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REGULATORY ASPECTS OF GLUTAMATE METABOLISM IN ASPERGILLUS

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

by James Robertson Kinghorn
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August 1973

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SUMMARY

The object of this study has been to extend our present knowledge of the control of gene action in eukaryotic cells. For the purpose of achieving this goal systems responsible for L-glutamate metabolism in the simple fungus *Aspergillus nidulans* have been chosen for investigation. These systems are L-glutamate transport, NADP L-glutamate dehydrogenase and NAD L-glutamate dehydrogenase.

A mutant, unable to grow on L-glutamate and L-aspartate as a carbon or nitrogen source and lacking L-glutamate and L-aspartate transport has been isolated. This *aauA1* grows normally on other amino acids as sole carbon or nitrogen sources and appears to have normal uptake of all other nitrogen sources tested indicating that there is a specific uptake system for L-glutamate and L-aspartate. This system is regulated by ammonium as are a number of other unrelated systems, e.g. nitrate reductase, acetamidase, etc. It is not clear whether this ammonium control is at the level of transcription or translation but for convenience referred to as ammonium repression.

The synthesis of L-glutamate from ammonium and α-oxoglutarate is catalysed by NADP L-glutamate dehydrogenase.

Mutants, unable to grow normally on ammonium and lacking NADP L-glutamate dehydrogenase activity, have been isolated. The mutants, designated *gdhA1* - *A9*, require a supplement of amino acid for normal wild type growth. In addition to losing their catalytic activity, *gdhA* mutants are insensitive to ammonium control of ammonium repressible systems including glutamate transport. A model for these results has been proposed. This suggests that NADP L-glutamate dehydrogenase plays a role in ammonium repression in addition to its catalytic function.

Aspergillus can utilise L-glutamate as a sole carbon source. Mutants, designated *gdhB1* - *B4*, unable to grow on glutamate as a sole carbon source and lacking NAD L-glutamate dehydrogenase activity, have been isolated. The *gdhB* mutants can still grow on ammonium or L-glutamate or other amino acids as sole nitrogen sources with glucose as the carbon source. NAD L-glutamate dehydrogenase activity is under repression by carbon metabolites, with glucose but not acetate, as a source of these metabolites. It may also be inducible but this is unclear at present. If induction plays a part in the control mechanism then it is probably subordinate to repression. A mutant which grows better than the wild type with glutamate as a nitrogen source has been isolated. This *gdhC1* mutant is partly insensitive to glucose repression of NAD L-glutamate dehydrogenase.

The *gdhC1* mutation is semi-dominant in the heterozygous diploid. Again a model has been proposed. This suggests that the *gdhC* locus codes for a regulatory product which has a positive effect on NAD L-glutamate dehydrogenase synthesis. The product of the *gdhC* locus is involved in repression by glucose or metabolites derived from glucose of NAD L-glutamate dehydrogenase.

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Section I Introduction

1. General remarks

The existence of many excellent reviews on the regulation of gene action including those of McFall and Maas (1967), Epstein and Beckwith (1968), Gross (1969), Clark (1971), Calvo and Fink (1971), indicates that a further comprehensive review would serve little purpose at this time. Therefore, I will emphasise only those areas of the literature which are directly relevant to the topics dealt with in this thesis with respect to the basic concepts of regulation in both procaryotes and eucaryotes.

2. Genetic regulatory systems in procaryotes

A great deal is known about the regulation of gene expression in bacteria in which the operon, as exemplified by the lactose operon in *Escherichia coli* is a functionally related group of genes. The group is transcribed as a complete unit from one end of the operon - the promoter - which is close to the first structural gene of the operon. The activities of the genes in the operon are controlled by a regulator molecule which is coded for by a gene which may be rather distant from the operon. This regulator molecule has allosteric properties and regulates the operon by attachment at an operator site which is next to the promoter site. The regulator molecule's affinity for the operator is lost when bound to its effector molecule. This basic model, proposed by Jacob and Monod (1961) has been supported by later biochemical studies. The product of the regulator gene (*i* gene) has since been isolated and shown to be a protein of molecular weight 200,000 (Gilbert and Muller-Hill, 1966). The lac operon is also repressed by glucose or a metabolite from glucose, a phenomenon known as catabolite repression (Magasanik, 1961). Recent results suggest that cyclic 3^1, 5^1 AMP plays a role in the initiation of transcription of the lac operon and is also involved in catabolite repression (Perlman and Pastan, 1968).

The essential feature of this model is the existence of a regulator gene, the product of which is an allosteric protein, which switches off the activity of the operon i.e. the control is negative. In addition to the negative action mechanisms there are positive control systems in which the operon is turned off and requires the presence of a protein effector to turn it on. The most novel feature of positive operons is the existence of recessive mutations which prevent the activity of all the genes of the operon. Perhaps the best studied is the arabinose system in Escherichia coli; a regulator gene C appears to code for a protein with both negative and positive action (Englesberg, Sheppard, Squires and Meronk, 1969). The regulator protein, in the absence of inducer, blocks gene expression, while it is modified and no longer has this negative action while the inducer is present. Instead, the protein has positive action.

Positively controlled operons have been reported in other inducible systems for the utilisation of rhamnose (Power, 1967) and maltose (Schwartz, 1967). Moreover, a positive effector may play a role in penicillinase production in Staphylococcus aureus (Richmond, 1967).

Positive control is also found in repressible systems. For instance, the repressible alkaline phosphatase system in E. coli

is reported to be controlled by a positive regulator, a product of the RI. gene (Echols, Garen, Garen and Torriani, 1961).

3. Genetic regulatory systems in eucaryotes

Although general principals of genetic regulatory mechanisms have been impressively well established for bacteria, our present state of knowledge of eucaryotic regulation is far from clear. The basic differences between procaryotes and eucaryotes, e.g. the arrangement of chromosomes within nuclear membranes and the random scattering of physiologically related genes throughout the eucaryotic genome may profoundly alter the nature of the mechanisms that regulate physiologically co-ordinated activity in eucaryotes.

Control of gene action studies in higher plants, e.g. maize (McClintock, 1965) and animals, e.g. human haemoglobin (Zuckerkandle, 1964) have met with little success due to the disadvantage that such organisms are not so amenable technically to genetic and biochemical analysis. Recently, the investigation of control phenomena in fungi has been intensified. Apart from the fact that fungal chromosomal arrangements and number are more akin to higher organisms than bacteria and therefore, models from such

fungal studies are more likely to be applicable to higher organisms, fungi offer many technical advantages which lend these organisms to detailed genetic studies. The dominance or recessivity of mutant alleles in heterokaryons and diploids may be ascertained with ease in certain fungi such as Aspergillus nidulans. In bacteria, the production of partial or complete diploids is usually more technically difficult.

In the limited number of investigations carried out in fungi, certain outstanding features emerge, e.g. there appears to be a lack of clustering of genes of related function compared with frequency of clustering in E. coli. The few 'examples' of clustering of genes of related function found in fungi are more likely to be genes coding for a single protein with multiple functions. In connection with this, the best example is the hist-3 locus in Neurospora crassa. Giles and his co-workers (Ahmed, Case and Giles, 1964), isolated polar mutants deficient in one or more of the three enzymes coded for by the hist-3 region. They postulated on the basis of complementation data that the hist-3 region coded for 3 separate enzymes and these were co-ordinately regulated. However, studies by Catcheside (1965) have suggested that the hist-3 gene is only one product. A similar sort of situation has been found in the cases of other reports of clustering, e.g. arom region in

Neurospora (Giles, Case, Partridge and Ahmed, 1967), and hi-4 regions in Saccharomyces (Fink, 1966). There are therefore major differences in the degree of clustering between bacteria and simple eucaryotes.

Another interesting feature which emerges is the resemblance of most of the fungal systems with the positive controlled arabinose system rather than with the negatively controlled lactose system.

One of the more informative control systems studied in fungi has been the enzymes involved in nitrate utilisation in Aspergillus nidulans (Pateman and Cove, 1967). The reduction of nitrate to ammonium, mediated by nitrite and hydroxylamine is catalysed by nitrate reductase and nitrite reductase which are induced by nitrate and repressed by ammonium. Control of the system by ammonium will be mentioned later (see page 13). Mutants have been isolated lacking either nitrate or nitrite reductase and map at the niaD and niiA loci respectively. These cistrons are probably about two units apart on linkage group VIII and although closely linked, the distance is large enough to suggest that an operon is not involved. The fact that attempts to isolate polar mutants have failed lends support to this contention. In addition,

two regulatory classes of mutants affecting the synthesis of nitrate and nitrite reductase were obtained by Pateman and Cove (1967). Firstly, semi-dominant constitutive mutants termed $\underline{nir}^{c}$, which synthesise nitrate and nitrite reductase in the absence of inducer. Secondly, recessive pleiotrophic mutants lacking both nitrate and nitrite reductase activities which also map at the nir locus and designated $\underline{nir}^{-}$ (formerly niiB). Pateman and Cove postulated that $\underline{nir}^{c}$ and $\underline{nir}^{-}$ were mutations in one gene which controls induction of the two enzymes, nitrate and nitrite reductase. The nir cistron maps at about 40 units away from niiA (Pateman, Rever and Cove, 1967). Pateman and Cove proposed a model involving a control system of the regulation of nitrate and nitrite reductase with a positive action. The most striking features of this system, and in general are typical of fungal regulatory systems, are that the genes are not clustered and the positive control type of regulation.

Further, semi-dominant constitutive mutants affecting the synthesis of xanthine dehydrogenase (Scazzocchio and Darlington, 1968) and amidases (Hynes and Pateman, 1970a, b) in *Aspergillus* have been found indicating further systems regulated by positive control.

Douglas and Hawthorne (1966) have studied the regulation of enzymes of galactose utilisation in yeast and proposed a control

system that is both positive and negative in action. The three enzymes, galactokinase, galactose-1-phosphate-UDP-glucose transferase and UDP-galactose epimerase, coded for by three closely linked genes ga 1, ga 7 and ga 10 respectively, are induced by galactose. Two types of constitutive mutants have been isolated; mutants at gene i which were found to be recessive and ga-4 semi-dominant. They proposed that gene i codes for a repressor which exerts negative control on ga-4 which in turn codes for a regulator molecule. This regulator molecule controls the synthesis of the three galactose enzymes in a positive fashion.

Metzenberg and his associates (Metzenberg and Parson, 1966, Marzluf and Metzenberg, 1968) report that there are two genes which regulate the three enzymes of sulphur metabolism in Neurospora crassa. These are cys-3 and eth-1^r. They propose that cys-3 exerts positive control while eth-1^r negative.

The regulation of the three enzymes of leucine synthesis i.e. β-isopropylmalate dehydrogenase, α-isopropylmalate isomerase and α-isopropylmalate synthetase coded for by leu-1, leu-2 and leu-4 respectively, may also be by positive as well as negative control mechanisms. Gross (1965, 1969) proposed that the product of a fourth gene, leu-3, a regulator protein, exerts negative and positive control.

Of particular importance to this discussion are the genes, the products of which have both regulatory and catalytic activity. This type of control appears to be relatively frequent in fungi. Adenylosuccinate synthetase in Saccharomyces cerevisiae is an instance of a system which appears to have combined regulatory and catalytic functions (Dorfman, 1969). Other systems which may have a dual role are ornithine transcarbamylase (Wiame, 1969; Bechet, Grenson and Wiame, 1970), iso-2 cytochrome c (Slonimski, Acher, Pere, Sels and Somlo, 1965) and hypoxanthine phosphoribosyl transferase (Lomax and Woods, 1970) in yeast and nitrate reductase in Aspergillus nidulans (Cove and Pateman, 1969).

It will be evident from these studies that the control of gene action in fungi does not conform with the relatively simple Jacob-Monod model. Moreover, in no case is the classic model of gene cluster and straightforward negative control applicable. Regulation in the fungi appears to be more complex than in procaryotes. Clues from experimentation are difficult to piece together to make a generalised theory since multiple control is often involved. Clearly, more research is required in this area to embellish our present knowledge of the significance of this fundamental difference with respect to gene regulatory mechanisms. This is the main aim of this thesis.

Systems which are important for intermediary metabolism were chosen for regulatory studies since models derived from such studies are more likely to be applicable to higher organisms than ones from studies of peripheral systems, such as nitrate reductase, etc., which are in most cases, not possessed by higher organisms. Such systems which would likely fulfil such a requirement include one involved in L-glutamate metabolism since L-glutamate is a key branch point between carbon and nitrogen metabolism. A number of metabolites, e.g. amino acids, purines and secondary metabolites are synthesised directly or indirectly from L-glutamate. Conversely, a number of amino acids are likely to be broken down via L-glutamate to carbon skeletons under conditions of carbon deficiency. The systems involved in L-glutamate metabolism chosen for regulatory studies are : L-glutamate transport, NADP L-glutamate dehydrogenase (E.C.1.4.1.4), and NAD L-glutamate dehydrogenase (E.C.1.4.1.2). These systems will now be briefly discussed individually.

4. The regulation of L-glutamate transport

The regulation of metabolism not only demands the control of the biosynthesis and degradation of amino acids, etc., but also control of their movement through the cell membrane. Indeed, a number of transport systems in eucaryotes have been found to be regulated by small molecular weight metabolites. For instance, ammonium regulates purine (Darlington and Scazzocchio, 1967), urea (Dunn and Pateman, 1972) and ammonium transport (Pateman, Dunn and Kinghorn, 1973) in *Aspergillus nidulans*; the general uptake system in yeast (Grenson, Hou and Crabeel, 1970) and in *N. crassa* (Sanchez, Martinez and Mora, 1972). In *P. chrysogenum* it has been shown that the specific system for L-methionine transport (Benko, Wood and Segel, 1967) and L-cystine (Skye and Segel, 1970) are regulated by sulphur. Moreover, a number of amino acid transport systems appear to be regulated by feedback inhibition, e.g. histidine uptake in yeast (Crabeel and Grenson, 1970). Evidence is presented in this thesis that there is a specific active transport system for L-glutamate and L-aspartate in *Aspergillus* and this is regulated by ammonium repression. Furthermore, this repression is annulled when L-glutamate is required as a carbon source. In contrast with L-glutamate uptake it appears that the transport of certain other amino acids is not subject to ammonium repression.

5. NADP L-Glutamate dehydrogenase and ammonium control

In *Aspergillus nidulans* a number of enzyme and uptake systems are regulated by ammonium. The activities of these systems are extremely low when wild type cells are grown or kept in the presence of ammonium. This phenomenon is known as ammonium repression. Systems repressed by ammonium include nitrate reductase, nitrite reductase (Pateman and Cove, 1967), xanthine dehydrogenase (Scazzocchio and Darlington, 1968), adenine-guanine-hypoxanthine uptake system (Darlington and Scazzocchio, 1967), thiourea uptake (Dunn and Pateman, 1972), extracellular protease (Cohen, 1972), acetamidase and formamidase (Hynes and Pateman, 1970*a*,*b*). In addition to these systems data will be presented in this thesis to show that the L-glutamate transport system is under ammonium control as discussed. There is then a considerable number of unrelated systems under ammonium repression and this is rather analogous to catabolite repression.

A number of mutants have been isolated, insensitive to ammonium repression, in an attempt to explain some of the molecular features of ammonium control. Arst and Cove (1969) found that mutation in two loci *meaA* and *meaB* resulted in simultaneous

resistance to the toxic ammonium analogue, methylammonium, and derepression for many ammonium repressed systems. Cohen (1972) isolated a mutant xprD1 that was derepressed for extracellular protease release and other systems. Pateman (personal communication) has isolated other classes of derepressed mutants DER-3 and amrA1. The results presented in this thesis show that some of these mutants are also derepressed for the L-glutamate uptake system and asparaginase.

Grenson and Hou (1972) have described a mutant in Saccharomyces cerevisiae that lacks NADP L-glutamate dehydrogenase activity and is abnormal with respect to the regulation by ammonium of the general amino acid permease. In this thesis it is reported that mutants lacking NADP-GDH are simultaneously ammonium derepressed for a number of enzyme and uptake systems including L-glutamate uptake. A regulatory role is suggested for NADP-GDH which accounts for most of the experimental observations. The results demonstrate the complexity of control systems in eucaryotic organisms.

6. NAD L-Glutamate dehydrogenase and catabolite repression

It has been known for over 30 years that the syntheses of certain enzymes by micro-organisms can be reduced by including glucose in the medium. For example, Klebsiella aerogenes synthesises histidine-ammonia lyase when grown on a medium containing glycerol as a carbon source. When the medium contains glucose, either instead of or in addition to glycerol, synthesis of this enzyme is completely prevented. This phenomenon was originally called the 'glucose effect' or 'glucose repression' but is now usually referred to as 'catabolite repression' (Magasanik, 1961). The lac operon (described above) is also subject to catabolic repression as well as lactose induction (Magasanik, 1961). The operation of this dual role most likely confers a competitive advantage on a micro-organism. A strain of E. coli with lactose induction only growing in the presence of glucose and lactose would synthesise β-galactosidase unnecessarily. Catabolite repression would prevent unnecessary synthesis of β-galactosidase by cells growing in such mixed carbon sources. The nature of the repressing catabolite or effector is in general, poorly defined and seems to vary with each system. Although relatively well documented for bacteria, e.g. galactosidase (Cohn and Monod, 1953), histidase (Neidhardt and Magasanik, 1956, 1957),

inositol dehydrogenase (Magasanik, 1961) and certain systems responsible for amino acid oxidation (Jacoby, 1964), there are few known instances of catabolite repression in eucaryotes. Perhaps the best studied system is the glyoxylate pathway in *Neurospora crassa* (Flavell and Woodward, 1970*a*,*b*, 1971). Glyoxylate shunt enzymes, isocitrate lyase and malate synthase are repressed when grown on glucose but derepressed on acetate as sole carbon source.

In *Aspergillus nidulans* Hynes and Pateman (1970*a*,*b*) found that some compounds such as acetamide are capable of being utilised as a carbon and nitrogen source. The enzyme responsible for the utilisation of acetamide, acetamidase, is repressed by glucose or metabolic products derived from glucose. Cohen (manuscript in preparation) has shown that extracellular protease release is under catabolite repression. However these systems have additional complications in that they are also under ammonium control.

The results presented in this thesis suggest that NAD L-glutamate dehydrogenase (NAD-GDH) is under catabolite repression, but unlike the protease and acetamidase, NAD-GDH seems to be free from ammonium control. NAD-GDH activity is maximal in wild type cells grown on glutamate or other amino acids as sole carbon source. This work suggested that the utilisation of glutamate as a carbon

source and the syntheses of NAD-GDH might constitute a suitable system for the investigation of catabolite repression. A priori, the main disadvantage would be that only one enzyme is likely to be involved in the pathway of utilisation rather than a number of different enzymes. Thus, regulation of only one structural gene could be studied. The chief advantage would be the possible regulation of enzyme synthesis by only repression and the fact that glutamate is a relatively poor carbon or nitrogen source, which might be useful for isolating control mutants.

The results have borne out some of these possibilities. A general outline of glutamate utilisation by *Aspergillus nidulans* has been established and a significant beginning made on the genetic analysis of the regulation of NAD-GDH synthesis.

Section II Materials and Methods

1. Media and supplements

(a) Media

The media used were based on those described by Pontecorvo, Roper, Hemmons, MacDonald and Bufton (1953).

Nitrogen and carbon less minimal medium (-CN medium)

nitrogen - less salts solution	20 ml
agar (for solid media)	12 gm

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

Nitrogen - less minimal medium (-N medium)

nitrogen - less salts solution	20 ml
D-glucose	10 gm
agar (for solid medium)	12 gm

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

Complete medium

D-glucose	10 gm
peptone	2 gm
casein hydrolysate	1.5 gm
yeast extract	1 gm
nitrogen - less salts solution	20 ml
vitamin solution	10 ml
agar (for solid medium)	12 gm
ammonium tartrate	10 mM

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

Nitrogen - less salts solution

potassium chloride (KCl)	26 gm
magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	26 gm
potassium dihydrogen phosphate (KH_2PO_4)	76 gm
trace element solution	50 ml

Made up to 1 litre with water. 2 ml chloroform added as a preservative and solution stored at 4^oC.

Trace elements solution

sodium borate ($Na_2B_4O_7.10H_2O$)	40 mg
copper sulphate ($Cu\ SO_4.5H_2O$)	400 mg
ferric orthophosphate ($Fe\ PO_4.1H_2O$)	800 mg
manganese sulphate ($Mn\ SO_4.4H_2O$)	800 mg
sodium molybdate ($Na\ Mo\ O_4.2H_2O$)	800 mg
zinc sulphate ($ZnSO_4.7H_2O$)	8 gm

Made up to 1 litre with distilled water.

Vitamin solution

P. aminobenzoic acid	40 mg
aneurin HCl	50 mg
biotin	1 mg
inositol	400 mg
nicotinic acid	100 mg
calcium D-pantothenate	200 mg
riboflavin	100 mg
pyridoxine	50 mg

Made up to 1 litre with distilled water.

(b) Supplements

These were kept as sterile concentrated equeous solutions at 4°C.

The appropriate amount of supplement was added to the medium before pouring.

Supplement	conc. of stock solution	amount per 100 ml medium	final conc.
(a) Vitamins			
biotin	40 γgm/ml	0.5 ml	0.2 γgm/ml
nicotinic acid	2000 γgm/ml	0.5 ml	10 γgm/ml
pyrodoxine HCl	1000 γgm/ml	0.5 ml	5 γgm/ml
riboflavin	200 γgm/ml	0.5 ml	.1 γgm/ml
P-aminobenzoic acid	1 mM	0.5 ml	.005 mM
(b) Toxic agents			
acriflavin	0.5%	1 ml	.005%
P-fluorophenyl-alanine	1%	2.5 ml	.025%
D-serine	1M	0.5 ml	5 mM
aspartate hydroxamate	16 mM	2 ml	0.32 mM
methylammonium	-	6.75 gm	1 M
thiourea	1M	1 ml	10 mM

chlorate	-	1.2 gm	100 mM
bromate	-	gm	100 mM
1 3 amino-tyrosine HCl	1M	1 ml	10 mM
glutamate hydroxamate	1M	0.5 ml	5 mM
azaguanine	-	10 gm	100 mg/ml
2.4 dinitrophenol	100 mM	1 ml	1 mM
sodium azide	100 mM	1 ml	1 mM
acridine yellow	-	6 mg	0.06 mg/ml
2-thioxanthine	100 mM	0.5 ml	0.5 mM
actidione	-	0.2 mg	0.2 µgm/ml

(c) Nitrogen sources

nitrate	1M	as specified in text
nitrite	1M	"
ammonia (ammonium tartrate)	1M	"
L-sodium glutamate	1M	"
L-sodium aspartate	1M	"
L-alanine	1M	"
L-arginine	1M	"
L-methionine	1M	"
L-lysine HCl	1M	"
L-phenylalanine	1M	"
L-proline	1M	"
L-cysteic acid	1M	"

(d) Others

sodium thiosulphate	0.2 M	1 ml	2 mM
putrescine	2000 γ/ml	1 ml	20 γ/ml
sodium deoxycholate	16%	0.5 ml	0.08%
sodium acetate	50%	2.5 ml	1.25%
galactose	20%	2.5 ml	0.5%
lactose	20%	2.5 ml	0.5%

(e) 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.

(f) Chemicals

Analytical grade chemicals were used whenever possible. ^{14}C-L-glutamate, ^{14}C-thiourea, ^{14}C-L-serine, ^{14}C-L-phenylalanine, ^{14}C-methylammonium, ^{14}C-L-arginine, ^{14}C-L-alanine and ^{14}C-L-arginine, were obtained from the Radiochemical Centre, Amersham.

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 on plates containing complete medium (solid) for 14-20 days. Conidia were scraped off the surface and resuspended in 10 ml of sterile distilled water. A few drops of Tween 80 were added and clumps of conidia separated by vigorous shaking. 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 3 ml of thick conidial suspensions were added to each flask. The mycelium was grown at 30°C in a Gallenkamp orbital incubator. After approximately 18 h 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 5 - 9 gm/l. The mycelium was quickly frozen and stored at -20°C for not more than 7 days until required for extraction. When the mycelium was required for amino acid pool analysis, it was used immediately after harvesting. The

glutamate dehydrogenase enzymes showed little sign of instability to cold storage.

When mycelium was pregrown before transfer to a treatment medium (carbon free, nitrogen free, etc), it was grown on glucose - 10 mM ammonium medium for 18 hr, 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 for amino acid pool analysis or/and frozen as above.

(b) Preparation of cell-free extracts

0.5 gm of frozen pressed mycelium was ground in a cold mortar with approximately 0.1 gm cold sand (M & B acid washed sand - medium fine) and 5 ml of cold buffer (0.05 phosphate buffer + 1 mM ethylenediaminetetraacetic acid (EDTA), + 1 mM mercaptoethanol, pH 7.75) for 2 min. The slurry was centrifuged for 15 min at 15,000 g at 4°C in an MSE Superspeed 65 refrigerated centrifuge. The supernatant was used for protein determinations and enzyme assays.

(c) <u>L-glutamate dehydrogenase assay</u>

NAD-GDH and NADP-GDH were assayed following the reductive amination of α-oxoglutarate in the presence of ammonium and reduced NAD or NADP. The initial reaction velocity was estimated from the change in optical density at 340 nm in a Unicam SP 800 spectrophotometer.

The reaction mixture for the NADP-GDH assay was : 0.4 M NH_4Cl in 0.05 M phosphate buffer pH 7.75, 0.4 ml; 0.2 M α-oxoglutarate in 0.05 M phosphate buffer pH 7.75, 0.2 ml; reduced NADP 2 mg/ml, 0.2 ml; 0.05 M 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 0.05 M phosphate buffer pH 8.0, 0.4 ml; 0.2 M α-oxoglutarate in 0.05 M phosphate buffer pH 8.0, 0.2 ml; reduced NAD 2 mg/ml, 0.2 ml; 0.05 M phosphate buffer pH 8.0 with 0.1 mM β-mercaptoethanol and 0.5 mM EDTA 2 ml. The assay temperature in both cases was 37°C and the results expressed as nanomole substrate transformed/min/mg protein.

(d) Nitrate reductase assay

The method used was that basically described by Nason and Evans (1953, 1955) and modified slightly by Cove (1966). Nitrite which is formed from nitrate by the catalytic action of nitrate reductase (E.C.1.6.6.3.), is determined colorimetrically. The results are expressed as nanomole substrate transformed/min/mg protein.

(e) L-Malate dehydrogenase assay

L-Malate : NAD oxidoreductase (E.C.1.1.1.37); trival name malate dehydrogenase, was assayed following the reduction of oxalacetate in the presence of oxalacetate and reduced NAD. The method was basically that of Munkres and Richards (1965). The reaction mixture was :
0.2 M oxalacetate in 0.05 M phosphate buffer pH 7.5, 0.2 ml; reduced NAD 2 mg/ml, 0.2 ml; 0.05 M phosphate buffer pH 7.5, 2.4 ml; enzyme extract 1 - 2 mg protein/ml, 0.2 ml. The assay temperature was 37°C and the results expressed in nanomoles substrate transformed/ml extract.

(f) Protein determination

All protein determinations were carried out by the procedure of Lowry, Rosebrough, Farr and Randall (1951). Serum albumin was used as a standard. The protein concentration of most extracts were in the range 1 - 2 mg/ml.

3. Amino acid transport assays

(a) Preparation of cells

Flasks were inoculated as above (page 25) and incubated at 25°C for 18 - 20 h in a New Brunswick Controlled Environmental orbital incubator. The nitrogen or carbon source is as specified in text. After this period of incubation, the cells were harvested on a nylon net cloth and washed twice with -CN medium, preheated to 25°C, and pressed dry with absorbent paper towels and weighed. As before, the pressed wet weight was usually in the range 5 - 9 gm/l. Finally, these cells were assayed. In certain cases it was necessary to treat the cells before harvesting and therefore the cells were resuspended in preheated (25°C) treatment medium (e.g. -N medium for nitrogen free treatment) for certain periods of time as specified in text. Where necessary, cells were repeatedly transferred to treatment medium to maintain the nitrogen and/or carbon concentrations close to the original value.

(b) Uptake assays

When grown under the 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 directly or after treatment, 1 gm of cells (pressed wet weight) was resuspended in a 250 ml Erlenmeyer flask in a shaking water bath at 25°C. The uptake flasks contained as follows :

uptake system under investigation	final concentration (^{12}C and ^{14}C)	approx. radioactivity
L-alanine	100 µM	1.5 µC
L-arginine	100 µM	1.5 µC
L-aspartate	100 µM	1.0 µC
L-glutamate	100 µM	0.5 µC
L-glutamine	200 µM	2.5 µC
L-phenylalanine	200 µM	2.0 µC
L-serine	100 µM	1.5 µC
methylammonium	500 µM	2.5 µC
thiourea	200 µM	2.5 µC

At 0, 2.5, 5.0, 7.5 and 10.0 min, 5 ml aliquots of the cell suspensions were filtered with two washes of water on a Millipore filter. The resultant pad of cells was weighed and transferred to 5 ml Bray's scintillation fluid and the radioactivity

measured in a Beckman Liquid Spectrometer. The rate of uptake of radioactivity into the cells is linear for all chemicals for the first 10 min. The uptake capacity of the cells is expressed as nmole of substrate taken up per wet weight cells except for the kinetic experiment, where dry weight was the standard used. This is found by taking half (by wet weight) of the pad after filtration and heating at 100°C in an oven overnight. The growth conditions and treatments used for transport experiments are specified in the text.

(c) Extraction and chromatography of accumulated ^{14}C L-glutamate

Following a 30 min incubation in 100 μM ^{14}C L-glutamate the cells were filtered, washed and pressed dry in absorbant towels. 1 gm wet weight of cells was suspended in 10 ml boiling water. After 30 min, 50 μl of the extract was spotted on to 3 MM Whatman chromatography paper (breadth 3 cm). The separation of L-glutamate from other amino acids was carried out by low voltage vertical ionophoresis; the method is basically that of Evered (1959). After ionophoresis for 1 h at 200 volts (current 30 mA) in tank buffer pH 1.9 (50 ml acetic acid and 25 ml 25% w/w formic acid) the

chromatogram was dried and cut into 1 cm sections (3 cm x 1 cm). The sections were transferred to vials containing 5 ml Bray's scintillation fluid and the radioactivity measured as before. The results are expressed as counts/min/section of chromatogram. A histogram was constructed to show the position of the counts.

4. Ammonium derepression and related tests

(a) Uptake tests for ammonium derepression

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

(1) presence of ammonium: Cells were grown on -N medium plus 0.15 casamino acids and 10 mM ammonium. After 18 hr growth cells were transferred as above to -N medium + 10 mM ammonium for 3 h.

(2) absence of ammonium: In this case, -N medium plus 0.15 casamino acids only was used for growth during the first 18 h. After this period the cells were transferred to -N medium (nitrogen free) for 3 h.

(b) Plate tests for ammonium derepression

Since *gdhA* mutants grow poorly on ammonium as a sole nitrogen source, the plate tests for derepression were modified by adding 10 mM alanine to the test medium; *gdhA* mutants grow as wild type with alanine as sole nitrogen source.

(1) nitrate reductase: Ammonium by repressing nitrate reductase synthesis protects the wild type against chlorate toxicity; poor growth on -N medium plus 100 mM $KClO_3$, plus 10 mM ammonium, plus 10 mM alanine indicates derepression of nitrate reductase (Arst and Cove, 1969).

(2) nitrite reductase: Ammonium, by repressing nitrite reductase synthesis alleviates bromate toxicity, poor growth on -N medium plus 100 mM $KBrO_3$, plus 10 mM ammonium, plus 10 mM alanine indicates derepression of nitrite reductase (Arst and Cove, 1969).

(3) xanthine dehydrogenase: A green-spored wild type develops yellow conidia in the presence of 2-thioxanthine due to the action of xanthine dehydrogenase. Ammonium prevents the development of yellow conidia by repressing xanthine dehydrogenase. The production of yellow conidia by a genotypically green-spored strain grown on -N medium plus 0.5 mM 2-thioxanthine, plus 10 mM ammonium, plus 10 mM alanine indicates derepression of xanthine dehydrogenase (Arst and Cove, 1969).

(4) extracellular protease: Ammonium derepression of protease release is indicated by a milk clearing halo around a colony growing on turbid -N medium plus milk plus 10 mM ammonium plus 10 mM alanine (Cohen, 1972).

(5) urea uptake: Ammonium by repressing the urea uptake system protects the wild type against thiourea toxicity; poor growth on -N medium plus 5 mM thiourea plus 10 mM ammonium plus 10 mM alanine indicates derepression of thiourea uptake (Dunn and Pateman, 1972).

(6) purine uptake: 8-azaquanine is a toxic substrate of the adenine-quanine uptake system and its toxicity is partially relieved by ammonium (Darlington and Scazzocchio, 1967). Poor growth on -N medium plus 100 mg/ml 8-azaquanine plus 10 mM ammonium plus 10 mM alanine indicates derepression of the uptake system.

(7) asparaginase: Aspartate hydroxamate, an analogue of asparagine, is extremely toxic for A. nidulans. This toxicity is reversed by ammonium as well as asparagine. Protection may be gained by the regulation by ammonium of a system(s) responsible for asparagine utilisation. There are several systems at which ammonium repression may operate.

i. Asparagine transport. The data presented in figure 10 (page 71) tends to disprove this hypothesis.

ii. Asparaginase. Initial experiments (Kinghorn, unpublished results) show that asparaginase is repressed or inhibited by ammonium in A. nidulans. This suggests that aspartate

hydroxamate protection is gained by ammonium repression or inhibition of asparaginase. The situation may be rather similar to that found in Pseudomonas fluorescens where aspartic hydroxamate is a substrate of asparaginase (Degroot, 1960) - the products of the reaction being aspartate and hydroxylamine. This latter product is a very potent poison to A. nidulans (Kinghorn, unpublished work). Ammonium repression or inhibition of asparaginase stops the production of hydroxylamine. Therefore poor growth on -N medium + 0.32 mM aspartate hydroxamate + 10 mM ammonium plus 10 mM alanine indicates derepression of asparaginase.

(8) methylammonium resistance: 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 chloride and 10 mM L-arginine, methylammonium resistant strains grow well.

(9) methylammonium super-sensitivity: The wild type strain has an appreciable level of growth when a low concentration of methylammonium (50 mM) and 10 mM L-arginine is present. Methylammonium super-sensitive strains grow extremely poorly on this medium.

5. Cellular localization of NADP L-glutamate dehydrogenase

The method was essentially that described by Flavell and Woodward (1971). Wild type cells were grown as above in shake flask culture on -N medium + 10 mM ammonium at 30°C for 18 h. After harvesting as above (page 25) 2 gm of pressed wet weight of mycelium was ground gently with approximately 1 gm sand for 30 sec in a precooled mortar with 10 ml of a 0.8 M sucrose solution (pH 8.0) containing 1 mM EDTA. The slurry was centrifuged lightly at approx. 1000 - 1500 g for 10 min at 4°C. The supernatant fraction was carefully pipetted off and recentrifuged at 20,000 g for 60 min. The supernatant was carefully withdrawn and examined for enzyme activity as the soluble fraction. The resulting pellet was resuspended in 0.05 M phosphate buffer, pH 7.75 and subjected to ultrasonic waves for 1 min from a 55 mm probe of a Mullard Ultrasonic Disintegrator. The ultrasonic probe and glass tube containing the resuspended pellet were intimately sealed so that they could be immersed in an ice/salt bath during the ultrasonic treatment. This was assayed as the mitochondrial fraction.

6. Preparation of cell-free extracts for free amino acid pool analysis

0.5 gm pressed mycelium of wild type cells grown as above was ground in a cold mortar with 10 ml 0.2 M phosphate buffer for 30 sec. 0.2 ml aliquots were taken and added to 5 mg sulphosalicylic acid in a small test tube and allowed to stand at 2 - 5°C with intermittent shaking to allow the protein fraction to precipitate (Mechanic, Efron and Shik, 1966). After 30 min the slurry was centrifuged for 15 min at 2°C at 10,000 g in a MSE Superspeed 65 refrigerated centrifuge. 0.2 ml of the supernatant was taken and transferred to ampules (Gallenkamp FM 520) and freeze dried.

The amino acid separation procedure was that of Spackman, Stein and Moore (1958) using a Locarte Amino Acid Analyser. This part of the work was carried out in collaboration with Dr. B. Wunner of the Virology Department, Glasgow University.

7. Electrophoretic studies of NADP L-glutamate dehydrogenase

Vertical starch-gel electrophoresis was carried out according to the method of Smithies (1955). Approximately 9.1 gm hydrolysed starch (Connaught Medical Laboratories, Toronto, Ontario) was dissolved in 100 ml .02 M borate buffer (pH 7.5). The buffer used in the electrode compartment was 0.15 M borate (pH 7.95). The protein content of the cell extracts were approximately 10 mg/ml. The wild type extract was diluted 1.4. Electrophoresis was carried out at 420 volts (15 mA) at 4 - 5°C for 16 hours. Straining for enzyme activity was carried out by transferring the gels into plastic sandwich dishes containing 10 ml of the following mixture (based on the method of Markert and Moller, 1959) :

0.05 M Tris-HCl buffer pH 8.0	50 ml
sodium L-glutamate	850 mg
NADP	10 mg
neotetrazolium chloride	15 mg
phenazine methosulphate	5 mg
0.5 M hydrazine (pH 7.5)	5 ml

The gel was incubated at 37°C with intermittent shaking. A band appeared after 10 min on the gel surface and this marked the position of NADP-GD^H.

8. Genetic techniques

(a) Strains: The wild type strain used in this study was a biotin auxotroph, bi1 (Glasgow No. 042) and a biotin and putrescine auxotroph bi1 puA2 (Glasgow No. 0171) both known to be translocation free. A multiply marked strain-master strain F (M.S.F. Glasgow No. 95) fpaD43, fpaD11 (Glasgow Nos. 090 and 091), p-fluorophenylalanine resistant, amino acid uptake mutants (Sinha, 1969) 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 and meaB6 (formerly meaA6) are methylammonium resistant, ammonium derepressed mutants (Arst and Cove, 1969) kindly supplied by Dr. H. N. Arst. DER3 is one of a series of ammonium derepressed mutants obtained by Professor J. A. Pateman selecting directly for ammonium derepression of nitrate reductase using a replica plating technique similar to that described by Pateman and Cove (1967). amrA1 was obtained by Professor J. A. Pateman selecting for mutants with poor growth on ammonium as a nitrogen source. xprD1 is an ammonium derepressed mutant obtained by selecting directly for ammonium derepression of extracellular protease production (Cohen, 1972) supplied by Dr. B. L. Cohen. uruA1 obtained from Mrs. Etta Dunn is a urea transport

mutant (Dunn and Pateman, 1972). ahyA1 ghyA1 and SER-9 are mutants resistant to aspartate hydroxamate, glutamate hydroxamate and D-serine respectively (Kinghorn, unpublished work. Figure 1 (page 50) shows a simplified linkage map of Aspergillus nidulans. An explanation of symbols and a complete linkage map are given by Clutterbuck and Cove (1973). Mutations located during this project and ones which affect regulatory systems and referred to in this thesis are marked in red.

(b) Naming of mutants: Three lower case letter symbols followed by capital letters have been used to designate loci (as in bacterial nomenclature - see Demerec, Adelberg, Clark and Hartman, 1966). The locus, mutation at which abolishes NADP L-glutamate dehydrogenase enzyme activity, has been called gdhA while the locus affecting NAD L-glutamate dehydrogenase, gdhB. The locus which affects carbon repression of NAD L-glutamate dehydrogenase has been designated gdhC. The amino acid uptake mutants are aauA, aauB, aauC and aauD.

This system of nomenclature follows that proposed for Aspergillus by Clutterbuck (1970).

(c) Isolation of mutants: N-methyl-N'nitro-nitrosoquanidine (NTG) was used as a mutagen for all mutational experiments (Adelberg, Mendel and Chen, 1965). A heavy conidial suspension was made up in

10 ml 100 mM tris-maleate buffer, pH 6.0 and shaken vigorously to break up clumps of conidia. 2.5 mg of the mutagen were added and the suspension was incubated for 30 min at 37°C. The suspension was lightly centrifuged and the pellet of precipitated conidia was resuspended in 10 ml of sterile distilled water. The suspension was again centrifuged and the conidial precipitate resuspended in 10 ml sterile distilled water. This procedure was repeated several times in an effort to dilute out N.T.G. Mutants abnormal with respect to NADP-GDH activity were selected by the method of Mackintosh and Pritchard (1963) while the NAD-GDH and amino acid uptake mutants by the method of Herman and Clutterbuck (1966).

(d) The method of Mackintosh and Pritchard (1963): This was used in the search for mutants unable to grow on high concentrations of ammonium as sole nitrogen source. The treated conidial suspension was counted and serial dilutions plated on to complete medium to determine viability. When viability was estimated the treated conidial suspension was spread on -N medium with biotin plus 2 mM urea, as sole nitrogen source. The plates contained 0.08% deoxycholate which reduces the size of the colonies and allows velvet replication to be carried out. Appropriate dilutions of the suspension were made in distilled water and spread over the surface

of the medium. 150 - 200 colonies per plate were used. The plates were incubated for 3 days and then velvet replicated to plates containing -N medium plus biotin plus deoxycholate plus 200 mM ammonium as sole nitrogen source. Colonies which grow on 2 mM urea but not 200 mM ammonium as sole nitrogen sources were picked off, purified and re-tested. Some of these proved to be mutants lacking NADP-GDH (Section IV).

(e) The method of Herman and Clutterbuck (1966): The above workers first observed that growth of bil puA2 (biotin and putrescine auxotroph) on minimal medium supplemented with biotin and only a limited concentration of putrescine (2.7×10^{-7}M) resulted in very compact and slow growth. In addition, from these minute compact colonies, faster growing sectors with a spidery growth form could be seen radiating out.

Such sectors were isolated and shown to be unable to utilise nitrate as a sole nitrogen source. It was concluded that an additional mutation had occurred resulting in the inability to use nitrate. Therefore at limiting putrescine concentration this inability results in a change from compact minute growth to spidery growth in a bil puA2 strain. Sinha and Clutterbuck (Hermann and Clutterbuck, 1966) also made use of this morphological phenomenon to isolate mutants unable to utilise lactose as a carbon source.

A successful attempt was made to isolate mutants lacking NAD L-glutamate dehydrogenase by the putrescine technique, using L-glutamate as a sole carbon and nitrogen source. NTG treated conidia were point inoculated onto -CN medium plus biotin plus limiting putrescine (9.4×10^{-7}M) plus 100 mM L-glutamate. After 3 - 4 days incubation at 37°C sectors showing spidery growth were isolated and purified. These (53 in number) proved to be strains unable to utilise L-glutamate as a sole carbon source. Later it was found that four of these strains lacked NAD-GDH (section V) and another seven were impaired in the transport of L-glutamate (section III).

Twenty-six mutants were also generated unable to utilise L-glutamate as a sole source of nitrogen on -N medium plus biotin plus limiting putrescine (8.1×10^{-7}M). No further work has been carried out to determine their physiological defect.

Rather gratuitously, during one of the experiments designed to generate mutants, unable to utilise L-glutamate as a nitrogen source, excess putrescine (8.1×10^{-3}M) was erroneously added. Although the bil puA2 strain grew as wild type on this, it was noticed that one of the colonies was significantly larger than the others. This exception was isolated, purified and re-tested

and consistently found to have better growth than the wild type. This proved to be a mutant which elaborates NAD-GDH under certain conditions in which the wild type does not. It was designated *gdhCl* (section V).

(f) *The isolation of temperature sensitive mutations in the gdhA gene*: Conidia of the *gdhA* mutant were treated with NTG (as above) and used to inoculate cool molten -N medium plus 200 mM ammonium. This mixture was used to pour agar plates. The plates were incubated at 25°C. Revertants were picked off after 3 days and sub-cultured on to fresh -N medium plus 200 mM ammonium agar plates and incubated at 37°C. Revertants which were unable to grow at 37°C were assayed for NADP-GDH at both temperatures.

(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 a further 8 - 14 days during which perithecia (sexual bodies) form.

Perithecia were isolated on to 3% minimal medium and cleaned from attached conidia and cell debri by rolling on the surface of the agar by prodding 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 $4^{\circ}C$.

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 heterokaryons: 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 transferred to similar plates of medium. This procedure was repeated until a heterokaryon, characterized by an equal mixture of conidial colours, was established.

(i) Production of diploids (Roper, 1952): Heterokaryons were established as described above. Dilute conidial suspensions were made from the heterokaryon and these were inoculated into molten minimal medium and plated out. On incubation, diploid colonies, characterized by light green conidia where the component strains were a yellow and a green strain, grew and were purified. Diploid strains were stored on minimal slopes.

(j) Haploidisation of diploids (Lhoas, 1961; McCully and 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-fluorophenylalanine. Acridine yellow was used where one of the component strains was p-fluorophenylalanine resistant. p-fluorophenylalanine and acridine yellow greatly increases the yield of haploid sectors. Haploid sectors were picked off after 5 - 7 days incubation, purified and growth tested.

The unknown mutant locus is then assigned to the linkage group of the marker to which it is apparently linked (Pontecorvo *et al.*, 1953). In all haploidisation tests the master strain used was M.S.F. This strain is marked as follows :

linkage group	I	II	III	IV	V	VI	VII	VIII
markers	yA2	AcraA1	galA1	pyroA4	facA3	sB3	nicB8	riboB2

Figure 1 : Linkage Map of *Aspergillus nidulans*

I aroC — 26 — trypB — 12 — adA — 10 — galD — 19 — SulA — 19 — riboA — 18 — anA — 7 — adG - - 9 — pycF — 4 — luA — 21 — stuA — 21 — proA — 6 — lusF — 5 — pabaA — 11 — uvsA — 4 — yA — 6 — biA — 40 — sgpO

- - 30 - aauC - - 24 - - - - ygA

II IodA — 36 — AcrA — 21 — methA — 4 — wA — 19 — palcA — 18 — riboE — 13 — drkB - - 50 - - - thiA — 34 — puA — 6 — abA — 12 — cnxE — 25 — rB — 29 — adC — 0.1 — adD — 24 — lacB

xprD - - 17 - - - - 26 - - - - 12 - - - - gdhA - - 23 - - -

- - - - 30 - - - - 16 - - - meaB

III alX — 32 — sorB — 32 — galE — 31 — adI — 20 — palcB — 17 — methH — 6 — argB — 23 — palA - - 14 - - galA — 5 — smA — 7 — ActA — 5 — phenA — 26 — suCpro — 21 — sC — 3 — sA — 23 — cnxH

amdT

gdhC

meaA - - 40 - - - mauA - - 11 - - - -

IV methC — 22 — hisA — 13 — frA — 28 — palC — 29 — pabaB — 10 — pyroA — 25 — apsA

amrA

gdhB

V nicA — 28 — v-1 — 15 — v-11 — 28 — facA — 14 — hxA — 34 — ribcD

VI sB — 35 — lysA   lacA — 29 — tsB — 0.4 — methB — 2 — apsB — 11 — nicC   pacC — 15 - - - uaX — 33 — molA

- - 5 - aauB - 24 - - -

VII wetA — 20 — malA — 14 — lysD — 13 — hisJ — 19 — chcA   palD — 17 — nicB - - - - 21 - v-10 — 12 — flA   drkA — 18 — phenB

aauA

VIII - - - tsD — 33 — sD — 7 — ornB — 18 — fwA — 10 — veA — 6 — facC — 26 — niiA — 2 — niaD - - 10 - - - brlA — 6 — cnxB (A, C) — 7 — uaY — 5 — fpaD — 4 — pyrD — 10 — palcC — 12 — argC — 15 — hisC — 10 — facB — 3 — riboB — 6 — trypC

aauD

Section III L-Glutamate transport in *Aspergillus nidulans*

1. Characteristics of L-glutamate transport

(a) Kinetics of L-glutamate transport

The effect of concentration on the rate of L-glutamate was investigated over the range of 1×10^{-5}M to 8×10^{-4}M as shown in figure 2.

A Lineweaver-Burk double reciprocal plot (Lineweaver and Burk, 1934) of the data was constructed (figure 3) and this shows that L-glutamate uptake follows Michaelis-Menten kinetics with a Michaelis Constant (Km) of 1.10×10^{-4}M and a maximum velocity of transport (Vmax) of 14 nanomoles/min/mg dry weight. The Km and Vmax values for L-aspartate uptake were found to be 1.30×10^{-4}M and 15.0 nanomoles/min/mg dry weight respectively.

Figure 2 : Influence of concentration on L-glutamate transport

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

V = nanomole/min/mg dry weight

S = 10^{-4}M L-glutamate

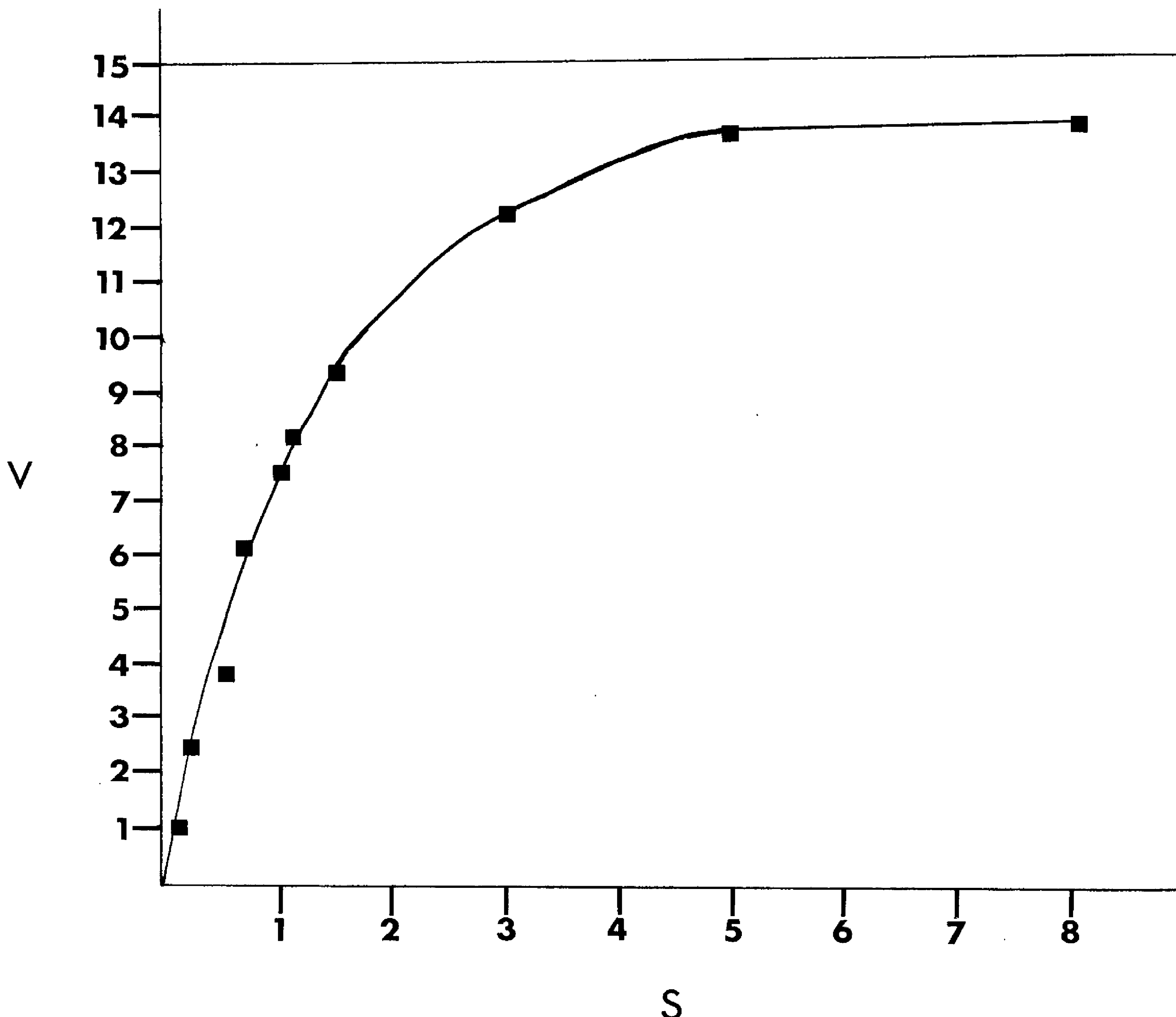

15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
V
1
2
3
4
5
6
7
8
S

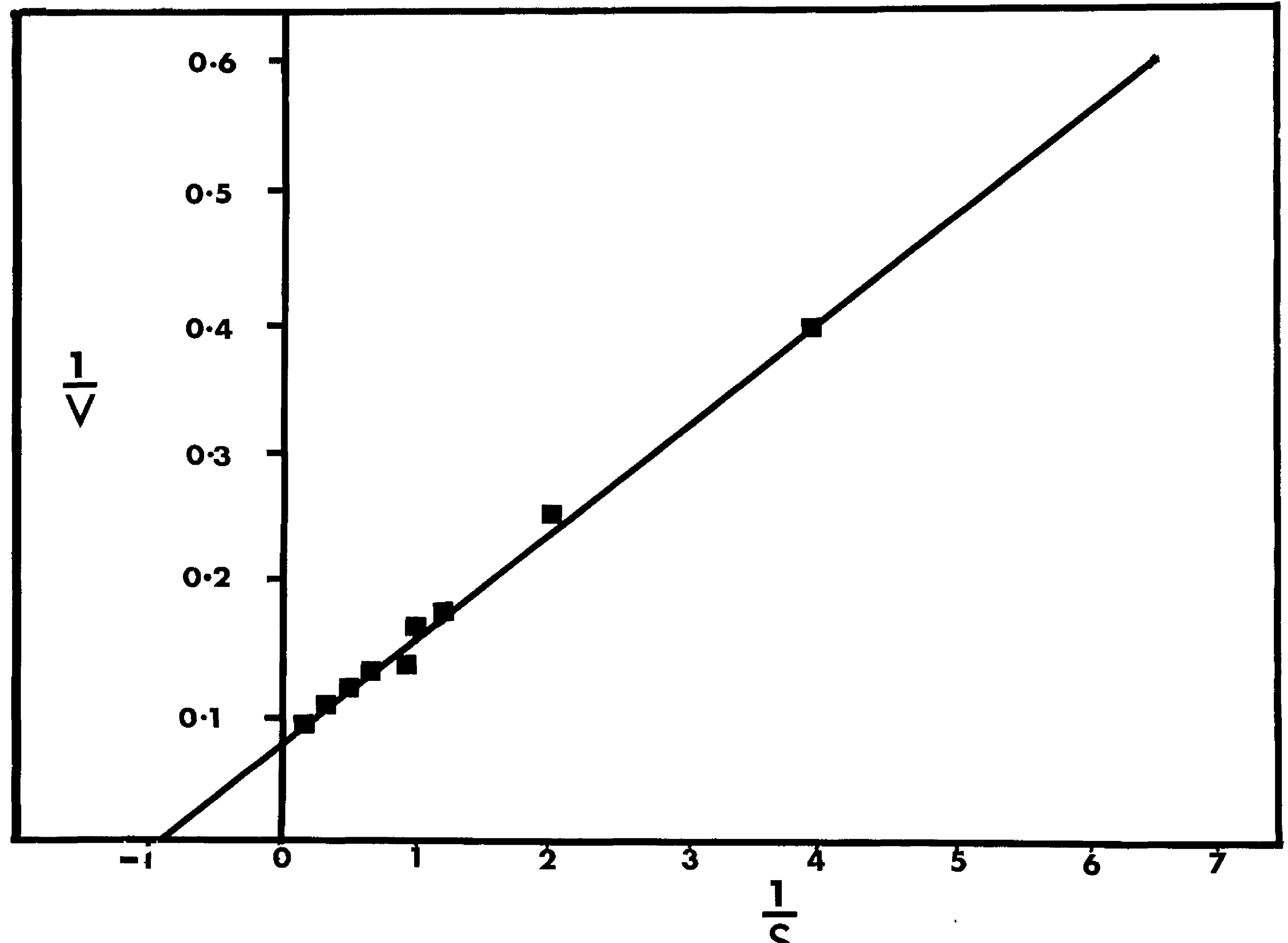
Figure 3 : Influence of concentration on L-glutamate transport (Lineweaver-Burk reciprocal plot)

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

V = nanomole/min/mg dry weight

S = 10^{-4}M L-glutamate


0·6
0·5
0·4
0·3
0·2
0·1
1/V
-1
0
1
2
3
4
5
6
7
1/S

(b) Competition data

The specificity of the L-glutamate transport system was investigated by determining the effect of a number of amino acids and inorganic nitrogen on L-glutamate transport (table 1). It can be seen that most of the substances tested had no significant effect on L-glutamate uptake. Only L-aspartate, L-cysteic acid and ammonium show significant inhibition.

A more detailed study was carried out in the case of the substances exerting an inhibition in order to determine whether this is competitive or non-competitive. The inhibition of L-glutamate by L-aspartate was measured under standard conditions. L-glutamate was used at levels ranging from 4×10^{-5}M to 8×10^{-4}M in the presence of 2×10^{-4}M and 6×10^{-4}M L-aspartate. Figure 4 shows a Lineweaver-Burk reciprocal plot of the results. It can be seen from this that there was a decrease in uptake as the concentration of L-aspartate was increased. The three lines intercepted at the same point on the Y-axis (1/Vmax) indicating that L-aspartate competed with L-glutamate for transport. The Ki value for L-aspartate was found to be 1.4×10^{-4}M.

Similarly, L-cysteic acid was found to be a competitive inhibitor of L-glutamate transport with a Ki value of 5.5×10^{-6}M. Conversely, L-glutamate and L-cysteic acid were found to be competitive inhibitors of L-aspartate uptake; the Ki's were 9.5×10^{-5}M and 5.0×10^{-5}M respectively.

No detectable L-cysteic acid uptake was recorded and it was therefore not possible to determine (a) the Km for L-cysteic acid uptake and (b) inhibition effects of other amino acids on L-cysteic acid uptake.

The results of Pateman (personal communication) show that ammonium is a non-competitive inhibitor of L-glutamate and L-aspartate uptake.

Table 1 : The effect of certain nitrogen sources on L-glutamate transport

nitrogen source added (2×10^{-3}M)	% inhibition
ammonium	35
L-aspartate	86
L-asparagine	10
L-cysteic acid	59
L-glutamate	90
L-glutamine	14
L-leucine	4
L-phenylalanine	2
L-proline	11
L-serine	3
L-tyrosine	9
L-valine	7
urea	5

Figure 4 : The effect of the presence of L-aspartate on L-glutamate transport

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

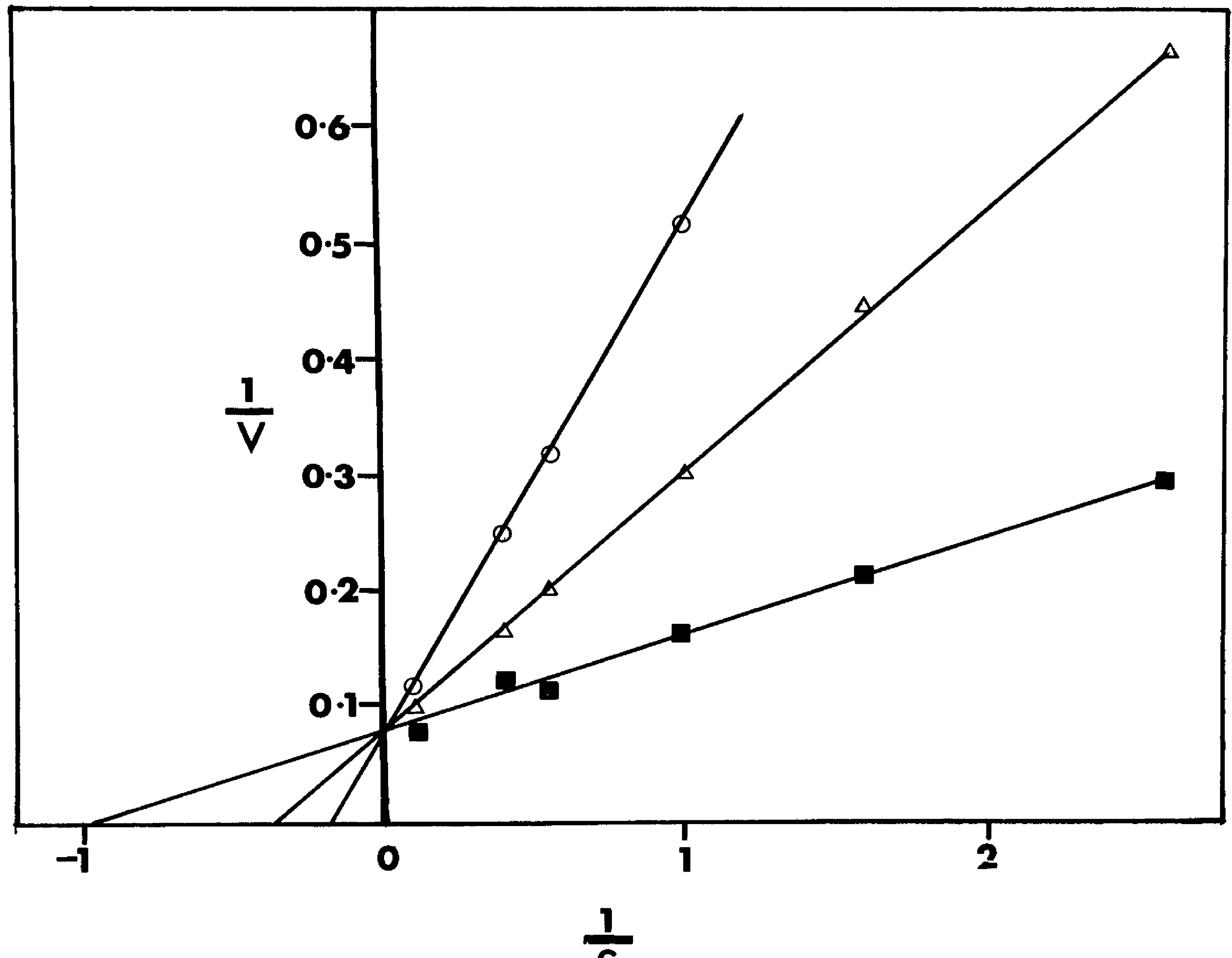
curve ■——■ L-aspartate absent

curve △——△ 2×10^{-4}M L-aspartate present

curve ○——○ 6×10^{-4}M L-aspartate present

V = nanomole/min/mg dry weight

S = 10^{-4}M L-glutamate


0·6
0·5
0·4
0·3
0·2
0·1
1/V
−1
0
1
2
1/S

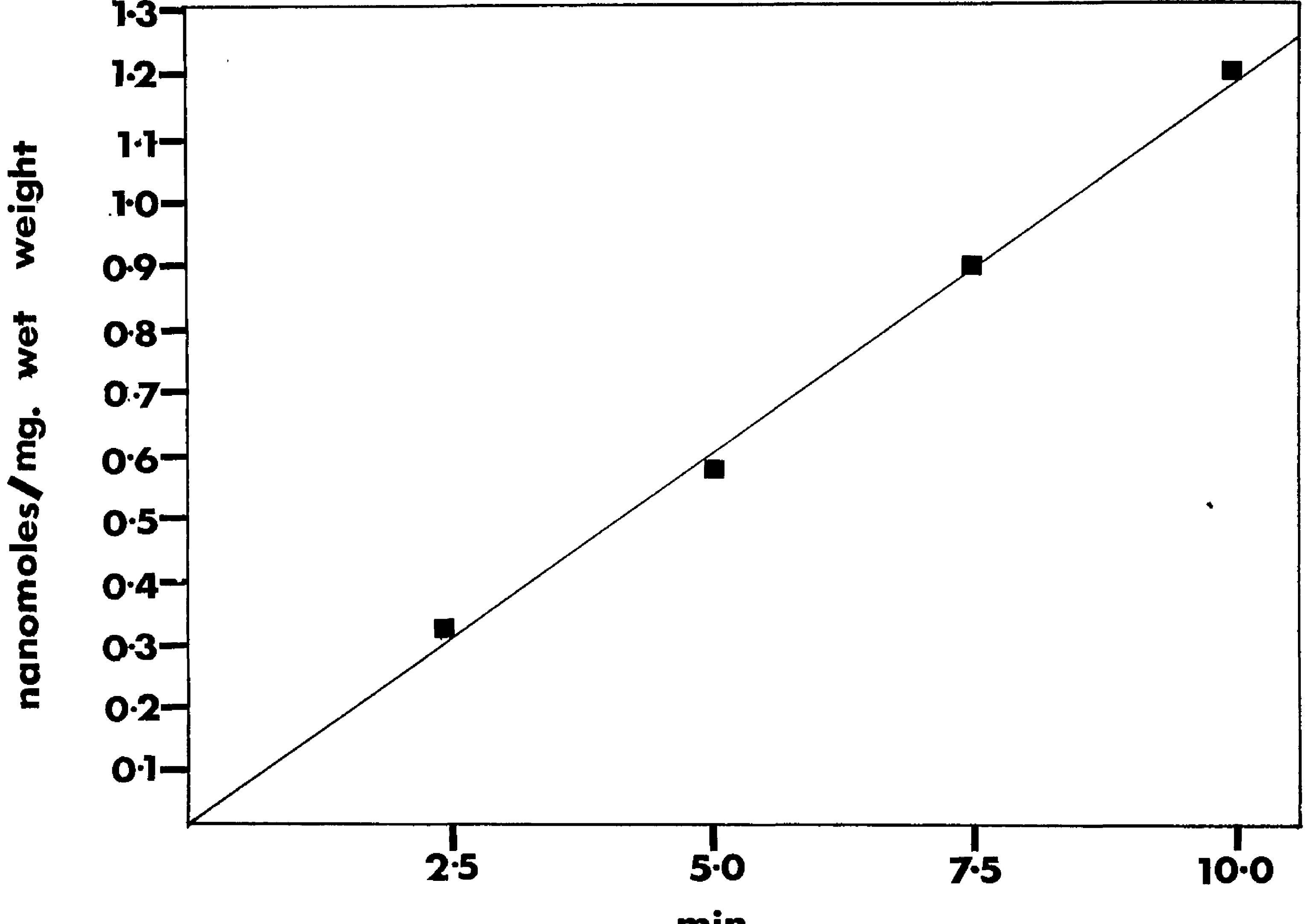
(c) Initial velocity of L-glutamate transport

The uptake of L-glutamate into wild type cells of *Aspergillus nidulans* was studied in young cells during the exponential growth phase. The kinetics of L-glutamate transport are linear for at least 10 min after the addition of cells to the test medium (figure 5). This test time was adhered to throughout the uptake experiments.

Figure 5 : Initial velocity of L-glutamate transport

Growth conditions :- -N medium + 10 mM urea

Treatment :- none



nanomoles/mg. wet weight
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min.

(d) Energy requirements of L-glutamate transport

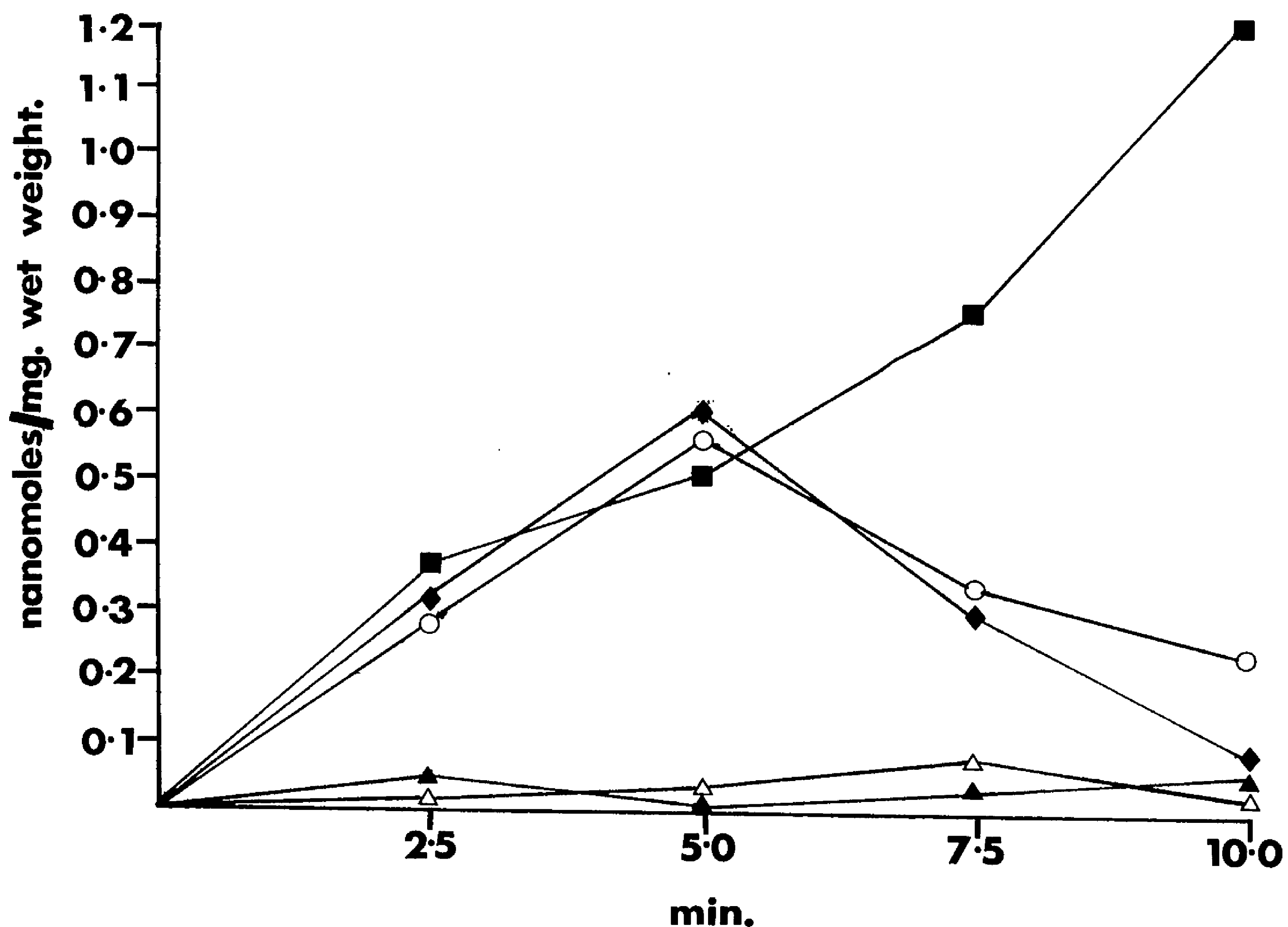
The data presented in figure 6 shows the effect of two metabolic uncoupling agents 2.4. dinitrophenol and sodium azide. Both toxic agents prevent the transport of L-glutamate when added at zero time. If the agents are added after 5 min incubation time, there is an immediate cessation of uptake. These results suggest (but do not prove) that energy is in some way required in the transport process.

Figure 6 : The effect of metabolic uncoupling agents on L-glutamate transport

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

curve	■——■	control
curve	▲——▲	2.4.dinitrophenol added at zero time.
curve	△——△	sodium azide added at zero time.
curve	○——○	2.4.dinitrophenol added after 5 min.
curve	◆——◆	sodium azide added after 5 min.



nanomoles/mg. wet weight.
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min.

(e) Recovery of accumulated intracellular L-glutamate

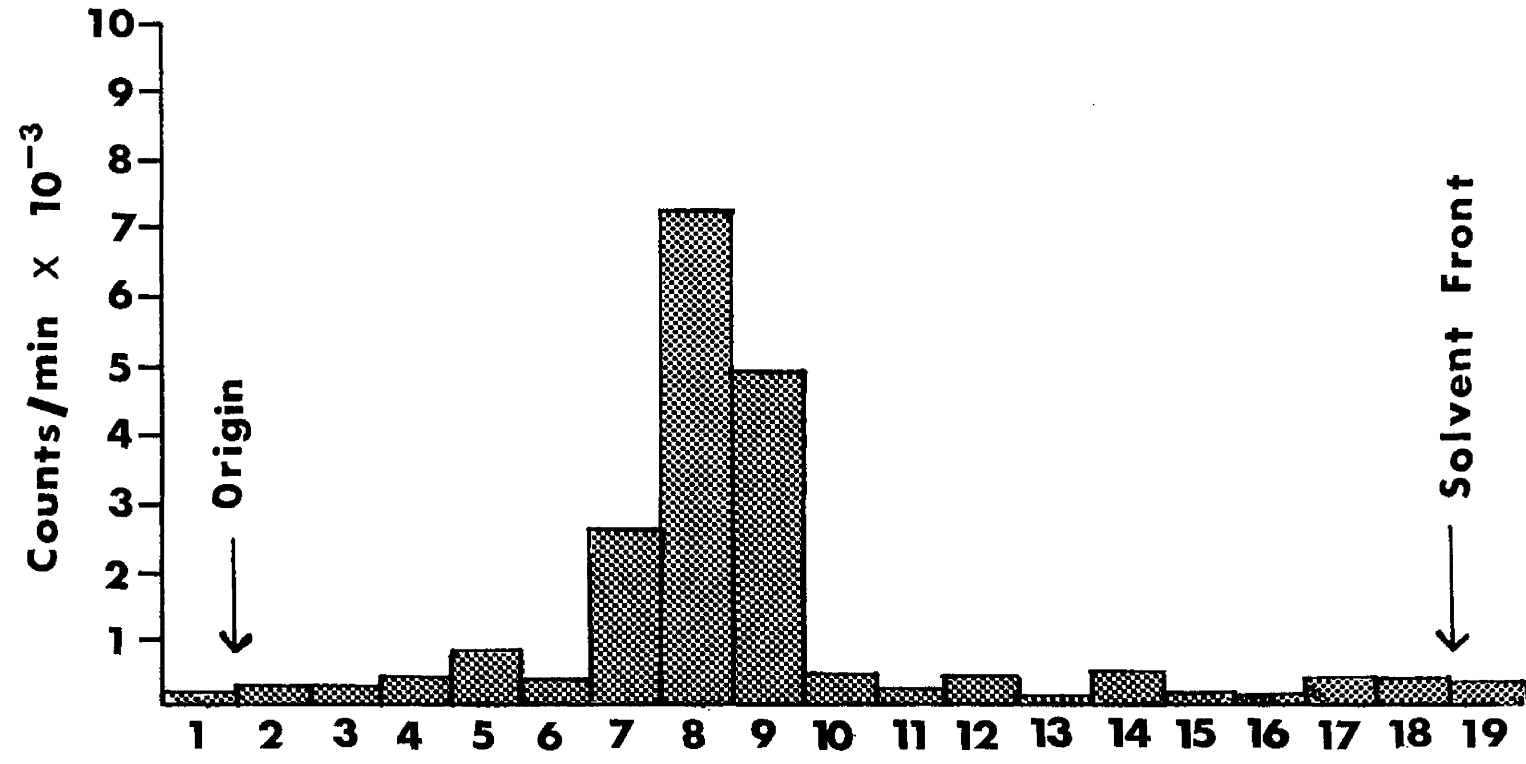
Hot water extracts of cells, which had been allowed to transport ^{14}C -L-glutamate for 30 min under the test conditions, were chromatographed. Only one radioactive spot was detected and this corresponded to L-glutamate (figure 7). The peak formed constituted approximately 90% of the total label extracted. Calculation of the intracellular concentration of L-glutamate was based on a mycelial wet weight of 1 gm pressed wet weight of cells per 1 ml water. Comparison of labelled L-glutamate concentration inside the cell to outside the cell indicated an approximate 30 fold increase of free L-glutamate inside the cells.

Figure 7 : Chromatogram of accumulated intracellular L-glutamate

Histogram representing 3 x 1 cm sections of the chromatograph.

Growth conditions :- -N medium + 10 mM urea

Treatment :- none


10
9
8
7
6
5
4
3
2
1
Counts/min X 10⁻³
Origin
Solvent Front
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Section of Chromatogram

2. Regulation of L-glutamate transport

(a) L-Glutamate transport in wild type cells grown under various nitrogen conditions

L-glutamate transport was studied in wild type cells grown on various nitrogen sources at a concentration of 10 mM (in the case of casamino acids the concentration was 0.15%) in an attempt to elucidate its regulatory patterns.

High levels of L-glutamate uptake were found in cells grown on -N medium plus one of the following nitrogen sources - L-glutamate, L-aspartate, L-alanine, L-arginine, L-asparagine, L-glutamine, L-ornithine, nitrate, urea or casamino acids (for clarity only one is shown in figure 8 (■—■)). In contrast, when ammonium was the sole nitrogen source there was a 20-fold decrease in L-glutamate uptake (O—O). However high levels of uptake were found in cells grown on ammonium and transferred to nitrogen free medium (-N medium) for 3 hours (△—△).

It was important to establish if low uptake was attributable to ammonium inhibition/repression or due to a secondary effect, e.g. lack of energy when ammonium is used as a sole nitrogen

source. Cells were grown on both ammonium and glutamate to gain evidence on this point. Figure 8 (△—△) shows that L-glutamate uptake is minimal under such conditions.

Figure 8 : L-glutamate transport in wild type cells grown on various nitrogen sources

curve ▣——▣ Growth conditions :- -N medium + 10 mM L-glutamate (or L-aspartate, or nitrate or L-asparaginine or L-glutamine or L-arginine or urea or 0.15% casamino acids.)

Treatment :- none

curve ○——○ Growth conditions :- -N medium + 10 mM ammonium

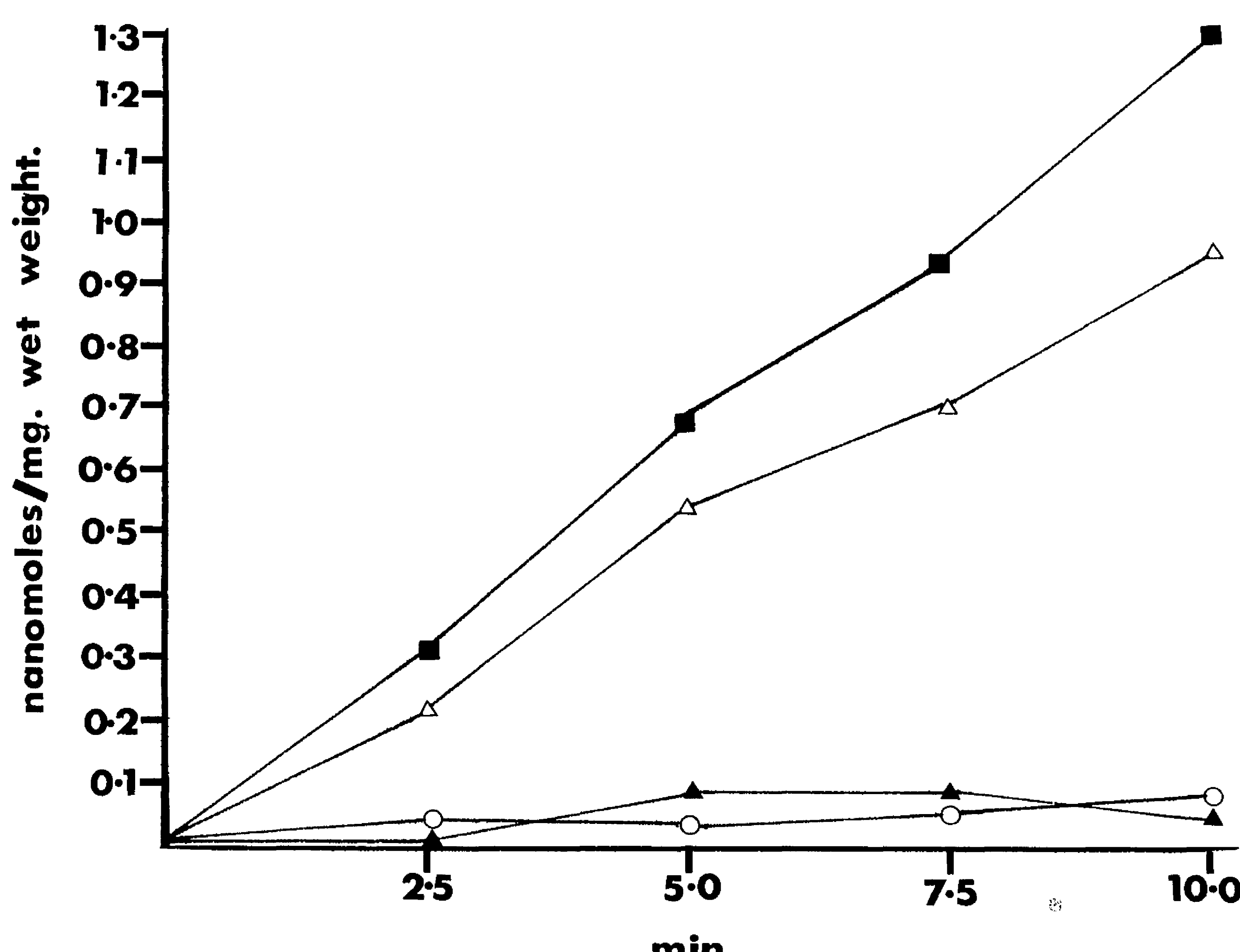
Treatment :- none

curve △——△ Growth conditions :- -N medium + 10 mM ammonium

Treatment :- -N medium for 3 h.

curve ▲——▲ Growth conditions :- -N medium + 10 mM L-glutamate + 10 mM ammonium

Treatment :- none



nanomoles/mg. wet weight.
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min

(b) L-glutamate transport in cells treated with various carbon conditions

The data presented in figure 9 shows that cells that have been deprived of carbon for 3 h have minimal L-glutamate uptake activity (▽—▽). If L-glutamate is used as a carbon source high levels of activities result (○—○). The addition of ammonium to L-glutamate as a carbon source (△—△) did not significantly influence the rate of transport. This is in contrast to the situation with glucose as the carbon source; the addition of ammonium results in low activity.

Figure 9 : <u>L-glutamate transport in cells treated under various carbon conditions</u>

curve ▽——▽ Growth conditions :- -N medium + 10 mM ammonium

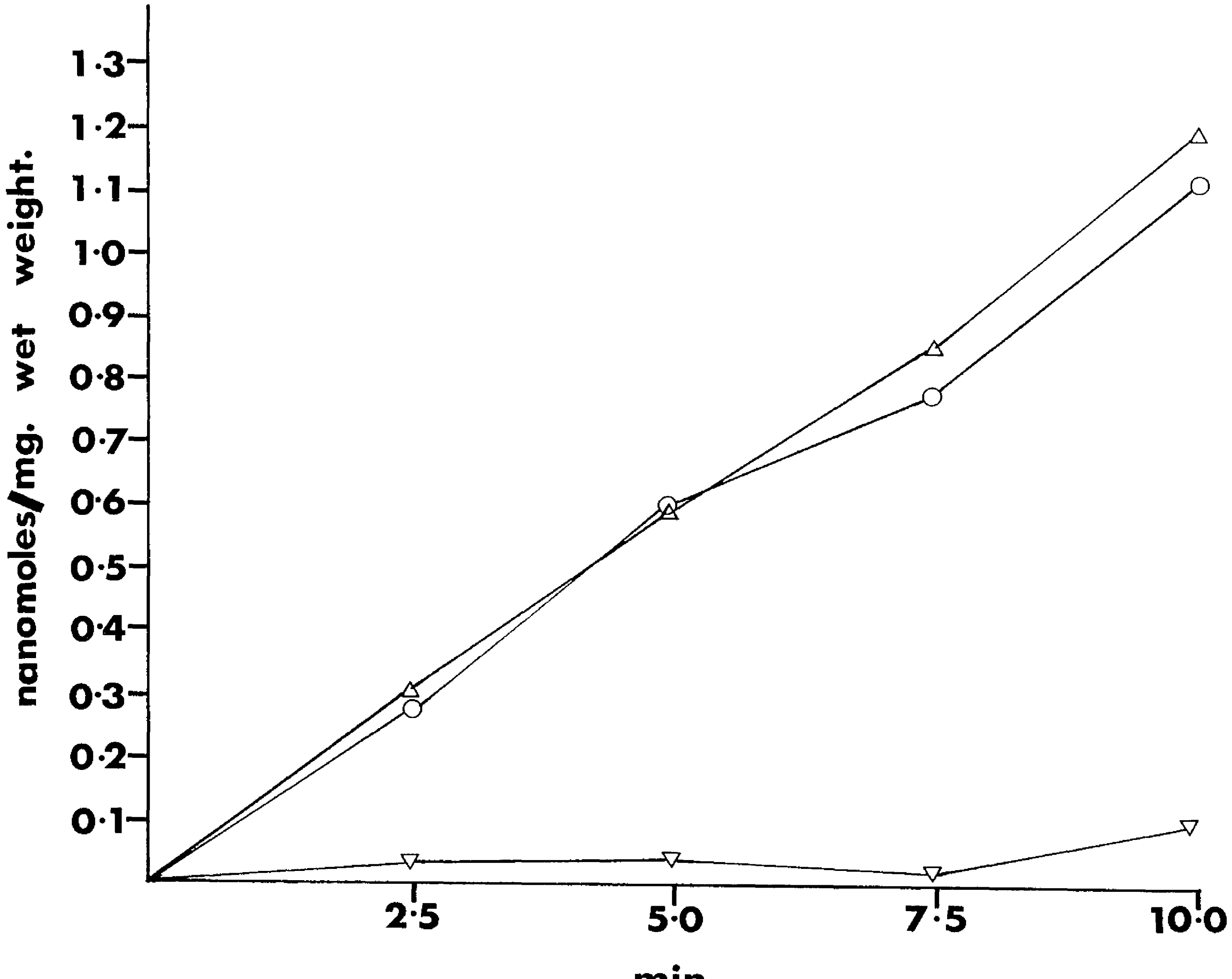
Treatment :- -CN medium for 3 hours

curve ○——○ Growth conditions :- -N medium + 10 mM ammonium

Treatment :- -CN medium + 100 mM L-glutamate for 3 hours

curve △——△ Growth conditions :- -N medium + 10 mM ammonium

Treatment :- -CN medium + 100 mM L-glutamate + 10 mM ammonium for 3 hours


nanomoles/mg. wet weight.
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min.

(c) Transport of other L-amino acids grown on ammonium as the sole nitrogen source

Since ammonium was found to profoundly influence the rate of uptake of L-glutamate, tests were carried out to investigate the possibility that other amino acid transport systems may be similarly affected by ammonium. Cells were grown under two nitrogen conditions, one ammonium and the other urea. It can be seen that L-aspartate uptake is minimal in cells grown on ammonium (figure 10). In contrast, and perhaps rather surprising in the light that general uptake systems are repressed or inhibited by ammonium in *N. crassa* and *S. cerevisiae*, L-phenylalanine and L-serine uptake are not affected by ammonium. Neither are L-alanine, L-asparagine, or L-glutamine (for clarity, not shown in figure 10).

The ineffectiveness of ammonium to inhibit or repress L-phenylalanine or L-serine is confirmed by toxicity reversal tests (table 2) in which it is shown that ammonium does not reverse p-fluorophenylalanine, 1,3-amino tyrosine or D-serine toxicity. Aspartic hydroxamate toxicity, an analogue of asparagine, is reversed by ammonium but this is not due to repression/inhibition of L-asparagine uptake, but probably to repression of asparaginase activity (see page 36).

Figure 10 : L-phenylalanine, L-serine and L-aspartate transport in the presence and absence of ammonium

curve ▣———▣ L-aspartate uptake

curve △———△ L-phenylalanine uptake

curve ▲———▲ L-serine uptake

curve 1 Growth conditions :- -N medium + 10 mM ammonium

Treatment :- none

curve 2 Growth conditions :- -N medium + 10 mM urea

Treatment :- none

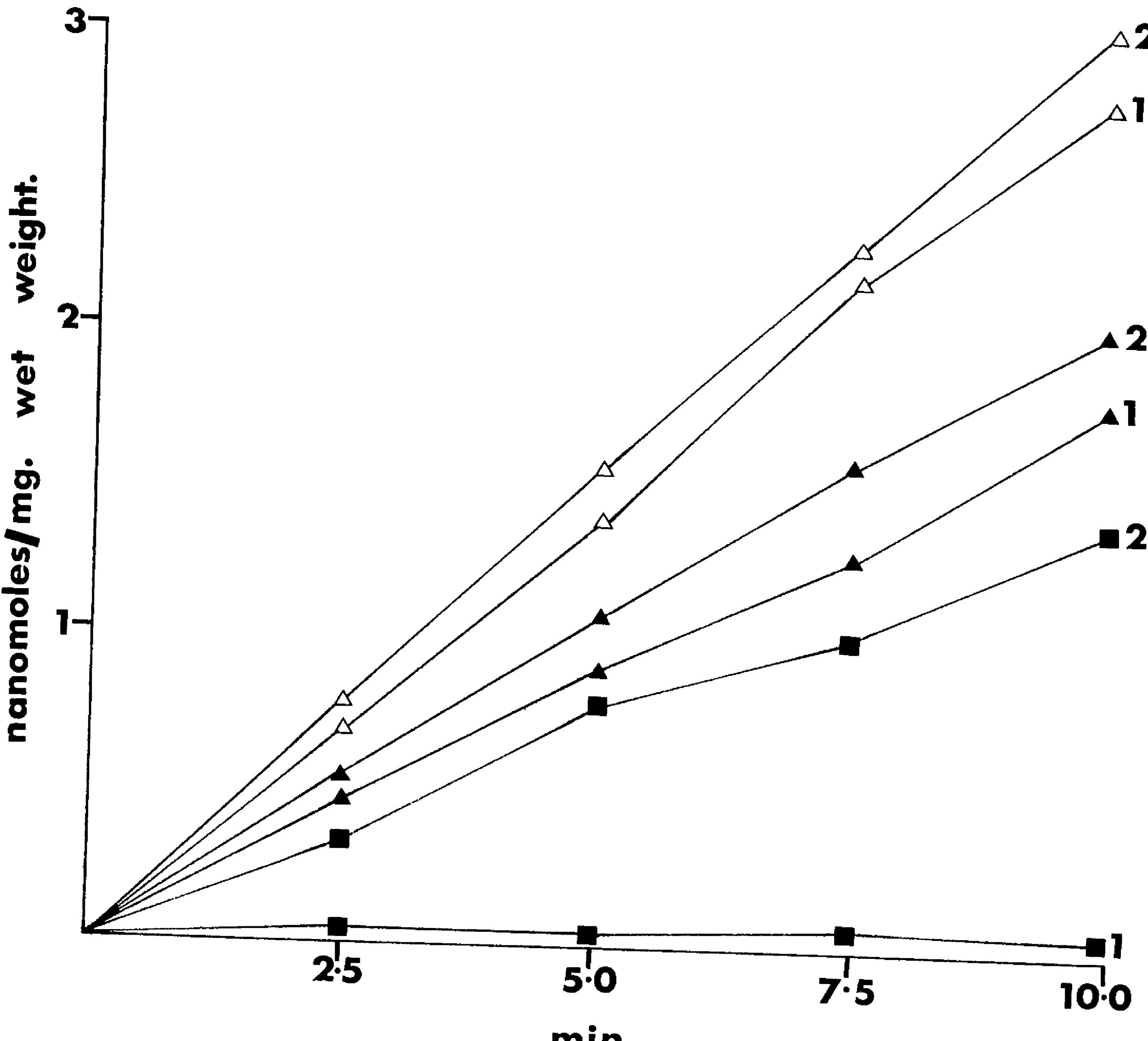

nanomoles/mg. wet weight.
3
2
1
2·5
5·0
7·5
10·0
min.
2
1
2
1
2
1

Table 2 : The reversal of certain amino acid analogue toxicities by ammonium

-N medium (solid) +	toxic analogue	Growth of wild type
10 mM nitrate	D-serine	-
10 mM ammonium	D-serine	-
10 mM L-serine	D-serine	+
10 mM nitrate	p-fluorophenylalanine	-
10 mM ammonium	p-fluorophenylalanine	-
10 mM L-phenylalanine	p-fluorophenylalanine	+
10 mM nitrate	aspartate hydroxamate	-
10 mM ammonium	aspartate hydroxamate	+
10 mM L-asparagine	aspartate hydroxamate	+
10 mM nitrate	1,3-amino tyrosine	-
10 mM ammonium	1,3-amino tyrosine	-
10 mM L-tyrosine	1,3-amino tyrosine	+

(d) L-glutamate transport by mutants insensitive to ammonium repression

As discussed in the introduction a number of mutants have been isolated which are insensitive to ammonium repression for a number of ammonium repressible systems. Experiments were carried out to investigate the possibility that such mutants are insensitive to ammonium control of the L-glutamate uptake system. Since several of the mutants cannot utilise certain compounds efficiently as sole nitrogen sources, cells in all cases, were grown on -N medium plus 0.15% casamino acids and 10 mM ammonium and transferred to -N medium plus 10 mM ammonium for 3 h. The results plotted in figure 11 show the L-glutamate uptake status of derepressed mutants grown on ammonium. It was also necessary to check the possibility that some of the ammonium derepressed mutants may have low L-glutamate uptake activity in the absence of ammonium. In this case, the cells were grown on -N medium plus 0.15% casamino acids and nitrogen starved for 3 h (figure 12).

The degree of ammonium derepression was expressed as a percentage; $\% \text{ derepression} = \frac{\text{activity on 10 mM ammonium}}{\text{activity on N free}} \times 100.$
From the computed percentages shown in table 3 it can be seen that

the degree of derepression for L-glutamate uptake varies for the different mutants. Taking the wild type percentage as being fully repressed (4%) gdhA1, meaA8, xprD1,amrA1 and DER-3 appear to be significantly derepressed while meaB6 remains repressed.

Another noteworthy point is that amrA1 has an impaired ability to transport L-glutamate in the absence of ammonium.

Figure 11 : L-glutamate transport by mutants insensitive to ammonium repression, grown in the presence of ammonium

Growth conditions and treatment as discussed in text.

curve	■———■	wild type
curve	●———●	*xprD1*
curve	▲———▲	*meaB6*
curve	○———○	*gdhA1*
curve	△———△	*meaA8*
curve	□———□	DER-3
curve	▽———▽	*amrA1*

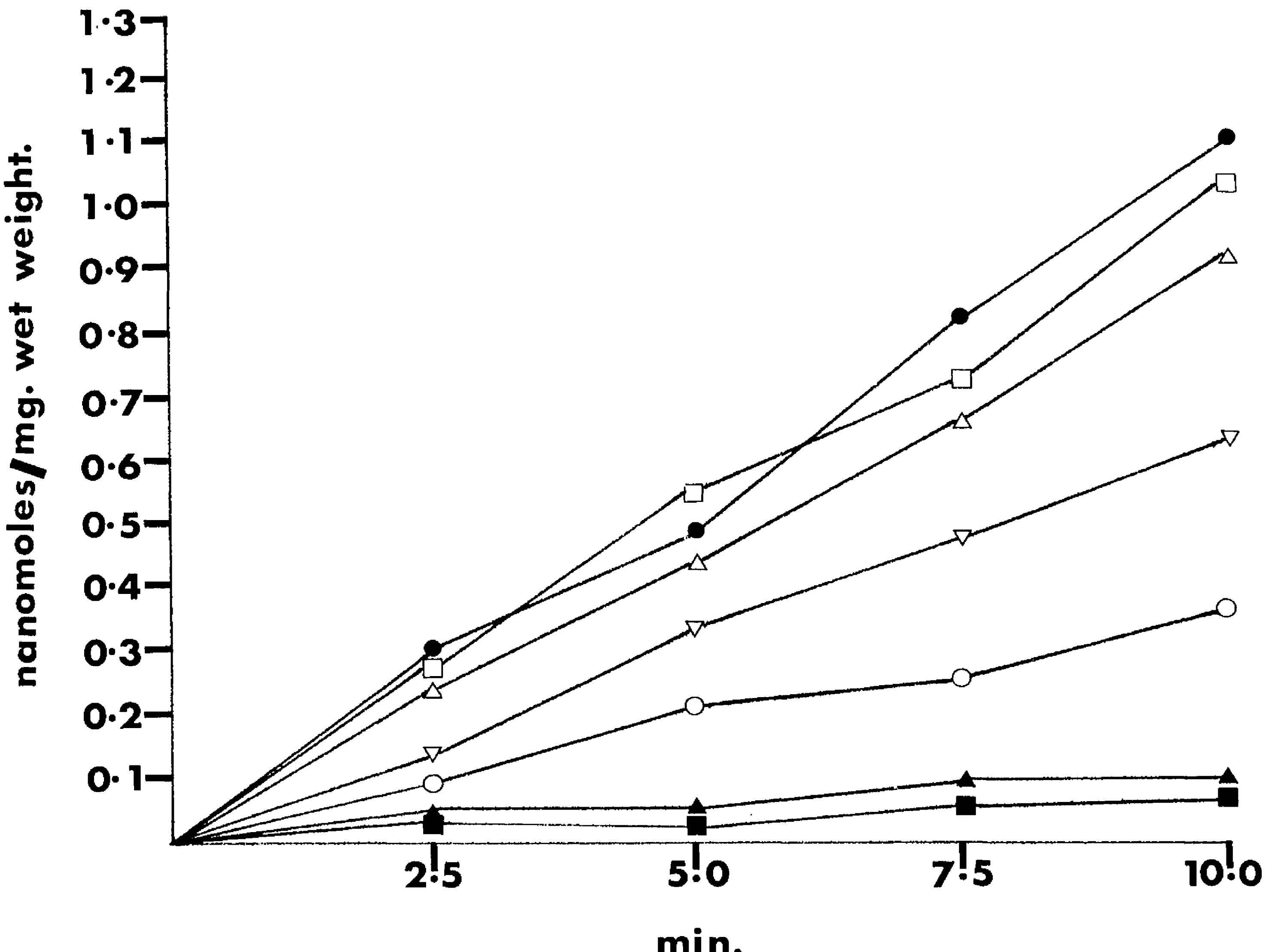

nanomoles/mg. wet weight.
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min.

Figure 12 : L-glutamate transport by mutants insensitive to ammonium repression grown in the absence of ammonium

Growth conditions and treatment as discussed in text.

curve	■——■	wild type
curve	●——●	xprD1
curve	▲——▲	meaB6
curve	○——○	gdhA1
curve	△——△	meaA8
curve	□——□	DER-3
curve	▽——▽	amrA1

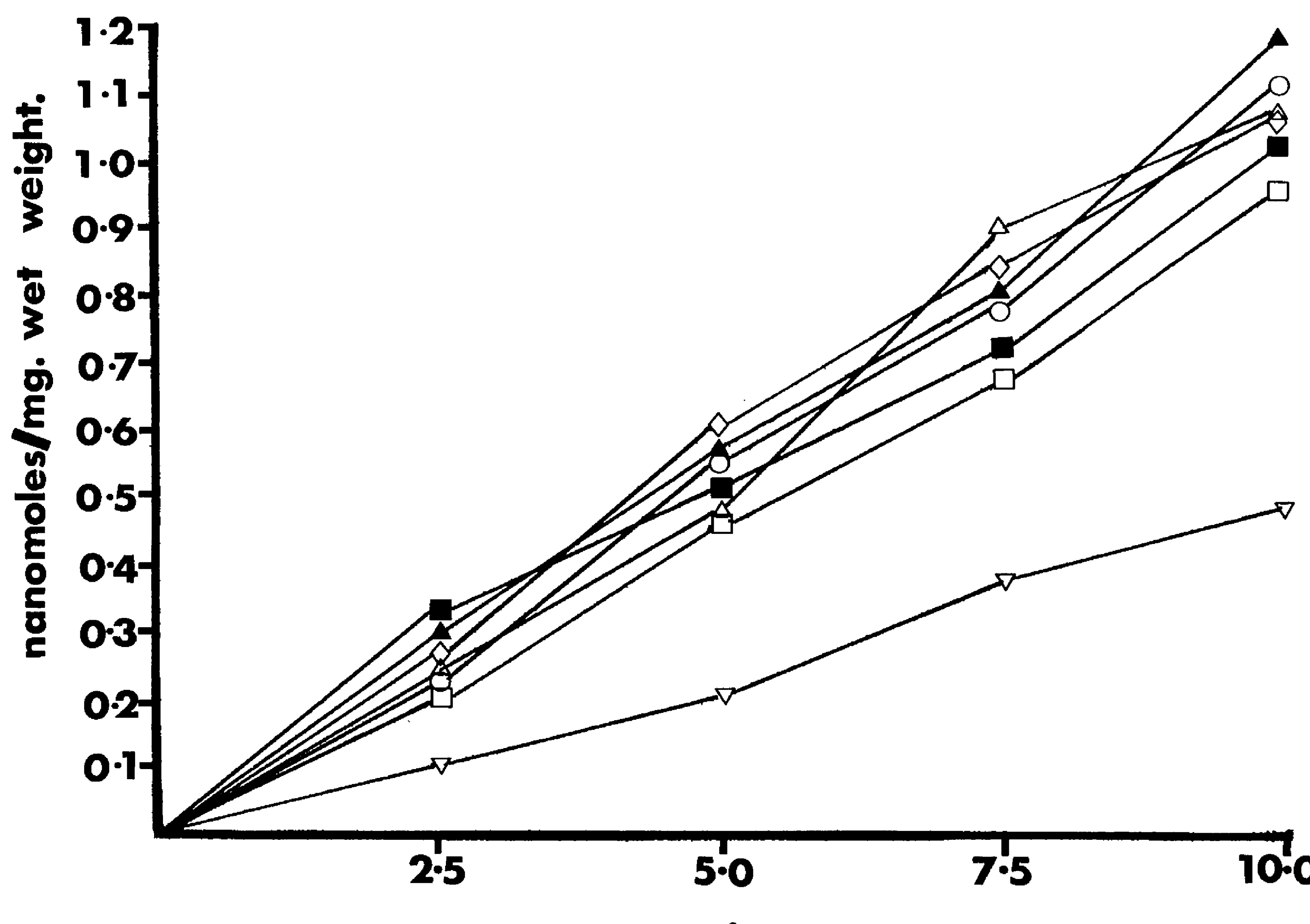

nanomoles/mg. wet weight.
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min.

Table 3 : Percentage derepression of L-glutamate transport shown by certain ammonium insensitive mutants

genotype	% derepression
wild type	4
gdhA1	32
meaA8	77
meaB6	7
DER-3	100
xprD1	100
amrA1	59

(e) The effect of actidione on L-glutamate transport

The results of the previous sub-sections show that L-glutamate uptake is minimal in cells grown on ammonium. Consequently, experiments were carried out in an attempt to investigate the nature of this effect. In particular, whether low activity is brought about by inhibition of already synthesised permease or by repressing protein synthesis.

The control (■——■) in figure 13 shows L-glutamate transport in wild type cells which have been grown on -N medium plus 10 mM ammonium and transferred to nitrogen free. The addition of actidione, an anti-metabolite, which stops protein synthesis in fungi (Segel and Johnston, 1963), prevents an increase in L-glutamate uptake.

Figure 13 : The effect of actidione on L-glutamate transport

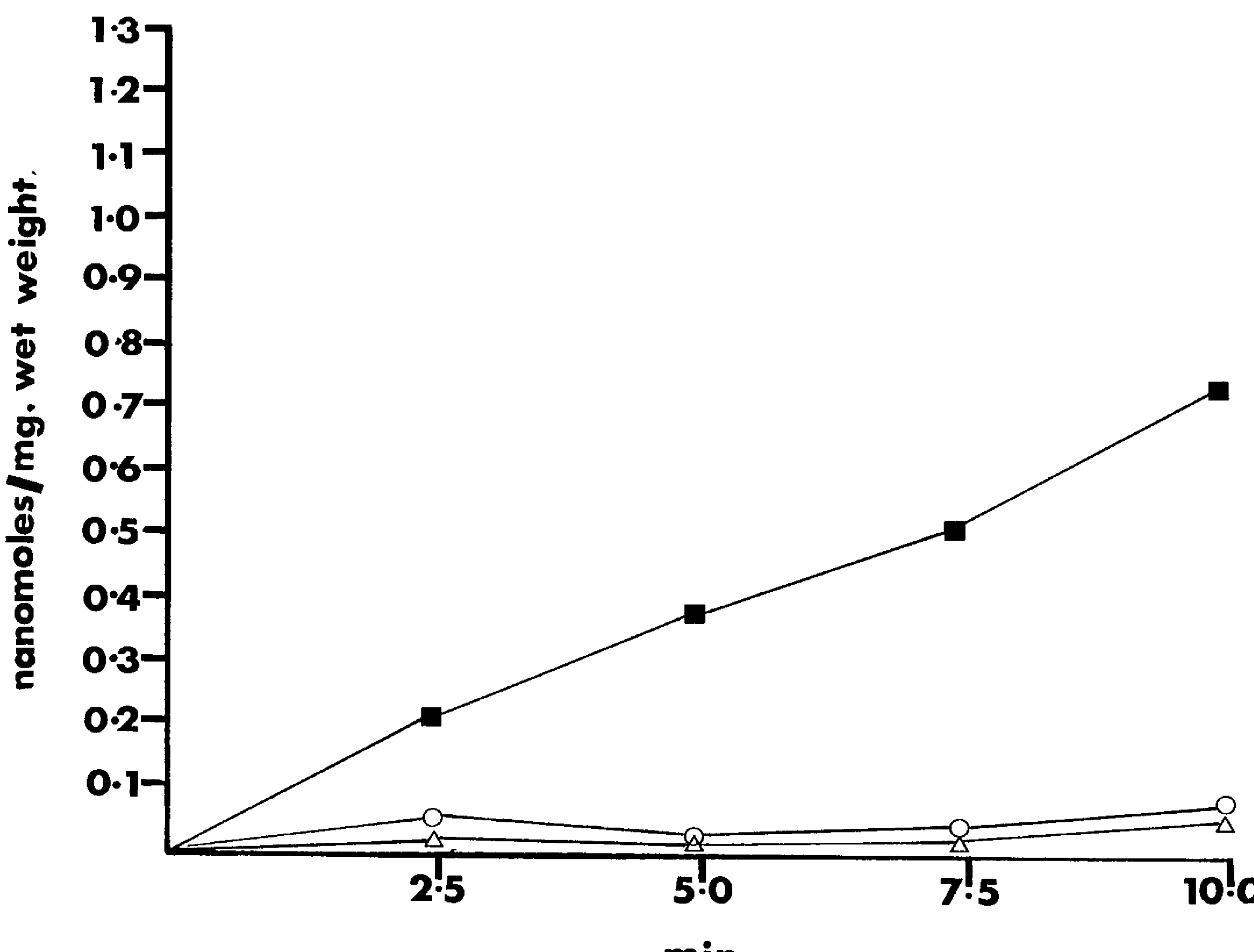
Growth conditions :- -N medium + 10 mM ammonium

Treatment :- -N medium for 3 h

curve ■——■ no actidione added (control)

curve ○——○ actidione added to treatment medium at time of transfer

curve △——△ actidione added to treatment medium at $1\frac{1}{2}$ h after transfer



nanomoles/mg. wet weight.
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min.

3. Mutations which affect L-glutamate transport

(a) Isolation and genetic characterisation of mutants

A large number of mutants, unable to utilise L-glutamate as a sole carbon and nitrogen source, were isolated by the putrescine technique of Herman and Clutterbuck (1966). Seven of these mutants were later found to have low uptake of L-glutamate (see page 98). Mitotic and meotic analyses revealed that there were no less than four loci involved. These loci were designated *aauA*, *aauB*, *aauC* and *aauD*. The genetic analysis of these will be treated separately.

aauA locus

By the technique of haploidisation of diploids it was found that one of the seven mutants assorts freely with all markers except *nicB* (table 4). Therefore, this mutant, later designated *aauA1* was assigned to linkage group VII.

Table 4 : Haploidisation analysis of aauA1

Diploids		aauA1 / M.S.F.	
linkage group	gene marker	$aauA^-$	$aauA^+$
I	y^+	31	18
	y^-	23	19
II	$Acra^S$	23	20
	$Acra^R$	17	21
III	gal^+	16	20
	gal^-	19	36
IV	$pyro^+$	14	19
	$pyro^-$	26	32
V	fac^+	21	21
	fac^-	19	30
VI	s^+	25	22
	s^-	26	18
VII	nic^+	43	0
	nic^-	0	48
VIII	$ribo^+$	26	19
	$ribo^-$	20	26

Conclusion :- The strain bil aauA1 is translocation free and the locus can be assigned to linkage group VII.

aauA1 showed approximately 50% recombination with the other markers of this linkage group.

cross I	bil	puA2	+	+	+	+	+	aauA1
	+	+	yA2	palD	nicB	malA	wetA	+

Markers bil puA2 yA2 being on other linkage groups were not classified.

Segregation of markers and allele ratios

	palD	+	
aauA1	36	52	88
+	45	28	73
	81	60	161

Recombination fraction :- palD - aauA1 = 39.8% $\pm$ 3.7

	wetA	+	
aauA1	42	30	72
+	54	45	99
	96	75	161

Recombination fraction :- aauA1 - wetA = 54.0% ± 4.1

	malA	+	
aauA1	41	50	91
+	35	37	72
	76	87	161

Recombination fraction :- aauA1 - malA = 48.4% ± 3.9

	nicB	+	
aauB1	24	42	66
+	41	54	95
	65	96	161

Recombination fraction :- aauA1 - nicB = 49.0% ± 3.9

aauB locus

It was also found that two of the secen mutants assort freely with all markers except nicB (table 5). Similarly, these mutants, late designated aauB1 and aauB2 were assigned to linkage group VII. Crosses between aauB mutants and other markers on linkage group VII were carried out. In this way, the aau locus was assigned to the wetA —— malA interval of linkage group VII.

Table 5 : Haploidisation analysis of aauB⁻ mutants

	Diploids	aauB1 M.S.F.		aauB2 M.S.F.	
linkage group	gene marker	$aauB^-$	$aauB^+$	$aauB^-$	$aauB^+$
I	y^+	25	18	12	16
	y^-	20	8	10	23
II	$Acra^S$	16	22	15	21
	$Acra^R$	5	18	11	12
III	gal^+	4	16	9	9
	gal^-	31	19	21	20
IV	$pyro^+$	16	18	15	10
	$pyro^-$	21	16	16	18
V	fac^+	17	19	12	24
	fac^-	25	10	14	9
VI	s^+	19	11	11	11
	s^-	23	18	18	19
VII	nic^+	41	0	28	0
	nic^-	0	30	2	29
VIII	$ribo^+$	15	20	13	9
	$ribo^-$	9	27	16	21

Conclusions :- The strains bil aauB1 and bil aauB2 are translocation free and the locus can be assigned to linkage group VII.

Cross I

bil	puA2	+	+	+	+	+	aauB1
+	+	yA2	palD	nicB	malA	wetA	+

Cross II

bil	puA2	+	+	+	+	+	aauB2
+	+	yA2	palD	nicB	malA	wetA	+

Markers bil puA2 yA2 being on other linkage groups were not classified.

Segregation of markers and allele ratios

Cross I

	palD	+	
aauB1	26	25	51
+	28	24	52
	54	49	103

Cross II

	palD	+	
aauB2	20	17	37
+	24	19	43
	44	36	80

Recombination fraction :-

palD - aauB1 = 48.6% $\pm$ 5.0

palD - aauB2 = 48.7% $\pm$ 5.6

Cross I

	wetA	+	
aauB1	1	47	48
+	52	3	55
	53	50	103

Cross II

	wetA	+	
aauB2	2	44	46
+	31	3	34
	33	47	80

Recombination fraction :-

wetA - aauB1 = 3.8% ± 1.9

wetA - aauB2 = 6.2% ± 2.7

Cross I

	malA	+	
aauB1	10	38	48
+	43	12	55
	53	50	103

Cross II

	malA	+	
aauB2	8	25	33
+	34	13	47
	42	38	80

Recombination fraction :-

malA - aauB1 = 21.3% ± 4.0

malA - aauB2 = 26.1% ± 4.9

Cross I

	nicB	+	
aauB1	13	31	44
+	28	41	69
	41	72	103

Cross II

	nicB	+	
aauB2	29	14	43
+	14	23	37
	43	37	80

Recombination fraction :-

nicA - aauB1 = 52.7% $\pm$ 4.9

nicA - aauB2 = 65.1% $\pm$ 5.3

Cross I

	wetA	+	
malA	11	45	56
+	35	13	48
	46	58	103

Cross II

	wetA	+	
malA	9	36	45
+	25	10	35
	34	46	80

Recombination fraction :-

wetA - malA = 23.3% $\pm$ 4.1

wetA - malA = 22.3% $\pm$ 4.7

The position of aauB mutants with respect to other markers on linkage group VII.

3.8 ± 1.9		21.3 ± 4.0
————————	aauB1	————————
6.2 ± 2.7		26.1 ± 4.9
————————	aauB2	————————

wetA - - - - - - - - - aauB - - - - - - malA palD - - - - nicB

5 (wetA–aauB) 24 (aauB–malA)

———————————————

22.8

The map positions suggest that aauB1 and aauB2 map very close to each other or are allelic.

aauC locus

Haploid segregants from the diploids between *bil puA2 aauC1/bil puA2 aauC2* and the tester strain 'MSF' were carried out on CM plus acridine yellow since *aauC* strains are p-fluoro-phenylalanine-resistant. These strains were classified and tabulated to show that the *aauC* locus is in linkage group II (table 6).

Table 6 : Haploidisation analysis of aauC mutants

Diploids		aauC1 M.S.F.		aauC2 M.S.F.	
linkage group	gene marker	aauC^{-}	aauC^{+}	aauC^{-}	aauC^{+}
I	y^{+}	12	22	8	12
	y^{-}	13	15	10	4
II	Acra^{+}	35	1	10	0
	Acra^{-}	0	26	0	24
III	gal^{+}	14	17	6	11
	gal^{-}	19	12	9	8
IV	pyro^{+}	10	22	12	10
	pyro^{-}	18	12	6	6
V	fac^{+}	6	23	5	16
	fac^{-}	9	14	8	5
VI	s^{+}	18	27	11	14
	s^{-}	5	12	4	5
VII	nic^{+}	14	26	3	10
	nic^{-}	13	8	8	10
VIII	ribo^{+}	19	11	8	10
	ribo^{-}	21	11	9	7

Conclusion :- The strains bil puA2 aauC1 and bil puA2 aauC2 are translocation free and the locus can be assigned to linkage group VII.

By appropriate crosses aauC1 and aauC2 were located in the cnxE - ygA interval in linkage group VII. aauC1 and aauC2 are either allelic or are located very close to each other. The results of the various crosses are presented as follows :-

Meiotic location of aauC1 and aauC2

cross 1	bi1	puA2	aauC1	+	+	+	+	+	+
	+	+		AcraA	WA	thiA	abA	cnxE	ygA

cross 2	bi1	puA2	aauC2	+	+	+	+	+	+
	+	+		AcraA	WA	thiA	abA	cnxE	ygA

Segregation of markers and recombination fraction :-

Cross I

	cnxE	+	
aauC1	10	10	
+	32	18	90

Recombination fraction :-

cnxE - aauC1 = 31.1% ± 4.9 .

	ygA	+	
aauC1	11	31	
+	37	11	90

Recombination fraction :-

ygA - aauC1 = 24.4% ± 4.5

	riboE	+	
aauC1	20	19	39
+	31	26	57
	51	45	90

Recombination fraction :-

riboE - aauC1 = 51.1% ± 5.3

Cross II

	cnxE	+	
aauC2	15	36	
+	61	18	130

cnxE - aauC2 = 29.9% ± 4.0

	ygA	+	
aauC2	11	59	
+	51	19	130

ygA - aauC2 = 23.0% ± 3.7

	riboE	+	
aauC2	29	26	55
+	44	31	75
	73	57	130

riboE - aauC2 = 46.1% ± 4.4

	cnxE	+	
ygA	25	18	43
+	32	15	47
	57	33	90

	cnxE	+	
ygA	41	25	66
+	32	32	64
	73	57	130

Recombination fraction :-

cnxE - ygA = 44.4% ± 5.2

cnxE - ygA = 56.1% ± 5.3

Linkage map

31.1 ± 4.9 —— aauC1 —— 24.4 ± 4.5

29.9 ± 4.0 —— aauC2 —— 23.0 ± 3.7

cnxE - - - - - aauC - - - - - - - - - ygA

30   24

50

aauD locus

Haploid segregants were made from bil puA2 aauD1 and tester strain 'M.S.F.' diploid using acridine yellow as before. The aauD strain was assigned to linkage group VIII (table 7).

Sinha (1969) reported the isolation of uptake mutants, designated fpaD11 and fpaD43, which also locate in linkage group VIII. To check for allelism crosses were set up between bil puA2 aauD1 and nicA1 riboA fpaD11; bil puA2 aauD1 and nicA1 riboA fpaD43. Approximately 10% recombinants from each of the crosses were wild types, i.e. sensitive to p-fluorophenylalanine, showing that the aauD mutants are not allelic with the fpaD alleles. The aauD locus therefore maps approximately 20 units on either side of the fpaD locus. More detailed genetic analysis is necessary to locate the position of aauD1 on linkage group VIII.

Meotic analysis

Cross	genotypes	genotypes considered	No. of segregants analysed	segregation of p-fluorophenyl-alanine resistance	
				sensitive	resistant
I	nicA1 riboA2 fpaD11 x bil puA2 aauD1	fpaD11 x aauD1	231	26	205

Recombination fraction :- xprD11 - aauD1 = 23.3 $\pm$ 1.7%

2	nicA1 riboA2 fpaD43 x bi1 puA2 aauD1	fpaD43 x aauD1	195	18	167

Recombination fraction :- fpaD43 - aauD1 = 18.7% $\pm$ 2.1

Table 7 : Haploidisation analysis of aauD1

Diploid		aauD1 M.S.F.	
linkage group	gene marker	aauD$^-$	aauD$^+$
I	y$^+$	16	23
	y$^-$	21	40
II	Acra$^+$	31	21
	Acra$^-$	16	22
III	gal$^+$	30	25
	gal$^-$	19	16
IV	pyro$^+$	21	20
	pyro$^-$	23	24
V	fac$^+$	21	24
	fac$^-$	12	23
VI	s$^+$	29	30
	s$^-$	11	20
VII	nic$^+$	25	13
	nic$^-$	26	26
VIII	ribo$^+$	41	0
	ribo$^-$	0	49

Conclusion :- The strain bil puA2 aauD1 is translocation free and the locus aauD can be assigned to linkage group VIII.

(b) Transport of L-glutamate and other amino acid by aau mutants

The uptake of ^{14}C-labelled amino acid was determined during the exponential growth phase in an attempt to study uptake in young cells under as standard and reproducible conditions as possible. The strains under investigation were grown on -N medium plus 10 mM urea as the sole nitrogen source. Representative amino acids, and certain inorganic nitrogen compounds were used to determine the uptake characteristics of the mutants (figures 14-25). These were L-glutamate (acidic), L-phenylalanine (aromatic), L-alanine and L-serine (neutral), L-arginine and L-glutamine (basic), thiourea and methylammonium (inorganic). From the data presented in figure 14, it can be seen that all classes of uptake mutants have low uptake of L-glutamate (< 10% of the wild type). The heterozygous diploids aauA1/+ and aauB1/+ appear to have normal uptake indicating that these mutations are recessive to their wild type alleles while aauC1 and aauD1 mutations are dominant (figure 15).

The data presented in figures 16 and 18 show that aauB1, aauC1 and aauD1, but not aauA1 have impaired neutral amino acid uptake - L-serine and L-alanine. Figures 17 and 19 show the recessivity of aauB1 and dominance of aauC1 and aauD1.

Figure 20 shows the uptake of the aromatic representative, L-phenylalanine, by the various genotypes. *aauA1* and *aauB1* show normal uptake while *aauC1* and *aauD1* impaired uptake. Again *aauC1* and *aauD1* appear to be dominant (figure 21).

All genotypes appear to have normal uptake of basic amino acids, L-arginine (figure 22), L-glutamine (figure 23), inorganic nitrogen, thiourea (urea) (figure 24) and methylammonium (ammonium) (figure 25).

In summary, *aauA1* shows low uptake of only acidic amino acids and is recessive. *aauB1* shows low uptake of acidic and neutral and is recessive. *aauC1* and *aauD1* have low acidic, neutral and aromatic but in contrast to *aauA1* and *aauB1* are dominant.

Figure 14 : L-glutamate transport by wild type and aau mutants

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

curve ■——■ wild type

curve △——△ *aauA1*

curve ○——○ *aauB1*

curve ◇——◇ *aauC1*

curve ▲——▲ *aauD1*

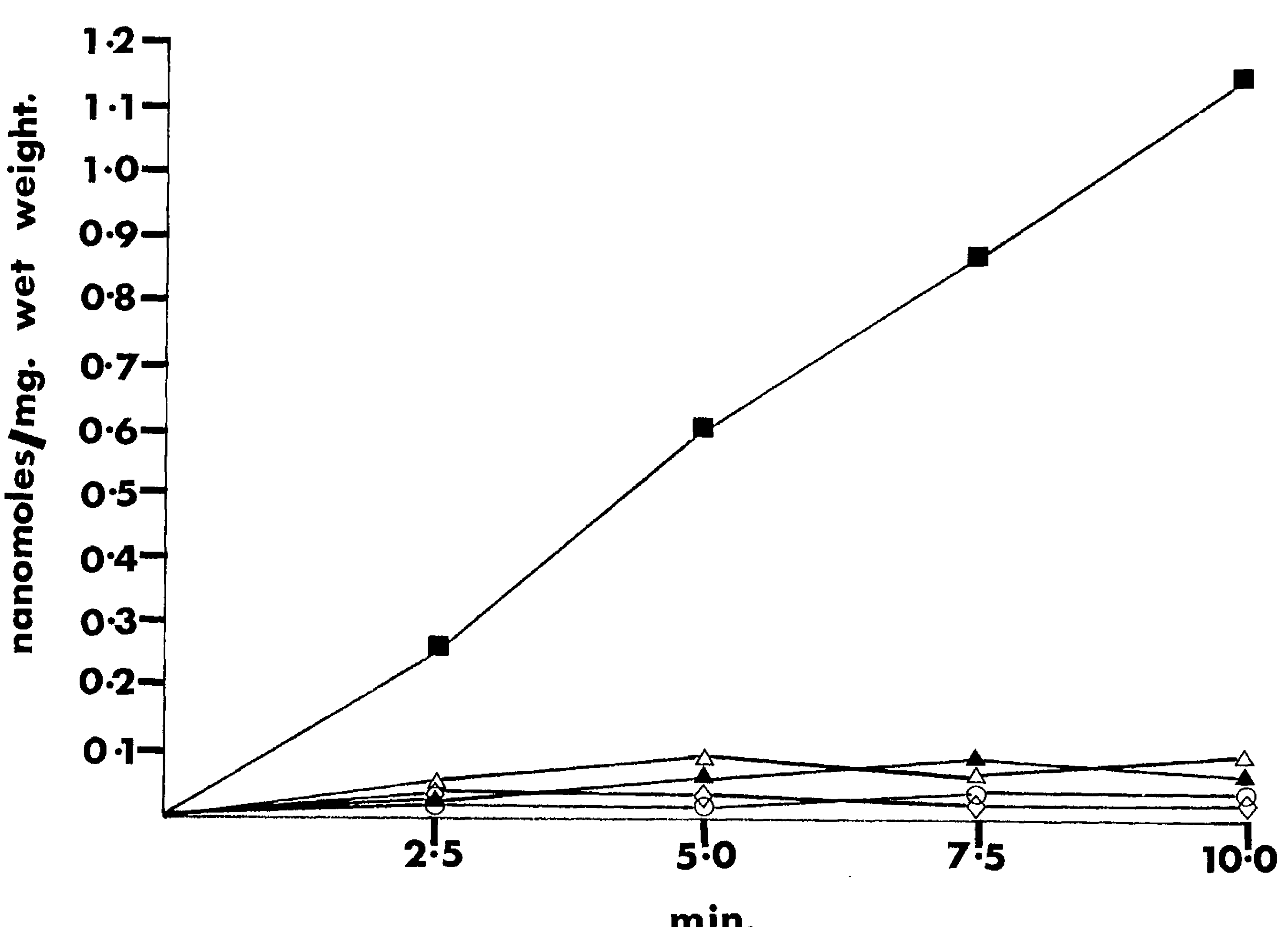

1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
nanomoles/mg. wet weight.
2·5
5·0
7·5
10·0
min.

Figure 15 : L-glutamate transport by heterozygous diploids of wild type and aau mutants

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

curve	■——■	wild type / wild type
curve	△——△	aauA1 / +
curve	○——○	aauB1 / +
curve	◇——◇	aauC1 / +
curve	▲——▲	aauD1 / +

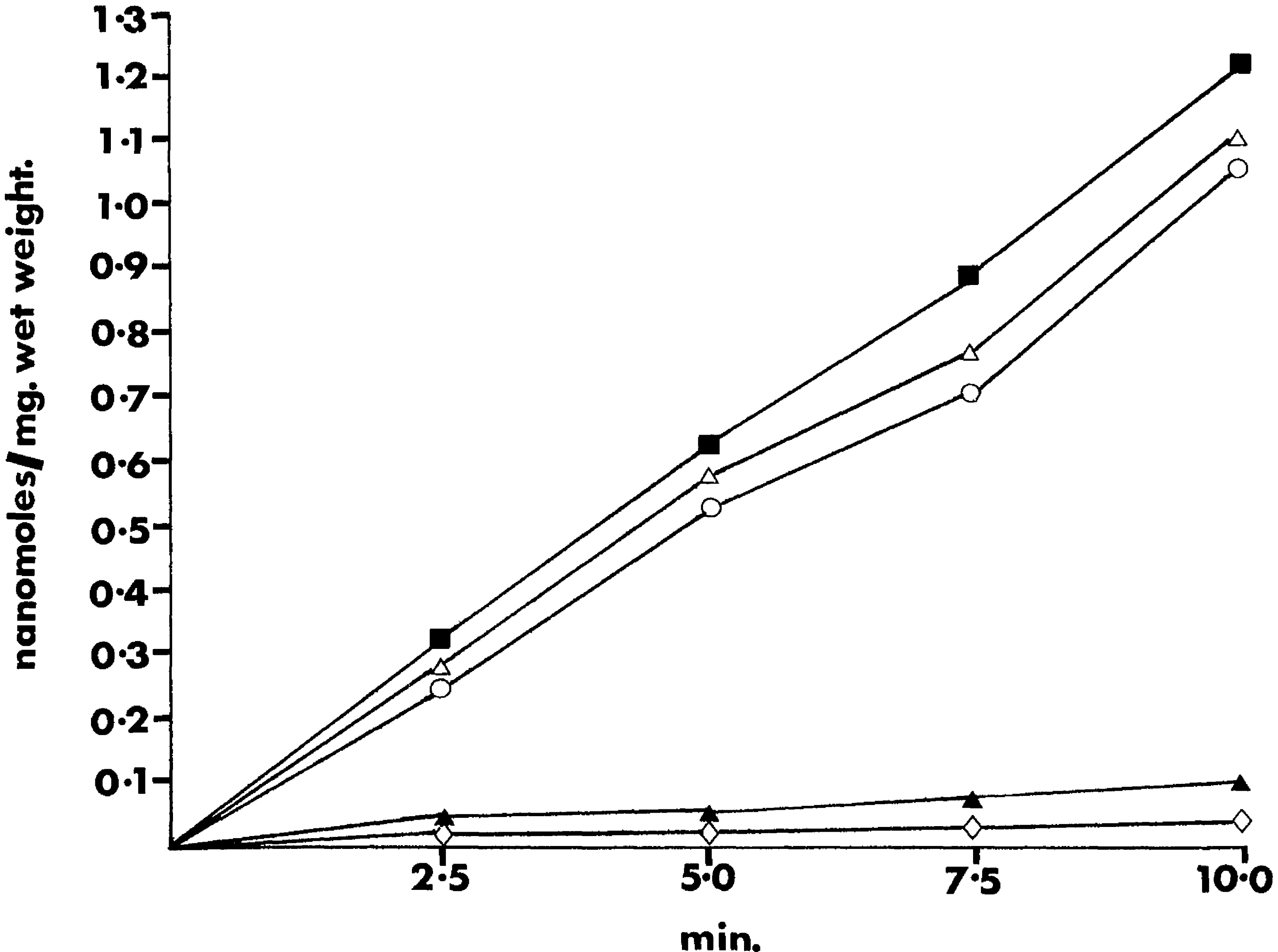

nanomoles/mg. wet weight.
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2·5
5·0
7·5
10·0
min.

Figure 16 : Transport of L-serine by wild type and aau mutants

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

curve	■——■	wild type
curve	△——△	aauA1
curve	○——○	aauB1
curve	◇——◇	aauC1
curve	▲——▲	aauD1

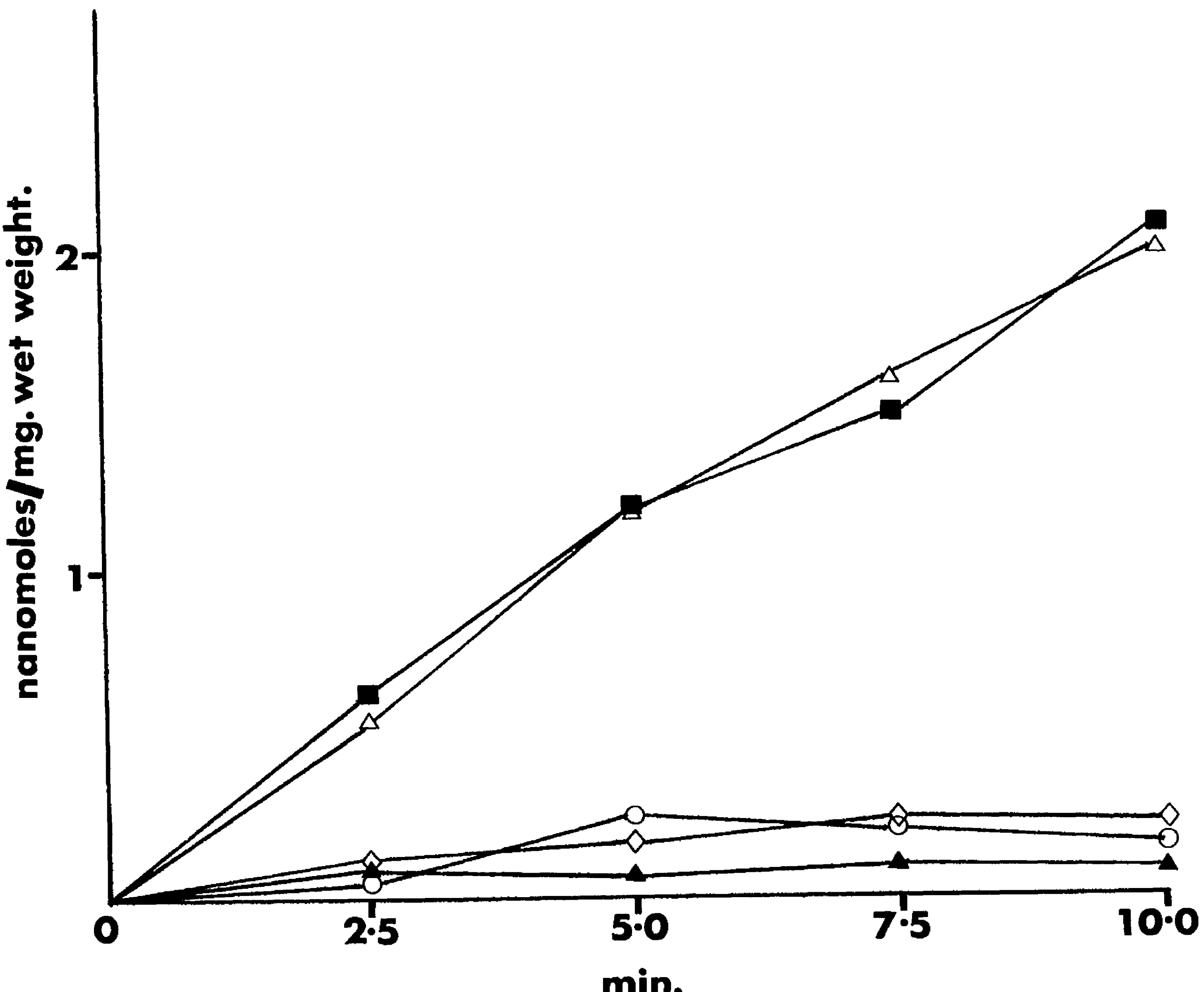
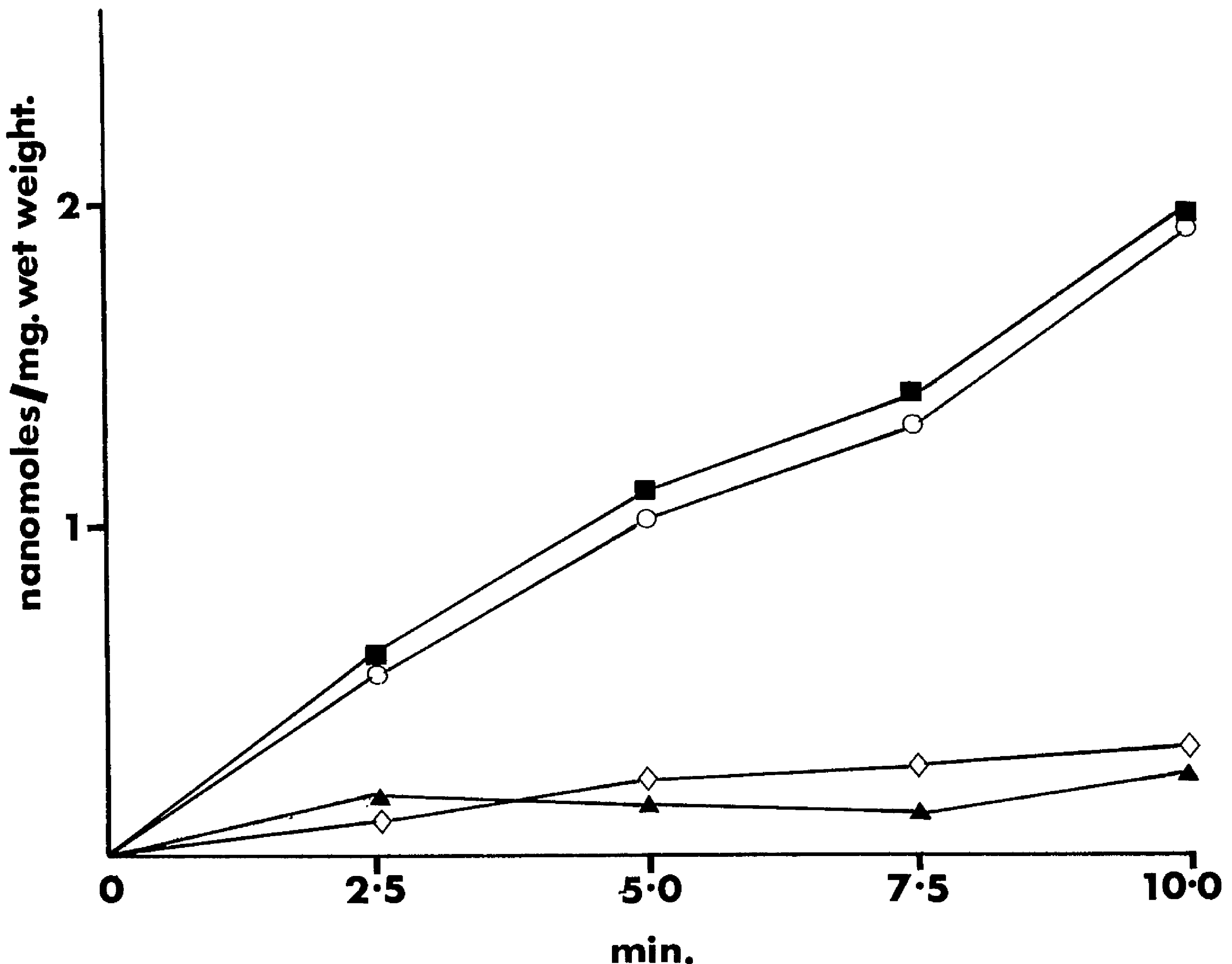

nanomoles/mg. wet weight.
2
1
0
2·5
5·0
7·5
10·0
min.

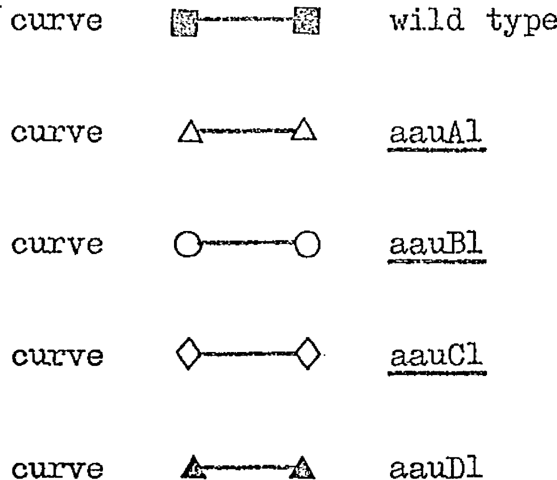
Figure 17 : Transport of L-serine by wild type and aau heterozygous diploids

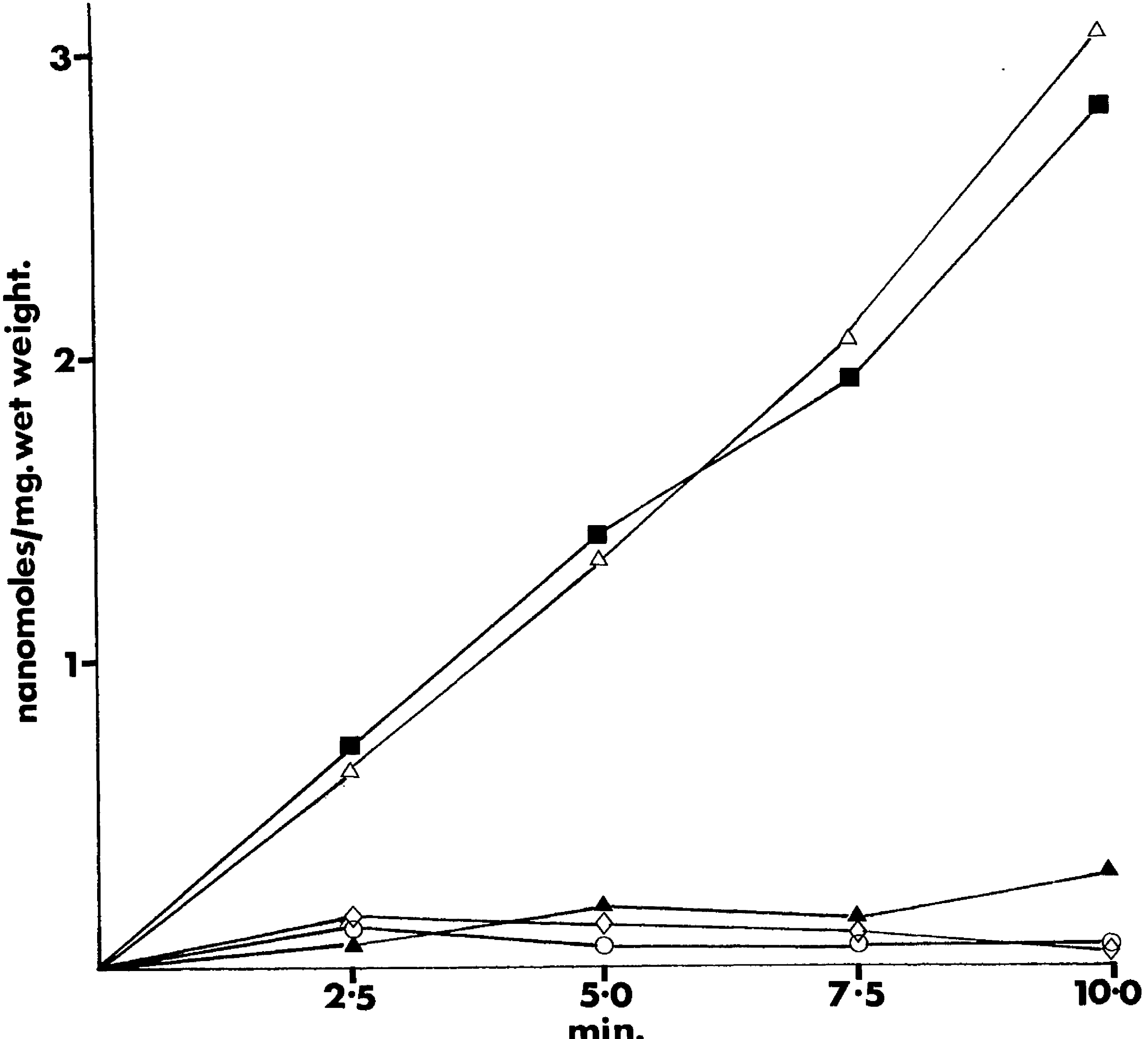
curve	■——■	wild type / wild type
curve	○——○	aauB1 / +
curve	◇——◇	aauC1 / +
curve	△——△	aauD1 / +



nanomoles/mg. wet weight.
2
1
0
2·5
5·0
7·5
10·0
min.

Figure 18 : Transport of L-alanine by wild type and aau mutants

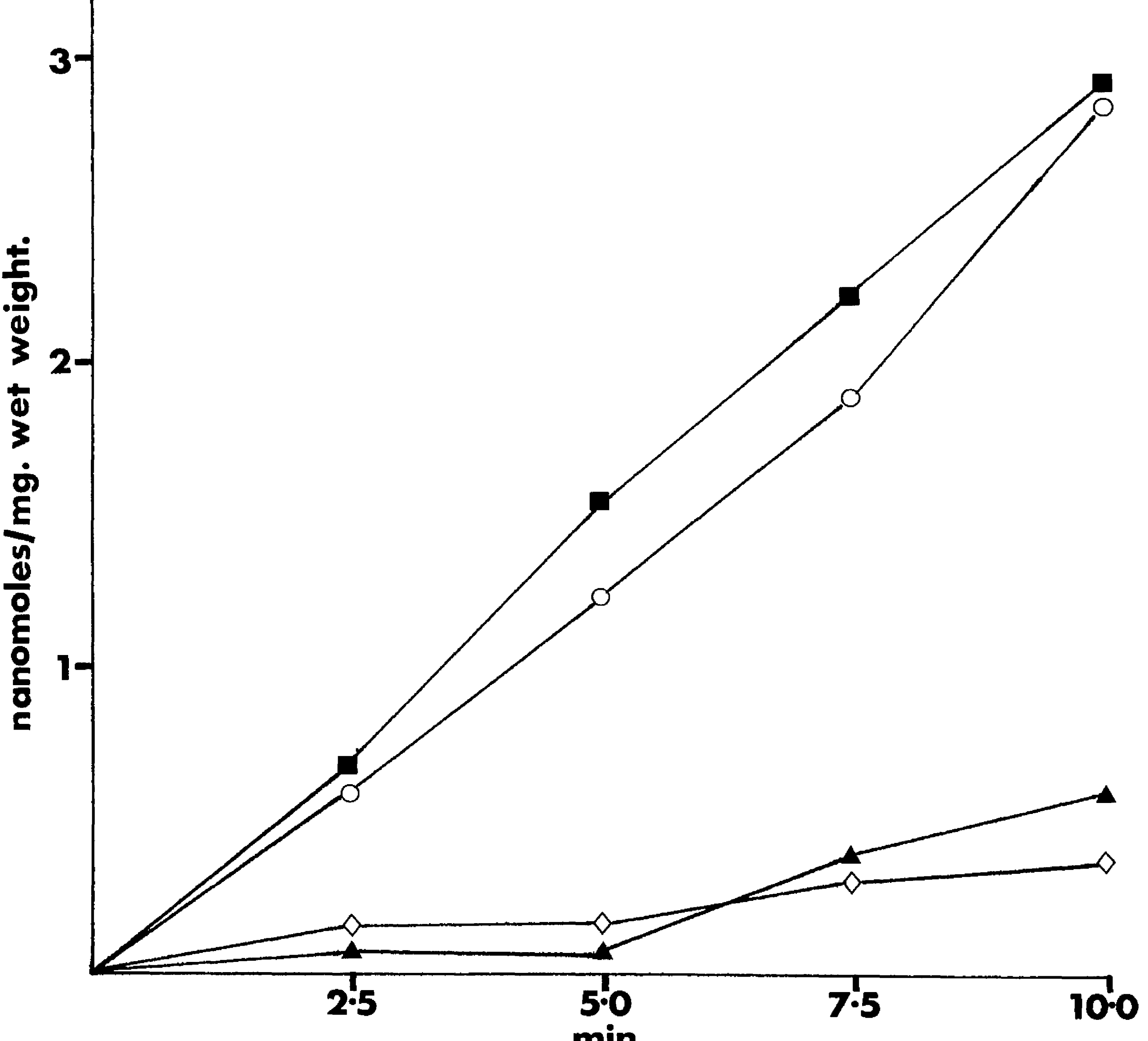




nanomoles/mg. wet weight.
3
2
1
2·5
5·0
7·5
10·0
min.

Figure 19 : Transport of L-alanine by wild type and aau heterozygous diploids

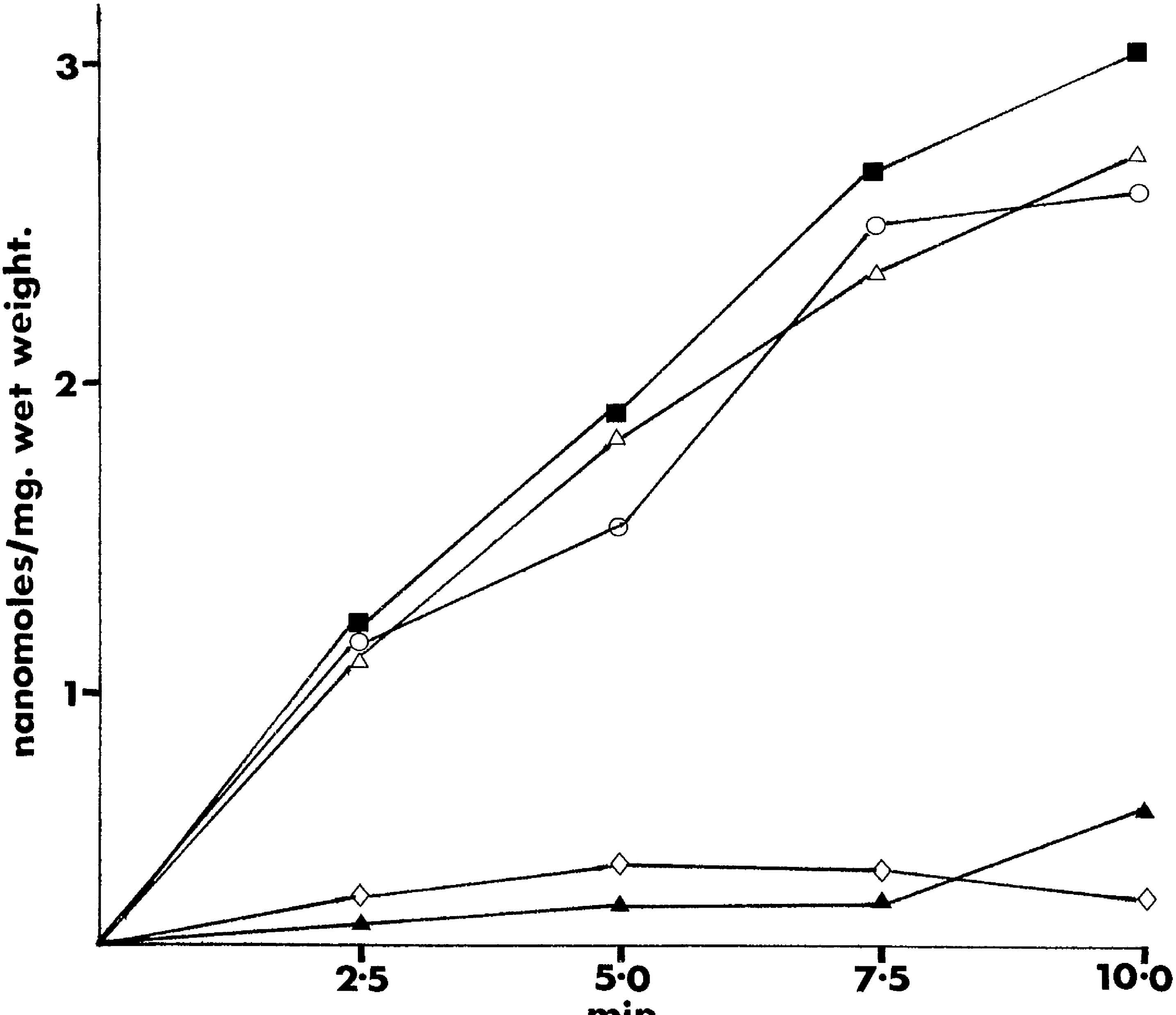
curve	■——■	$\frac{\text{wild type}}{\text{wild type}}$
curve	○——○	$\frac{\text{aauB1}}{+}$
curve	◇——◇	$\frac{\text{aauC1}}{+}$
curve	▲——▲	$\frac{\text{aauD1}}{+}$



3
2
1
nanomoles/mg. wet weight.
2·5
5·0
7·5
10·0
min.

Figure 20 : Transport of L-phenylalanine by wild type and aau mutants

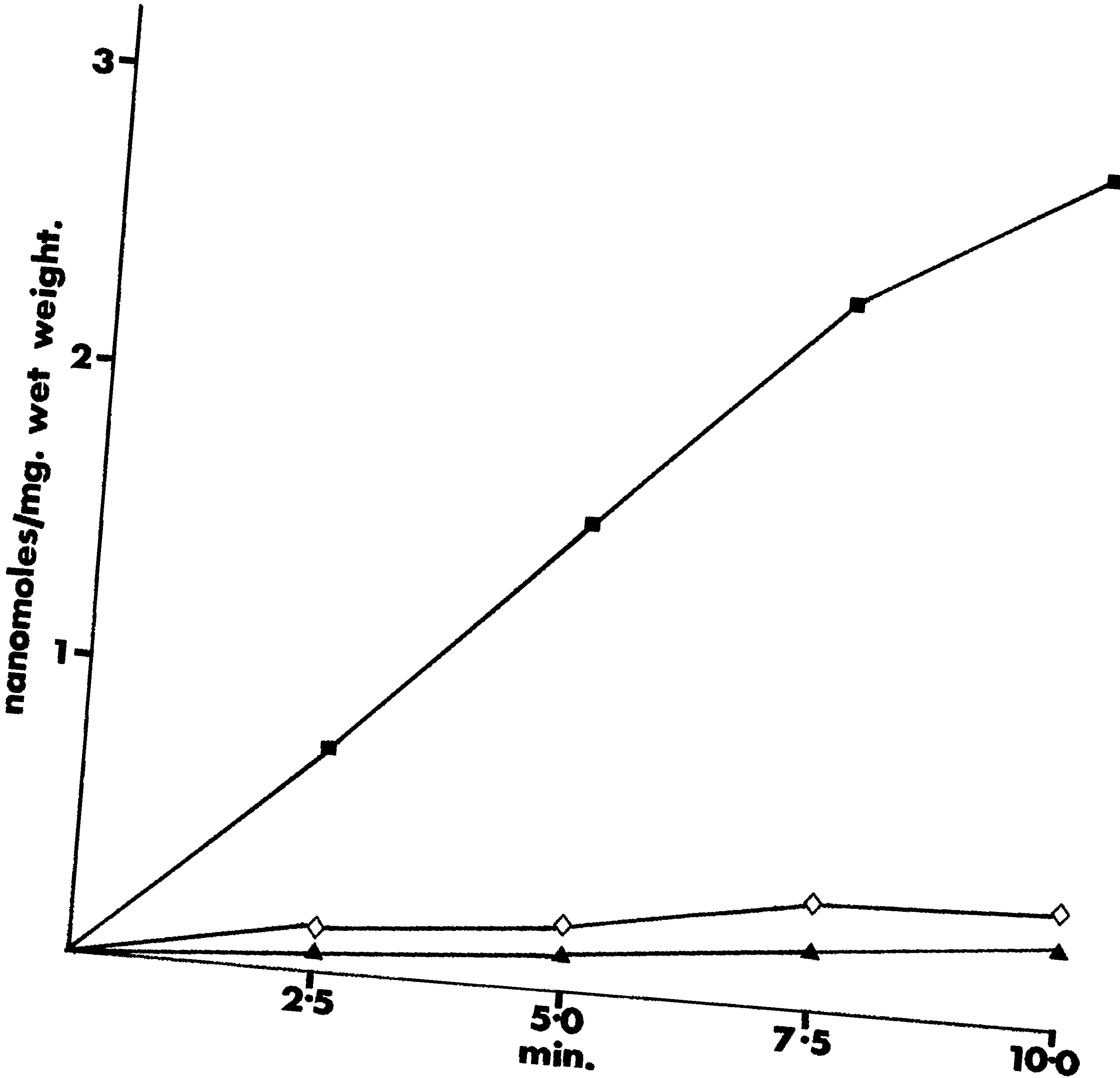
curve	■———■	wild type
curve	△———△	aauA1
curve	○———○	aauB1
curve	◇———◇	aauC1
curve	▲———▲	aauD1



nanomoles/mg. wet weight.
3
2
1
2·5
5·0
7·5
10·0
min.

Figure 21 : <u>L-phenylalanine transport by heterozygous diploids of wild type and aau mutants</u>

curve	■——■	$\frac{\text{wild type}}{\text{wild type}}$
curve	◇——◇	$\frac{\text{aauC1}}{+}$
curve	▲——▲	$\frac{\text{aauD1}}{+}$



nanomoles/mg. wet weight.
3
2
1
2·5
5·0
min.
7·5
10·0

Figure 22 : L-arginine transport by wild type and aau mutants

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

curve ▣——▣ wild type

curve △——△ aauA1

curve ○——○ aauB1

curve ◇——◇ aauC1

curve ▲——▲ aauD1

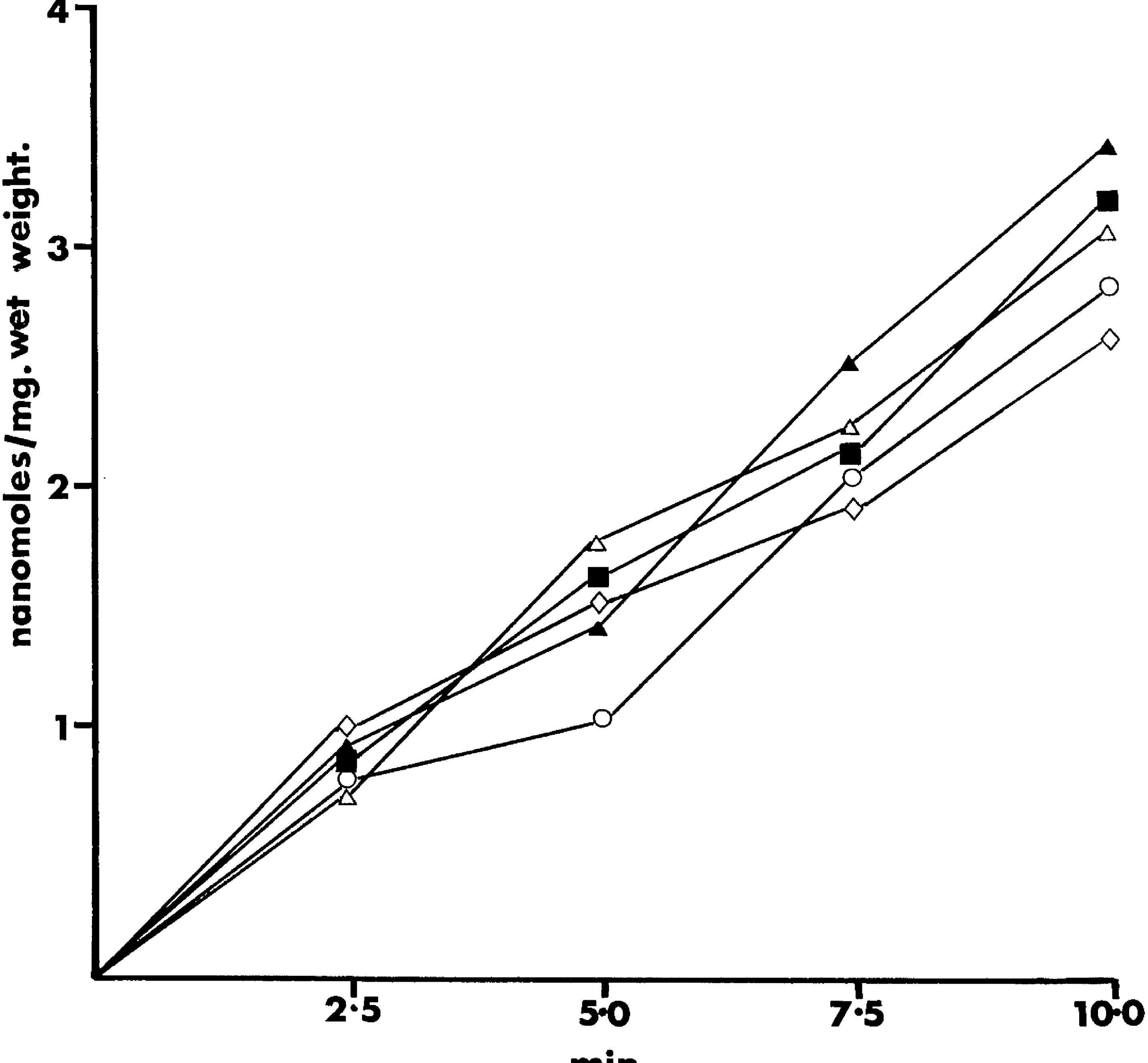

nanomoles/mg. wet weight.
4
3
2
1
2·5
5·0
7·5
10·0
min.

Figure 23 : L-glutamine transport by wild type and aau mutants

Growth conditions :- -N medium + 10 mM urea

Treatment :- none

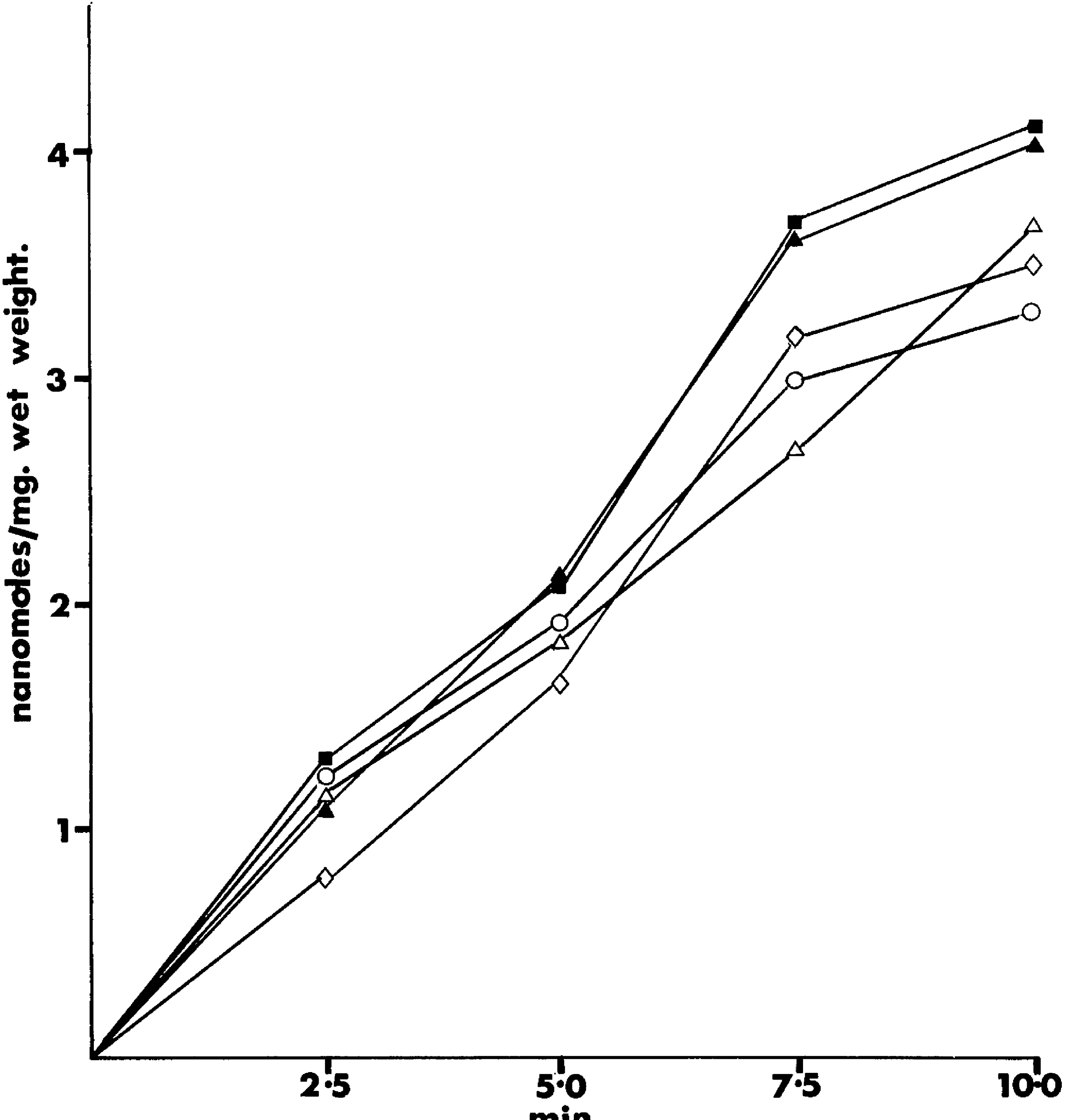
curve ◻——◻ wild type

curve △——△ aauA1

curve ○——○ aauB1

curve ◇——◇ aauC1

curve ▲——▲ aauD1



4
3
2
1
nanomoles/mg. wet weight.
2·5
5·0
7·5
10·0
min.

Figure 24 : Thiourea transport by wild type and aau mutants

curve	■———■	wild type
curve	△———△	aauA1
curve	○———○	aauB1
curve	◇———◇	aauC1
curve	▲———▲	aauD1

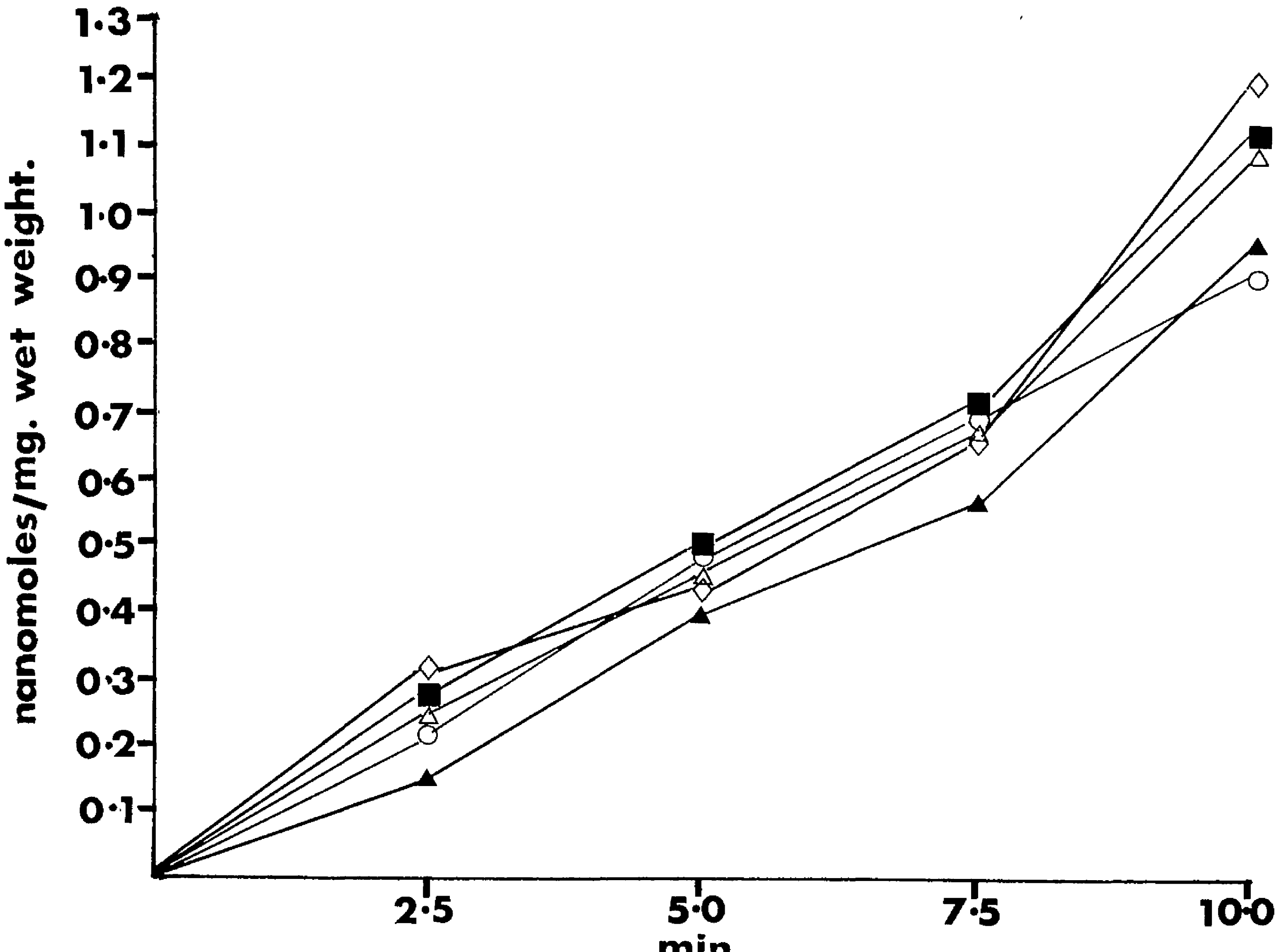

1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
nanomoles/mg. wet weight.
2·5
5·0
7·5
10·0
min.

Figure 25 : Methylammonium transport by wild type and aau mutants

Growth conditions :- –N medium + 10 mM urea

Treatment :- none

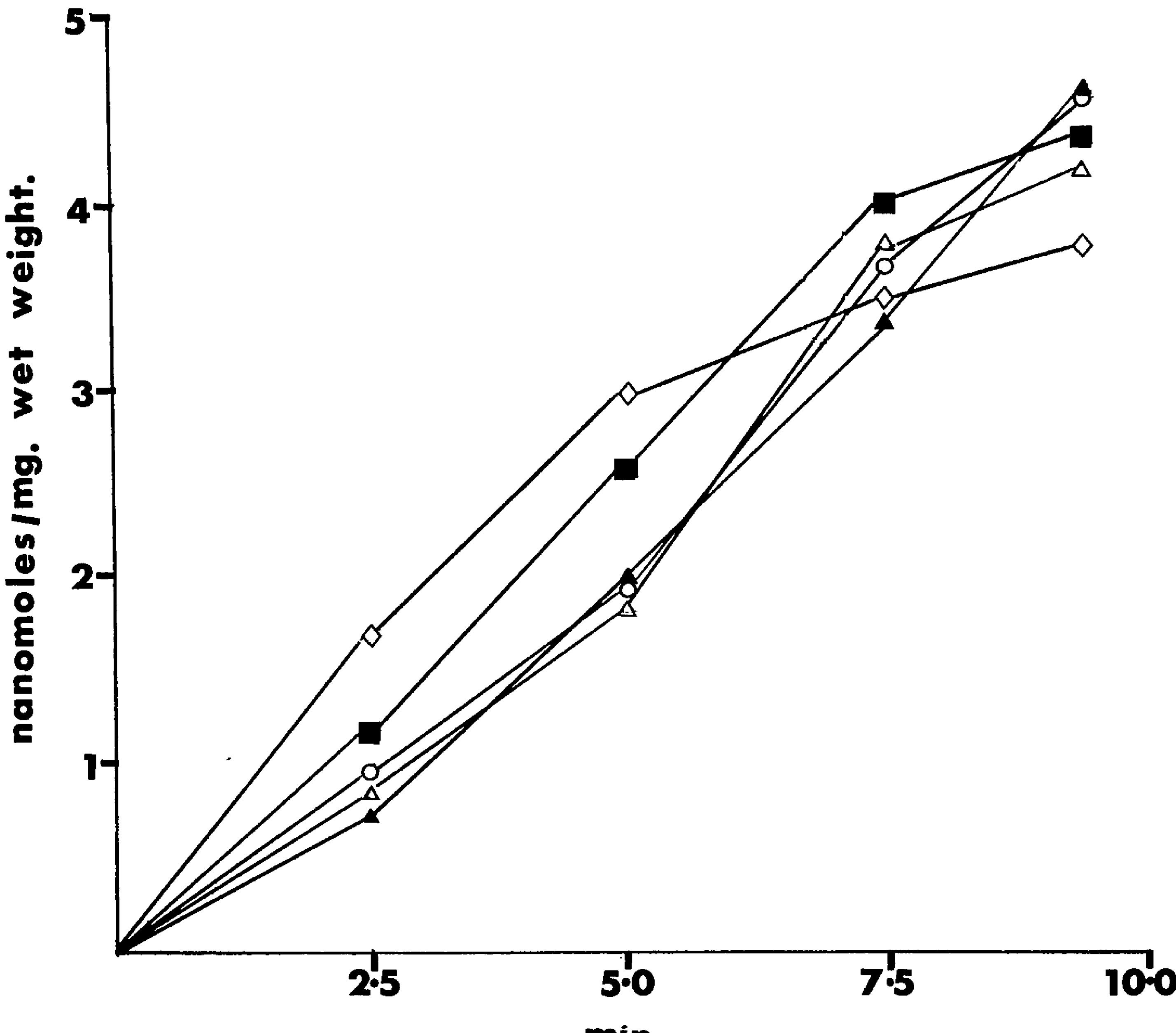
curve ■——■ wild type

curve △——△ aauA1

curve ○——○ aauB1

curve ◇——◇ aauC1

curve ▲——▲ aauD1



nanomoles/mg. wet weight.
5
4
3
2
1
2·5
5·0
7·5
10·0
min.

(c) Growth responses of aau mutants

The results presented in table 8 show the ability or inability of the four classes of uptake mutants to grow on a spectrum of amino acids as sole nitrogen. The wild type growth on all nitrogen sources is designated by a plus sign, although growth of the wild type varies greatly on these sources.

aauA1 grows as wild type on inorganic nitrogen sources, e.g. nitrate ammonium (plate 1) or urea, neutral, e.g. L-serine, L-alanine (plate 3) or L-valine, aromatic, e.g. L-phenylalanine (plate 7), L-tryptophan or L-tyrosine, and basic amino acids such as L-arginine (plate 2), L-ornithine, L-asparagine or L-glutamine but poorly on acidic amino acids - L-glutamate (plate 5) and L-aspartate. The heterozygous diploid aauA1/+ grows as wild type on L-glutamate and L-aspartate indicating that the aauA1 mutation is recessive (plate 6).

Mutation in the aauB gene results in poor growth on acidic (plate 5) and neutral (plate 3) amino acids. The mutation also appears to be recessive (plates 4 and 6).

aauC1 and aauD1 are rather similar with respect to

growth responses in that they grow poorly on acidic (plate 5), neutral (plate 3) and aromatic (plate 7), but as wild type on basic amino acids (plate 2). Moreover, these mutations are semi-dominant or dominant in the heterozygous diploid (plates 4, 6 and 8).

All classes of mutants appear to have wild type growth on certain sugars tested (table 9). However, the utilisation of L-glutamate (plates 9 and 10), L-aspartate and L-alanine as sole carbon sources by the mutants was relatively poorer than their utilisation of these amino acids as sole nitrogen sources. This is not surprising since larger quantities of the amino acid would be necessary to provide a sufficient energy source.

Table 8 : Growth Responses of aau mutants on certain nitrogen sources.

-N medium (solid)	wild type	aauA1	aau A1 +	aauB1	aauB1 +	aauC1	aauC1 +	aauD1	aauD1 +
10 mM urea	+	+	+	+	+	+	+	+	+
10 mM ammonium	+	+	+	+	+	+	+	+	+
10 mM nitrite	+	+	+	+	+	+	+	+	+
10 mM L-aspartate	+	-	+	-	+	-	-	-	-
10 mM L-glutamate	+	-	+	-	+	-	-	-	-
10 mM L-alanine	+	+	+	-	+	-	-	-	-
10 mM L-serine	+	+	+	-	+	-	-	-	-
10 mM L-valine	+	+	+	-	+	-	-	-	-
10 mM L-phenylalanine	+	+	+	+	+	-	-	-	-
10 mM L-tryptophan	+	+	+	+	+	-	-	-	-
10 mM L-tyrosine	+	+	+	+	+	-	-	-	-
10 mM L-glutamine	+	+	+	+	+	+	+	+	+
10 mM L-asparagine	+	+	+	+	+	+	+	+	+
10 mM L-arginine	+	+	+	+	+	+	+	+	+
10 mM L-ornithine	+	+	+	+	+	+	+	+	+

+ denotes wild type growth (this varies with the nitrogen source)

- denotes very poor growth

Table 9 : Growth Responses of aau mutants on certain carbon sources

CN medium + 10 mM ammonium (solid)	wild type	aauA1	aauA1 +	aauB1	aauB1 +	aauC1	aauC1 +	aauD1	aauD1 +
1% galactose	+	+	+	+	+	+	+	+	+
1% maltose	+	+	+	+	+	+	+	+	+
1% sedoheptulose	+	+	+	+	+	+	+	+	+
1% acetate	+	+	+	+	+	+	+	+	+
100 mM L-glutamate	+	-	+	-	+	-	-	-	-
100 mM L-aspartate	+	-	+	-	+	-	-	-	-
100 mM L-alanine	+	+	+	-	+	-	-	-	-

+ denotes wild type growth (this varies with the carbon source)

- denotes very poor growth

Plate 1 Growth response of aau mutants with ammonium as the sole nitrogen source

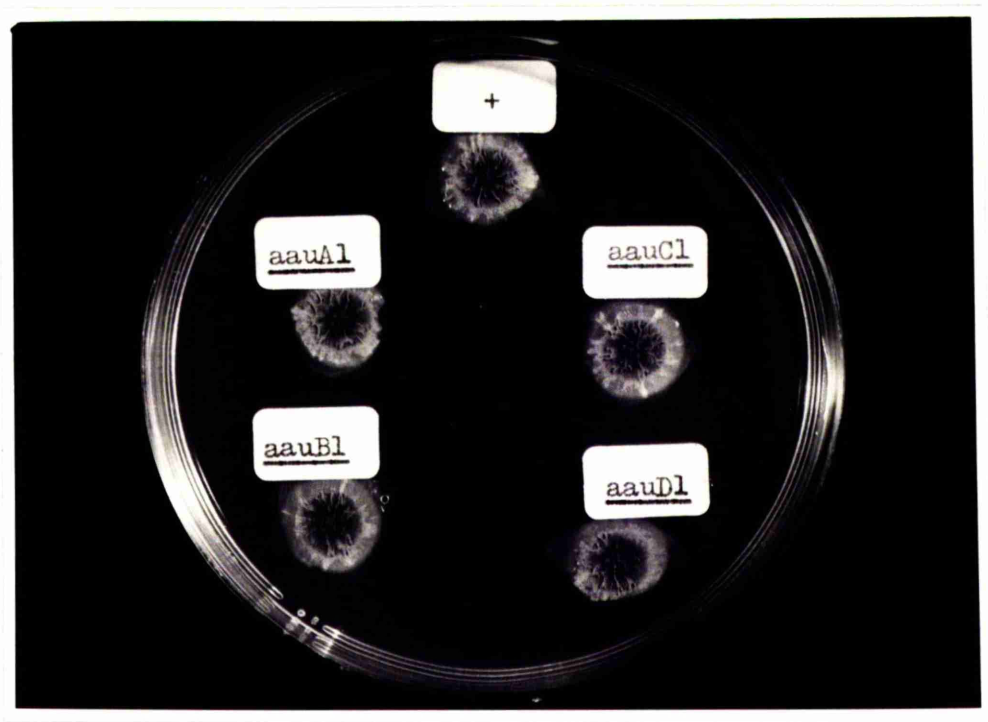


Plate 2 Growth response of aau mutants with L-arginine as the sole nitrogen source

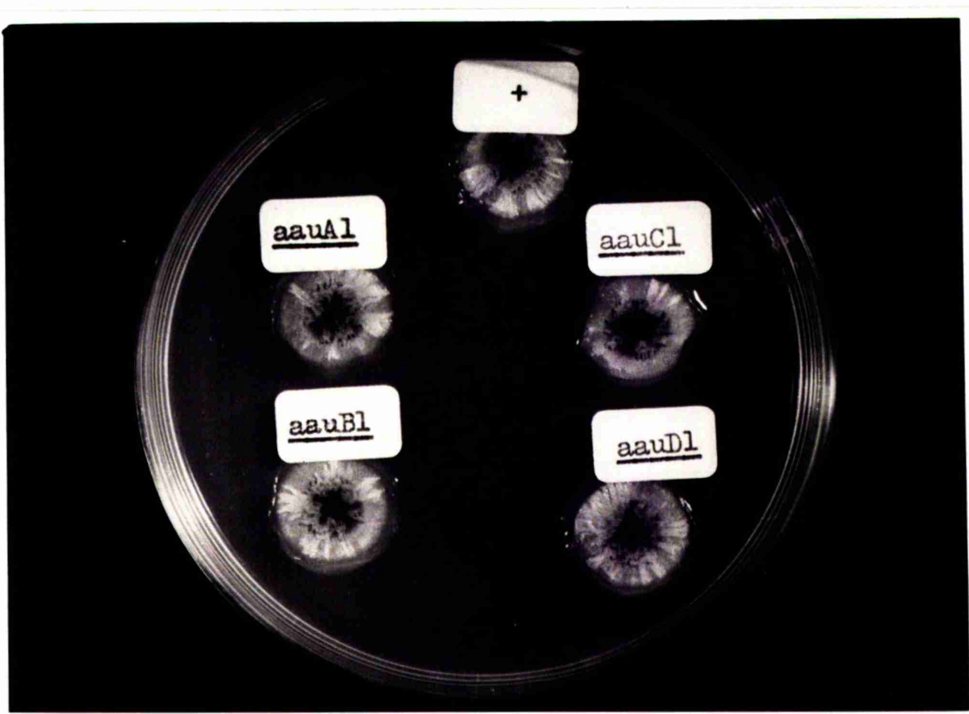


Plate 3 Growth response of aau mutants with L-alanine as sole nitrogen source

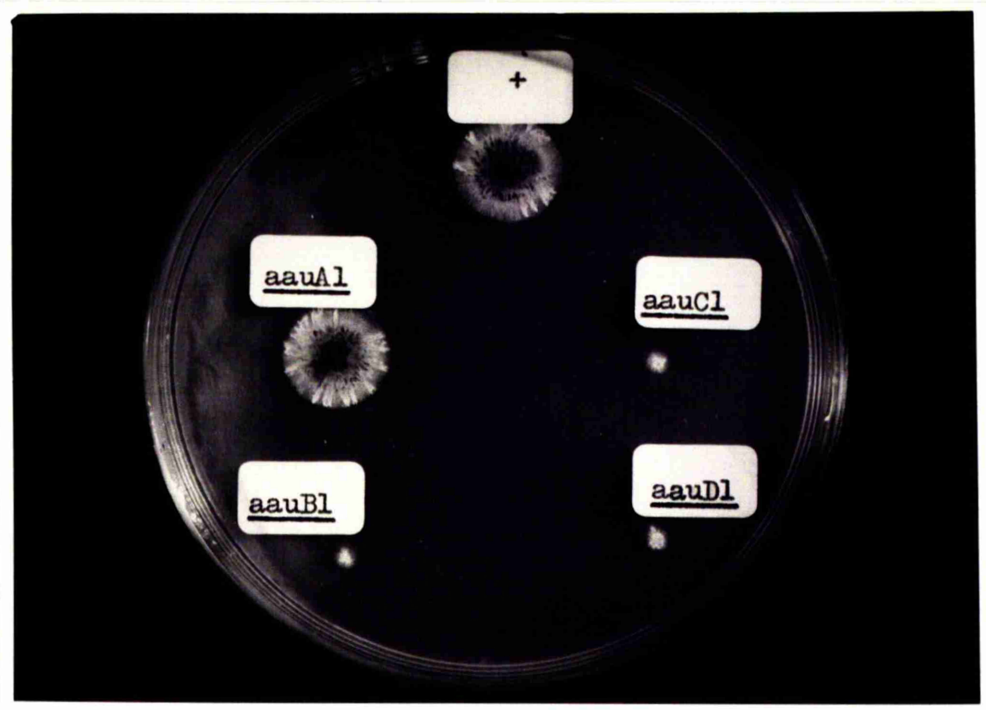


Plate 4 Growth response of aau heterozygous diploids with L-alanine as sole nitrogen source

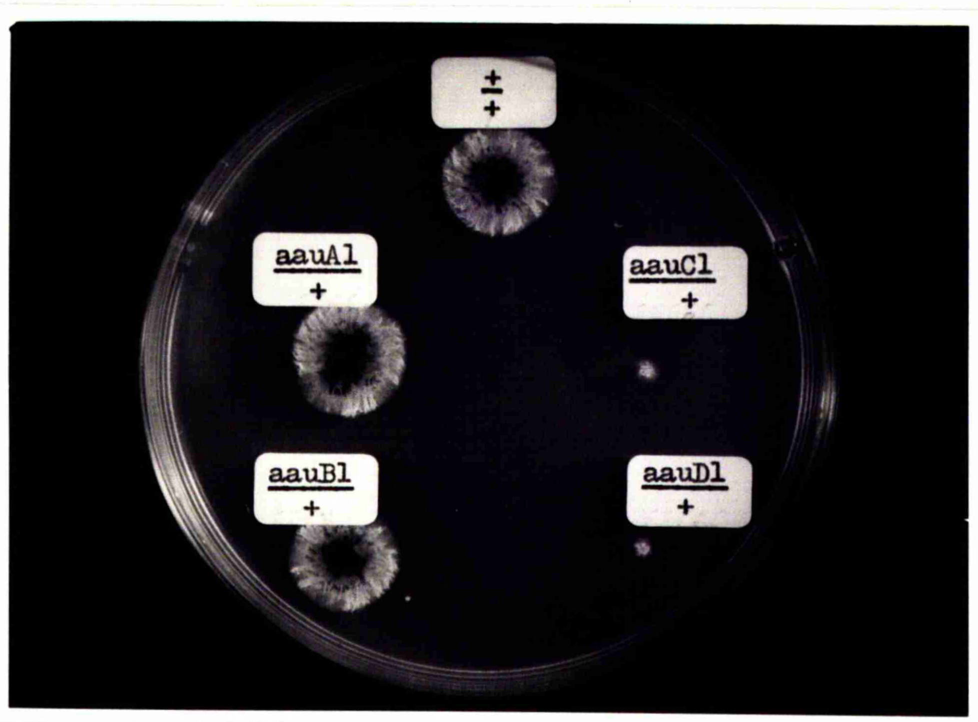


Plate 5 Growth response of aau mutants with L-glutamate as sole nitrogen source

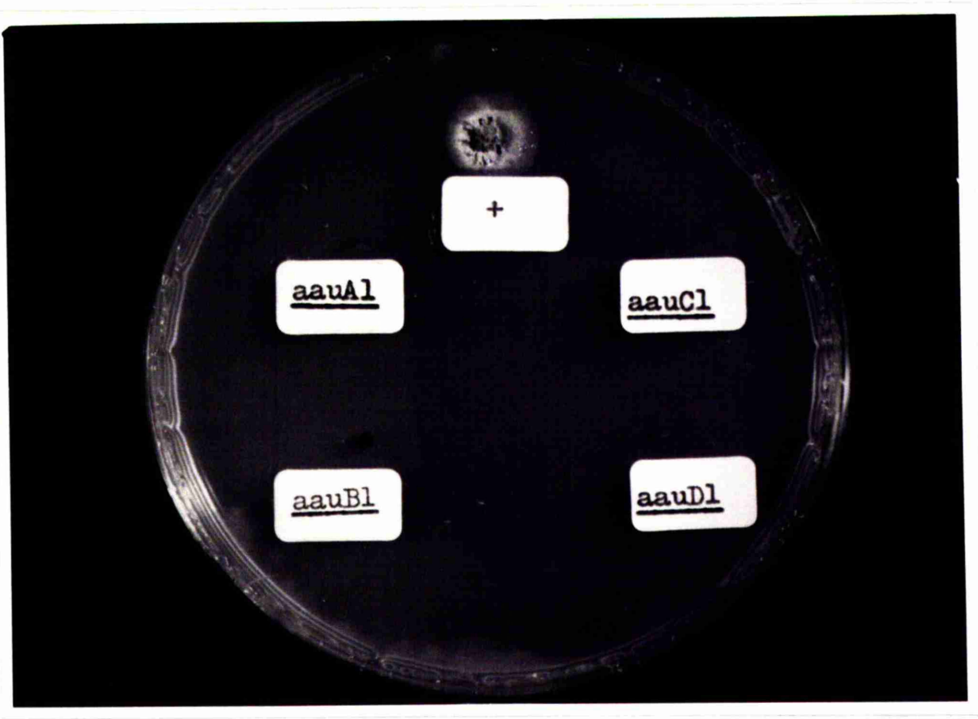


Plate 6 Growth response of aau heterozygous diploids with L-glutamate as sole nitrogen source

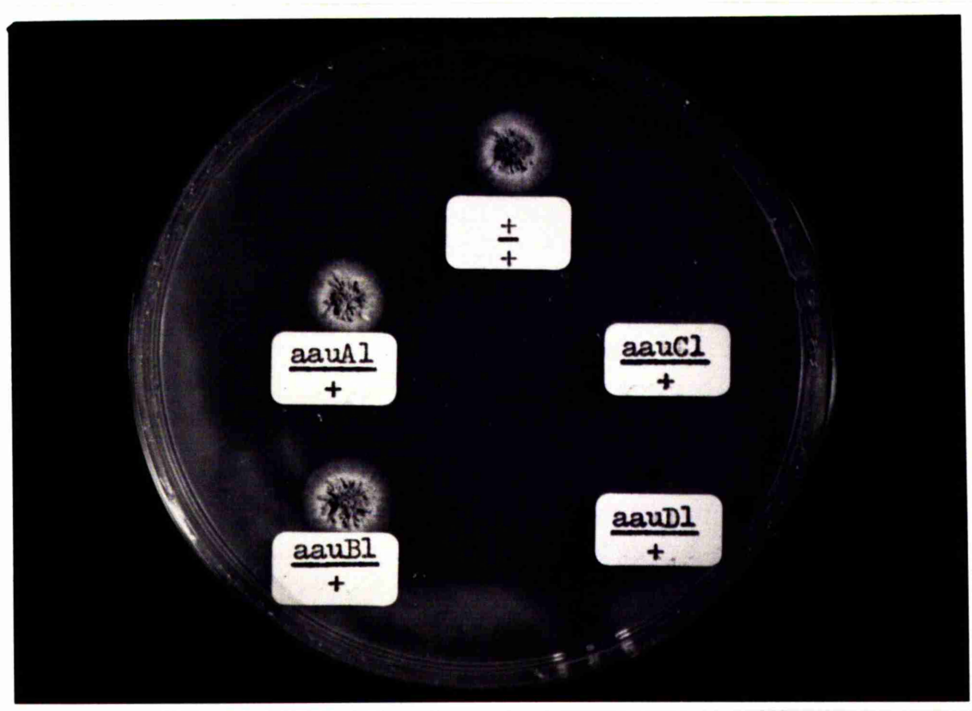


Plate 7 Growth response of aau mutants with L-phenylalanine as the sole nitrogen source

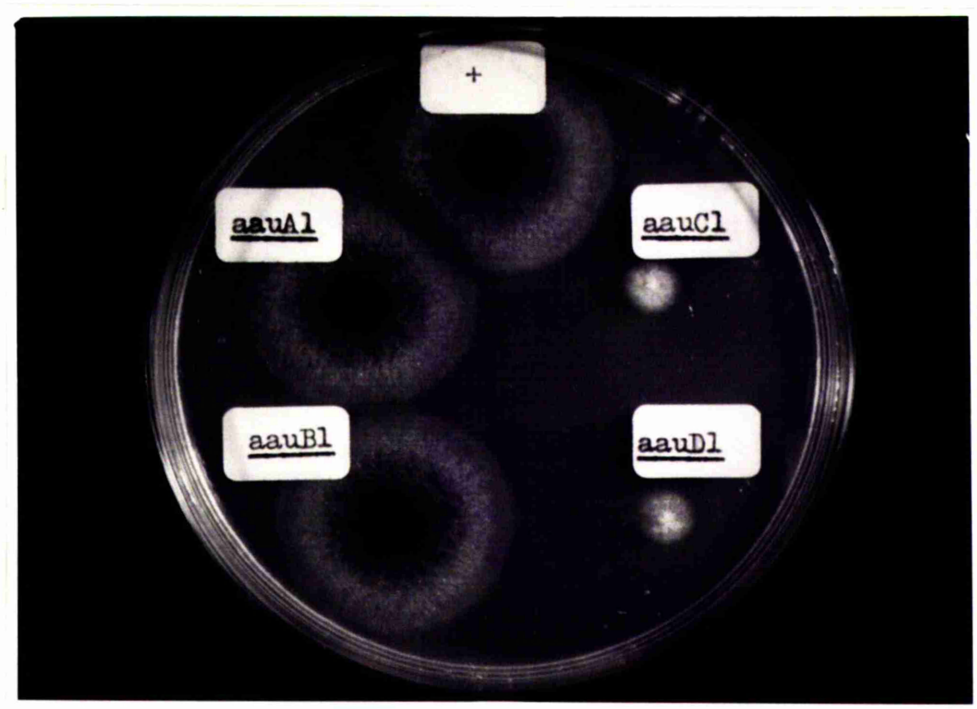


Plate 8 Growth response of aau heterozygous diploids with L-phenylalanine as sole nitrogen source

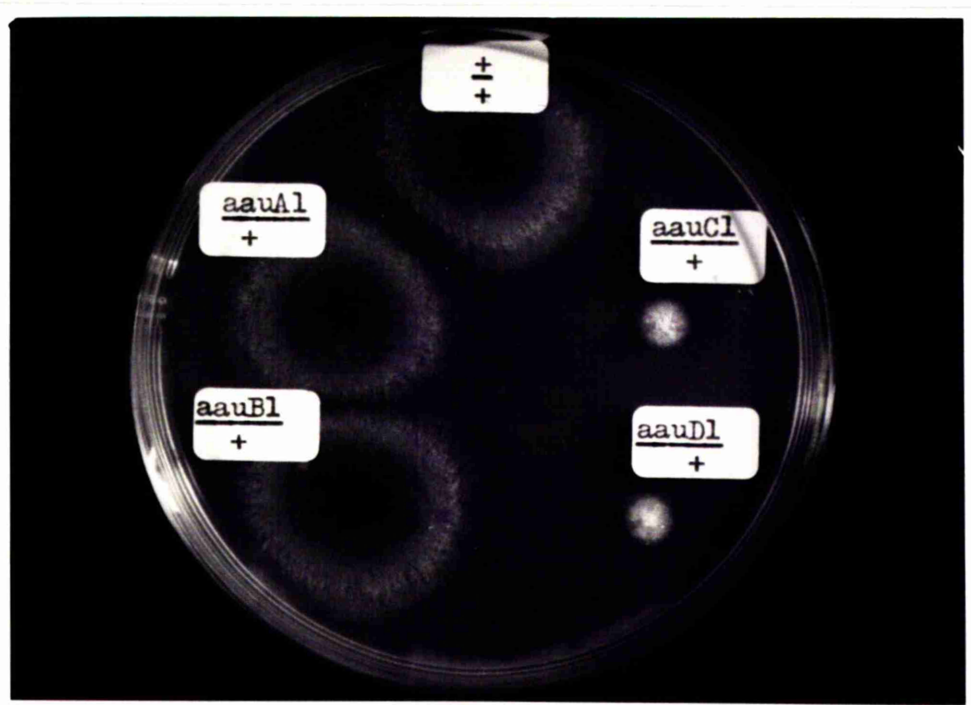


Plate 9 Growth response of aau mutants with L-glutamate as the sole carbon source

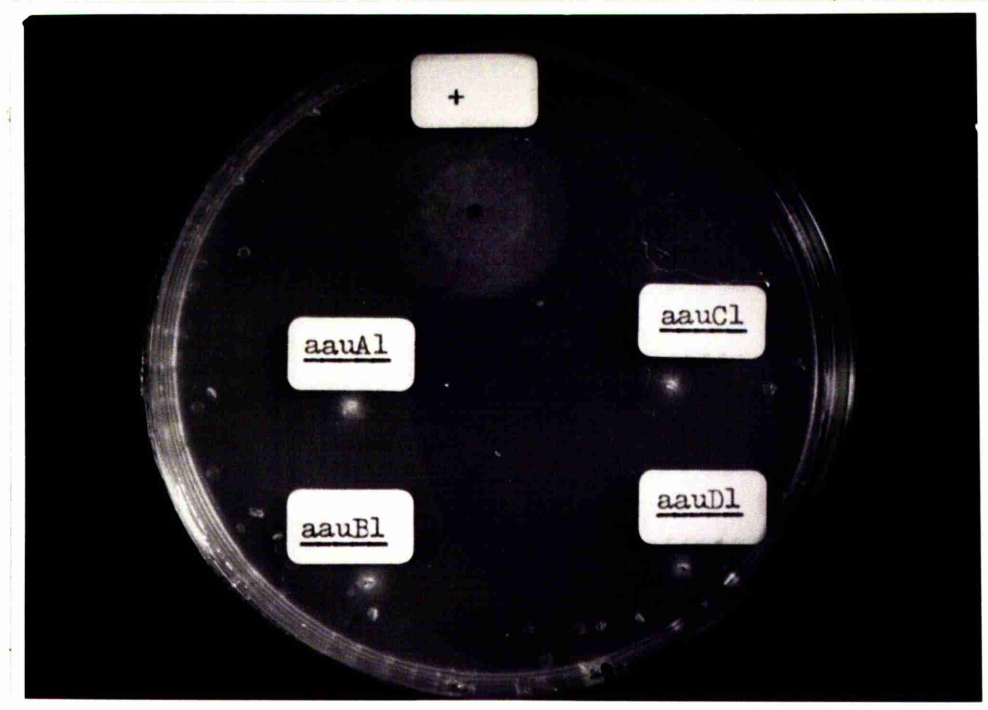
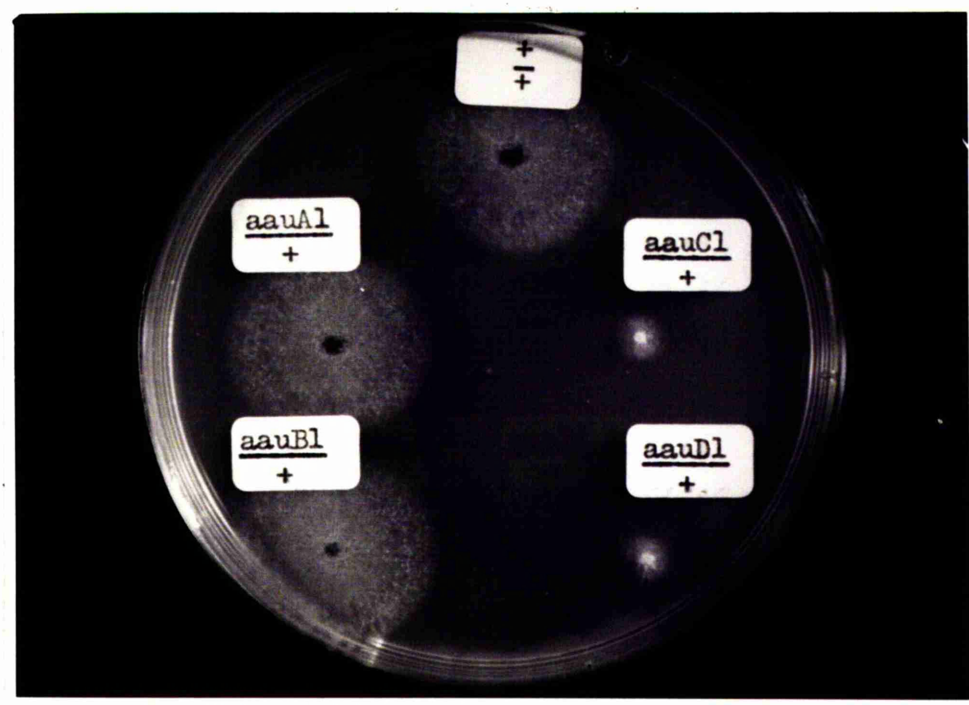


Plate 10 Growth response of aau heterozygous diploids with L-glutamate as a carbon source



(d) Resistance of aau mutants to certain toxic analogues of amino acids and inorganic nitrogen

An attempt was made to correlate uptake deficiencies with resistance to certain toxic analogues. Unfortunately, this survey was rather limited since most of the analogues reported to be inhibitory to bacteria and yeast were found to be ineffective against wild type cells of *Aspergillus nidulans*. These include analogues of L-glutamate (D-glutamate), L-aspartate (fluoro-aspartate, D-aspartate), L-arginine (canavanine), L-proline (azetidine-2-carboxylic acid), L-lysine (thiosine), L-methionine (ethionine) and glutamine (glutamyl-hydrazine).

However, three analogues were found to inhibit the growth of *A. nidulans* viz., aspartate hydroxamate, glutamate hydroxamate and D-serine. Auxanographic tests of a wild type strain indicated that inhibition of growth is reversed by L-asparagine, L-glutamine and L-serine respectively.

In addition, p-fluorophenylalanine, 1.3-amino tyrosine HCl (Sinha, 1967), methylammonium (Arst and Cove, 1969) and thiourea. (Dunn and Pateman, 1972) toxic analogues of L-phenylalanine, L-tyrosine, ammonium and urea respectively were used.

Mutants, meaA8 uruA1 SER-9 fpaD43 ahyA1 glyA1, known to be resistant to at least one of these analogues, were tested as controls. The results of these tests are shown in table 10 and plates 11 - 16. aauA1, which has low uptake of L-glutamate and L-aspartate, is found to be sensitive to all seven toxic analogues, p-fluorophenylalanine, D-serine, aspartate hydroxamate, glutamate hydroxamate, 1.3-amino tyrosine, methylammonium and thiourea. aauB1 is resistant to only D-serine and aauB1/+ sensitive to this indicating again the recessivity of the aauB1 mutation. aauC1 and aauD1 are resistant to p-fluorophenylalanine, 1.3-amino tyrosine and D-serine. Again, as judged by resistance tests these mutants appear to be semi or fully dominant.

Of the six control mutants three are known to be uptake mutants; meaA8 - low uptake of methylamine (Pateman, personal communication, Arst and McDonald, 1973), uruA1 - low uptake of urea (Dunn and Pateman, 1972) and fpaD43 - low uptake of certain amino acids including phenylalanine (Sinha, 1969). meaA8, uruA1 and aau mutants have no common resistance pattern. fpaD43 shares some common resistance features, e.g. resistance to p-fluorophenylalanine and 1.3-amino tyrosine. However, fpaD43 differs in that it is not resistant to D-serine, unlike aauB1, aauC1 and aauD1.

Table 10 : Resistance of aau mutants to toxic analogues of certain nitrogen sources

Toxic analogue	analogue of	-N medium	wild type	amino acid uptake mutants								control mutants					
				aauA1	aauA1 +	aauB1	aauB1 +	aauC1	aauC1 +	aauD1	aauD1 +	meaA8	uruA1	SER-9	fpaD43	ahyA1	glyA1
p-fluorophenyl-alanine	phenylalanine	10 mM nitrate	-	-		-		+	+	+	+	-	-	-	+	-	-
D-serine	L-serine	10 mM nitrate	-	-		+	-	+	+	+	+	-	-	+	-	-	-
L-aspartate hydroxamate	L-asparagine	10 mM nitrate	-	-		-		-		-		-	-	-	-	+	-
L-glutamate hydroxamate	L-glutamine	10 mM nitrate	-	-		-		-		-		-	-	-	-	-	+
1,3-amino tyrosine HCl	tyrosine	10 mM nitrate	-	-		-		+	+	+	+	-	-	-	+	-	-
methylammonium	ammonium	10 mM arginine	-	-		-		-		-		+	-	-	-	-	-
thiourea	urea	10 mM nitrate	-	-		-		-		-		-	+	-	-	-	-

Control mutants

meaA8 - resistant to methylamine only (Arst and Cove, 1969)
fpaD43 - resistant to p-fluorophenylalanine and 1,3-amino tyrosine HCl only (Sinha,196
SER-9 - resistant to D-serine only (Kinghorn, unpublished work)
ahyA1 - resistant to aspartate hydroxamate only (Kinghorn, unpublished work)

Plate 11 Resistance of aau mutants to D-serine

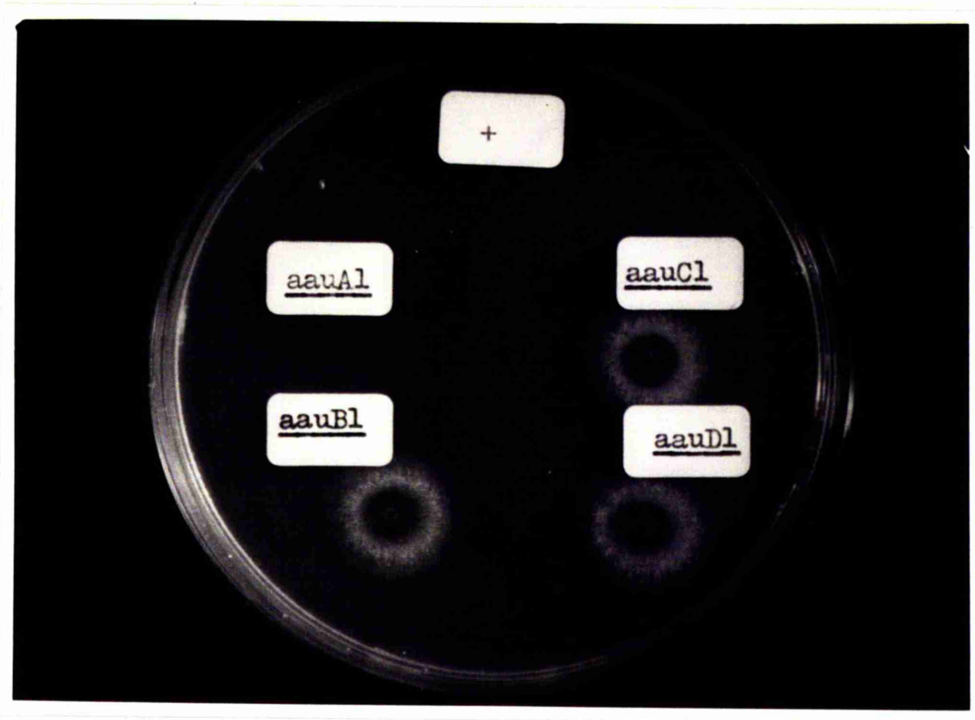


Plate 12 Resistance of aau heterozygous diploids to D-serine

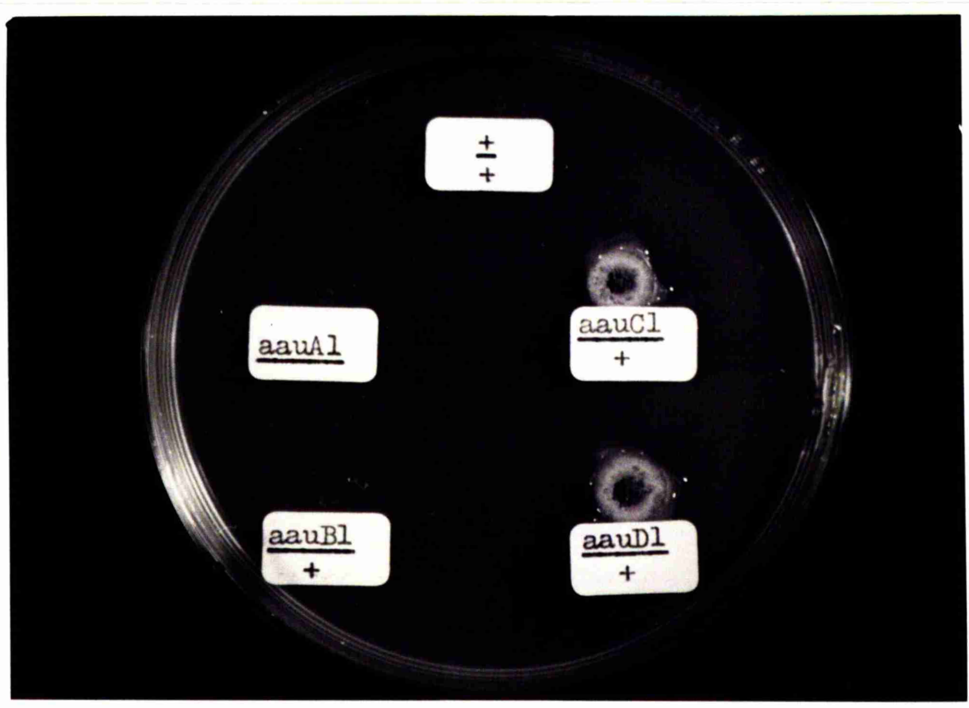


Plate 13 . Resistance of aau mutants to p-fluorophenylalanine

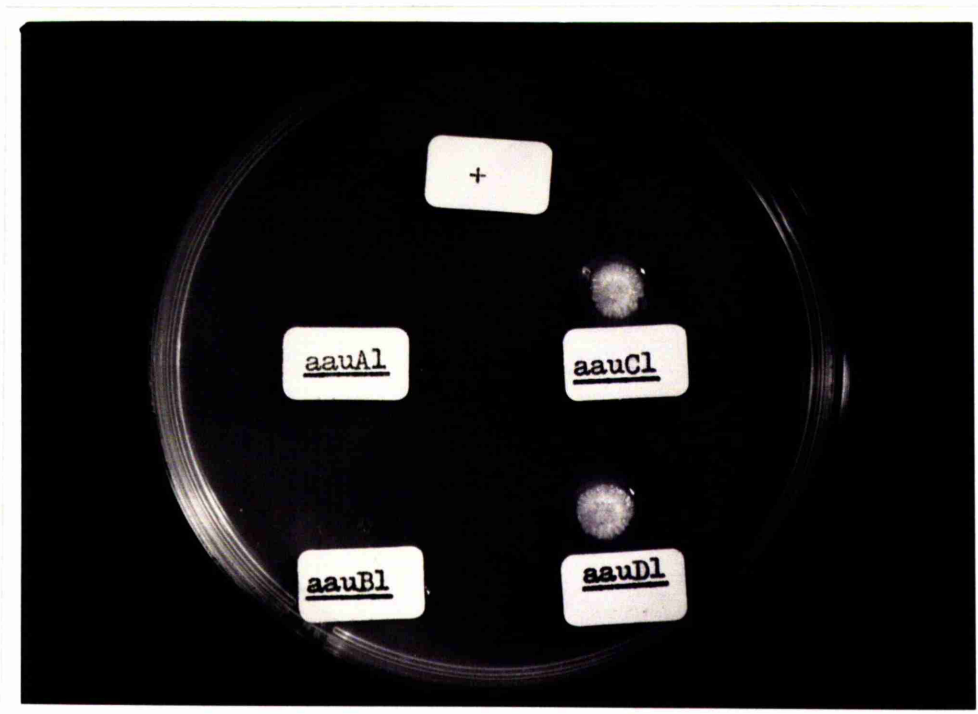


Plate 14 Resistance of aau heterozygous diploids to p-fluorophenylalanine

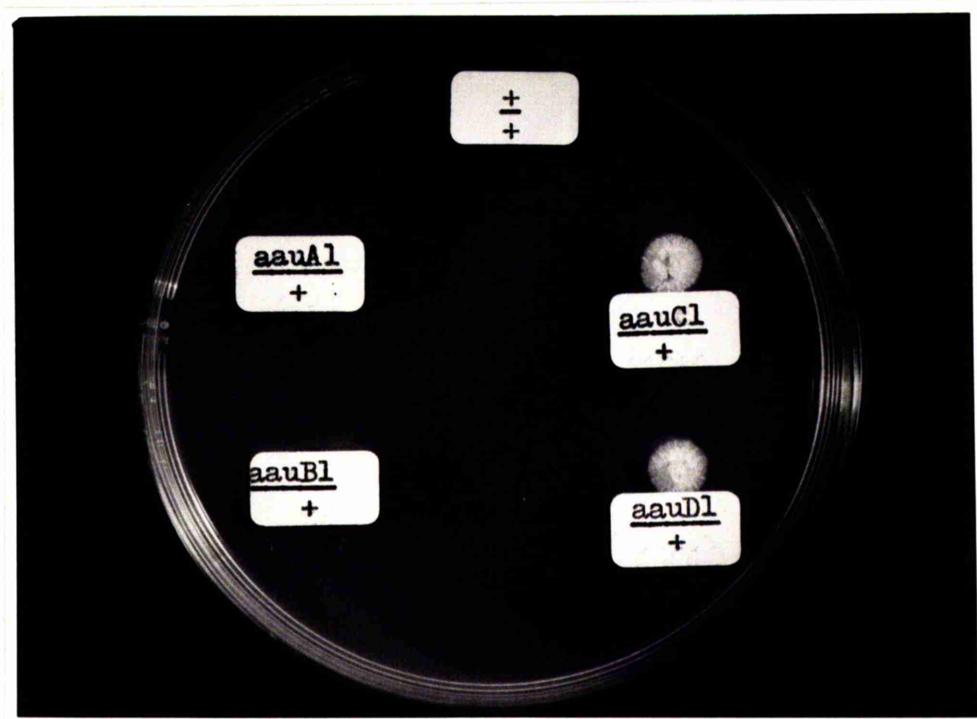


Plate 15 . Resistance of aau mutants to thiourea

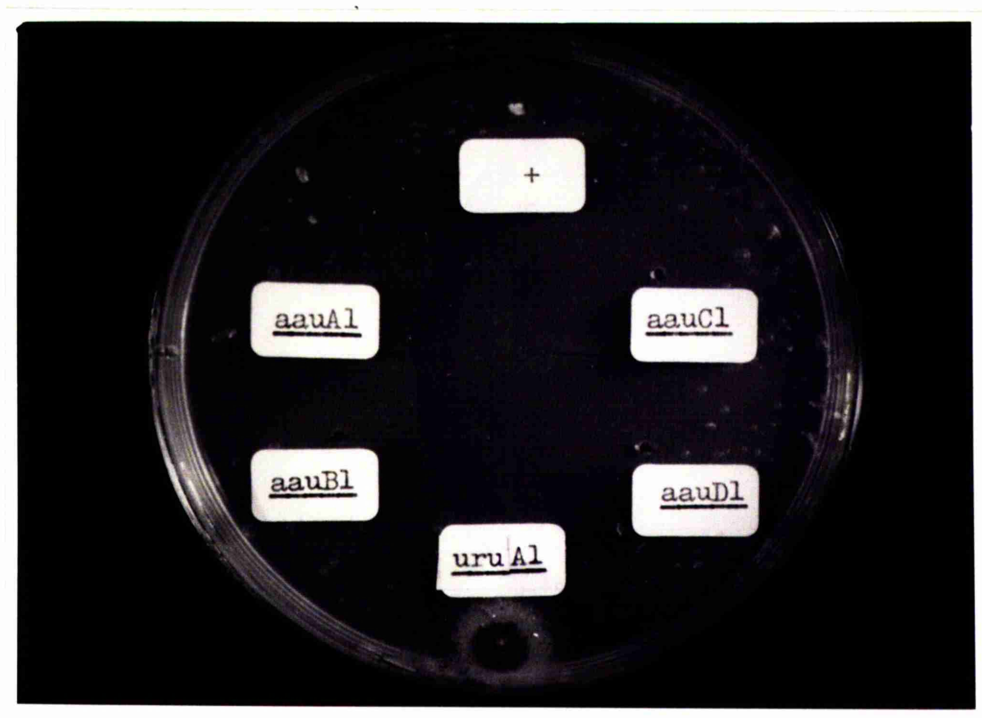
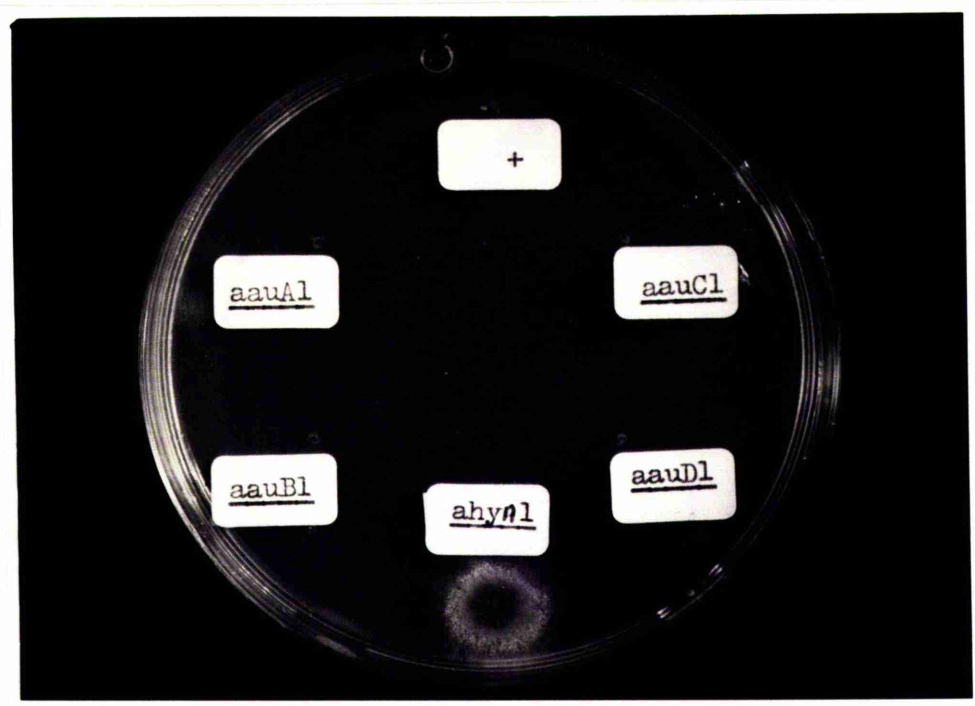


Plate 16 Resistance of aau mutants to L-aspartate hydroxamate



4. Discussion

The results presented in this section suggest that *Aspergillus nidulans*, in the wild state, elaborates an uptake system specific for certain acidic amino acids. This system, actively concentrates L-glutamate and L-aspartate against a concentration gradient. Cysteic acid may also be at least partly transported by the L-glutamate transport system since it was found to be a competitive inhibitor of L-glutamate and L-aspartate transport. Since only low levels of uptake of this amino acid were found under all conditions tested, it was not possible to compare the Km and Ki value. Therefore the question remains unanswered and indeed there is the possibility that L-cysteic acid uptake is an independent specific system, regulated by sulphur as has been found in *P. chrysogenum* (Skye and Segel, 1970). Unfortunately, all cells were grown in minimal medium which contains excess sulphur (2.6% magnesium sulphate.) Since the question of L-cysteic acid uptake remains unresolved, the system will be referred to as the L-glutamate uptake system, as opposed to the acidic amino acid uptake system. Apart from this anomaly, there is support for the contention that the system is specific for L-glutamate and L-aspartate; first there is

agreement between the Km for L-glutamate and its Ki when an inhibitor of L-aspartate uptake, and vice versa, and secondly, similar Km values for L-glutamate and L-aspartate.

Evidence is presented in this section that the L-glutamate transport system is regulated by ammonium, although it is not clear if this is due to inhibition of an already synthesised system or repression of the synthesis of such a system. It was shown that ammonium inhibits L-glutamate transport by approximately 35%, but this could be inhibition by an indirect non-specific type, rather than by a direct control type. On the other hand, the addition of actidione, an anti-metabolite, which stops protein synthesis in fungi (Segel and Johnston, 1963) prevents an increase in L-glutamate uptake when cells are derepressed. This may imply that the appearance of the uptake system in such derepressed cultures is due to *de nova* synthesis of the uptake system. However the possibility cannot be ruled out that inhibition of L-glutamate transport, by actidione, is due to a high pool of a certain metabolite(s), most likely an amino acid, which accumulates as a result of the inhibition of protein synthesis. Therefore the question remains open and bearing these points in mind the phenomenon will be referred to as ammonium repression in this thesis. Indeed a number of uptake systems in *Aspergillus nidulans* and other micro-organisms have been found

regulated by ammonium as discussed before. A number of mutants have been isolated which are insensitive to ammonium repression of certain other ammonium repressible systems. These are meaA8, meaB6, gdhA1, amrA1, xprD1 and DER-3. These mutants are also ammonium derepressed to various degrees for L-glutamate uptake lending support to the argument that L-glutamate transport is yet another system regulated by ammonium. As well as ammonium derepression the amrA1 mutation simultaneously results in impaired transport of L-glutamate. These results will be discussed in more detail in Section VI (General discussion).

Although the regulation of the L-glutamate uptake system by ammonium can clearly be seen from the data, the role of L-glutamate and the carbon status of the cells is not so evident. Maximal activity of L-glutamate uptake is found in cells grown in -N medium plus one of a number of nitrogen sources including nitrate, urea, L-glutamate, L-aspartate or L-alanine. This strongly suggests that L-glutamate does not induce the system. Nevertheless, high L-glutamate transport activity is found in cells grown on -CN medium plus 100 mM L-glutamate or -CN medium plus 100 mM L-glutamate plus 10 mM ammonium. This indicates that ammonium repression of L-glutamate transport does not operate when L-glutamate is used as a carbon source. In fact,

this implies that ammonium repression may be annulled when the substrate is required as a carbon source. This view is supported by the findings that, in A. nidulans, extracellular protease (Cohen, manuscript in preparation) and acetamidase (Hynes, 1970) become insensitive to ammonium repression when protein and acetamide, substrates of these systems respectively, are the carbon sources. The tenet, that ammonium repression is annulled in those cases where the substrate can be used as a carbon source may be tested by investigating a system in which the substrate cannot be used as a carbon source. Such a system is urea uptake since urea cannot be used as an effective carbon source. It was found that in contrast to L-glutamate transport, urea transport in cells grown on -CN medium + 100 mM L-glutamate and 10 mM ammonium was minimal lending support to the above hypothesis (Dunn, personal communication).

Apart from the kinetic data there is then a second argument in favour of a specific system for L-glutamate and L-aspartate. Only this system appears to be regulated by ammonium. Regulatory aspects of the other amino acid transport systems have been investigated in some detail and from such studies it can be concluded that the presence of ammonium does not significantly alter the transport of other amino acids tested. This is supported by the fact

that toxicities of certain amino acid analogues (e.g. D-serine, p-fluorophenylalanine and 1.3-amino tyrosine) are not reversed by ammonium. In contrast the toxicities of analogues of uptake systems, which are repressed by ammonium are reversed by the presence of ammonium, e.g. urea uptake (thiourea), purine uptake (8 azaquanine).

Furthermore, the existence of a mutant aauAl in which only L-glutamate and L-aspartate transport is abnormal strongly suggests the existence of a specific transport system. The phenotype of this mutant under a number of growth conditions is consistent with the above conclusion, i.e. wild type growth on all nitrogen sources except for L-glutamate and L-aspartate. The growth of aauAl is even poorer relative to the wild type when L-glutamate or L-aspartate is the sole carbon source.

The case for specific transport systems in fungi have been argued by several workers. Pall has carried out detailed kinetic analyses of amino acid uptake in *Neurospora crassa* and this indicates the presence of three specific systems each of which transports L-neutral and L-aromatic (Pall, 1969; Wiley and Matchett, 1966), L-basic (Pall, 1970a; Bauerle and Garner, 1964), acidic

amino acids (Pall, 1970b). Grenson and co-workers in a series of communications present evidence for specific uptake systems for individual amino acids in yeast, i.e. lysine (Grenson, 1966), L-methionine (Gits and Grenson, 1967), L-arginine (Grenson, Mousset, Wiame and Becket, 1966) and L-histidine (Crabeel and Grenson, 1970). Moreover, it has been reported that ammonium is transported by a specific system in Aspergillus nidulans and certain other fungi (Pateman, Dunn and Kinghorn, 1973; Hackette, Skyle, Burton and Segel, 1970) as is urea uptake (Dunn and Pateman, 1972). In Pencillum chrysogenum there appear to be specific systems for L-methionine (Benko, Wood and Segel, 1967), L-cystine (Skyle and Segel, 1970), L-proline, L-lysine, L-arginine as well as distinct acidic and basic systems (Hunter and Segel, 1971).

The isolation of uptake mutants in a number of micro-organisms has tended to support the case for specific uptake systems. For instance, the uruA1 mutation in Aspergillus nidulans results in impaired urea uptake (Dunn and Pateman, 1972) as does meaA8 ammonium uptake (Arst and Page, 1973). In Neurospora crassa, Thwaites and Pendyala (1969) described the properties of a mutant, designated bat (formerly UM535) which specifically is impaired in the uptake of basic amino acids. However, other mutations which cause an

alteration in basic amino acid transport include hlp-1 (Choke, 1969) and CR-10(Roess and Debusk, 1968). Pall (1969) reported that mtr mutants of Lester (1966) and Stadler (1966) appear to lack the neutral and aromatic amino acid system.

Mutants of S. cerevisiae have been isolated in which the uptake of certain amino acids is specifically impaired. For example, specific permease mutants for arginine (arg-pl), lysine (lys-pl) and methionine (met-pl) are resistant to the corresponding toxic analogues canavanine, thiosine and arginine, respectively (Grenson et al., 1970 and references therein). A mutant specifically affecting L-histidine uptake (his-pl) was isolated on the basis of poor growth on L-histidine as a nitrogen source (Grabeel and Grenson, 1970).

Thus, uptake systems specific for (a) groups of 'families' of amino acids, i.e. acidic, neutral or basic, (b) specific amino acids, appear to be present in most fungi studied. Transport of acidic amino acids i.e. L-glutamate and L-aspartate in Aspergillus nidulans, therefore, appears to be rather similar to specific systems in other simple eucaryotes. Moreover, the L-glutamate uptake system described in this thesis resembles the acidic acid transport systems described in N. crassa (Pall, 1970b) and P. chrysogenum (Hunter and Segel, 1971).

In addition to specific systems, general amino acid transport systems have also been described in fungi. For instance, Grenson and co-workers have described a 'general' amino acid transport system in yeast (Grenson *et al.*, 1970). The presence of a 'general' transport system has also been reported for *Botrytis fabae* (Jones, 1963), *Arthrobotrys conoides* (Gupta and Pramer, 1970), *Neurospora crassa* (Pall, 1969) and *Penicillium chrysogenum* (Benko *et al.*, 1970).

As in the cases of specific systems mutants have been isolated which have lost the ability to transport a number of amino acids. In yeast mutation in the *gap* locus results in loss of the general permease system and the uptake of certain neutral and basic amino acids but not acidic are impaired (Grenson *et al.*, 1970). Grenson and Hennault (1971) reported that mutation in the *apf* locus (not allelic with *gap*) resulted in low uptake of basic, neutral and acidic amino acids. Another mutation allelic with *apf* (designated *aap*) has been reported resulting in low uptake of a number of amino acids (Surdin, Sly, Sire, Borden and De-Robichon-Szulmayster, 1965).

Several genes are also known to affect amino acid transport in *Neurospora crassa*. Kappy and Metzenberg (1965) have shown that *un-t* has decreased transport of acidic, aromatic and neutral

amino acids. Moreover, it was found that mutation at the *nap* locus results in a similar phenotype (Jacobson and Metzenberg, 1968).

In *Aspergillus nidulans* Sinha (1969) has described uptake mutants originally isolated by Morpurgo (1962a, 1962b) and McCully (Dorn, 1967). These mutations at the *fpaD* locus result in low uptake of aromatic neutral and acidic amino acids.

The data presented in this thesis shows that mutation at no less than another three loci, *aauB*, *aauC* and *aauD* results in impaired uptake of a number of amino acids. The selection technique, i.e. inability to grow on L-glutamate as a sole carbon and nitrogen source, was designed to select uptake mutants with low uptake of the L-glutamate ~~uptake system~~. Rather gratuitously, this method generated a number of mutants which appear to be defective in the uptake of a number of amino acids including L-glutamate and L-aspartate. Although the study of these mutants did not yield very much information with respect to regulatory aspects of L-glutamate metabolism, nevertheless may be of value in providing information regarding other aspects of transport activities, e.g. specificity etc., and will be briefly discussed.

The data presented in this section shows that mutation at the aauB locus reduces the permeability of the cell membrane to acidic and neutral amino acids. The growth as well as resistance properties, is probably confered by decreased uptake of these amino acids. Uptake of inorganic nitrogen, aromatic and basic amino acids is not affected and this is in agreement with growth and resistance tests : aauB mutants are sensitive to thiourea, p-fluorophenylalanine, 1,3-amino tyrosine, aspartate hydroxamate and glutamate hydroxamate. Since the growth rate of aauB1 is similar to that of the wild type when grown on inorganic nitrogen sources, aromatic or basic amino acids, it is concluded that the physiological defect is limited to the transport function.

Several explanations can be advanced to explain such mutations including the hypothesis that certain fungi have systems with wide specificities. These systems in some cases, e.g. yeast, may embrace aromatic, basic and neutral and in others, e.g. N. crassa, acidic, neutral and aromatic amino acid transport. At first sight it might seem that A. nidulans possesses a similar wide specificity transport system since aauB1 is rather similar to nap and un-t mutations in N. crassa in uptake patterns. There is however, one important difference in A. nidulans since the data suggests that

there is only one important system for L-glutamate transport. The existence of aauA1, which has low L-glutamate uptake but normal neutral and aromatic uptake and the fact that only the L-glutamate system is regulated by ammonium are strong evidence on this point. Another possibility is that the locus aauB specifies a protein which is shared by both acidic aromatic and neutral systems and which plays an important role in membrane function. For instance, neutral, aromatic and basic systems may share a common energy coupling system such as D(-) lactic dehydrogenase. This enzyme has been found to generate energy for the transport of amino acids in bacteria (Kaback and Milner, 1970). The fact that the aauB mutation is recessive does not disagree with this theory.

Evidence has been presented which suggests that aauC and aauD loci also play an important role in amino acid transport. Mutations at these loci result in impairment in the transport of acidic, neutral and aromatic amino acids while basic amino acids and inorganic nitrogen uptake is not affected. The mutants grow poorer than wild type when grown on acidic neutral and aromatic amino acids as sole nitrogen source. In addition, they are resistant to D-serine, p-fluorophenylalanine and 1.3-amino tyrosine. Thus, again, as in the case of the aauB1 mutant, the uptake data, growth tests and resistance patterns agree closely. The mutants grow as wild type

on minimal medium indicating that the defect is mainly in transport. The *aauC1* and *aauD1* are rather similar to the *fpaD* mutants of Sinha (1969) in their uptake characteristics and dominance over the wild type allele. *fpaD*43 can be distinguished from *aauC1* and *aauD1* by its D-serine sensitivity. Moreover, the *aau* mutants rather resemble *un-t* (Kappy and Metzenberg, 1965) and *nap* (Jacobson and Metzenberg, 1968) mutants in *N. crassa* which have decreased ability to transport L-acidic, L-neutral and L-aromatic amino acids as discussed above.

Sinha (1969) discussed the possibility that *fpaD* plays a regulatory role in amino acid uptake in *A. nidulans*. However, the finding that dominant mutations at another two loci result in similar phenotypic expressions tends to discredit this theory. It is unlikely that all three loci are involved in regulation. A more plausible explanation is that the loci code for structural proteins which are intimately associated with and shared by acidic, neutral and aromatic amino acid uptake. The products of these three loci are required for normal uptake of these amino acids. As mentioned before this is rather similar to the *Neurospora* situation where mutation at *un-t* or *nap* results in a decreased uptake of neutral, aromatic and acidic amino acids. Moreover the gene products of

bat, hlp-1 and cr-10 are required for basic amino acid transport in Neurospora crassa and gap and apf for general amino acid in yeast. Indeed, it may be that mutation at any one of these loci aauC, aauD or fpaD (and perhaps others presently unknown) results in the production of an abnormal protein which is incorporated into the membrane in such a way that there is distortion of the membrane leading to malfunctioning of certain uptake systems. Moreover, it is also plausible that this type of mutation would be dominant since such an abnormal protein might also be incorporated into the membrane in the heterozygous diploid. Another theory is that aauC, aauD and fpaD code for systems which provide transport energy. This is thought to be improbable since it is likely that this type of mutation would be recessive.

Another noteworthy point is that all classes of uptake mutants isolated, i.e. aauA1, aauB1, aauC1 and aauD1 have normal uptake of basic amino acids. This is probably due to the selection method chosen, i.e. poor growth on L-glutamate as a carbon source. This suggests that basic amino acid uptake is rather similar to the Neurospora situation where basic amino acid seems to be relatively independent of acidic, neutral and aromatic uptake as judged by the uptake patterns of mtr, un-t and nap. It may be possible to isolate a mutant of Aspergillus nidulans rather similar to bat by selecting for resistance to aspartate and/or glutamate hydroxamate.

As a cautionary note in studies of this kind, it should be kept in mind that transport systems may exist which facilitate the exit of nitrogenous compounds as has been found recently in the case of L-glutamate efflux in E. coli (Halpern, Barash and Druck, 1973) and ammonium in Aspergillus nidulans (Pateman and Forbes, 1972). Moreover recent studies (Halpern, Barash, Dover and Druck, 1973) have shown that there is a dependence of L-glutamate transport on the concentration of Na^+ and K^+ ions.

Future research on aau mutants along these lines may invalidate some of the above arguments but clearly similar investigations are required in eucaryotes.

A preliminary report of this work was presented to the Genetical Society (Kinghorn and Pateman, 1972).

Section IV NADP L-glutamate dehydrogenase and ammonium control

1. Isolation and genetic characterisation of gdhA mutants

Nine mutants designated gdhA1 to gdhA9 were isolated by the method of MacKintosh and Pritchard (1963) on the basis of sensitivity to high concentrations of ammonium (see Materials and methods, page 43). Diploids between gdhA1 and other gdhA alleles, and heterokaryons of all possible combinations of gdhA alleles, were made. The mutants were shown to be allelic by their failure to complement in these diploids and heterokaryons. By the technique of haploidisation, it was found that gdhA1 gdhA2 and gdhA9 mutants assort freely with all markers except gal indicating that gdhA is on linkage group III (table 11).

Table 11 : Haploidisation analysis of gdhA⁻ mutants

linkage group	Diploids gene marker	gdhA1 M.S.F. gdhA⁻	gdhA1 M.S.F. gdhA⁺	gdhA2 M.S.F. gdhA⁻	gdhA2 M.S.F. gdhA⁺	gdhA9 M.S.F. gdhA⁻	gdhA9 M.S.F. gdhA⁺
I	y^+	23	5	11	29	20	26
	y^-	10	22	3	9	3	13
II	$Acra^S$	10	6	14	19	18	30
	$Acra^R$	19	25	7	5	4	7
III	gal^+	25	0	34	0	30	0
	gal^-	0	35	1	10	1	26
IV	$pyro^+$	19	6	25	8	16	23
	$pyro^-$	9	26	6	1	1	26
V	fac^+	38	5	16	18	10	20
	fac^-	10	7	4	3	5	22
VI	s^+	39	3	14	19	14	23
	s^-	11	7	6	1	6	20
VII	nic^+	30	11	11	19	23	10
	nic^-	9	10	6	4	10	13
VIII	$ribo^+$	25	14	5	25	19	20
	$ribo^-$	5	16	4	11	1	17

Conclusion :- The strains bil gdhA1, bil gdhA2 and bil gdhA9 are translocation free and the locus gdhA can be assigned to linkage group III.

Mapping gdhA1 mutant by meiotic analysis

By appropriate crosses gdhA1 was located in the argB - galD interval in linkage group III. Results of various crosses are presented here:

cross 1	bil	+	+	+	+	+	gdhA1
				methH	argB	galA	+

Markers bil, y-2 and w-3 being on linkage groups I, I and II respectively were irrelevant for the purpose of this cross, and were, therefore not classified. The results from a hybrid perithecium show the following segregation of markers:

Segregation of markers and allele ratios:

	methH	+	
argB	65	6	71
+	3	81	84
	68	87	155

Recombination fraction :- argB - methH = 5.8% $\pm$ 1.9

	methH	+	
gdhA	10	78	88
+	59	8	57
	69	86	155

Recombination fraction :- methH - gdhA = 18.0% ± 3.1

	argB	+	
gdhA	4	65	69
+	62	14	76
	66	79	155

Recombination fraction :- argB - gdhA = 11.6% ± 2.7

	galA	+	
gdhA	18	79	97
+	51	17	68
	69	96	155

Recombination fraction :- galA - gdhA = 23.2% ± 3.3

	galA	+	
argB	20	61	81
+	53	21	74
	73	82	155

Recombination fraction :- galA - argB = 26.1% ± 3.5

cross 2 $\frac{+}{bil}$ $\frac{y}{+}$ $\frac{paba}{+}$ $\frac{gdhA1 \quad +}{+ \quad meaB6}$

	meaB6	+	
gdhA	4	10	14
+	13	23	36
	17	23	50

Recombination fraction :- meaB - gdhA = 54.1% ± 7.1

cross 3 $\frac{bil}{+}$ $\frac{+}{y\text{-}2}$ $\frac{+}{pabaA}$ $\frac{gdhA1 \quad +}{+ \quad xprD1}$

	xprD	+	
gdhA	1	30	33
+	20	9	29
	21	39	73

Recombination fraction :- xprD - gdhA = 24.6% ± 5.1

A linkage map shows the position of the gdhA locus with respect to other markers in linkage group III.

xprD - - - - methH - - - - argB - - - - gdhA - - - - galA   meaB6

methH ⟷ argB: 5.8 ± 1.9

argB ⟷ gdhA: 11.6 ± 2.7

gdhA ⟷ galA: 23.2 ± 3.3

methH ⟷ gdhA: 18.0 ± 3.1

argB ⟷ galA: 26.1 ± 3.5

xprD ⟷ gdhA: 24.6 ± 5.1

gdhA ⟷ meaB6: 54.1 ± 7.1

It was concluded that the gdhA locus is in the argB - - - galA interval of the linkage group. Derepression of ammonium-repressible activities has also been shown to be a pleiotropic effect of mutations at two loci which are also located in linkage group III (meaB and xprD). The data presented shows that gdhA1 maps independently of these loci; gdhA1 showed approximately 50% recombination with meaB6 and 24% with xprD1. Therefore, it was concluded that gdhA1 is not allelic with meaB6 or xprD1. The phenotypes of the double mutants will be discussed later.

2. NADP-GDH activity in gdhA mutants

Table 12 shows NADP-GDH activity in *gdhA* mutants grown under different conditions. Pateman (1969) has shown that high NADP-GDH activity is found in wild type cells when grown on 10 mM ammonium as a sole nitrogen source. Since *gdhA* mutants grow poorly on this, -N medium plus 10 mM ammonium was supplemented with 0.15% (approx. 10 mM) casamino acids and transferred to -N medium plus 10 mM ammonium only for a period of 3 h. While the wild type has high activity (approximately 2000 nanomoles/min/mg) the *gdhA1*, *gdhA2*, *gdhA3*, *gdhA4* undetectable activity ($<$100 nanomoles/min/mg) and *gdhA5* to *A9* from 10% to 20% wild type NADP-GDH activity.

The heterozygous diploid *gdhA1*/+ has similar NADP-GDH activity to the haploid $gdhA^{+}$ indicating that the *gdhA1* mutation is recessive.

When wild type cells are grown on 1.5% casamino acids as a sole carbon and nitrogen source there is undetectable NADP-GDH activity (see page 201). The *gdhA* mutants also have similar activities under these conditions.

Table 12 : NADP-GDH activity in *gdhA mutants*

(a) Growth conditions :- -N medium + 0.15% casamino acids + 10 mM ammonium

Treatment :- -N medium + 10 mM ammonium for 3 h

(b) Growth conditions :- -CN medium + 1.5% casamino acids

Treatment :- none

Strains	Enzyme activity nanomole/min/mg protein	
	Growth condition and greatment (a)	Growth condition and treatment (b)
wild type	1940	<100
gdhA1	<100	<100
gdhA2	<100	<100
gdhA3	<100	<100
gdhA4	<100	<100
gdhA5	310	<100
gdhA6	350	<100
gdhA7	320	<100
gdhA8	210	<100
gdhA9	390	<100
gdhA1/+	1810	<100

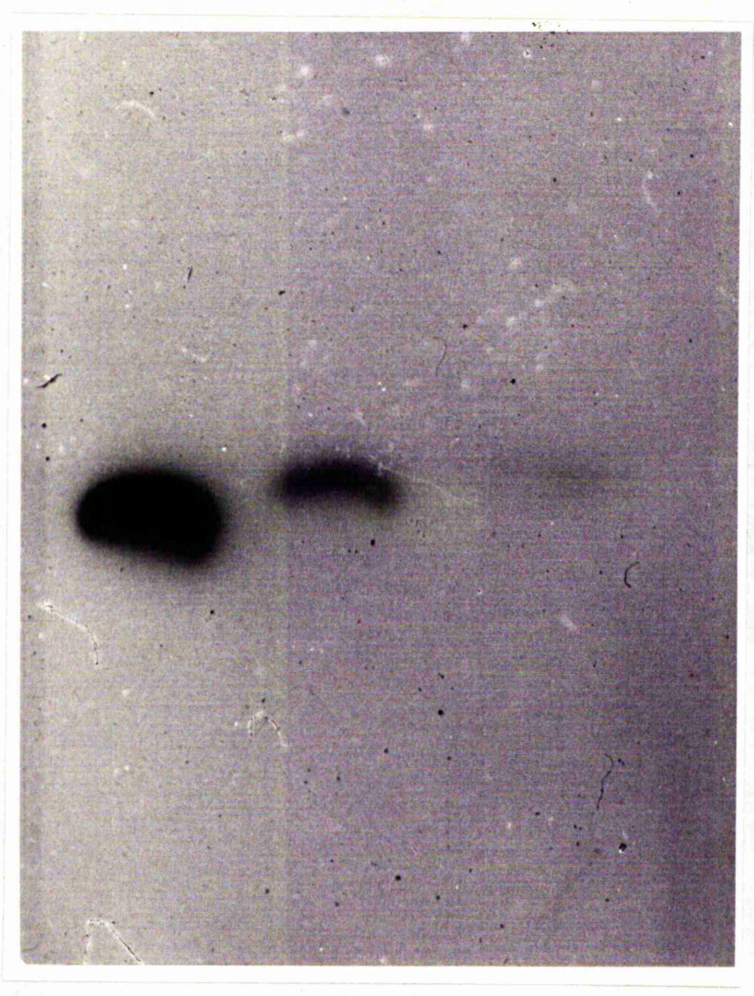
3. Electrophoretic mobility of mutant NADP-GDH proteins

From plate 17 depicting a starch gel stained specifically for NADP-GDH activity, it can be seen that there is only one band of activity in the wild type strain. Only the mobilities of three mutant proteins are shown for clarity, namely gdhA1 which shows no detectable NADP-GDH as judged by enzyme assays, gdhA8 - the least detectable activity and gdhA9 - maximum detectable activity. No band was observed for gdhA1 while a faint band for gdhA8 and a heavier band for gdhA9. A direct comparison of activity with wild type cannot be made since the concentration of mutant extracts were approximately four times that of the wild type. The bands of mutant activity appeared at approximately the same position as the wild type, thus showing that the wild type, gdhA9 and gdhA8 (and also gdhA7, gdhA6 and gdhA5) have similar NADP-GDH electrophoretic mobilities.

Plate 17 Electrophoretic mobility of mutant NADP-GDH protein

growth conditions :- -N medium + 0.15% casamino acids + 10 mM ammonium

treatment :- -N medium + 10 mM ammonium



wild type *gdhA9* *gdhA8* *gdhA1*

4. NAD-GDH activity in *gdhA* mutants

Table 13a shows that NAD-GDH activity in *gdhA* mutants is unaffected. Grown under a condition which gives maximum wild type activity (see page 200) namely on 1.5% casamino acids as a sole carbon and nitrogen source the mutants have wild type activity. When grown on -N medium 0.15% casamino acids plus 10 mM ammonium and transferred to -N medium plus 10 mM ammonium for periods up to 24 h the mutants have undetectable levels of activity like the wild type (table 13b). This is quite different from the situation in *Neurospora crassa* where *am* mutants 'adapt' after a lag by synthesising NAD-GDH (Ahmed and Sanwal, 1967).

Table 13a : NAD-GDH activity in *gdhA* mutants

Growth conditions :- -CN medium + 1.5% casamino acids

Treatment :- none

Strains	Enzyme activity nanomole/min/mg protein
wild type	2520
gdhA1	2610
gdhA2	2600
gdhA3	2300
gdhA4	2490
gdhA5	2590
gdhA6	2210
gdhA7	2430
gdhA8	2630
gdhA9	2690
gdhA1/+	2630

Table 13b : NAD-GDH activity in gdhA mutants

Growth conditions :- -N medium + 0.15% casamino acids
+ 10 mM ammonium

Treatment :-
(a) -N medium + 10 mM ammonium for 3 h
(b) -N medium + 10 mM ammonium for 6 h
(c) -N medium + 10 mM ammonium for 12 h
(d) -N medium + 10 mM ammonium for 24 h

Strains	Enzyme activity nanomole/min/mg protein			
	Treatments			
	(a)	(b)	(c)	(d)
wild type	<100	<100	<100	<100
gdhA1	<100	<100	<100	<100
gdhA2	<100	<100	<100	<100
gdhA3	<100	<100	<100	<100
gdhA4	<100	<100	<100	<100
gdhA5	<100	<100	<100	<100
gdhA6	<100	<100	<100	<100
gdhA7	<100	<100	<100	<100
gdhA8	<100	<100	<100	<100
gdhA9	<100	<100	<100	<100
gdhA1/+	<100	<100	<100	<100

5. Growth responses of gdhA mutants

The gdhA strains were tested for growth on a number of certain inorganic and amino acids as sole nitrogen sources. The results presented in table 14 show that the gdhA mutants grow equally poorer than wild type on inorganic nitrogen sources, i.e. 10 mM nitrate urea or ammonium (plate 18). The addition of 100 mM glycine to 10 mM ammonium did not significantly change the growth responses of gdhA mutants with the exception of gdhA4; glycine has an inhibitory effect on NADP-GDH minus mutants in Neurospora crassa (Pateman and Fincham, 1965). The growth difference between gdhA and wild type was more extreme on 200 mM ammonium or urea (plate 19). gdhA mutants grow as wild type on 10 mM L-glutamate (plate 20) and certain other amino acids, e.g. L-aspartate, L-alanine, L-arginine, L-asparagine, or L-glutamine as sole nitrogen source. gdhA mutants are repaired by the addition of 10 mM glutamate to -N medium + 10 mM ammonium, but not to -N medium + 200 mM ammonium. gdhA/+ grows as wild type with 10 mM ammonium and 200 mM ammonium again indicating the recessivity of the gdhA1 mutation.

Table 14 : Growth responses of gdhA mutants with certain nitrogen sources

-N medium (solid)	genotypes										
	wild type	gdhA1	gdhA2	gdhA3	gdhA4	gdhA5	gdhA6	gdhA7	gdhA8	gdhA9	gdhA1/+
10 mM ammonium	+	±	±	±	±	±	±	±	±	±	+
10 mM nitrate	+	±	±	±	±	±	±	±	±	±	+
10 mM urea	+	±	±	±	±	±	±	±	±	±	+
10 mM L-glutamate	+	+	+	+	+	+	+	+	+	+	+
10 mM L-aspartate	+	+	+	+	+	+	+	+	+	+	+
10 mM L-alanine	+	+	+	+	+	+	+	+	+	+	+
10 mM L-arginine	+	+	+	+	+	+	+	+	+	+	+
10 mM L-asparagine	+	+	+	+	+	+	+	+	+	+	+
10 mM L-glutamine	+	+	+	+	+	+	+	+	+	+	+
200 mM ammonium	+	−	−	−	−	−	−	−	−	−	+
200 mM urea	+	−	−	−	−	−	−	−	−	−	+
10 mM ammonium + 10 mM L-glutamate	+	+	+	+	+	+	+	+	+	+	+
200 mM ammonium + 10 mM L-glutamate	+	−	−	−	−	−	−	−	−	−	+
10 mM ammonium + 100 mM glycine	+	±	±	±	−	±	±	±	±	±	+

+ = wild type growth

± = approximately 25 - 50% of the wild type

− = extremely poor growth

Plate 18 Growth response of gdhA1 with 10 mM ammonium as sole nitrogen source

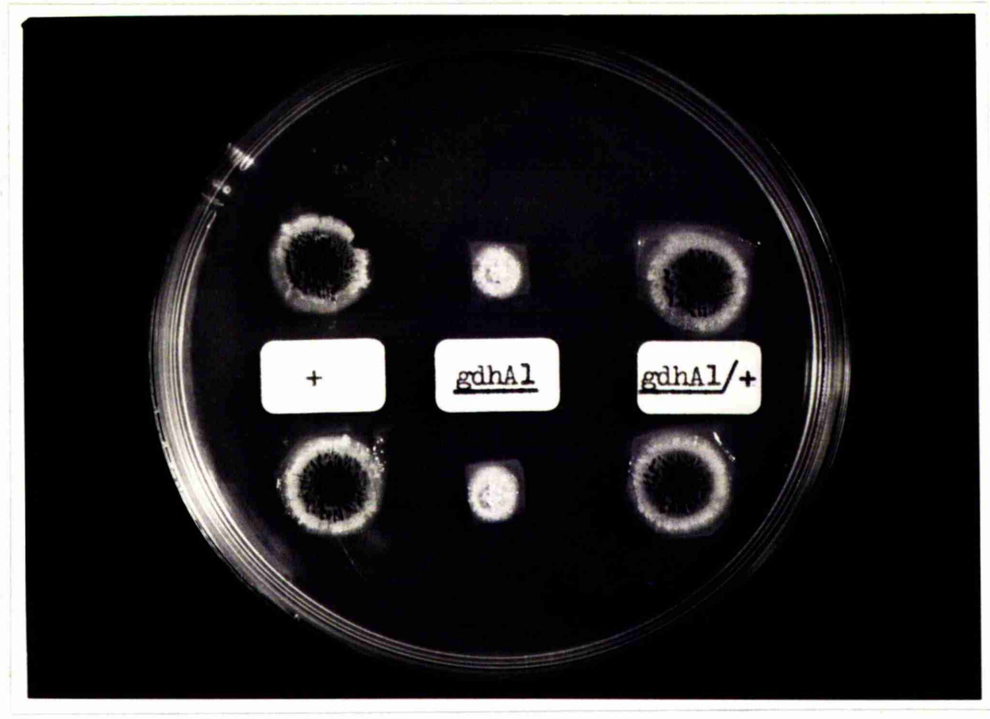


Plate 19 Growth response of gdhA1 with 200 mM ammonium as sole nitrogen source

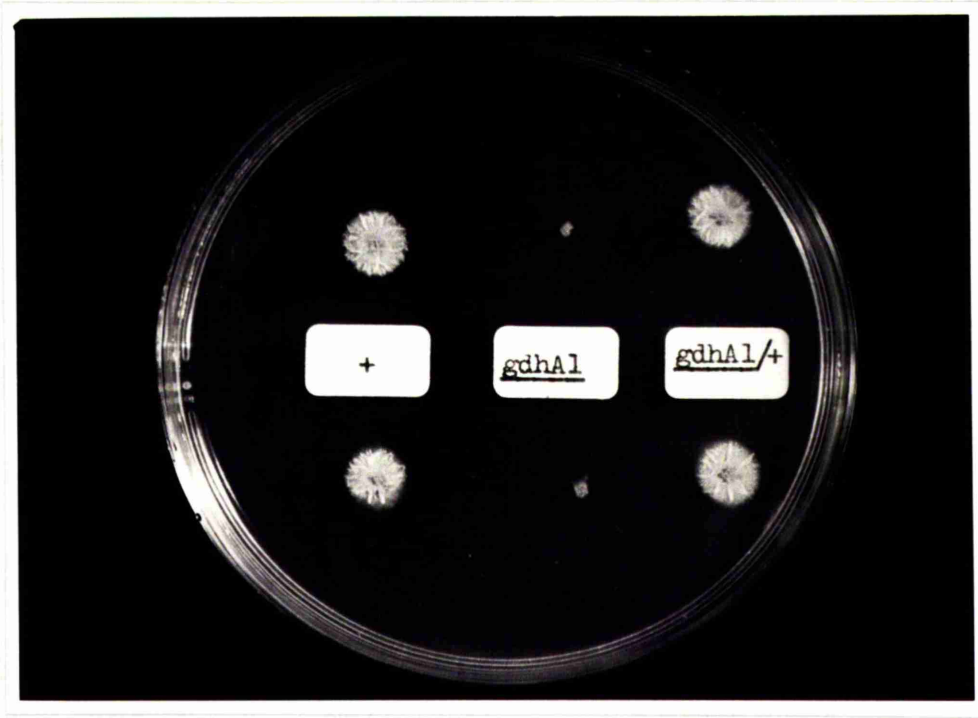
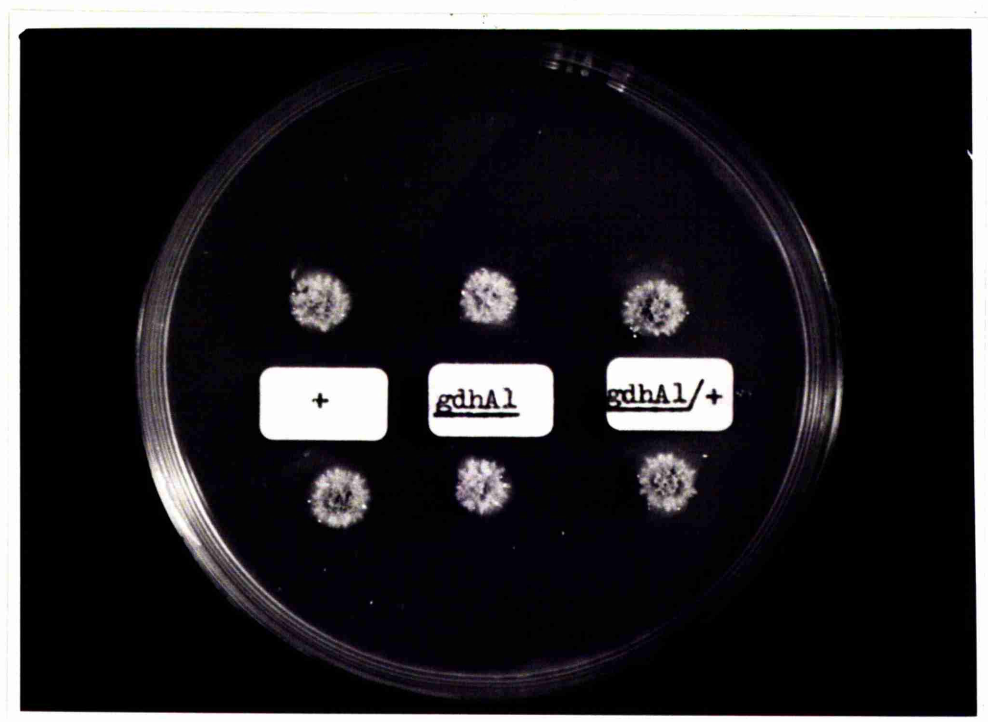


Plate 20 Growth response of gdhA1 with 10 mM L-glutamate as sole nitrogen source



6. The gdhA mutation and ammonium derepression

(a) Plate tests

A number of plate tests have been devised (see Materials and methods, page 34) to enable quick screening of the ammonium repression status of nitrate reductase, nitrite reductase, xanthine dehydrogenase, extracellular protease, asparaginase, adenine-quanine uptake system, and the thiourea uptake system. The results presented in table 15 show that the gdhA mutants are derepressed for nitrate reductase (plate 21), nitrite reductase, xanthine dehydrogenase, asparaginase, adenine-quanine uptake system and urea uptake (plate 22) but are repressed for extracellular protease. gdhA1 appears to be recessive with respect to ammonium control as judged by plate tests.

Table 15 : Plate tests of ammonium derepression in gdhA mutants

	genotypes										
	wild type	gdhA1	gdhA2	gdhA3	gdhA4	gdhA5	gdhA6	gdhA7	gdhA8	gdhA9	gdhA1/+
nitrate reductase ⊙	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	+(R)
nitrite reductase ⊙	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	+(R)
extracellular protease *	+(R)	+(R)	+(R)	+(R)	+(R)	+(R)	+(R)	+(R)	+(R)	+(R)	+(R)
xanthine dehydrogenase o	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	+(R)
adenine-quanine uptake system ⊙	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	+(R)
urea uptake system ⊙	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	+(R)
asparaginase ⊙	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)	+(R)

⊙ nitrate reductase, nitrite reductase, asparaginase, urea and adenine-quanine uptakes.

+ = wild type growth, repressed by ammonium (R)
- = extremely poor growth, not repressed by ammonium (D)

* extracellular protease

+ = no halo produced, repressed by ammonium (R)
- = halo produced, not repressed by ammonium (D)

o xanthine dehydrogenase

+ = green conidia, repressed by ammonium (R)
- = yellow/white conidia, not repressed by ammonium (D)

Plate 21 Protection against chlorate toxicity by ammonium

(Plate test for ammonium regulation of nitrate reductase)

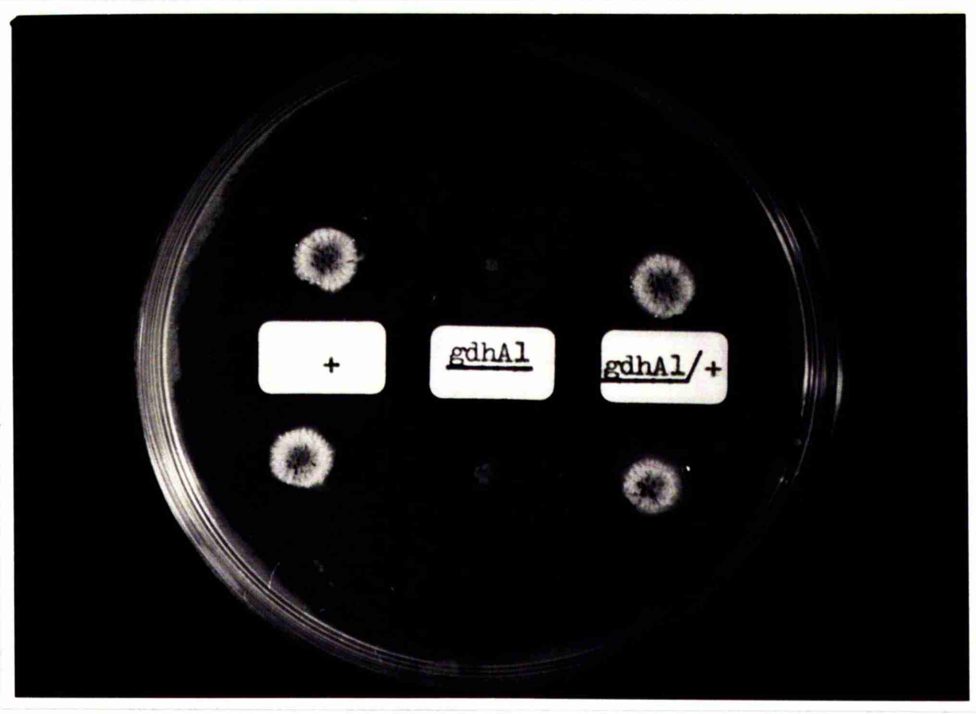
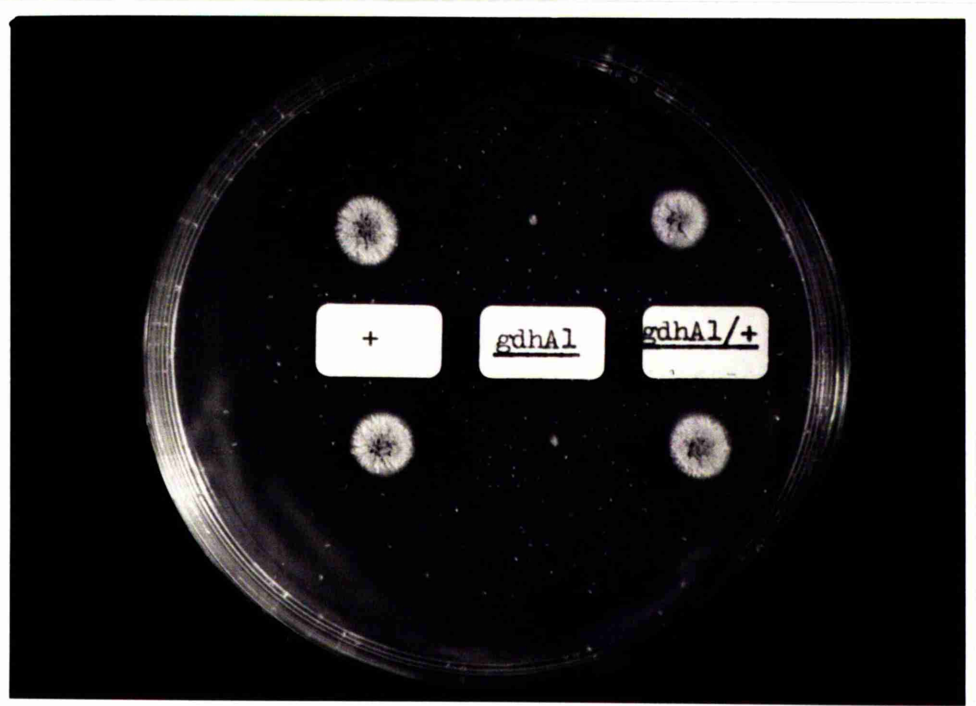


Plate 22 Protection against thiourea toxicity by ammonium

(Plate test for ammonium regulation of urea uptake)



(b) Enzyme and uptake assays

The wild type strain was shown to be repressed for nitrate reductase, urea and L-glutamate uptake (tables 16 and 17). The *gdhA* strains are approx. 40 - 50% ammonium derepressed for nitrate reductase and L-glutamate and 80% for urea uptake. The difference in derepressed levels between *gdhA1* and *gdhA9* do not appear to be significant.

The heterozygous diploid *gdhA1*/+ again is recessive.

Table 16 : Ammonium repression of nitrate reductase, glutamate and urea uptake in wild type, gdhA1 and gdhA9 strains

Growth conditions	Treatment	System	genotypes			
			wild type	gdhA1	gdhA9	gdhA1/+
-N medium + .15% casamino acids + 10 mM nitrate	-N medium + 10 mM nitrate	nitrate + reductase	120	98	84	111
+ .15% casamino acids + 10 mM nitrate + 10 mM ammonium	+ 10 mM nitrate + 10 mM ammonium	nitrate + reductase	4	45	39	4
+ .15% casamino acids	-N medium	L-glutamate uptake *	1.05	1.09	1.20	1.02
+ .15% casamino acids + 10 mM ammonium	+ 10 mM ammonium	L-glutamate uptake *	.06	0.41	0.56	0.07
+ .15% casamino acids	-N medium	urea uptake *	1.0	1.1	1.25	1.02
+ .15% casamino acids + 10 mM ammonium	+ 10 mM ammonium	urea uptake *	.05	.90	1.02	.09

+ results expressed as nanomoles/min/mg protein

* results expressed as nanomoles/mg wet weight after 10 min.

Table 17 : Percentage ammonium derepression of nitrate reductase urea and L-glutamate uptake

genotypes / system	wild type	gdhA1	gdhA9	gdhA1/+
nitrate reductase	3.3	40	52	3.6
L-glutamate uptake	5.7	37	53	6.8
urea uptake	5.0	81	81	8.8

7. Methylammonium toxicity and sensitivity

Two of the five classes of ammonium derepressed mutants, meaA and meaB are resistant to the toxic ammonium analogue methylammonium (Arst and Cove, 1969). The results presented in table 18 show that gdhA mutants are not resistant to 1M methylammonium. Moreover, at 50 mM methylammonium, a concentration at which the wild type is resistant, gdhA mutants are hypersensitive.

Table 18 : Methylammonium toxicity

Strains	Resistance (1M methyl-ammonium)	Hypersensitivity (50 mM methyl-ammonium)
wild type	-	+
meaA8	+	+
meaB6	+	+
gdhA1	-	-
gdhA2	-	-
gdhA3	-	-
gdhA4	-	-
gdhA5	-	-
gdhA6	-	-
gdhA7	-	-
gdhA8	-	-
gdhA9	-	-
gdhA1/+	-	+

+ = growth
- = poor growth

8. NADP-GDH activity in other classes of ammonium derepressed mutants

Since it was found that mutations at the *gdhA* locus resulted in loss of NADP-GDH and simultaneous loss of ammonium control, it was necessary to establish the level of NADP-GDH activity in other classes of derepressed mutants. It can be seen from table 19 that *meaA8*, *meaB6*, *xprD1*, *amrA1* and DER-3 have relatively wild type activities. The only class of derepressed mutants which has low NADP-GDH is the ammonium sensitive class, the *gdhA* mutants.

Table 19 : NADP-GDH activity in other classes of ammonium derepressed mutants

Growth conditions	-N medium + 0.15% casamino acids + 10 mM ammonium	-CN medium + 1.5% casamino acids
treatment	-N medium + 10 mM ammonium for 3 h	-
Strains	Enzyme activity nanomole/min/mg	
wild type	1900	<100
gdhA1	<100	<100
meaA8	1790	<100
meaB6	1520	<100
xprD1	1840	<100
amrA1	2050	<100
DER-3	1620	<100

9. Interaction between gdhA and other ammonium derepressed loci

(a) meaA locus. The results of a cross (gdhA x meaA8) presented in table 20 show that there is some interaction between methylammonium resistance (due to mutation at the meaA locus) and ammonium sensitivity (due to mutation at gdhA). The phenotype of the double mutant class is resistance to methylammonium and partial resistance to high ammonium. Table 23 shows that there is no detectable NADP-GDH in this class of recombinants. Moreover each phenotype class was recovered when one recombinant of this was outcrossed. The growth responses of the double mutant are the same as that of gdhA except for high ammonium sensitivity (table 21). The double mutant is similar to gdhA and meaA8 in ammonium repression characteristics as shown by plate tests (table 22).

Table 20 : Segregation of methylammonium resistance and ammonium sensitive markers in a gdhA1 x meaA8 cross

Genotypes of parents	No. of colonies analysed	genotypes considered	Segregation to parental		Segregation to non-parental	
			+ −	+ −	+ +	− −
			R/R	S/S	R/S	PS/R
			meaA8	gdhA	wild type	double mutant
bil gdhA1 x y pyro meaA8	153	gdhA-meaA8	35	41	39	38

Table 21 : Growth responses of the gdhA1 meaA8 double mutant

-N medium (solid)	wild type	gdhA1	meaA8	recombinant 1
10 mM urea	+	±	+	±
10 mM ammonium	+	±	+	±
10 mM nitrate	+	±	+	±
10 mM L-glutamate	+	+	+	+
10 mM L-aspartate	+	+	+	+
200 mM ammonium	+	-	+	±

\+ = wild type growth

± = approximately 50% of the wild type

\- = extremely poor growth

Table 22 : Ammonium repression in gdhA1, meaA8 double mutant

(plate tests)

System		wild type	gdhA	meaA8	recombinant 1
nitrate reductase	⊚	+(R)	-(D)	-(D)	-(D)
nitrite reductase	⊚	+(R)	-(D)	-(D)	-(D)
extracellular protease	*	+(R)	+(R)	+(R)	+(R)
xanthine dehydrogenase	o	+(R)	-(D)	-(D)	-(D)
adenine-guanine uptake system	⊚	+(R)	-(D)	-(D)	-(D)
urea uptake system	⊚	+(R)	-(D)	-(D)	-(D)
asparaginase	⊚	+(R)	-(D)	-(D)	-(D)

⊚ nitrate reductase, nitrite reductase, asparaginase, urea and adenine-guanine uptake

+ = wild type growth, repressed by ammonium (R)
- = extremely poor growth, not repressed by ammonium (D)

* extracellular protease

+ = no halo produced, repressed by ammonium (R)
= = halo produced, not repressed by ammonium (D)

o xanthine dehydrogenase

+ = green conidia repressed by ammonium (R)
- = yellow conidia not repressed by ammonium (D)

Table 23 : NADP-GDH activity in one class of recombinants recovered from a gdhA1 x meaA8 cross

Growth conditions :- -N medium + .15% casamino acids + 10 mM ammonium

Treatment :- -N medium + 10 mM ammonium for 3 hours

Strain	NADP-GDH activity nanomoles/min/mg protein
wild type	2100
gdhA1	<100
recombinant 1	<100
" 10	<100
" 52	<100
" 63	<100
" 68	<100
" 94	<100
" 103	<100
" 119	<100
" 121	<100

(b) meaB locus. Recombinants were isolated which had the double phenotype methylammonium resistance and high ammonium sensitivity (table 24). This class of recombinants lacked NADP-GDH activity (table 27) and when outcrossed each phenotype class was recovered. The gdhA1, meaB6 double mutant grows as gdhA1 on inorganic nitrogen (table 25). It is also apparently derepressed for ammonium repressible systems including extracellular protease release (table 26), unlike meaB6 and gdhA1 mutants which retain the ability to repress protease (plate 23 - xprD1 is a control for ammonium derepression.) However, it is difficult to differentiate between loss of ammonium regulation with respect to extracellular protease and a premature autolytic state since the double mutants autolyse relatively quickly leaving a large hole in the centre of the colony (plate 24). This may be a result of an early release of proteases, ribonucleases, etc.

The recombination fraction (approx. 44%) found by this cross confirms an earlier result (page 146) that meaB and gdhA are unlinked loci.

Table 24 : Segregation of methylammonium resistant and ammonium sensitive markers in a gdhA1 x meaB6 cross

Genotypes of parents	No. of colonies analysed	genotypes considered	Segregants: parental		non-parental	
			+ − R/R meaB6	− + S/S gdhA1	+ + R/S wild type	− − S/R double mutant
bi1 meaB6 x y pyro gdhA1	92	gdhA1−meaB6	27	31	35	9

Recombination fraction :− 44.4% ± 5.2

Table 25 : Growth responses of gdhA1,meaB6 double mutants

-N medium (solid)	wild type	gdhA1	meaB6	recombinants 2	5	22	34
10 mM urea	+	∓	+	±	±	±	±
10 mM ammonium	+	∓	+	±	±	±	±
10 mM nitrate	+	∓	+	±	±	±	±
10 mM L-glutamate	+	+	+	+	+	+	+
10 mM L-aspartate	+	+	+	+	+	+	+
10 mM L-alanine	+	+	+	+	+	+	+
10 mM L-asparagine	+	+	+	+	+	+	+
10 mM L-glutamine	+	+	+	+	+	+	+
200 mM ammonium	+	-	+	-	-	-	-

+ = wild type growth

± = approximately 50% of the wild type

\- = extremely poor growth

Table 26 : Plate tests for ammonium repression in gdhA, meaB6 double mutants

test	wild type	gdhA1	meaB6	recombinants 2	5	22	34
nitrate reductase ⊚	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)
nitrite reductase ⊚	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)
extracellular protease *	+(R)	+(R)	+(R)	-(D)	-(D)	-(D)	-(D)
xanthine dehydrogenase o	+(R)	-(D)	-(D)	-(D)	-(D)	-(D)	-(D)
adenine-quanine uptake system ⊚	+(R)	-(D)	+(D)	-(D)	-(D)	-(D)	-(D)
urea uptake system ⊚	+(R)	-(D)	+(R)	-(D)	-(D)	-(D)	-(D)
asparaginase ⊚	+(R)	-(D)	+(R)	-(D)	-(D)	-(D)	-(D)

⊚ nitrate reductase, nitrite reductase, asparaginase, urea and adenine-quanine uptake

+ = wild type growth, repressed by ammonium (R)
- = extremely poor growth, not repressed by ammonium (D)

* extracellular protease

+ = no halo produced, repressed by ammonium (R)
- = halo produced, not repressed by ammonium (D)

o xanthine dehydrogenase

+ = green conidia repressed by ammonium (R)
- = yellow conidia not repressed by ammonium (D)

Table 27 : NADP-GDH activity in recombinants with the gdhA, meaB double phenotype

Growth conditions :- -N medium + 0.15% casamino acids + 10 mM ammonium

Treatment :- -N medium + 10 mM ammonium for 3 h

Strain	NADP-GDH activity nanomole/min/mg protein
recombinant No. 22	< 100
" 5	< 100
" 34	< 100
" 2	< 100
wild type	2100
gdhA1	< 100

Plate 23 Ammonium repression of extracellular protease in the gdhA1, meaB6 double mutant

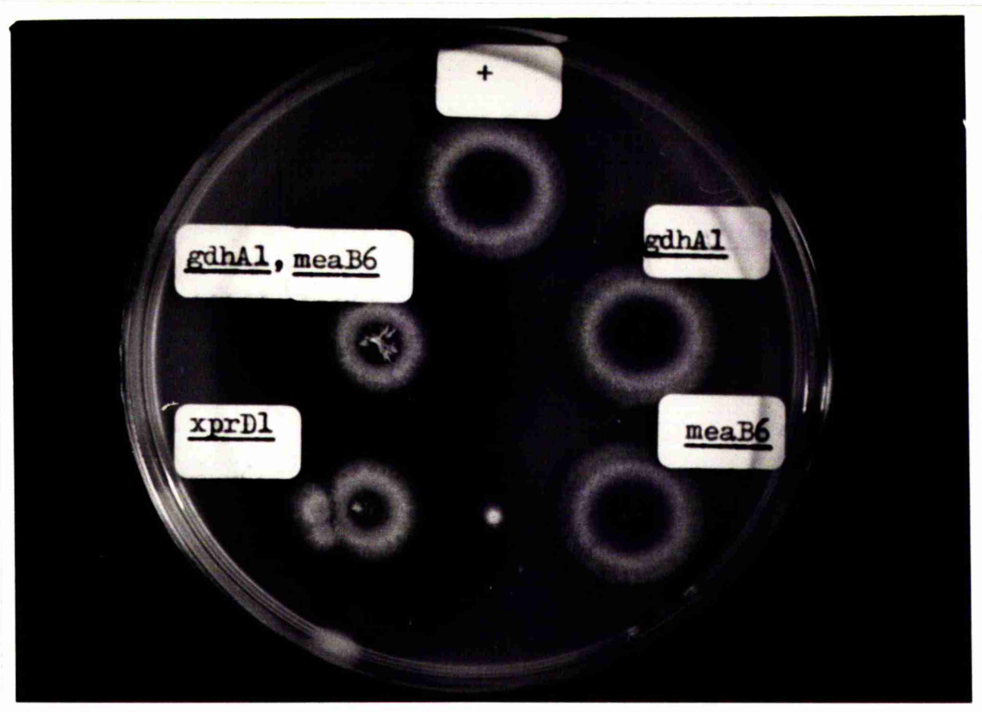


Plate 24 Morphology of the gdhA1, meaB6 double mutant

(c) *xprD* locus. From the results of a cross between *y paba xprD* x *bil gdhA1* (page 146) *gdhA1* showed approximately 24% recombination with *xprD1*. However since 20% of the progeny were 'grotty' colonies, i.e. colonies which are very small and lack asexual reproductive structures, little confidence is placed upon this recombination figure. These 'grotties' only grew on complete medium and were impossible therefore to investigate any further. Only one recombinant with the double phenotype was recovered. This proved to be lacking in NADP-GDH activity. The *gdhA1*, *xprD1* double mutant grew extremely poorly on complete medium and is ammonium derepressed for extracellular protease and also sensitive to high concentrations of ammonium. Cohen (personal communication) also found similar low recoveries of double mutants in *gdhA1* x *xprD1* crosses.

Another cross was set up between *nicB8 riboA2 xprD1* and *bil gdhA1* to investigate the possibility that the 'grotties' are a property of the *y paba xprD1* strain. Again, grotties at a frequency of 20% of the total progeny appeared, but recombinants with the double phenotype were not recovered (table 28). The recovery of 'grotties' may be an aberration in the *xprD1* strain and is likely to be separate from the *xprD* phenotype (Cohen, 1973 - manuscript in preparation).

Table 28 : Segregation of extracellular protease derepression and ammonium sensitivity in a gdhA1 x xprD1 cross

genotypes of parents	No. of colonies analysed	genotypes considered	Segregants: parental + − R/Der. gdhA1	parental − + S/Rep. xprD1	non-parental + + R/Rep. wild type	non-parental − − R/Deer. double mutant
bi1 gdhA1 x ribo2 nicB8 xprD1	96	gdhA1-xprD1	44	41	11	0

Recombination fraction = 22.9% ± 4.3

(d) amrA locus. Mutation at the amrA1 locus results in pleiotropic loss of ammonium regulation for a number of systems regulated by ammonium and simultaneous impaired uptake of certain nitrogen sources such as thiourea (urea) and methylammonium (Pateman - personal communication) and L-glutamate (see page 73). amrA1, as a consequence of low uptake of thiourea, is able to grow better than the other ammonium derepressed mutants on -N medium plus 10 mM thiourea and 10 mM ammonium. This characteristic was made use of when scoring for amrA1. Table 29 shows that the double phenotype is not recovered in a gdhA1 x amrA1 cross. Since there was a significantly high number of single amrA1 phenotype recombinants recovered from this cross a number of these were screened for gdhA activity (table 30). Some were found to lack NADP-GDH and it was concluded that the amrA1 mutation confers resistance upon gdhA1 to high concentrations of ammonium.

Table 29 : Segregation of thiourea resistance and ammonium sensitivity in a gdhA1 x amrA1 cross

genotypes of parents	No. of colonies analysed	genotypes considered	Segregants: parental + + gdhA1	Segregants: parental + − amrA1	Segregants: non-parental + + wild type	Segregants: non-parental − − double mutant
bi1 gdhA1 x yA2 pyroA4 amrA1	128	gdhA1-amrA1	21	66	41	0

Table 30 : NADP-GDH activity in the recombinant class with the amrA1 phenotype

Growth conditions :- -N medium + 0.15% casamino acids + 10 mM ammonium

Treatment :- -N medium + 10 mM ammonium for 3 h

Recombinant No.	NADP-GDH activity nanomole/min/mg protein
wild type	2150
gdhA1	<100
6	<100
8	<100
20	175
34	1620
91	1950
73	<100
52	<100

10. Attempts to isolate temperature sensitive mutations in the *gdhA* gene

The approach was to isolate new *gdhA* strains which were able to grow on 200 mM ammonium at 25°C but not at 37°C (see Materials and methods, page 43). This work was carried out to establish if (1) temperature sensitive *gdhA* mutants are also temperature sensitive for ammonium derepression and (2) the *gdhA* locus is the structural gene locus for NADP-GDH. 600 revertants which were able to grow on 200 mM ammonium at 25°C were isolated, but all except one were able to grow on this medium at 37°C. It was found that this exception did not have temperature sensitive NADP-GDH activity. I have been unable, therefore, to find any evidence which would establish any of the above objectives. However, a noteworthy point yielded by this line of investigation was that a large number of revertants on high concentrations of ammonium were also methylamine resistant and remained derepressed for ammonium regulated systems. Further investigation revealed that these were double mutants *gdhA*, *meaA8*. This find adds support to the claim (see page 167) that the *meaA8* mutation confers resistance upon *gdhA1* to high concentrations of ammonium.

11. Cellular localisation of NADP L-glutamate dehydrogenase

To determine if the NADP-GDH activity locates in the mitochondrial or cytoplasmic fraction, mycelium was disrupted and a cell fractionation carried out. Table 31 shows the activities of NADP-GDH in soluble and insoluble fractions. Malate dehydrogenase, (MDH), reported to have been found in mitochondria of certain organisms including fungi (Munkres, Giles and Case, 1965) was used as a mitochondrial fraction control. It was found that the insoluble fraction contained relatively high malate dehydrogenase activity but undetectable NADP-GDH activity. Most of the NADP-GDH activity appeared to reside in the cytoplasm and this is rather similar to the situation found in *Neurospora crassa* (Flavell and Woodward, 1971).

Table 31 : Cellular location of NADP-GDH

fraction	nanomoles/ml extract	
	MDH	NADP-GDH
soluble	220	2820
insoluble	520	<100

12. The free amino acid pool in wild type cells

The possible involvement of glutamate itself in regulation was investigated by determining the glutamate pool size under varying carbon and nitrogen conditions. The aspartate and L-alanine pool sizes were also computed from the results for two reasons - (a) these were more than 5 μmole/mg dry weight cells. (All other amino acids except glutamate and arginine were found below 1 μmole/gm dry weight except when grown on that amino acid as a sole nitrogen source). (b) changes in aspartate and alanine pools are more likely to affect the size of the glutamate pool than other amino acids since aspartate and alanine can be converted to glutamate by one step transamination reactions.

One of the interesting features which emerged from this survey was that the concentration of free intracellular glutamate was higher than any other amino acid (table 32). Another point which is relevant to later discussion is the relative consistency of the glutamate pool in cells grown on -N medium plus 10 mM ammonium and grown on -N medium plus 10 mM L-glutamate. One discrepancy in the glutamate pool size may have arisen over spontaneous breakdown of glutamine to glutamate and ammonium during the extraction procedure.

However, this is not thought to be serious and rather overestimates the glutamate pool size in all cases. In contrast to glutamate, the pool sizes of aspartate and alanine could be increased considerably, in some cases 10-12 fold when the cells were grown in aspartate and alanine respectively.

Table 32 : The free amino acid pool in wild type cells

Growth conditions	Treatment	µ moles/gm dry weight		
		aspartate	alanine	glutamate
-N medium +				
10 mM urea	-	9	31	65
10 mM ammonium	-	8	28	58
10 mM nitrate	-	7	47	54
10 mM L-glutamate	-	7	58	69
10 mM L-glutamine	-	10	38	74
10 mM L-arginine	-	15	35	76
10 mM L-aspartate	-	98	55	30
0.15% casamino acids	-	5	40	60
10 mM L-alanine	-	6	95	99
	-CN medium +			
10 mM urea	100 mM aspartate for 5 h	142	35	27
10 mM urea	100 mM L-alanine for 5 h	29	92	85
10 mM urea	100 mM L-glutamate for 5 h	8	58	78
10 mM urea	10 mM urea for 5 h	7	38	59

13. Discussion

The gdhA mutants, isolated on the basis of sensitivity to high concentrations of ammonium are defective in NADP-GDH activity. Four of the mutants gdhA1-A4 have activity below the limit of detection while low but detectable activity can be demonstrated in gdhA5-A9. Indeed, gdhA9 has 20% activity of that of the wild type. The NADP-GDH activity of gdhA5-A9 can be demonstrated by starch gel electrophoresis and this activity shows that the mutant proteins have wild type electrophoretic mobilities. All the gdhA mutants grow less than wild type on inorganic nitrogen sources, e.g. nitrate, ammonium and urea, presumably because they are unable to utilise ammonium efficiently for the synthesis of L-glutamate and require a supplement of amino acid for growth. This explanation is supported by the fact that the mutants grow as wild type on all amino acids tested as sole nitrogen sources. When gdhA mutants are grown on -N medium plus an inorganic nitrogen source, NAD-GDH is not synthesised and therefore does not substitute for the lack of NADP-GDH activity. Thus, it is difficult to explain the leakiness of gdhA mutants when grown on 10 mM ammonium. Perhaps there are residual levels of other amination reactions, e.g. aspartase, which help the mutants to grow in the absence of the primary amination step.

The gdhA mutants are completely unable to grow on high concentrations of ammonium. Moreover, whereas the addition of 10 mM L-glutamate to low concentrations of ammonium results in wild type growth of gdhA strains, this addition to high concentrations does not. This suggests that 200 mM ammonium is positively toxic to gdhA strains. Perhaps sensitivity to ammonium is connected with the fact that gdhA mutants have a higher intracellular ammonium pool size than the wild type in the presence of high concentrations of extracellular ammonium or urea (Pateman, Kinghorn, Dunn and Forbes, 1972; Pateman and Kinghorn, 1972). This high ammonium pool size may be due to one or more of the following: (a) inability of gdhA mutants to utilise ammonium efficiently as discussed above; (b) the fact that gdhA mutants are derepressed for ammonium uptake itself (Pateman et al., 1973); (c) impairment in the ammonium efflux system. Pateman and Forbes (1972) have shown that A. nidulans wild type can efflux ammonium and at least one class of ammonium derepressed mutants is impaired in this process. However, some other ammonium derepressed mutants, e.g. xprD1 and amrA1 also have high intracellular ammonium pools but are not ammonium-sensitive (Pateman and Kinghorn, 1972).

Unlike the other classes of ammonium derepressed mutants gdhA strains are super-sensitive to low concentrations of methylammonium.

In contrast, two classes of derepressed mutants, namely meaA8 and meaB6 are resistant to high concentrations. The reason for methylammonium ~~resistance~~ toxicity is unknown at present and therefore it is not possible to give a reasonable explanation of this super-sensitivity effect. It is likely that it is again connected with the fact that gdhA mutants have higher ammonium and therefore methylammonium pool sizes than the wild type resulting in higher sensitivity to methylammonium.

It is of more than peripheral interest to compare the phenotype of gdhA mutants with NADP-GDH deficient mutants (designated am) in Neurospora crassa. Beadle and Tatum, more than twenty years ago, isolated amination deficient mutants in Neurospora crassa. The phenotypes, genetics and complementation patterns of these mutants have been described by Fincham and co-workers in a series of papers (Fincham, 1950; Fincham, 1959; Fincham and Coddington, 1963; Fincham and Stadler, 1965; Pateman and Fincham, 1965). These mutants, which map at the am-1 locus show a growth lag on ammonium and Ahmed and Sanwal (1967) suggest that this adaptive phenomenon is due to an increase of NAD-GDH activity. The growth difference between wild type and am-1 strains can be sharpened up by the addition of 100 mM glycine to the ammonium medium (Pateman and Fincham, 1965). Aspergillus nidulans gdhA differ from am-1 mutants in certain respects:

(a) the *gdhA* strains do not appear to have a well defined growth lag on low concentrations of ammonium; (b) *gdhA* strains fail to synthesise NAD-GDH even after 38 hours growth; (c) the *gdhA* mutants are not sensitive to glycine except for *gdhA4*; (d) the *am-1* strains are not so sensitive to high concentrations of ammonium as the *gdhA* mutants (Kinghorn, unpublished work). It is possible that the differences in growth responses of mutants lacking NADP-GDH activity in these evolutionarily rather similar micro-organisms may be due to the differences in NAD-GDH activity.

As mentioned above *gdhA4* appears to be sensitive to glycine unlike the other *gdhA* mutants. The reason for this is not clear. Moreover no other differences between this and the other mutants were observed.

The *gdhA* mutation, as well as resulting in abnormal NADP-GDH activity, simultaneously results in loss of ammonium control for most systems regulated by ammonium including asparaginase, nitrate reductase, nitrite reductase, xanthine dehydrogenase, L-glutamate, purine and urea uptake. The mutation also results in derepression of ammonium uptake itself (Pateman *et al.*, 1973). However, ammonium repression of extracellular protease is unaffected by the *gdhA* mutation and this may be connected with the fact that

protease is under multiple control; it is also regulated by carbon sulphur and phosphorous repression (Cohen, manuscript in preparation).

Since the *gdhA* mutants are derepressed for systems regulated by ammonium, this suggests that NADP-GDH may play some role in ammonium regulation. There are several theories which would account for most of the facts. Firstly, the NADP-GDH protein specified by the *gdhA* locus has only catalytic function, but low NADP-GDH itself results in certain metabolic changes, e.g. pool sizes of important metabolites which then result in derepression. The chief candidates for this regulatory role are perhaps ammonium itself or L-glutamate, the product of the NADP-GDH catalytic reaction. At the present time, there is evidence concerning the relationship between ammonium pool size which makes this unlikely one. This is that wild type cells can be either fully repressed or fully derepressed with respect to ammonium regulation of ammonium repressible systems and yet have the same concentration of intracellular ammonium (Pateman *et al.*, 1973). For a similar reason, it is thought unlikely that L-glutamate is the repressor. *gdh*$^{+}$ has similar L-glutamate pool values when grown under fully repressed or fully derepressed conditions. Another possibility is that the product of the *gdhA*

gene itself is involved in the regulation of ammonium repressible systems in addition to its catalytic function of synthesising L-glutamate from α-oxoglutaric acid and ammonium. If this protein is involved it can only be part of the regulatory system since deficient NADP-GDH is not an essential requirement for ammonium derepression. Five other classes of ammonium derepressed mutants *xprD1*, *meaA8*, *meaB6*, *amrA1* and DER-3 all have normal NADP-GDH.

Double mutants between *gdhA1* and the other classes of derepression with the exception of DER-3 (already a double mutant) were made. While the phenotypes of the double mutants did not prove very informative with respect to elucidating the mechanism of ammonium repression they nevertheless yielded some interesting interactions. It was shown that the addition of *meaA8* or *amrA1* genotype confers resistance upon *gdhA1* to high concentrations of ammonium. The reason for this is presumably ~~due to the fact~~ that *meaA8* and *amrA1* have low uptake of methylammonium and therefore ammonium itself under certain conditions (Arst and Page, 1972; Pateman, personal communication); this low methylammonium transport conferring resistance upon *gdhA1*. The double mutants *gdhA*, *meaB6* and *gdhA1*, *xprD1* are sensitive to high ammonium. All 4 classes of double mutants are derepressed for ammonium repressible systems similar to

their haploid parents with the exception of gdhA, meaB6. This strain appears to be abnormal with respect to extracellular protease release and perhaps other extracellular enzymes. Even in the presence of ammonium very high levels of protease are released. Indeed, after 4-5 days growth the centre of the colony is digested completely leaving a 'gap' or 'hole'. This effect is difficult to explain since again the molecular basis of methylammonium resistances conferred by mutation at the meaB6 locus is not known.

To return to the possibility that the protein defined by the gdhA locus itself is involved in ammonium regulation. If this is so then there are several explanations as to how this would be effected. The first hypothesis is that NADP-GDH is a multi-functional protein which has catalytic activity and also plays a direct role in the repression of a number of activities. Mutation in the structural gene for NADP-GDH, i.e. gdhA, would result in both abnormal catalytic and control activity. Secondly, the gdhA gene specifies a product which has some fundamental but at present unknown regulatory function with respect to a number of metabolic systems. Mutation in the gdhA locus can simultaneously result in repression of NADP-GDH and derepression of other systems. This is perhaps the more unlikely for the following reason. A common class of NADP-GDH

minus mutants should be due to mutation in the structural gene(s) for the protein. On this hypothesis such structural gene mutants should have abnormal enzyme activity but normal ammonium repression. However, all nine known NADP-GDH deficient mutants are ~~all~~ ammonium derepressed. Therefore the hypothesis requires the assumption that there are no NADP-GDH structural gene mutations among the nine known GDH-deficient mutants, although structural gene mutants should be the most common class exhibiting enzyme deficiency.

However the point may be made that the selection method (high ammonium sensitivity) selects only this type of control mutant and not structural gene mutants. Arst and MacDonald (1973) have recently isolated another *gdhA* mutant (designated *gdhA10*) on the basis of inability to grow as wild type on low concentrations of ammonium. The existence of this mutant which simultaneously lacks NADP-GDH and is derepressed for ammonium repressible systems, annuls the above argument.

In order to distinguish between the first and second hypothesis it is necessary to determine if the *gdhA* locus is in fact the structural gene for NADP-GDH. Preliminary attempts at making temperature sensitive NADP-GDH mutants have failed and lack

of time prevented other attempts at making these as well as osmotic sensitives and electrophoretic variants. Studies will be made along these lines and in addition, on the kinetic properties of the NADP-GDH from *gdhA5* to *gdhA9* to provide evidence on this point.

If it were shown conclusively that *gdhA* is the structural gene and NADP-GDH played a regulatory role in ammonium regulation it would prove extremely interesting, indeed exciting, since in this case the regulatory molecule is relatively stable, easy to purify and therefore amenable to intensive biochemical studies, e.g. configuration, amino acid sequence, etc. In most cases the nature of the regulator is quite unknown. At present there is no biochemical data published for NADP-GDH in *A. nidulans* which supports or rejects such a possibility. However it is likely that *A. nidulans* is similar to *N. crassa* in that NADP-GDH in the latter organism has been found to have allosteric properties (West, Tuveson, Barratt and Fincham, 1967). The regulatory role is not contradicted by the find that NADP-GDH is found in the cytoplasm. It would be more difficult to allocate a regulatory role to a mitochondrial based system.

As mentioned before, in the latter stages of this project I learned that Arst and MacDonald (1973) have isolated a mutant

(designated *gdhA10*) which has similar properties to *gdhA1-A9*. Their findings also suggest that NADP-GDH plays a role in ammonium repression.

A preliminary report of this work was given to the Genetical (Kinghorn and Pateman, 1973a)and the Biochemical Societies (Kinghorn and Pateman, 1973b). A full account is published in the Journal of General Microbiology (Kinghorn and Pateman, 1973c).

Section V The regulation of NAD L-glutamate dehydrogenase

1. NAD-GDH activity in wild type cells grown on various nitrogen sources

The results presented in table 33 show that wild type cells of *Aspergillus nidulans* grown on -N medium with any one of 10 mM ammonium, nitrate, L-glutamate, L-aspartate, L-alanine, L-glutamine, L-arginine, L-asparagine or 0.15% casamino acids as sole nitrogen source possessed undetectable NAD-GDH activity. A similar result was obtained when wild type cells were deprived of nitrogen for periods up to 6 h. However, after growth on 1.5% (~ 100 mM) casamino acids there were appreciable levels of activity. Significant levels of activity were obtained in cells grown on 100 mM L-glutamate, L-aspartate or L-alanine but not on 100 mM ammonium or nitrate.

Table 33 : NAD-GDH activity in wild type cells grown with various nitrogen sources

Growth conditions	Treatment	NAD-GDH nanomoles/min/mg protein
-N medium +		
10 mM ammonium	-	< 100
10 mM nitrate	-	< 100
10 mM L-glutamate	-	< 100
10 mM L-aspartate	-	< 100
10 mM L-alanine	-	< 100
10 mM L-arginine	-	< 100
0.15% casamino acids	-	< 100
10 mM L-glutamine	-	< 100
10 mM L-asparagine	-	< 100
100 mM ammonium	-	< 100
100 mM nitrate	-	< 100
100 mM L-glutamate	-	425
100 mM L-aspartate	-	395
100 mM L-alanine	-	505
1.5% casamino acids	-	859
10 mM ammonium	-N medium for 1.5 h	< 100
10 mM ammonium	-N medium for 3.0 h	< 100
10 mM ammonium	-N medium for 6.0 h	< 100

2. NAD-GDH activity in wild type cells grown on various carbon sources

The results presented in table 34 show NAD-GDH levels in wild type cells grown on various carbon sources. Little activity was found in cells grown on 1% glucose but appreciable levels on 1% acetate. L-glutamate, L-aspartate and L-alanine are relatively poor carbon sources especially when used in shake flask culture. Consequently, 0.1% glucose was added to supplement these amino acids as carbon sources and high levels of NAD-GDH were found on all three amino acids as the main carbon and nitrogen source. High levels of activity were also found in cells grown in a similar way but with the exception that 10 mM ammonium was added. The highest NAD-GDH activity was found in cells grown on 1.5% casamino acids as the sole carbon and nitrogen source.

Table 34 : NAD-GDH activity in wild type cells grown with various carbon sources

Growth conditions	NAD-GDH nanomoles/min/mg protein
-CN medium +	
1% glucose + 10 mM ammonium	< 100
1% acetate + 10 mM ammonium	850
0.1% glucose + 100 mM L-glutamate	1855
0.1% glucose + 100 mM L-aspartate	1620
0.1% glucose + 100 mM L-alanine	2050
1.5% casamino acids	2740
0.1% glucose + 100 mM L-glutamate + 10 mM ammonium	1955
0.1% glucose + 100 mM L-aspartate + 10 mM ammonium	1760
0.1% glucose + 100 mM L-alanine + 10 mM ammonium	1830

3. NAD-GDH levels in cells held in various carbon sources

When wild type cells, after growth on -N medium with 10 mM ammonium were carbon starved they developed low levels of activity which were maximal after 3 h (table 35). The level of activity decreased again if the carbon starvation was continued for 6 h. If instead the cells were transferred to 100 mM L-glutamate, L-aspartate or L-alanine or 1.5% casamino acids they developed extremely high levels of NAD-GDH activity.

Table 35 : NAD-GDH activity in wild type cells held in various carbon sources

Growth conditions	Treatment	NAD-GDH nanomoles/min/mg protein
-N medium +	-CN medium +	
10 mM ammonium	10 mM ammonium for 1.5 h	430
10 mM ammonium	10 mM ammonium for 3.0 h	510
10 mM ammonium	10 mM ammonium for 6.0 h	100
10 mM ammonium	100 mM L-glutamate for 1.5 h	560
10 mM ammonium	100 mM L-glutamate for 3.0 h	850
10 mM ammonium	100 mM L-glutamate for 6.0 h	1630
10 mM ammonium	100 mM L-aspartate for 1.5 h	370
10 mM ammonium	100 mM L-aspartate for 3.0 h	1150
10 mM ammonium	100 mM L-aspartate for 6.0 h	2020
10 mM ammonium	100 mM L-alanine for 1.5 h	660
10 mM ammonium	100 mM L-alanine for 3.0 h	1600
10 mM ammonium	100 mM L-alanine for 6.0 h	2425
10 mM ammonium	1.5% casamino acids for 1.5 h	290
10 mM ammonium	1.5% casamino acids for 3.0 h	1050
10 mM ammonium	1.5% casamino acids for 6.0 h	1930

4. Isolation and characterisation of mutants with abnormal NAD-GDH activity

Four mutants, designated *gdhB1* - *gdhB4* were isolated by the putrescine method of Herman and Clutterbuck (see Materials and methods, page 44). These were unable to utilise glutamate as a sole carbon and nitrogen source. Heterokaryons between all possible combinations of *gdhB* alleles, were made. These mutants were shown to be allelic by their failure to complement in these heterokaryons. Haploidisation of the diploid between *bi1 puA2 gdhB1* and master strain F yielded segregants which showed free assortment between *gdhB1* and all markers except *pyroA4* which is in linkage group IV (table 36). The locus *gdhB* is therefore defined in this group.

A further mutant, designated *gdhC1* was isolated which grew better than the wild type strain when L-glutamate was the sole nitrogen source. A diploid was set up between *gdhC1* and master strain F. By the technique of haploidisation and mitotic analysis, *gdhC* locus was assigned to linkage group III (table 37).

Table 36 : Haploidisation analysis of gdhB1 mutant

Diploid		gdhB1 M.S.F.	
Linkage group	gene marker	gdhB$^-$	gdhB$^+$
I	y$^+$	24	25
	y$^-$	15	16
II	Acra^S	18	24
	Acra^R	10	28
III	gal$^+$	24	26
	gal$^-$	8	22
IV	pyro$^+$	35	1
	pyro$^-$	0	44
V	fac$^+$	24	11
	fac$^-$	25	20
VI	s$^+$	9	26
	s$^-$	11	34
VII	nic$^+$	21	23
	nic$^-$	16	20
VIII	ribo$^+$	25	19
	ribo$^-$	14	22

Conclusion : The strain bil gdhB1 is translocation free and the locus gdhB can be assigned to linkage group IV.

Table 37 : Haploidisation analysis of gdhC1 mutant

Diploid		gdhC1 M.S.F.	
linkage group	gene marker	gdhC⁻	gdhC⁺
I	y^+	14	9
	y^-	16	10
II	$Acra^S$	9	30
	$Acra^R$	6	14
III	gal^+	20	0
	gal^-	0	29
IV	$pyro^+$	6	19
	$pyro^-$	11	13
V	fac^+	26	14
	fac^-	5	4
VI	s^+	12	13
	s^-	9	15
VII	nic^+	11	6
	nic^-	14	17
VIII	$ribo^+$	9	19
	$ribo^-$	5	16

Conclusion : The strain bil gdhC1 is translocation free and the locus gdhC can be assigned to linkage group III.

5. NAD-GDH activity in gdhB and gdhC mutants

Table 38 shows that mutation in the *gdhB* locus results in undetectable NAD-GDH activity in cells held under conditions which result in high activity in the wild type. These conditions are 100 mM L-glutamate or L-aspartate or L-alanine or 1.5% casamino acids as the sole carbon and nitrogen source. There is also undetectable NAD-GDH activity in *gdhB* mutants, when grown on 1.5% casamino acids as the sole carbon and nitrogen source. Wild type NAD-GDH activity is found in the heterozygous diploid *gdhB1*/+ after these treatments indicating the recessivity of the *gdhB* mutation.

Table 38 also shows that the *gdhC1* mutant has appreciable levels of NAD-GDH activity in the presence of glucose, while the wild type has activity below the limit of detection. Wild type cells and *gdhC1* held in the presence of L-glutamate or L-aspartate or L-alanine or casamino acids or grown on casamino acids as the sole carbon and nitrogen source have similar levels of NAD-GDH activity. The *gdhC1* mutation is semi-dominant in the heterozygous diploid with respect to NAD-GDH activity.

Table 38 : NAD-GDH activity in gdhB and gdhC mutants

Initial growth conditions	treatment	wild type	gdhB1-B4	gdhB1 +	gdhC1	gdhC1 +
		NAD-GDH nanomoles/min/ mg protein				
-N medium +						
10 mM ammonium	-	<100	<100	<100	645	420
10 mM nitrate	-	<100	<100	<100	810	530
10 mM L-glutamate	-	<100	<100	<100	710	415
10 mM L-aspartate	-	<100	<100	<100	595	390
10 mM L-alanine	-	<100	<100	<100	830	555
.15% casamino acids	-	<100	<100	<100	610	315
-N medium +	-CN medium for 6 h +					
10 mM ammonium	100 mM L-glutamate	1610	<100	1420	1645	1725
10 mM ammonium	100 mM L-aspartate	2010	<100	1715	1950	1950
10 mM ammonium	100 mM L-alanine	2215	<100	2095	2200	2305
10 mM ammonium	1.5% casamino acids	2035	<100	1920	1975	2350
-CN medium +						
1.5% casamino acids	-	2615	<100	2510	2755	2835

6. NADP-GDH activity in *gdhB* and *gdhC* mutants

Mutants have been described in this thesis which lack NADP-GDH. These mutants, designated *gdhA1-A9*, appear to have wild type NAD-GDH activity (section iv).

It was desirable to study the effect of *gdhB* and *gdhC* mutations on NADP-GDH activity. Table 39 shows that NADP-GDH levels in *gdhB1* and *gdhC1* grown on various nitrogen sources were similar to those of the wild type. However when grown or treated on various carbon sources *gdhB1* differed from that of the wild type with respect to NADP-GDH activity (table 40). The *gdhB1* mutant has appreciable levels of NADP-GDH activity under conditions in which the wild type has activity below the levels of detection, i.e. when grown with casamino acids as the carbon source. A similar result was obtained with *gdhB1* treated with 100 mM L-aspartate, L-alanine or L-glutamate as the sole carbon source for a period of 6 h. Cells were treated rather than grown with these amino acids as carbon sources since the *gdhB1* mutant is unable to use these as sole carbon sources (see page 211). When deprived of carbon for a period of 6 h both wild type and *gdhB1* genotypes have undetectable activity.

NADP-GDH levels in *gdhC* were similar to those of the wild type under all conditions tested.

Table 39 : NADP-GDH activity in the gdhB and gdhC mutants grown with various nitrogen sources

Growth conditions	wild type	gdhB1	gdhC1
	NADP-GDH	nanomoles/min/mg protein	
-N medium +			
10 mM ammonium	2100	1890	1950
10 mM nitrate	2150	1915	1995
10 mM L-glutamate	405	495	420
10 mM L-aspartate	625	830	710
10 mM L-alanine	820	755	785

Table 40 : NADP-GDH activity in gdhB and gdhC mutants held or grown with various carbon sources

Growth condition	treatment	wild type	gdhB1	gdhC1
		NADP-GDH.	nanomoles/min/mg protein	
-CN medium + 1.5% casamino acids	-	<100	495	<100
-N medium +	-CN medium +			
10 mM ammonium	10 mM L-aspartate for 6 h	<100	390	<100
10 mM ammonium	100 mM L-glutamate for 6 h	<100	510	<100
10 mM ammonium	100 mM L-alanine for 6 h	<100	420	<100
10 mM ammonium	100 mM ammonium	<100	<100	<100

7. Growth responses of gdhB1 and gdhC1 mutants

The results of growth tests carried out on solid media show that the *gdhB1* grows as wild type on all nitrogen sources tested but poorly on L-glutamate, L-aspartate or L-alanine as the sole carbon source (table 41). However, *gdhB1* grows as wild type on 1.5% casamino acids as a carbon source. Unlike mutation at the *gdhA* locus mutation at the *gdhB* locus does not result in high ammonium sensitivity. The heterozygous diploid *gdhB1*/+. grows as well as the haploid wild type with L-glutamate (plate 25), L-aspartate or L-alanine as the sole carbon and nitrogen source.

Mutation at the *gdhC* locus results in better growth than the wild type on 10 mM L-glutamate (plate 26) or L-aspartate or L-alanine but wild type growth on 10 mM L-arginine or L-asparagine or L-glutamine or ammonium or nitrate as sole nitrogen source. The *gdhC* mutant is not sensitive to high concentrations of ammonium. Moreover, it shows normal growth on L-glutamate or L-aspartate or L-alanine as sole carbon and nitrogen source. The heterozygous diploid *gdhC1*/+ shows mutant growth on certain nitrogen sources including L-glutamate (plate 26) indicating the semi-dominance or dominance of the *gdhC1* mutation.

Table 41 : Growth responses of gdhB and gdhC mutants

Strains / Growth conditions	wild type	gdhB1	gdhB1/+	gdhC1	gdhC/+
-N medium (solid) +					
10 mM ammonium	+	+	+	+	+
10 mM nitrate	+	+	+	+	+
10 mM L-glutamate	+	+	+	++	++
10 mM L-alanine	+	+	+	++	++
10 mM L-aspartate	+	+	+	++	++
10 mM L-arginine	+	+	+	+	+
10 mM L-glutamine	+	+	+	+	+
10 mM L-arginine	+	+	+	+	+
200 mM ammonium	+	+	+	+	+
-CN medium (solid) +					
100 mM L-glutamate	+	-	+	+	+
100 mM L-alanine	+	-	+	+	+
100 mM L-aspartate	+	-	+	+	+
1.5% casamino acids	+	+	+	+	+

++ = better than wild type growth

\+ = wild type growth

\- = extremely poor growth

Plate 25 Growth response of gdhB1 with L-glutamate as sole carbon and nitrogen source

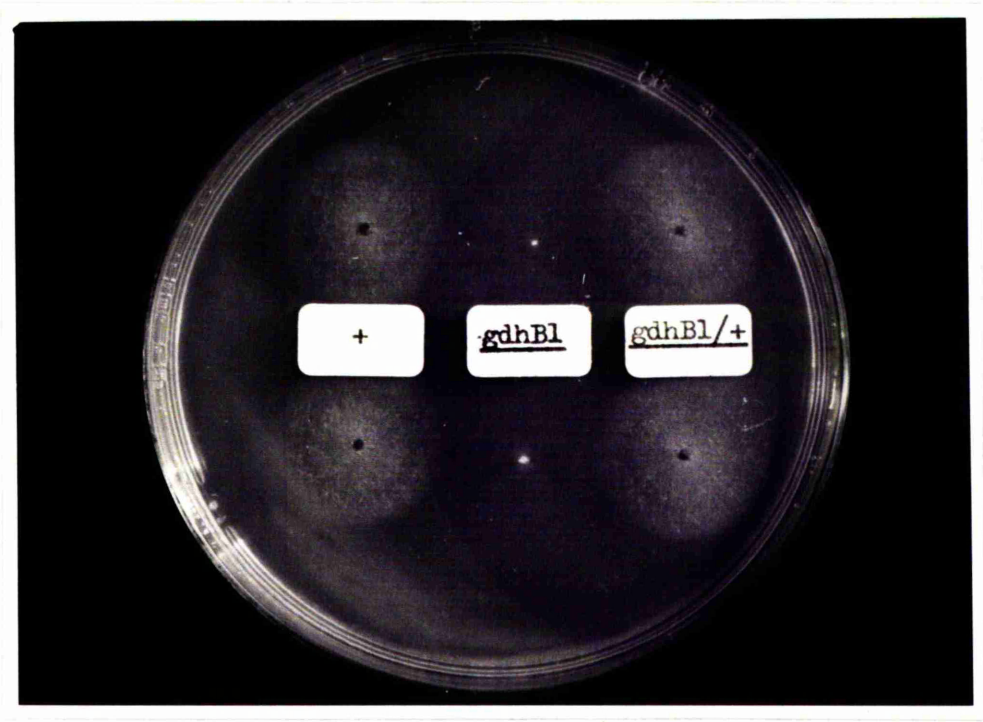
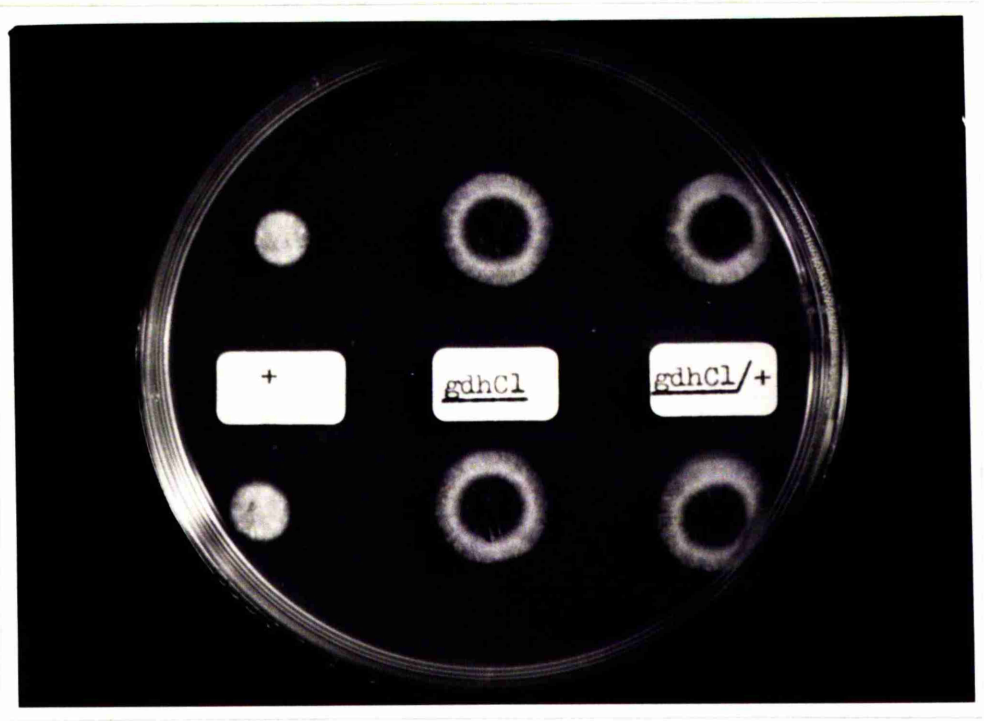


Plate 26 Growth response of gdhC1 with L-glutamate as sole nitrogen source



8. Discussion

It is clear from the results that wild type cells of *Aspergillus nidulans* elaborate under certain conditions a glutamate dehydrogenase which is dependent upon the co-enzyme NAD. Moreover, the results suggest that this enzyme is at least partly regulated by glucose or a metabolite derived from glucose. The main facts which support this argument are -

1) carbon starvation treatment results in appreciable NAD-GDH activity. This activity was maximal after 3 h. If the carbon starvation treatment was continued for 6 h the level of NAD-GDH activity decreased again. This decrease is probably due to energy or/and carbon skeleton deficiency.

2) maximal activity was found in cells in which the carbon status is low but probably sufficient for protein synthesis, e.g. when acetate, L-glutamate, L-aspartate or casamino acids is the main or only carbon source.

There is also the possibility that NAD-GDH is at least partly inducible since appreciable activity is found in cells grown on high concentrations of amino acids, e.g. 100 mM L-glutamate, when glucose is present.

Moreover, Arst and MacDonald (1973) have reported that growth of wild type A. nidulans cells on L-proline as a sole nitrogen source results in appreciable NAD-GDH activity again in the presence of glucose. Perhaps the most likely candidate for the inducer role in theory is L-glutamate itself. However, since NAD-GDH activity is undetectable in cells grown in 10 mM L-glutamate and only just above detection on 100 mM L-glutamate, L-glutamate may be ruled out as a possible inducer. L-proline is another possibility and clearly this point requires further investigation but it is at least possible that higher energy is required to transport L-proline into the cell and this extra energy requirement interferes with the carbon status of the cell with the result that NAD-GDH is derepressed. This argument is not contradicted by the fact that proline uptake can be carried out by a specific proline transport system in yeast (Schwencke and Magana-Schwencke, 1969) and in certain fungi e.g. P. chrysogenum (Hunter and Segel, 1971). A similar argument may be used to explain NAD-GDH activity with high concentrations of L-glutamate, L-alanine, L-aspartate or casamino acids used as sole nitrogen source. In the light of the fact that NAD-GDH activity is found in cells after a short period of carbon starvation and in cells grown on acetate and ammonium as the carbon and nitrogen source respectively, it is the view of the author that the system is more ~~either~~, likely to be only regulated either by glucose repression or by

repression and induction; induction being subordinate to repression. This seems to be similar to the situation in *N. crassa* (Kapoor and Glover, 1970; Strickland, 1971) but rather different to the one in *Saccharomyces cerevisiae* where ammonium represses NAD-GDH activity (Hierholzer and Holzer, 1963). The appearance of NAD-GDH may be as a result of *de nova* enzyme synthesis or activation of enzyme molecules. Experiments were not carried out to distinguish between these possibilities. It is thought that control would be effected at the level of transcription since control at the translation level of catabolic enzymes would seem to be rather inefficient. Any theory of regulation of enzyme activity would be more complex than one based on repression of synthesis. Therefore, it will be assumed that regulation is at the level of protein synthesis.

It has been demonstrated that mutation in the *gdhB* gene results in the abolition of NAD-GDH activity. Although it is probable that the *gdhB* is the structural gene for NAD-GDH, it is far from certain; *gdhB* mutants elaborate NADP-GDH, albeit low activity, under conditions which the wild type does not, i.e. when glutamate is used as a sole carbon and nitrogen source. Moreover, Ahmed and Sanwal (1967) have claimed to have isolated structural gene mutants lacking NAD-GDH called *am-2* in *Neurospora* and this is almost certainly incorrect, since these mutants are alleles of *nit-2* locus (D.A. Catcheside,

personal communication) which is not the structural gene for NAD-GDH. Clearly, more detailed investigation will have to be carried out to establish this important point of whether the gdhB locus is a structural or regulatory gene.

The gdhB mutants can utilise inorganic nitrogen and amino acids as sole nitrogen sources for normal growth. They are unable to utilise a number of amino acids including L-glutamate as a sole carbon source and clearly this is due to impaired NAD-GDH activity. Probably the NADP-GDH which develops in the gdhB mutants is insufficient for the effective deamination of relatively large concentrations of L-glutamate required for carbon skeletons in the absence of glucose. It is rather surprising that the gdhB mutants grow as wild type on 1.5% casamino acids as a sole carbon source, since they are unable to grow on L-glutamate, L-aspartate or L-alanine. One explanation is that one or several amino acids are not broken down to glutamate and deaminated, but deaminated by other mechanisms and incorporated into the tricarboxylic acid cycle. The other possibility is that there is sufficient carbon in casamino acids to enable gdhB to grow. If the former theory is correct it should be possible to determine which amino acid(s) is not oxidised via L-glutamate.

A mutant at a third locus gdhC has appreciable NAD-GDH activity when grown in the presence of glucose, that is, significantly derepressed. This activity appears to be irrespective of the nitrogen source and is found in cells grown on ammonium or nitrate or L-glutamate or L-aspartate or L-alanine or L-arginine or L-glutamine as sole nitrogen. Mutation at the gdhC locus confers better than wild type growth on L-glutamate or L-aspartate or L-alanine as sole nitrogen source. This is probably due to the bonus of derepressed NAD-GDH activity which makes ammonium more easily available for other amination reactions. The wild type under these conditions may only possess repressed levels of NADP-GDH activity (Pateman, 1969). Moreover the formation of ammonium may also be impeded by the anabolic function of NADP-GDH itself. This line of argument is supported by the fact that gdhC1 grows as wild type on nitrogen sources which provide easily accessible ammonium, e.g. ammonium, L-arginine and L-glutamine. The properties of this mutant could be explained if the gdhC codes for a regulatory product which on interaction with the carbon metabolite co-repressor represses enzyme synthesis. Moreover, because of the semi-dominance of gdhC1 the gdhC product may have a positive regulatory function and this point will be enlarged upon in the next section.

Further studies are being carried out to determine if the *gdhC1* mutant has altered glucose repression of other systems, e.g. amylase, glyoxylate enzymes, etc. Initial studies show that the *gdhC1* mutant has wild type carbon repression of extracellular protease (Cohen, personal communication).

Recent studies by Hynes (1972) have shown that mutation at the *amdT* locus results in loss of carbon regulation of acetamidase. Since *gdhC* and *amdT* loci are both defined in linkage group III it is of importance that allelism tests are carried out. This is at present being done in collaboration with Hynes. However, early indications are that *gdhC1* and *amdT* are at least different alleles of the same gene since *amdT* mutants have wild type NAD-GDH glucose repression (Hynes, personal communication).

A preliminary report of this work was given to the Biochemical Society (Kinghorn and Pateman, 1973d). A short communication is published in Genetical Research (Kinghorn and Pateman, 1973e).

Section VI General Discussion

General Discussion

The chief objective of this work was to extend our present understanding of control phenomena in eucaryotic cells. In an effort to achieve this goal systems connected with L-glutamate metabolism were chosen since these lie at a key branch point between nitrogen and carbon metabolism, and likely to be subject to genetic control, and moreover perhaps even exerting control over other systems. As discussed earlier, models derived from studies of systems connected with intermediate metabolism are more likely to be applicable to higher organisms than models from the regulation of peripheral systems. The results obtained bear out some of these possibilities.

1. L-glutamate transport

In section III an active specific transport system is described for the uptake of L-glutamate and L-aspartate and the activity of which is lost by the aauA1 mutation. This system is regulated by ammonium as are a number of other systems in wild type cells of *Aspergillus nidulans* such as asparaginase, nitrate reductase, nitrite reductase, extracellular protease, acetamidase,

urea and purine uptake, and perhaps others presently unknown. L-glutamate transport then is only one of a battery of unrelated systems regulated by ammonium. It is not clear whether this effect of ammonium is on protein synthesis, on inhibition of activity, or a combination of both. However the phenomenon is usually termed ammonium repression and is for convenience referred to as this in this thesis. One anomaly of this ammonium control phenomenon is that the fully repressed level of L-glutamate uptake is found in cells grown on 10 mM ammonium and fully derepressed in cells grown on 10 mM urea, yet urea is converted to ammonium by urease (Darlington and Scazzocchio, 1967) which is present at high levels in cells grown in the presence or absence of ammonium. Similarly, 10 mM ammonium, but not 10 mM urea provides protection against the toxic effects of certain analogues, which are substrates of ammonium repressible systems - aspartate hydroxamate, chlorate, bromate, 2-thioxanthine, thiourea and 8-azaguanine, providing evidence that the activities of asparaginase, nitrate reductase, nitrite reductase, xanthine dehydrogenase, urea and purine uptake respectively, are also repressed in cells grown on ammonium and derepressed on urea. Moreover it was found that the internal ammonium pool is not significantly different in cells grown on either of these two nitrogen sources (Pateman _et al._, 1973; Pateman

and Kinghorn, 1972). These findings lead to the postulate that L-glutamate and the other ammonium repressible systems are dependent on the concentration of extracellular ammonium and not intracellular ammonium. In contrast it was found that ammonium transport itself is regulated by intracellular ammonium (Pateman *et al.*, 1973; Pateman and Kinghorn, 1972).

2. NADP L-glutamate dehydrogenase

The possibility was discussed in section IV that the NADP-GDH protein itself plays some role in ammonium repression. Several models may be constructed to account for the characteristics of *gdhA* mutations and the possible regulatory nature of NADP-GDH. Perhaps the simplest type of theory which covered most of the facts was one proposed by Pateman, Kinghorn, Dunn and Forbes (1973). The main features are that NADP-GDH, in addition to its catalytic function, plays a dual regulatory role in ammonium regulation. These roles are that -

a) NADP-GDH can locate in a regulatory site in the cell membrane and in juxaposition complexes with extracellular ammonium. This complex is now in a suitable form to effect the repression of ammonium repressible systems such as L-glutamate uptake.

b) NADP-GDH can combine with intracellular ammonium to form a second type of regulatory complex which is in a suitable form to effect the repression of ammonium transport itself.

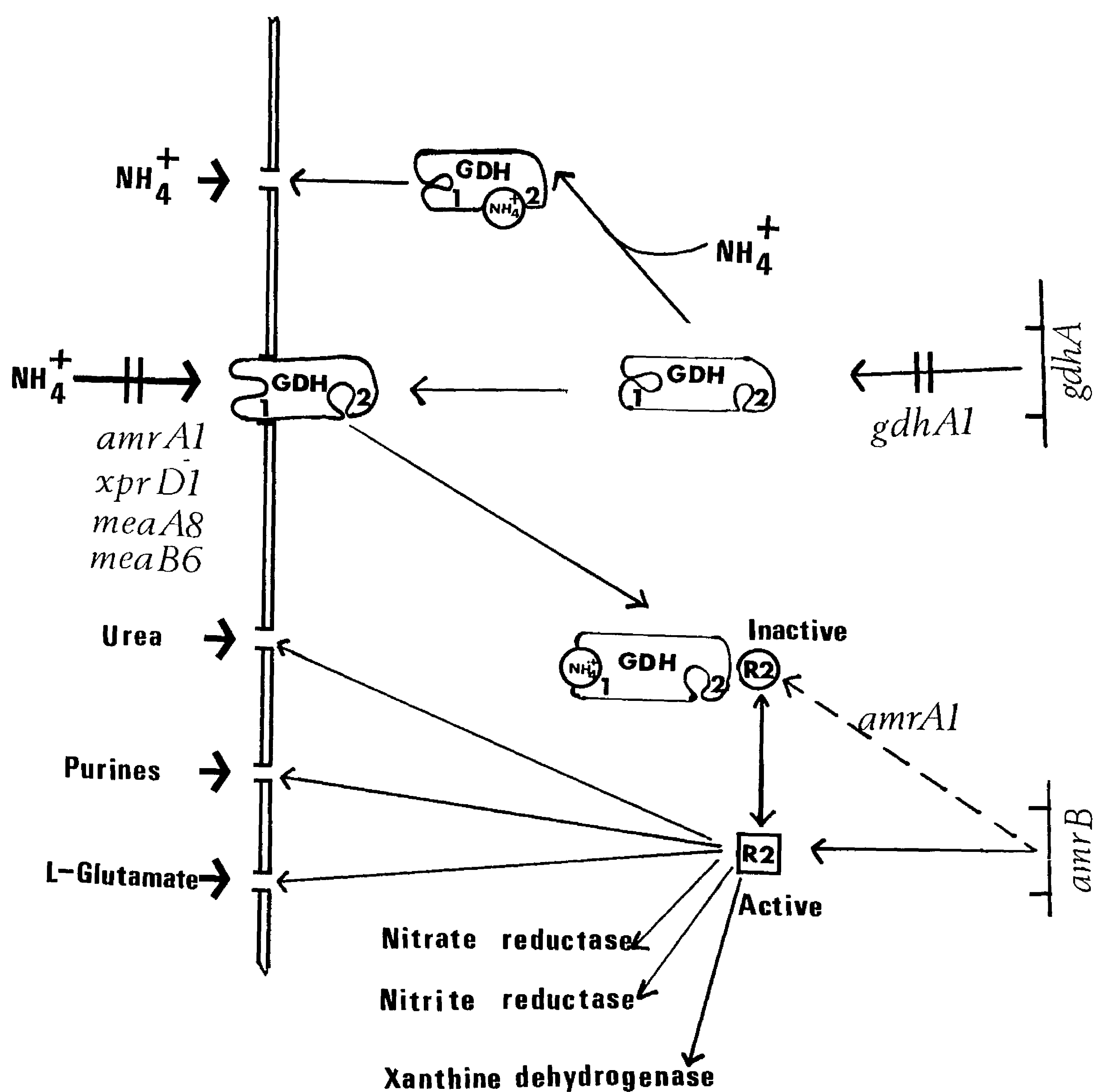
It was shown that all ammonium derepressed mutants except gdhA have normal NADP-GDH. Some of these are known to be transport mutants; meaA8 (Arst and Page, 1973) amrA1 and DER-3 (Pateman, Dunn and Kinghorn, 1973) have a lower uptake of methylammonium and hence ammonium itself than the wild type. The fact that amrA1 and meaA8 are epistatic to gdhA1 with respect to ammonium sensitivity supports the contention that the transport of ammonium is impaired thereby relieving gdhA1 of high ammonium 'sickness'. xprD1 is partially resistant to thiourea (Kinghorn, unpublished work) and this is probably due to impaired urea uptake (Dunn, personal communication). Little is known about meaB6. It is at least possible that this too is a transport mutant since it is resistant to p-fluorophenylalanine (Arst, 1972). There is then evidence to support the hypothesis that most if not all classes of derepressed mutants except gdhA1 are transport mutants. To account for the above transport and derepressed properties of all derepressed mutants, except gdhA, it was proposed that the NADP-GDH site in the membrane is altered in such a way that the regulatory complex cannot be formed resulting in loss of control of systems regulated by external ammonium. The

gdhA mutants which have no known transport abnormalities is the only class of derepressed mutants which is derepressed with respect to ammonium uptake itself. Therefore a second non-membrane based regulatory NADP-GDH controlling the uptake of ammonium by monitoring and complexing with internal ammonium was proposed.

More recently, several classes of mutants have been isolated whose properties, when fully analysed, will probably require amendments to the model proposed by Pateman *et al.*, (1973). These mutants appear to be repressed for ammonium repressible systems in the absence of ammonium (Kinghorn and Pateman, 1973f; Arst, personal communication). Moreover one class of these mutants (designated *amrB*) appear to synthesise wild type NADP-GDH activity and is not allelic with *gdhA*, *meaA8*, *meaB6* or *amrA1* (at the present time it is not known whether *amrB1* is allelic with *xprD1*). The *amrB1* mutants are epistatic to *meaA8*, *meaB6*, *amrA1* and *gdhA*.

To account for these findings it is perhaps best to formulate a second model (figure 26). This is basically an embellishment of the first and also proposes that NADP-GDH plays a regulatory role in ammonium regulation as well as a catalytic one. The salient points are that -

Figure 26 : Proposed hypothesis for ammonium regulation



NH_4^+
GDH
1
2
NH_4^+
NH_4^+
NH_4^+
GDH
1
2
GDH
1
2
gdhA
gdhA1
amrA1
xprD1
meaA8
meaB6
Urea
Inactive
GDH
NH_4^+
1
2
R2
amrA1
Purines
L-Glutamate
R2
Active
amrB
Nitrate reductase
Nitrite reductase
Xanthine dehydrogenase

a) NADP-GDH can complex with intracellular ammonium and this complex represses ammonium uptake.

b) NADP-GDH locates at a special regulatory site in the membrane and complexes with external ammonium.

c) another regulator, the nature of which is unknown and for convenience is referred to as R2 is involved in allowing expression of ammonium regulated systems in the absence of this latter NADP-GDH ammonium regulatory complex. In the presence of the NADP-GDH ammonium complex, R2 is inactivated and so expression of ammonium regulated systems cannot proceed.

As in the case of the former model and for the same reasons it is proposed that all classes of ammonium derepressed mutants, except gdhA are altered in membrane structure in such a way that NADP-GDH cannot complex with external ammonium. Therefore a regulatory form of NADP-GDH is not made and cannot complex with R2 with consequent expression of ammonium regulated systems in the presence of ammonium. The gdhA mutants are derepressed for one or both of the following reasons. The mutant NADP-GDH (a) fails to complex with external ammonium (b) complexes with external ammonium but fails to inactivate R2. The simplest explanation of the super-repressed mutants is that amrB codes for this second regulatory

molecule R2. Mutation at the *amrB* locus results in the production of an altered R2 effector molecule which represses even in the absence of the NADP-GDH ammonium regulator molecule. The fact that the *amrB1* mutation is recessive (Kinghorn and Pateman, 1973f) indicates that the system is subject to control by positive action. This type of model would explain the epistasis of *amrB* to *meaA*, *meaB*, *amrA* and *gdhA* since it is proposed that the altered R2 molecule does not require to complex with NADP-GDH + ammonium.

There are of course other models which could be propounded but the above is perhaps the simplest which covers most of the facts known about a very complex control situation. It is hoped that further studies of this system will elucidate some of the mechanisms associated with this ammonium control. For instance, if the product of the *amrB* gene plays such a role in ammonium regulation it should be possible to make derepressed mutations in the *amrB* gene. Furthermore such a hypothesis predicts the existence of super-repressed mutations in the *gdhA* locus.

The results in Section III show that ammonium regulation is inoperative in cases where the substrate of the ammonium repressible system can be used as a carbon source, e.g. L-glutamate. When grown on L-glutamate as a carbon source wild type cells

synthesise NAD-GDH while NADP-GDH activity is below the limit of detection. From this it can be concluded that under these conditions the absence of NADP-GDH even in the presence of ammonium results in loss of ammonium control. Conversely, it is clear that NAD-GDH (which develops rapidly in cells grown on L-glutamate as a carbon source), does not 'fill in' for NADP-GDH and therefore does not play any significant regulatory role in ammonium repression.

This absence of NADP-GDH in cells grown on L-glutamate as sole carbon source and the fact that NADP-GDH activity is quickly lost upon carbon starvation are interesting. There are several theories which may explain this. Firstly, NADP-GDH may be subject to glucose induction as well as L-glutamate repression (Pateman, 1969) and consequently inactivation may result from a combination of

1) a fast turn over of enzyme molecules

and 2) a diminished concentration of inducer substance.

Another possibility is that in the absence of a carbon source an intracellular proteolytic enzyme inactivates NADP-GDH activity to ensure that not only synthesis but also activity (regulatory and catalytic) is stopped. This view is supported by the finding in *Aspergillus nidulans* (Hynes, personal communication) that inactivation of NADP-GDH requires protein synthesis. It is

difficult to explain why gdhB synthesises NADP-GDH under these conditions. It may be that the gdhB mutation results in a defect of the synthesis of this proteolytic system as well as NAD-GDH, thus resulting in NADP-GDH activity under such conditions. Pateman (1969) observed high levels of NADP-GDH activity in cells grown on glucose plus organic nitrogen sources such as ammonium nitrate or urea and approximately 20% of this activity when grown on glucose plus L-glutamate. As a consequence he postulated that NADP-GDH is regulated by L-glutamate. It would be of obvious interest to study further the regulation of NADP-GDH itself and in particular isolate control mutants with respect to NADP-GDH levels.

3. NAD L-glutamate dehydrogenase

In addition to NADP-GDH which plays an anabolic role and is responsible for the synthesis of L-glutamate from ammonium *Aspergillus nidulans* also synthesises NAD-GDH. Both systems have been found in a number of fungi (Le John, 1971). NAD-GDH probably plays a catabolic role as has been found in *Neurospora crassa* (Kapoor and Glover, 1970; Strickland, 1971) deaminating L-glutamate for carbon skeletons when the carbon status of the cell is low. The different *in vivo* functions of the two enzymes are clearly

demonstrated by the conditions which determine the synthesis of the two enzymes and the growth properties of strains carrying *gdhA* and *gdhB* mutations.

As discussed in the previous section the carbon status of the culture affects NAD-GDH activity and therefore there appears to be at least one mechanism controlling NAD-GDH levels - carbon metabolite repression. This type of control exerted upon amino acid degradation systems is not uncommon; for instance, it has been found that the systems for tyrosine, histidine (Jacoby, 1964), serine (McFall and Broom, 1971) and proline (Newell and Brill, 1972) breakdown are regulated by carbon repression. This type of control is particularly advantageous since it prevents the destruction of amino acids after their biosynthesis.

The *gdhC* locus is defined by only one mutant *gdhC1* which grows better than the wild type on L-glutamate. When grown on glucose the mutant produces significant levels of NAD-GDH. These properties indicate that carbon metabolite repression is not fully effective in this mutant. The properties of the mutant could be partly explained if the *gdhC* locus is a structural one determining an enzyme involved in carbon metabolism, and this enzyme activity results in the production of a carbon compound which is the co-repressor of

NAD-GDH synthesis. The main objections to this hypothesis are 1) *gdhC1* grows as wild type on glucose minimal medium, 2) the heterozygote *gdhC1*/+ should be recessive, but it is in fact semi-dominant. By analogy with the L-arabinose system in *E. coli*, (Englesberg *et al.*, 1969) the nitrate reductase (Pateman and Cove, 1967) and xanthine dehydrogenase systems (Scazzocchio and Darlington, 1968) in *Aspergillus nidulans*, such semi-dominance suggests that glucose repression of NAD-GDH may be a positive control system. Several theories can be advanced to explain some of the above observations. The simplest explanation is that the *gdhC* gene product is a regulator molecule with a necessary function in the synthesis of NAD-GDH. This regulator molecule in the absence of glucose performs an essential function at the translation or transcription level in the synthesis of NAD-GDH (figure 27a). In the presence of glucose a complex of the *gdhC* product and the effector molecule is formed. This complex of regulatory molecule and effector is inactive with respect to the synthesis of NAD-GDH (figure 27b). Mutation in the *gdhC* locus can result in the production of an altered regulator molecule which is active functionally with respect to the synthesis of NAD-GDH even in the presence of the effector molecule (figure 27c). If the *gdhC* product is a regulator molecule with a positive action, this would imply

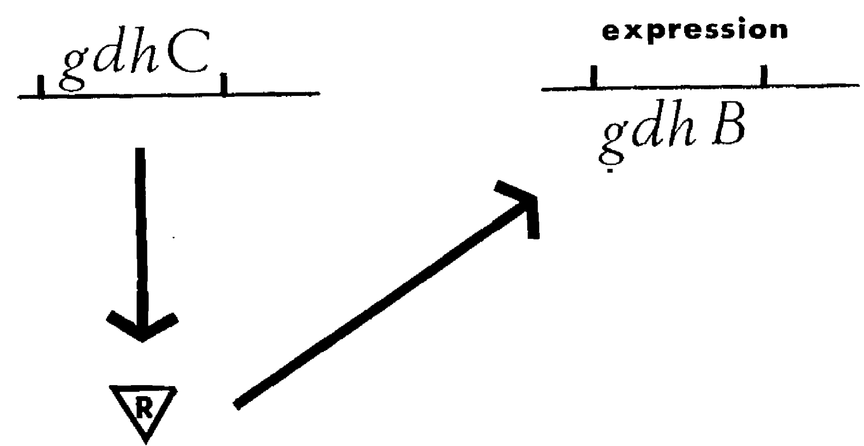
the existence of recessive mutations in the *gdhC* locus which result in the production of inactive regulatory molecules and consequent failure to synthesise NAD-GDH. It would be of obvious interest to make a further series of more mutants lacking NAD-GDH activity to see if any of these map in the *gdhC* gene. Moreover the above speculations are only based on one mutant and further isolation and studies of *gdhC* mutants are required.

The nature of the effector(s) is unknown but it is probably derived from glucose or at least the concentration of the effector is affected by the presence of glucose. It is unlikely that it is derived from or affected by acetate, since acetate does not repress NAD-GDH. No attempt was made to identify the effector since this is a major project in itself. Such investigations are fraught with difficulties, e.g. interpretation of experimental results is made difficult by the network of pathways of carbohydrate metabolism. For example, formation of glyceraldehyde-3-phosphate from glucose can proceed via the Embden-Meyerhof pathway or/and the hexose monophosphate shunt in *Aspergillus* (Smith, Valenzuela and Ng, 1971), providing ATP and pyruvate as well as ribose-5-phosphate for nucleic acid synthesis, hexosamines for cell wall synthesis and glucose-1-phosphate for glycogen synthesis. Therefore changing the rate of such reaction by changing the carbon source will have marked

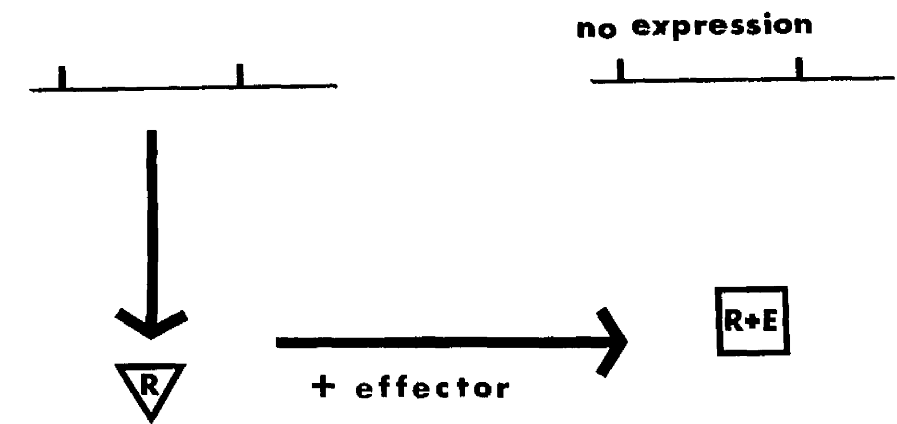
effects on the concentrations of intermediates of carbohydrate metabolism. Despite these difficulties there has been success in some cases. For instance Kornberg (1966) studied the repression of the glyoxylate enzymes in *E. coli* and determined that phospho-enol-pyruvate is the probable co-repressor of these enzymes. This investigation was carried out using mutants blocked at various stages in carbon metabolism. This type of analysis would be necessary to determine the co-repressor of NAD-GDH synthesis.

Figure 27 : Simple model to explain the properties of the gdhC1 mutation

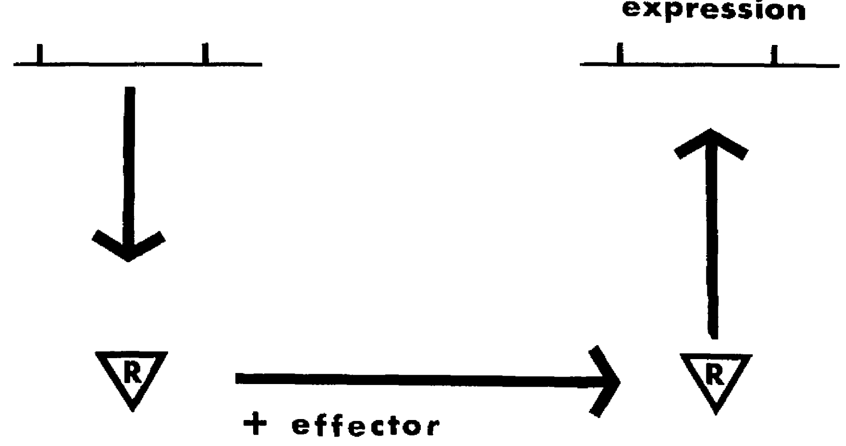
a Wild type cells grown in the absence of glucose.



b Wild type cells grown in the presence of glucose.



c *gdhC1* grown in the presence of glucose.



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