar and Schwartz 1968). L-Asparaginase II of E. coli is a tetramer of four 65,000 dalton subunits (Kirschbaum et al., 1969). Ho et al. (1969 and 1970) crystallized partially purified L-asparaginase from E. coli.

An intracellular L-asparaginase have been found in Saccharomyces cerevisiae cell extracts. It is apparently synthesized constitutively and the level is not affected by ammonia or L-aspartate. The synthesis of L-asparaginase in S. cerevisiae is necessary for the utilization of L-asparagine as nitrogen source as indicated by mutants lacking L-asparaginase activity which are unable to grow on asparagine (Jones and Mortimer 1970, 1973 and Jones 1973). These mutants map at the aspl locus which is the structural gene for S. cerevisiae L-asparaginase. In addition, S. cerevisiae has an extracellular asparaginase. The appearance of the extracellular L-asparaginase is stimulated by nitrogen starvation and requires energy sources and protein synthesis (Dunlop and Roon 1975). Arima et al. (1972) also detected extracellular asparaginase in other yeasts and certain fungi.

In Pseudomonas sp. asparaginase is induced by L-glutamic acid and inhibited by a number of amino acids and especially by L-alanine and L-proline (Mardashev et al., 1969). Bascomb and Bettelheim (1975) working with E. coli, Erwinia carotovora, Citrobacter sp. and Chromobacterium violeceum found immunologically related asparaginases in E. coli and Citrobacter sp.

Antitumor activity and toxic effects: Great interest was aroused by the discovery that asparaginase may have antitumor activity. First Kidd (1953 a & b) observed that guinea pig serum had antilymphoma activity against lymphosarcoma 6C3HED in C3H mice and lymphoma II in albino A mice; other lymphomas were not affected. He recognized the importance of the discovery by noting "The finding

provides an example, unique this far, of a naturally occurring substance that brings about regression of cancer cells in living animals without doing obvious harm to the latter." Broome (1963 a & b and 1965) proved that asparaginase was responsible for the antitumor activity. Mashburn and Wriston (1963 and 1964) described the tumor inhibitory effects of asparaginase. Khan and Levine (1974) found that asparaginase inhibited the hyperacute form of the experimental allergic encephalitis in rats.

Sobin and Kidd (1965) observed that in vitro incorporation of ^{14}C L-valine into cells of the Gardner lymphosarcoma was dependent on the level of L-asparagine in the medium, while an asparaginase resistant subline did not require exogenous asparagine for protein synthesis. In further experiments Kidd and Sobin (1966) and Sobin and Kidd (1966) showed that protein synthesis in Gardner's lymphosarcoma, grown in the ascites form, stopped sharply within 15 minutes after the intraperitoneal injection of guinea pig serum; this is due to the conversion of the available L-asparagine of the host to L-aspartic acid which cannot be assimilated by the lymphoma cells. Asparaginases with high affinity for asparagine are those which possess antitumor activity, such as the asparaginase II from E. coli (Campbell et al., 1967) and Erwinia carotovora (Cammack et al., 1972). Later many toxic effects have been noted during clinical trials with L-asparaginase, including falls in serum albumin, hemoglobin, lipoprotein and fibrinogen (Haskell et al., 1969) and in liver function (Gross et al., 1969, Canellos et al., 1969).

In the genus Aspergillus, asparaginase with antitumor activity has been found in Aspergillus terreus (De-Angeli et al., 1970). Assuming that A. nidulans has an asparaginase with relatively high affinity for asparagine, then high yielding strains might have some therapeutic application.

c. L-Asparagine transport

The transport system of L-asparagine has not been extensively studied so far. Reports have been published about active transport systems of asparagine in Escherichia coli, Lactobacillus plantarum and Streptococcus faecalis. In E. coli a highly specific constitutive system has been described (Willis and Woolfolk 1970 and 1975), characterized by two kinetic components with different Km values. The E. coli transport system is energy dependent and strains lacking asparaginase activity could accumulate asparagine intracellularly some 100-fold above the external medium. Diazo-oxo-norvaline and aspartic hydroxamate proved to be competitors of asparagine.

In L. plantarum and S. faecalis the L-asparagine transport system was stimulated by glucose (Holden and Bunch 1973). The concentration of L-asparagine in the cells was at least 8-fold compared with the extracellular concentration. It was proposed that there is more than one system in the above micro-organisms catalysing the entry of asparagine in the cell. The transport systems of the two bacterial species differ in structure specificity. In L. plantarum L-glutamine acts as a competitor with L-asparagine, while in

S. faecalis neutral amino acids were effective competitors with L-asparagine. There are no reports for L-asparagine transport in A. nidulans so far. Forbes studying the asparagine transport system (personal communication) found that it was active and that approximately 10 nanomoles of asparagine were taken up within 10 minutes per mg of dry weight by cells grown on nitrate as the sole nitrogen source.

CHAPTER II MATERIALS AND METHODS

MATERIALS AND METHODS

1. Media and supplements

a. Media

The media used were essentially those described by Pontecorvo, Roper, Hemmons, MacDonald and Bufton (1953) and Cove (1966).

Minimal medium (MM)

Sodium nitrate	6.00 g
Potassium chloride	0.52 g
Magnesium sulphate	0.52 g
Ferrous sulphate	2.00 g
Zinc sulphate	2.00 g
Potassium dihydrogen orthophosphate	1.52 g
D-glucose	10.00 g
Agar No. 3-oxoid	12.00 g

Volume made up to 1 litre with distilled water and pH was adjusted to 6.5 with 1 N sodium hydroxide.

Complete medium (CM)

Sodium nitrate	6.00 g
Potassium chloride	0.52 g
Magnesium sulphate	0.52 g
Potassium dihydrogen orthophosphate	1.52 g
Distilled water	250 ml

To the above mixture was added

peptone	2 g
yeast extract	1 g
casein hydrolysate	5 ml
D-glucose	10 g
Agar No. 3-oxoid	12 g
vitamin solution	1 ml

Made up to 1 litre with distilled water and the pH adjusted to 6-6.2 with 1 N sodium hydroxide. The vitamin solution was added after the adjustment of the pH. The medium was supplemented with ammonia at a final concentration of 10 mM before pouring when appropriate.

Carbon less base minimal medium (BM)

As described for minimal medium without adding the D-glucose component.

Sulphate less base minimal medium (-S medium)

As described for minimal medium without adding the sulphate components.

Nitrogen and carbon less minimal medium (-CN medium)

Nitrogen less salts solution	20 ml
agar No. 3-oxoid	12 g

Volume made up to 1 litre with distilled water and pH adjusted to 6.5 with 1 N sodium hydroxide.

Nitrogen less minimal medium (-N medium)

nitrogen less salts solution	20 ml
D-glucose	10 g
agar No. 3-oxoid	12 g

Volume made up to 1 litre with distilled water and the pH adjusted to 6.5 with 1 N sodium hydroxide.

All media were sterilised by autoclaving, kept solid in conical flasks and melted before use.

Nitrogen less salts solution

potassium chloride (KCl)	26 g
magnesium sulphate ($MgSO_4$ $7H_2O$)	26 g
potassium dihydrogen phosphate (MH_2SO_4)	76 g
trace elements solution	50 ml

Made up to 1 litre with distilled water.

2 ml chloroform was added as a preservative, and the solution stored at 4°C.

Trace elements solution

sodium borate ($Na_2B_2O_7.10H_2O$)	40 mg
copper sulphate ($CuSO_4.5H_2O$)	400 mg
ferric orthophosphate ($FeSO_4.1H_2O$)	800 mg
manganese sulphate ($MnSO_4.4H_2O$)	800 mg
sodium molibdate ($NaMoO_4.2H_2O$)	800 mg
zinc sulphate ($ZnSO_4.7H_2O$)	8 mg

Made up to 1 litre with distilled water.

Vitamin solution

para-aminobenzoic acid	40 mg
aneurin HCl	50 mg
biotin	1 mg
inositol	400 mg

nicotinic acid	100 mg
calcium D-panothenate	200 mg
riboflavin	100 mg
pyridoxine	50 mg

Made up to 1 litre with distilled water, kept sterile at 4°C.

b. Supplements

These were kept as sterile concentrated aqueous solutions at 4°C. The appropriate amount of supplement was added to the molten medium before pouring.

Supplement	conc. of stock solution	amount per 100 ml medium	final conc.
Vitamins			
Biotin	40 ng/ml	0.1 ml	0.04 ng/ml
nicotinic acid	2000 ng/ml	0.1 ml	2.00 ng/ml
pyrodoxine HCl	1000 ng/ml	0.1 ml	1.00 ng/ml
riboflavin	200 ng/ml	0.1 ml	0.20 ng/ml
p-aminobenzoic acid	1 mg/ml	0.1 ml	1.00 µg/ml
Toxic agents			
aspartic hydroxamate	20 mM	as specified in the text	

glutamic hydroxamate	20 mM	as specified in the text	
p-fluoro-phenylalanine (for haploidization)	1%	1.75 ml	0.0125%
p-fluoro-phenylalanine (for toxicity tests)	1%	2.5 ml	0.025 %
D-serine	1 M	0.5 ml	5 mM
methylammonium	-	6.75 g	1 M
thiourea	1 M	1 ml	10 mM
chlorate	-	1.2 g	100 mM
sodium azide	100 mM	1 ml	1 mM
cycloheximide	-	1 mg	10 µg/ml

Nitrogen sources

nitrate	1 M	as specified in the text
nitrite	1 M	"
ammonia (ammonium tartrate)	1 M	"
ammonia (ammonium chloride)	1 M	"
L-sodium glutamate	1 M	"
L-sodium aspartate	1 M	"
L-alanine	1 M	"
L-arginine	1 M	"
L-proline	1 M	"
L-glutamine	0.2 M	"
L-asparagine	0.2 M	"
urea	1 M	

For growth or test on ammonia as nitrogen source ammonium tartrate was used, unless otherwise specified in the text.

Others

sodium thiosulphate	200 M	1 ml	2 mM
sodium deoxycholate	16%	0.5 ml	0.08%
sodium acetate	50%	2.5 ml	1.25%
galactose	20%	2.5 ml	0.5 %
glycerol	-	0.8 ml	1%

Solid media in petri dishes

Disposable plastic petri dishes were used throughout. For most purposes 20 ml of solid media was added to each petri dish.

Chemicals

Analytical grade chemicals were used whenever possible.

^{3}H-L-asparagine	500 µCi/ml stock solution
^{14}C-L-asparagine	50 µCi/ml stock solution

2. Enzyme assays

a. Growth of mycelium

The technique was basically that described by Cove (1966). Strains for the inoculation of the growth flasks were grown either on plates or on slopes containing complete medium (solid) for 14-20 days. Conidia were scraped off the surface and resuspended in 10 ml of sterile distilled water containing a few drops of Tween 80. Clumps of conidia were separated by vigorous shaking. Conidia from one plate or two slopes were used for suspension in 10 ml of water. This was used to inoculate 1 litre Ehrlenmeyer flasks containing 200 ml of -N medium or -CN medium. The nitrogen and carbon sources and growth supplements were added at the time of inoculation and described in the text. Approximately 1 ml of thick conidial suspensions were added to each flask. The mycelium was grown at 30°C in a Gallenkamp orbital incubator. After approximately 17 hours the mycelium was harvested by filtration through a nylon net cloth, washed with distilled water, blotted dry on absorbant paper towels and weighed. The pressed wet weight was usually in the range 3-8 g/l. The mycelium was used immediately for the enzyme assays.

When mycelium was pregrown before transfer to a treatment medium (carbon free, nitrogen free, etc.) it was grown on glucose -N medium, supplemented as specified in the text, for 17 hours, harvested through a cheese cloth, washed with treatment medium and transferred to this fresh treatment medium. After the desired time of treatment, the mycelium was harvested and used as above.

b. Preparation of cell free extracts

0.5 g of frozen pressed mycelium was ground in a cold mortar with approximately 0.1 g cold sand (M & B acid washed sand medium fine) and 5 ml of cold buffer (0.5 M tris hydroxymethyl (amino methane)-HCl buffer pH 7.2 + 1 mM Clealand's reagent (dithiothreitol)) for 2 min. The slurry was centrifuged for 20 min. at 20,000 RPM (31,748 g) at $4^{o}C$ in a Beckman model L-2 ultracentrifuge. The supernatant was kept cold in ice and used for protein determinations and enzyme assays.

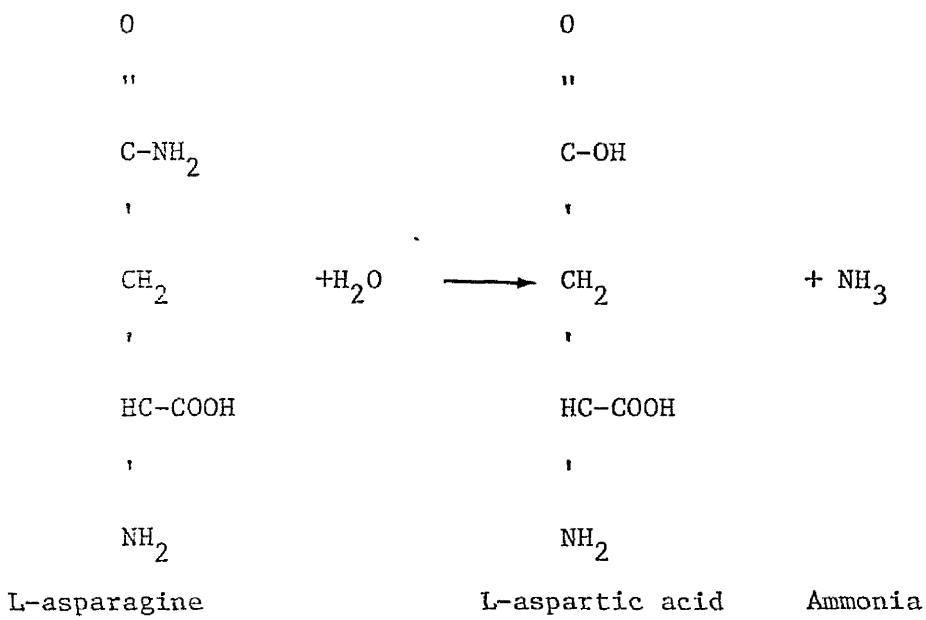
c. Asparaginase activity and assay methods

The normal catalytic action of L-asparaginase is to decompose L-asparagine, producing L-aspartic acid and ammonia. In addition DeGroot and Lichtenstein (1960 a and b) and Ermann et al. (1971) reported an aspartyl-transferase activity of asparaginase, forming aspartic hydroxamate from L-asparagine and hydroxylamine as well as producing hydroxylamine from aspartic hydroxamate. Miller and Earlbalis (1969) proposed that asparaginase possesses glutaminase activity. Recently Resnick and Magasanik (1976) reported that asparaginase from Klebsiella aerogenes possesses aspaspartyl-transferase, glutamyl-transferase and glutaminase activity as well. Its formation is activated by glutamine synthetase; is not reduced by L-asparagine and is not carbon catabolite repressible. Cedar and Schwartz (1967) have proposed that asparaginase inhibits protein synthesis in cell free extracts.

Many methods have been devised to assay asparaginase activity. Briefly they are based on a) ammonia determination (Meister 1955, Wriston 1970, Schwartz et al., 1970, Cammack et al., 1972), b) L-asparagine disappearance (Howard and Carpenter 1972) and c) on disappearance or formation of aspartic hydroxamate (Ermann et al., 1971).

Amino-hydrolase activity

This activity was assayed following the deamination of L-asparagine and estimating the amount of ammonia or L-aspartic acid produced:



In the case of aspartic acid production only a qualitative determination was made. (Fig. 6).

Ammonia determination by Nessler's reagent. The reaction mixture was: 0.5 ml of 200 mM aqueous solution of L-asparagine (final concentration 50 mM); 1 ml of 0.5 M tris-hydroxymethyl (amino methane) buffer pH adjusted to 8 with HCl (Tris-HCl buffer final concentration 250 mM); 0.5 ml of cell free extract at a final volume 2 ml. The reaction mixture was incubated in a water bath with the temperature set up at 37°C and was let to run for 30 min. The reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA) and the precipitated protein was removed by centrifuging at 5,000 revolutions per minute (RPM, 2,000 g) in an MSE bench centrifuge. The determination of the ammonia produced was made by colour developing with the Nessler's reagent (Imada et al., 1973). In 3.7 ml of distilled water 0.2 ml of sample and 0.1 ml of Nessler's reagent were successively added. Colour was left to be developed for 20 min in room temperature and reading took place at 450 nm in a Unicam SP 1800 spectrophotometer. The results were expressed as nanomoles of ammonia/30 min. Serial concentrations of ammonium tartrate were used to make a standard curve.

Aspartic acid production: The reaction mixture and the time and temperature of incubation were as above, except ^{14}C-L-asparagine was added (10 µl of 50 µCi/ml stock solution, to give about 2,500 CPM). The reaction was stopped by transferring the reaction tubes into ice water. 50 µl samples were spotted on 3mm Whatman chromatography paper (breadth 3 cm). The separation of aspartic acid from asparagine was carried out by low voltage vertical

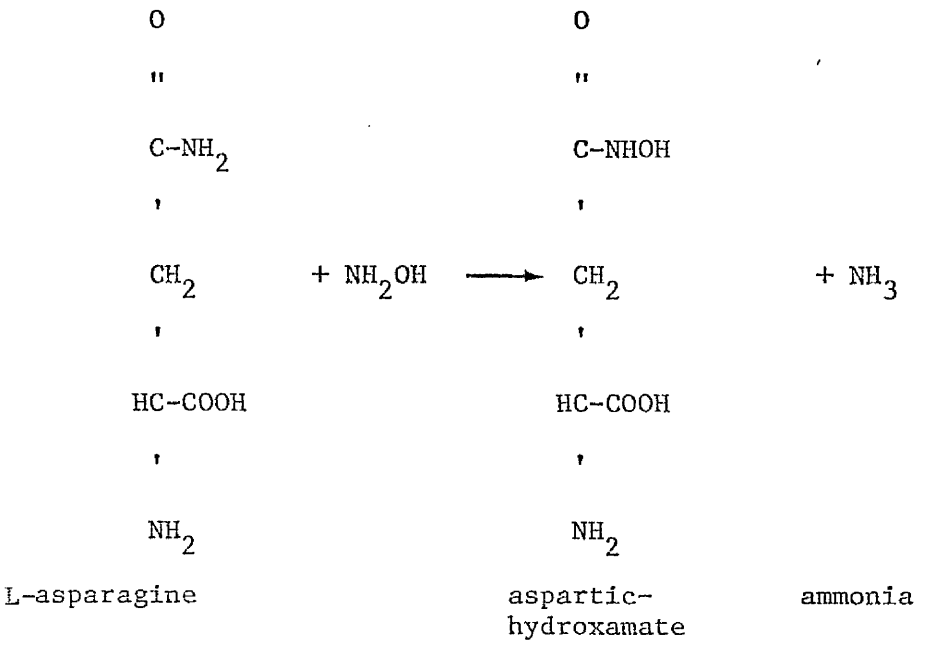
ionophoresis: the method was basically that of Evered (1959). After ionophoresis for 4 hours at 150 volts (current 20 mA) in tank buffer pH 4 (40 mM potassium hydrogen phthalate) the chromatogram was dried and the chromatography paper cut into equal portions in vials with scintillation fluid NE 250 and the radioactivity was counted in a Beckman scintillation counter. The results were plotted in a histogramme (Fig. 6).

Change of conductivity: As it is mentioned above, ammonia is produced by the catalytic action of L-asparaginase from L-asparagine. In aqueous solutions the ammonia molecules form ammonium ions (NH_4^+). The production of these ions results in change of the conductivity of the aquatic mixture.

Based on this principle, asparaginase activity in cell extracts can be detected and accurately measured. The method of measuring enzyme activity by the change of the conductivity due to the ionic form of the products, is described by Lawrence and Moores (1972) and Lawrence et al. (1974).

To measure this asparaginase activity, cells were extracted as described above by using a 10 mM Tris-HCl extraction buffer, pH 8. The reaction mixture was 1 ml of the extraction buffer, degased before use, 30 µl of 20 mM stock solution L-asparagine (to give 0.6 mM final concentration), 5 µl of cell extract. Aspartic hydroxamate was also used as substrate in place of L-asparagine at a concentration 1.2 mM (70 µl/ml of a stock solution 20 mM).

Aspartyl-transferase activity. This activity was assayed following the formation of aspartic hydroxamate by replacement of the β-amino group of L-asparagine with hydroxylamine (DeGroot and Lichtenstein 1960 a & b, Ehram et al., 1971):



The reaction mixture was: 0.2 ml of 200 mM aqueous solution of L-asparagine (final concentration 20 mM); 0.2 ml of 1 M aqueous solution of hydroxylamine (final concentration 100 mM) neutralized just before use with 5N potassium hydroxide; 1.5 ml of 20 mM tris-hydroxymethyl (amino methane)-HCl buffer, pH 8; 0.1 ml of cell free extract in a total volume of 2 ml. The reaction mixture was incubated in a water bath set up at 37°C. After 30 min the reaction was stopped and colour was developed by adding 0.5 ml of ferric chloride reagent (2/3 N HCl, 10% $FeCl_3$, 5% trichloroacetic acid). The brown colour due to aspartic hydroxamate formation in reaction mixtures with and without asparagine was measured at 500 nm in a Unicam SP 1800 spectrophotometer and enzyme units were

expressed as nanomoles of aspartic hydroxamate/min/mg of protein. Serial concentrations of DL-aspartic acid-β-hydroxamate were used to make a standard curve. In my hands the above assay was the most accurate and convenient to investigate the regulation of asparaginase synthesis and it was routinely used.

d. L-asparagine synthetase assays

These were based on the aspartic hydroxamate formation from 1) asparagine, hydroxylamine and ADP, 2) aspartic acid, hydroxylamine and ATP.

1) This assay was a modification of that used by Pateman (1969) to assay glutamine synthetase (see below) by using L-asparagine in place of L-glutamine. The reaction mixture was the following: 0.2 ml of 200 mM aqueous solution of L-asparagine (final concentration 20 mM); 0.2 ml of 1 M aqueous solution of hydroxylamine (final concentration 100 mM neutralized before use with 5N potassium hydroxide; 0.1 ml of 0.4 M sodium arsenate (final concentration 20 mM); 0.1 ml of 60 mM manganous chloride ($MnCl_2$ final concentration 3 mM); 0.1 ml of 20 mM ADP (final concentration 1 mM); 1.2 ml of 20 mM tris hydroxymethyl (aminomethane)-HCl buffer pH 7.8; 0.1 ml of cell free extract at a final volume 2 ml. The reaction mixture was incubated as above. After 30 min the reaction was stopped and colour was developed by adding 0.5 ml of ferric chloride reagent and reading took place as above.

2) This assay was essentially that described by Ravel, Norton, Humphreys and Shive (1962). The reaction mixture was:

0.1 ml of 60 mM aqueous solution of sodium aspartate (final concentration 3mM); 0.2 ml of 0.5 M aqueous solution of hydroxylamine (final concentration 50 mM) neutralized before use as above; 0.2 ml of 100 mM magnesium acetate (final concentration 10 mM); 0.2 ml of 200 mM mercaptoethanol (final concentration 20 mM); 0.2 ml of 30 mM ATP (final concentration 3 mM); 1 ml of 100 mM tris-hydroxymethyl (aminomethane)-HCl buffer pH 7.8; 0.1 ml of cell free extract in a total volume of 2 ml. The extraction buffer was 100 mM tris-hydroxymethyl (aminomethane) - HCl pH 8 plus 1 mM mercaptoethanol plus 20% glycerol. Incubation temperature and time was as above. Colour was developed by adding 3 ml of ferric chloride reagent into 0.5 ml sample of the reaction mixture and reading took place as above.

e. Glutamine synthetase assay

The method applied to assay glutamine synthetase activity was that described by Pateman (1969). The reaction mixture was: 0.6 ml of 0.3 M aqueous solution of L-glutamine (final concentration 90 mM) for more sensitive assays L-glutamine was added to a final concentration 0.2 M; 0.1 ml of 60 mM manganous chloride (final concentration 3 mM); 0.1 of 0.4 M sodium arsenate (final concentration 20 mM); 0.2 ml of 1 M neutralized hydroxylamine solution (final concentration 50 mM); 0.1 ml of 20 mM ATP (final concentration 1 mM); 0.8 ml of 20 mM tris-hydroxymethyl (aminomethane) buffer, pH 8; 0.1 ml of cell free extract at a final volume 2 ml. Incubation took place at 37^{o}C for 20-30 min. The reaction was

stopped and colour developed by adding 0.5 ml of ferric chloride reagent. The brown colour due to γ-glutamic-hydroxamate formation in the reaction mixture with and without L-glutamine was measured at 500 nm in a Unicam SP 1800 spectrophotometer. Specific activity is expressed as nanomoles of glutamic hydroxamate/min/mg protein.

f. L-glutamate dehydrogenase assay

NAD-GDH and NADP-GDH were assayed following the reductive amination of a α-oxoglutarate in the presence of ammonia and reduced NAD or NADP (Kinghorn and Pateman 1973). The initial reaction velocity was estimated from the change in optical density at 340 nm in a Unicam SP 1800 spectrophotometer.

The reaction mixture for the NADP-GDH assay was: 0.4 M ammonium chloride (NH_4Cl) in 50 mM phosphate buffer pH 7.75, 0.4 ml; 200 mM α-oxoglutarate in 50 mM phosphate buffer pH 7.75, 0.2 ml; reduced NADP 2 mg/ml, 0.2 ml; 50 mM phosphate buffer pH 7.75, 2 ml; enzyme extract 1-2 mg protein/ml, 0.2 ml. The reaction mixture for the NAD-GDH was: 0.4 M NH_4Cl in 50 mM phosphate buffer pH 8.0; 0.4 ml; 200 mM α-oxoglutarate in 50 mM phosphate buffer pH 8.0, 0.2 ml; reduced NAD 2 mg/ml, 0.2 ml; 50 mM phosphate buffer pH 8.0 with 0.1 mM 2-mercaptoethanol and 0.5 mM EDTA, 2 ml. The assay temperature in both cases was 37°C and the results expressed as nanomoles substrate transformed/min/mg/protein.

g. Protein determination

All protein determinations were carried out by the procedure of Lowry, Rosebrough, Farr and Randall (1951). In 0.2 ml of cell free extract diluted 1:4 1 ml of copper alkaline reagent (100 part of 2% Na_2CO_3 in 100 mM NaOH, 1 part of 2% sodium tartrate, 1 part of 1% copper sulphate, the mixture never kept for more than one day) was added. After 10 min, 0.1 ml of Folin's reagent (Folin and Ciocalteu's phenol reagent, BDH laboratory reagent) was added and colour was left to develop at room temperature for 30 min. The developed blue colour was read at 700 nm at a Unicam SP 800 spectrophotometer. Bovine serum albumin serial dilutions were used as a standard. The protein concentrations of most extracts were in the range 0.7 - 1 mg/ml.

h. Linear regression

All straight lines of graphs representing initial velocities or reciprocal plots, were drawn from a regression analysis. Any straight line graph can be expressed as the equation: $y = mx + b$, where x is a value on the x axis, y is a value on the y axis, m is the slope of the line and b is the y intercept. The slope (m) of the line is the ratio of its "rise" to its "run" and the intercept (b) is where the line crosses the y axis. When the slope and the intercept of a line is known the line can easily be drawn. The slope and the intercept of the lines to be drawn were calculated from the experimental data using a TI Programmable 57 calculator (Texas Instruments) by the program described on pages 9.14-9.19 of the manual.

3. Asparagine transport assays

a. Preparation of cells

Flasks were inoculated as above (page 22) and incubated at 30°C for 17 hours in a New Brunswick Controlled Environmental orbital incubator. After this period of incubation the cells were filtered through a millipore filtering apparatus of approximate volume 250 ml, washed with -CN medium preheated to 30°C, pressed gently with absorbent paper and weighed. As above the pressed wet weight was usually in the range 3-8 g/l. Finally these cells were used for the uptake assays. In certain cases it was necessary to treat the cells before harvesting and therefore the cells were resuspended in preheated (30°C) treatment medium for certain periods of time, as specified in the text. For the uptake assays 0.5 g of mycelium were suspended in uptake medium before use.

b. Uptake assay

When grown under conditions described above, Aspergillus nidulans is largely in the form of small colonies less than 1 mm in diameter. These colonies can be kept in suspension by shaking and quantitative samples can be withdrawn from the suspension. After growth or after treatment, 0.5 g of cells pressed wet weight was resuspended in a 250 ml Erlenmeyer flask in a shaking water bath at 25°C. The uptake flask contained a final concentration of L-asparagine 100 µM with approximate radioactivity 0.125 µCi. At 0, 2, 4, 6, 8 and 10 min. 10 ml aliquots of the cell suspensions were filtered with two washes of distilled water on a Millipore filter. The resultant

pad of cells was weighed and transferred to 5 ml NE 250 scintillation fluid and the radioactivity was measured in a Beckman Liquid Spectrometer. The rate of uptake of radioactivity into the cell is linear for the first 10 min. The counts per μmole were calculated by transferring 50 μl of the uptake medium (before the addition of the suspended cells) into the scintillation fluid and counting as above. The uptake capacity of the cells is expressed as nanomoles of substrate taken up per dry weight of cells. The dry weight is found by taking half (by wet weight) of the pad after filtration and heating at 100°C in an oven overnight (about 12 hours). Each piece of mycelium was taken out of the oven and weighed immediately to avoid any hydration of the dried mycelium, which results in increase of weight. The measured weight corresponds to the dry weight of the part of the mycelium used to count the radioactivity of the taken up substrate. To calculate the uptake capacity of the cells the following procedure was used:

i. Medium sample: Let us assume that 1800 are the counts per min (CPM) which correspond to the 50 μl of the uptake medium with the hot plus cold substrate. To find the CPM in 40 ml of medium we multiply 1800 x 40 x 20. The uptake medium before the addition of the suspended cells is 40 ml and the sample to be counted is 50 μl.

ii. To calculate nanomoles of substrate in 50 ml of medium:
100 μM = 100 μmoles/l = 5 μmoles/50 ml = 5×10^3 nanomoles/50 ml.

iii. To calculate the CPM equivalent to 1 nanomole of substrate:
1800 x 40 x 20 CPM/5×10^3 nanomoles = 1800 x 0.16 CPM/nanomole. 0.16 is a standard used routinely to calculate the CPM/nanomole.

iv. To calculate CPM/mg dry weight: divide CPM of each sample by the corresponding dry weight estimated as above.

v. To calculate nanomoles/mg dry weight: divide CPM/mg dry weight by the equivalent of CPM to 1 nanomole of substrate (the result of the calculation in paragraph iii).

The growth conditions and treatments used for the transport experiments are specified in the text.

c. Extraction and chromatography of accumulated ^{14}C-L-asparagine

Following a 30 min incubation in 100 mM L-asparagine + ^{14}C-L-asparagine (2.5 µCi in 50 ml test medium) the cells were filtered, washed and pressed dry in absorbant towels. 1 g wet weight of cells was suspended in 10 ml boiling water. After 30 min 50 µl of the extract was spotted on to 3mm Whatman chromatography paper (breadth 3 cm). The separation of L-asparagine from other amino acids was carried out by low voltage vertical ionophoresis (as above). After ionophoresis for 4 hours at 150 volts (current 20 mA) in tank buffer as above, the chromatogram was dried out and cut into 1 cm sections. The sections were transferred to vials containing 5 ml NE 250 scintillation fluid and the radioactivity measured as above. The results are expressed as counts/min/section of chromatogram. A histogram was constructed to show the position of the counts. All histograms represent 3 x 1 cm sections of the chromatograms.

4. Enzyme and uptake tests for ammonia derepression

For this series of tests the procedure was as above except that cells were grown in the presence and absence of ammonia.

a. Presence of ammonia

Cells were grown on -N medium plus 10 mM ammonia. After 17 hours growth cells were transferred as above to -N medium plus 10 mM ammonia for 3 hours.

b. Absence of ammonia

Cells were grown on -N medium plus 10 mM ammonia. After 17 hours growth cells were transferred to -N medium for 3 hours.

5. Plate tests for resistance on toxic agents

a. Aspartic hydroxamate

-N medium or -CN medium was supplemented by the appropriate nitrogen source or nitrogen and carbon source and concentration of aspartic hydroxamate as specified in the text.

b. Glutamic hydroxamate

The wild type will not grow on -N medium plus 1 mM glutamic hydroxamate and 10 mM nitrate as nitrogen source, glutamic hydroxamate resistant strains grow well.

c. Methylammonium

This is a modification of the test used by Arst and Cove (1969). Wild type will not grow on -N medium plus 1 M methylammonium and 10 mM L-arginine as nitrogen source.

d. Thiourea

Wild type will not grow on -N medium plus 10 mM thiourea plus 10 mM alanine as the nitrogen source (Dunn and Pateman 1972).

e. Chlorate

This is a modification of the test used by Cove (1976). Wild type will not grow on -N medium plus 100 mM $KClO_3$ plus 10 mM L-alanine as the nitrogen source.

f. D-serine

Wild type will not grow on -N medium plus 5 mM D-serine plus 10 mM nitrate as nitrogen source.

g. Hydroxylamine

Wild type will not grow on -N medium plus 1 mM hydroxylamine plus 10 mM nitrate as nitrogen source.

6. Genetic techniques

a. Strains

A biotin auxotroph biA1 known to be translocation free was used as the wild type (Glasgow No. 051). A multiply marked strain - master strain E (M.S.E. Glasgow No. 94) - was used for heterocaryon and diploid formation for gene assignment to a linkage group (see below). niaD17, nirA1 (Glasgow Nos. 0126 and 0128), nitrate reductase less and nitrate - nitrite reductase less mutants (Pateman and Cove 1967, Pateman, Rever and Cove 1967), and various recombinants (described in text) with markers for linkage studies were also obtained from the culture Stocks in the Institute of Genetics, University of Glasgow. meaA8 is methylammonium resistant, ammonia derepressed mutant (Arst and Cove 1969), supplied by Dr. H. N. Arst. DER-3 is one of a series of ammonia derepressed mutants obtained by Pateman selecting directly for ammonia derepression of nitrate reductase using a replica plating technique (Pateman, Kinghorn, Dunn and Forbes 1973). xprD1 is an ammonia derepressed mutant obtained by selecting directly for ammonia derepression of extracellular protease production (Cohen 1972) supplied by Dr. B. L. Cohen. $tamA^{r}119$, partially ammonia repressed mutant isolated on the base of simultaneous resistance on thiourea, aspartic hydroxamate, methylammonium (Kinghorn and Pateman 1975 c), $tamA^{r}50$ and $tamA^{d}1$ completely ammonia repressed and derepressed, respectively, alleles of the tamA locus (Pateman and Kinghorn manuscript in preparation). The tamA alleles were kindly

supplied by Dr. J. R. Kinghorn. areA^{r}510, areA^{r}551 partially repressed alleles of the locus areA (Arst and Cove 1973), areA^{r}550, areA^{d}520 completely repressed and derepressed alleles respectively (Arst and Cove 1973) supplied by Dr. H. N. Arst. gdhA, NADP-L-glutamate dehydrogenase less mutant (Kinghorn and Pateman 1973 and 1975 a).

b. Naming of mutants

The system of nomenclature follows that proposed for Aspergillus by Clutterbuck (1970 and 1973). Three small italic letter symbols followed by a capital italic letter have been used to designate loci. The locus in which mutation confers resistance to the toxic analogue aspartic hydroxamate and loss of asparaginase activity has been called ahrA. The locus in which mutation confers asparagine auxotrophy (hypothetically loss of asparagine synthetase activity) has been called asn^{-}A1 and the locus in which mutation abolishes glutamine synthetase activity has been called gln^{-}A1 (Kinghorn personal communication). The locus in which mutation can suppress tamA30 on asparagine and result in derepression of aspartic hydroxamate toxicity has been called aspA20.

c. Isolation of mutants

N-methyl-N-nitro-N-nitrosoquanidine (NTG) was used as a mutagen in all the mutational experiments. NTG may react with DNA and alter it in such a way as to promote template errors during subsequent replication (Adelberg, Mendel and Chen 1965). A heavy

conidial suspension was made up in 10 ml distilled water containing Tween 80 to separate clumps of conidia. The clumps were broken up by vigorous shaking. The distilled water was removed after centrifugation in a bench MSE centrifuge at 2,000 g for 5 min. 10 ml of 100 mM tris-maleate buffer pH 6.0 (Gomori's tris-maleate buffer), containing 2.5 mg of NTG, disolved thoroughly, were added to the pellet of precipitated conidia. The conidia were resuspended in the solvent by vigorous shaking and the suspension was incubated at 37°C. After 30 min of incubation the suspension was centrifuged as above and the conidial pellet was resuspended in 10 ml of sterile distilled water. The suspension was again centrifuged and the pellet resuspended in 10 ml sterile water. This procedure was repeated several times in order to remove the NTG. Care was taken in discarding the NTG because of its dangerous carcinogenic action. The supernatants containing NTG from the centrifuge washes were inactivated by adding a strong base (5 N NaOH 2 ml) and the liquid was discarded after one week. The above NTG treatment of the cells gives approximately 5% survival.

d. Isolation of aspartic hydroxamate resistant mutants

Wild-type cells do not grow on -N medium plus 0.3 mM of aspartic hydroxamate plus 10 mM nitrate, or with 0.1 mM of aspartic hydroxamate plus 10 mM L-alanine as nitrogen source. Aspartic hydroxamate resistant mutants were isolated by plating about 2×10^6 NTG treated live biA1 wild-type conidia after adding them directly to a flask with 180 ml -N medium, melted and cooled and supplemented with the appropriate nitrogen source and aspartic hydroxamate

concentration. In such a selective medium only colonies resistant to aspartic hydroxamate should grow. Growing colonies were purified by streaking on complete medium and re-tested on -N medium plus 10 mM nitrate plus various concentrations of aspartic hydroxamate. Strongly resistant mutants were isolated on 0.3 to 1.5 mM and a weakly resistant mutant on 0.3 to 0.5 mM aspartic hydroxamate. A total of 11 mutants resistant on aspartic hydroxamate were isolated and named ahrA mutants. The mutation rate was approximately 1 per 10^7 live mutagenised conidia. This is a relatively low rate compared to other mutations which occur at about $1:10^4$ (Pontevorvo et al., 1953).

e. Isolation of asparagine and glutamine auxotrophs

The treated conidial suspension was counted and after serial dilution plated on to complete medium to determine viability. The treated conidial suspension was spread after dilution on -N medium with biotin plus 10 mM L-asparagine or L-glutamine as the sole nitrogen source. The plates contained 0.08% sodium deoxycholate which reduces the size of the colonies (MacKintosh and Pritchard 1963) and allows velvet replication to be carried out. Appropriate dilutions of the suspensions were made in distilled water and spread over the surface of the medium to give about 150-200 colonies per plate. The plates were incubated for three days and then velvet replicated to plates containing -N medium plus biotin plus sodium-deoxycholate plus 10 mM ammonia as sole nitrogen source. Colonies which grow on 10 mM L-asparagine or 10 mM L-glutamine but not on 10 mM ammonia were picked off, purified and re-tested. One proved to be an asparagine auxotroph and one a glutamine auxotroph.

The gln⁻A1 auxotroph was isolated by Dr. J. R. Kinghorn (personal communications). The asparagine auxotrophic mutant was isolated after replicating about 10^4 colonies.

f. Isolation of the aspA20 mutant

tamA50 treated conidia were plated out on -N medium plus sodium desoxycholate plus 10 mM ammonia to give about 150-200 colonies. After three days of incubation the growing colonies were velvet replicated as above on -N medium plus 10 mM L-asparagine. tamA50 is unable to utilise L-asparagine as nitrogen source (Pateman and Kinghorn, unpublished results). Colonies which grew were picked off, purified and re-tested for growth on L-asparagine. One of them proved to be suppressor of tamA50, growing on L-asparagine but not on any other nitrogen sources. This mutant, named aspA20, was isolated after replicating 10^4 colonies. A selective technique was also used in an attempt to isolate tamA50 suppressors on L-asparagine by inoculating about 2×10^6 tamA50 NTG treated conidia per flask containing 180 ml -N medium supplemented with 10 mM L-asparagine as the sole nitrogen source. All the strains growing on the above selective medium proved to be tamA50 after purification and re-testing.

g. Meiotic mapping

A minimal agar plate was inoculated at the centre with a clump of conidia of one parent strain and then the other. The conidia were then mixed in a loopful of nutrient broth and the resulting mixture of conidia from parent strains streaked over the

surface of the minimal medium in four directions. The plates were taped up with adhesive tape, so as to create anaerobic conditions and incubated for further 8-14 days during which perithecia (sexual bodies) are formed.

The perithecia were isolated on 3% agar minimal medium and cleaned from attached conidia and cell debris by rolling on the surface of the agar with a dissecting needle. The cleaned perithecia were each squashed into 10 ml sterile distilled water, agitated and suspended. This ascospore suspension was stored at $4^{o}C$.

A loopful of the ascospore suspension was streaked on to complete medium and incubated. Perithecia, which resulted from a cross between parent strains, gave rise to segregation for conidial colour markers and thus could be readily identified. An ascospore suspension from crossed perithecia was diluted and spread on complete medium containing any extra supplement required by the parent strains, to give approximately 100 colonies of progeny per plate. These were picked off and growth tested for the markers under examination. In some cases, diluted ascospore suspensions were added directly to an appropriate molten selective medium and plated out.

h. Formation of heterocaryons

Two techniques were used to form heterocaryons:

1) A loopful of conidia from each of the appropriate strains was carefully layered upon the surface of 10 ml liquid complete medium in standard 6" x 3/4" test tubes. The tubes were incubated for two days and the resulting mycelium pad was repeatedly washed in sterile

distilled water and transferred to a plate of minimal medium, broken up and spread over the surface of the medium. The plate was incubated for two days and pieces of growing mycelium were transferred to similar plates of medium. This procedure was repeated until an heterocaryon, characterized by an equal mixture of conidial colours was established.

2) Conidia from each of the appropriate strains were streaked close to each other (about 1 mm distance) on the surface of complete medium and incubated for 4 days. Small pieces of mycelium, from the region where the two strains were mixed together, were transferred and inoculated on the surface of minimal medium and incubated. Pieces of growing mycelium were transferred as above to similar plates of medium until an heterocaryon was formed.

i. Production of diploids (Roper 1952)

Dilute conidial suspensions were made from the heterocaryon and these were inoculated into melted minimal medium and plated out. On incubation, diploid colonies, characterized by light green coloured conidia, where the component strains were a yellow and a green strain or the white MSE and a green strain, grew and were purified. After purification the diploid conidia were tested under the microscope. Diploid conidia are bigger than haploid ones. Diploid strains were stored on minimal slopes.

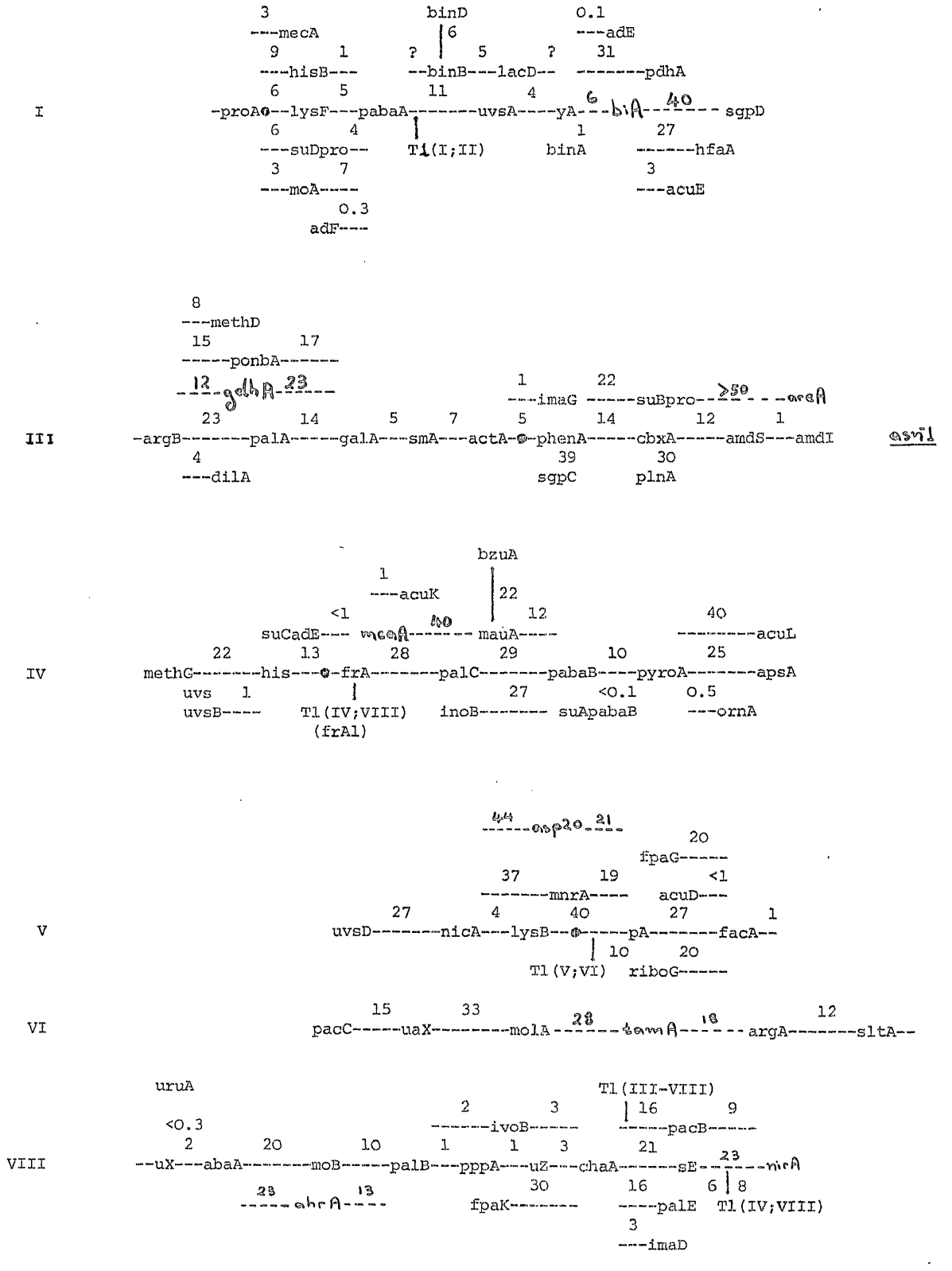


Figure 1. Partial linkage map of Aspergillus nidulans

j. Haploidization of diploids (Lhoas 1961, McCully & Forbes 1965)

Clumps of conidia from the diploid strain were point inoculated (9 inocula per plate) on to complete medium supplemented with riboflavin and p-fluoro-phenylalanine. Benlate was used when one of the component strains was hypersensitive to p-fluoro-phenylalanine. p-fluoro-phenylalanine and benlate greatly increase the yield of haploid sectors. Haploid sectors were picked off after 5-7 days incubation, purified and growth tested. The unknown mutant locus was then assigned to the linkage group of the marker to which it is apparently linked (Pontecorvo et al., 1953). In all haploidization tests the master strain used was the MSE marked as follows:

linkage group	markers
I	yA2 yellow spored, suladE20 suppressor of adE20, adE20 adenine requirement.
II	w-3 white spored (epistatic to yellow)
III	galA1 unable to utilize galactose as carbon source.
IV	pyroA4 pyridoxin auxotroph.
V	facA303 unable to utilize acetate as carbon source.
VI	sB3 thiosulphate auxotroph.
VII	nicB8 nicotinic acid auxotroph.
VIII	riboB2 riboflavin auxotroph.

A partial linkage map of Aspergillus nidulans is given in Figure 1. Loci, mutants in which were isolated during the course of the present thesis or alleles of which were used for the enzyme and uptake assays are marked in red on the linkage map.

CHAPTER III ASPARTIC HYDROXAMATE TOXICITY

nitrogen source (10 mM)	Concentration of aspartic hydroxamate (mM)								
	0	0.1	0.3	0.5	1	1.5	3	5	10
nitrate	+++	+++	-	-	-	-	-	-	-
nitrite	+++	+++	+	-	-	-	-	-	-
ammonia	+++	+++	+++	+++	+++	+++	+++	+++	+++
L-alanine	+++	-	-	-	-	-	-	-	-
L-asparagine	+++	+++	+++	++	++	++	$\overset{+}{-}$	-	-
L-aspartate	+++	-	-	-	-	-	-	-	-
L-glutamate	+++	-	-	-	-	-	-	-	-
L-glutamine	+++	+++	++	$\overset{+}{-}$	-	-	-	-	-
L-proline	+++	+++	+	-	-	-	-	-	-
urea	+++	+++	+	-	-	-	-	-	-

Table 1. The toxic effect of aspartic hydroxamate on the wild-type in the presence of various nitrogen sources.

+++: very good growth, ++: good growth, +: weak growth, $\overset{+}{-}$: very weak growth, -: no growth.

ASPARTIC HYDROXAMATE TOXICITY

DL-aspartic acid-β-hydroxamate is referred to throughout as aspartic hydroxamate.

1. Growth tests of the wild type on aspartic hydroxamate

The investigation of the growth of wild type colonies in presence of aspartic hydroxamate, showed that growth is inhibited if the concentration of the toxic analogue is raised to more than 0.3 mM in the growth medium with nitrate as the sole nitrogen source (table 1). When L-alanine or L-aspartate or L-glutamate are used as the sole nitrogen sources, the toxicity of aspartic hydroxamate is greater; wild type colonies will not grow at concentrations higher than 0.1 mM of the analogue when one of the above three amino acids is used as nitrogen source in the growth medium.

Table 1 shows that ammonia completely overcomes the toxicity of aspartic hydroxamate. Wild type colonies can grow at the highest concentration of aspartic hydroxamate used in the growth tests (10 mM) when ammonia present in the medium. The protecting effect of ammonia against aspartic hydroxamate toxicity is not affected by the presence of any other nitrogen source used. To carry out this growth tests ammonia was added in -N medium supplemented with one of the nitrogen sources L-asparagine, L-aspartate, L-alanine, L-glutamine, L-glutamate, L-proline, nitrate, urea.

L-Asparagine protects the wild type colonies against aspartic hydroxamate toxicity when the concentration of the analogue is not higher than 3 mM (plate 6).

When one of the nitrogen sources L-glutamine, L-proline, urea is present in the growth medium, aspartic hydroxamate is toxic at concentrations of 0.5 mM or more.

strains / nitrogen source (10mM)	concentration of aspartic hydroxamate (mM)											
	0			0.5			1.5			2.5		
	tamA119	tanA1	areA^{r}551	tamA119	tanA1	areA^{r}551	tamA119	tanA1	areA^{r}551	tamA119	tanA1	areA^{r}551
nitrate	+++	+++	+++	-	++	-	-	++	-	-	-	-
nitrite	+++	+++	+++	+	+	-	+	+	-	-	-	-
ammonia	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L-alanine	+++	+++	+++	++	-	-	-	-	-	-	-	-
L-asparagine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+
L-aspartate	+++	+++	+++	-	-	-	-	-	-	-	-	-
L-glutamate	+++	+++	+++	-	-	-	-	-	-	-	-	-
L-glutamine	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-
L-proline	+++	+++	+++	-	-	-	-	-	-	-	-	-
urea	+++	+++	+++	+++	+++	-	++	++	-	-	-	-

Table 2. The toxic effect of aspartic hydroxamate on ammonia repressed mutants in the presence of various nitrogen sources.

+++: very good growth, ++: good growth, +: weak growth, -: no growth.

2. Growth tests of ammonia repressed mutants on aspartic hydroxamate

tamA119 is a partially ammonia repressed mutant at the regulatory gene tamA, which is partly responsible for the regulation of ammonia repressible systems (Kinghorn and Pateman 1975). The mutant tamA119 is resistant to 0.5 mM aspartic hydroxamate when L-glutamine or L-alanine or urea are used as the sole nitrogen sources (table 2). On the same concentration of aspartic hydroxamate tamA119 is sensitive when nitrate or L-proline or L-aspartate or L-glutamate are used as the sole nitrogen sources. The mutant tanA1, an allele of the areA locus (Kinghorn, personal communication), is resistant on 0.5 mM aspartic hydroxamate with nitrate or L-glutamine or urea as the sole nitrogen sources. tanA1 is sensitive when L-alanine or L-aspartate or L-glutamate or L-proline is the sole nitrogen source. Neither of the two repressed mutants is resistant on concentrations of aspartic hydroxamate higher than 2.5 mM on any of the above nitrogen sources.

Another partially repressed mutant, the areA551 of the areA locus which is also regulating ammonia repressible systems (Arst and Cove, 1973), is as sensitive to aspartic hydroxamate as the wild type.

L-Asparagine and ammonia protect the three repressed mutants against the toxicity of the analogue.

strains / nitrogen source (10mM)	concentration of aspartic hydroxamate (mM)									
	0					0.5				
	meaA8	DER-3	tamA^{d}1	areA^{d}520	gdhA1	meaA8	DER-3	tamA^{d}1	areA^{d}520	gdhA1
nitrate	+++	+++	+++	+++	+	-	-	-	-	-
nitrite	++	++	+++	+++	+	-	-	-	-	-
ammonia	+++	+++	+++	+++	+	-	-	-	-	-
L-alanine	+++	+++	+++	+++	+++	-	-	-	-	-
L-asparagine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L-aspartate	++	++	+++	+++	++	-	-	-	-	-
L-glutamate	++	++	+++	+++	++	-	-	-	-	-
L-glutamine	+++	+++	+++	+++	+++	-	-	-	-	-
L-proline	+++	+++	+++	+++	+++	-	-	-	-	-
urea	+++	+++	+++	+++	+++	$\pm$	-	-	-	-

Table 3. The toxic effect of aspartic hydroxamate on ammonia derepressed mutants in the presence of various nitrogen sources.

+++: very good growth, ++: good growth, +: weak growth, $\pm$: very weak growth, -: no growth.

3. Growth tests of ammonia derepressed mutants on aspartic hydroxamate

The ammonia derepressed mutants tested were meaA8 a mutant with defective ammonia transport, DER-3 a modified meaA, tamA^{d}1 a mutant of the tamA locus derepressed for a number of ammonia repressible systems, areA^{d}250 a mutant of the areA locus derepressed for a number of ammonia repressible systems, and gdhA1 a mutant lacking NADP-GDH activity with a poor growth on all inorganic nitrogen sources and a possible regulatory role in ammonia repression (Kinghorn and Pateman, 1975). To test for ammonia derepression of aspartic hydroxamate toxicity in the gdhA1 mutant, L-alanine was added into the medium since it does not interfere with the effect of ammonia.

It was found that ammonia does not protect any of the ammonia derepressed mutants tested. All of the mutants were sensitive on all the nitrogen sources tested in presence of aspartic hydroxamate except for L-asparagine (table 3). L-Asparagine protects the ammonia derepressed mutants against the toxicity of aspartic hydroxamate to the same degree as the wild type (plate 6). This is an indication that the protection provided by L-asparagine is not due to the production of ammonia from it.

Plate 1. Growth of *ahrA* mutants and various heterozygous diploids on nitrate, L-alanine, L-aspartate and L-glutamate as the sole nitrogen sources.

Concentration of nitrogen sources: 10 mM

Growth medium: -N

Upper plates: Left, nitrate - Right, L-alanine

Lower plates: Left, L-aspartate - Right, L-glutamate

Strains on the plates in duplicate:

ahrA1/*ahrA*$^+$	*ahrA1*
ahrA2/*ahrA*$^+$	*ahrA2*
ahrA3/*ahrA*$^+$	*ahrA3*
wild-type	

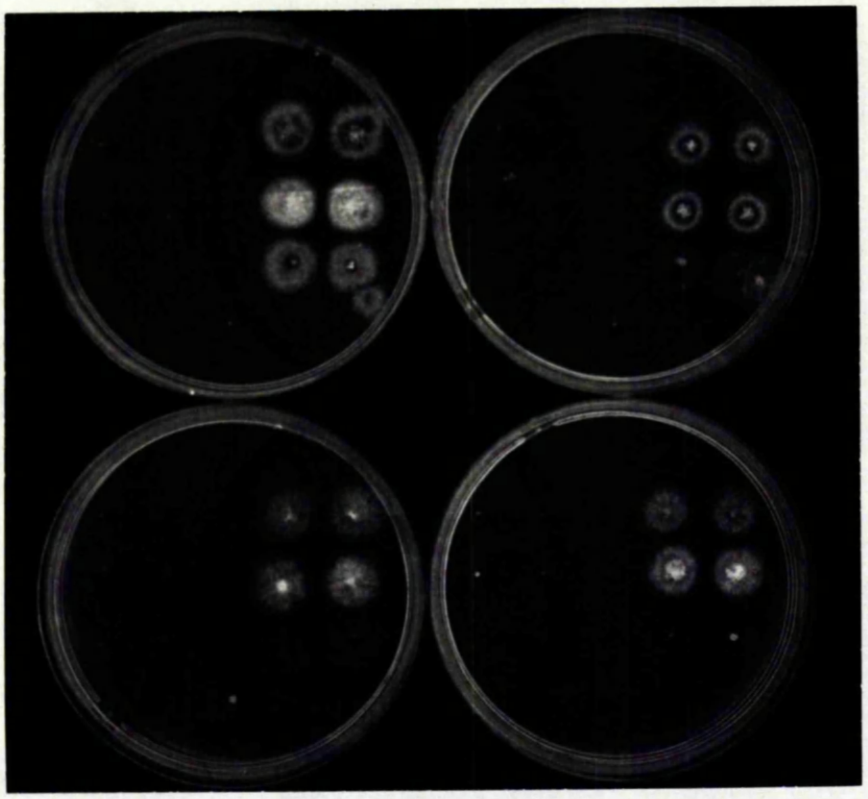


Plate 2. Growth of ahrA mutants and various heterozygous diploids on 0.5 mM of aspartic hydroxamate.

Concentration of nitrogen sources: 10 mM

Growth medium: -N

Upper plates: Left, nitrate - Right, L-alanine

Lower plates: Left, L-aspartate - Right, L-glutamate

Strains on the plates in duplicate:

ahrA1/ahrA$^+$	ahrA1
ahrA2/ahrA$^+$	ahrA2
ahrA3/ahrA$^+$	ahrA3

wild-type

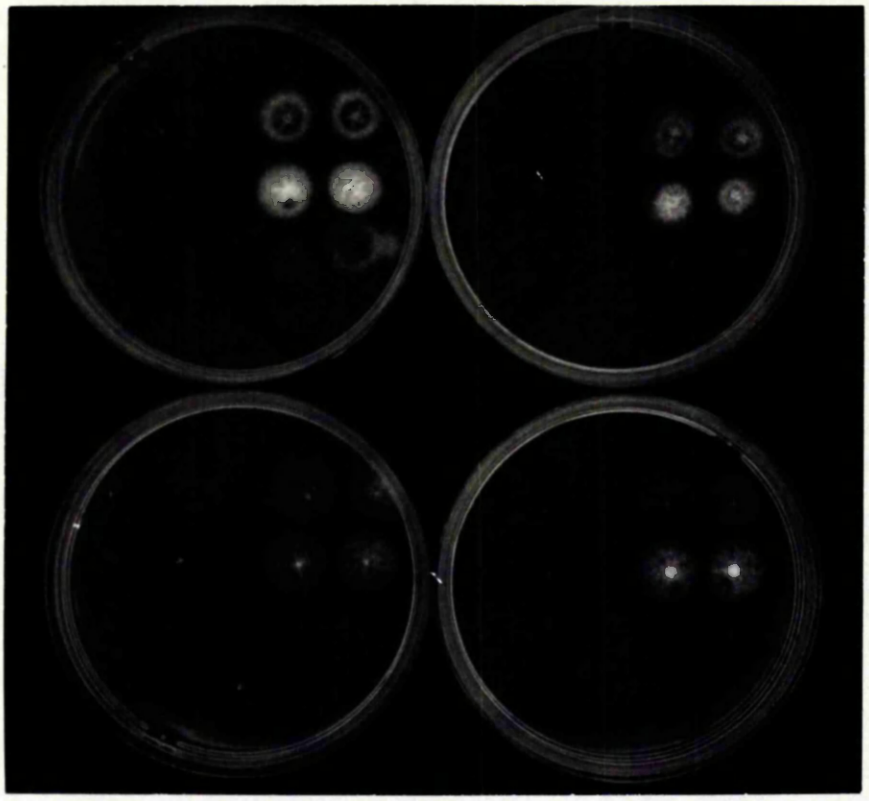


Plate 3. Growth of ahrA mutants and various heterozygous diploids on 1 mM of aspartic hydroxamate.

Concentration of nitrogen sources: 10 mM

Growth medium: -N

Upper plates: Left, nitrate - Right, L-alanine

Lower plates: Left, L-aspartate - Right, L-glutamate

Strains on the plates in duplicate:

ahrA1/ahrA$^+$	ahrA1
ahrA2/ahrA$^+$	ahrA2
ahrA3/ahrA$^+$	ahrA3

wild-type

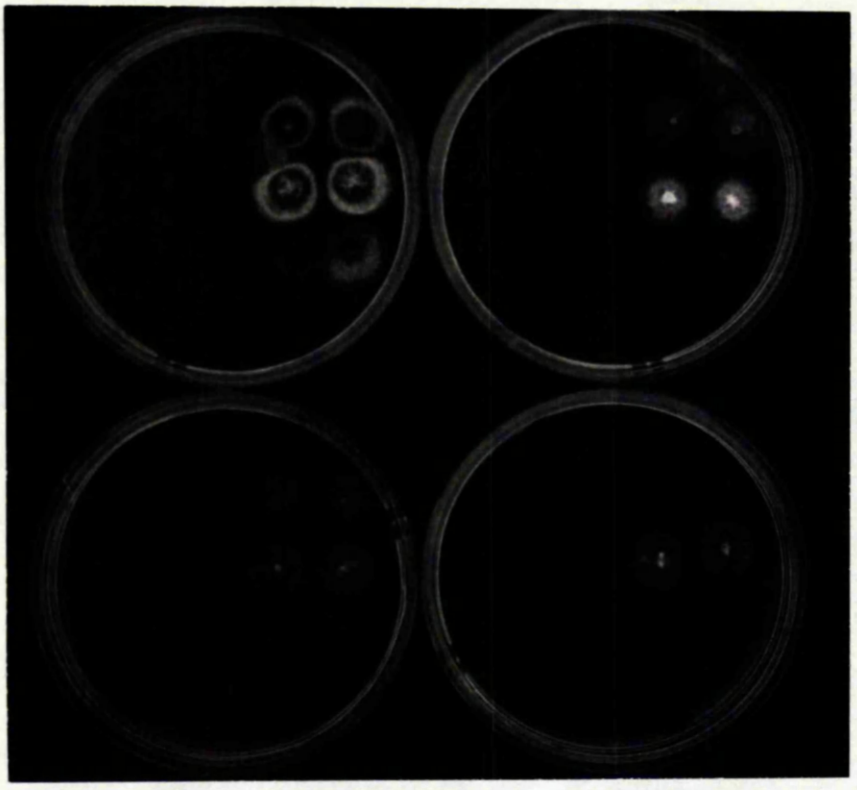


Plate 4. Growth of ahrA mutants and various heterozygous diploids on 3 mM of aspartic hydroxamate.

Concentration of nitrogen sources: 10 mM

Growth medium: -N

Upper plates: Left, nitrate - Right, L-alanine

Lower plates: Left, L-aspartate - Right, L-glutamate

Strains on the plates in duplicate:

ahrA1/ahrA$^+$	ahrA1
ahrA2/ahrA$^+$	ahrA2
ahrA3/ahrA$^+$	ahrA3

wild-type

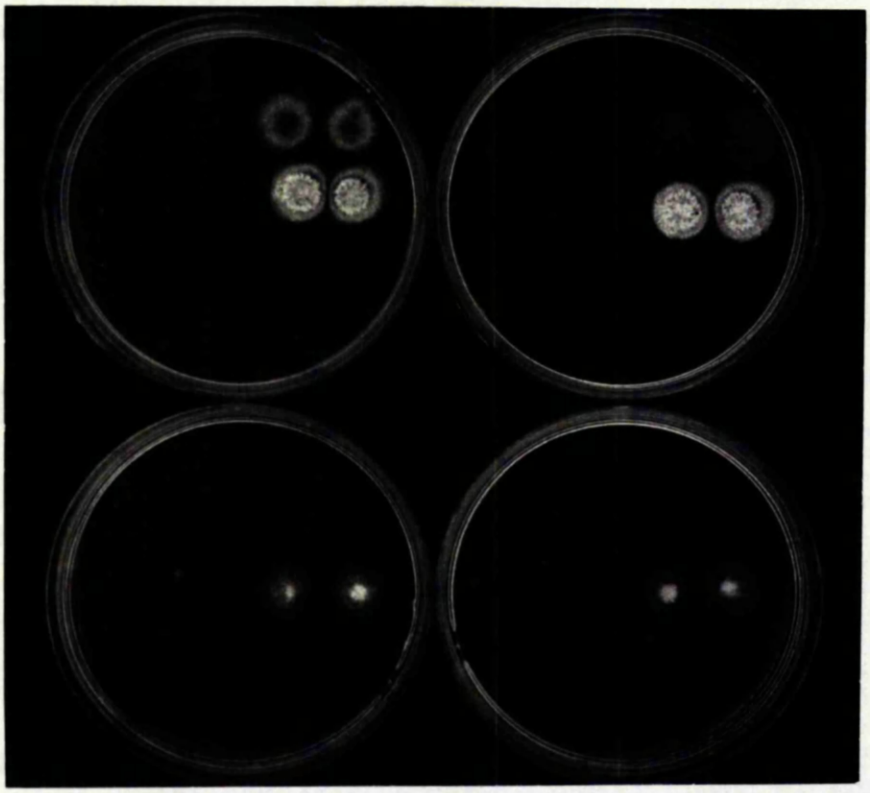


Plate 5. Differential growth of the mutants ahrA1 and ahrA2.

Growth medium: -N

Upper plates: Left, 5 mM aspartic hydroxamate

Right, 10 mM aspartic hydroxamate

Nitrogen source, 10 mM nitrate

Lower plates: Left, 5 mM aspartic hydroxamate

Right, 10 mM aspartic hydroxamate

Nitrogen source, 10 mM L-alanine

Strains on the plates in duplicate as in plates 1 - 4

4. Aspartic hydroxamate resistant mutants

a. Isolation

Eleven mutants designated ahrA (aspartic hydroxamate resistant) were isolated on the basis of resistance to the toxic analogue. The rate of occurrence of the ahrA mutants was relatively small (see materials and methods). Although the sample of the ahrA mutants is small, several levels of resistance were found. The ahrA mutants include strongly resistant (ahrA2, ahrA4-11), less strongly resistant (ahrA1) and weakly resistant (ahrA3).

The strongly resistant mutants were isolated by plating NTG treated wild type conidia on nitrogen free solid growth medium, containing 0.5 - 1.5 mM aspartic hydroxamate with nitrate (ahrA1, ahrA2, ahrA8, ahrA11) or L-alanine (ahrA4, ahrA5, ahrA6, ahrA7, ahrA9, ahrA10) as the sole nitrogen source. The weakly resistant ahrA3 mutant was isolated by plating wild type conidia, with the same treatment as above, on nitrogen free medium containing 0.5 mM aspartic hydroxamate with nitrate as the sole nitrogen source.

b. Resistance levels and growth responses

The different degrees of resistance of the various ahrA mutants is demonstrated in plates 1 - 5. In absence of aspartic hydroxamate the mutants and the wild-type grow similarly on all nitrogen sources tested (plate 1). On 0.5 mM of aspartic hydroxamate the wild type and the heterozygous diploids do not grow (plate 2). The mutant ahrA3 is characterized as weakly resistant because it

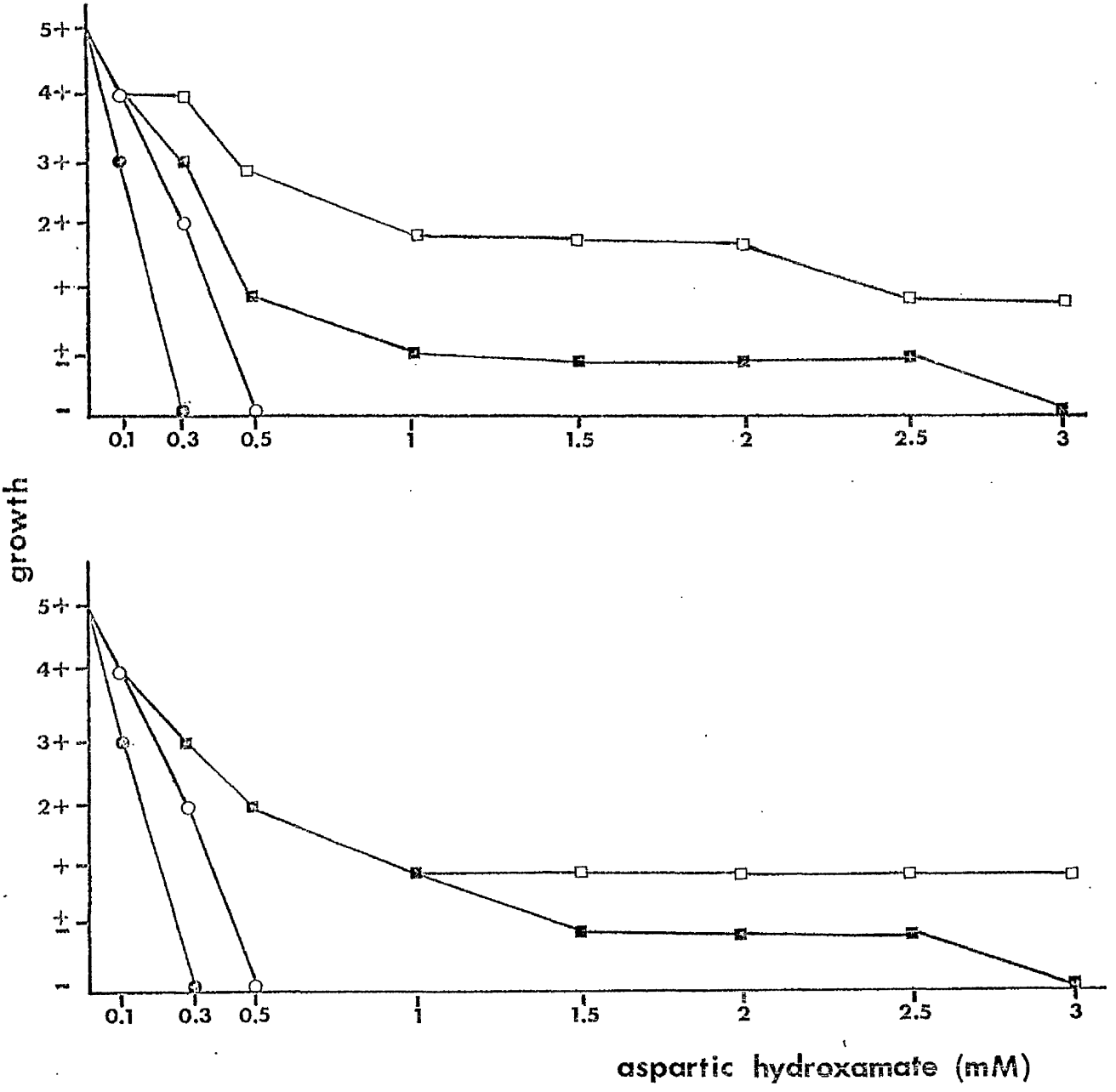


Figure 2. Growth of ahrA mutants on L-glutamate and L-aspartate in presence of various concentrations of aspartic hydroxamate.

(a): 10 mM L-glutamate, (b): 10 mM L-aspartate

5 +: very good mycelial growth (plate 1)

- : no growth (wild-type in plates 2 - 5)

Strains: ●: wild-type, ■: ahrA1, □: ahrA2, ○: ahrA3.

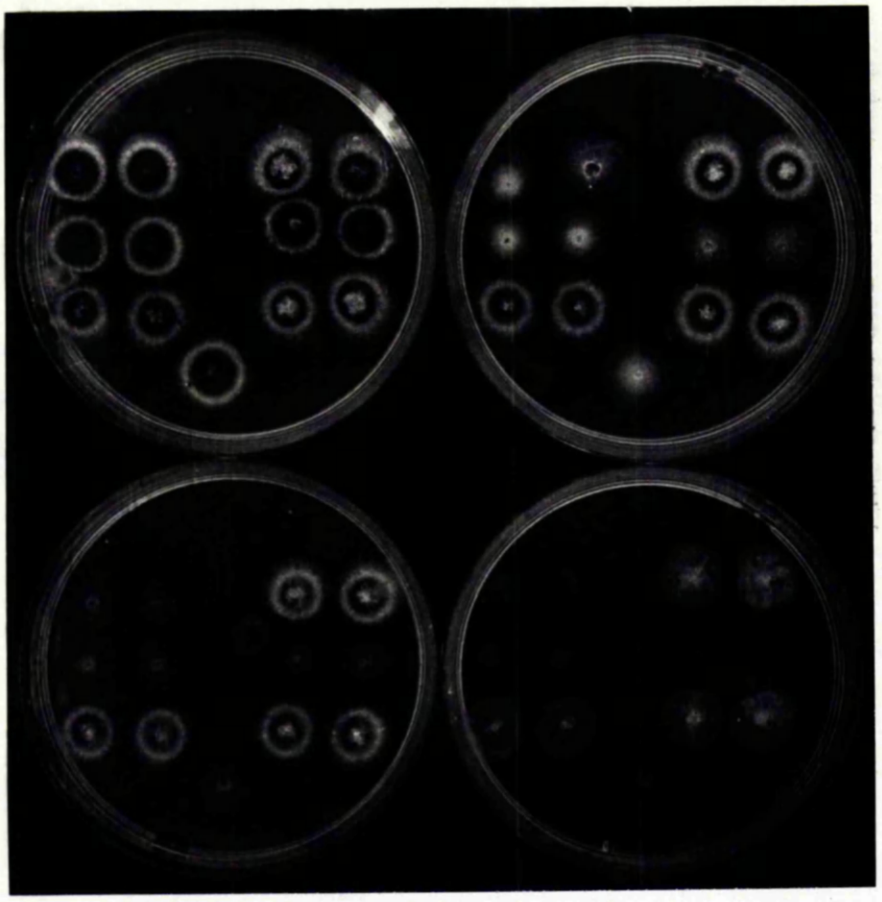


Plate 6. Aspartic hydroxamate toxicity with L-asparagine as the sole nitrogen source.

Growth medium: -N plus 10 mM L-asparagine

Upper plates: Left, 1 mM aspartic hydroxamate

Right, 3 mM aspartic hydroxamate

Lower plates: Left, 5 mM aspartic hydroxamate

Right, 10 mM aspartic hydroxamate

Strains on the plates in duplicate:

tamA^{d}1	ahrA2
meaA8	asn^{-}1
ahrA1	ahrA4

wild-type

cannot grow on concentrations of aspartic hydroxamate greater than 0.5 mM (plate 3), or 0.3 mM if the nitrogen source is nitrate or L-alanine respectively. All the other mutants are characterized as strongly resistant because of their ability to grow on higher concentrations of aspartic hydroxamate. The mutant ahrA2 is resistant on any concentration of aspartic hydroxamate tested, the highest being 10 mM. The ahrA1 mutant is not resistant on concentrations higher than 5 mM when nitrate is used as nitrogen source (plate 5) and on concentrations higher than 3 mM when L-alanine or L-aspartate or L-glutamate is used as the sole nitrogen source (plate 4).

The level of resistance is not the same on all the nitrogen sources tested, the resistance of the mutants is reduced when L-aspartate or L-glutamate or L-alanine is used as the sole nitrogen source (plate 3-5). In figure 2 the reduction of the growth of the mutants ahrA1, ahrA2 and ahrA3 is demonstrated, as the concentration of aspartic hydroxamate is raised in presence of L-aspartate or L-glutamate as the sole nitrogen sources. The mutants ahrA4-11 have similar growth to ahrA2.

All the ahrA mutants were tested for growth on L-asparagine as the sole nitrogen source or sole carbon and nitrogen source. They all grow on L-asparagine like the wild type (plate 6).

c. Dominance relationships

The ahrA mutations are recessive to the wild type allele with respect to resistance in heterozygous diploids since

the ahrA1/ahrA$^+$ diploids were sensitive to aspartic hydroxamate (plates 1-5). The heterozygous diploid ahrA2/ahrA3 was resistant on aspartic hydroxamate with a phenotype intermediate between those of the haploid strains since it was resistant at 1 mM and sensitive at 1.5 mM of aspartic hydroxamate.

d. Temperature sensitivity

All of the ahrA mutants were tested for temperature sensitivity. They were all incubated in 37°C, 30°C and 25°C on various concentrations of aspartic hydroxamate using various nitrogen sources such as ammonia, nitrate, L-asparagine, L-aspartate, L-glutamine, L-glutamate, L-alanine, L-proline and urea. Under all conditions and temperatures the ahrA mutants were resistant at the same degree as at 37°C therefore none are temperature sensitive.

Attempts were made to isolate temperature sensitive mutants by replication of colonies from NTG treated wild-type conidia on nitrogen free medium containing 10 mM nitrate as the sole nitrogen source and various concentrations of aspartic hydroxamate (range 0.3 - 1.5 mM) and incubation of three replica plates in 37°C, 30°C and 25°C. These attempts were not successful.

In addition attempts were made to obtain temperature sensitive mutants using NTG treated conidia from ahrA1, ahrA2 and ahrA3 strains. Starting with a mutant allele it might be possible for a second mutation within the ahrA gene to reverse the effect of the original mutation. Such secondary mutations whether at the site of the first mutation or elsewhere in the ahrA gene would usually result in an

	wild-type	ahrA strains				control strains			asp20
toxic analogues		ahrA1	ahrA2	ahrA3	ahrA4-11	TCA117	tamA119	ghrA1	
chlorate	-	-	-	-	-	+	+	-	-
glutamic hydroxamate	-	-	-	-	-	-	-	+	-
hydroxylamine	-	-	-	-	-	-	-	-	-
methylammonium	-	-	-	-	-	-	+	-	-
p-fluoro-phenylalanine	-	-	-	-	-	-	-	-	-
D-serine	-	-	-	-	-	-	-	+	-
thiourea	-	-	-	-	-	+	+	-	-

Table 4. Growth of the ahrA mutants on various amino acid and inorganic nitrogen toxic analogues.

Growth conditions and concentrations of the toxic analogues as specified in the materials and methods (chapter II).

+: growth, -: no growth

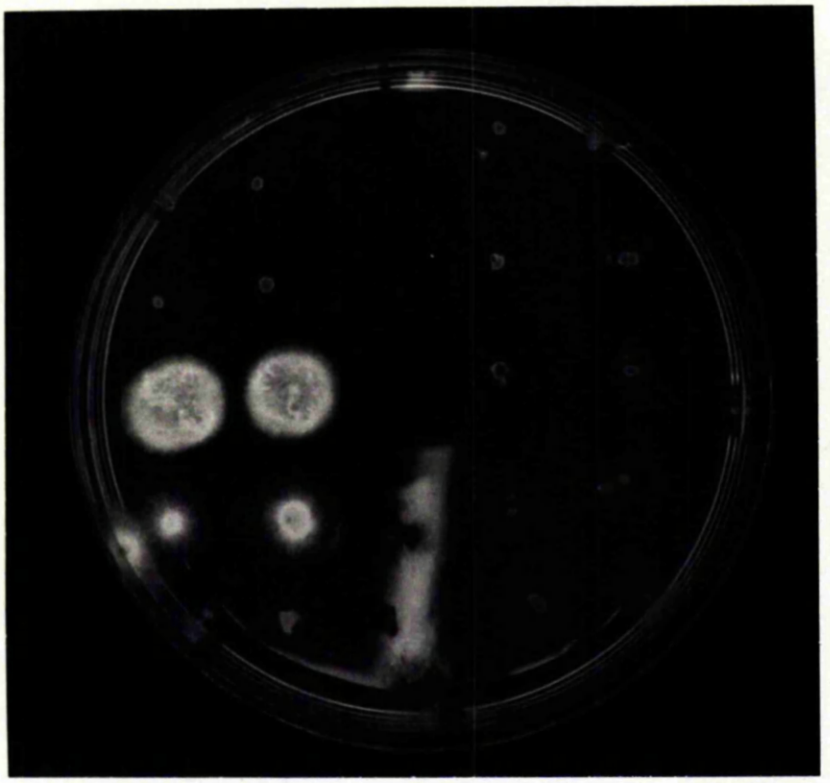


Plate 7. Growth tests of the ahrA mutants on thiourea.

Growth medium: -N plus 10 mM L-alanine plus 10 mM thiourea

Strains on the plate in duplicate:

ahrA1	ahrA3
ahrA2	ahrA4
TCA117	ahrA5
tamA119	ahrA6
biA1	ahrA7

altered gene product. Such an altered gene product, presumably a protein, might well be thermolabile. In fact the spectrum of backmutations due to a second mutation within a gene is likely to contain a higher proportion of temperature sensitives than that found in the spectrum of single mutations resulting in loss of function. In the case of the wild type there is no selective method available for the detection of temperature sensitive alleles induced in ahrA mutants. However, since there was a real possibility that detectable temperature sensitive alleles might be more readily induced in one of the ahrA mutants than in the $ahrA^+$ strain, a number of mutagenesis experiments were carried out.

These attempts to isolate a temperature sensitive mutant from the ahrA mutants were not successful.

e. Sensitivity of the ahrA mutants to other toxic analogues of amino acids and nitrogen metabolites

The ahrA mutants were tested for growth on thiourea, methylammonium, potassium chlorate, glutamic hydroxamate, D-serine and p-fluorophenylalanine. They were all sensitive on all of the toxic analogues (table 4). In plate 7 the sensitivity of the ahrA mutants on thiourea is demonstrated, only the thiourea resistant mutants TCA117 and tamA119 can grow. The ahrA mutants were sensitive to hydroxylamine to the same degree as the wild-type. The TCA117 is a mutant isolated by Dr. J. R. Kinghorn (personal communication) for simultaneous resistance on thiourea, chlorate and

Diploids		ahrA1 M.S.E.		ahrA2 M.S.E.	
linkage group	gene marker	$ahrA1^R$	$ahrA1^S$	$ahrA2^R$	$ahrA2^S$
	y^+	20	27	15	20
I	y^-	25	26	20	21
	w^+	19	29	18	22
II	w^-	26	24	17	19
	gal^+	18	31	16	18
III	gal^-	27	22	19	23
	$pyro^+$	25	25	21	19
IV	$pyro^-$	20	28	14	22
	fac^+	18	31	15	24
V	fac^-	27	22	20	17
	s^+	22	28	14	21
VI	s^-	23	25	21	20
	nic^+	19	30	18	21
VII	nic^-	26	23	17	20
	$ribo^+$	45	0	35	0
VIII	$ribo^-$	0	53	0	41

Table 5. Haploidization analysis of the ahrA1 and ahrA2 alleles.

+: wild-type allele, -: mutant allele

R: resistant, S: sensitive

Diploids		ahrA3 M.S.E.		ahrA4 M.S.E.	
linkage group	gene marker	$ahrA3^R$	$ahrA3^S$	$ahrA4^R$	$ahrA4^S$
	y^+	16	6	10	13
I	y^-	15	6	18	11
	w^+	19	7	13	10
II	w^-	12	5	15	14
	gal^+	18	5	17	11
III	gal^-	13	7	11	13
	$pyro^+$	19	8	16	9
IV	$pyro^-$	12	4	12	15
	fac^+	16	4	13	12
V	fac^-	15	8	15	12
	s^+	19	5	19	14
VI	s^-	12	7	9	10
	nic^+	16	5	12	15
VII	nic^-	15	7	16	9
	$ribo^+$	31	0	28	0
VIII	$ribo^-$	0	12	0	24

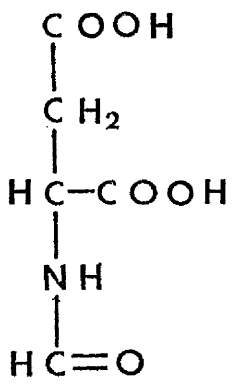
Table 6. Haploidization analysis of the ahrA3 and ahrA4 alleles.

+: wild-type allele, -: mutant allele

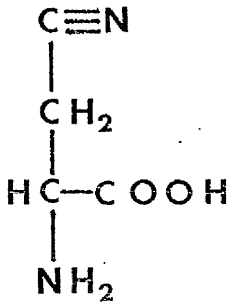
R: resistant, S: sensitive

cross	No. of progeny	recombinants
1. pabaA2-yA2-pyroA4-ahrA3 x biA1-ahrA1	475	none
2. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA1	550	none
3. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA3	625	none
4. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA4	500	none
5. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA5	475	none
6. pabaA2-yA2-pyroA4-ahrA3 x biA1-ahrA6	317	none
7. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA7	475	none
8. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA8	450	none
9. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA9	625	none
10. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA10	450	none
11. pabaA2-yA2-pyroA4-ahrA2 x biA1-ahrA11	425	none

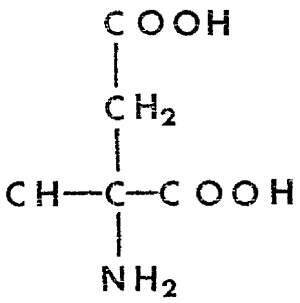
Table 7. Crosses between the ahrA mutants.



N-formyl-L-aspartic acid



cyano-L-alanine



Methyl-DL-aspartic acid

Figure 3. Chemical formulas of N-formyl-L-aspartic acid, cyano-L-alanine and methyl-DL-aspartic acid.

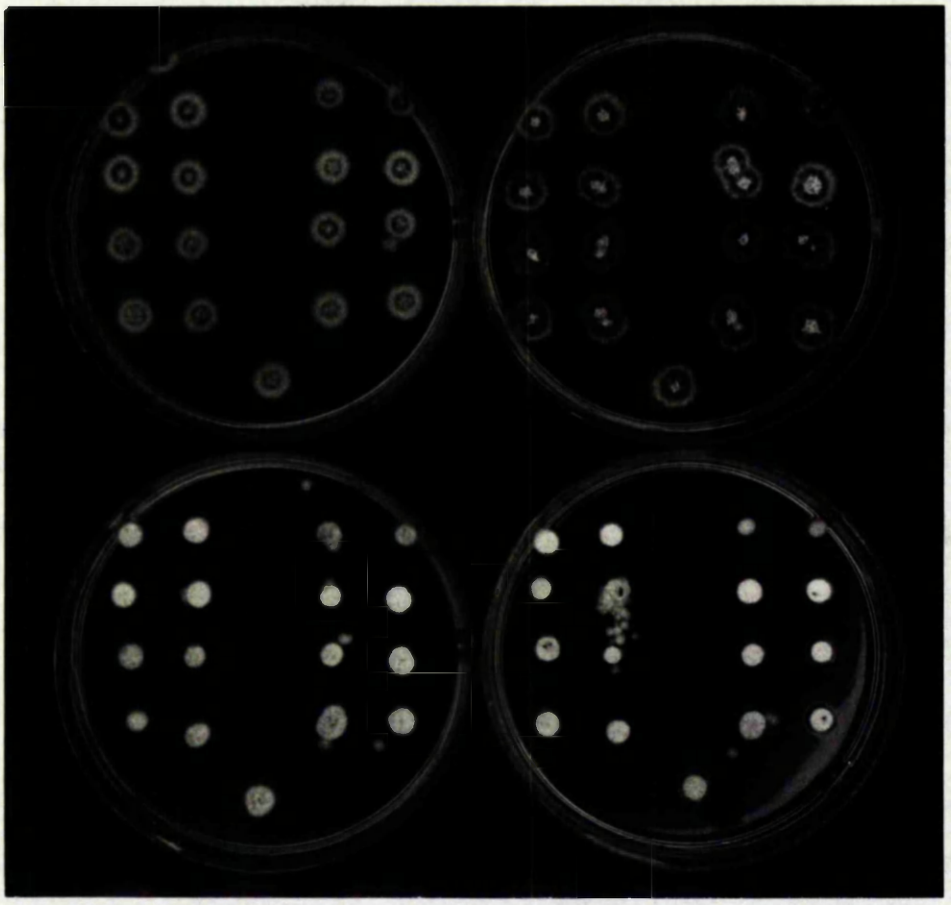


Plate 8. Growth inhibition by N-formyl-L-aspartic acid.

Growth medium: -N plus 10 mM L-alanine

Upper plates: Left, 5 mM formyl aspartic acid

Right, 0 mM formyl aspartic acid

Lower plates: Left, 10 mM formyl aspartic acid

Right, 8 mM formyl aspartic acid

Strains on the plates in duplicate:

ahrA5	ahrA1
ahrA6	ahrA2
ahrA7	ahrA3
ahrA8	ahrA4

wild-type

aspartic hydroxamate and it was used only as a control for the thiourea resistance tests.

A number of other L-asparagine analogues were tested for toxicity in A. nidulans. These were: N-formyl-L-aspartic acid, cyano-L-alanine, and methyl-DL-aspartic acid (chemical formulas given in figure 3). Only formyl-L-aspartic acid proved to inhibit hyphal growth at concentrations higher than 8 mM. The growth of the ahrA mutants was inhibited by the same concentration of N-formyl-L-aspartic acid as the wild-type (plate 8).

f. Genetic characterization of the ahrA mutants

Mitotic and meiotic analysis of the ahrA mutants revealed that only one locus was involved.

Four diploids of the ahrA mutants with the M.S.E. strain were haploidised by the technique of McCully and Forbes (1965). It was found that all the four mutants (later designated ahrA1, ahrA2, ahrA3, ahrA4) assort freely with all markers except riboB (tables 5 and 6). Therefore the mutants were assigned to linkage group VIII, and they were translocation free. They all showed approximately 50% recombination with the other markers of this linkage group.

The ahrA mutants were crossed to each other and no recombinants were obtained in approximately 500 progeny from each cross. The crosses between the ahrA mutants carried out are represented in table 7.

g. Mapping of the ahrA locus

Five crosses were carried out to map the ahrA locus. These crosses were extensively analysed as follows:

Cross I $\frac{+}{biA1}$ $\frac{pabaA2}{+}$ $\frac{yA2}{+}$ $\frac{pyroA4}{+}$ $\frac{ahrA2}{+}$ $\frac{+}{niaD17}$

The markers biA1, pabaA2, yA2, pyroA4 on the other linkage groups are not classified.

Segregation of markers and allele ratios:

	niaD17	+
ahrA2	36	51
+	50	48

Recombination fraction: ahrA2 - niaD17 = 45.4% ± 3.6.

Cross II: $\frac{pabaA2}{pabaA2}$ $\frac{+}{yA2}$ $\frac{+}{pyroA4}$ $\frac{fwA}{+}$ $\frac{facB}{+}$ $\frac{riboB}{+}$ $\frac{galC}{+}$ $\frac{+}{ahrA2}$

The markers pabaA, yA and pyroA on other linkage groups are not classified.

Segregation of markers and allele ratios:

	fwA	+
ahrA2	29	33
+	41	44

Recombination fraction: ahrA2 - fwA = 49.6% ± 4.1.

Segregation of markers and allele ratios:

	riboB	+
ahrA2	35	26
+	60	26

Recombination fraction: ahrA2 - riboB = 41.5% ± 4.

	facB	+
ahrA2	38	24
+	53	32

Recombination fraction: ahrA2 - facB = 47.6% ± 4.1.

	galC	+
ahrA2	39	23
+	42	43

Recombination fraction: ahrA2 - galC = 55.8% ± 4.1.

Cross III: $\frac{\text{pabaA2}}{+}$ $\frac{\text{yA2}}{+}$ $\frac{\text{pyroA4}}{+}$ $\frac{+}{\text{adH}}$ $\frac{\text{ahrA2}}{+}$ $\frac{+}{\text{chaA}}$ $\frac{+}{\text{abaA}}$

The markers pabaA, yA, pyroA and adH on other linkage groups are not classified.

Segregation of markers and allele ratios:

	chaA	+
ahrA2	28	136
+	109	25

Recombination fraction: ahrA2 - chaA = 17.6% ± 1.7.

	abaA	+
ahrA2	48	116
+	94	40

Recombination fraction: ahrA2 - abaA = 23.8% ± 1.9.

Cross IV: $\frac{+}{yA2}$ $\frac{pabaA2}{+}$ $\frac{+}{pyroA4}$ $\frac{facB}{+}$ $\frac{riboB}{+}$ $\frac{palB}{+}$ $\frac{chaA}{+}$ $\frac{galC}{+}$ $\frac{+}{ahrA1}$ $\frac{+}{nirA1}$

The markers yA, pabaA and pyroA on other linkage groups are not classified.

	facB	+
ahrA1	56	56
+	57	52

Recombination fraction: ahrA1 - facB = 48.8% ± 3.3.

	riboB	+
ahrA1	57	55
+	52	57

Recombination fraction: ahrA1 - riboB = 51.6% ± 3.4.

	palB	+
ahrA1	15	97
+	94	15

Recombination fraction: ahrA1 - palB = 13.5% ± 2.3.

	chaA	+
ahrA1	24	90
+	91	18

Recombination fraction: ahrA1 - chaA = 19% ± 2.6.

	galC	+
ahrA1	55	57
+	62	47

Recombination fraction: ahrA1 - galC = 46.2% ± 3.3.

	F1 genotypes	No. of segregants	
P	$abaA^+$ ahrA $chaA^+$	182	
	abaA $ahrA^+$ chaA	120	
R	$abaA^+$ $ahrA^+$ chaA	44	s.cr.o.
	abaA ahrA $chaA^+$	51	
	$abaA^+$ ahrA chaA	33	
	abaA $ahrA^+$ $chaA^+$	32	
	$abaA^+$ $ahrA^+$ $chaA^+$	10	d.cr.o.
	abaA ahrA chaA	10	

Table 8. Three gene analysis of cross III.

$abaA^+$, $ahrA^+$, $chaA^+$: wild type alleles

abaA, ahrA, chaA : mutant alleles

P: parental types, R: recombinant types

s.cr.o.: single crossovers

d.cr.o.: double crossovers

Conclusion: The ahrA locus is located between the abaA and chaA markers.

	F1 genotypes	No. of segregants	
P	palB chaA ahrA$^+$	88	
	palB$^+$ chaA$^+$ ahrA	87	
R	palB chaA ahrA	14	s.cr.o.
	palB$^+$ chaA$^+$ ahrA$^+$	12	
	palB$^+$ chaA ahrA	10	
	palB chaA$^+$ ahrA$^+$	6	
	palB$^+$ chaA ahrA$^+$	3	d.cr.o.
	palB chaA$^+$ ahrA	1	

Table 9. Three gene analysis of cross IV.

palB$^+$, chaA$^+$, ahrA$^+$: wild-type alleles

palB, chaA, ahrA : mutant alleles

P: parental types, R: recombinant types

s.cr.o.: single crossovers

d.cr.o.: double crossovers

Conclusion: The ahrA locus is not located between the palB and chaA markers.

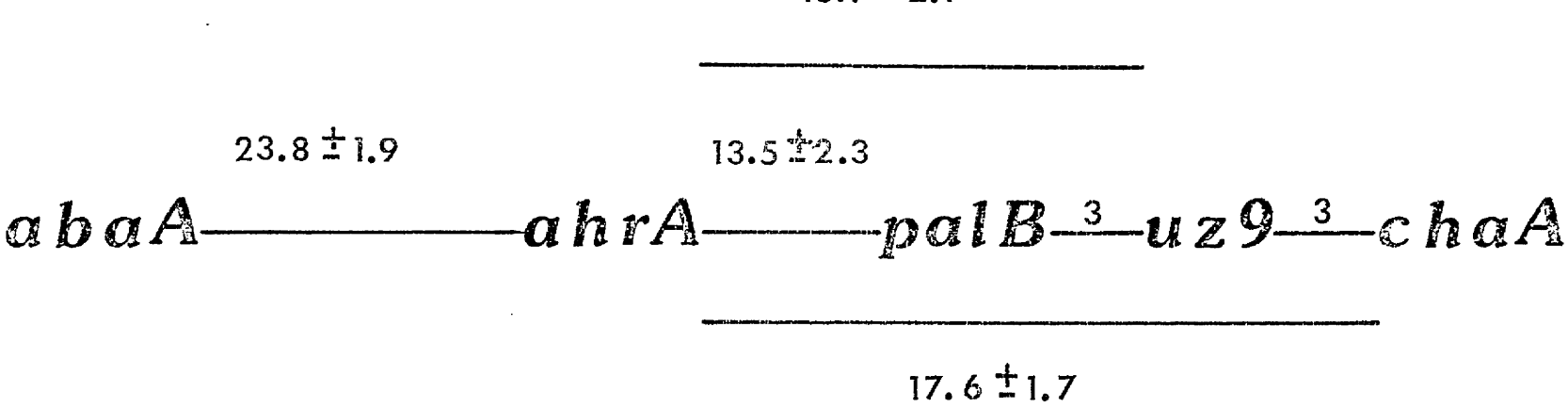


Figure 4. Linkage relationships of the ahrA locus.

Distances between the markers are given in cM.

	nirA	+
ahrA1	53	59
+	66	43

Recombination fraction: ahrA1 - nirA = 43.4% ± 3.3.

Cross V: $\frac{\text{pabaA2}}{+}$ $\frac{\text{yA2}}{+}$ $\frac{\text{pyroA4}}{+}$ $\frac{+}{\text{biA1}}$ $\frac{\text{ahrA2}}{+}$ $\frac{+}{\text{uZ9}}$

The markers pabaA, yA, pyroA and biA on other linkage groups are not classified.

Segregation of markers and allele ratios:

	uZ9	+
ahrA2	8	81
+	85	14

Recombination fraction: ahrA2 - uZ9 = 11.7% ± 2.3.

Three gene analysis of the cross III and IV located the ahrA gene between the abaA and palB markers (tables 8 and 9). The linkage relationships of the ahrA locus are summarised in figure 4.

5. Discussion

Aspartic hydroxamate, compared with other toxic analogues, is extremely toxic for Aspergillus nidulans. Hyphal growth is inhibited at a concentration of the analogue of 0.3 mM or 0.1 mM or more when nitrate or L-alanine is used as the sole nitrogen source. In comparison thiourea is toxic at 10 mM (Dunn and Pateman 1972), glutamic hydroxamate is toxic at 1 mM (Drainas and Kinghorn unpublished work), methylammonium is toxic at 100 mM (Arst and Cove 1973, Cove 1970), and hydroxylamine is toxic at 1 mM. The toxicity of aspartic hydroxamate, as is shown at the next chapter, is due to the production of hydroxylamine from it by the catalytic action of asparaginase. An observation which can be made here is that aspartic hydroxamate is more toxic than hydroxylamine. This is probably because hydroxylamine is produced intracellularly from aspartic hydroxamate.

Aspartic hydroxamate is more toxic in presence of poor nitrogen sources and less toxic in presence of rich ones. When L-alanine or L-aspartate or L-glutamate are the nitrogen sources, aspartic hydroxamate is toxic at 0.1 mM while in presence of nitrate or urea or L-glutamine or L-proline the analogue is toxic at 0.3 mM or more.

Wild type colonies are protected against the toxicity of aspartic hydroxamate when ammonia is present in the growth medium. Ammonia controls the expression of a number of enzyme and uptake systems (see introduction). These systems in the presence of ammonia have low or undetectable levels. Consequently ammonia may

afford protection from an analogue when the toxicity is due to the activity of an ammonia controlled metabolic system.

Protection against the toxicity of aspartic hydroxamate, in some degree, is provided by L-asparagine if the analogue is not present in the growth medium at concentrations higher than 3 mM. The fact that ammonia derepressed mutants are protected by L-asparagine to the same degree as the wild type, proves that this protection is not due to the production of ammonia from L-asparagine. Ammonia derepressed mutants have altered ammonia control for a number of metabolic systems. These mutants are not protected by ammonia from a number of toxic analogues. For example, the ammonia derepressed mutants meaA8, DER-3 and $tamA^{d}1$ are not protected against the toxicity of thiourea. All the ammonia derepressed mutants tested are sensitive to aspartic hydroxamate in the presence of ammonia.

Conversely, mutants repressed for a number of ammonia controlled metabolic systems were found to be resistant, in some degree, to aspartic hydroxamate. These mutants are the tamA119 and tanA1 (see pages 37 and 47) which are also resistant to thiourea and methylammonium (Kinghorn and Pateman 1975 c, Kinghorn personal communication).

The protection of wild type strains against the toxicity of aspartic hydroxamate, the sensitivity of ammonia derepressed mutants in presence of ammonia and the resistance of repressed mutants to the toxic analogue is some evidence that the toxicity of aspartic hydroxamate is due to the action of a metabolic system

which is controlled by ammonia repression. This system as is shown and discussed in the next chapter is probably the asparaginase I.

Resistant mutants were selected on various concentrations of aspartic hydroxamate. A spectrum of mutants was obtained, strongly resistant and weakly resistant. The strongly resistant ones are mutants resistant to aspartic hydroxamate up to a concentration 5 mM and others resistant up to a concentration 10 mM (10 mM was the highest aspartic hydroxamate concentration tested); the weakly resistant is the mutant ahrA3 which is resistant to 0.5 mM aspartic hydroxamate and sensitive to any higher concentration of it.

All of the above mutants, named ahrA mutants, proved to be allelic, located on the VIII linkage group. The linkage map of the ahrA locus is shown on figure 4. The ahrA locus is located between the abaA and palB markers and can be used as an excellent marker for further genetic analysis.

The ahrA mutants are recessive to the wild-type allele, since heterozygous diploids between the mutants and the wild-type allele are sensitive to aspartic hydroxamate to the same degree as the wild-type. Heterozygous diploids between the two phenotype classes (strongly resistant and weakly resistant), result in phenotype intermediate between those of the haploid strains.

The ahrA mutants can grow as the wild-type on all the nitrogen sources tested. In particular the ahrA mutants can grow as wild-type on L-asparagine as the sole nitrogen or nitrogen and

carbon source. This is some evidence that the ahrA mutants are not defective in a system essential for the utilization of L-asparagine as nitrogen source, and also that they are not defective for the L-asparagine transport system.

The ahrA mutants are sensitive to all other toxic analogues tested. The ahrA mutants are also sensitive to hydroxylamine, when it is present in the growth medium, to the same degree as the wild-type. Therefore, the ahrA mutants must be defective in the production rather than the metabolism of hydroxylamine.

CHAPTER IV REGULATION OF ASPARAGINASE SYNTHESIS

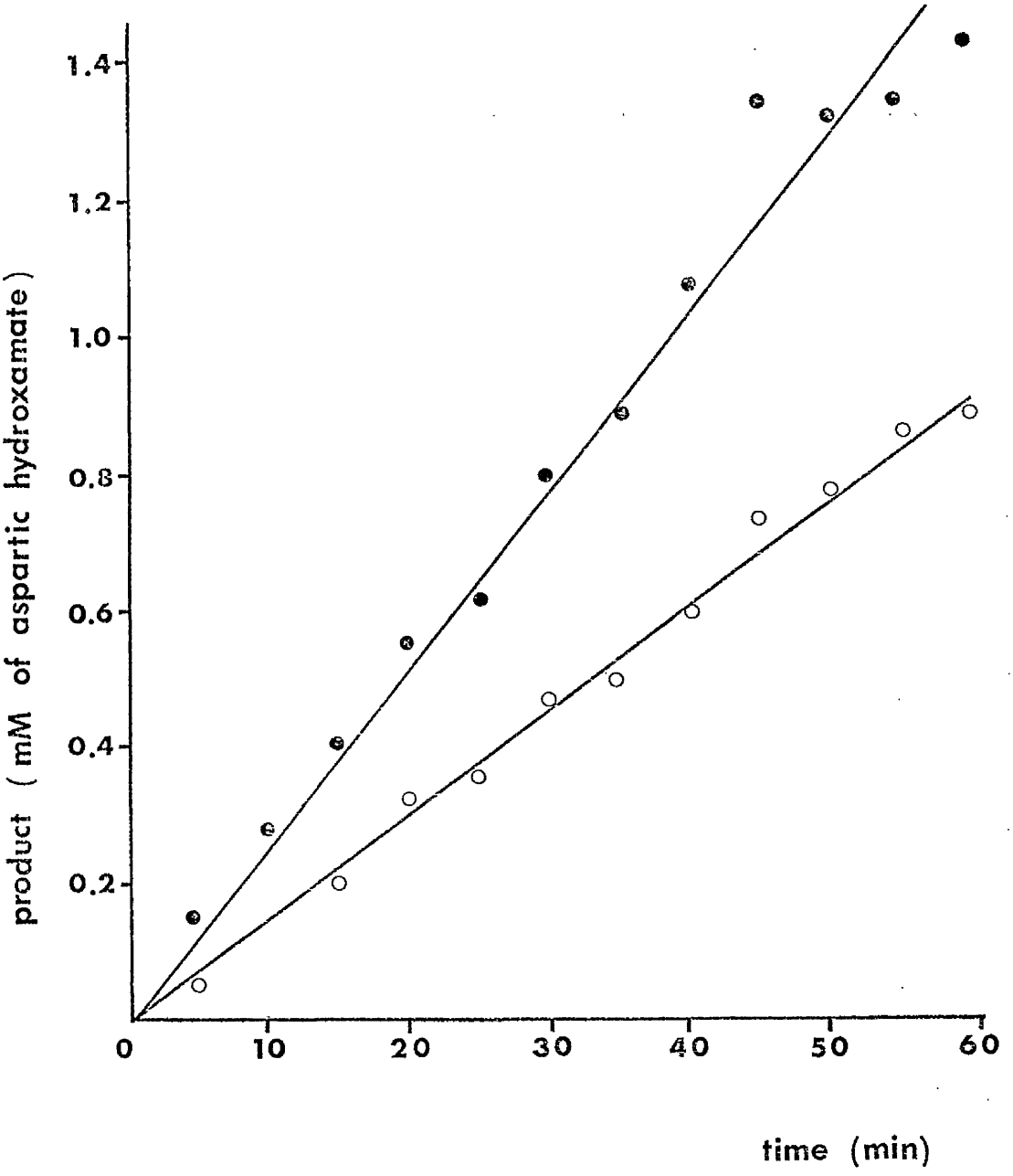


Figure 5. Asparaginase activity.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extract: wild-type

Assay method: ●: aspartic hydroxamate determined by the ferric chloride reagent (aspartyl-transferase activity)

○: ammonia determined by the Nessler's reagent (amino-hydrolase activity)

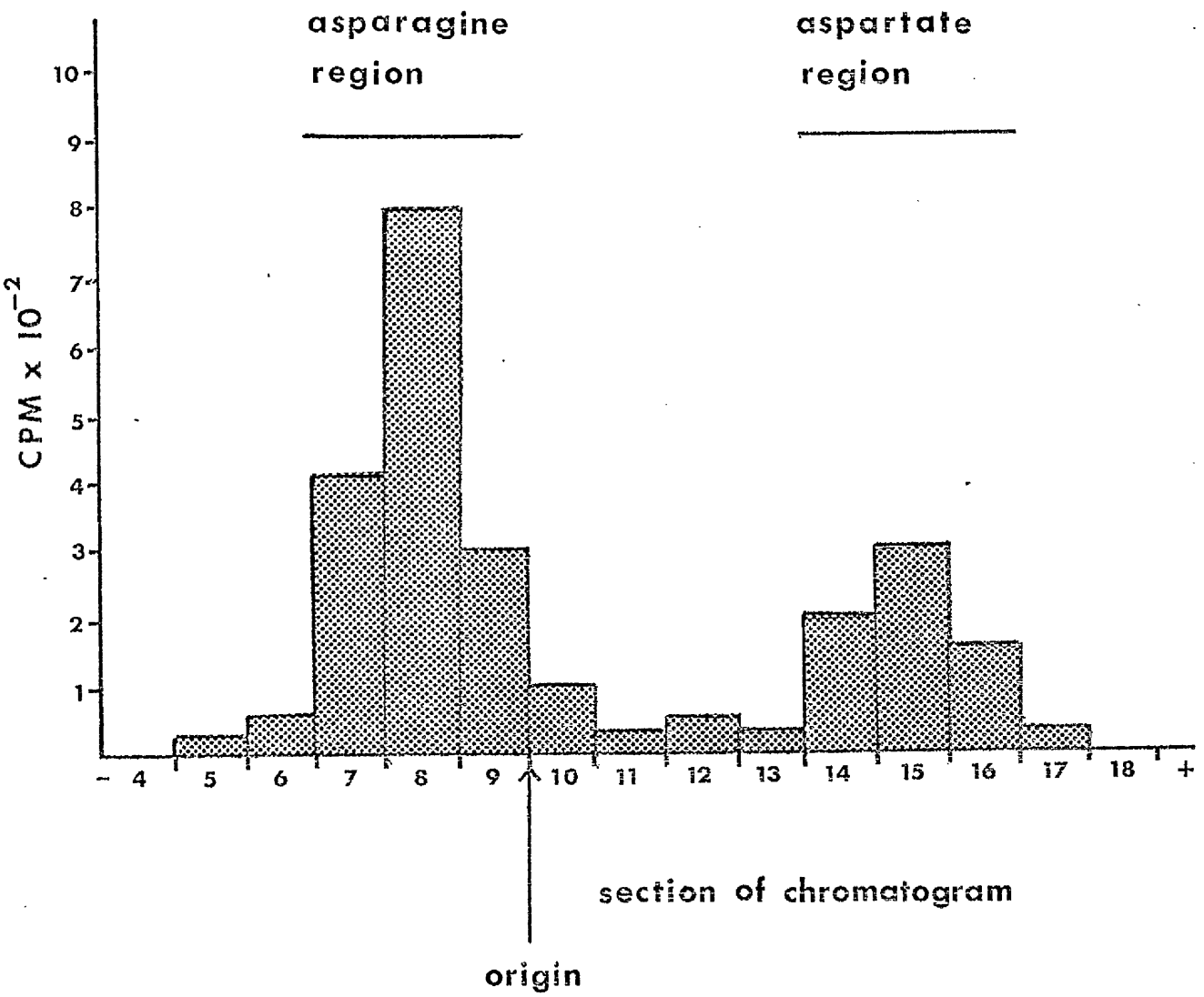


Figure 6. Separation of aspartate on paper chromatography.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extract: wild-type

Histogram representing 2 x 1 cm sections of the chromatography paper.

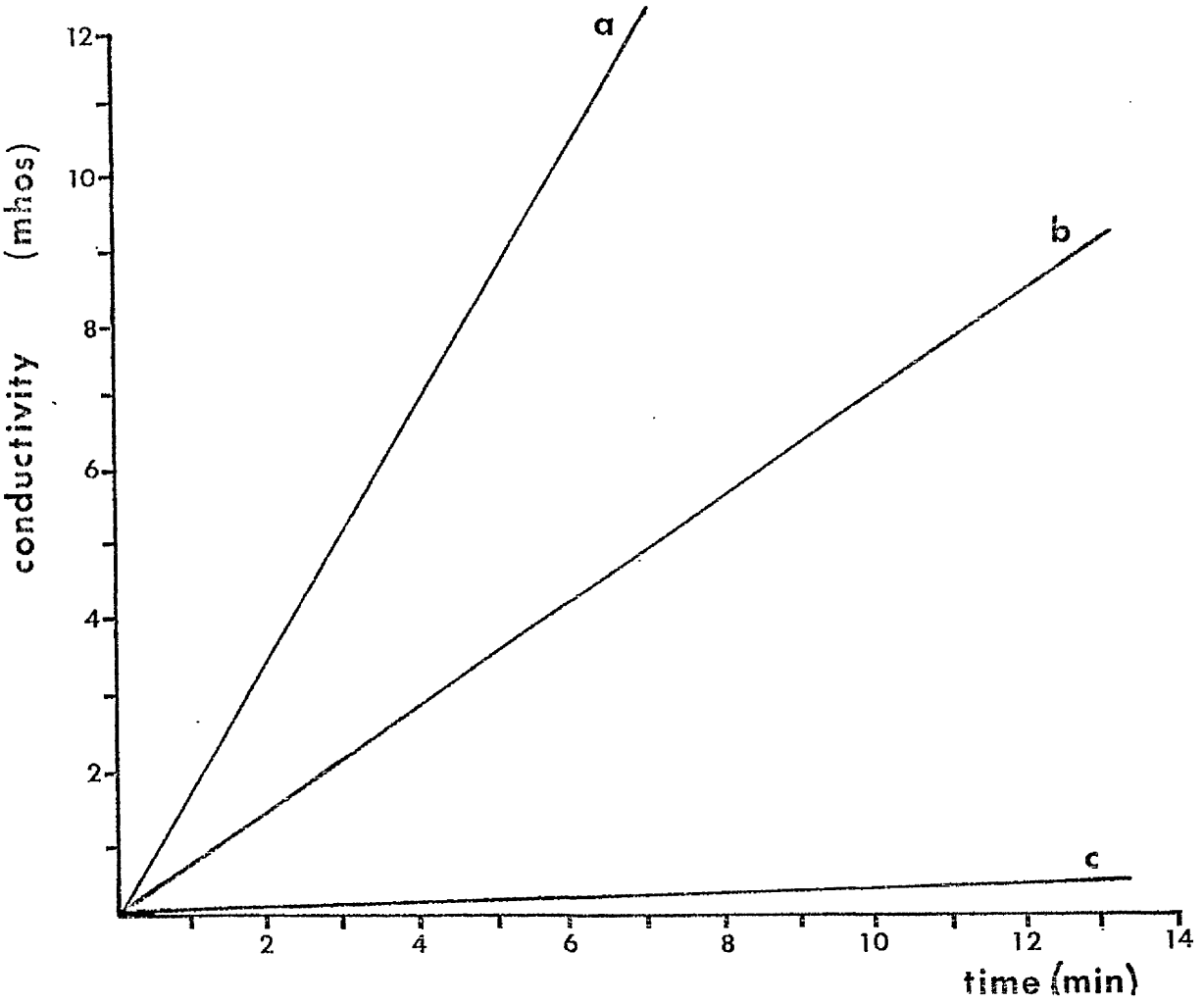


Figure 7. Conductimetric method of detecting asparaginase activity.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extract: wild-type

Substrates: curve (a): 30 µM L-asparagine

curve (b): 30 µM aspartic hydroxamate

curve (c): control (no substrate)

The increase of conductivity is due to production of NH_4^+ ions (when L-asparagine was used as substrate) or H^+ ions (when aspartic hydroxamate was used as substrate) and is determined qualitatively.

mhos: unit of conductivity.

REGULATION OF ASPARAGINASE SYNTHESIS

1. Assaying asparaginase activity

Asparaginase activity was assayed on the basis of:
i) the deamination of L-asparagine (amidohydrolase activity of asparaginase) and,
ii) the formation of aspartic hydroxamate from L-asparagine and hydroxylamine (aspartyl-transferase activity of asparaginase).
The assay techniques are described in chapter II (materials and methods).

The amidohydrolase activity of asparaginase has been detected by:

i) the determination of the produced ammonia by colour reaction with the Nessler's reagent (figure 5),
ii) the separation on paper chromatography of ^{14}C-L-aspartate produced from ^{14}C-L-asparagine (figure 6) and
iii) the measurement of the change of the conductivity due to the production of ammonium (NH_4^+) ions from L-asparagine (figure 7).

The aspartyl-transferase activity of asparaginase has been detected by:
i) the determination of the aspartic hydroxamate produced from L-asparagine and hydroxalamine by a colour reaction with the ferric chloride reagent (figure 5), and
ii) the measurement of the change of the conductivity, due to the

production of H^+ ions from aspartic hydroxamate by asparaginase (figure 7).

The method of detecting asparaginase activity by measuring the aspartyl-transferase activity with the ferric chloride reagent is more sensitive than the method detecting asparaginase activity with the Nessler's reagent (figure 5) and moreover is cheap, quick and reproducible. All the results concerning asparaginase activity have been obtained by this method.

Measuring asparaginase activity by the change of the conductivity is also a very sensitive method as well as cheap and reproducible. The high sensitivity of this method has a main disadvantage: the use of very weak buffer (10 mM tris-hydroxmethyl-aminomethan) to extract the enzyme. This probably results in loss of part of the activity, since the usual extraction buffer is of the level of 500 mM. Owing to technical difficulties, this method was not used in the present research. However this assay method was used, as described in chapter II (materials and methods), to prove, only qualitatively, that asparaginase can utilise aspartic hydroxamate as a substrate as well as L-asparagine. In figure 7 the increase of the contactivity of three reaction mixtures by the time is demonstrated. All the reaction mixtures consisted of 1 ml of 10 mM tris-HCl buffer and 5 µl of wild-type cell extract, extracted in the same buffer. Reaction mixture (a) contained L-asparagine as substrate, reaction mixture (b) contained aspartic hydroxamate as substrate and reaction mixture (c) was used as a control containing

no substrate. The three reaction mixtures were placed in three cells containing electrodes connected to a point recording machine. The three cells were adjusted using a magnetic field and temperature control device (Lawrence and Moores 1972, Lawrence et al., 1974). The cell extract was added to the reaction mixtures with the temperature set at 37^{o} and the ionic capacity of the cells had equilibrated (the point recording parallel to the time axe). The recording of the three reaction mixtures took place simultaneously and is represented by the three lines (a,b,c) on figure 7. It is shown that the cell extract possesses some enzyme which can utilise L-asparagine and aspartic hydroxamate as substrate and causes an increase of the conductivity of the reaction mixtures (a) and (b). In the reaction mixture (c) where no substrate is present, the change of the conductivity is almost undetectable.

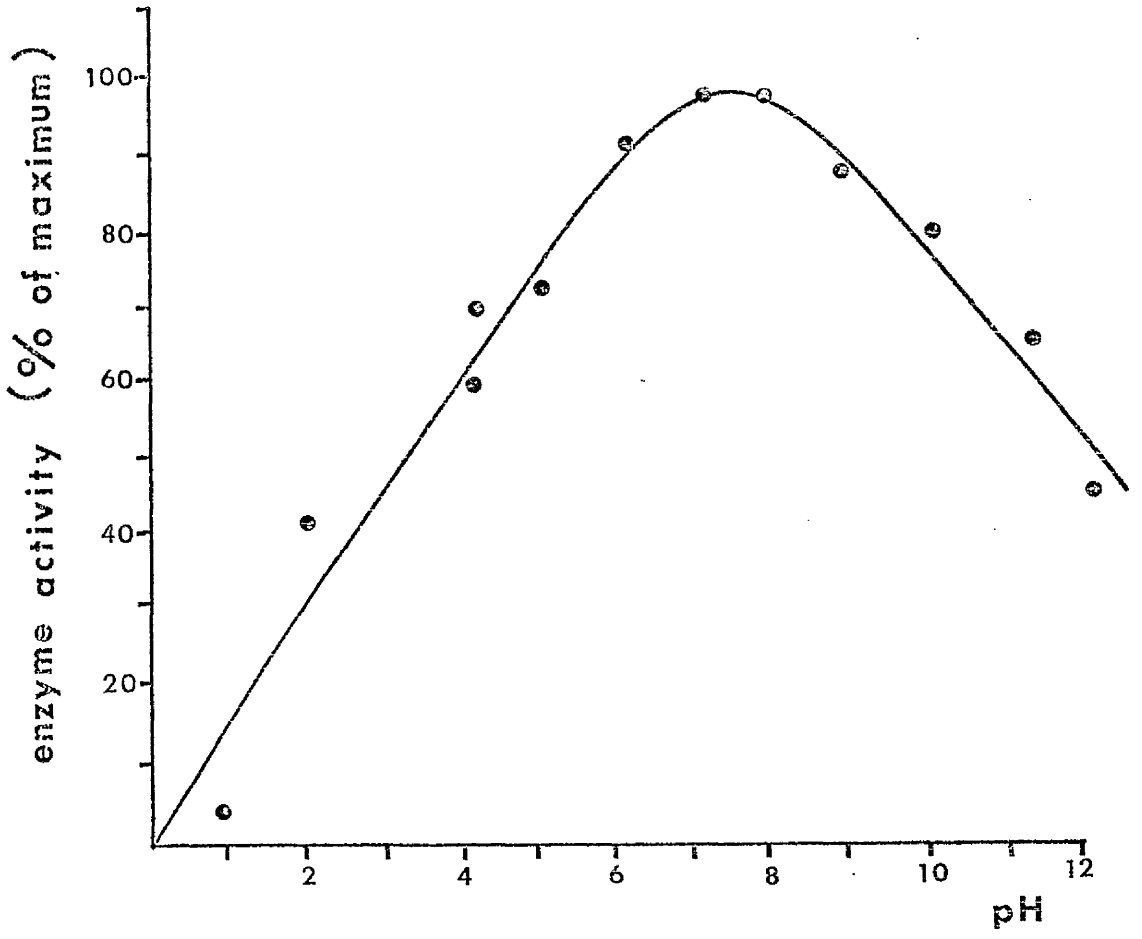


Figure 8. pH optimum of asparaginase activity.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extract: wild-type

Assay method: aspartyl-transferase activity with ferric chloride reagent

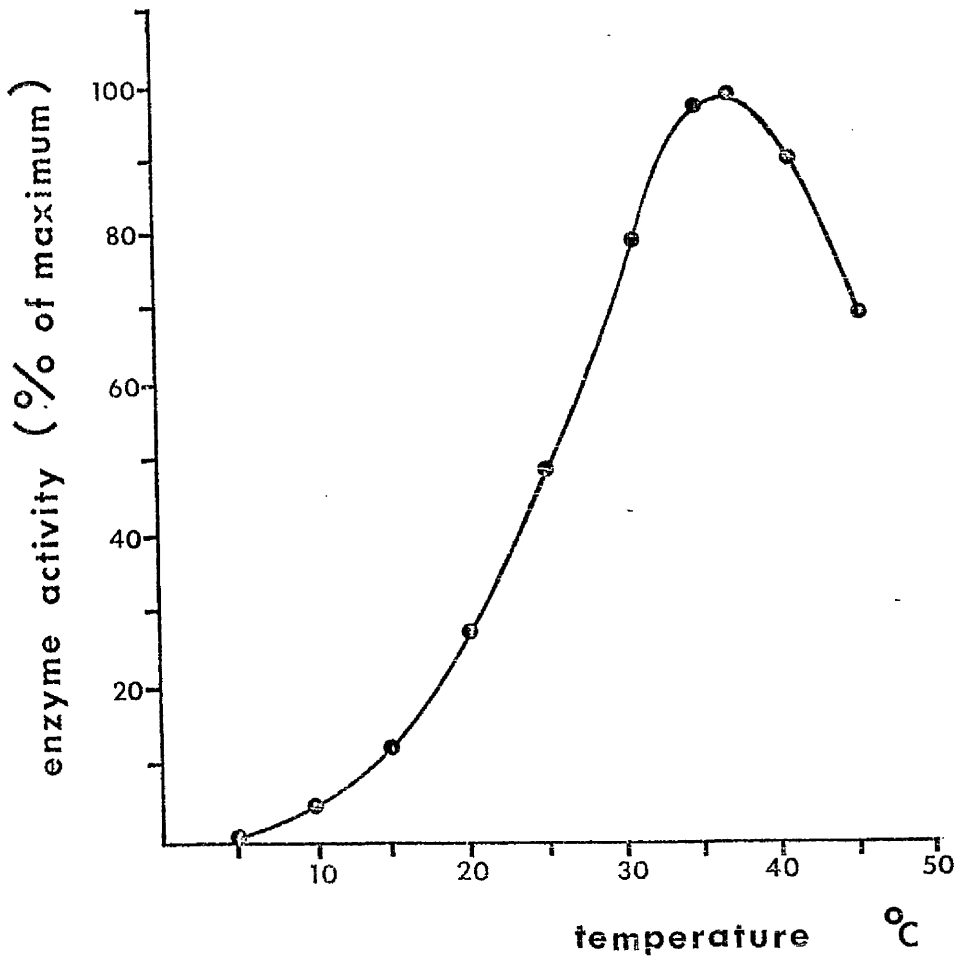


Figure 9. Temperature optimum of asparaginase activity.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extract: wild-type

Assay method: aspartyl-transferase activity with ferric chloride reagent

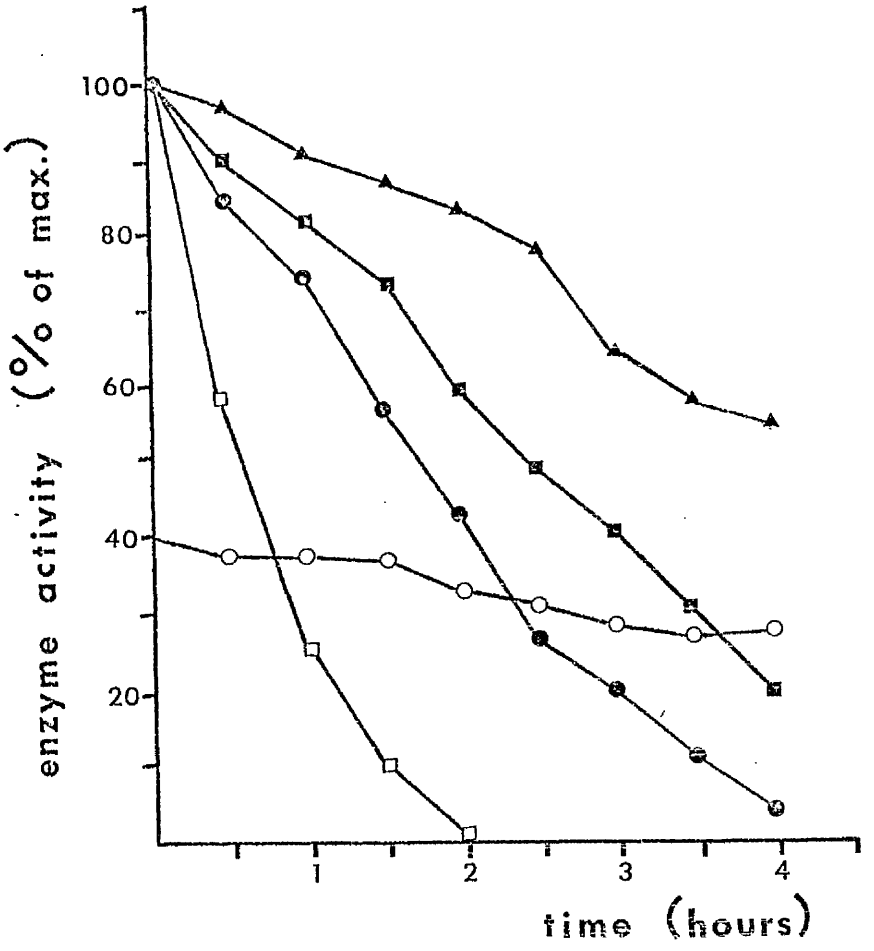


Figure 10. Loss of asparaginase activity of extracts incubated at high temperatures.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extract: wild-type

Incubation conditions

●: 25°C

▲: 25°C with 50 mM L-asparagine

■: 25°C with 1 mM cleland's reagent

○: 25°C with 50 mM L-asparagine added when the 60% of the activity was already lost

□: 37°C

Assay method: aspartyl-transferase activity with ferric chloride reagent

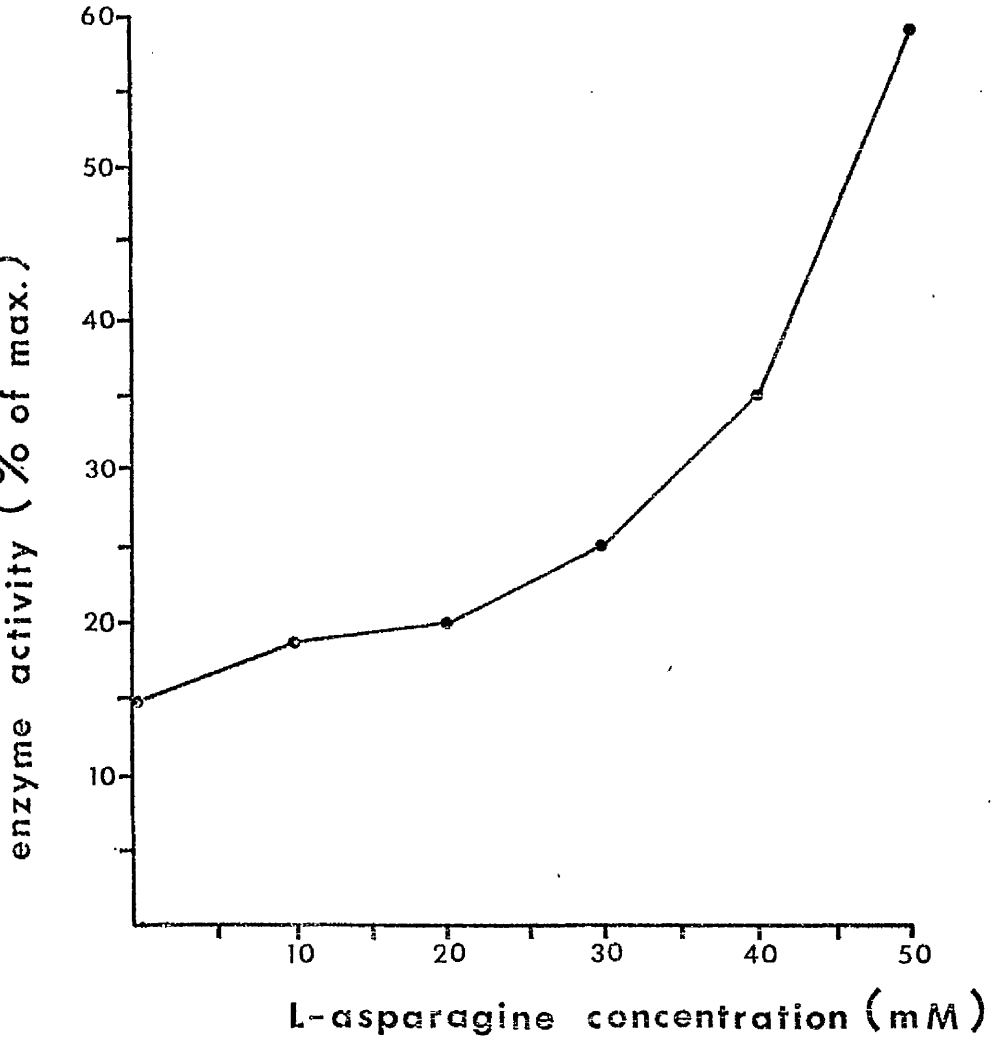


Figure 11. Protection of asparaginase activity by L-asparagine

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extract: wild-type

Incubation conditions: 25°C in presence of various concentrations of L-asparagine for 4 hours

Assay method: aspartyl-transferase activity with ferric chloride reagent

2. Biochemical properties of asparaginase

The pH and temperature optima of asparaginase have been estimated (figures 8 and 9). The pH optimum is 8 and the temperature optimum 37°C.

Extracts with the maximum asparaginase activity (see below), maintain their activity for at least 6 months, if they are stored at -20°C. The same extracts lose the 50% of their activity within 24 hours if they are stored in 4°C, and they lose all of their activity if they are stored in the same temperature for more than 48 hours.

Extracts with maximum activity incubated in higher temperatures (25°C or 37°C) gradually lose their activity in shorter time (figure 10). Incubation in 25°C results in complete loss of the activity after 4½ hours, while incubation in 37°C results in complete loss of the activity after 2 hours.

L-asparagine reduces the loss of enzyme activity in extracts held at high temperatures (figure 10). The protection against loss of the activity at high temperatures is proportional to the amount of L-asparagine added into the extract (figure 11). To ensure that the protection against the loss of the activity is not due to the increase in molarity of the extract, controls were set up with tris-HCl buffer of the same molarity added to the extracts. The loss of the activity in these controls was similar to that in extracts with no L-asparagine added.

Ammonia or L-aspartate added in the reaction mixture at the time of assaying asparaginase activity, had not any effect on the activity. The assay method was that used to detect aspartyl-transferase activity with ferric chloride reagent. The reaction mixture was that as described for this method with ammonia or L-aspartate added. The concentration of ammonia and L-aspartate in the reaction mixture was a range of 5-40 mM. The fact that ammonia or L-aspartate do not reduce asparaginase activity is some evidence that asparaginase is not subject to product inhibition.

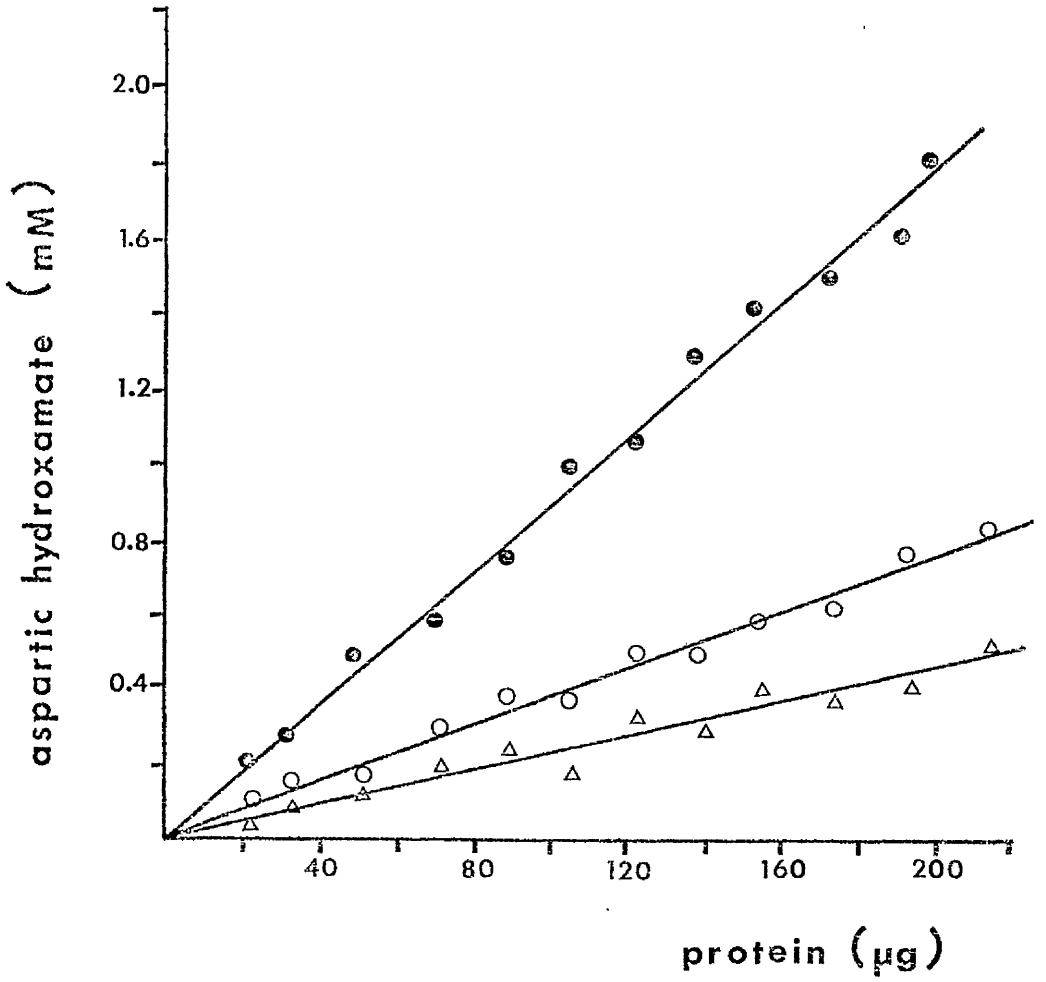


Figure 12. Asparaginase activity of the ahrA3 and tamA119 mutants (activity versus enzyme concentration).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Extracts: ●: wild-type, ○: ahrA3, △: tamA119

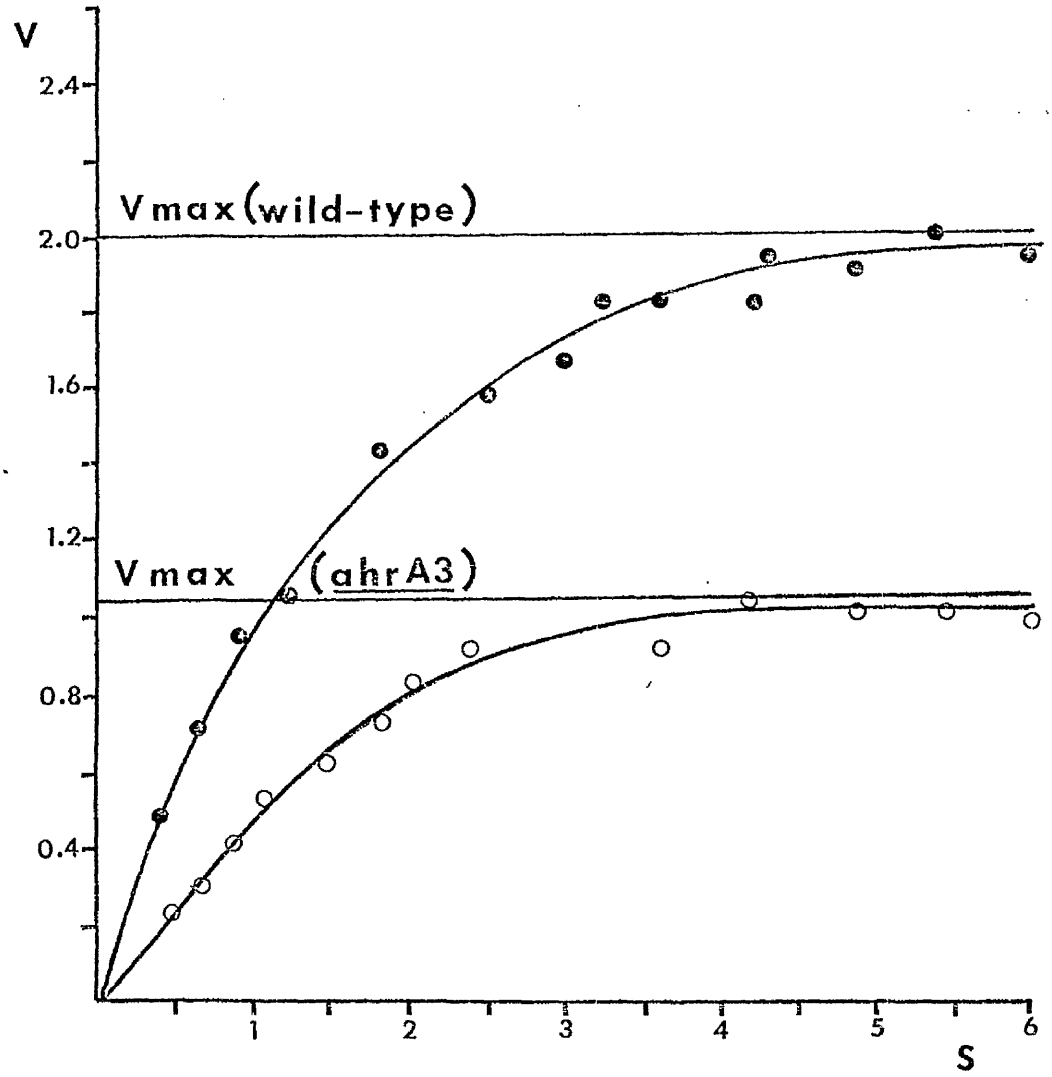


Figure 13. Effect of L-asparagine concentration on asparaginase activity.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

V: µmoles of aspartic hydroxamate/30 min

S: µmoles of L-asparagine

Extracts: ●: wild-type, ○: ahrA3

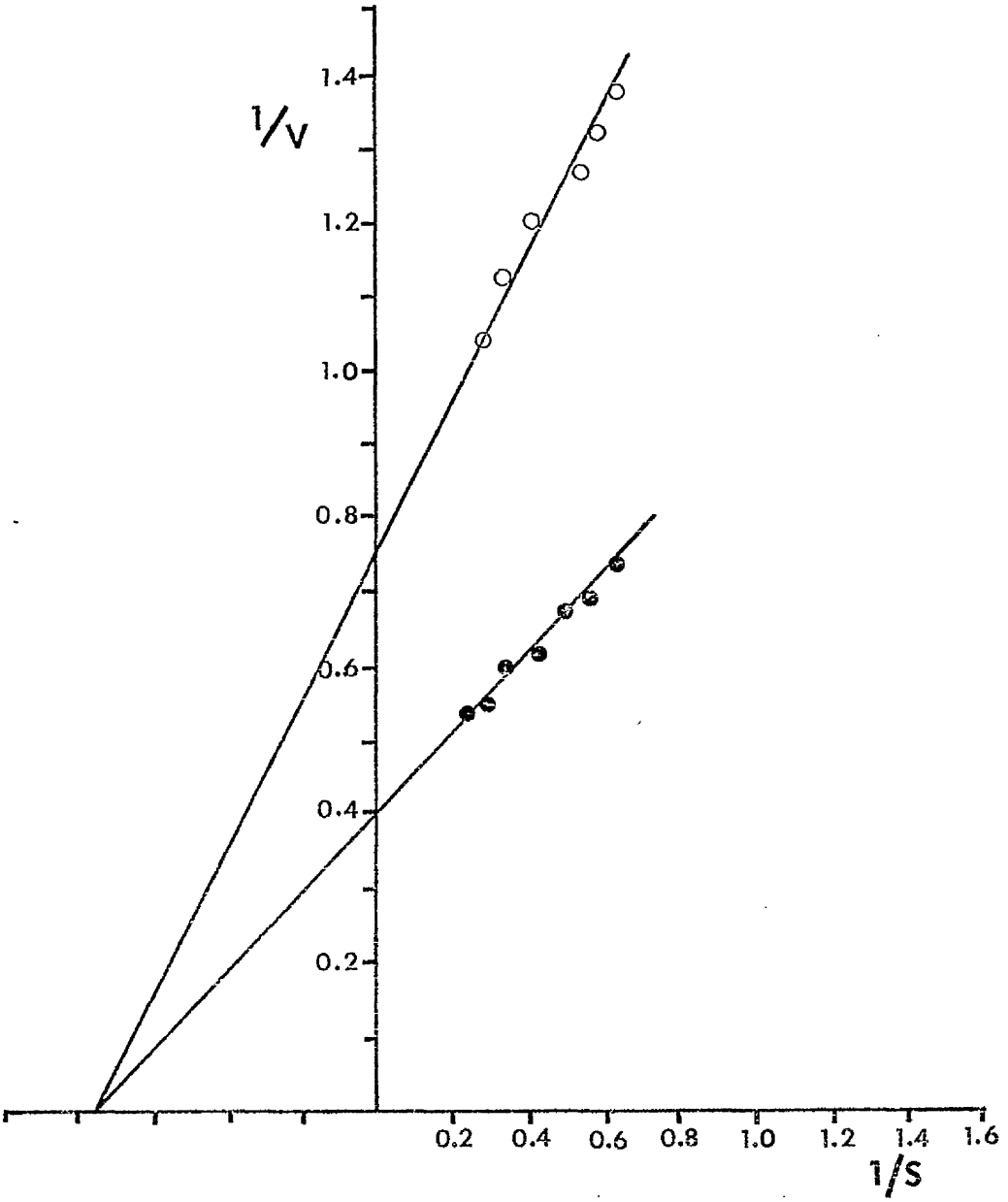


Figure 14. Effect of L-asparagine concentration on asparaginase activity (Lineweaver-Burk reciprocal plot).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

V: µmoles of aspartic hydroxamate/30 min

S: µmoles of L-asparagine

Extracts: ● : wild-type, ○ : ahrA3

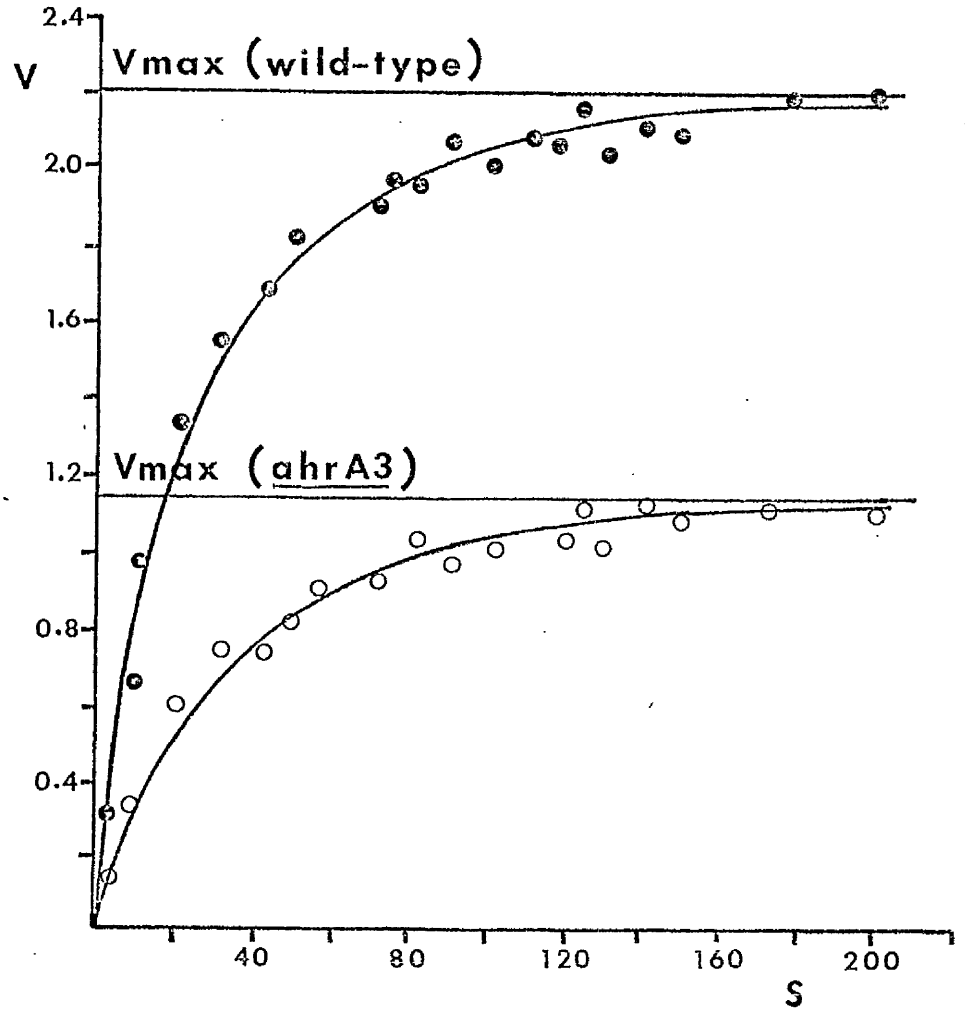


Figure 15. Effect of hydroxylamine concentration on asparaginase activity.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

V: µmoles of aspartic hydroxamate/30 min

S: µmoles of hydroxylamine

Extracts: ●: wild-type, ○: ahrA3

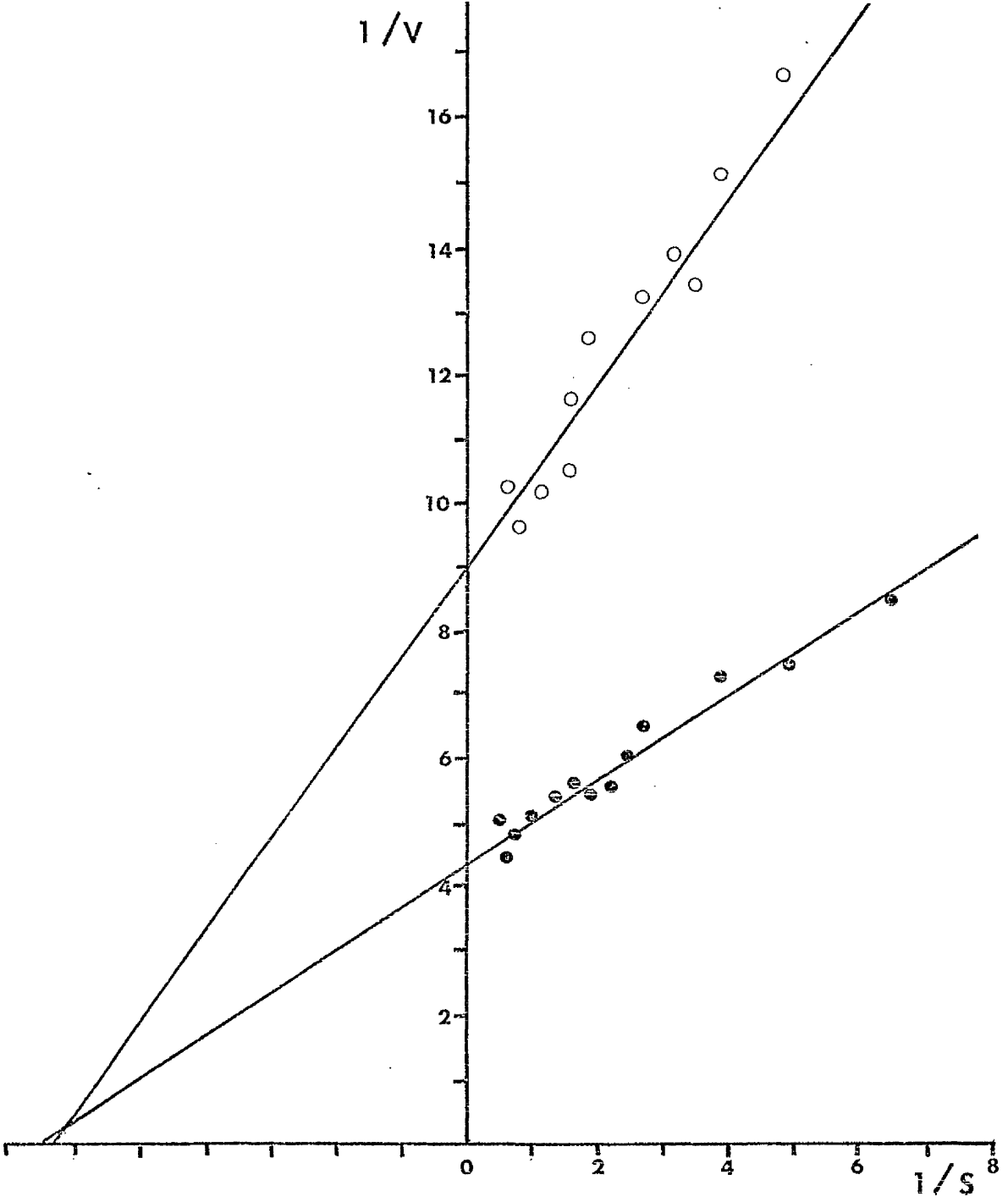


Figure 16. Effect of hydroxylamine concentration on asparaginase activity (Lineweaver-Burk reciprocal plot).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

V: 10^{-1} µmoles aspartic hydroxamate/30 min

S: 10^{-2} µmoles hydroxylamine

Extracts: ● : wild-type, ○ : ahrA3

3. Kinetics of asparaginase activity

A number of variables affecting the assay of asparaginase activity were studied: It was found that asparaginase activity is linear for at least the first hour of the reaction time (figure 5), and the activity is proportional to the enzyme concentration (figure 12). The enzyme concentration was taken as proportional to the total protein concentration of the extract.

The effect of substrate concentration on asparaginase activity was investigated over a range of 0.5 - 6 µmoles L-asparagine (figure 13) and 0.5 - 200 µmoles hydroxylamine (figure 15). A Lineweaver-Burk double reciprocal plot (Lineweaver and Burk 1934) of the data was made (figures 14 and 16) and this shows that asparaginase activity follows Michaelis-Menten kinetics with a Michaelis constant (km) of 0.6 mM for asparagine and 8.3 mM for hydroxylamine and a maximum velocity of about 2.2 µmoles of aspartic hydroxamate/30 min.

nitrogen source (10 mM)	asparaginase activity nanomoles/min/mg protein	
	no treatment	transfer to -N medium for 3 h
L-alanine	250	500
ammonia	< 5	1200
L-asparagine	100	750
L-aspartate	250	225
L-glutamate	200	230
L-glutamine	< 5	900
nitrate	50	800
nitrite	<5	950
urea	173	850

Table 10. Wild-type asparaginase activity with various nitrogen sources.

<5: undetectable activity

4. Regulation of asparaginase synthesis

a. Wild type asparaginase activity with various nitrogen sources

Wild type cells were grown on various nitrogen sources and their cell free extracts were assayed for asparaginase activity, either without any further treatment or after transferring the cells to nitrogen free conditions (-N medium). Cells grown on ammonia as the sole nitrogen source had no detectable asparaginase activity. Cells grown on ammonia and transferred to nitrogen free conditions for 3 hours had the maximum asparaginase activity (table 10). Cells grown on one of the following nitrogen sources L-asparagine, L-glutamine, nitrate, nitrite, urea, had low or undetectable asparaginase activity (table 10). The activity was increased (derepressed) when the cells were transferred to nitrogen free conditions after growth on one of the above nitrogen sources. Cells grown on L-alanine or L-aspartate or L-glutamate had significantly higher asparaginase activity compared to the activity of cells grown on any other nitrogen source with no further treatment. The activity of these cells remained at about the same level when they were transferred to nitrogen free conditions. The activity of cells grown on L-alanine was increased two fold after transfer to -N medium.

b. Kinetics of the derepression of asparaginase activity

Wild-type cells grown on ammonia were transferred to nitrogen free conditions and the increase of asparaginase activity

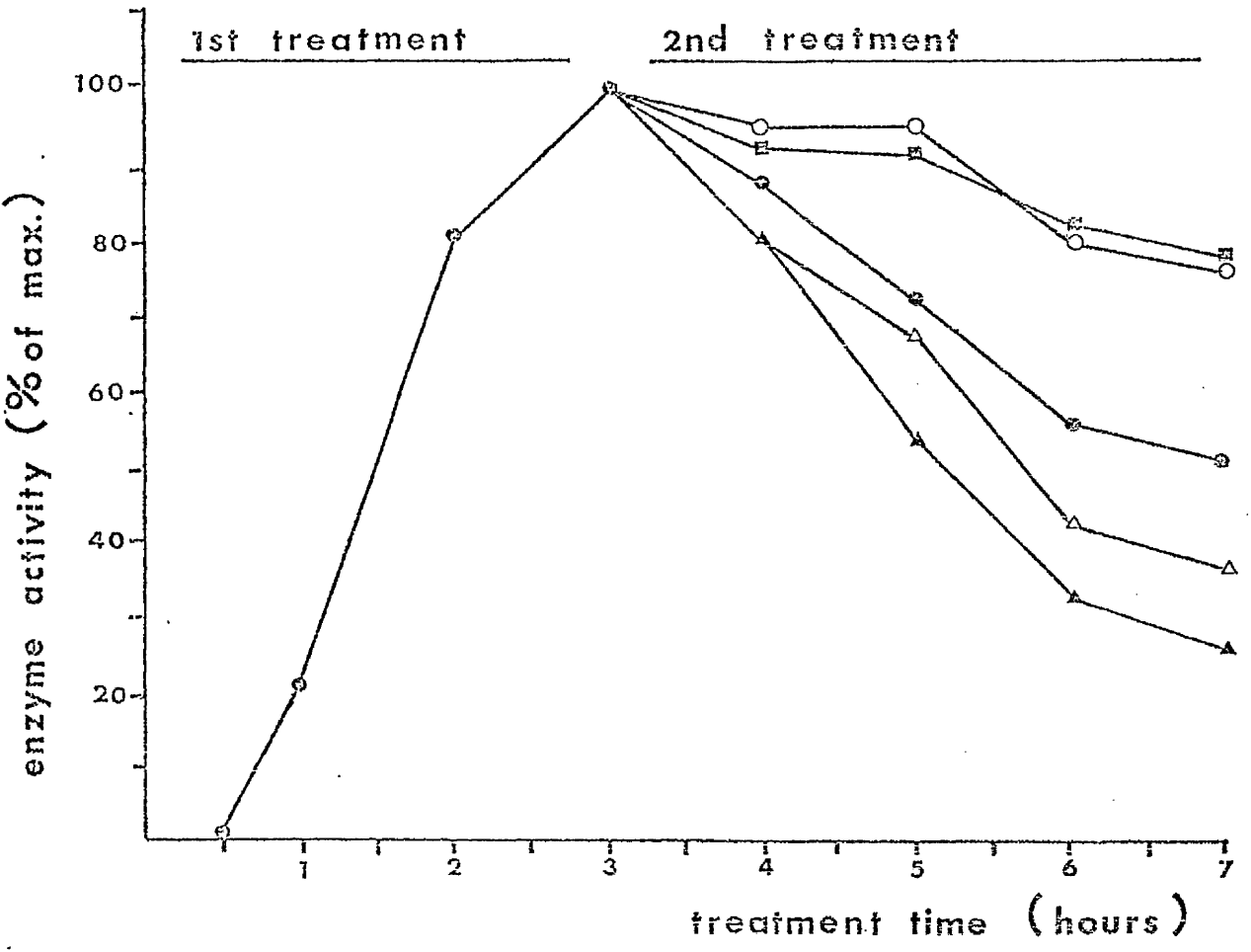


Figure 17. The effect of ammonia on asparaginase synthesis.

Growth conditions: 17 hours on 10 mM ammonia

1st treatment: nitrogen starvation (-N medium)

2nd treatment: ⊖: continuation of nitrogen starvation

○: continuation of nitrogen starvation in presence of cycloheximide (10 µg/ml)

▲: 10 mM ammonia

△: 10 mM nitrate or L-asparagine

▣: 10 mM ammonia or nitrate or L-asparagine in presence of cycloheximide

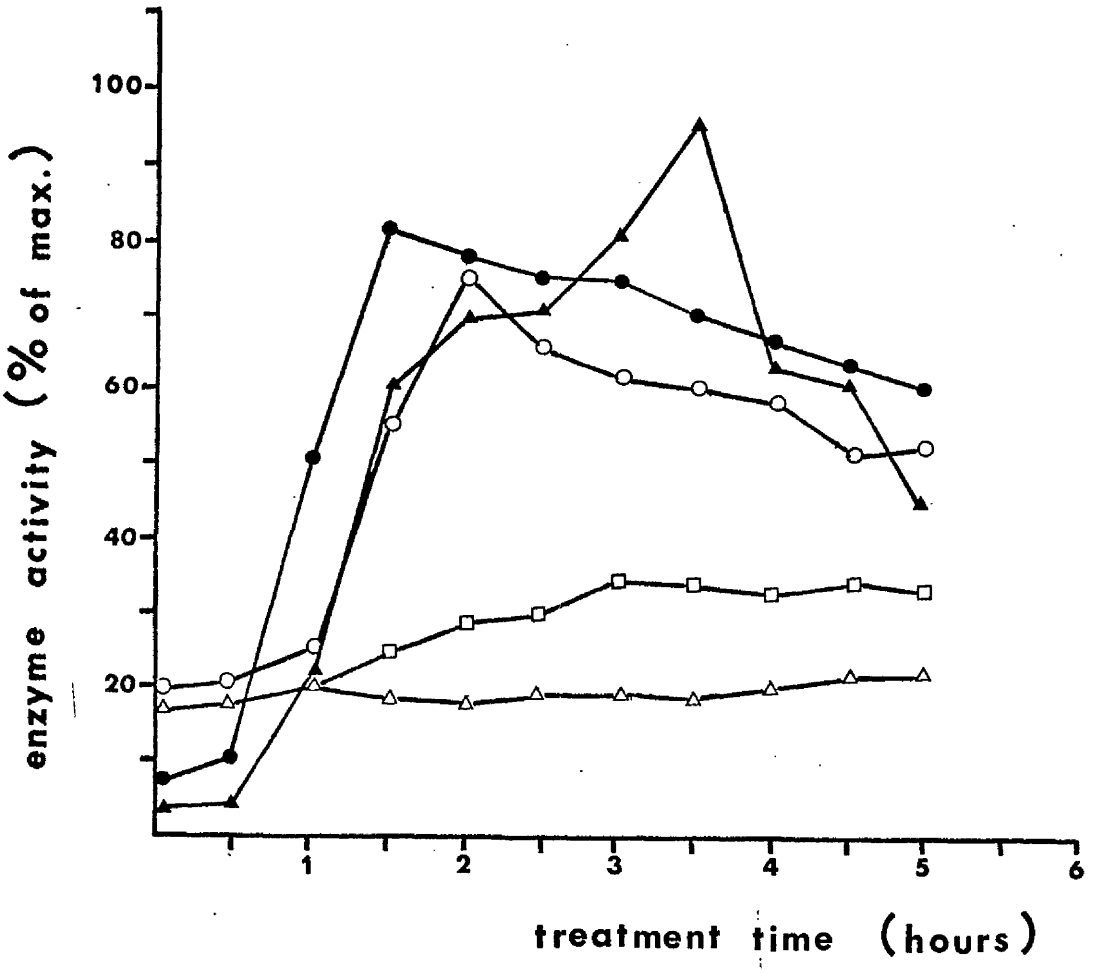


Figure 18. Asparaginase activity of nitrogen starved cells grown on various nitrogen sources before starvation.

Growth conditions: 17 hours on 10 mM of:

●: urea

○: nitrate

Δ: L-aspartate or L-glutamate

□: L-alanine

▲: L-asparagine

Treatment: nitrogen starvation (-N medium)

after various times of nitrogen starvation was determined (figure 17). Asparaginase activity could be detected approximately 30 minutes after transfer and increased to a maximum at 3 hours. After 3 hours the activity decreased. The addition of cycloheximide (inhibitor of protein synthesis, Siegel and Sisler 1963) at the time of transfer when derepression starts, prevented any increase of the enzyme activity. Cycloheximide added to the growth medium at 3 hours (maximum activity) had less effect on activity and reduced the loss of the activity during nitrogen starvation.

Addition of ammonia at 3 hours (maximum activity) resulted in rapid loss of activity (figure 17). When cycloheximide was added at the same time as ammonia, the effect was the same as if ammonia had not been added at all. Addition of nitrate at 3 hours (maximum activity) had the same effect as ammonia but to a lesser extent. Addition of cycloheximide at the same time, similarly prevented the effect of nitrate. Addition of L-asparagine at 3 hours had the same effect as nitrate and it was also prevented by cycloheximide. The effect of L-asparagine for clarity is not presented on figure 17.

Cells grown on nitrate or L-asparagine or urea and transferred to nitrogen free conditions had a maximum increase of asparaginase activity after 3½, 1½ and 2 hours respectively (figure 18). The activity of cells grown on L-aspartate or L-glutamate transferred to nitrogen free conditions remained at about the same level during the time of treatment (figure 18). Cells grown on L-alanine had some increase of asparaginase activity after transfer to the same conditions.

	asparaginase activity nanomoles/min/mg protein	
growth conditions	wild-type	*nirA1*
10 mM ammonia	5	5
transfer to -N medium	1200	1200
transfer to -N medium + 10 mM nitrate	325	1200

Table 11. Asparaginase activity of the *nirA* mutant.

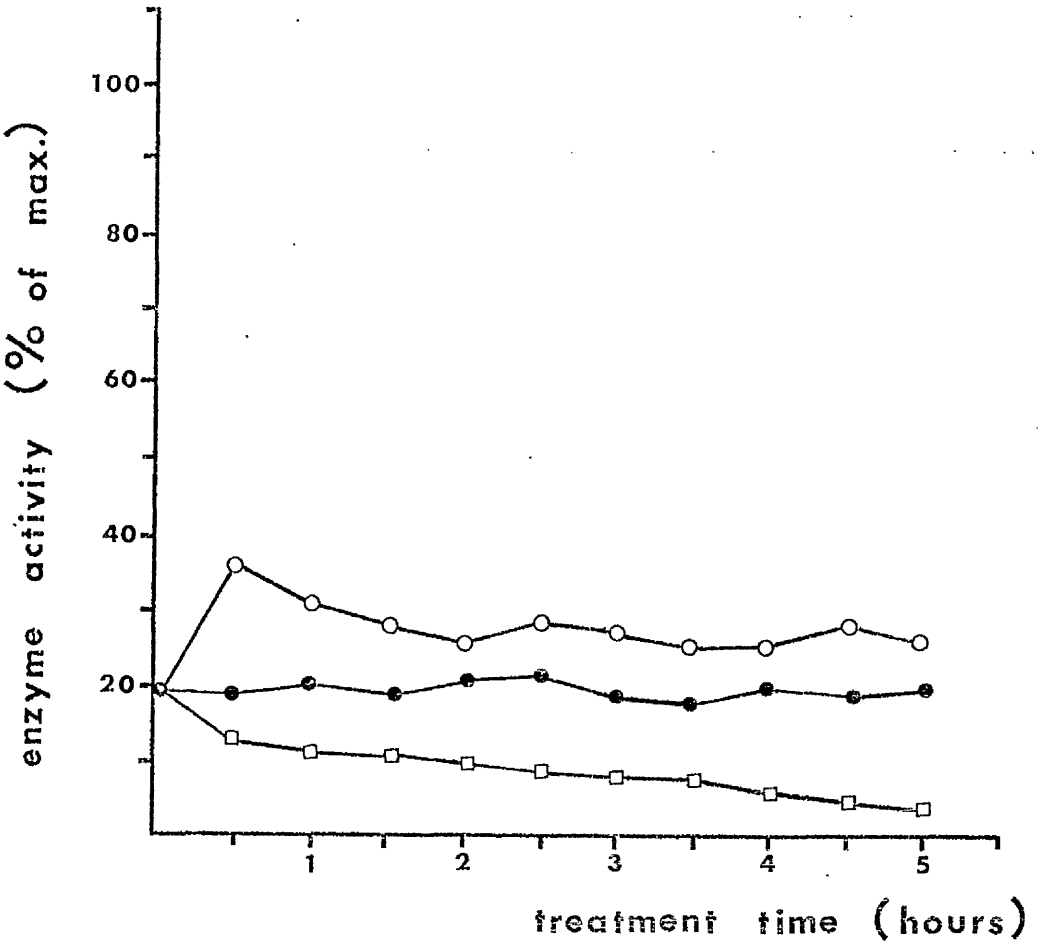


Figure 19. The effect of carbon and nitrogen starvation on asparaginase activity.

Growth conditions: 17 hours on 10 mM urea

Treatment: ●: nitrogen and carbon starvation

○: 10 mM L-asparagine as sole nitrogen and carbon source

□: 10 mM L-asparagine as sole nitrogen source.

Transfer of wild type cells into carbon or nitrogen and carbon free conditions does not result to increase of asparaginase activity (figure 19). The activity remains undetectable if cells grown on ammonia are transferred to the above conditions. The activity remains at the same level if cells grown on urea are transferred to the same conditions (figure 19).

Cells grown on ammonia and transferred to L-asparagine as sole nitrogen or sole nitrogen and carbon source have no detectable asparaginase activity. Cells grown on urea and transferred to the same conditions have decreased and increased to a limited extent asparaginase activity respectively (figure 19).

c. Asparaginase activity of the nirA mutant

nirA is a regulatory mutant lacking both nitrate and nitrite reductase activity (see introduction). This mutant cannot grow on nitrate or nitrite as sole nitrogen sources and is unable to reduce them to ammonia. nirA cells grown on ammonia have no detectable asparaginase activity, but they have wild-type activity when they are transferred to nitrogen free conditions. Nitrate added at the time of derepression has no effect on the increase of asparaginase activity (table 11), but nitrate added to wild-type cells when derepression starts prevents the production of asparaginase and the activity is very low. This suggests that in wild-type cells nitrate represses asparaginase because of the production of ammonia.

	asparaginase activity nanomoles min^{-1} $(mg\ protein)^{-1}$	
	10 mM ammonia	transfer to -N medium 3 h
tamA119	< 5	200
tamA50	< 5	< 5
$areA^{r}551$	< 5	1100
$areA^{r}510$	< 5	1200
$areA^{r}550$	< 5	< 5
tanA1	< 5	220
$tamA^{d}1$	460	1100
$areA^{d}520$	200	530
gdhA1	125	700
meaA8	163	169
DER-3	254	810
xprD1	< 5	300

Table 12. Asparaginase activity of ammonia repressed and derepressed mutants.

5. Asparaginase activity of ammonia repressed and derepressed mutants

Partially repressed mutants of the tamA locus proved to have low asparaginase activity (table 12). The partially repressed mutants tamA119 and tan1 have low asparaginase activity of the level 200 nanomoles/min/mg of protein. The completely repressed mutant tamA50 has no detectable asparaginase activity. Partially repressed mutants of the areA locus (areA551 and areA510) have wild-type asparaginase activity but the areA550 completely repressed mutant has no detectable asparaginase activity (table 12).

All the ammonia derepressed mutants tested produced asparaginase activity when grown on ammonia as the sole nitrogen source (table 12). Specifically the $tamA^{d}1$ yields twice as much activity as the $areA^{d}520$ levels of 460 nanomoles/min/mg of protein and 200 nanomoles/min/mg of protein respectively. The activity of the gdhA and the ammonia transport deficient mutant meaA8 when grown on ammonia is low compared with the others (125 nanomoles/min/mg of protein and 163 nanomoles/min/mg of protein respectively), but the activity of DER-3 is higher (254 nanomoles/min/mg of protein). The xprD1 mutant grown on ammonia has no detectable asparaginase activity.

The activity of the ammonia derepressed mutants is increased when the cells are transferred to nitrogen free conditions after growth on ammonia (with an exception of meaA8 where the activity remains the same). In order to assay the gdhA mutant for asparaginase

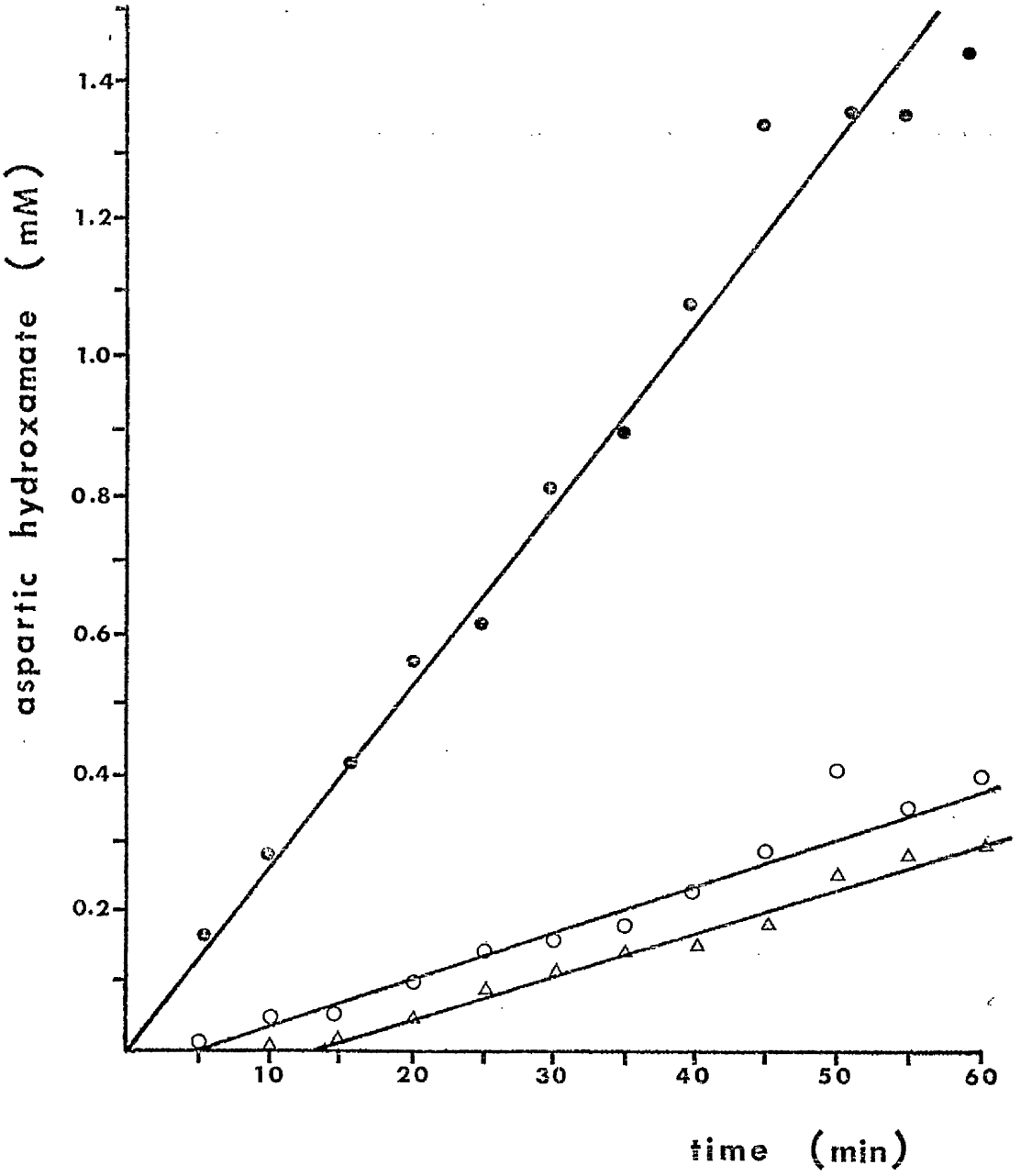


Figure 20. Asparaginase activity in ahrA3 and tamA119 extracts (activity versus time).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

Assay method: aspartyl-transferase activity with ferric chloride reagent.

●: wild-type, ○: ahrA3, △: tamA119

activity after growth on ammonia L-alanine was added to the growth medium as gdhA has poor growth on ammonia as the sole nitrogen source.

The rate of enzyme activity of the partially repressed mutant tamA119 is linear during the time of the assay and is proportional to enzyme concentration (figures 20 and 12). tamA119 asparaginase activity follows Michaelis-Menten kinetics and has a Km similar to the wild-type for asparagine and hydroxylamine. On the figures 13-16 the tamA119 curve for clarity is not presented, since it is similar to the ahrA3 curve.

Strains	Asparaginase activity		Percentage of maximum activity in haploid wild-type
	amino-hydrolase nmoles NH_4/min/mg	aspartyltransferase nmoles NH_4/min/mg	
ahrA$^+$	350	1200	100
ahrA$^+$/ahrA$^+$	-	1320	110
ahrA1	<30	<5	0
ahrA1/ahrA$^+$	-	640	53
ahrA2	<25	<5	0
ahrA2/ahrA$^+$	-	630	52
ahrA3	85	420	35
ahrA3/ahrA$^+$	-	990	82
ahrA4	-	<5	0
ahrA4/ahrA$^+$	-	690	57
ahrA2/ahrA3	-	230	18

Table 13. Asparaginase activity of wild-type and ahrA haploid and heterozygous diploid strains.

Cells were grown for 17 hours on -N medium supplemented with 10 mM ammonia and then transferred to -N medium for three hours. <30 activity for amino-hydrolase activity is considered as undetectable since the same activity is detected in wild-type cells grown on ammonia (see table 25). The percentage of maximum activity refers to aspartyl-transferase activity.

6. Asparaginase activity of the ahrA mutants. Gene-dosage effect.

The ahrA mutants were assayed for both of the activities of asparaginase, amino-hydrolase and aspartyl-transferase (table 13). The strongly resistant mutants ahrA1 and ahrA2 have undetectable levels of both activities. The weakly resistant ahrA3 has detectable but low levels of both activities. The strongly resistant mutants ahrA4-A11 were assayed for aspartyl-transferase only and had undetectable activity. In table 13 the mutants ahrA5-A11 for economy are not represented since they were very similar to ahrA4. The heterozygous diploids of the mutants ahrA1, ahrA2 and ahrA4 with the wild-type allele possessed about 50% of the enzyme activity of the homozygous wild-type diploid. The heterozygous diploid of the weakly resistant ahrA3 with the wild-type had about 80% of the wild-type diploid activity. The heterozygous diploid between the strongly resistant ahrA2 and the weakly resistant ahrA3 had about 18% of the enzyme activity of the wild-type diploid (50% of the activity of the haploid ahrA3).

The rate of asparaginase activity of the ahrA3 mutant was linear during the time of the assay and proportional to enzyme concentration (figures 20 and 12), and follows Michaelis-Menten kinetics with a Km for L-asparagine and hydroxylamine similar to that of the wild-type (figures 13, 14, 15 and 16). The asparaginase activity of the heterozygous diploids ahrA/ahrA^{+} also had a wild-type Km for both the substrates.

strains	enzyme		
	NADP-GDH	NADPH-GDH	Glutamine synthetase
wild-type	1560	1450	1100
ahrA1	1200	1100	1700
ahrA2	1250	1100	1850
ahrA3	1320	1150	1500

Table 14. Enzyme activities of the ahrA mutants.

NADP-GDH activity measured in nanomoles of NADP reduced/min/mg protein.

NADPH-GDH activity measured in nanomoles of NADPH oxidised/min/mg protein.

Glutamine synthetase activity measured in nanomoles of glutamate hydroxamate/min/mg protein.

The ahrA mutants were also assayed for other enzyme activities such as NADP and NADPH glutamate dehydrogenase activity and glutamine synthetase activity. All the ahrA mutants tested had approximately wild-type activities for the other enzymes. The specific activities are shown in table 14.

7. Discussion

Asparaginase activity was assayed in wild-type cells of Aspergillus nidulans by various methods based on amino-hydrolase and aspartyl-transferase activity. The data presented show a parallelism between the two activities which indicates that both are probably catalytic activities of the same enzyme. Wild-type cells grown on ammonia as the sole nitrogen source have undetectable levels of both amino-hydrolase and aspartyl-transferase activity. Wild-type cells grown on ammonia as the sole nitrogen source have maximum levels of both activities after transfer to -N medium for three hours. Similar results are obtained using the conductimetric method. The above hypothesis is supported by the fact that the ahrA mutants lack both amino-hydrolase and aspartyl-transferase activities (see discussion below). It was found that the assay based on the aspartyl-transferase activity of asparaginase by estimating the produced aspartic hydroxamate from L-asparagine and hydroxylamine, was both sensitive and accurate and it was routinely used to investigate the regulation of asparaginase synthesis. This enzyme, which, as discussed below, is presumably coded by the ahrA locus, will from now on be referred to as asparaginase I.

The level of asparaginase I activity is reduced by ammonia if it is present in the growth medium or if it is produced intracellularly. Cells grown on ammonia or ammonia-producing amino acids or inorganic nitrogen sources, have low or undetectable asparaginase I activity. On transfer of the cells to nitrogen free conditions the enzyme activity is derepressed, increasing to a maximum

after about 3 hours and then it starts gradually to decrease. Presence of cycloheximide at the time when derepression starts, results in a lack of asparaginase I activity. Therefore protein synthesis is apparently necessary for the production of asparaginase I activity.

Ammonia added at the time of maximum enzyme activity results in rapid loss of the activity. This loss of activity is prevented by cycloheximide if added at the same time as ammonia. There are two main types of explanation for the role of ammonia in the control of asparaginase I activity:
First, ammonia inactivates asparaginase I *in vivo* and this inactivation, possibly mediated by a protease induced by ammonia, needs protein synthesis. Ammonia does not inhibit asparaginase I *in vitro*.
Second, ammonia represses asparaginase I synthesis and the requirement for protein synthesis is due to the ammonia mediated synthesis of one or more control proteins which block the synthesis of asparaginase I. It is possible that ammonia both represses the synthesis of asparaginase I and inactivates the enzyme *in vivo* by stimulating the synthesis of a protease which can use asparaginase as a substrate.

Nitrate added at the time of maximum activity has the same effect as ammonia but to a lesser extent. The decrease in enzyme activity caused by nitrate is probably due to the ammonia produced from it. This is indicated by the fact that nitrate prevents derepression in wild-type cells but does not prevent derepression in *nirA*$^-$ cells (which are unable to produce ammonia from nitrate).

Asparaginase I activity did not increase when cells growing on ammonia were transferred to carbon-free or nitrogen- and carbon-free conditions.

Asparaginase I activity is not induced by L-asparagine. The cells synthesise asparaginase I when nitrogen is in short supply and not just when L-asparagine is present in the growth medium.

Mutants generally repressed for a number of systems involved in nitrogen metabolism, are repressed for asparagine I activity. Partially repressed alleles of the tamA locus have low asparaginase I activity and the completely repressed allele has no detectable enzyme activity. Partially repressed alleles of the areA locus have almost wild-type enzyme activity, while completely repressed alleles of the same locus have no detectable asparaginase I activity. General ammonia derepressed mutants all have significantly detectable asparaginase I activity in the presence of ammonia. These derepressed mutants have also proved to be derepressed for other ammonia controlled systems (tamA^{d}1 and areA^{d}520) or they are deficient in ammonia transport (mea8 and DER-3).

The aspartic hydroxamate resistant mutants have asparaginase I activity in parallel with their resistance to aspartic hydroxamate. The strongly resistant mutants have no detectable asparaginase I activity and the weakly resistant one (ahrA3) has low asparaginase I activity. The ahrA mutants have wild-type activities for other enzymes tested. The anomaly is that the ahrA mutants are resistant to aspartic hydroxamate but can grow on L-asparagine as nitrogen source. There are two main possible explanations.

First, the asparaginase-like activity, determined by the ahrA gene is not in vivo responsible for the utilisation of L-asparagine. Second, this enzyme is responsible for the utilisation of L-asparagine, but when we select for resistance to aspartic hydroxamate, we select for a modified protein which retains the ability to use L-asparagine as substrate but can no longer use aspartic hydroxamate as a substrate. The extreme form of the hypothesis that the enzyme in the ahrA mutants has lost the ability to use aspartic hydroxamate but has retained activity with L-asparagine is disproved because some mutants were assayed for both activities (production of aspartic hydroxamate and production of ammonia from L-asparagine) and they had lost both. Moreover the ahrA3 mutant which is weakly resistant to aspartic hydroxamate can produce small amounts of ammonia from L-asparagine, indicating that it can utilise L-asparagine as a substrate and it also has low aspartyl-transferase activity. Thus the most probable explanation for the ability of the ahrA mutants to grow on L-asparagine as nitrogen source is that there exists a second protein (asparaginase II) with asparaginase-like activity coded by a separate locus from ahrA. This hypothesis is discussed in chapter VII (general discussion).

The levels of asparaginase I activity show a very clear-cut gene dosage effect in various haploids and diploids (table 13). The haploid $ahrA^+$ and the homozygous diploid $ahrA^+/ahrA^+$ show similar enzyme levels. The heterozygous diploids $ahrA1/ahrA^+$, $ahrA2/ahrA^+$ and $ahrA4/ahrA^+$ have about 50% of the enzyme activity of the homozygous wild-type. The diploid ahrA2/ahrA3 has about 50% of the activity of the hapolid ahrA3. Clutterbuck (1968) has shown that in A. nidulans

gene dosage is the same in haploids and diploids. Therefore the simplest explanation is that gene expression under derepression is limiting with consequent gene dosage effects in the various diploids.

The lack of asparaginase I activity in the strongly resistant ahrA mutants, the low enzyme activity of the weakly resistant ahrA3 mutant, the strict gene dosage effect and the allelism of all the ahrA mutants isolated, strongly suggests that the ahrA gene is the structural gene of asparaginase I in Aspergillus nidulans.

Part of this work has been presented to the 180th Genetical Society Meeting (Drainas, Kinghorn and Pateman 1976) and published in the Journal of General Microbiology (Drainas, Kinghorn and Pateman 1977). Another part has been presented to the 566th Biochemical Society Meeting (Drainas and Pateman 1977).

CHAPTER V A MUTATION AFFECTING L-ASPARAGINE UTILISATION

Cross	$\frac{pabaA^+\ yA^+\ biA1\quad pyroA^+\quad asp20\quad tamA50}{pabaA2\ yA2\ biA^+\quad pyroA4\quad asp20^+\ tamA^+}$				
	growth conditions (10mM of)				
genotypes	ammonia	L-alanine	L-asparagine	ammonia + 1mM aspartic hydroxamate	No. of segregants
$asp20^+\ tamA^+$	+	+	+	+	22
$asp20\ tamA^+$	+	+	+	-	26
$asp20^+\ tamA50$	+	-	-	+	30
$asp20\ tamA50$	+	-	+	-	18
				Total	96

Table 15. Isolation of the asp20 mutant from segregants of a cross of the asp20-tamA50 double mutant with the wild type.

+: growth, -: no growth.

The asp20 mutant was isolated from segregants sensitive on aspartic hydroxamate in presence of ammonia

A MUTATION AFFECTING L-ASPARAGINE UTILISATION

1. Isolation of the aspA20 mutant

Constitutive mutants of various enzyme or uptake systems can be isolated on the basis of suppression of regulatory repressed mutants of the corresponding system (Arst and Cove 1973). On this basis the tamA50 mutant which is completely repressed for a number of systems (Kinghorn and Pateman manuscript in preparation) was used for the isolation of such a derepressed mutant specific for the utilisation of L-asparagine.

tamA50 is unable to grow on any inorganic or amino acid nitrogen source except ammonia. Consequently it is unable to grow on any concentration of L-asparagine tested (5 - 50 mM). A mutant which specifically suppressed tamA50 on L-asparagine would allow growth of the double mutant as a sole nitrogen source. Such a mutant was isolated as described in the materials and methods by NTG treated tamA50 conidia. A cross of the suppressed tamA50 strain with the wild-type showed that it was a double mutant which contained the tamA50 allele and a mutation at a new locus (temporarily designated aspA20), which allowed the utilisation of L-asparagine as nitrogen source (table 15) in the presence of the tamA50 allele.

Both the double mutant aspA20-tamA50 and the single mutant aspA20 are ammonia derepressed for aspartic hydroxamate toxicity. Therefore phenotypically the aspA20 mutant could be isolated on the basis of sensitivity on aspartic hydroxamate in the presence of

ammonia and growth on any nitrogen source which distinguishes it from the aspA20-tamA50 double mutant which is also sensitive on aspartic hydroxamate but is able to grow only on L-asparagine (or ammonia) as a sole nitrogen source.

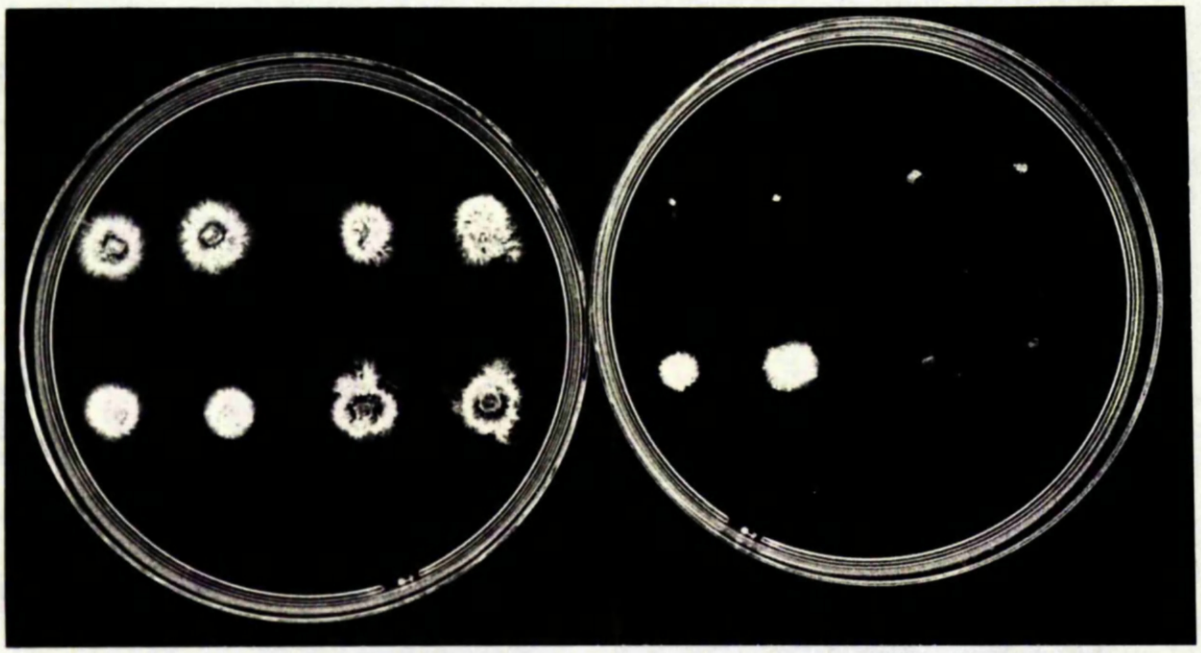


Plate 9. Growth of heterocaryons (*asp20* + *asp20*$^+$) on aspartic hydroxamate in presence of ammonia.

Growth medium: -N plus 10 mM ammonia

Left plate: no aspartic hydroxamate

Right plate: 1 mM aspartic hydroxamate

Heterocaryons on the plates in duplicate:

(*asp20* + *asp20*$^+$)1 (*asp20* + *asp20*$^+$)2

(*asp20*$^+$ + *asp20*$^+$) (*asp20* + *asp20*$^+$)3

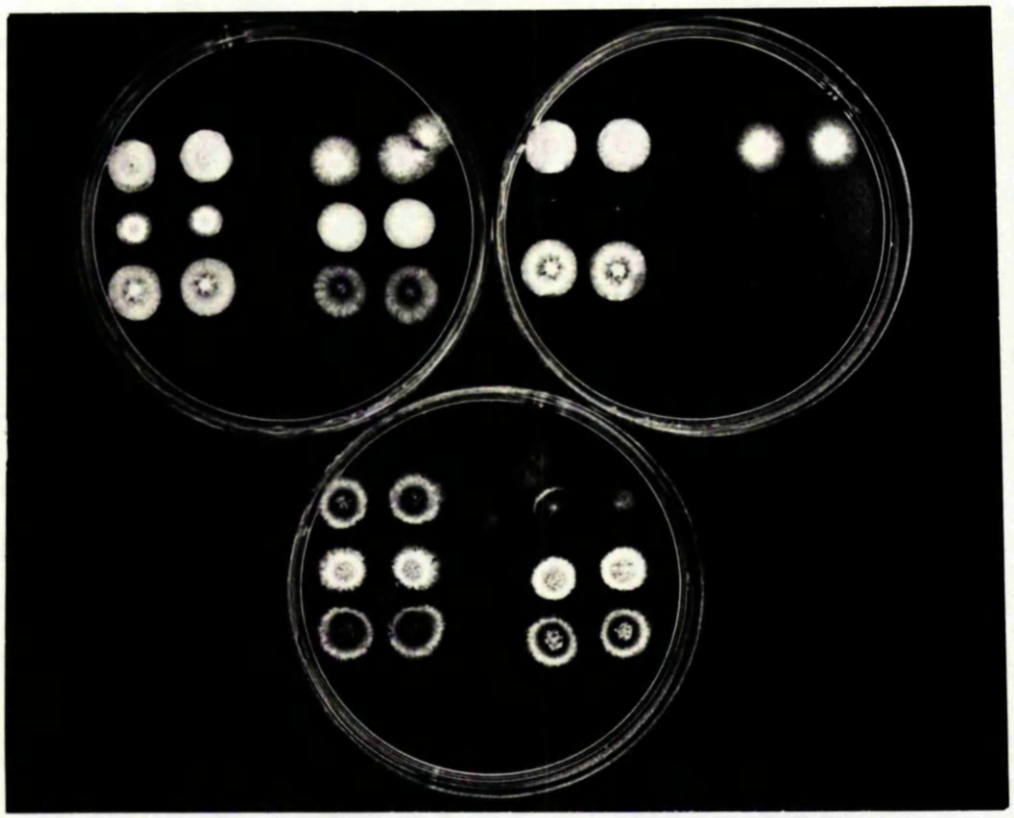


Plate 10. Phenotypic differences of asp20, tamA50 and wild-type on ammonia plus aspartic hydroxamate and on L-asparagine.

Growth medium: -N

Upper plates: Left, 10 mM ammonia

Right, 10 mM ammonia plus 1 mM aspartic hydroxamate

Lower plate: 10 mM L-asparagine

Strains on the plates in duplicate:

Wild-type	tamA50
meaA8	asp20
asp20^{+}/asp20	asp20-tamA50

2. Genetic characterisation of the aspA20 mutant

a. Dominance relationships

Forced heterozygous heterocaryons of the aspA20 allele with the wild-type allele were made. The aspA20 strain was carrying the biA1 marker and the strain carrying the aspA20$^+$ allele also carried the markers of the M.S.E. strain. Three different heterocaryons were made with the same forcing markers and were growth tested on -N medium plus ammonia plus aspartic hydroxamate (plate 9). All three heterocaryons were sensitive to aspartic hydroxamate in the presence of ammonia and therefore phenotypically have the characteristic of the mutant allele. In the plate 9 the homozygous wild-type heterocaryon was carrying the biA1 marker from the one strain and the M.S.E. markers from the other. The heterozygous diploids aspA20$^+$/aspA20 produced from the above heterocaryons are resistant in the same conditions and therefore have the character of the wild-type allele (plate 10). In plate 10 the difference in phenotype of the wild-type and the mutant strain on aspartic hydroxamate in the presence of ammonia is shown. As a conclusion the aspA20 mutation is expressed as dominant in heterocaryons and recessive in diploids.

The suppression of tamA50 by aspA20 was investigated in the haploid double mutant aspA20-tamA50 only with respect to growth on L-asparagine as the sole nitrogen source. There was no investigation of the relationship of the aspA20 locus to the tamA50 allele with respect to growth on other nitrogen and carbon sources, or of the

Diploid		asp20 M.S.E.	
Linkage group	gene marker	asp20	asp20$^+$
	y^+	22	26
I	y^-	25	23
	w^+	24	24
II	w^-	23	25
	gal^+	27	23
III	gal^-	20	26
	$pyro^+$	24	22
IV	$pyro^-$	23	27
	fac^+	47	0
V	fac^-	0	49
	s^+	16	17
VI	s^-	31	32
	nic^+	35	31
VII	nic^-	12	18
	$ribo^+$	19	29
VIII	$ribo^-$	28	20

Table 16. Haploidization analysis of the asp20 mutant

Conclusion: asp20 is linked with the V linkage group and is translocation free

relationship of aspA20 with areA alleles. It was considered that such investigations should have second priority with respect to work for this thesis. The major interest was put on the investigation of the nature of the enzyme(s) regulated by the aspA20 locus, concerned with the utilisation of L-asparagine, and the relationships with other L-asparagine utilising enzymes. Further knowledge of the relationship of the aspA20 locus with the tamA and areA loci would be obtained from data on the dominance of aspA20 with respect to the suppression of tamA50 (using forced heterocaryons and diploids homozygous for tamA50), and on the relationship between aspA20 and various alleles in the areA locus.

b. Gene assigning on linkage group and chromosome mapping

The haploidisation of the aspA20 mutant with the M.S.E. strain revealed linkage with the facA marker and therefore the aspA20 mutation was assigned to the linkage group V (table 16).

Cross V: $\frac{biAI\ yA^{+}\quad meth^{+}\quad aspA20\ nicA^{+}\ pA^{+}\ facA^{+}\ riboD^{+}}{biA^{+}\ yA2\quad meth\quad aspA20^{+}\ nicA\ pA\ facA\ riboD}$

The markers biA, yA and meth on the other linkage groups are not classified.

Segregation of markers and allele ratios.

	nicA	nicA$^+$
aspA20	15	32
aspA20$^+$	22	29

Recombination fraction: aspA20 - nicA = 44.9% $\pm$ 5.

	facA	facA$^+$
asp20	13	34
asp20$^+$	21	30

Recombination fraction: aspA20 - facA = 43.8% $\pm$ 5.

	pA	pA$^+$
aspA20	9	34
aspA20$^+$	39	12

Recombination fraction: aspA20 - pA = 21.4% $\pm$ 4.1.

	riboD	riboD$^+$
aspA20	17	30
aspA20$^+$	27	24

Recombination fraction: aspA20 - riboD = 41.8% $\pm$ 4.9.

	F1 genotypes	No. of segregants	
P	facA$^+$ asp20 pA$^+$	31	
	facA asp20$^+$ pA	20	
R	facA$^+$ asp20 pA	3	d.cr.o.
	facA asp20$^+$ pA$^+$	3	
	facA$^+$ asp20$^+$ pA	19	s.cr.o.
	facA asp20 pA$^+$	7	
	facA$^+$ asp20$^+$ pA$^+$	9	
	facA asp20 pA	6	

Table 17. Three gene analysis between the markers asp20, pA, facA of cross V.

facA$^+$, asp20$^+$, pA$^+$: wild-type alleles

facA, asp20, pA : mutant alleles

Conclusion: The pA marker is located between asp20 and facA.

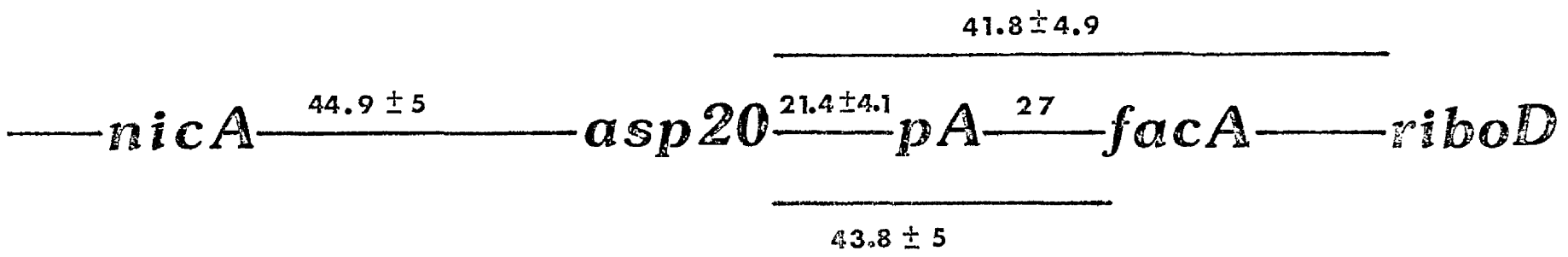


Figure 21. Linkage relationships of the asp20 locus.

Distances between the markers are given in cM.

conclusion: the aspA20 mutation is linked with the pA marker at a distance of 21.4 cM. Three gene analysis located aspA20 at the left side of the pA marker on the linkage map (table 17).

The linkage relationships of the aspA20 mutant are given in figure 21.

strains	wild-type	asp20	asp20-tamA50	asp20-ahrA	asp20-asn⁻1	asn⁻1-ahrA
growth conditions						
-N medium +						
10 mM L-asparagine	+	+	+	+	+	+
10 mM L-aspartate	+	+	-	+	-	-
10 mM L-glutamine	+	+	-	+	-	-
10 mM L-glutamate	+	+	-	+	-	-
10 mM L-alanine	+	+	-	+	-	-
10 mM L-proline	+	+	-	+	-	-
10 mM urea	+	+	-	+	-	-
10 mM ammonia	+	+	+	+	-	-
-CN medium +						
50 mM L-asparagine	+	++	+	+		
50 mM L-aspartate	+	+	+	+	not tested	
50 mM L-glutamine	+	+	+	+		
50 mM L-glutamate	+	+	+	+		

Table 18. Growth responses of the asp20 mutant.

++: growth better than the wild type, +: wild type growth, -: no growth

Cross		$\frac{pabaA^+\ yA^+\ biA1}{pabaA^+\ yA2\ biA^+}$	$\frac{pyroA^+}{pyroA4}$	$\frac{asp20}{asp20^+}$	$\frac{ahrA^+}{ahrA2}$	
		growth conditions (10 mM of)				
genotypes	L-proline	L-proline + 1 mM aspartic hydroxamate	ammonia		ammonia + 1 mM aspartic hydroxamate	No. of segregants
$\frac{asp20}{asp20}\ \frac{ahrA^+}{ahrA^+}$	+	-	+		-	52
$\frac{asp20^+}{asp20}\ \frac{ahrA2}{ahrA2}$	+	+	+		+	23
$\frac{asp20}{asp20}\ \frac{ahrA2}{ahrA2}$ (double mutant)						
$\frac{asp20^+}{asp20^+}\ \frac{ahrA^+}{ahrA^+}$ (wild type)	+	-	+		+	21
					Total	96

Table 19. Cross of the asp20 mutant with an ahrA strain

+: growth, -: no growth.

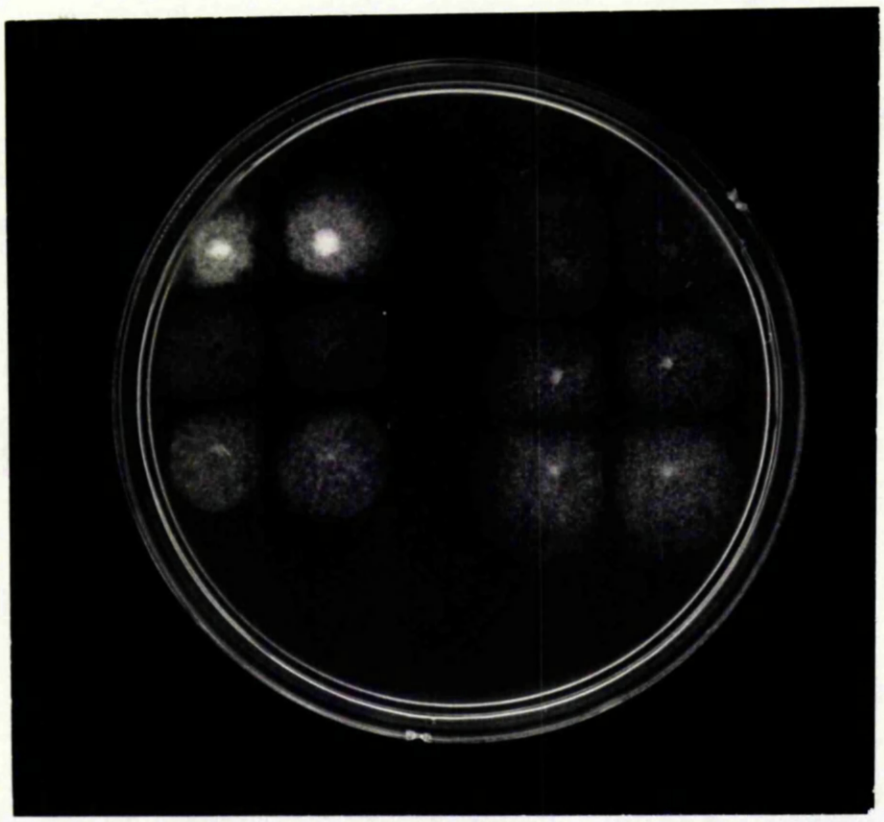


Plate 11. Growth of the asp20 mutant on L-asparagine as the sole carbon source.

Growth medium: -CN plus 10 mM ammonia (ammonium chloride as nitrogen source) plus 50 mM L-asparagine (as carbon source)

Strains on the plate in duplicate:

asp20	wild-type
asp20-tamA50	tamA50
asp20-ahrA2	ahrA2

3. Growth responses of the aspA20 mutant and various double mutants

a. Growth responses of the aspA20 mutant

As mentioned above aspA20 is not protected by ammonia from the toxicity of aspartic hydroxamate and it suppresses tamA50 for growth on L-asparagine and sensitivity on aspartic hydroxamate (plate 10). aspA20 grows as the wild-type on all the nitrogen sources tested and it grows better than the wild type on L-asparagine as carbon source (plate 11, table 18). aspA20 is sensitive on all the amino acid and inorganic nitrogen toxic analogues tested (table 4).

b. The aspA20-ahrA double mutant

The cross between the aspA20 strain and an ahrA strain revealed that the two mutations recombine freely (table 19). The expected ratio of the four classes is 1:1:1:1. Wild-type recombinants comprise about 1/4 of the total progeny. The aspA20 phenotypes comprise about 1/2 of the total progeny. This indicates that the aspA20 phenotypic class includes the aspA20-ahrA^{+} and the aspA20-ahrA genotypes.

10 segregants of the aspA20 phenotype were picked off and assayed for asparaginase I activity. It was found that 6 of them had wild-type activity and 4 had no detectable activity. This indicates that the double mutant has no asparaginase I activity, so aspA20 is epistatic to ahrA. One of the double mutant strains was out-crossed with the wild type and ahrA segregants were recovered.

Diploid		asn⁻1 M.S.E.	
Linkage group	gene marker	asn⁻1	asn⁺
	y^+	20	28
I	y^-	22	26
	w^+	18	25
II	w^-	24	29
	gal^+	42	0
III	gal^-	0	54
	$pyro^+$	15	20
IV	$pyro^-$	27	34
	fac^+	20	21
V	fac^-	22	33
	s^+	25	30
VI	s^-	17	24
	nic^+	19	25
VII	nic^-	23	29
	$fibo^+$	26	26
VIII	$ribo^-$	16	28

Table 20. Haploidization analysis of the asn⁻1 mutant

Conclusion: asn⁻1 is in the linkage group III and is translocation free

Cross					
	pabaA2 yA2 biA$^+$	pyroA4	asp20	asn$^+$	
	X				
	pabaA$^+$ yA$^+$ biA1	pyroA$^+$	asp20$^+$	asn$^-$1	

genotypes	growth conditions (10 mM of)			No. of segregants
	ammonia	ammonia + 1 mM aspartic hydroxamate	L-asparagine	
asp20 asn$^+$	+	-	+	20
asp20$^+$ asn$^-$1	-	-	+	61
asp20$^+$ asn$^+$	+	+	+	17
asp20 asn$^-$1				
			Total	98

phynotypic classes	out-cross of the asp20$^+$ asn$^-$1 segregants with the wild type				
	out - cross				
	I	II	III	IV	V
asp20	0	17	0	14	0
asn$^-$1	50	61	49	62	42
wild-type	46	18	47	20	54

Table 21. Formation of the asp20-asn$^-$1 double mutant.

+: growth, -: no growth.

The number of the out-crosses indicate the number of segregants.

Conclusion: The asp20 asn$^-$1 double mutant is included in the asp20$^+$ asn$^-$1 class of segregants.

The asparaginase I activity of the aspA20 mutant is similar to the wild-type (see next section). The aspA20-ahrA and aspA20-tamA50 double mutants can utilise L-asparagine as carbon source in a similar fashion to the wild-type (plate 11, table 18).

c. The aspA20-asn⁻A1 double mutant

asn⁻A1 is an asparagine auxotrophic mutant. It grows only in presence of 1 mM or more L-asparagine and this indicates that the strain is deficient in the system responsible for the synthesis of asparagine, probably asparagine synthetase. All the attempts to assay asparagine synthetase activity in vitro in A. nidulans were not successful.

The asn⁻A1 mutant was isolated on the basis of growth in presence of L-asparagine by replica plating on nitrogen less (-N) medium, supplemented by ammonia. Colonies growing on L-asparagine but not on ammonia were picked off, purified and retested. In that way asn⁻A1 was found to be an asparagine auxotrophic strain.

asn⁻A1 in diploids with the wild-type allele is L-asparagine independent, so the mutant allele is recessive to the wild-type. Haploidization of the diploid asn⁻A1 M.S.E. revealed linkage with linkage group III (table 20).

The cross between the aspA20 mutant and the asn⁻A1 mutant revealed that the two mutations recombine freely (table 21). Four classes are expected from this cross in a ratio of 1:1:1:1. Wild-type recombinants comprise about 1/4 of the total progeny. The phenotype

Cross	$\frac{pabaA2\ yA2\ biA^{+}}{pabaA^{+}\ yA^{+}\ biA1}$ X $\frac{asn^{+}}{asn^{-}1}$ $\frac{ahrA2}{ahrA^{+}}$			
	growth conditions (10 mM of)			
genotypes	ammonia	L-asparagine	L-asparagine + 1 mM aspartic hydroxamate	No. of segregants
$\frac{asn^{+}}{asn^{-}1}$ $\frac{ahrA2}{ahrA^{+}}$	+	+	+	22
$\frac{asn^{-}1}{asn^{-}1}$ $\frac{ahrA^{+}}{ahrA^{+}}$	-	+	-	28
$\frac{asn^{+}}{asn^{+}}$ $\frac{ahrA^{+}}{ahrA^{+}}$	+	+	-	25
$\frac{asn^{-}1}{asn^{-}1}$ $\frac{ahrA2}{ahrA2}$	-	+	+	21
			Total	96

Table 22. Formation of the $asn^{-}1$-ahrA double mutant

+: growth, -: no growth.

Conclusion: The $asn^{-}1$ and ahrA characteristics in double mutants are additive.

Cross	*pabaA2 yA2 biA*$^{+}$ *pyroA4 asp20 gln*$^{+}$ *puA*$^{+}$ X *pabaA*$^{+}$ *yA*$^{+}$ *biA1 pyroA*$^{+}$ *asp20*$^{+}$ *gln*$^{-}$*1 puA1*			
	growth conditions (10mM of)			
genotypes	ammonia	ammonia + 1mM aspartic hydroxamate	L-glutamine	No. of segregants
asp20 gln^{+}	+	-	+	18
asp20^{+} *gln*$^{-}$*1*	-	-	+	65
asp20^{+} *gln*$^{+}$	+	+	+	15
asp20 gln^{-}*1*				
			Total	98

phenotypical classes	cross of the *asp20*$^{+}$*gln*$^{-}$*1* segregants with the wild-type				
	out-cross				
	I	II	III	IV	V
asp20	0	16	0	0	0
gln^{-}*1*	55	67	45	61	44
wild-type	41	13	51	35	52

Table 23. Formation of the *asp20-gln*$^{-}$*1* double mutant.

+: growth, -: no growth.

The number in the out-crosses indicate the number of segregants.

Conclusion: The *asp20-gln*$^{-}$*1* double mutant is included in the *asp20*$^{+}$ *gln*$^{-}$*1* class of segregants.

class of the asn⁻A1 mutation comprises about 1/2 of the total progeny, this indicates that this class includes the aspA20-asn⁻A1 double mutant.

A double mutant between asn⁻A1 and ahrA was made (table 22). This double mutant has the phenotype characteristics of both the single mutants; it requires L-asparagine to grow, is resistant on aspartic hydroxamate (on high concentrations of aspartic hydroxamate in presence of L-asparagine since on small concentrations L-asparagine protects against its toxicity) and it has no asparaginase I activity.

d. The aspA20-gln⁻A1 double mutant

gln⁻A1 is a glutamine auxotrophic strain isolated and kindly provided by Dr. J. R. Kinghorn. The cross between the aspA20 mutant and the gln⁻A1 mutant proved that the two mutations recombine freely. The expected ratio of the four classes is 1:1:1:1 and wild-type recombinants comprise about 1/4 of the total progeny. The phenotypic class of the gln⁻A1 mutation comprises about 1/2 of the total progeny, this indicates that this class includes the aspA20-gln⁻A1 double mutant. 5 segregants of this class were crossed with the wild-type and from one of them aspA20 phenotypes were recovered. This proves that the double mutant aspA20-gln⁻A1 has the same phenotype as the gln⁻A1 mutant. The aspA20 mutation is not epistatic to the glutamine auxotrophy. On table 23 the formation of the aspA20-gln⁻A1 double mutant is demonstrated.

genotypes	Growth conditions (10mM)					
	NH_4^+	NH_4^+ + 1mM AH	pro	pro + 1mM AH	asn	No. of segregants
ahrA2 aspA20$^+$tamA$^+$	+	+	+	+	+	9
ahrA$^+$ aspA20 tamA50	+	-	-	-	+	10
ahrA2 aspA20 tamA50	+	-	-	-	+	
ahrA$^+$ aspA20$^+$tamA$^+$	+	+	+	-	+	11
ahrA2 aspA20$^+$tamA50	+	+	-	-	-	
ahrA$^+$ aspA20 tamA$^+$	+	-	+	-	+	39
ahrA2 aspA20 tamA$^+$	+	-	+	-	+	
ahrA$^+$ aspA20$^+$tamA50	+	+	-	-	-	27
				Total		96

Table 24. Formation of the aspA20-tamA50-ahrA2 triple mutant.

cross: pabaA2 yA2 biA$^+$ pyroA4 ahrA2 aspA20$^+$ tamA$^+$

pabaA$^+$ yA$^+$ biA1 pyroA^+_X ahrA$^+$ aspA20 tamA50

NH_4^+ : ammonia

AH : aspartic hydroxamate

pro : L-proline

asn : L-asparagine

e. The aspA20-tamA50-ahrA2 triple mutant

The aspA20-tamA50-ahrA2 triple mutant was made by crossing an ahrA2 strain with the aspA20-tamA50 double mutant (table 24). 8 genotype classes are expected from this cross, as listed on table 24. The cross revealed 5 phenotype classes. The triple mutant is expected to belong to that phenotype class which is sensitive to aspartic hydroxamate in presence of ammonia and can grow on L-asparagine but not on L-proline as the sole nitrogen source. Segregants from phenotype classes with the above characteristics were out crossed with the wild-type and all single mutant phenotype classes (aspA20, ahrA, tamA50) were recovered. This proves that the triple mutant is the same as the double mutants aspA20-ahrA2, aspA20-tamA50 for sensitivity on aspartic hydroxamate.

Enzyme	Enzyme activities (nanomoles/min/mg protein)			
	repressed conditions		induced (or derepressed) conditions	
	wild-type	asp20	wild-type	asp20
asparaginase				
a. amino-hydrolase	< 30	< 30	350	325
b. aspartyl-transferase	< 5	< 5	1200	1250
glutamine synthetase	230	60	1100	1150
NADP GDH	50	45	1560	1520
NAD GDH	25	37	850	830

Table 25. Enzyme activities of the asp20 mutant.

repressed conditions:	asparaginase	10 mM ammonia
	glutamine synthetase	50 mM glutamine
	NADP GDH	1.5% casamino acids
	NAD GDH	10 mM ammonia
induced (or derepressed) conditions:	asparaginase	3 h on -N medium
	glutamine synthetase	50 mM glutamate
	NADP GDH	10 mM ammonia
	NAD GDH	1.5% casamino acids

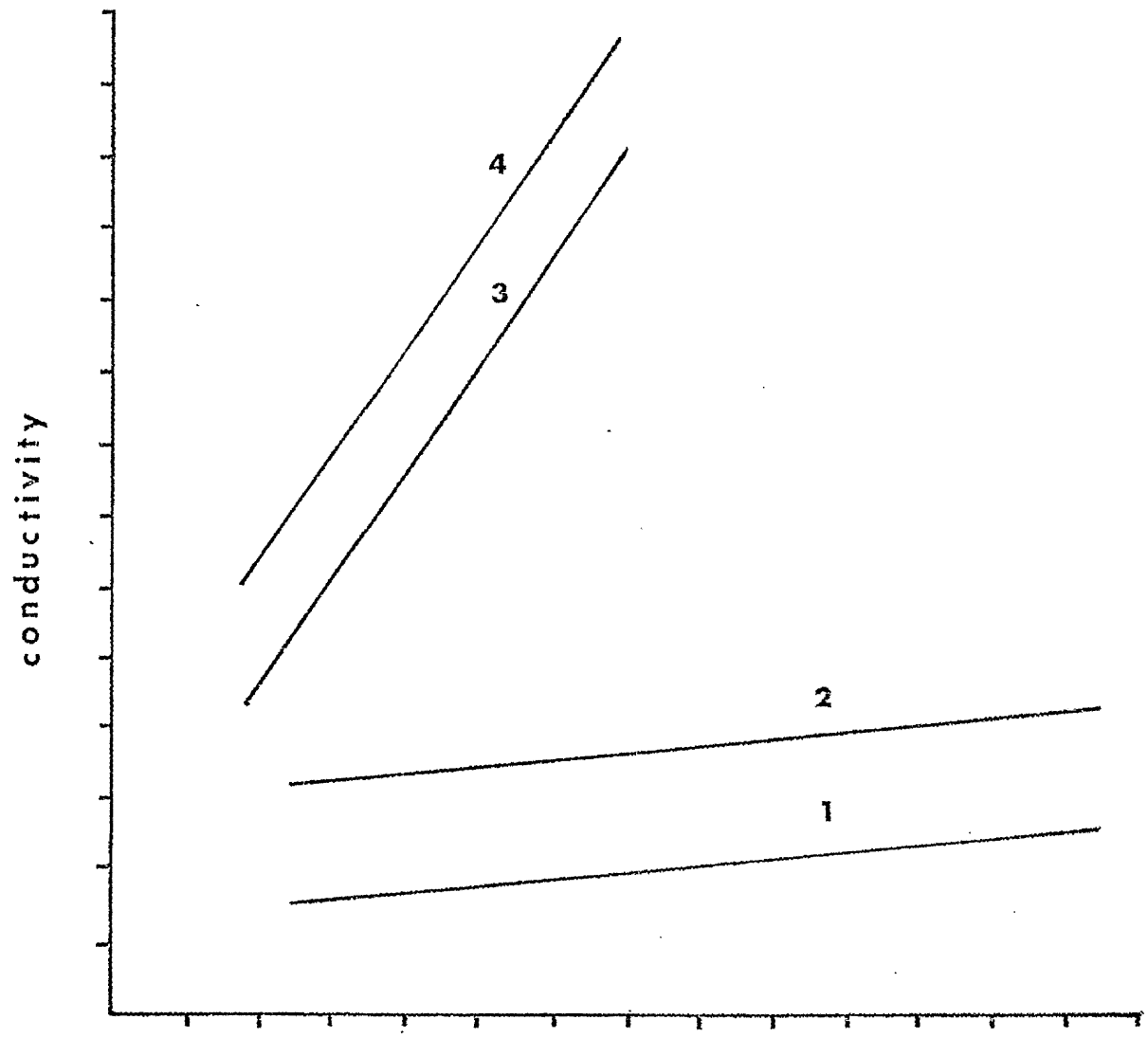


Figure 22. Change of conductivity by asparaginase activity from wild-type and asp20 extracts.

Qualitative demonstration of the increase of conductivity, due to the production of NH_4^+ ions in the reaction mixtures, by the time. The lines represent the pen recording from the reaction cells.
Extracts in cells 1&3: wild-type, 2&4: asp20
Growth conditions: 17 hours on 10 mM ammonia
Treatment: for extracts in 1&2: none, for extracts in 3&4: 3 hours on -N medium
Assay: 1 ml of 10 mM tris-HCl buffer, 30 µM L-asparagine, 5 µl cell free extract.

4. Enzyme activities of the aspA20 mutant

As mentioned above, the aspA20 mutation results in better utilisation of L-asparagine as a carbon source and ammonia derepression of aspartic hydroxamate toxicity. A possible hypothesis is that aspA20 possesses an enzyme activity in presence or absence of ammonia which can produce hydroxylamine or some other toxic substance from aspartic hydroxamate. In addition this enzyme activity enables the aspA20 strains to utilise L-asparagine as a carbon source better than the wild-type.

A number of enzymes were assayed as possible candidates responsible for the phenotype of the aspA20 mutation. These enzymes were: asparaginase (amino-hydrolase and aspartyl-transferase activity), glutamine synthetase, NADP and NAD glutamate dehydrogenase and glutaminase.

a. Asparaginase activity

aspA20 cells grown on ammonia were assayed for L-asparagine amino-hydrolase and aspartyl-transferase activity by estimating the ammonia and aspartic hydroxamate produced with Nessler's and $FeCl_3$ reagent respectively. In both cases no enzyme activity was detected. The aspA20 mutant under ammonia derepressed conditions has wild-type asparaginase activity (table 25).

The L-asparagine amino-hydrolase activity under ammonia repressed and derepressed conditions was also assayed by the conductivity method (figure 22). The assay used was the same as

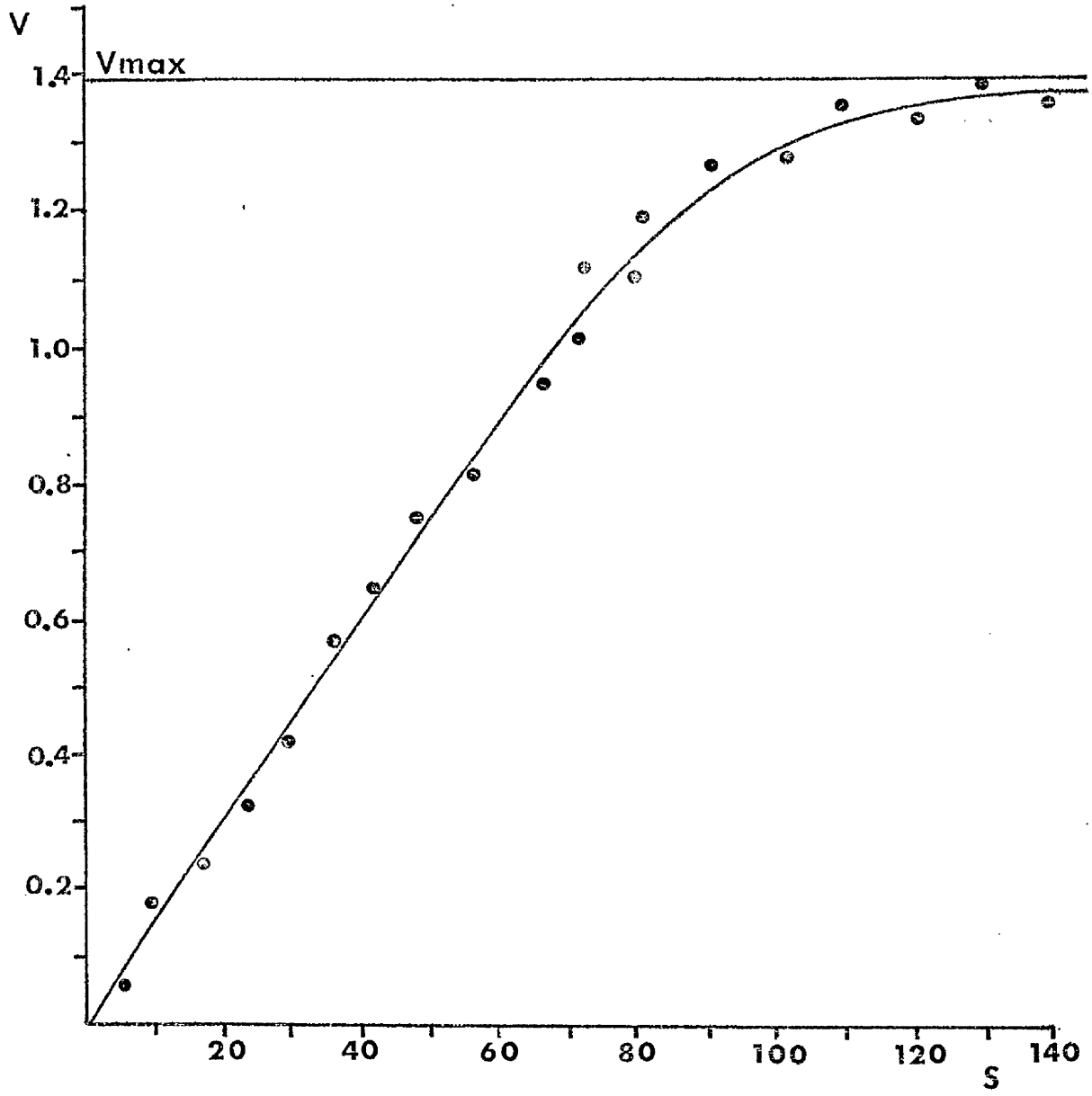


Figure 23. Effect of L-glutamine concentration on glutamine synthetase activity.

Growth conditions: 17 hours on 50 mM L-glutamate

Treatment: none

Extract: wild-type

V: mM glutamic hydroxamate/30 min

S: mM L-glutamine

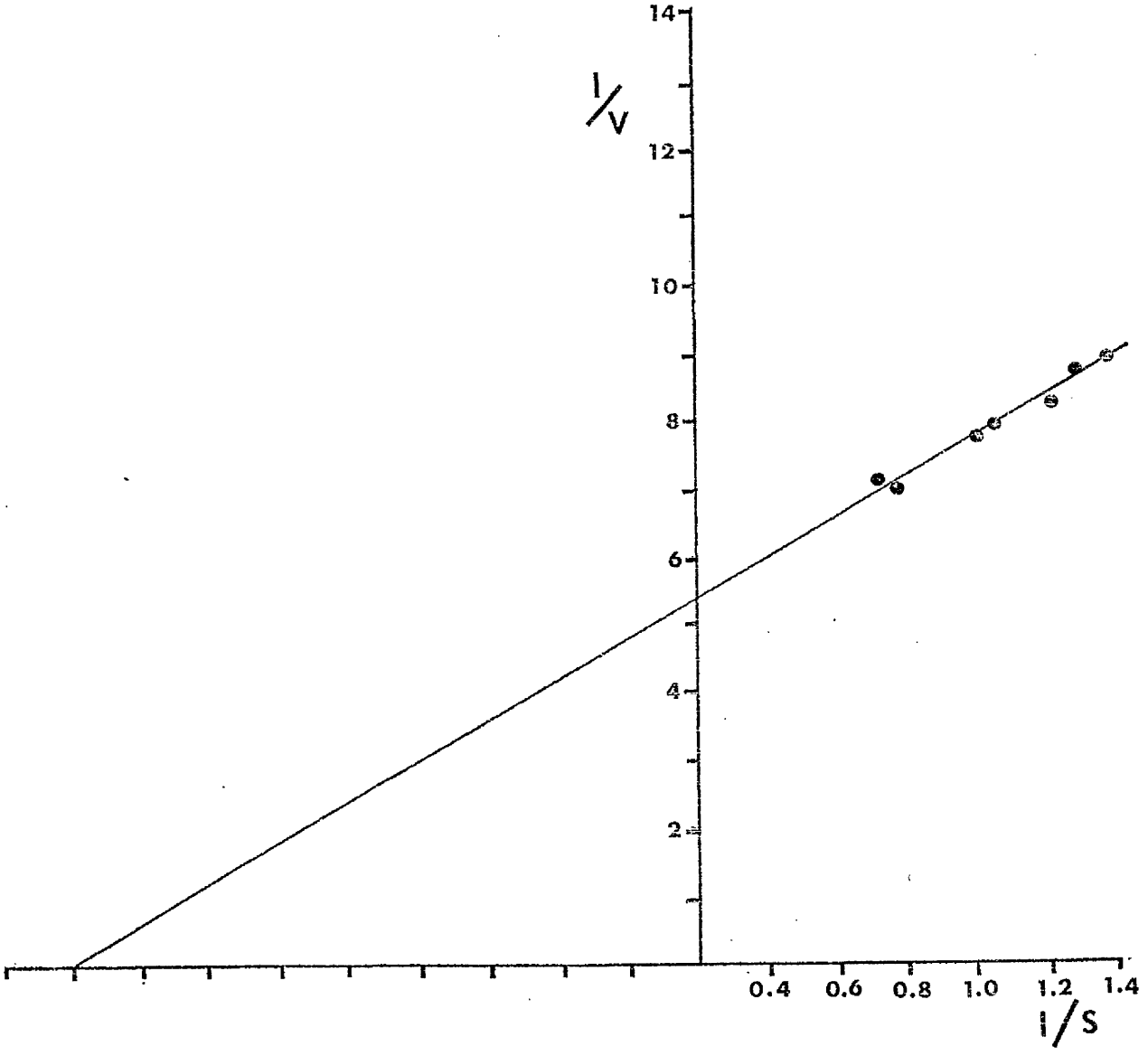


Figure 24. Effect of L-glutamine concentration on glutamine synthetase activity (Lineweaver-Burk reciprocal plot).

Growth conditions: 17 hours on 50 mM L-glutamate

Treatment: none

Extract: wild-type

V: mM glutamic hydroxamate/30 min x 10^{-1}

S: mM L-glutamine x 10^{-2}

described in chapter IV (page 64). Here again no differences between the wild-type and aspA20 activities under any conditions were detected. The rate in the increase of the conductivity in both the extracts is shown to be the same (figure 22).

All attempts to assay glutaminase activity by estimating ammonia produced from L-glutamine with Nessler's reagent or by change of conductivity method (using L-glutamine as substrate in place of L-asparagine) were not successful.

Nitrogen less (-N) medium containing ammonia as the sole nitrogen source in which aspA20 cells had been cultured for 17 hours, was assayed for extracellular asparaginase activity. There was no detectable extracellular asparaginase activity. All attempts to assay enzyme activity by the disappearance of aspartic hydroxamate in reactions with extracts of the above cells were not successful.

b. Glutamine synthetase activity

Glutamine synthetase activity in wild-type and aspA20 cell-free extracts was assayed by glutamyl-transferase activity, estimating the glutamic hydroxamate produced in the presence of ADP, manganous chloride and sodium arsenate (Pateman 1969). The effect of substrate concentration on glutamine synthetase activity was investigated in the range 5-140 mM l-glutamine (figure 23). A Lineweaver-Burk double reciprocal plot of the data was constructed (figure 24) and this shows that glutamine synthetase activity follows Michaelis-Menten kinetics with a Michaelis constant (Km) of 45 mM L-glutamine. Glutamine synthetase activity of the aspA20

strains	growth conditions						
	10 mM of ammonia	10 mM of L-glutamate	10 mM of L-glutamine	10 mM of L-asparagine	50 mM of ammonia	50 mM of L-glutamate	50 mM of L-glutamine
wild-type	830	1100	330	810	500	1150	230
asp20	660	1150	160	670	350	1100	60
asp20 ahrA2	800	1050	380	850	330	1200	100
asp20 tamA50	750	1000	320	720	370	-	-
tamA50	630	970	250	650	450	-	-
ahrA2	1100	1800	550	950	650	1750	310
gln⁻1	<5	<5	<5	<5	<5	<5	<5
asn⁻1	720	1120	450	820	470	1250	370

Table 26. Glutamine synthetase activity.

The enzyme activity is given in nanomoles of glutamic hydroxamate/min/mg protein. The gln⁻1 and asn⁻1 auxotrophs were grown in presence of 1mM L-glutamine or L-asparagine respectively. Glutamine synthetase activity of cells grown on L-glutamine (10mM or 50mM) was increased after transfer to L-glutamate (10mM or 50mM) for 3 hours.

mutant has a Km similar to the wild-type.

Glutamine synthetase is low after growth on L-glutamine and high after growth on L-glutamate (table 26). The aspA20 mutant has approximately wild-type glutamine synthetase activity under all conditions. The double mutants aspA20-ahrA and aspA20-tamA50 have wild-type glutamine synthetase activity as well. The glutamine auxotrophic strain gln⁻A1 has no detectable glutamine synthetase activity. No activity was detected under any growth conditions in any of the extracts when ADP or manganous ions or arsenate ions were not added in the reaction mixture.

c. NADP and NAD glutamate dehydrogenase activity

The assays used for NADP and NAD-GDH activities were those described by Kinghorn and Pateman (1973). In table 25 is shown that the aspA20 mutant has wild-type NADP and NAD-GDH activities.

Attempts were made to assay L-asparagine transaminase activity by coupling the reaction with NADP-GDH activity. In the reaction mixture L-asparagine and a-oxoglutarate were used as substrates instead of L-glutamate. As a result of L-asparagine transaminase activity glutamate would be produced which would provide a substrate for the NADP-GDH, thus the transaminase activity could be estimated by the amount of the reduced NADP. No enzyme activity was detected in the experiments.

carbon source	wild-type	asp20	tamA50	asp20-tamA50	ahrA2	asp20-ahrA2	meaA8
acetate	+	-	+	-	+	-	+
adonitol	+	-	+	-	+	-	+
*L-alanine	+	-	+	-	+	-	+
ammonium tartrate	+	-	+	-	+	-	+
arabinose	+	-	+	-	+	-	+
arabitol	+	-	+	-	+	-	+
*L-asparagine	+	-	+	-	+	-	-
*L-aspartate	-	-	-	-	-	-	-
ethanol	+	-	+	-	+	-	+
galactose	+	-	+	-	+	-	+
D-glucono-γ-lactose	+	-	+	-	+	-	+
glucoronic acid	+	-	+	-	+	-	+
glucose	+	-	+	-	+	-	-
*L-glutamate	-	-	-	-	-	-	-
*L-glutamine	+	-	+	-	+	-	+
glycerol	+	-	+	-	+	-	+
inositol	+	-	+	-	+	-	+
lactose	+	-	+	-	+	-	+
lyxose	+	-	+	-	+	-	+
mannose	+	-	+	-	+	-	+
methanol	+	-	+	-	+	-	+
*L-proline	+	-	+	-	+	-	-
ribose	*	-	+	-	+	-	+
sorbitol	+	-	+	-	+	-	+
sucrose	+	-	+	-	+	-	-
xylitol	+	-	+	-	+	-	+
xylose	+	-	+	-	+	-	+

Table 27. The effect of the carbon source on the suppression by ammonia of aspartic hydroxamate toxicity.

Concentration of carbon sources: 1%, *: 50 mM

Concentration of aspartic hydroxamate: 1 mM

Growth medium: -CN medium

nitrogen source: 10 mM ammonium chloride

+: growth, -: no growth

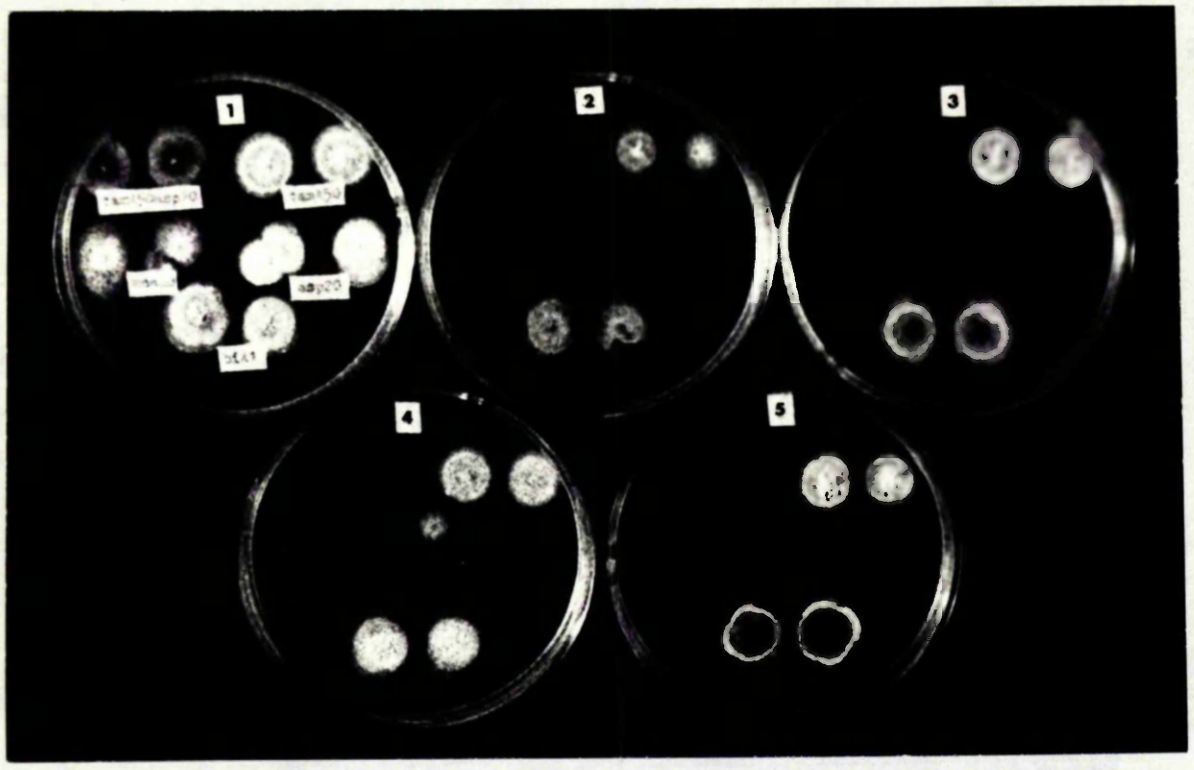


Plate 12. The effect of carbon source (glucose) on aspartic hydroxamate toxicity.

Growth medium: -CN plus 1% glucose (carbon source) plus ammonium chloride (NH_4Cl as nitrogen source)

Plates: (1) 10 mM NH_4Cl, (2) 1 mM NH_4Cl plus 1 mM aspartic hydroxamate, (3) 5 mM NH_4Cl plus 1 mM aspartic hydroxamate, (4) 10 mM NH_4Cl plus 1 mM aspartic hydroxamate, (5) 10 mM NH_4Cl plus 10 mM glutamate plus 1 mM aspartic hydroxamate

Strains on the plates in duplicate:

asp20-tamA50	tamA50
meaA8	asp20

wild-type

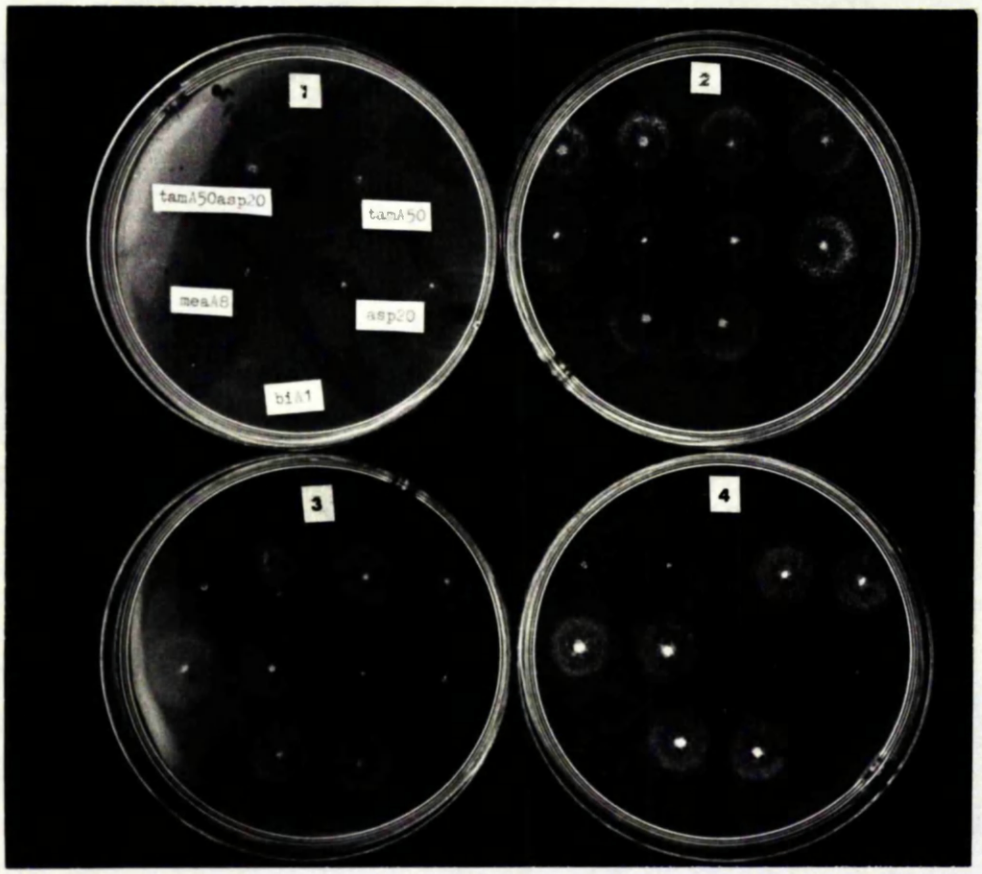


Plate 13. The effect of carbon source (ammonium tartrate) on aspartic hydroxamate toxicity.

Growth medium: -CN plus ammonium tartrate (NH_4T as carbon and nitrogen source)

Plates: (1) no NH_4T (control), (2) 10 mM NH_4T, (3) 5 mM NH_4T plus 1 mM aspartic hydroxamate, (4) 10 mM NH_4T plus 1 mM aspartic hydroxamate

Strains on the plates in duplicate:

asp20-tamA50	tamA50
meaA8	asp20

wild-type

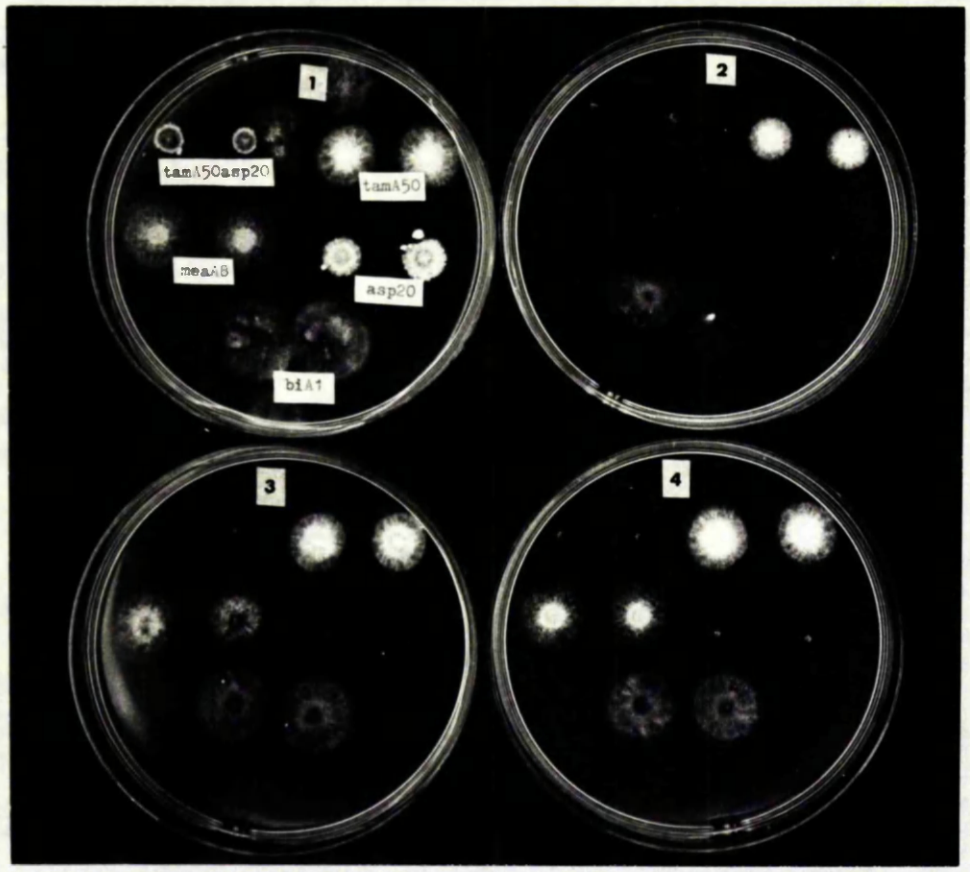


Plate 14. The effect of carbon source (glycerol) on aspartic hydroxamate toxicity.

Growth medium: -CN plus 1% glycerol (carbon source) plus ammonium chloride (NH_4Cl as nitrogen source)

Plates: (1) 10 mM NH_4Cl, (2) 1 mM NH_4Cl plus 1 mM aspartic hydroxamate, (3) 5 mM NH_4Cl plus 1 mM aspartic hydroxamate, (4) 10 mM NH_4Cl plus 1 mM aspartic hydroxamate

Strains on the plates in duplicate:

asp20-tamA50	tamA50
meaA8	asp20

wild-type

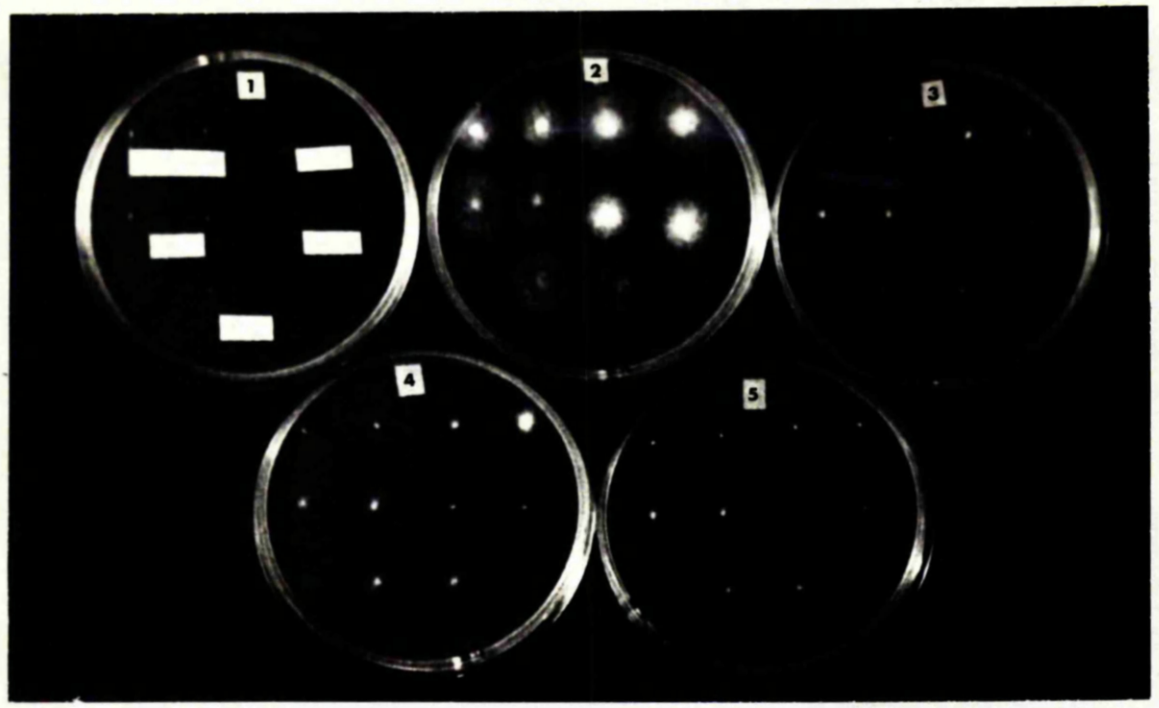


Plate 15. The effect of carbon source (L-glutamate) on aspartic hydroxamate toxicity.

Growth medium: -CN plus 50 mM L-glutamate (carbon source) plus ammonium chloride (NH_4Cl as nitrogen source)

Plates: (1) no glutamate plus 10 mM NH_4Cl (control) (2) 10 mM NH_4Cl, (3) 1 mM NH_4Cl plus 1 mM aspartic hydroxamate, (4) 5 mM NH_4Cl plus 1 mM aspartic hydroxamate, (5) 10 mM NH_4Cl plus 1 mM aspartic hydroxamate

Strains on the plates in duplicate:

asp20-tamA50	tamA50
meaA8	asp20

wild-type

5. Effect of carbon source on aspartic hydroxamate toxicity

In chapter III it is mentioned that the wild-type is protected by ammonia against the toxicity of aspartic hydroxamate. In the present chapter it is demonstrated that ammonia protection is dependent on the carbon source present. Wild-type, aspA20, tamA50, ahrA2 and the double mutants of aspA20 with tamA50 and ahrA2 were tested for growth on aspartic hydroxamate in presence of ammonia with various carbon sources. It was found that wild-type colonies are sensitive to aspartic hydroxamate in presence of ammonia if L-aspartate or L-glutamate are used as the sole carbon sources (table 27, plate 15). All the other carbon sources tested have the same effect as glucose (table 27, plates 12-14). The aspA20 mutant is sensitive to the toxic analogue in presence of ammonia on all the carbon sources tested. This indicates that aspA20 possesses some constitutive or carbon derepressed enzyme activity, able to produce some toxic substance from aspartic hydroxamate.

The mutants tamA50 and ahrA2 which have no detectable asparaginase I activity are both sensitive to aspartic hydroxamate in presence of ammonia when L-aspartate or L-glutamate are used as the sole carbon sources (table 27, plate 15). The double mutants aspA20 tamA50 and aspA20 ahrA2 phenotypically are sensitive on all the carbon sources tested.

The ammonia derepressed mutant meaA8 was used as a control on the above growth tests. It was found that on several carbon sources the mutant is protected by ammonia against the

toxicity of aspartic hydroxamate (table 27, plates 13 and 14). Possibly this is due to some elevation of the ammonia transport system of this mutant in the presence of certain carbon sources, so that the higher amount of ammonia which enters the cells protects them against the toxicity of the analogue.

6. Discussion

The aspA20 mutant suppresses the tamA50 allele for growth on L-asparagine as nitrogen source but not on any other nitrogen source tested (i.e. nitrate, L-alanine, L-proline, L-glutamine, L-aspartate, L-glutamate). The aspA20 mutant can utilise L-asparagine as a carbon source more effectively than the wild-type and can metalolise aspartic hydroxamate in the presence of ammonia. The isolation of the aspA20 mutants is an indication that A. nidulans possibly can utilise L-asparagine as a nitrogen source, as well as a carbon source, by a third system, different than the already discussed asparaginase I or the hypothetical asparaginase II. This system is not associated with the ahrA gene or the tamA regulator gene, as indicated by the facts that: a) the ahrA mutants lack both amino-hydrolase and aspartyl-transferase activities of asparaginase but they are still able to grow on L-asparagine as a nitrogen source, b) the tamA50, completely repressed allele, has undetectable asparaginase I activity and is unable to grow on L-asparagine as nitrogen source, indicating that it possibly lacks the activities of both asparaginase I and asparaginase II, c) the aspA20 mutant supresses tamA50 for growth on L-asparagine as nitrogen source and is epistatic to ahrA for sensitivity to aspartic hydroxamate, indicating the possibility of the existence of a third enzyme.

tamA50 is the only allele known (Kinghorn and Pateman personal communication) at the tamA locus which results in the inability to grow on L-asparagine as the sole nitrogen source. In the absence

of other tamA alleles unable to utilise L-asparagine as the sole nitrogen source, it is not possible to know if aspA20 is an allele specific or locus specific suppressor. What can be stated is that aspA20 suppresses tamA50 allele for growth on L-asparagine but not on any other nitrogen source tested. An investigation of such characteristics as the suppression of tamA50 and areA alleles by aspA20 would provide further information concerning the involvement of aspA20 in carbon catabolite repression. However, such work was accorded second priority and it was not possible to carry it out in the time available.

The wild-type as well as the ahrA and tamA mutants lacking asparaginase I activity are sensitive to aspartic hydroxamate in the presence of ammonia when L-aspartate or L-glutamate are used as the sole carbon sources. This indicates that ammonia repression of aspartic hydroxamate toxicity is overruled when L-aspartate or L-glutamate are the carbon sources. Similar results have been reported by Cohen (1972) for extracellular protease and Hynes (1970) for acetamidase where ammonia repression is ineffective when protein or acetamide are the carbon sources. This ineffectiveness of ammonia repression refers to the phenotype in growth tests in the presence of ammonia and certain carbon sources. It is not meant to imply that components of the mechanism of ammonia and carbon regulation necessarily interact or in any way are interdependent. It may be that the molecular mechanisms of nitrogen and carbon control are independent. It has been argued that ammonia repression and carbon catabolite repression are two independent mechanisms with respect to the regulation of proline oxidase, which is controlled by both ammonia

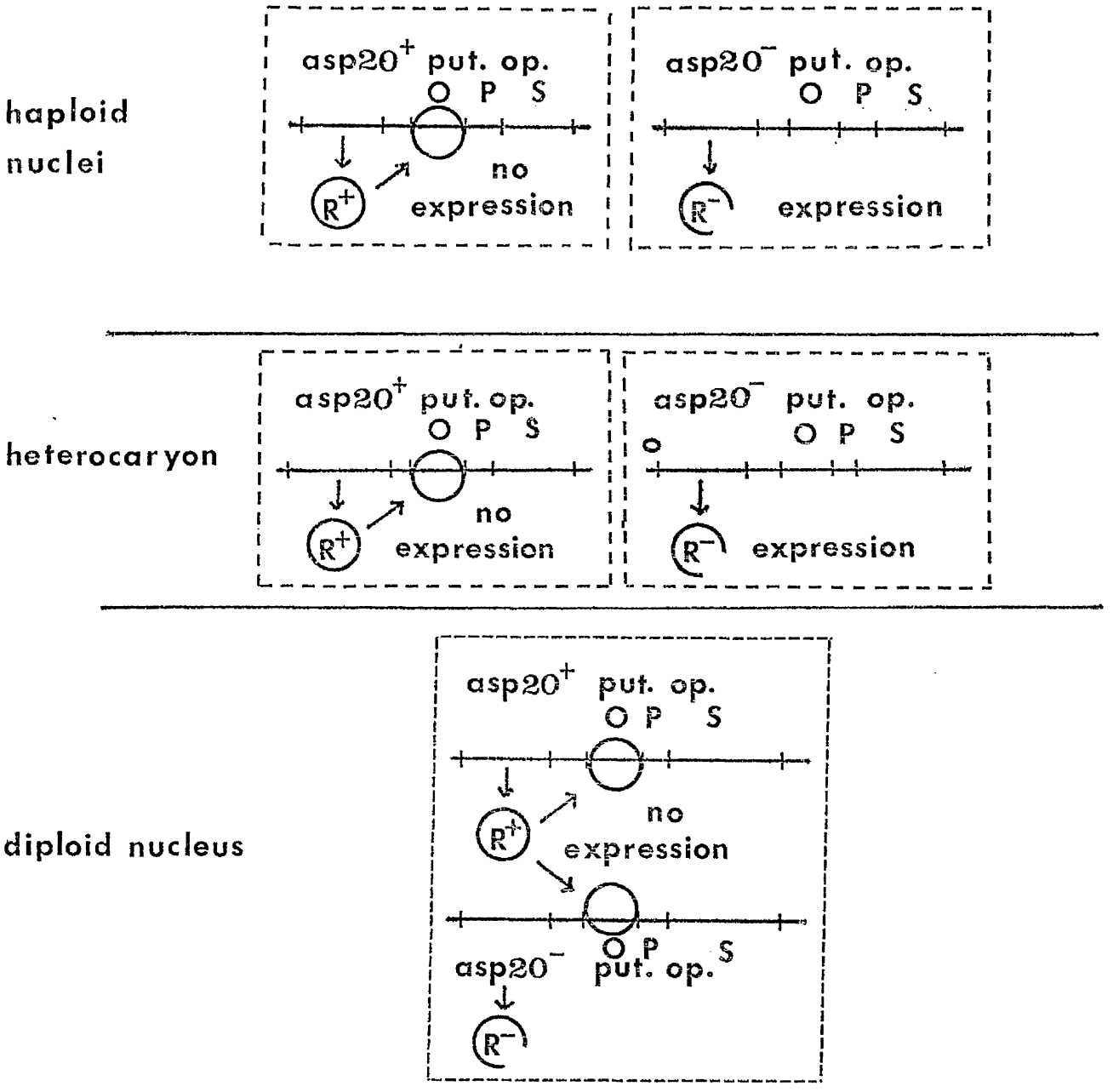


Figure 25. Diagrammatic presentation of the possible operation of the asp20 gene in haploid, heterocaryons and diploid cells.

asp20$^+$: wild-type allele, asp20$^-$: mutant allele, R^+: wild-type regulator molecule, R^-: mutant regulator molecule, put.op.: putative operon, O: operator site, P: promotor site, S: structural gene(s).

repression and carbon catabolite repression (Arst and MacDonald 1975).

aspA20 appears to be dominant to the wild-type allele in heterocaryons and recessive in diploids, since the heterocaryon (aspA20 + aspA20^{+}) is sensitive to aspartic hydroxamate in the presence of ammonia while the heterozygous diploid aspA20/aspA20^{+} is resistant. A possible explanation for the role of the aspA20 mutant is the following: aspA20^{+} is a regulator gene, the product of which (probably a protein molecule) is intranuclear and stops the synthesis of an enzyme which allows the utilisation of L-asparagine as nitrogen and carbon source. This enzyme is also responsible for the production of a toxic metabolite from aspartic hydroxamate and may be the third enzyme mentioned above responsible for the utilisation of L-asparagine. The aspA20 mutant allele of this gene results in the production of a deficient product and consequently in derepressed synthesis of the unknown enzyme (figure 25). In heterocaryons of the mutant with the wild-type allele, where haploid nuclei of both the parental strains are individually present in the hyphae, the mutant nuclei can allow the expression of the gene determining the unknown enzyme. As a result the heterocaryons are phenotypically similar to the aspA20 mutant. In the heterozygous diploid the regulator molecule, coded by the wild-type allele, is able to stop the expression of the genes for the unknown enzyme on both the chromosomes.

An alternative explanation is that the different phenotype of the heterocaryon may be due to a dilution of gene products in the

cytoplasm of the heterocaryon and is not due to intranuclear action (Casselton and Lewis 1967). A dilution of the product of the aspA20^{+} gene below a critical threshold during passage in the cytoplasm could result in failure of its regulatory function in a heterocaryon. Two factors which would increase the probability that the concentration of the regulator molecule might be too low for effective function in the heterocaryon are: 1) the production of limiting quantities of the regulator molecule, 2) an unequal distribution of nuclei resulting in clustering of nuclei of the same type and/or unequal ratios of the two types of nuclei in the heterocaryon. In the heterozygous diploid, where all the nuclei are of one type, at least the unequal distribution or unequal ratios of nuclei would not apply and this might be sufficient to account for the difference of phenotype between the heterozygous diploid and the heterocaryon.

At present it is not possible to decide which of the two hypotheses are true. In either event it is assumed that the action of the carbon catabolite effector may be to induce or repress the synthesis of the wild-type regulator molecule or to activate it. In absence of this effector the regulator product either is not synthesised or is inactive. In the mutant aspA20 either the regulator molecule is not produced or it cannot be activated.

The enzyme regulated by the aspA20 gene does not possess asparagine and glutamine synthetic activities, since the aspA20 mutation is unable to recover asparagine and glutamine auxotrophies in double mutants with the asn^{-}A1 and gln^{-}A1 auxotrophs. All the efforts made to assay the unknown enzyme were unsuccessful.

CHAPTER VI L-ASPARAGINE TRANSPORT SYSTEM

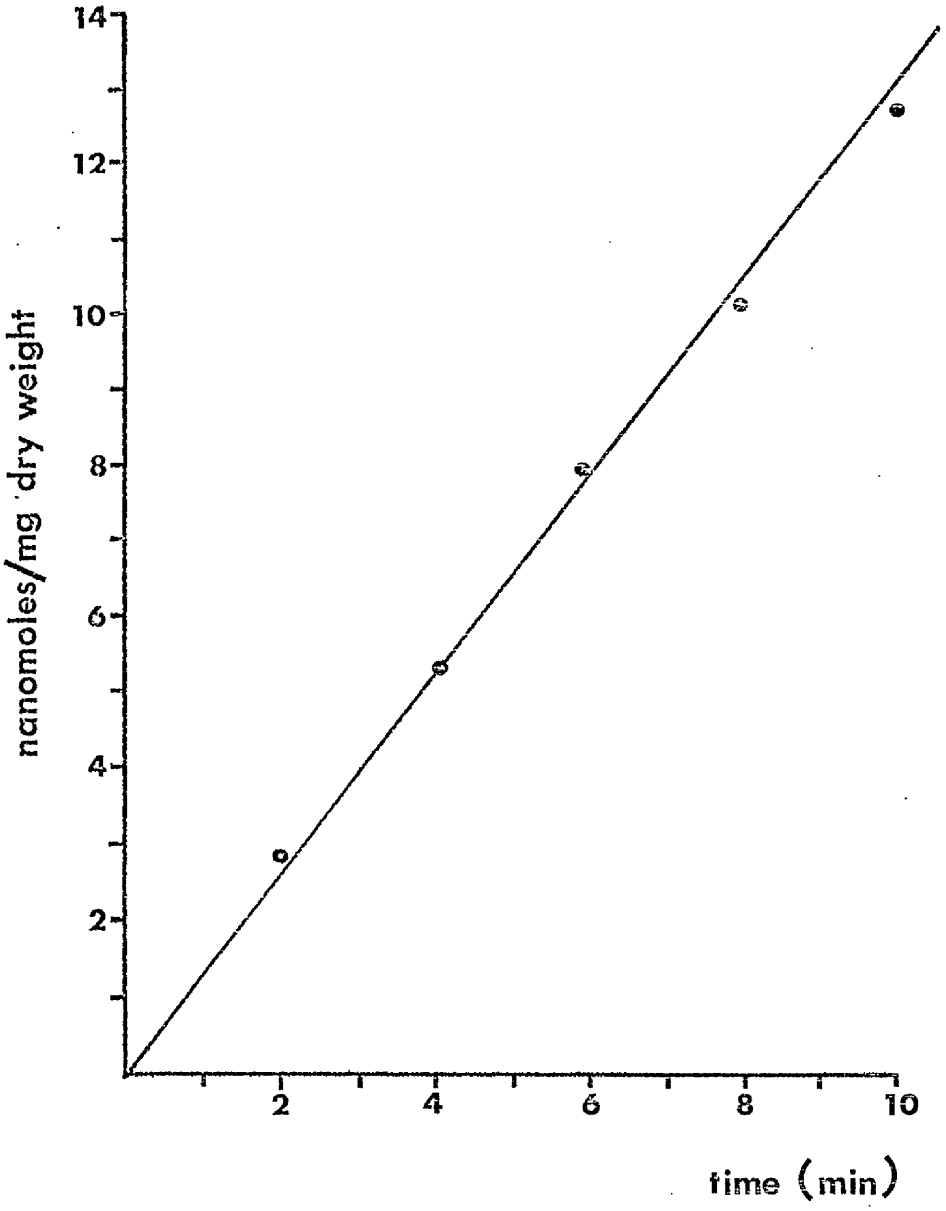


Figure 26. Initial velocity of L-asparagine transport.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -CN medium

Cells: wild-type

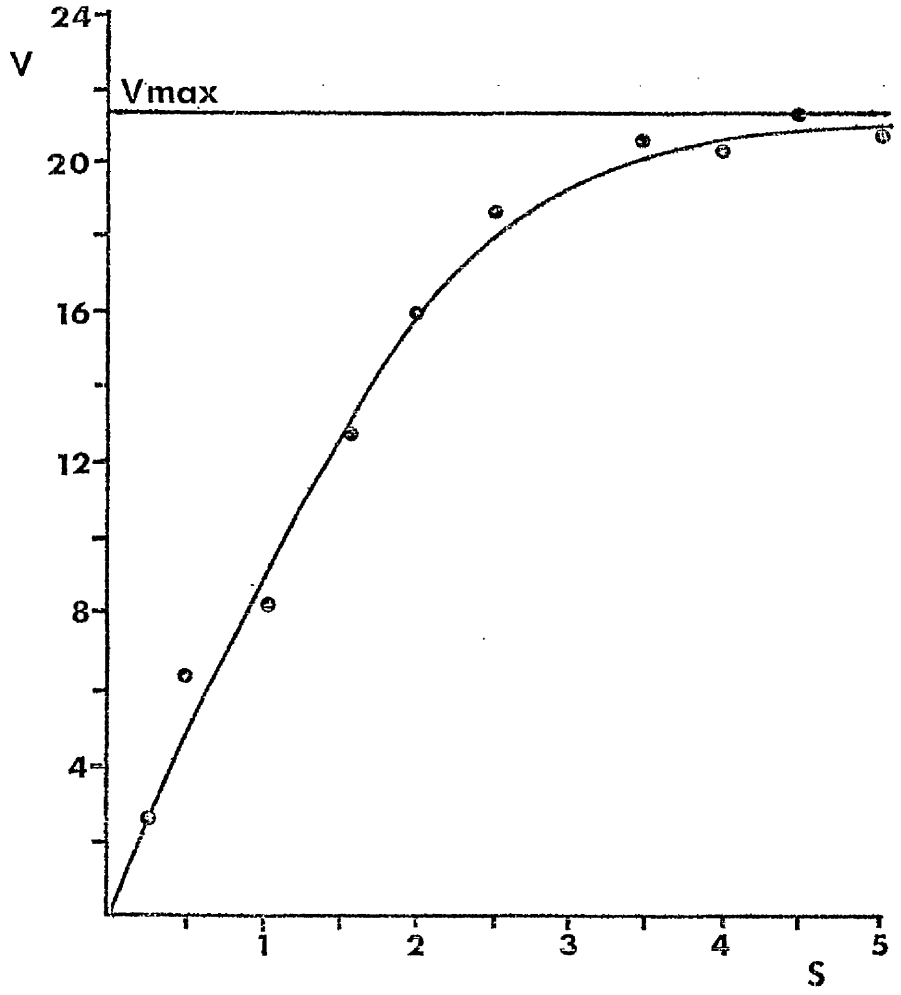


Figure 27. The dependence of L-asparagine transport on L-asparagine concentration.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -CN medium

Cells: wild-type

V: nanomoles/mg dry weight

S: 10^{-4}M L-asparagine

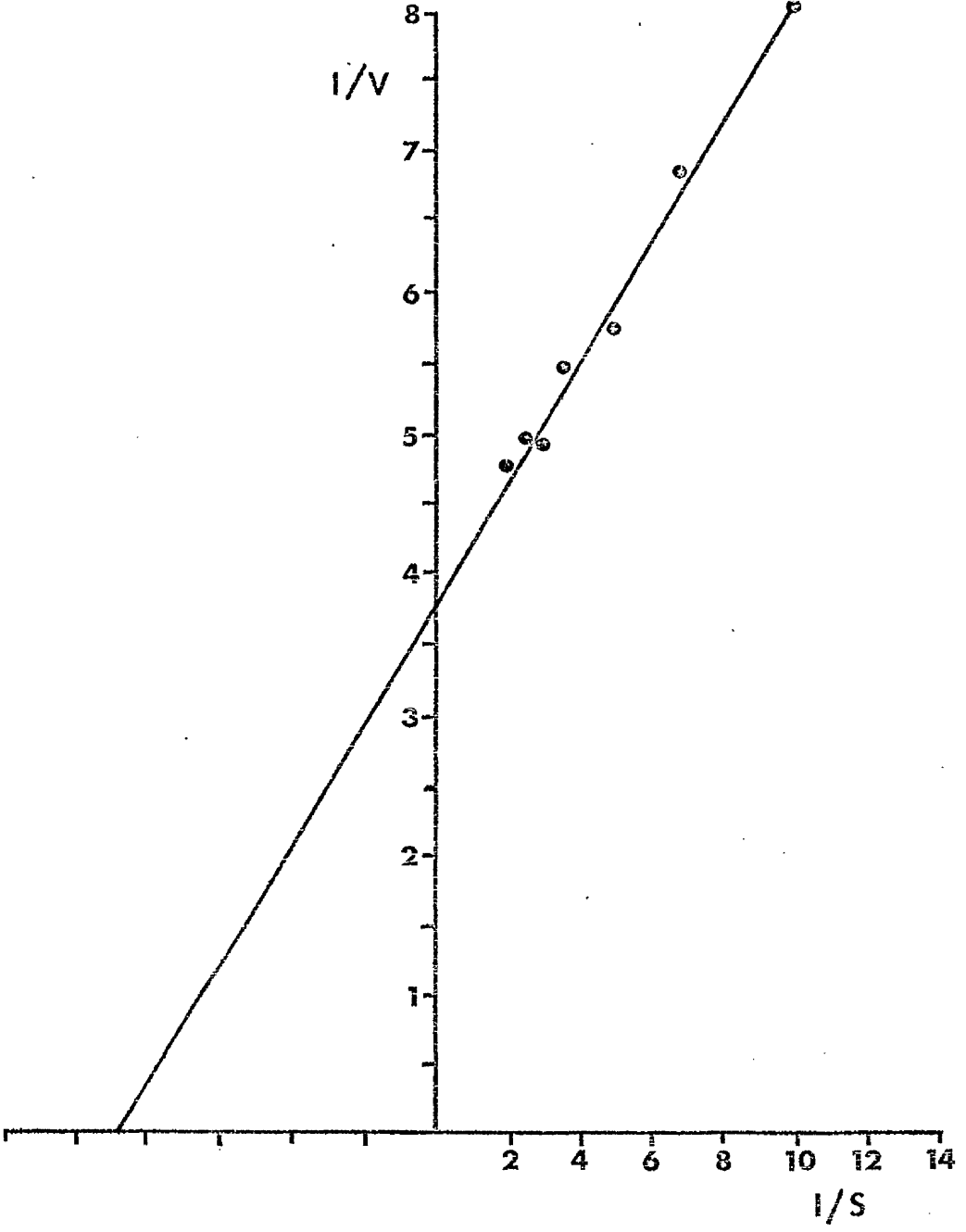


Figure 28. The dependence of L-asparagine transport on L-asparagine concentration (Lineweaver-Burk reciprocal plot).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -CN medium

Cells: wild-type

V: nanomoles/mg dry weight

S: 10^{-4}M L-asparagine

L-ASPARAGINE TRANSPORT SYSTEM

1. Characterization of the L-asparagine transport system

a. Initial velocity

Wild-type A. nidulans cells have a maximum capacity for L-asparagine uptake after growth on -N medium plus 10 mM ammonia and transfer for three hours to -CN medium (see regulation of L-asparagine transport page 103). The rate of uptake is linear for at least 10 minutes after the addition of the cells into the test medium (figure 26). Therefore 10 min was used as the standard time for all the uptake experiments.

b. Kinetics of the L-asparagine uptake

The effect of concentration on the rate of L-asparagine uptake was investigated over the range 1×10^{-5}M to 5×10^{-4}M as shown in figure 27. A Lineweaver-Burk double reciprocal plot (Lineweaver and Burk 1934) of the data was constructed (figure 28) and this shows that L-asparagine uptake follows Michaelis-Menten kinetics with a Michaelis constant (Km) of 1.1×10^{-4}M L-asparagine and a maximum velocity (Vmax) of 2.6 nanomoles/min/mg dry weight.

nitrogen source added 2 x 10^{-2}M	% of competition
ammonia	5
L-aspartate	4
L-asparagine	90
[x]aspartic hydroxamate	44
L-glutamate	2
L-glutamine	69
urea	3
L-proline	11
L-alanine	7

Table 28. The effect of various nitrogen metabolites on L-asparagine transport.

[x]aspartic hydroxamate was tested as being an analogue of L-asparagine.

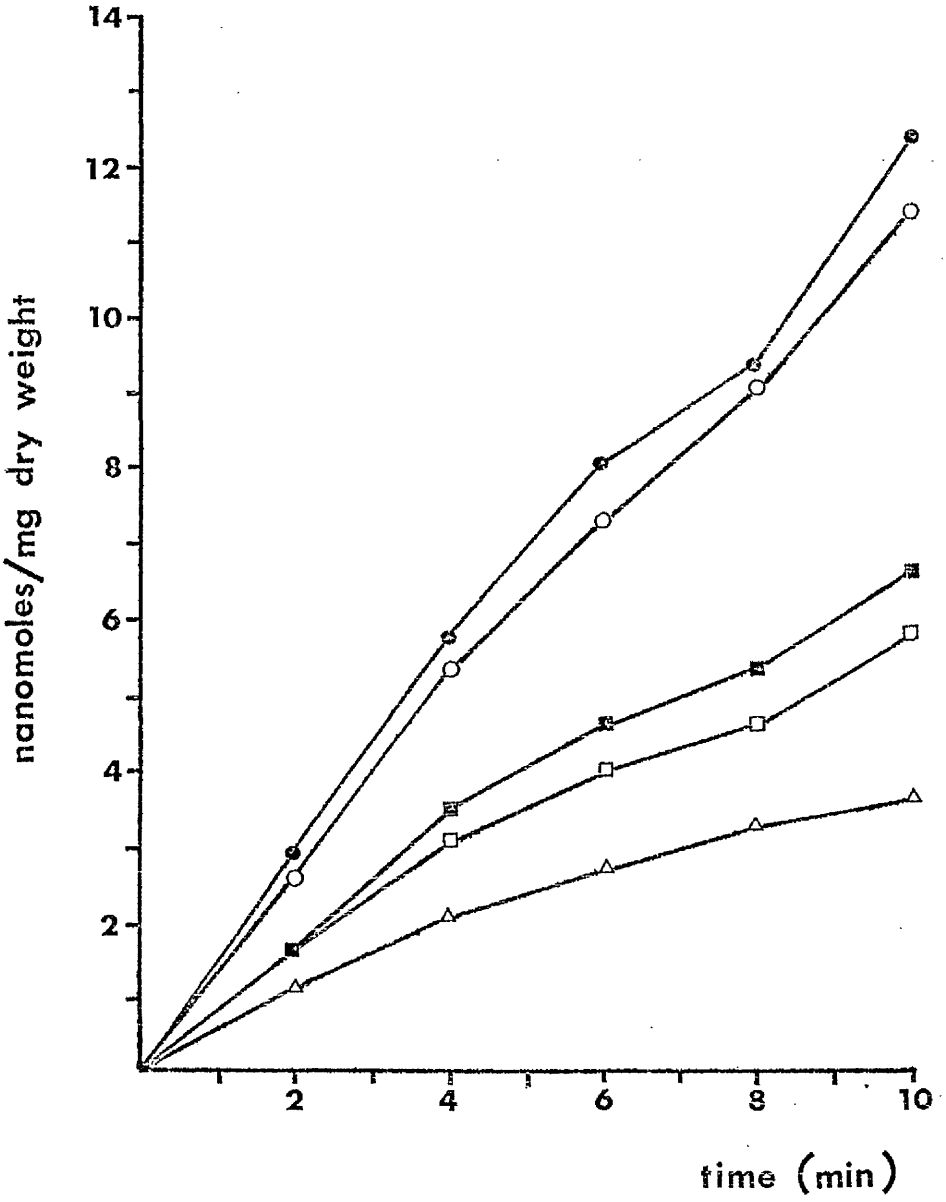


Figure 29. The effect of L-glutamine concentration on L-asparagine transport.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -CN medium

●: 0 mM L-glutamine

○: 0.1 mM "

■: 0.5 mM "

□: 1 mM "

△: 2 mM "

Cells: wild-type

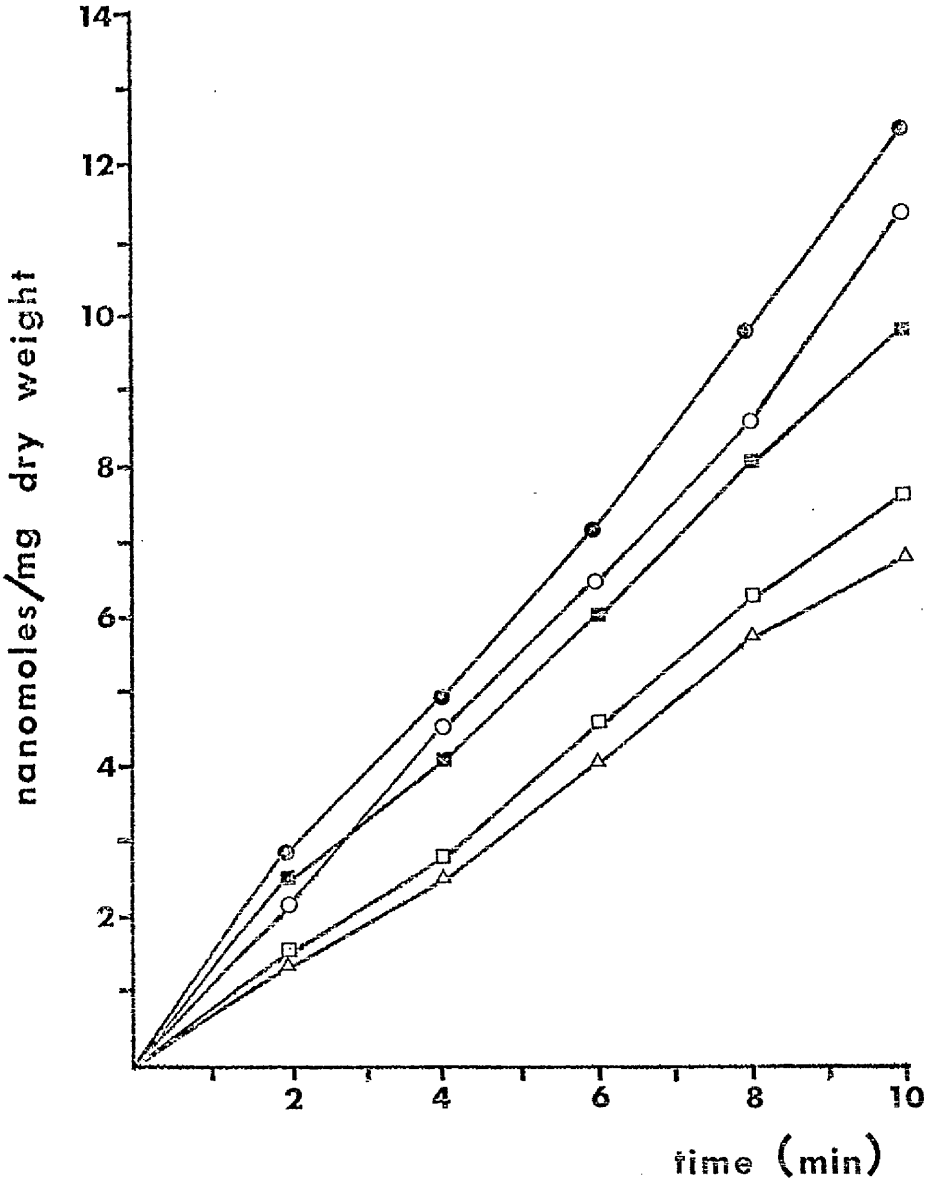


Figure 30. The effect of aspartic hydroxamate concentration on L-asparagine transport.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -CN medium

●: 0 mM aspartic hydroxamate

○: 0.1 mM " "

■: 0.5 mM " "

□: 1 mM " "

△: 2 mM " "

Cells: wild-type

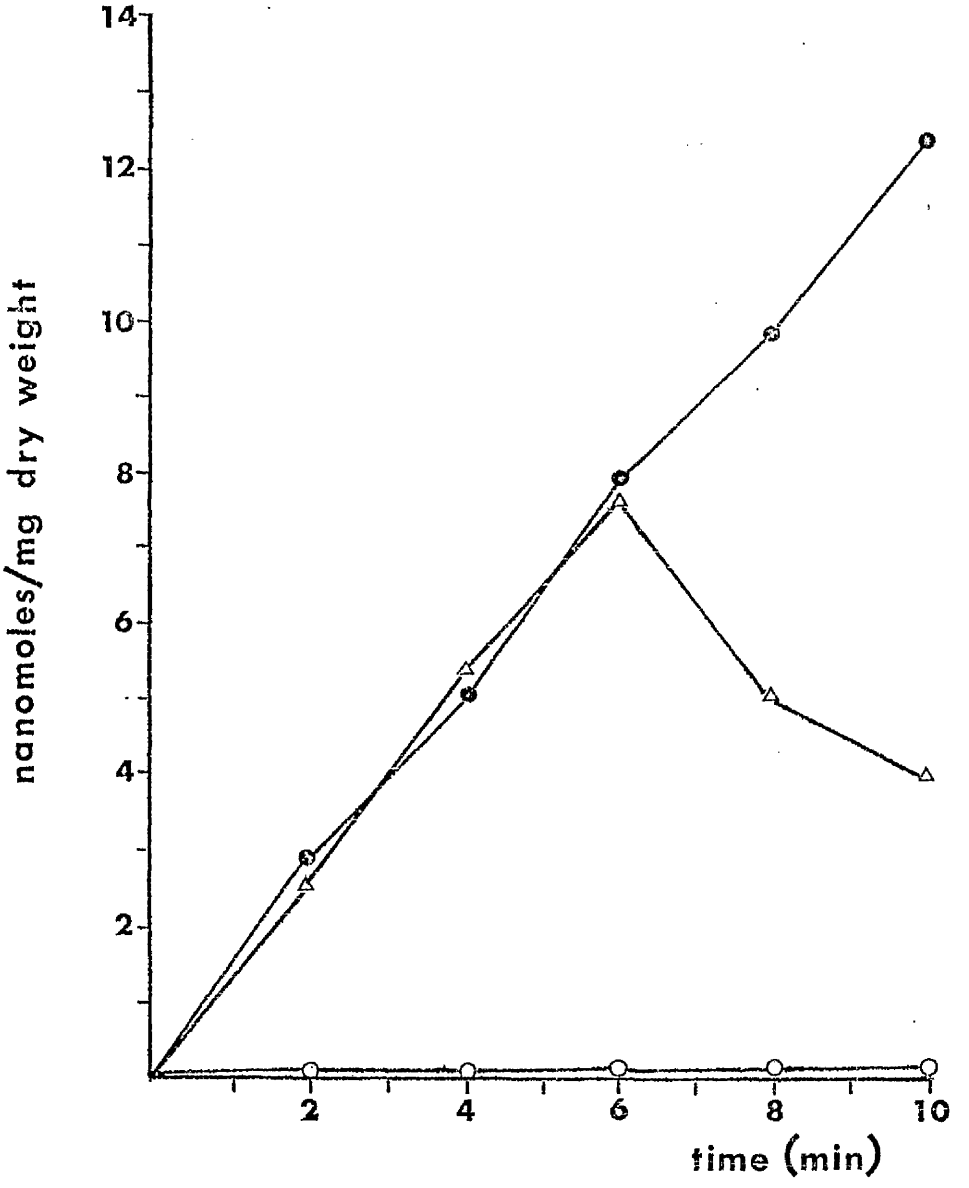


Figure 31. Effect of sodium azide on L-asparagine transport.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -CN medium

Cells: wild-type

●: control (uptake in absence of sodium azide)

○: 1 mM sodium azide added at zero time

△: 1 mM sodium azide added after 6 minutes

c. Competition data

The specificity of the L-asparagine transport system was investigated by determining the effect of a number of amino acids and inorganic nitrogen metabolites on L-asparagine transport (table 28). 2mM of the appropriate amino acid or inorganic nitrogen were added to the uptake medium and the rate of L-asparagine uptake estimated. Most of the nitrogen sources tested did not have any significant effect on L-asparagine transport. L-glutamine competition is significant when it is present in the uptake medium at a concentration of 0.5 mM or more (figure 29). The L-asparagine analogue, aspartic hydroxamate, competes with L-asparagine transport to a lesser degree than L-glutamine, and its competition is significant when it is present in the test medium at 0.5 mM or more (figure 30).

d. Energy requirement of the L-asparagine transport system

To study the energy requirement of the L-asparagine transport system the respiratory metabolic inhibitor sodium azide was added to the uptake assay at zero time and after 6 minutes (figure 31). When sodium azide is added at zero time the uptake of L-asparagine is totally prevented. If sodium azide is added after 6 minutes incubation time, there is an immediate cessation of the uptake. Sodium azide is supposed to inhibit respirator processes (in oxidative phosphorylation) by inhibiting the activity of ATP synthetase. Therefore these results suggest that energy is required for the L-asparagine uptake. In fact the alternative assumption that sodium azide might inactivate or inhibit the synthesis of the actual

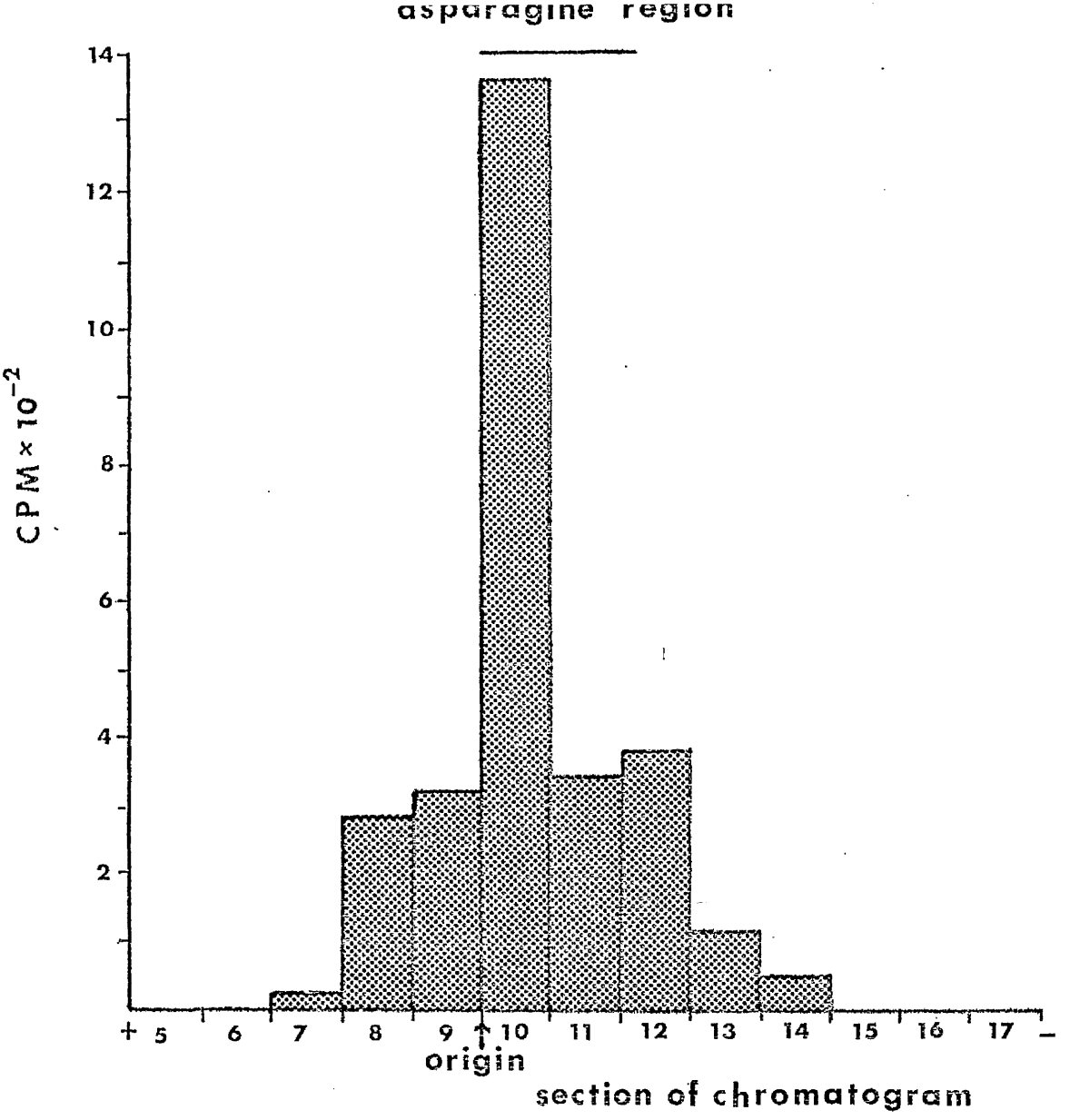


Figure 32. Chromatogram of accumulated intracellular ^{14}C-L-asparagine in wild-type cells.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: none

permease responsible for the transport of L-asparagine from the external environment into the fungal cell may also be true. Bearing in mind the effect of sodium azide on the L-asparagine transport suggests but does not prove that energy is in some way required in the transport process.

e. Recovery of accumulated intracellular L-asparagine

Hot water extracts of cells, which had been allowed to transport ^{14}C-L-asparagine for 30 minutes under the test conditions, were chromatographed. Only one radioactive spot was detected and this corresponded to L-asparagine (figure 32). The peak formed constituted approximately 87% of the total label extracted. Calculation of the intracellular concentration of L-asparagine was based on a mycelial wet weight of 1 gm pressed wet weight of cells being equivalent to 1 ml. The radioactivity corresponding to intracellular concentration of free L-asparagine was approximately 2300 CPM (corresponding to a volume of 1 ml), while the radioactivity corresponding to the extracellular concentration was approximately 3400 CPM (corresponding to a volume of 50 ml). Thus the L-asparagine transport system can concentrate L-asparagine at least 30-fold above the external concentration.

nitrogen source (10 mM of)	strains				
	wild-type	asp20	ahrA1	tam50	asn⁻1
ammonia	0.50	1.25	0.48	0.45	-
L-asparagine	0.45	1.30	0.40	-	0.60
L-aspartate	0.75	1.51	0.73	-	-
L-glutamine	0.55	1.35	0.68	-	-
L-glutamate	0.70	1.42	0.70	-	-
nitrate	0.75	-	0.65	-	-
urea	0.80	1.40	0.75	-	-

Table 29. The level of L-asparagine transport after growth on various nitrogen sources. (Uptake is nanomoles/min/mg dry weight).

Growth conditions before transfer to -N medium	strains				
	wild-type	asp20	ahrA	tam50	asn⁻1
ammonia	0.58	1.30	0.42	0.30	-
L-asparagine	0.40	1.35	0.42	-	0.55
L-aspartate	0.60	1.50	0.65	-	-
L-glutamine	0.52	1.42	0.50	-	-
L-glutamate	0.65	1.40	0.67	-	-
nitrate	0.70	-	0.55	-	-
urea	0.72	1.52	0.63	-	-

Table 30. L-Asparagine transport under nitrogen free conditions (3 hours on -N medium).

Uptake is nanomoles/min/mg dry weight.

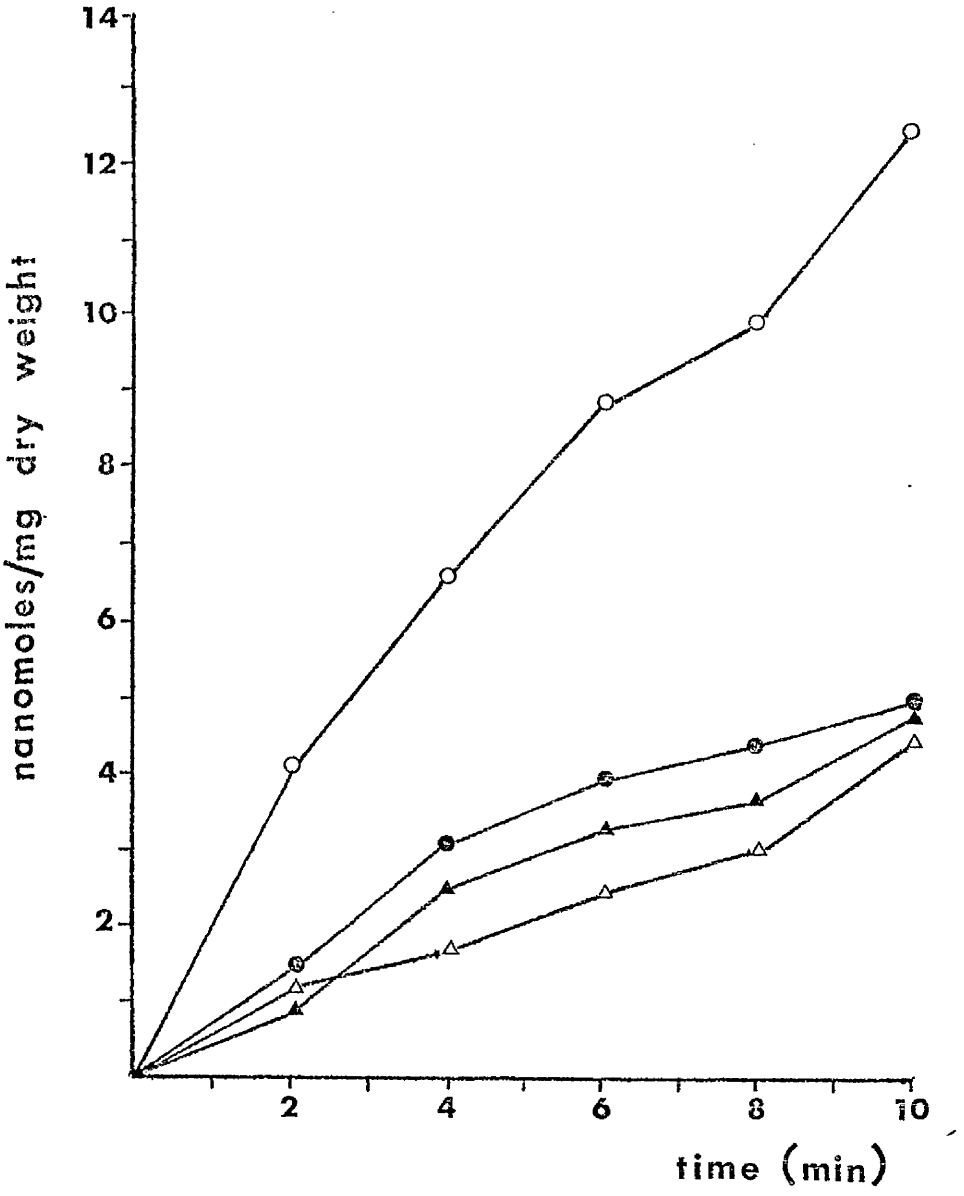


Figure 33. L-Asparagine transport of wild-type and various mutant strains (in presence of nitrogen).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: none

●: wild-type, ○: asp20, ▲: ahrA2, △: tamA50

2. Regulation of L-asparagine transport

The level of the L-asparagine uptake system of the wild-type and various mutants was estimated after growth on a variety of nitrogen and carbon sources. Attempts were made to isolate mutants with low uptake by using NTG treated wild-type or ahrA conidia and the subsequent isolation of colonies unable to grow on L-asparagine as sole nitrogen or sole nitrogen and carbon source. These efforts were not successful.

a. The effect of nitrogen source on L-asparagine transport

Table 29 shows that wild-type cells have lower levels of L-asparagine uptake after growth on ammonia, L-asparagine or L-glutamine and higher levels of L-asparagine uptake after growth on L-aspartate, L-glutamate, nitrate or urea.

The mutants ahrA1 - 11 appear to have about the same L-asparagine uptake levels as the wild-type. In figure 33 the levels of the uptake system in these mutants after growth on ammonia is shown. The level of the uptake of the aspA20 mutant is approximately 3 times greater than the wild-type. The ahrA1 - 11 alleles were all assayed and had similar uptake levels. For clarity only ahrA2 is represented in figure 33. The asparagine auxotroph asn⁻A1 has the same level of uptake as the wild-type.

Nitrogen starvation does not affect the level of L-asparagine uptake (table 30). All the strains tested have about the same value of L-asparagine uptake when they are transferred to

Growth conditions before transfer to -CN medium	strains				
	wild-type	asp20	ahrA	tam50	asn 1
ammonia (ammonium tartrate)	1.32	2.35	1.28	1.15	-
L-asparagine	1.40	2.40	1.30	-	1.42
L-aspartate	1.45	2.38	1.35	-	-
L-glutamine	1.38	2.39	1.33	-	-
L-glutamate	1.48	2.42	1.40	-	-
nitrate	1.29	-	1.32	-	-
urea	1.35	2.36	1.30	-	-

Table 31. L-Asparagine transport under carbon free conditions (3 hours on -CN medium).

Uptake is nanomoles/min/mg dry weight.

Treatment (-CN medium + 10 mM of)	strains				
	wild-type	asp20	ahrA	tamA50	asn⁻1
-CN medium	1.32	2.35	1.28	1.15	-
ammonia (ammonium chloride)	0.75	1.60	0.70	0.72	-
L-asparagine	0.40	1.35	0.38	0.30	-
L-aspartate	1.18	2.20	1.35	1.22	-
L-glutamine	0.50	1.75	0.60	0.52	-
L-glutamate	1.15	2.50	1.20	1.18	-

Table 32. The influence of ammonia and amino acid on L-asparagine transport.

Growth conditions before treatment:

-N medium + 10 mM ammonia (ammonium tartrate).

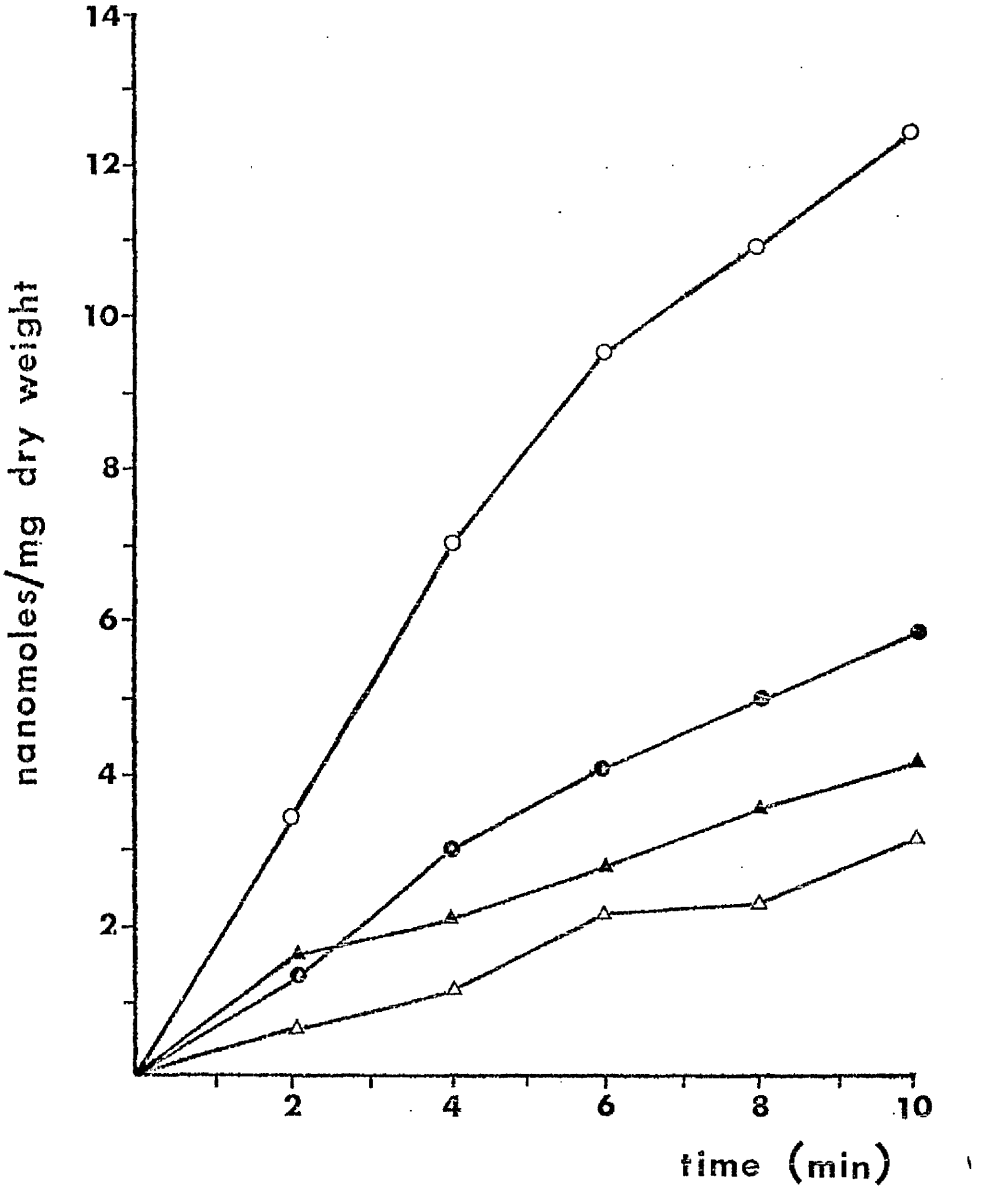


Figure 34. L-Asparagine transport of wild-type and various mutant strains (nitrogen free conditions).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -N medium

● : wild-type, ○ : asp20, ▲ : ahrA2, △ : tamA50

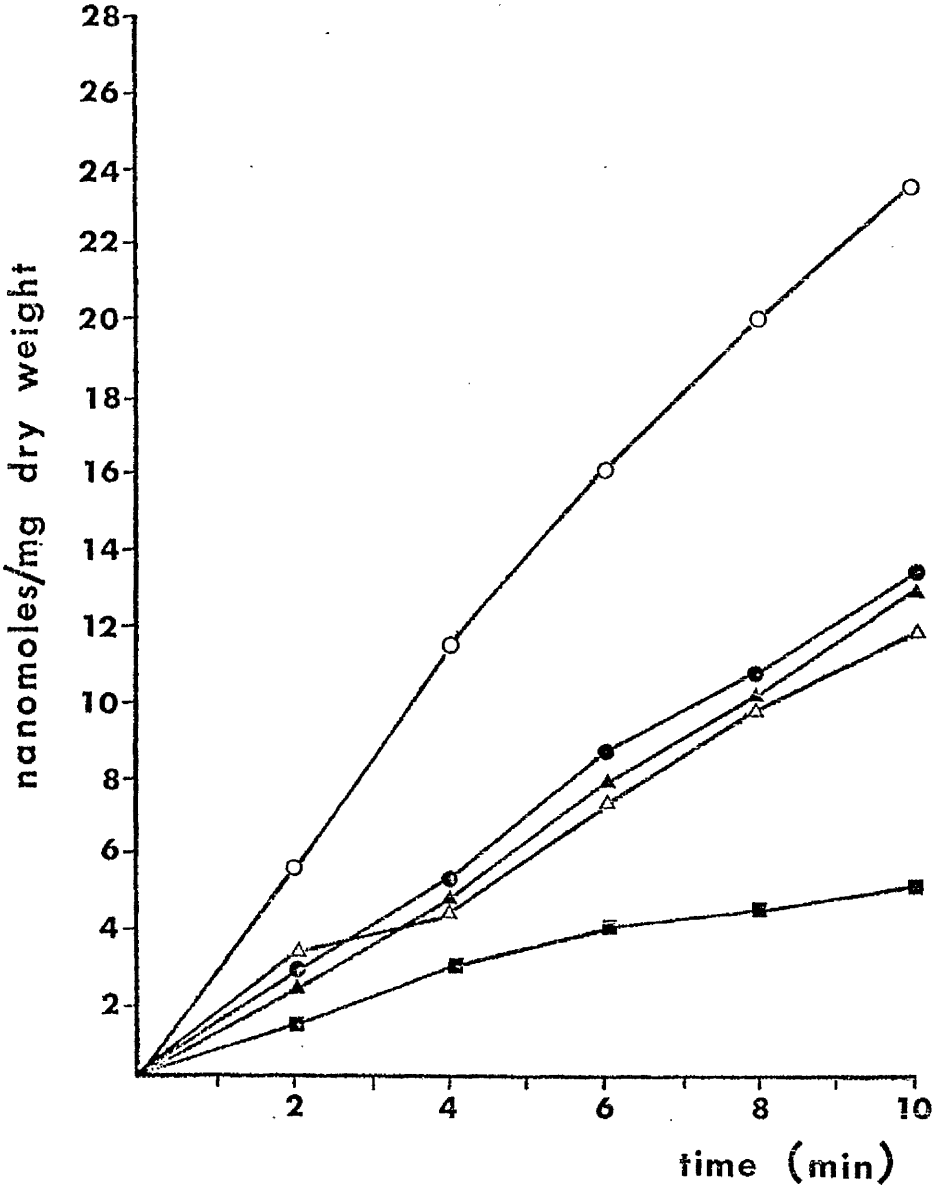


Figure 35. L-Asparagine transport of wild-type and various mutant strains (carbon and nitrogen free conditions).

Growth conditions: 17 hours on 10 mM ammonia

Treatment: 3 hours on -CN medium

● : wild-type, ○ : asp20, △ : tamA50, ▲:ahrA2

■ : control (wild-type with no treatment)

-N medium. In figure 34 is shown that the mutants ahrA2 and tamA50 have lower uptake than the wild-type under nitrogen free conditions after growth on ammonia as the sole nitrogen source. The mutant aspA20, under the same conditions, still has significantly higher uptake compared to all the other strains tested.

b. The effect of growth on various carbon sources on L-asparagine uptake

The level of L-asparagine uptake is considerably higher than the level mentioned above if the cells are held for a period in nitrogen and carbon free conditions (table 31). To carry out these experiments, cells were grown on to -N medium supplemented with the appropriate inorganic or amino acid nitrogen source and transferred to carbon and nitrogen free (-CN) medium for 3 hours.

The rate of L-asparagine uptake in wild-type cells is increased approximately 3 times when the cells are transferred to carbon and nitrogen free conditions (figure 35). The mutants ahrA and tamA50, under the same conditions, have increased uptake similar to that of the wild-type. The level of uptake of the aspA20 mutant is still greater than that of the wild-type, when this mutant is held under carbon and nitrogen free conditions (figure 35). The level of uptake of all the strains is similar as in figure 35 when L-aspartate or L-glutamate are present as sole carbon and nitrogen sources as when carbon and nitrogen are absent (table 32). In figure 35 the curves represent growth conditions on L-aspartate or L-glutamate as sole carbon and nitrogen source, for clarity, are not included. When

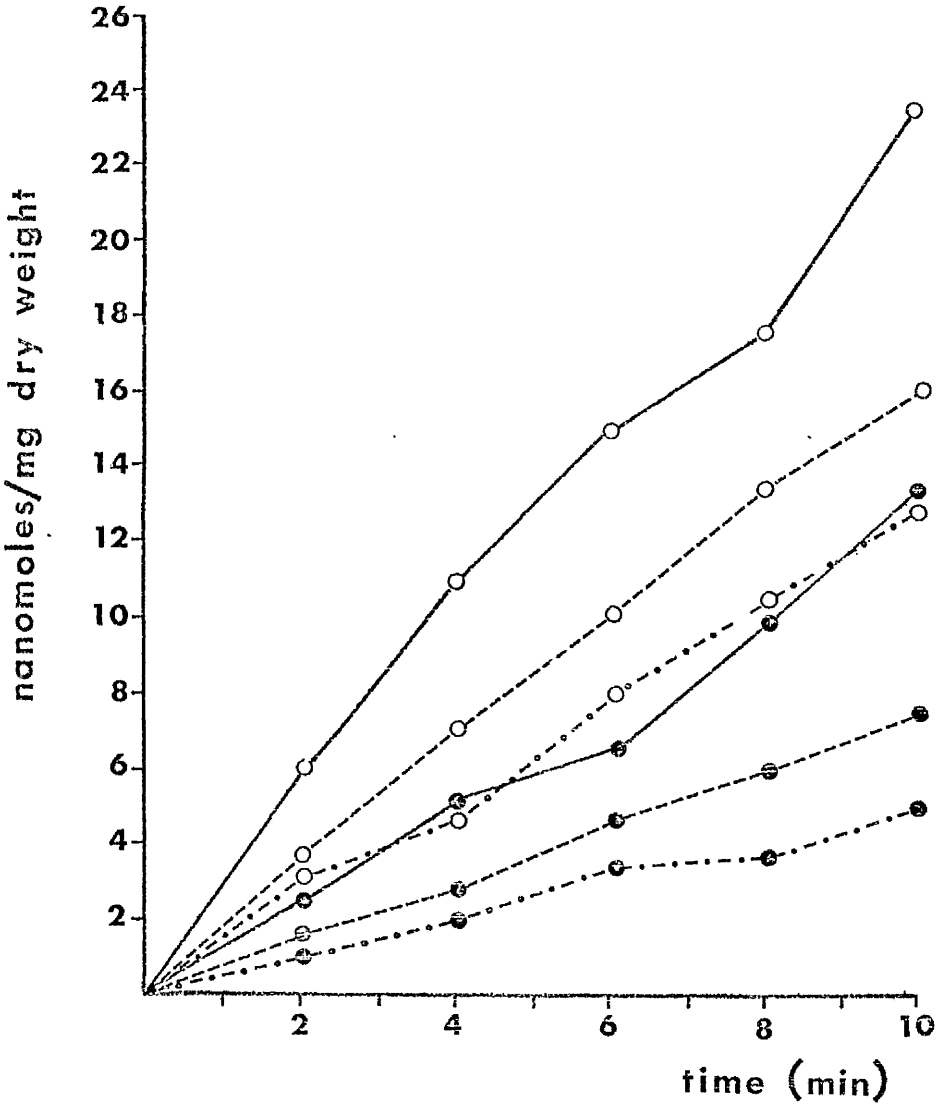


Figure 36. The effect of ammonia on L-asparagine transport.

Growth conditions: 17 hours on 10 mM ammonia

Treatment: (a) 3 hours on -CN medium (curve ——)

(b) 3 hours on -CN medium plus 10 mM ammonia (ammonium chloride, curve - -)

(c) 3 hours on -N medium or -N plus 10 mM ammonia (ammonium chloride, curve -·-·-)

●: wild-type, ○: asp20

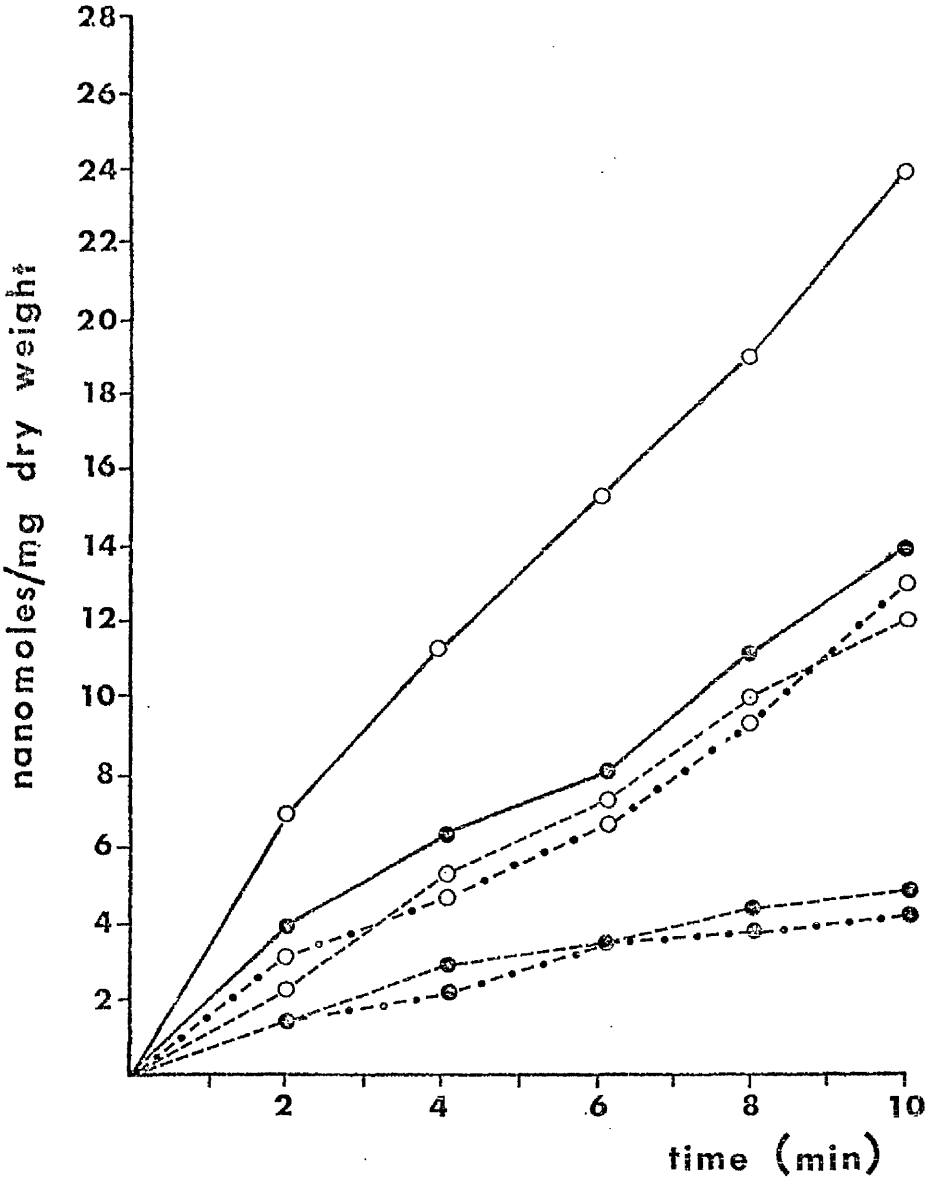


Figure 37. The effect of cycloheximide on L-asparagine transport.

Growth medium: 17 hours on 10 mM ammonia

Treatment: (a) 3 hours on -CN medium (curve ——)

(b) 3 hours on -CN medium plus 10 µg/ml cycloheximide (curve - - -)

(c) none (curve -•-•-)

●: wild-type, ○: asp20

L-asparagine or L-glutamine are present as the sole carbon and nitrogen sources the uptake of all the strains is similar to that after growth on L-asparagine or L-glutamine in presence of glucose (tables 29 and 32).

In the presence of ammonia and absence of carbon both the wild-type and aspA20 strains have lower levels of uptake than in the absence of both nitrogen and carbon (table 32, figure 36).

c. The effect of cycloheximide

If cycloheximide is added to the carbon and nitrogen free medium at the time of the transfer of the cells, there is no increase in the level of L-asparagine uptake (figure 37). The level of the uptake of wild-type and aspA20 cells remains the same in the presence of cycloheximide, as if the cells had not been transferred into the -CN medium. This indicates that protein synthesis is necessary for the increase in the level of L-asparagine transport.

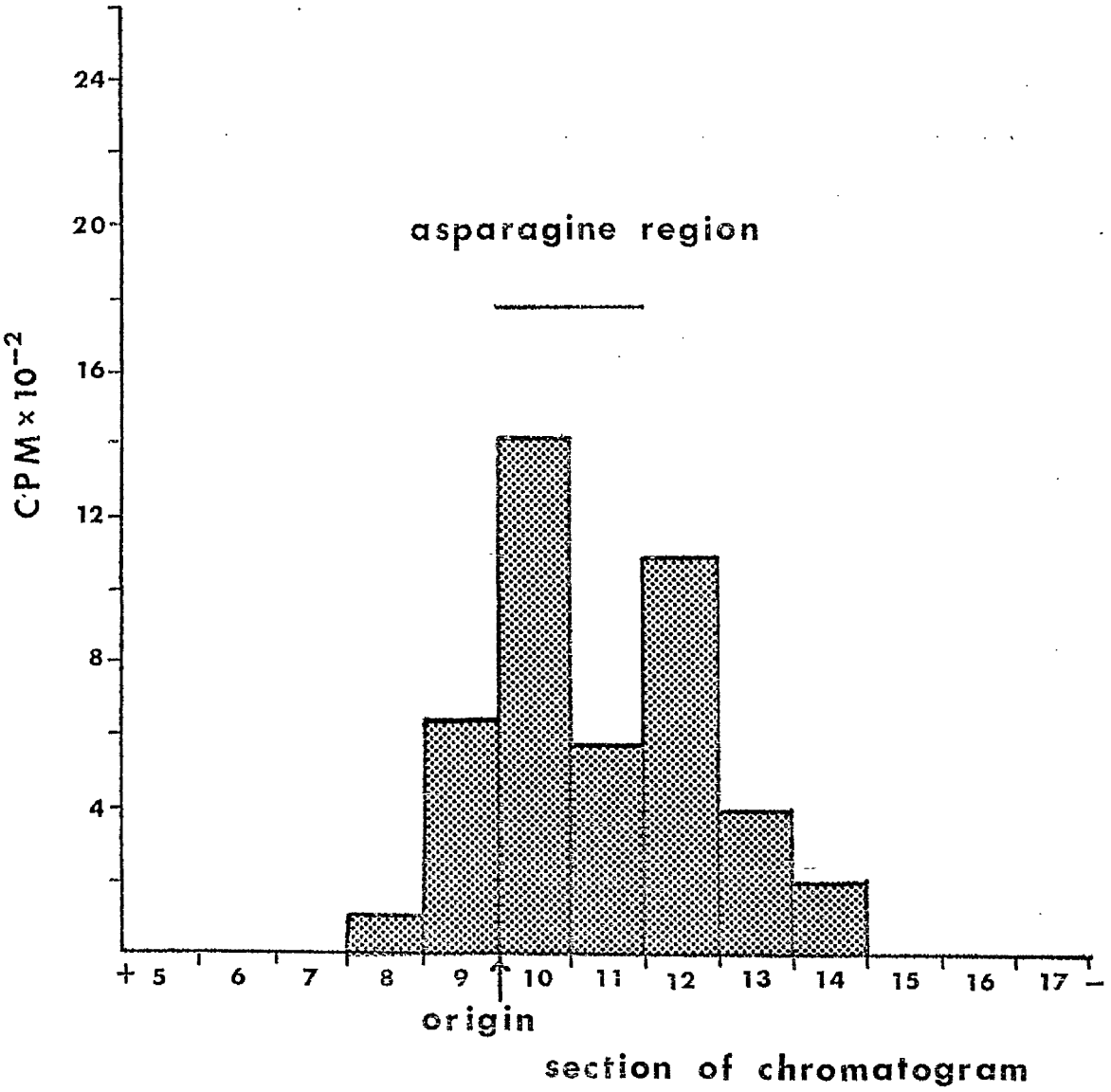


Figure 38. Chromatogram of accumulated intracellular ^{14}C-L-asparagine in asp20 cells.

Growth conditions: 17 hours on 10 mM ammonia

Treatment : none

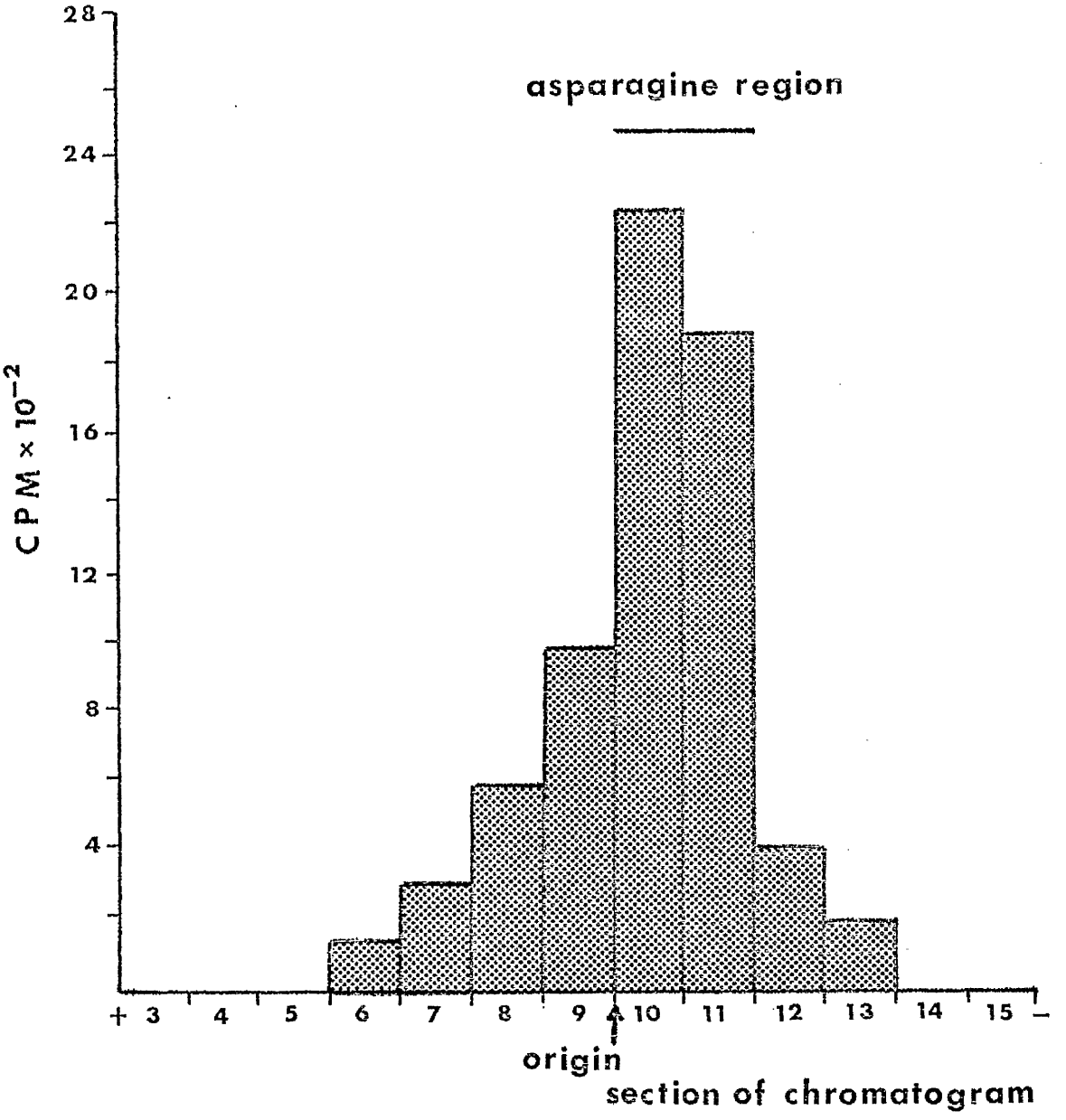


Figure 39. Chromatogram of accumulated intracellular ^{14}C-L-asparagine in wild-type cells on carbon and nitrogen free conditions.

Growth conditions: 17 hours on 10 mM ammonia

Treatment : 3 hours on -CN medium

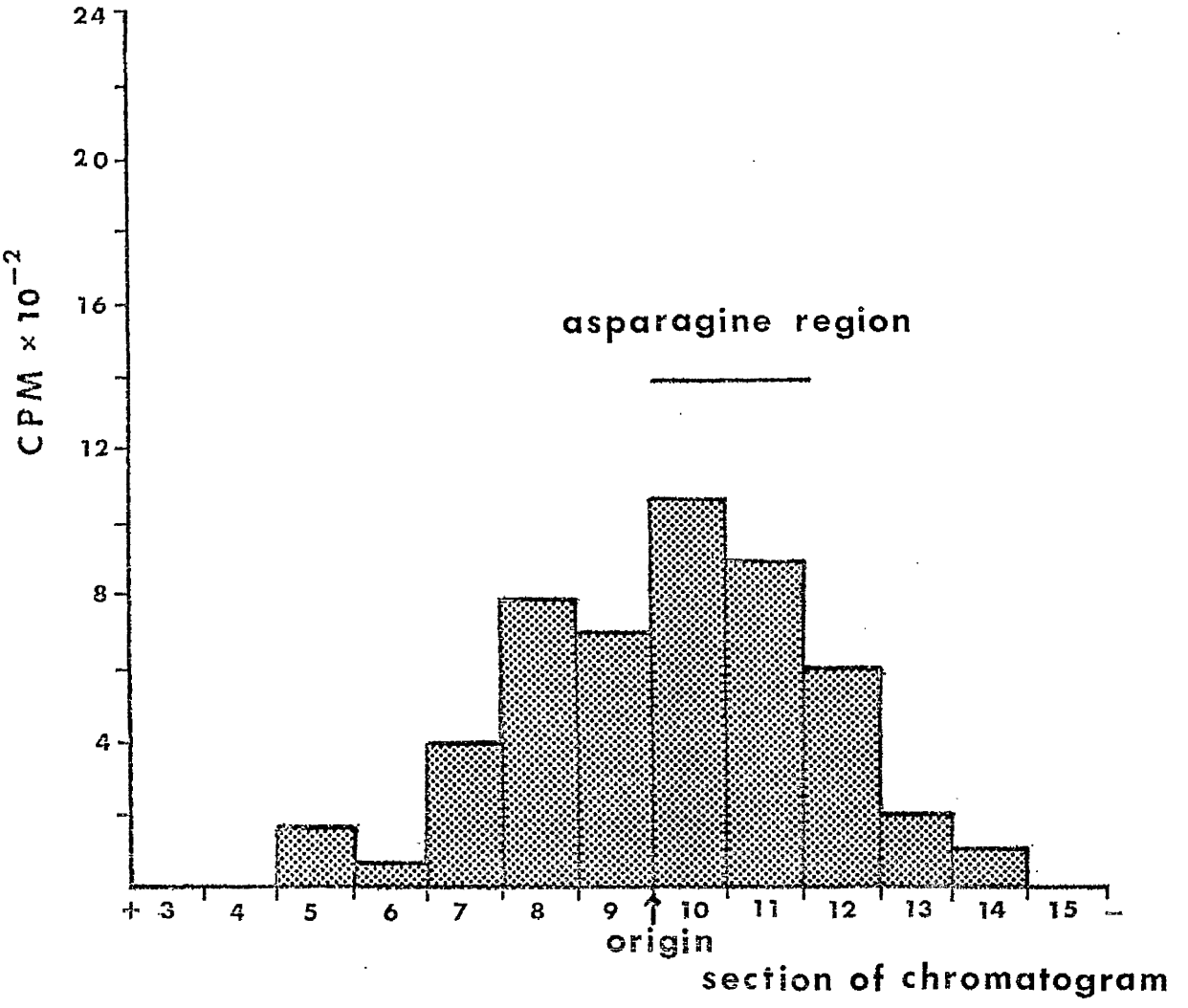


Figure 40. Chromatogram of accumulated intracellular ^{14}C-L-asparagine in asp20 cells on carbon and nitrogen free conditions.

Growth conditions: 17 hours on 10 mM ammonia

Treatment : 3 hours on -CN medium

3. The accumulation of intracellular ^{14}C-L-asparagine in the aspA20 mutant

The accumulation of intracellular L-asparagine in aspA20 cells was measured, in an effort to investigate the nature of the aspA20 mutant with respect to L-asparagine utilisation. aspA20 cells were assayed and extracted as described for the wild-type (see pages 34 and 102), and the results are presented in a histogram. After growth of aspA20 cells on ammonia the peak of radioactivity was in the region of L-asparagine (figure 38) as in the wild-type, and constituted approximately 65% of the total intracellular radioactivity. This is significantly lower than that of the wild-type (figure 32), in which the peak contained about 87% of the total intracellular radioactivity. Figures 39 and 40 show that wild-type cells under carbon and nitrogen free conditions accumulate 70% of the total intracellular radioactivity in the L-asparagine region, while aspA20 cells, under the same conditions, accumulate 47% of the total intracellular radioactivity in the L-asparagine region. This loss of ^{14}C-L-asparagine counts indicates that the aspA20 cells metabolise part of the L-asparagine taken up in a different fashion to the wild-type.

4. Discussion

L-asparagine uptake is an active transport system in Aspergillus nidulans responsible for the transport of L-asparagine from the external environment into the fungal cells. The inhibition of the transport system by the metabolic uncoupling agent sodium azide, together with the accumulation of L-asparagine against a concentration gradient, indicates that this system requires energy to operate.

Unlike the L-glutamate transport system, described by Pateman et al. (1974), L-asparagine transport does not appear to be regulated by straightforward ammonia repression since A. nidulans cells can take up about 0.4 nanomoles of L-asparagine/min/mg of dry weight of the cells, either after growth on ammonia or after transfer into nitrogen free conditions. In contrast the L-glutamate transport is increased about 8 fold when cells grown on ammonia are transferred in nitrogen free conditions (Pateman et al., 1974) An approximately 3 fold increase of the L-asparagine uptake takes place when the cells are transferred in carbon and nitrogen free conditions, after growth on ammonia. This increase is lower, about 2 fold, when ammonia is present in the carbon and nitrogen free treatment medium. This strongly suggests that the L-asparagine transport system is primarily under carbon catabolite control, but there is some degree, probably indirect, of nitrogen metabolite control.

The fact that no increase of the L-asparagine transport system takes place when cycloheximide is present in the treatment -CN medium, suggests that protein synthesis is necessary for this

increase. However, this does not prove that a permease protein is the product of this protein synthesis. It is possible that under carbon and nitrogen free conditions an enzyme(s) which rapidly metabolises L-asparagine might be produced and thus enhance L-asparagine uptake by reducing the intracellular pool. This explanation is made less likely by the data presented in figures 39 and 40. After 30 mins of nitrogen and carbon free treatment, although a large proportion of the counts do not correspond to L-asparagine, the intracellular concentration of L-asparagine is still quite large. This level of L-asparagine makes it unlikely that the enhancement of L-asparagine uptake is due to the action of an enzyme rapidly metabolising L-asparagine.

An alternative possible explanation is an intracellular compartmentation of L-asparagine in cellular organelles, which may interfere with L-asparagine uptake. Such an intracellular organelle has been described by Subramanian, Weiss and Davis (1973), Weiss (1976) and Weiss and Davis (1977). The above workers isolated the so-called "vesicle", serving as a reserve of the intracellular L-arginine and L-ornithine pools and possibly other amino acids and also suggested a possible regulatory role of this organelle in the transport and metabolism of these amino acids. A major amount of the intracellular L-asparagine might be stored in a similar organelle and not be concerned with the regulation of L-asparagine uptake, while the concentration of L-asparagine in the cytosol might determine the level of uptake. If the L-asparagine in the cytosol were rapidly metabolised by the putative enzyme this could result in enhancement of L-asparagine uptake. As a conclusion, the elevation of

L-asparagine uptake, under carbon and nitrogen free conditions, may be due to both the existence of a rapidly L-asparagine metabolising enzyme and a possible compartmentation of L-asparagine in cellular organelles. Alternatively, under these conditions, a permease, specific for L-asparagine transport, might be derepressed and result in elevation of the L-asparagine uptake.

The lack of transport deficient mutants makes the investigation of this L-asparagine transport system difficult. aspA20 is the only mutant found to be significantly altered in respect with L-asparagine transport. The transport system of this mutant is about 3 fold higher than the wild-type and the rest of the strains tested under all conditions. Under carbon and nitrogen free conditions the L-asparagine transport system of the aspA20 mutant is increased to a maximum of 2.4 nanomoles/min/mg of dry weight. This may be due to the hypothetical enzyme activity possessed by the aspA20 mutant reducing the intracellular L-asparagine pool and consequently elevating the L-asparagine transport. A second possible explanation may be that the putative aspA20 regulator gene controls the expression of the hypothetical L-asparagine permease(s). This permease(s) is derepressed in the aspA20 mutant and the optimum condition for derepression is carbon and nitrogen starvation. The wild-type under these conditions has derepressed L-asparagine transport, but to a lower level compared to aspA20, possibly because it cannot reach the maximum level of derepression possessing the normal product of the aspA20 locus.

CHAPTER VII GENERAL DISCUSSION

GENERAL DISCUSSION

L-Asparagine can be utilised by Aspergillus nidulans cells as a good nitrogen source as well as a poor carbon source. In the present thesis evidence is presented which suggests that L-asparagine metabolism includes at least three catalytic enzyme systems, a snythetic enzyme system and an active transport system.

1. Catalytic enzyme systems

L-Asparaginase activity is probably the main route of A. nidulans for the utilisation of L-asparagine as a nitrogen source. There are probably several distinct types of asparaginase activity in A. nidulans. The main evidence which indicates this is the nature of various mutations in the ahrA, tamA and areA loci.

a. Asparaginase I

The nature of the ahrA mutants and the fact that aspartic hydroxamate is more toxic on the presence of poor nitrogen sources in the growth medium, is less toxic on rich ones and its toxicity is reversed (repressed) when ammonia is present in the growth medium, support the theory that the toxicity of aspartic hydroxamate is due to the action of asparaginase I, since the ahrA mutants lack asparaginase I activity and are resistant to the toxic analogue, asparaginase I is higher on poor nitrogen sources and low or undetectable on rich ones.

A spectrum of mutants has been isolated of various degree of resistance to aspartic hydroxamate and the level of resistance of the mutants is correlated with their asparaginase activity. Specifically, strongly resistant mutants have no detectable asparaginase I activity, while the weakly resistant one has low activity. It is probable that the ahrA mutants identify a locus which is the structural gene for an asparaginase activity and this asparaginase for convenience has been called asparaginase I.

A somewhat similar situation has been described by Jones (1973 and 1977) who detected two different asparaginases in yeast cells (Saccharomyces cerevisiae) an intracellular, called asparaginase I, and an extracellular, called asparaginase II. Also in bacteria (Escherichia coli) different asparaginases have been detectected determined as I and II (see introduction). Unlike the yeast mutants lacking asparaginase I activity which are unable to grow on L-asparagine as a nitrogen source, the ahrA mutants can utilise L-asparagine as a good nitrogen source in a comparable fashion to the wild-type. This is strong evidence that the ahrA mutants possess some other enzyme activity (probably an asparaginase II) for which L-asparagine is a substrate. This enzyme must be unable to utilise aspartic hydroxamate as a substrate or at least unable to produce a toxic catabolite from it. In fact the ahrA mutants are sensitive to hydroxylamine to the same degree as the wild-type. This is evidence that the ahrA mutants are deficient in the production rather than the metabolism of hydroxylamine.

The investigation of the asparaginase I activity of various heterozygous diploids of the ahrA mutants with the wild-type, showed a strict gene dosage effect for asparaginase I activity. The dosage effect of the ahrA/wild-type heterozygous diploids is supporting evidence that the ahrA locus is the structural gene for asparaginase I.

Ammonia control (referred to by Pateman, Cove and other workers as ammonia regulation) has been extensively investigated in A. nidulans. A number of enzyme and transport systems including nitrate reductase, ammonia transport, acetamidase, extracellular protease, glutamate transport, urea transport, are regulated by ammonia. All of these systems are included in nitrogen metabolism or result in the production of nitrogen metabolites. Ammonia which is an end product of nitrogen catabolism is probably the effector and its presence results in repression and low levels of all the systems regulated. Two regulator loci are known to be involved with ammonia regulated systems, areA (Arst and Cove 1973) and tamA (Kinghorn and Pateman 1975 and Pateman and Kinghorn 1976). The areA locus can be interpreted as specifying a protein which is capable of allowing the synthesis of a number of systems of nitrogen metabolism but which cannot function in the presence of ammonia (positive control). A similar positive regulatory role is proposed for the tamA locus. In addition the product of the gdhA locus is proposed to have some regulatory role in ammonia regulation (Kinghorn and Pateman 1973). A possible explanation is that NADP-GDH (the product of the gdhA locus) is a multi-functional protein which has catalytic activity and also plays a role in either the repression or

inhibition, or both, of a number of metabolic activities. Mutation in the gdhA locus, the structural gene for NADP-GDH, would result in both abnormal enzyme and control activity.

Asparaginase I activity is under ammonia control. A. nidulans cells have no detectable asparaginase I activity when ammonia is present in the growth medium and they have maximum activity after nitrogen starvation. This ammonia control may be either inactivation or repression of enzyme synthesis or both. Strong evidence about the possible regulatory role of ammonia comes from the fact that when added to the growth medium at the time of maximum activity it results in rapid loss of the activity. This loss of activity is prevented by the protein synthesis inhibitor cycloheximide when added to the growth medium at the same time as ammonia. The two main types of explanation which are proposed are the following:

ammonia inactivates asparaginase I in vivo, inhibition of asparaginase I in vitro has not been observed. This inactivation, possibly mediated by a protease, needs protein systhesis. The second possible explanation is that ammonia represses asparaginase I synthesis, and this repression requires protein synthesis. The requirement for protein synthesis may be due to the ammonia mediated synthesis of one or more control proteins which block the synthesis of asparaginase I. It is also possible that the role of ammonia in the regulation of asparaginase I synthesis is due to both repression of synthesis and inactivation of enzyme activity in vivo. Other good nitrogen sources for A. nidulans, as nitrate and L-asparagine,

have the same effect as ammonia when added in the growth medium at the time of maximum activity but to a lesser extent. Their action is similarly prevented by cycloheximide added at the same time. Loss of activity caused by the presence of nitrate may be due to the ammonia produced from it, since no loss of activity is observed in mutants unable to metabolise nitrate (nirA).

Further evidence that asparaginase I activity is regulated, at least in part, by general ammonia repression, is provided by the properties of the mutant alleles in the tamA and areA regulatory loci. A partially repressed allele of the tamA regulatory gene, tamA119, has low asparaginase I activity and is also resistant to aspartic hydroxamate toxicity. $tamA^{d}1$ an ammonia derepressed allele of the same gene, has detectable asparaginase I activity in the presence of ammonia. tamA50, a completely repressed allele of the same locus, has no detectable asparaginase I activity. Completely repressed alleles ($areA^{r}550$) of the areA regulatory gene have no detectable asparaginase I activity and derepressed alleles of the same locus ($areA^{d}520$) have detectable asparaginase I activity in the presence of ammonia. In addition the ammonia derepressed mutants meaA8, DER-3 and gdhA1 have detectable asparaginase I activity in the presence of ammonia. Therefore, as a conclusion from the above data, mutants altered in respect with general ammonia regulation are altered in asparaginase I synthesis as well.

b. Asparaginase II

The tamA50 completely repressed allele of the tamA regulatory gene which has undetectable asparaginase I activity, is unable to utilise L-asparagine as nitrogen source. It is probable that the tamA50 mutant is not only repressed for asparaginase I activity but also for the enzyme activity that enables the ahrA mutants to utilise L-asparagine as nitrogen source (presumably asparaginase II). However, tamA50 is able to utilise L-asparagine as carbon source. This implies the existence of a third enzyme system in A. nidulans which can use L-asparagine as substrate.

c. Asparaginase III

Cohen (1972) and Hynes (1970) have reported that ammonia repression of certain systems is phenotypically inoperative when a substrate of the system is used as the sole carbon source. A similar observation has been made about L-aspartate and L-glutamate. When one of these two amino acids is present in the growth medium as the sole carbon source, aspartic hydroxamate is toxic for the wild-type and any other mutant tested in presence of ammonia. Under these conditions even the strongly resistant ahrA mutants and the completely repressed tamA50 are sensitive on aspartic hydroxamate and they do not grow at all. It is obvious that an enzyme system, different from asparaginase I and asparaginase II is operating which can metabolise aspartic hydroxamate and produce some toxic metabolite from it. It is not known if this system, responsible for the toxicity of aspartic hydroxamate, can also utilise aspartate and glutamate as substrates.

The nature of the aspA20 mutant supports the above suggestion about the probable existence of a third enzyme system. The aspA20 mutant is derepressed for aspartic hydroxamate toxicity on all carbon sources and nitrogen sources tested. This mutant can also utilise L-asparagine as carbon source in a fashion better than the wild-type, and is epistatic to ahrA and tamA mutants with respect to aspartic hydroxamate sensitivity in presence of ammonia.

A possible explanation for the above results is that A. nidulans possesses yet another enzyme system which can use L-asparagine as a substrate, in addition to asparaginase I and the putative asparaginase II. This enzyme for convenience may be named asparaginase III. However, this nomenclature might be wrong in that the so-called asparaginase III might not be an asparagine deaminating enzyme. It is probable that asparaginase III is under carbon catalobite repression and is regulated in some way by the product of the aspA20 locus. This aspA20 product is a regulatory molecule with a possible intranuclear negative action. This interpretation of the role of the aspA20 product is based on the fact that mutant alleles of the aspA20 locus are dominant to the wild-type in heterozygous heterocaryons and recessive in heterozygous diploids. Asparaginase III activity is normally repressed under all conditions and is derepressed when L-aspartate or L-glutamate are the sole carbon sources in the growth medium. The enzyme can utilise aspartic hydroxamate as a substrate and produce some toxic metabolite to which A. nidulans is sensitive. Attempts were made to identify this enzyme but they were all unsuccessful. It is obvious that L-asparagine metabolism is a more complicated mechanism than at first thought,

and more research is necessary to investigate the enzyme system (asparaginase II) which enables the ahrA mutants to utilise L-asparagine as nitrogen source, and the enzyme system (asparaginase III) which is regulated by the aspA20 locus.

2. Synthetic enzyme system

A preliminary investigation has been made of the L-asparagine synthetase system. A mutant has been isolated, named asn⁻A1, which requires L-asparagine to grow. Double mutants of the asn⁻A1 with the ahrA mutants and the aspA20 mutant, still require L-asparagine to grow. It is suggested that asn⁻A1 lacks L-asparagine synthetase activity and consequently requires L-asparagine for growth. This enzyme is not related to the asparaginase activities. All the attempts made to assay such an enzyme were unsuccessful.

3. Transport system

An active transport system for L-asparagine is present in A. nidulans, which is responsible for the active transport of L-asparagine from the external environment into the cells. The above conclusion derives from the fact that A. nidulans cells accumulate L-asparagine intracellularly about 30-fold above the external concentration. In addition the inhibition of the L-asparagine transport by the uncoupling agent sodium azide indicates a possible energy requirement of this system.

Unlike some other transport systems in A. nidulans such as these for L-glutamate, urea and ammonium, L-asparagine transport

is not simply determined by ammonia regulation. The level of the L-asparagine uptake system is about the same in cells grown on ammonia or nitrogen starved. In contrast the level of L-asparagine uptake is increased (more than two fold) when cells are transferred to nitrogen and carbon free conditions after growth on ammonia. When ammonia is present in the carbon free treatment medium the elevation of the L-asparagine uptake is half as much as in the absence of both ammonia and carbon. This is an indication that L-asparagine transport may be under both nitrogen metabolite and carbon catabolite repression.

The presence of L-aspartate or L-glutamate in the nitrogen and carbon free treatment medium does not prevent the elevation of L-asparagine uptake by this treatment. This observation together with the reversal of ammonia repression of aspartic hydroxamate toxicity when these two amino acids are used as carbon sources, suggests that the L-asparagine transport system is carbon repressed. The aspA20 mutant also supports this explanation since this mutant has elevated L-asparagine transport compared to the wild-type. Thus the aspA20 mutant appears to be a carbon derepressed mutant for asparaginase III and L-asparagine transport system. There are two main possible types of explanation for this situation. First the aspA20 locus plays a direct role in the carbon regulation of both asparaginase III and L-asparagine transport. Second, the aspA20 mutant is only carbon derepressed for asparaginase III and consequently has an increased capacity for metabolising L-asparagine. As a result the L-asparagine taken up by the aspA20 cells is not accumulated intracellularly to the same level as in the wild-type. This might

allow a higher uptake rate in aspA20. In fact experiments to measure the intracellular concentration of L-asparagine in wild-type and aspA20 cells showed that it was lower in the aspA20 cells.

However the lack of mutants deficient in L-asparagine uptake is a great obstacle in the investigation of the L-asparagine transport system. The isolation of such mutants is necessary to establish the specificity and the regulation of this transport system.

4. Conclusion

At least three enzyme systems in Aspergillus nidulans are able to use L-asparagine as substrate:

1) asparaginase I, ammonia regulated, probably determined by the ahrA structural gene,

2) asparaginase II, ammonia regulated, enabling the ahrA mutants to utilise L-asparagine as nitrogen source,

3) asparaginase III, carbon catabolite repressed, regulated in some fashion by the aspA20 locus.

4) There is an energy requiring low capacity L-asparagine transport system in A. nidulans which is subject to some form of carbon regulation and possibly also to some form of nitrogen regulation.

5) It is possible that an L-asparagine anabolic system operates in A. nidulans, the L-asparagine synthetase, which is probably determined by the asn⁻A1 gene.

REFERENCES

ADELBERG, E.A., MANDEL, M. AND CHEN, G.C.C. (1965)

Optimum conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichia coli K12.

Biochemical and Biophysical Research Communications. 18: 788.

AHMED, A., CASE, M.E. AND GILES, N.H. (1964)

The nature of complementation among mutants in the histidine-3 region of Neurospora crassa.

Brookhaven Symposium in Biology. 17: 53.

ALTENBERN, R.A. AND HOUSEWRIGHT, R.D. (1954)

Stereospecific asparaginases in smooth Brucella aborus strain 19.

Archives in Biochemistry and Biophysics. 49: 130.

ARFIN, S.M. (1967)

Asparagine synthesis in the chick embryo liver.

Biochimica et Biophysica Acta. 136: 233.

ARIMA, K.T., SAKAMOTO, T., ARAKI, C. AND TAMURA, G. (1972)

Production of extracellular L-asparaginases by microorganisms.

Agricultural and Biological Chemistry. 36: 356.

ARST, H.N. Jr., AND COVE, D.J. (1969)

Methylammonium resistance in Aspergillus nidulans.

Journal of Bacteriology. 95: 1284.

ARST, H.N. Jr. AND COVE, D.J. (1973)

Nitrogen metabolite repression in Aspergillus nidulans.

Molecular and General Genetics. 126: 111.

ARST, H.N. Jr. AND PAGE, M.M. (1973)

Mutants of Aspergillus nidulans altered in the transport of methylammonium and ammonium.

Molecular and General Genetics. 121: 239.

ARST, H.N. Jr. AND MACDONALD, D.W. (1975)

A gene cluster in Aspergillus nidulans with an internally located cis-acting regulatory region.

Nature. 254: 26.

ARST, H.N. Jr. AND SCAZZOCCHIO, C. (1975)

Initiator constitutive mutation with an "up-promoter" effect in Aspergillus nidulans.

Nature. 254: 31.

BAILEY, C. AND ARST, H.N. Jr. (1975)

Carbon catabolite repression in Aspergillus nidulans.

European Journal of Biochemistry. 51: 573.

BASCOMB, S. AND BETTELHEIM, K.A. (1975)

Immunological relationships of bacterial L-asparaginases.

Journal of General Microbiology. 92: 175.

BECKWITH, J. AND ROSSON, P. (1974)

Analysis of genetic regulatory mechanisms.

Annual Review of Genetics. 8: 1.

BRICKMAN, E., SOLL, L. AND BECKWITH, J. (1973)

Genetic characterization of mutations which affect catabolite-sensitive operons in Escherichia coli, including deletions of the gene for adenyl cyclase.

Journal of Bacteriology. 116: 582.

BROOME, J.D. (1963a)

Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects.

I. Properties of the L-asparaginase of guinea pig serum in relation to those on antilymphoma substance.

Journal of Experimental Medicine. 118: 99.

BROOME, J.D. (1963b)

Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects.

II. Lymphoma 6C3HED cells cultured in a medium devoid of L-asparagine lose their susceptibility to the effects of guinea pig serum in vivo.

Journal of Experimental Medicine. 118: 121

BROOME, J.D. (1965)

Antilymphoma activity of L-asparaginase in vivo: clearance rates of enzyme preparation from guinea pig serum and yeast in relation to their effect on tumor growth.

Journal of National Cancer Institute. 35: 967.

BUETTNER, M.J., SPITZ, E. AND RICKENBERG, H.V. (1973)

Cyclic adenosine 3', 5'-monophosphate in Escherichia coli.

Journal of Bacteriology. 114: 1068.

CALHOUN, D.H. AND HATFIELD, G.W. (1975)

Autoregulation of gene expression.

Annual Review of Microbiology. 29: 275.

CALVO, J.M. AND FINK, G.R. (1971)

Regulation of biosynthetic pathways in bacteria and fungi.

Annual Review of Biochemistry. 40: 943.

CAMMACK, K.A., MARLBOROUGH, D.I. AND MILLER, D.S. (1972)

Physical properties and subunit structure of L-asparaginase isolated from Erwinia carotovora.

Biochemical Journal. 126: 361.

CAMPBELL, H.A., MASHBURN, L.T., BOYSE, E.A. AND OLD, L.J. (1967)

Two L-asparaginases from Escherichia coli B. Their separation, purification and antitumor activity.

Biochemistry. 6: 721.

CANELLOS, G.P., HASKELL, G.M., ARSENEAN, J. AND CARBONE, P.P. (1969)

Hypoalbuminemic and hypocholesterolemic effect of L-asparaginase (NSC-109, 229) treatment in man: A preliminary report.

Cancer Chemotherapy Rep. 53(1): 67.

CAPIZZI, R.L., BERTINO, J.R. AND HANDSCHUMACHER, R.E. (1970)

L-Asparaginase.

Annual Review of Medicine. 21: 433.

CASSELTON, L.A. AND LEWIS, D. (1967)

Dilution of gene products in the cytoplasm of heterocaryons in Coprinus lagopus.

Genetical Research, Camb. 9: 63.

CEDAR, H. AND SCHWARTZ, J.H. (1967)

Localization of the two L-asparaginases in anaerobically grown Escherichia coli.

Journal of Biological Chemistry. 242: 3753

CEDAR, H. AND SCHWARTZ, J.H. (1968)

Production of L-asparaginase II by Escherichia coli.

Journal of Bacteriology. 96: 2043.

CEDAR, H. AND SCHWARTZ, J.H. (1969a)

The asparagine synthetase of E. coli.

I. Biosynthetic role of the enzyme, purification and characterization of the reaction products.

Journal of Biological Chemistry. 244(15): 4112.

CEDAR, H. AND SCHWARTZ, J.H. (1969b)

The asparagine synthetase of E. coli.

II. Studies on mechanism.

Journal of Biological Chemistry. 244(15): 4122.

CHALEFF, R. (1971)

Evidence for a gene cluster controlling the inducible quinate catabolic pathway in Neurospora crassa.

Genetics. 68: S10.

CLEMENTI, D.A. (1922)

La desamidasion enzymatique de l'asparagine chez les differentes especes animales, et la signification physiologique de sa presence dans l'organism.

Arch. Intern. Physiol. Biochim. 19: 369.

CLUTTERBUCK, A.J. (1968)

Cell volume per nucleus in haploid and diploid strains of Aspergillus nidulans.

Journal of General Microbiology. 55: 291.

CLUTTERBUCK, A.J. (1970)

Questionnaire on gene symbols.

Aspergillus News Letter. 10: 18.

CLUTTERBUCK, A.J. (1973)

Gene symbols in Aspergillus nidulans.

Genetical Research Camb. 21: 291.

CLUTTERBUCK, A.J. (1974)

Aspergillus nidulans.

Handbook of Genetics. R.C. King (ed.). Plenum Press New York. 1: 447.

COHEN, B.L. (1972)

Ammonium repression of extracellular protease in Aspergillus nidulans.

Journal of General Microbiology. 71: 293.

COONEY, D.A. AND HANDSCHUMACHER, R.E. (1970)

L-Asparaginase and L-asparagine metabolism.

Annual Review of Pharmacology. 10: 421.

COVE, D.J. (1966)

The induction and repression of nitrate reductase in the fungus Aspergillus nidulans.

Biochimica et Biophysica Acta. 113: 51.

COVE, D.J. (1976)

Chlorate toxicity in Aspergillus nidulans: The selection and characterization of chlorate resistant mutants.

Heredity. 36: 191.

COVE, D.J. AND PATEMAN, J.A. (1969)

Autoregulation of the synthesis of nitrate reductase in Aspergillus nidulans.

Journal of Bacteriology. 97: 1374.

DARLINGTON, A.J., SCAZZOCCHIO, C. AND PATEMAN, J.A. (1965)

Biochemical and genetical studies of purine breakdown in Aspergillus.

Nature (London). 206: 599.

DAVIS, R. (1975)

Compartmentation and regulation of fungal metabolism; genetic approaches.

Annual Review of Genetics. 9: 32.

DEANGELI, L.C., POCCHIARI, F., RUSSI, S., TONOLO, A. AND ZURITE, V.E. (1970)

Effect of L-asparaginase from Aspergillus terreus on ascites Sarcoma in rat.

Nature. 225: 549.

DEGROOT, N. AND LICHTENSTEIN, N. (1960a)

The action of mammalian liver enzyme preparation on asparagine and asparagine derivatives.

Biochimica et Biophysica Acta. 40: 92.

DEGROOT, N. AND LICHTENSTEIN, N. (1960b)

The action of mammalian liver enzyme preparation on asparagine and asparagine derivatives.

Biochimica et Biophysica Acta. 40: 99.

DOUGLAS, H.C. AND HAWTHORNE, D.C. (1966)

Regulation of genes controlling synthesis of the galactose pathway enzymes in yeast.

Genetics. 54: 911.

DOX, A.W. (1909)

The intracellular enzymes of lower fungi, especially those of Penicillium camemberti.

Journal of Biological Chemistry. 6: 461.

DRAINAS, C., KINGHORN, J.R. AND PATEMAN, J.A. (1976)

Aspects of asparaginase regulation in the fungus Aspergillus nidulans.

180th Genetical Society Meeting. Heredity. 37: 153.

DRAINAS, C., KINGHORN, J.R. AND PATEMAN, J.A. (1977)

Aspartic hydroxamate resistance and asparaginase regulation in the fungus Aspergillus nidulans.

Journal of General Microbiology. 98: 493.

DRAINAS, AND PATEMAN, J.A. (1977)

L-Asparaginase activity in the fungus Aspergillus nidulans.

Biochemical Society Transactions. 5(1): 259.

DUNLOP, P.C. AND ROON, R.J. (1975)

L-Asparaginase of Saccharomyces cerevisiae: an extracellular enzyme.

Journal of Bacteriology. 122(3): 1017.

DUNN, E. AND PATEMAN, J.A. (1972)

Urea and thiourea uptake in Aspergillus nidulans.

Heredity. 29: 129.

EHRMAN, M., CEDAR, H. AND SCHWARTZ, J.H. (1971)

L-Asparaginase II of Escherichia coli.

Journal of Biological Chemistry. 246: 88.

ENGLESBERG, E., SHEPPARD, D., SQUIRES, C. AND MERONK, F. (1969)

An analysis of "revertants" of a deletion mutant in the C gene of the arabinose gene complex in Escherichia coli B/r_2.

Isolation of initiator constitutive mutants (I^c).

Journal of Molecular Biology. 43: 281.

ENGLESBERG, E. AND WILCOX. (1974)

Regulation; positive control.

Annual Review of Genetics. 8: 219.

EPSTEIN, W. AND BECKWITH, J.R. (1968)

Regulation of gene expression.

Annual Review of Biochemistry. 37: 411.

EVERED, D.G. (1959)

Ionophoresis of acidic and basic amino acids on filter paper using low voltages.

Biochimica et Biophysica Acta. 36: 14.

FINCHAM, J.R.S. AND DAY, P.R. (1963)

Outline of the Biology of fungi of genetic interest.

(Chapter 2).

Fungal Genetics. Blackwell Scientific Publications Oxford.

FURTH, O. AND FREIDMANN, M. (1910)

Uberdie verbreitung asparaginspaltender organfermente.

Biochemische Zeitschrift. 26: 435.

GILES, N.H., CASE, M.E., PARTRIDGE, C.W.H. AND AHMED, S.I. (1967)

A gene cluster in Neurospora crassa coding for an aggregate of five aromatic synthetic enzymes.

Proceedings of National Academy of Sciences U.S.A. 58: 1453.

GILBERT, W. AND MULLER-HILL, B. (1966)

Isolation of the lac repressor.

Proceedings of National Academy of Sciences U.S.A. 56: 1891.

GROSS, S.R. (1969)

Genetic regulatory mechanisms in the fungi.

Annual Review of Genetics. 3: 395.

GROSS, M.A., SREER, R.J. AND HILL, J.M. (1969)

Hepatic lipidosis associated with L-asparaginase treatment.

Proceedings of the Society for Experimental Biology and Medicine. 130(3): 733.

GROVER, C.E. AND CHIBNAL, A.C. (1927)

The enzymatic deamination of asparagine in the higher plants.

Biochemical Journal. 21: 857.

HASKELL, C.M., CANELLOS, G.P., LEVENTHAL, B.G., CARBONE, P.P., SERPICK, A.A. AND HANSEN, H.H. (1969)

L-Asparaginase toxicity.

Cancer Research. 29: 974.

HO, P.P.K., BRUCE, H.F. AND BURCK, P.J. (1969)

Crystalline L-asparaginase from E. coli B.

Science. 165: 510.

HO, P.P.K., MILIKIN, E.B., BOBBITT, J.L., GRINMAN, E.L., BURCK, P.J., FRANK, B.H., BOECK, L.D. AND SQUIRES, R.W. (1970)

Crystalline L-asparaginase from E. coli B. I. Purification and chemical characterization.

Journal of Biological Chemistry. 245: 3708.

HOLDEN, J.T. AND BUNCH, J.M. (1973)

Asparagine transport in Lactobacillus plantarum and Streptococcus faecalis.

Biochimica et Biophysica Acta. 307: 640.

HOWARD, J.B. AND CARPENTER, F.H. (1972)

L-Asparaginase from Erwinia carotovora.

Journal of Biological Chemistry. 247: 1020.

HYNES, M.J. (1970)

Induction and repression of amidase enzymes in Aspergillus nidulans.

Journal of Bacteriology. 103: 482.

HYNES, M.J. (1975)

A cis-dominant regulatory mutation affecting enzyme induction in the eucaryote Aspergillus nidulans.

Nature. 252: 210.

HYNES, M.J. AND PATEMAN, J.A. (1970a)

The genetic analysis of regulation of amidase synthesis in Aspergillus nidulans. I. Mutants unable to utilise acrylamide.

Molecular and General Genetics. 108: 97.

HYNES, M.J. AND PATEMAN, J.A. (1970b)

The genetic analysis of regulation of amidase synthesis in Aspergillus nidulans. II. Mutants resistant to fluoroacetamide.

IMADA, S., IGARASKI, K., NAKAHAMA, AND ISONO, M. (1973)

Asparaginase and Glutaminase activities of microorganisms.

Journal of General Microbiology. 76: 85.

JACOB, F. AND MONOD, J. (1961)

On the regulation of gene activity.

Symposia on Quantitative Biology. 26: 193.

JONES, G.E. (1973)

A fine structure map of the yeast asparaginase gene.

Molecular and General Genetics. 121: 9.

JONES, G.E. (1977)

Genetic and physiological relationships between L-asparaginase I and L-asparaginase II in Saccharomyces cerevisiae.

Journal of Bacteriology. 130: 128.

JONES, G.E. AND MORTIMER, R.K. (1970)

L-Asparaginase deficient mutants of yeast.

Science. 167: 181.

JONES, G.E. AND MORTIMER, R.K. (1973)

Biochemical properties of yeast L-asparaginase.

Biochemical Genetics. 9: 131.

KHAN, A. AND LEVINE, S. (1974)

Further studies on the inhibition of allergic encephalomyelitis by L-asparaginase.

Journal of Immunology. 113(1): 367.

KIDD, J.G. (1953a)

Regression of transplanted lymphomas induced *in vivo* by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum or rabbit serum.

Journal of Experimental Medicine. 98: 565.

KIDD, J.G. (1953b)

Regression of transplanted lymphomas induced *in vivo* by means of normal guinea pig serum. II. Studies of the nature of the active serum constituent. Histological mechanism of the regression: tests for effects of guinea pig serum on lymphoma *in vitro*. Discussion.

Journal of Experimental Medicine. 98: 583.

KIDD, J.G. AND SOBIN, L.H. (1966)

The incorporation of L-asparagine-^{14}C by lymphoma GC3HED cells: its inhibition by guinea pig serum.

Cancer Research. 26: 208.

KINGHORN, J.R. AND PATEMAN, J.A. (1972)

Regulation of glutamate transport in *Aspergillus nidulans*.

Heredity. 29: 128.

KINGHORN, J.R. AND PATEMAN, J.A. (1973)

NAD and NADP L-glutamate dehydrogenase activity and ammonium regulation in *Aspergillus nidulans*.

Journal of General Microbiology. 78: 39.

KINGHORN, J.R. AND PATEMAN, J.A. (1975a)

The structural gene for NADP-L-glutamate dehydrogenase in Aspergillus nidulans.

Journal of General Microbiology. 86: 294.

KINGHORN, J.R. AND PATEMAN, J.A. (1975b)

Mutations which affect amino acid transport in Aspergillus nidulans.

Journal of General Microbiology. 86: 174.

KINGHORN, J.R. AND PATEMAN, J.A. (1975c)

Studies of partially repressed mutants at the tamA and areA loci in Aspergillus nidulans.

Molecular and General GEnetics. 140: 137.

KINGHORN, J.R. AND PATEMAN, J.A. (1976)

A gene cluster in a simple eucaryote.

Heredity. 37: 154.

KIRSCHBAUM, J., WRISTON, J.C. AND RATYCH, O.J. (1969)

Subunit structure of L-asparaginase from E. coli B.

Biochimica et Biophysica Acta. 194: 161.

LAWRENCE, A.J. AND MOORES, G.R. (1972)

Conductimetry in enzyme studies.

European Journal of Biochemistry. 24: 538.

LAWRENCE, A.J., MOORES, G.R. AND STEELE, J. (1974)

A conductimetric study of erythrocyte lysis by lysolecithin and linoleic acid.

European Journal of Biochemistry. 48: 277.

LEE, M.B. AND BRIDGES, J.M. (1968)

L-Asparaginase activity in human and animal sera.

Nature. (Lon.) 217: 758.

LINEWEAVER, H. AND BURK, D. (1934)

The determination of enzyme dissociation constants.

Journal of American Chemical Society. 56: 658.

LHOAS, P. (1961)

Mitotic haploidization by treatment of Aspergillus niger diploids with para-fluorophenylalanine.

Nature. 190: 744.

LOWRY, O.H., ROSEBROUGH, N.I., FARR, A.L. AND RANDALL, R.J. (1951)

Protein measurement with the Folin phenol reagent.

Journal of Biological Chemistry. 193: 265.

LUKASZKIEWUZ, Z. AND PASZEWSKI, A. (1976)

Hyper-repressible operator-type mutant in sulphate permease gene in Aspergillus nidulans.

Nature. 259: 337.

MACINTOSH, M.E. AND PRITCHARD, R.H. (1963)

The production and replica plating of micro-colonies of Aspergillus nidulans.

Genetical Research. 4: 320.

MARDASHEV, S.R., SOKOLOV, N.N., EYSEEV, L.P. AND NIKOLAEV, A.Ya. (1969)

Effect of amino acids on the induced formation of asparaginase, glutaminase and glutamine synthetase in Pseudomonas sp.

Biokhimiya. 34(3): 529.

MASHBURN, L.T. AND WRISTON, J.C., Jr. (1963)

Tumor inhibitory effect of L-asparaginase.

Biochemical and Biophysical Research Communication. 12: 50.

MASHBURN, L.T. AND WRISTON, J.C., Jr. (1964)

Tumor inhibitory effect of L-asparaginase from Escherichia coli.

Archives in Biochemistry and Biophysics. 105: 451.

McCULLY, K.S. AND FORBES, E. (1965)

The use of p-fluorophenylalanine with "master strains" of Aspergillus nidulans for assigning genes to linkage groups.

Genetical Research. 6: 352.

MEISTER, A. (1955)

Glutaminase, asparaginase, and α-keto-w-amidase. B.

L-Asparaginase from guinea pig serum.

Methods in Enzymology. 2: 383.

METZENBERG, R.L. (1972)

Genetic regulatory systems in Neurospora 3037.

Annual Review of Genetics. 6: 111.

MILLER, H.K. AND EARLBALIS, M. (1969)

Glutaminase activity of L-asparagine amidohydrolase.

Biochemical Pharmacology. 18(9): 2225.

MONOD, J. AND COHEN-BASIRE, G. (1953)

L'effect d'inhibition specifique dans la biosynthese de la tryptophane-des-mase chez Aerobacter aerogenes.

Comptes Rendus de l'Academie des Sciences. 236: 530.

PAGE, M.M. (1971)

Genetic and biochemical studies on the catabolism of amines and alcohols in Aspergillus nidulans.

Ph.D. Thesis, University of Cambridge.

PAGE, M.M. AND COVE, D.J. (1972)

Alcohol and amine catabolism in the fungus Aspergillus nidulans.

Biochemical Journal. 127: 17 P.

PATEMAN, J.A. (1969)

Regulation of synthesis of glutamate dehydrogenase and glutamine synthetase in microorganisms.

Biochemical Journal. 115: 769.

PATEMAN, J.A. AND COVE, D.J. (1967)

Regulation of nitrate reduction in Aspergillus nidulans.

Nature. 215: 1234.

PATEMAN, J.A., DUNN, E., KINGHORN, J.R. AND FORBES, E. (1974)

The transport of ammonium and methylammonium in wild-type and mutant cells of Aspergillus nidulans.

Molecular and General Genetics. 133: 225.

PATEMAN, J.A. AND KINGHORN, J.R. (1976a)

Nitrogen metabolism.

The Filamentous Fungi: Biosynthesis and Metabolism.

Chapter 7. John E. Smith, David R. Berry (ed.) II: 159.

PATEMAN, J.A. AND KINGHORN, J.R. (1976b)

The urea utilization operon in Aspergillus nidulans.

Proceedings of the Society for General Microbiology. 3: 188.

PATEMAN, J.A. AND KINGHORN, J.R. (1977)

Genetic regulation of nitrogen metabolism.

Symposium: Physiology and Genetics of Aspergillus.

Edward Amed.

PATEMAN, J.A., KINGHORN, J.R. AND DUNN, E. (1974)

Regulatory aspects of L-glutamate transport in Aspergillus nidulans.

Journal of Bacteriology. 119: 534.

PATEMAN, J.A., KINGHORN, J.R., DUNN, E. AND FORBES, E. (1973)

Ammonium regulation in Aspergillus nidulans.

Biochemical Society Transactions. 537th Meeting, Canterbury. 1: 674.

PATEMAN, J.A., REVER, B.M. AND COVE, D.J. (1967)

Genetic and Biochemical studies of nitrate reduction in Aspergillus nidulans.

Biochemical Journal. 104: 103.

PATTERSON, M.K. Jr. AND ORR, G.R. (1968)

Asparagine biosynthesis by the Novikoff Hepatoma.

Journal of Biological Chemistry. 243: 376.

POLKINGHORNE, E.M. AND HYNES, M.J. (1974)

Mutants affecting histidine utilization in Aspergillus nidulans.

Genetical Research Camb. 25: 119.

PONTECORVO, G., FORBES, E. AND ADAM, O.B. (1949)

Genetics of the homothallic ascomycete Aspergillus nidulans.

Heredity. 3: 385.

PONTECORVO, G. AND ROPER, J.A. (1952)

Genetic analysis without sexual reproduction by means of polyploidy in Aspergillus nidulans.

Proceedings of the Journal of General Microbiology. 6: vii.

PONTECORVO, G., ROPER, J.A., HEMMONS, C.M., MACDONALD, K.D. AND BUFTON, A.W.J. (1953)

The genetics of Aspergillus nidulans.

Advances in Genetics. 5: 141.

RAVEL, J.M., NORTON, S.J., HUMPHREYS, J.S. AND SHIVE, W. (1962)
Asparagine biosynthesis in Lactobacillus arabinosus and its control by asparagine through enzyme inhibition and repression.
Journal of Biological Chemistry. 237: 2845.

RESNICK, A.D. AND MAGASANIK, B. (1976)
L-Asparaginase of Klebsiella aerogenes.
Journal of Biological Chemistry. 251: 2722.

RINES, H.W. (1968)
The recovery of mutants in the inducible quinic acid catabolic pathway in Neurospora crassa.
Genetics. 60: 215.

ROPER, J.A. (1952)
Production of heterozygous diploids in filamentous fungi.
Experimentia. 8: 14.

SCAZZOCCHIO, C., HOLL, F.G., FOGUELMAN, A.I. (1973)
The genetic control of molybdoflavoproteins in Aspergillus nidulans. I. Allopurinol-resistant mutants constitutive for xanthine dehydrogenase.
European Journal of Biochemistry. 46: 428.

SCAZZOCCHIO, C. (1973)
The genetic control of molybdoflavoproteins in Aspergillus nidulans. II. Use of the NADH dehydrogenase activity associated with xanthine dehydrogenase to investigate substrate and product induction.
Molecular and General Genetics. 125: 147.

SCAZZOCCHIO, C. AND DARLINGTON, A.A. (1968)

The induction and repression of the enzymes of purine breakdown in Aspergillus nidulans.

Biochimica et Biophysica Acta. 166: 557.

SCHWARTZ, J.H., REEVES, J.Y. AND BROOME, J.P. (1966)

Two L-asparaginases from E. coli and their action against tumors.

Proceedings of National Academy of Sciences U.S.A. 56: 1516.

SCHWARTZ, M.K., LASH, E.D., OETTGEN, H.F. AND TOMAO, F.A. (1970)

L-Asparaginase activity in plasma and other biological fluids.

Cancer. 25: 244.

SHAPIRO, B.M. AND STADTMAN, E.R. (1970)

The regulation of glutamine synthesis in microorganisms.

Annual Review of Microbiology. 24: 501.

SIEGEL, M.R. AND SISLER, H.D. (1963)

Inhibition of protein synthesis in vitro by cycloheximide.

Nature. 200: 675.

SINHA, U. (1969)

Genetic control of the uptake of amino acids in Aspergillus nidulans.

Genetics. 62: 495.

SOBIN, L.H. AND KIDD, J.G. (1965)

A metabolic difference between two lines of lymphoma 6C3HED cells in relation to asparagine.

Proceedings of the Society for Experimental Biology and Medicine. 119: 325.

SOBIN, L.H. AND KIDD, J.G. (1966)

Alterations in protein and nucleic acid metabolism of lymphoma 6C3HED-OG cells in mice given guinea pig serum.

Journal of Experimental Medicine. 123: 55.

SUBRAMANIAN, K.N., WEISS, R.L. AND DAVIS, R.H. (1973)

Use of external, biosynthetic, and organellar arginine by Neurospora.

Journal of Bacteriology. 115: 284.

VALONE, J.A. Jr., CASE, M.E. AND GILES, N.H. (1971)

Constitutive mutants in a regulatory gene exerting positive control of quinic acid catabolism in Neurospora crassa.

Proceedings of National Academy of Sciences U.S.A. 68: 1555.

WEISS, R.L. (1976)

Compartmentation and control of argine metabolism in Neurospora.

Journal of Bacteriology. 126: 1173.

WEISS, R.L. AND DAVIS, R.H. (1977)

Control of arginine utilization in Neurospora.

Journal of Bacteriology. 129: 866.

WILLIS, R.C. AND WOOLFOLK, C.A. (1970)

Transport of asparagine in E. coli K12.

Bacteriological Proceedings (American Society of Microbiology). 127, P27.

WILLIS, R.C. AND WOOLFOLK, C.A. (1975)

L-Asparagine uptake in E. coli.

Journal of Bacteriology. 123: 937.

WRISTON, J.C. Jr. (1970)

Asparaginase.

Methods in Enzymology. 17(A): 732.

WRISTON, J.C. Jr. AND YELLIN, T.D. (1973)

L-Asparaginase: a review.

Advances in Enzymology. 39: 185.