GENETIC AND BIOCHEMICAL STUDIES OF UREA UTILIZATION IN ASPERGILLUS NIDULANS

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by

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DECLARATION

The work in this thesis is my own, except where explicit reference to the results of others is made in the text. No part of this work has previously been submitted by me, or to the best of my knowledge, by anyone else, to this, or any other university.

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SUMMARY

Aspergillus nidulans can utilize urea as a sole source of nitrogen but not of carbon. At high external concentrations sufficient urea for normal growth can enter the cell by diffusion. In addition there is an active transport system probably for scavenging low concentrations of urea when nitrogen is in short supply. The enzyme which degrades urea is a urease. A very sensitive conductimetric assay for urease activity in crude extracts was developed.

The metabolic factors controlling the activity of urease and of the urea uptake system were studied. The activities of both urease and the urea uptake system are subject to nitrogen regulation. For both systems, the signal which results in nitrogen regulation, the effector, appears to be the intracellular concentration of L-glutamine and not the intracellular concentration of ammonia.

A high intracellular concentration of L-glutamine appears to result in an inactivation of urease which is independent of protein synthesis and probably does not involve a direct interaction between L-glutamine and urease. The occurrence of inactivation makes it difficult to judge to what extent, if any, urease synthesis is repressed. L-glutamine appears to inhibit the urea uptake system. In addition, a high intracellular concentration of L-glutamine either results in repression of the synthesis of the uptake system or causes the system to be inactivated in such a way that protein synthesis is required to reverse the inactivation.

Though urease has no obvious connection with

carbon metabolism, the activities of both urease and the urea uptake system decrease during carbon starvation. These decreases appear to require protein synthesis.

Other authors have noted that the presence of L-histidine in growth medium severely decreases urease activity. The present work indicates that this L-histidine effect occurs irrespective of the nitrogen or carbon status of the cell and affects strains either repressed or derepressed with respect to nitrogen regulation. Uptake activity appears also to be affected by L-histidine. It is unclear whether this effect is one of inhibition by L-histidine itself or results from the intracellular accumulation of urea through the effect of L-histidine on urease activity.

Four loci, ureA, ureB, ureC and ureD, are known each to have a role in urea utilization. The loci, ureA and ureB, are closely linked. It was intended that a deletion map of this region would be produced to form a basis for study of the control of the two linked genes. This did not prove possible. Crosses between ureB alleles were generally infertile and no deletion running through both ureA and ureB was detected.

It is probable that ureA codes for the urea-specific transport protein. It may be that ureB is the structural gene for the major urease subunit : urease is probably essentially homomeric but may contain a small cofactor protein. The ureD locus is probably involved in the synthesis or incorporation of a nickel cofactor essential for urease activity. It may be that ureC also has a role concerning nickel and urease.

CHAPTER I

INTRODUCTION

INTRODUCTION

1. The Use of Aspergillus nidulans for Investigating Metabolic Regulation

Aspergillus nidulans, (Eidam) Winter, is an Ascomycete belonging to the sub-class Euascomycetes, of the order Plectomycetes (Fincham and Day, 1963). This filamentous fungus is a true eukaryote having a membrane-bound nucleus, a nucleolus, a microtubular mitotic spindle and mitochondria. The Aspergillus chromatin, histones and tubulin are similar to those of higher eukaryotes (Morris *et al.*, 1977).

Pontecorvo and his co-workers established Aspergillus nidulans as a suitable organism for genetic research and documented its parasexual life cycle, growth requirements and many of the genetic techniques which are still employed (Pontecorvo *et al.*, 1953). The particular advantages of Aspergillus for research have been reviewed by Clutterbuck (1974), and Cove (1977). These advantages include the following: unlike many fungi, Aspergillus does not have mating types and, with a few exceptions, any strain may be crossed to any other; stable diploids may be quite easily produced for complementation studies; the conidia are uninucleate and so are ideal for the production of mutant strains; the existence of easily distinguishable colour markers greatly facilitates all crossing and complementation studies.

Knowledge of the physiology of Aspergillus has been accumulating since the mid 1960's. Like other fungi, Aspergillus has a great nutritional versatility and is

able to utilize a wide range of carbon and nitrogen sources. Combined biochemical and genetic studies of strains showing irregularities in the utilization of particular substances can be used to investigate the mechanisms which regulate Aspergillus metabolism.

As a simple filamentous eukaryote Aspergillus represents an important link between prokaryotes and the multicellular condition of higher organisms. It is reasonable to expect that study of the control of metabolism and gene expression in Aspergillus will clarify the differences between prokaryotic and eukaryotic control and give insight into the mechanisms of metabolic control in higher organisms.

2. Control of Carbon and Nitrogen Metabolism in Micro-organisms

Many micro-organisms can utilize a wide range of carbon and nitrogen sources. The information necessary for the synthesis of uptake systems for transporting the carbon and nitrogen sources into the cell and the information for the synthesis of catabolic and anabolic enzymes is encoded in their DNA. These systems for the utilization of carbon and nitrogen sources are not, however, all continuously active in the cell. The DNA also contains information which allows the micro-organism to sense its metabolic status and to control the activities of individual uptake and enzyme systems in response to changes in the supply of carbon and nitrogen. This control of activity could, in principle, be exerted at, broadly, five levels:

(i) Transcription. RNA polymerase might be prevented from transcribing particular structural genes.

(ii) Post-transcription. Under some metabolic conditions, some mRNA molecules might be less stable than others.

(iii) Translation. The rate of polypeptide initiation might be specifically decreased for some mRNA molecules.

(iv) Post-translation. The translated polypeptide might not be processed correctly.

(v) Inhibition and Inactivation. The protein would have been made correctly but might be specifically prevented from continuing to perform its particular function.

Prokaryotes appear to largely exert control at levels (i) and (v), transcription and inhibition and inactivation. Controls at these two levels are not mutually exclusive and may both be operative under some metabolic conditions. There are several parallels between prokaryotic and fungal systems and the evidence from fungal systems so far suggests that they may also largely exert control at these levels (Fincham *et al.*, 1979).

a. Transcriptional control in bacteria

In many instances in bacteria, structural genes with related function are grouped together in a cluster with an adjacent control region. The clustered genes are transcribed into one mRNA molecule, a polycistronic message. The unit comprising structural genes and regulatory genes is called an operon (Jacob and Monod, 1961). The

control region has a promoter site at which RNA polymerase binds to begin transcription and a second regulatory region. These regulatory regions are of two types, operator and initiator genes. The control region of a negatively controlled operon is an operator. This type of region allows the passage of RNA polymerase molecules unless regulatory proteins bind to it. The control region of a positively controlled operon is called an initiator. This type of region does not allow the passage of RNA polymerase molecules unless regulatory proteins are bound to it. In both cases the mechanism for altering the frequency of transcription involves both a specific allosteric regulatory protein and a specific small molecule. In the case of repressible operons, (e.g. the *E. coli* tryptophan operon) the regulatory protein can only bind to the operator when the protein is itself bound to a specific small molecule, typically the end product, or closely related metabolite, of the biosynthetic pathway to which the enzymes encoded by the operon belong. The regulatory protein of an inducible operon is itself the active repressor. The inducer, typically the substrate or closely related metabolite, of a catabolic pathway, binds to the repressor and thus either renders the protein unable to bind to the operator (e.g. as occurs in the *lac* operon in *E. coli*) or, through binding, allows it to then bind to an initiator (this occurs with the arabinose operon *E. coli* which has both an operator and an initiator).

Strains with abnormal operon regulation can be used to determine the mechanism of the regulation. Though ideally the mRNA transcripts in such strains would be

followed, it is possible to at least tentatively deduce the mechanism of regulation from the characteristics of mutant strains. Mutations in operator or initiator genes are cis-acting; cis-dominant, trans-recessive. Such mutations only affect expression of the structural genes adjacent to the control region and cannot affect expression of the same structural genes on a separate chromosome region in a diploid or partial diploid. The characteristics of a strain carrying a mutation in a gene coding for a regulatory protein depends on whether the protein acts negatively (as a repressor) or positively (as an activator of transcription) or in both capacities at different times. If the regulatory protein is a repressor, regulatory mutants may be constitutive-produce enzyme regardless of nutritional conditions - and be recessive in diploids, or, mutants may be enzyme negative and dominant in diploids (super-repressed mutants). If the regulatory protein is an activator of transcription regulatory mutants might be constitutive and dominant or might be enzyme negative and recessive. The above criteria apply to both operon systems and single structural genes with adjacent control regions.

A further type of transcriptional control, attenuation, has been found in some operons such as the his and leucine operons S. typhimurium and the thr operon of E. coli. Attenuation was first discovered in the tryptophan operon of E. coli. In this operon there is a leader region between the operator and the beginning of the first structural gene. Certain mutants with a deletion starting in the leader region and ending in one

of the five structural genes showed significantly increased expression of the remaining genes. These deletions were cis-acting and were removing a transcriptional block in the leader region, the attenuator (Morse and Morse, 1976). In conditions of tryptophan sufficiency, signalled by the accumulation of, probably charged, tryptophan tRNA the majority of RNA polymerase molecules that initiate transcription at the promoter, terminate transcription in the leader region. Thus in conditions of tryptophan sufficiency two types of transcriptional control are operative: the binding of the regulatory protein plus tryptophan to the operator decreases the number of RNA polymerase molecules initiating transcription, and the majority of those which do initiate transcription terminate at the attenuator.

The DNA sequence in the leader transcription termination region of E. coli contains GC-rich and AT-rich blocks. Such sequences, which can form stable stem and loop secondary structures occur in all known transcription termination sites recognised by E. coli RNA polymerase *in vitro* (Rosenberg *et al.*, 1978). Though translation of the leader transcript may be involved in transcription termination at the attenuator, it is this mRNA secondary structure which is recognised as the regulatory signal (Crawford and Stauffer, 1980).

In addition to the individual control associated with a substrate or end product, operons and single structural genes may be subject to more general controls determined by the cells overall carbon or nitrogen status. These general controls are carbon catabolite repression

and nitrogen repression.

b. Carbon catabolite repression in bacteria

In some bacteria, such as E. coli, when glucose or some other good carbon source is available, the activities of the uptake systems and enzymes for the utilization of poorer carbon sources are decreased. This decrease is mediated by the level of cAMP in the cell. When carbon is in short supply the intracellular concentration of cAMP is high and cAMP together with cAMP receptor protein binds to the operator site of relevant operons and promotes transcription. Carbon catabolic repression is therefore a lack of activation of transcription by cAMP (Pastan and Adhya, 1976, review).

c. Nitrogen repression in bacteria

In a similar fashion to carbon catabolite repression, when ammonia or some other good nitrogen source is available, the activities of many of the systems involved in the utilization of poor nitrogen sources are decreased.

The main route for ammonia assimilation is to glutamate. Glutamate dehydrogenase catalyses the reductive amination of oxoglutarate by ammonia, in an NADPH-dependent reaction. Another means of forming glutamate was first discovered in bacterial mutants lacking NADP-GDH activity. Ammonia is assimilated to glutamine and glutamate produced as the result of a coupled reaction catalysed by glutamine synthetase and glutamate synthase. Glutamate synthase catalyses the transfer of the amide group of glutamine to oxoglutarate. This NADPH-dependent reaction results in the formation of two molecules of

glutamate (Tyler, 1978). Glutamate dehydrogenases are found in both prokaryotes and fungi. Glutamate synthase has been identified in prokaryotes and some strains of yeast (Brown *et al.*, 1973, Roon *et al.*, 1974) but despite considerable effort, this activity has not been identified in Aspergillus (Kinghorn and Pateman, 1977).

In virtually all cells, glutamate and glutamine are the nitrogen donors for biosynthetic reactions. In enteric bacteria (and probably in a wider range of prokaryotes) glutamine synthetase, in addition to catalysing the synthesis of glutamine from glutamate, acts as a regulatory protein. In this role, glutamine synthetase is involved in the control of the synthesis of a wide array of enzymes involved in nitrogen metabolism.

In Klebsiella, it appears that glutamine synthetase can activate the synthesis of the enzymes responsible for the utilization of proline, histidine, arginine, putrescine, urea, asparagine, tryptophan and molecular nitrogen, and may repress the synthesis of NADP-glutamate dehydrogenase. In addition, it appears that the rate of glutamine synthetase synthesis itself is determined by some form of autogenous regulation.

It appears that in conditions of nitrogen sufficiency, possibly measured by the ratio of glutamine to oxoglutarate, glutamine synthetase is adenylated and when nitrogen is limiting is unadenylated. The unadenylated form can bind to DNA and activate the transcription of the other enzyme systems. This subject has been reviewed by Tyler (1978).

d. Transcriptional control in fungi

Clustering of genes with related function is much less common in fungi than in bacteria. Some apparent clusters whose products appeared to form aggregates, are now interpreted as being single genes coding for multi-functional proteins folded into separate domains with different enzyme activities. This is the situation with the aro system in Neurospora crassa (Lumsden and Coggins, 1977) and the urea degradation system encoded by dur 1,2 in S. cerevisiae (Cooper *et al.*, 1980). Such systems can be misleading as they initially suggest coordinate control of the various activities and the possibility of operon organisation. These systems involve the transcription as a single unit of more than one activity but not polycistronic transcription of individual enzymes. The existence of a polycistronic message has not been convincingly demonstrated in any fungal system. No eukaryotic gene cluster has been shown to have all the characteristics of the bacterial operon. Fungi do, however, contain some of the elements of bacterial transcriptional control.

Cis-acting regulatory sites have been identified beside some single structural genes such as yeast arginase, ornithine transaminase, carbamoyl phosphate synthase and ornithine carbamoyltransferase (reviewed by Fincham *et al.*, 1979). Cis-acting regulatory regions in Aspergillus are described in Chapter I.3.d. Genes coding for proteins concerned with regulating individual systems are also known such as qa-1 in Neurospora and gal-4 in S. cerevisiae. These systems are described below.

There are many fungal examples of increased

enzyme activity in the presence of an enzyme's substrate and this is generally referred to as induction. There have been some demonstrations that this induction probably does represent activation of transcription. Studies using inhibitors of transcription and translation, such as lomofungin and cycloheximide, have shown that both processes are required for induction and that in the presence of the inducer and an inhibitor of translation, cells develop the capacity to produce the particular enzyme. The capacity presumably corresponds to the accumulation of the mRNA for the particular enzyme. This has been found for the induction of nitrate reductase by nitrate in Neurospora (Subramanian and Sorger, 1972), arginase by arginine in Aspergillus and in S. cerevisiae (Cybis and Weglenski, 1972; Bossinger and Cooper, 1977), allophanate hydrolase activity by urea in S. cerevisiae (Lawther and Cooper, 1975), isopropylmalate isomerase by isopropylmalate in Neurospora (Reichenbecher *et al.*, 1978) and uricase by uric acid in Neurospora (Wang and Marzluf, 1979).

Development of the techniques for cloning fungal genes should provide the means of following specific mRNA levels under differing metabolic conditions and of demonstrating the extent of transcriptional control more convincingly than has previously been possible. In S. cerevisiae orotidine-5-phosphate (OMP) decarboxylase coded for by ura-3, is the last enzyme in the pyrimidine biosynthetic pathway. It was found some years ago that a mutant strain accumulating dihydroorotic acid had a high level of OMP decarboxylase activity which suggested that dehydroorotic acid induced OMP decarboxylase synthesis

(Lacroute, 1968). Recent cloning of the ura-3 gene has made possible a comparison of the levels of ura-3 mRNA in the mutant strain and in wild-type (both grown in excess uracil) by hybridisation of labelled mRNA from each strain to cloned ura-3 DNA. The mutant strain had five times the wild-type OMP decarboxylase activity and five times the wild-type ura-3 mRNA level. No difference was observed between the decay rates of ura-3 mRNA in wild-type and the mutant. It was therefore concluded that induction of OMP decarboxylase occurs at the level of transcription (Bach *et al.*, 1979).

In N. crassa four genes necessary for the catabolism of quinate to protocatechuic acid are tightly linked and map in the order qa-1, qa-3, qa-4, qa-2. qa-1 is a regulatory gene and the others code for, respectively, quinate dehydrogenase, dehydroshikimate dehydrase and catabolic dehydroquinase (Case and Giles, 1976). Quinate, as sole carbon source, causes the induction of the three enzymes. The first qa-1 mutants isolated were recessive in diploids and deficient in all three enzyme activities. Constitutive semi-dominant qa-1 mutants have also been isolated. This suggests that the qa-1 product exerts positive control over the synthesis of the three enzymes. The possibility that the three were synthesised constitutively but in an inactive form and that the qa-1 product, synthesised only in the presence of quinate, was required to activate them, has been eliminated. Density labelling with D_2O demonstrated that two of the enzymes, dehydroquinase and quinate dehydrogenase are synthesised *de novo* in the presence of quinate (Reinert and Giles, 1977).

Thus it seems likely that in the presence of quinate the qa-1 product activates the transcription of the three structural genes.

There is also a specific regulatory protein involved in the control of the gal cluster concerned with galactose catabolism in yeast. The product of gal-4 is thought to be a positive acting regulatory protein. The gal-4 locus is not linked to the cluster. The cluster comprises the three genes gal-7, gal-10 and gal-1 coding for respectively, galactose-1-phosphate uridyl transferase, uridine diphosphogalactose-4-epimerase and galactokinase. Since several yeast clusters have been found to encode a single polypeptide with more than one enzyme activity, this possibility was specifically investigated with the gal cluster. Two of the enzymes purified as separate polypeptides, no precursor polypeptide was found and in strains carrying a nonsense mutation in one of the genes, only the product of that gene was affected (Broach, 1979).

The three enzymes are induced during growth on galactose. It has been suggested that it is a product of galactose metabolism rather than galactose itself which induces the enzymes and that this metabolite is produced by the product of the gal-3 locus (which is unlinked to the other gal loci) (Broach, 1979). However, the increases in enzyme activity during growth on galactose will be referred to here as galactose induction.

An immunoprecipitation assay which enables detection of the gal-7 product, the transferase, in yeast extracts and among the polypeptides synthesised in an RNA

dependent *in vitro* translation system, was used to compare the levels of transferase and transferase mRNA in induced and uninduced cultures. The level of transferase mRNA in a strain with an amber mutation in the gal-4 gene was also measured, under inducing conditions. It was concluded that induction of transferase activity occurs at the level of transcription, though the possibility of post-transcriptional control was not excluded, and that the gal-4 product is necessary for the appearance of functional transferase mRNA (Hopper *et al.*, 1978).

The level at which galactose induction of galactokinase occurs has also been investigated (though not the involvement of gal-4 in galactose induction). The percent of total polysomes immunoprecipitated from galactose induced and uninduced cells was compared, as was the amount of labelled galactose mRNA (purified from the precipitated polysomes) hybridising to total DNA in the presence of total unlabelled mRNA from induced and uninduced cultures. It was found that the induced cells had a large increase in the level of galactokinase mRNA. It was confirmed that the purified mRNA (from the polysomes) did code for galactokinase by using it to detect a clone of yeast DNA containing the galactokinase gene. *E. coli* strains containing this plasmid had a low level of yeast galactokinase activity and hybridisation of the purified galactokinase mRNA to the plasmid DNA was unaffected by excess mRNA from uninduced cells (Schell and Wilson, 1979). Thus galactose induction of transferase activity and galactokinase activity probably result from increased transcription of gal-7 and gal-1 and this increased

transcription of, at least, gal-7 requires the presence of the gal-4 product.

The details of the mechanism of the gal-4 protein's involvement in induction are unclear. It was previously thought that gal-4 was itself regulated by repression mediated by the product of gal-80 (unlinked to the other gal genes) and that this repressor protein bound to an operator, gal-81 adjacent to gal-4. Recent evidence indicates that gal-4 is expressed constitutively, that in the absence of inducer gal-4 protein activity is prevented by a direct cytoplasmic interaction with the gal-80 product, and that gal-81 is not an operator but is a part of the gal-4 gene, the part coding for the site of interaction with the gal-80 product (Matsumoto *et al.*, 1978, 1980).

In fungi as in bacteria, in addition to the controls associated with individual systems, there are more general controls determined by the cell's overall carbon or nitrogen status - carbon catabolite repression and nitrogen repression.

e. Carbon catabolite repression in fungi

In fungi, carbon catabolite repression appears to be as prevalent as in bacteria as indicated by the decreases in the activities of enzymes responsible for the utilization of poor carbon sources in the presence of a good carbon source like glucose. No evidence has been found to implicate the level of cAMP in the carbon catabolite effect. Though referred to as 'repression', it has not been generally shown that control is exerted at the level of transcription.

Cloning of cyc-1, the structural gene for cytochrome C in S. cerevisiae (Montgomery *et al.*, 1978) has enabled a convincing demonstration that carbon catabolite regulation of cytochrome C activity does occur at the level of transcription. The rates of cyc-1 mRNA synthesis in cells grown under derepressing and repressing conditions were assayed by hybridising total mRNA from each culture to cloned cyc-1 DNA. The derepressed cells synthesised cyc-1 mRNA at six times the rate of repressed cells and the half-life of cyc-1 mRNA was the same under repressing and derepressing conditions (Zitomer *et al.*, 1979). This 6-fold difference equals the difference in the rates of cytochrome C synthesis in derepressed and repressed cells, measured by immunoprecipitation (Zitomer *et al.*, 1976). Regulation at the level of transcription is therefore sufficient to account for the difference in the levels of cytochrome C protein in derepressed and repressed cells.

In Aspergillus, three loci are known which are somehow involved in carbon catabolite repression. Mutation in any one of these loci, creA (Arst and Cove, 1973; Bailey and Arst, 1975), creB and creC (Hynes and Kelly, 1977) can cause derepression of a number of systems involved in carbon metabolism.

f. Nitrogen repression in fungi

In fungi, like bacteria, the activities of many of the enzymes and uptake systems for the utilization of poorer nitrogen sources are decreased in the presence of better ones, most notably, ammonia. It is not known at which level the 'repression' acts, nor has the metabolic

effector (or effectors) been established. Nitrogen regulation will be outlined in a later section (Chapter I.3.b) with reference to Aspergillus.

g. Enzyme inhibition and inactivation in prokaryotes and fungi

(i) Inhibition

In both prokaryotic and fungal cells there are many examples of enzyme activity being decreased by the loose binding of a product of the pathway, usually the end-product, in which the enzyme acts (Fincham *et al.*, 1979). As the concentration of the product increases the loose binding causes a conformational change in the enzyme such that it can no longer function so efficiently. This feed-back inhibition is particularly important when the enzyme acts at a branch point in two or more pathways. A decrease in the activity of the enzyme will then affect the rate of production of all the pathways.

(ii) Inactivation

Comparatively recently, it has become appreciated that selective inactivation is an important enzyme activity control in micro-organisms. This includes both modification inactivation in which the enzyme protein remains intact but loses activity either through a change in physical state caused by the tight binding of ligands or attachment or removal of a covalent modifying group, and degradative inactivation in which at least one polypeptide bond is cleaved. This latter is distinct from turnover though as with turnover this first nick may result in the enzyme being completely degraded by less specific proteases.

Selective inactivation of enzymes, following a change in the carbon or nitrogen status of the cell, provides a quick means of preventing an enzyme from continuing to act in a pathway which may have become futile or wasteful. In yeast and bacteria, some enzymes involved in the utilization of poor carbon sources are inactivated when a preferred source like glucose becomes available (Holzer, 1976; Feldman and Datta, 1975). In bacteria at least, therefore, in addition to carbon catabolite repression of synthesis there is also some carbon catabolite inactivation.

The adenylation of glutamine synthetase in some bacteria when transferred from conditions of nitrogen limitation to sufficiency is an example of a general effect of enzyme inactivation on nitrogen metabolism. In Candida utilis, too, glutamine synthetase is inactivated when cells are transferred to ammonia or glutamine medium.

Inactivation may occur through changes in the conformation of the octameric enzyme with its separation into tetramers, followed by dissociation into component monomers and dimers (Ferguson and Sims, 1974b; Sims *et al.*, 1974).

While the mechanisms of some inactivations are understood, many more have not yet been sufficiently explained. Switzer (1977) has reviewed the subject and lists many examples of inactivations, and mechanisms, where known. The proteolytic type of mechanism and its importance in both micro-organisms and mammalian cells has been reviewed by Holzer and Heinrich (1980).

3. Control of Nitrogen Metabolism in Aspergillus

a. Induction

A number of systems involved in nitrogen catabolism may be subject to induction as indicated by increased activity of the system in the presence of its substrate or a related metabolite. Examples of this include: the induction of nitrate and nitrite reductases by nitrate (Pateman *et al.*, 1964; Cove, 1979, review); the induction by proline of proline permease, proline oxidase and pyrroline-5-carboxylate dehydrogenase (Arst and MacDonald, 1975, 1978); the induction of most of the enzymes of the purine degradation pathway by uric acid and in some cases allantoin (Scazzocchio and Darlington, 1968); the induction of acetamidase by acetamide and other sources of acetyl-CoA and by ω-amino acids (Hynes, 1970, 1978a); the induction by arginine of arginase and ornithine amino-transferase (Cybis and Weglenski, 1972; Bartnik *et al.*, 1977).

The work of Cybis and Weglenski (1972) indicates that the increased activity of arginase in the presence of arginine does correspond to increased synthesis of arginase mRNA. Cells were grown up in a non-inducing medium and transferred to medium containing arginine and a protein synthesis inhibitor. After transfer to a further medium lacking both arginine and the protein synthesis inhibitor the cells had increased arginase activity. This implies that the synthesis of the arginase mRNA was increased in the first transfer medium which contained arginine and the mRNA was translated in the second transfer medium which lacked arginine. The increase in arginase activity did not occur when an inhibitor of transcription was also present in the arginine medium.

b. Nitrogen regulation

A number of enzyme and uptake systems in Aspergillus are subject to nitrogen regulation. In the presence of good nitrogen sources, nitrogen sources which will support the most growth, the activities of the enzyme and uptake systems for the utilization of poorer nitrogen sources tend to be reduced. This effect is generally most pronounced when ammonia is present in the growth medium at a concentration sufficient for it to serve as the sole nitrogen source. In the presence of ammonia as sole nitrogen source the activities of many enzyme and uptake systems are reduced to basal levels (Pateman and Kinghorn, 1977, review).

This effect has been termed 'nitrogen repression' or, more commonly, 'ammonia repression' as it appears that either extracellular or intracellular ammonia may mediate

the decreases in activity. It has also been suggested, however, that glutamine or a related metabolite may be the true effector (Hynes, 1974a; Cook and Antony, 1978, 1980). The term 'repression' implies, in analogy with prokaryotic systems, that transcription of the structural gene for the enzyme or permease is prevented. There is no strong evidence to support this.

The effector of nitrogen regulation is not known and it is not known if nitrogen regulation acts at the level of protein synthesis or at the level of inhibition or inactivation or at both levels. In the present work the term 'nitrogen regulation' will be used wherever possible but for clarity it will also be necessary to frequently use the terms 'nitrogen repression' and 'ammonia repression'.

c. Genes with broad effects on nitrogen regulation

(i) areA. Strains carrying a mutation in areA show general pleiotropic effects on nitrogen regulation. Repressed and derepressed alleles are known. The repressed strains cannot, or can only poorly, utilize a wide range of nitrogen sources including amino acids, purines, amides, nitrate and nitrite, while growth on ammonia in unaffected. The derepressed strains can be derepressed for one or more activities which are subject to ammonia repression in wild-type.

Utilization of acetamide and most amino acids as carbon sources or carbon and nitrogen sources is not affected in $areA^r$ strains. These strains are also able to use acetamide and proline (but not other amino acids) as nitrogen sources in the presence of non-catabolite repressing

carbon sources (Arst and Cove, 1973).

The existence of both repressed and derepressed phenotypes indicates that areA is a regulatory gene. In heterozygous combinations of areA mutants with wild-type or mutants with each other, higher enzyme or uptake activity is dominant or semi-dominant to poorer activity or growth. Arst and Cove have suggested that the areA locus specifies a protein capable of allowing or essential for the synthesis of a number of enzymes involved in nitrogen metabolism but which cannot function in the presence of ammonia.

(ii) tamA. The tamA locus is analogous to areA. Repressed tamA alleles are known which have lowered or undetectable levels of ammonia controlled systems in the absence of ammonia. One derepressed tamA allele has also been isolated. This mutant is ammonia derepressed for a number of ammonia controlled systems.

The existence of alleles at the tamA locus which can determine either repression or derepression indicates that tamA, like areA, is a regulatory gene involved in ammonia repression (Kinghorn and Pateman, 1975a; Pateman and Kinghorn, 1977).

(iii) meaA. Mutation in this gene can result in simultaneous resistance to the ammonia analogue, methylammonia, and derepression of several ammonia repressed activities (Arst and Cove, 1969). The meaA strains have decreased methylammonia uptake (Arst and Page, 1973; Pateman *et al.*, 1973) and it is likely that meaA specifies a component of the ammonia transport system (Pateman *et al.*, 1974). Decreased activity of the ammonia transport system does

not, however, account for the ammonia derepression of meaA strains.

(iv) gdhA. Strains carrying a mutation at this locus have reduced NADP-glutamate dehydrogenase activity and it has been shown that gdhA is the structural gene for the enzyme. The gdhA mutants are derepressed for a number of ammonia repressible systems. These strains are more sensitive than wild-type to high external ammonia concentrations and have high intracellular concentrations of ammonia (Kinghorn and Pateman, 1973, 1975b; Pateman *et al.*, 1973).

Strains deficient in NAD-glutamate dehydrogenase activity and unable to utilize L-glutamate as a carbon source, designated gdhB, do not show abnormalities with respect to ammonia control (Kinghorn and Pateman, 1973).

d. Cis-acting regulatory regions

Three cis-acting regulatory regions have been identified in Aspergillus. In each case an $areA^r$ strain was subjected to mutagenic treatment and a colony regaining the ability to grow on a particular nitrogen source was isolated. The genes known to have a closely linked control region are:

(i) The structural gene for acetamidase, amdS. The amdI9 mutation results in increased induction of acetamidase by acetate, acetamide and other sources of acetyl-CoA. Both amdI9 and another mutation, amdI18, which may define a promoter site for amdS, map at one end of the amdS gene, outside the apparent coding region (Hynes, 1975, 1978b).

(ii) The putative structural gene for the uric acid and xanthine permease, uapA. The uap-100 mutation is adjacent

to uapA and is described as initiator constitutive. The fully induced level of activity is higher than in wild-type, as is the uninduced level (Arst and Scazzocchio, 1975).

(iii) A proline permease structural gene, prnB. Mutations in the adjacent prn^d regulate the expression of prnB and suppress $areA^r$ mutations for proline utilization (Arst and MacDonald, 1975; Arst *et al.*, 1980).

e. Clusters

(i) prn cluster. The prnB locus and control region described above are part of a gene cluster concerned with proline utilization. In addition to prnB which probably specifies a component of the proline uptake system, the cluster also contains the structural gene for proline oxidase, prnD and the structural gene for Δ'-pyrroline-5-carboxylate (P5C) dehydrogenase. There is another gene, prnA, which may code for a positive-acting regulatory protein necessary for the expression of prnD, prnC and to a lesser extent, prnB. The gene order is prnA-prnD-regulatory region (prn^d)-prnB-prnC.

Proline uptake, proline oxidase and P5C dehydrogenase are all induced by proline. Uptake and proline oxidase are subject to carbon catabolite repression. All three activities are repressed by ammonia. Evidence has been presented which suggests that under some conditions prnB and prnC may be transcribed as a single unit (Arst and MacDonald, 1975, 1978; Arst *et al.*, 1980).

(ii) Nitrate and nitrite reductases

The structural genes for nitrate reductase, niaD, and nitrite reductase, niiA, are closely linked (Cove,

1979, review). A cis-acting regulatory mutation affecting niiA has been mapped between the two genes. This mutation is most probably caused by a translocation and it may be that niiA along with its normal promoter has been fused to a promoter normally in a different linkage group. In strains carrying this nis-5 mutation, niiA and niaD are separated by a large number of genes. The new promoter affects the regulation of nitrite reductase but has no effect on nitrate reductase. This provides some evidence that the expression of niiA does not solely occur by synthesis of a dicistronic message of niaD and niiA (Arst *et al.*, 1979).

f. Enzyme inactivation

There is evidence that enzyme inactivation has a role in controlling the activity of at least two systems involved in nitrogen metabolism in Aspergillus.

(i) Nitrate reductase

Carbon starvation causes a rapid decrease in nitrate reductase activity even in the presence of inducer, nitrate, and this decrease is not dependent on protein synthesis. Transfer of cells to medium containing ammonia causes a slightly less severe protein synthesis independent decrease (Hynes, 1973). Dunn-Coleman and Pateman (1977), found that NADPH could protect nitrate reductase *in vitro* and suggested that inactivation of nitrate reductase *in vivo* was in part dependent on the ratio of NADPH: NADP.

(ii) NADP-L-glutamate dehydrogenase

NADP-GDH activity is rapidly lost in cells transferred from a medium containing a good carbon source to

medium containing L-glutamate as the carbon source, or no carbon source. This decrease in activity is dependent on protein synthesis suggesting it may be mediated by a newly synthesised protease (Hynes, 1974b; Kinghorn and Pateman, 1974).

4. Urea Utilization

a. Enzymes responsible for urea degradation

Many micro-organisms can utilize urea as a source of nitrogen. In over 200 species of bacteria, several algae, and several species of fungi, urea is hydrolysed by the enzyme urease (E.C.3.5.1.5.) (Varner, 1961). The seeds of higher plants contain a large quantity of urease and this contributed to its being the first enzyme to be crystallised (Sumner, 1926). Jack bean urease is a polymeric enzyme but despite considerable work on the subject the number of subunits in the active enzyme is still not generally agreed. It is, however, generally agreed that jack bean urease contains only one type of subunit (Reithel, 1971).

The control of urease activity has not been studied in many micro-organisms. In Klebsiella urease is present at a high level when the cells are starved for nitrogen and urease synthesis is repressed when the external ammonium concentration is high. Urease synthesis is not induced by urea and is not subject to carbon catabolite repression. The synthesis of urease, like several other enzymes in Klebsiella, is controlled by glutamine synthetase. Mutants lacking glutamine synthetase fail to produce urease and mutants forming glutamine synthetase at

a high constitutive level also form urease constitutively (Friedrich and Magasnik, 1977).

In Neurospora, four loci are known which are involved in urea utilization. Two of these, ure1 and ure2, are closely linked on one chromosome (Kolmark, 1969) and the other two are unlinked on a different chromosome. Mutation at any one of the four loci may result in loss of urease activity (Haysman and Branch Howe, 1971).

In some species of algae and yeast, urea is degraded by a different enzyme activity (Roon and Levenberg, 1968; Leftley and Syrett, 1973). This activity is induced by urea and repressed by ammonia (Roon and Levenberg, 1972). Whitney and Cooper (1972) have shown that the activity actually results from a complex of two enzymes, urea carboxylase and allophanate hydrolase. The structural genes for these two enzymes are closely linked (Lam and Cooper, 1977).

It has been noted that organisms appear to contain either urease or the urea carboxylase-allophanate hydrolase complex, not both. In assays of cell-free extracts the two are readily distinguished. The enzyme complex requires ATP, Mg^{2+}, and K^{+} and is inhibited by avidin (Roon and Levenberg, 1968). Urease does not require the addition of cations or ATP and is insensitive to avidin.

b. Urea uptake

Transport of urea into the cell has been studied in very few micro-organisms. In Saccharomyces cerevisiae urea transport occurs by two methods: an inducible active transport system which is sensitive to nitrogen repression

and which can transport low concentrations of urea; passive or facilitated diffusion by which higher concentrations of urea can enter the cell (Cooper and Sumrada, 1975).

c. Aspergillus nidulans

In Aspergillus, urea is formed as a purine degradation product and as a product of arginine catabolism. Urea can also enter the cell from the surrounding environment and Aspergillus, like many other micro-organisms, can utilize urea as a sole source of nitrogen but not of carbon. It has been supposed by previous workers that the Aspergillus enzyme responsible for degrading urea to ammonia is a urease (E.C.3.5.1.5.) and for clarity it should be said now that the present work supports this.

Urease is the final enzyme in the purine degradation pathway. Mutants at three loci, ureB, ureC and ureD (formerly uX, uY and uZ) were isolated on the basis of their failure to utilize purines as a sole source of nitrogen. These strains cannot grow on urea as a sole source of nitrogen and lack urease activity (Scazzocchio and Darlington, 1968). A further class of urease deficient mutant, putatively at a fourth locus, ureE has also been isolated. The strain, ureE3 grew about half as well as wild-type on urea but had undetectable urease activity *in vitro* (Kinghorn and Pateman, 1977).

An active transport system for urea has previously been partially characterised in Aspergillus and it appears that the locus ureA specifies a component of this system (Dunn and Pateman, 1972 and unpublished results).

Investigation of the metabolic regulation of the urea transport system is a subject of this thesis and the preliminary work of Dunn and Pateman forms a basis for this investigation. Their unpublished results are given in the introductions to relevant results chapters.

At the onset of this work urea utilization in Aspergillus seemed a favourable system for investigating the mechanism of eukaryotic gene regulation at the molecular level. It was intended that this work would be the foundation for a further study which would involve cloning the ureA and ureB genes, and investigating their control at the molecular level, e.g. the genes and their products would be sequenced and compared to determine coding regions, control mutants would be isolated and control regions orientated with respect to the structural genes and the effects of regulatory mutations on the expression of the structural genes studied by following mRNA production.

The system seemed favourable for such an eventual study for the following reasons:

(i) Metabolic regulation of urease was expected to be relatively simple. It was expected that urease would not be subject to carbon catabolite repression but would be governed by nitrogen regulation and possibly induction.

(ii) ureA and ureB were believed to be closely linked, and it was expected that being linked to the likely structural gene for uptake, ureB would be a structural gene for urease. Uptake and urease activities were believed both to be subject to nitrogen regulation. This suggested that the two genes might be subject to some form

of joint regulation and that the clustering of the genes might have significance with respect to the mechanism of control of the region. A cis-acting mutant isolated for its effect on expression of one of the two genes might also affect expression of the other.

(iii) It was thought that a deletion map of the ureA ureB region, to be used in conjunction with *in vitro* studies in determining coding regions, orientating newly isolated mutations including control mutations, could be quite easily produced. Thiourea-resistant mutants could be readily isolated and urease activity detected using a plate test. It was expected that the existence of cis-acting control regions might be revealed amongst the deletions.

(iv) Urease deficient strains were (with one exception) clean, non-leaky mutants. Complementation studies and crossing programmes were therefore expected to be straightforward.

(v) Aspergillus has high urease activity. It was therefore thought likely that there would be a considerable amount of both urease protein and urease mRNA. Consequently it was expected that through fractionation of total mRNA utilizing the wild-type and a ureB deletion strain, subsequent production of a probe for the ureB gene would be feasible. As ureA and ureB were believed to be linked, it would be possible to isolate the ureA gene also from the gene bank and therefore be possible to clone not only an enzyme structural gene but also that of a transport protein.

(vi) By analogy with urease having been the first enzyme

to be crystallised, it was expected, especially in the light of developments in protein chemistry in the last fifty years, that purifying Aspergillus urease would be quite straightforward. As it was thought that Aspergillus probably made quite a lot of urease and that this could be easily purified, it was also believed that antibodies could be readily made by injecting rabbits with the purified protein and that purified urease could be readily sequenced. Antibodies and the amino acid sequence of urease could be used to identify the *in vitro* translation product of a putative ureB probe before cloning.

(vii) It was known that conductimetry provides a very sensitive means of assaying commercially purified urease. It was considered likely that a conductimetric assay could be developed for urease in crude extracts. This could be used, for example, to determine the level of wild-type urease activity after growth under various conditions, to assay mutant strains, and to follow urease activity during purification procedures.

Towards the eventual aims stated above, the work in this thesis is mainly concerned with:

(i) confirming and extending the genetic characterisation of the ure loci and investigating their roles;

(ii) developing a sensitive assay for urease activity and partially characterising wild-type urease activity;

(iii) assaying urease and urea uptake activities of wild-type and mutant strains after growth under various conditions to: discover what factors appear to control urease activity; discover whether regulation appears to be at the level of protein synthesis; find ways of

specifically increasing or decreasing urease activity; (iv) confirming the close linkage of ureA and ureB and attempting to isolate strains carrying deletions in the region, to be used in constructing a deletion map and in isolating a probe for ureB.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Media and Supplements

a. Media

The media are essentially those described by Pontecorvo *et al.* (1953), as modified by Cove (1966).

Complete medium (CM)

nitrogen-less salts solution	20ml
peptone	2g
yeast extract	1g
casamino acids	5ml
ammonium tartrate	5ml
D-glucose	10g

The volume was made up to 1 litre with distilled water and, if necessary, the pH was adjusted to 6 with 5N sodium hydroxide. For solid medium, No.3 Oxoid agar was added at 1.2%. Vitamin solution, 1ml/l, was added after autoclaving.

Minimal medium (MM)

nitrogen-less salts solution	20ml
sodium nitrate	6g
D-glucose	10g

The volume was made up to 1 litre with distilled water and the pH was adjusted to 6.5 with 5N sodium hydroxide. For solid medium, No.3 Oxoid agar was added at 1.2%.

The following media, used either in solid or liquid form, are all based on the recipe for minimal medium:

Carbon-less minimal medium (-C MM)

The glucose was omitted from the recipe for MM.

Nitrogen-less minimal medium (-N MM)

The nitrate was omitted from the recipe for MM.

Carbon-less and nitrogen-less minimal medium (-C-N MM)

The glucose and nitrate were omitted from the recipe for MM.

All media were sterilised by autoclaving for 20 min at 20lb/in^2.

Nitrogen-less salts solution

potassium chloride (KCl)	26g
magnesium sulphate ($MgSO_4$ $7H_2O$)	26g
potassium dihydrogen phosphate (KH_2PO_4)	76g
trace elements solution	50ml

The volume was made up to 1 litre with distilled water. Chloroform, 2ml, was added as a preservative and the solution was stored at 4^{o}C.

Trace elements solution

sodium tetraborate	$Na_2B_2O_7 . 10H_2O$	40mg
copper sulphate	$CuSO_4 . 5H_2O$	400mg
ferric orthophosphate	$FePO_4 . 4H_2O$	800mg
manganese sulphate	$MnSO_4 . 4H_2O$	800mg
sodium molybdate	$NaMoO_4 . 2H_2O$	800mg
zinc sulphate	$ZnSO_4 . 7H_2O$	8g

The volume was made up to 1 litre with distilled water and the solution stored at 4^{o}C.

Vitamin solution

riboflavin	1g
nicotinamide	1g
p-aminobenzoic acid	100mg
pyridoxin-HCl	500mg
thiamine-HCl	500mg

biotin	10mg
inositol	4g
calcium D-pantothenate	2g

The volume was made up to 1 litre with distilled water and the solution was autoclaved and stored at 4°C in a dark bottle.

b. Supplements

supplement	concentration of stock solution	final concentration
Vitamins		
biotin	0.1mg/ml	0.1µg/ml
p-aminobenzoic acid	0.1mg/ml	0.1µg/ml
pyridoxin-HCl	0.5mg/ml	0.5µg/ml
riboflavin	1.0mg/ml	1.0µg/ml
nicotinic acid	1.0mg/ml	1.0µg/ml
Carbon sources		
D-glucose	1M	50mM
D-glycerol	1M	50mM
sodium acetate	1M	50mM
L-arabinose	1M	50mM
Nitrogen sources		
ammonium tartrate	1M	as specified in test
ammonium chloride	1M	"
sodium nitrate	1M	"
L-glutamine	0.2M	"
L-proline	1M	"
L-arginine	1M	"
L-ornithine-HCl	1M	"
L-alanine	1M	"
L-histidine-HCl	0.2M	"

supplement	concentration of stock solution	final concentration
Nitrogen sources (cont'd)		
L-sodium glutamate	1M	as specified in text
urea	1M	"
casamino acids	1M	"
adenine	5mg/ml	"
allantoin	1mg/ml	"
sodium urate	-	"
Toxic agents and urea analogues		
thiourea	1M	as specified in text
hydroxyurea	0.5M	"
allylurea	1M	"
selinourea	20mM	"
p-fluoro-phenylalanine	1%	0.0125%
sodium deoxycholate	5%	0.08%
cycloheximide	-	10µg/ml
Metal salts		
nickel acetate	0.1M	as specified in text
nickel sulphate	0.1M	"
copper sulphate	0.1M	"
manganese sulphate	0.1M	"
Others (for haploidisation)		
sodium thiosulphate	0.2M	2mM
sodium acetate	50%	1.25%
galactose	20%	0.5%

Chemicals

Analytical grade chemicals were used whenever possible.

Radioisotopes

^{14}C-thiourea was obtained from Radiochemicals Amersham. The stock solution was 50µCi/ml.

Mutagens

1,2,7,8-Diepoxyoctane (Aldrich)

N-methyl-N'-Nitro-N-Nitrosoguanidine (Sigma)

Solid media in petri dishes

Glass petri dishes were used for master-plates for the analysis of cross or haploidisation progeny. Disposable plastic petri dishes, usually containing 20ml medium, were used for all other purposes.

2. Enzyme Assays

a. Growth of mycelium

The procedure was basically that described by Cove (1966). Strains to be enzyme assayed were first grown up on complete medium slopes (test-tubes, 6in x 1in with about 20ml medium, sloped while the medium solidified) at 37^{o}C for about 4 days and then stored at room temperature. When used for assays, these cultures were between 10 and 42 days old.

The conidia were scraped off the surface of the slope with a wire loop and suspended in 10ml sterile distilled water containing a few drops of tween-80 solution (0.1ml tween-80 detergent in 100ml distilled water, sterilised). The conidia were separated by vigorous agitation (with a 'vortex' or 'whirlimix'). The suspension was filtered through non-absorbent cotton wool to remove any debris, and counted with a haemocytometer. An inoculum of about 130 x 10^{6} conidia was added to a 1 litre Ehrlenmeyer flask containing 200ml medium. The nitrogen and/or carbon source was added to the -C, -N or -C-N minimal medium just prior to inoculation, as was vitamin solution.

The mycelia were grown at 30^{o}C in a Gallenkampf orbital incubator, generally for 18h. To harvest the mycelia, the contents of the growth flask was filtered through a nylon net, (nytal nylon screen, 149 microns) washed with about 100ml distilled water and dried by pressing between absorbent paper towels. The pressed weight of cells was usually 0.5-5.0g/l. The mycelium was either used directly for assay or was wrapped in aluminium

foil, put into liquid nitrogen, then stored in a liquid nitrogen tank.

When cells were grown up for 18h then transferred to a second medium, they were harvested through the nylon net, washed with about 100ml of sterile distilled water at 25°C and spooned into the second flask, also at about 25°C. After the appropriate number of hours incubation in the second medium, the mycelia were harvested and used as above.

b. Preparation of cell-free extracts

Mycelium, 0.05-0.5g, was ground for 1.5 min in a cold mortar with about 0.1g acid-washed sand and cold extraction buffer, 0.5-5ml. The slurry was centrifuged for 20 min at 15,000 rpm at 4°C in a Sorval Superspeed RC2-B. The supernatant was kept on ice for enzyme assay and protein determination.

Extraction buffer:

disodium hydrogen orthophosphate	7g
potassium dihydrogen orthophosphate	6.8g
dithiothreitol	15.4mg
glycerol	200ml

The phosphate salts were disolved in 500ml distilled water and the pH adjusted to 7.75. Glycerol and dithiothreitol were added and the volume made up to 1 litre.

c. Urease assay with Muftic method for ammonia determination (Muftic, 1964).

The reaction mixture, 0.8ml Gomories Buffer, 100µl cell-free extract, 100µl 2M urea, was incubated in sealed test-tubes in a shaking water bath at 30°C for 20 min. The reaction was stopped by the addition of 0.5ml

20% phenol in ethanol. Precipitated protein was removed by spinning in a bench centrifuge. Calcium hypochlorite solution, 1ml, was added and the mixture vigorously agitated with a 'vortex'. The resulting blue colour was allowed to develop for 40 min at room temperature. The optical density was then read at 655nm in a Unicam spectrophotometer. The total 'blank' value to be subtracted from each reading was calculated by adding the individual contributions of 100µl urea, 100µl extract and that of the buffer and solutions mixture.

Serial dilutions of ammonium chloride were used to make a standard curve. Urease activity is expressed in nmoles urea hydrolysed/min/mg.

Gomories Buffer:

25ml of a solution of 0.2M tris-HCl and 0.5M maleic acid was added to 24ml 0.2N sodium hydroxide and the volume made up to 100ml with distilled water. The pH is 7.

Calcium Hypochlorite solution:

135ml of a 20% solution of potassium carbonate was added to 25g calcium hypochlorite in 300ml hot water. The mixture was stirred well while heated gently and the volume made up to 500ml. The filtered solution was stored in a dark bottle at $4^{o}C$.

d. Conductimetric urease assay

This assay measures urease activity by a direct recording conductimetric method. The apparatus which measures conductance changes was designed and built by Dr. A.J. Lawrence, in the Cell Biology Department of the University of Glasgow. This was connected to a Rikadenki

six channel pen recorder.

The conductimetric apparatus is similar to that described by Lawrence (1971), and Lawrence and Moores (1972). The apparatus basically consists of six glass cells of 1ml capacity with platinum electrodes bonded to their inner walls. These cells sit in a thermostatically controlled water bath. Each cell contains a small magnetic stirring pellet which is activated by a large revolving magnet in a compartment underneath the cells. Each cell is individually connected such that any change in conductance is measured and amplified and can be recorded on the chart of a connected pen recorder.

When not in use the cells were filled with a solution of detergent. This prevents the gradual accumulation of a film of protein on the electrodes. Before each assay, the cells were emptied by suction and cleaned thoroughly with distilled water followed by assay buffer.

The routine assay mixture was:

1ml 10mM tricine (N-tris (hydroxymethyl) methyl glycine), (BDH), pH 7.5, degassed

10µl cell-free extract

10µl 2M urea

Reactions were followed at 37^{o}C unless otherwise specified. The buffer and extract were added to the glass cells first and the system allowed to equilibrate before the addition of urea.

The system was calibrated by measuring the conductance change which occurred when a concentrated solution of Sigma urease type IX, about 100 units/ml,

completely hydrolysed known amounts, 20, 30, 40 and 50 nmoles of urea. From this, the conductance change representing the total hydrolysis of 1nmole of urea was established. Urease activity is expressed in nmoles urea hydrolysed/min/mg total protein.

e. Protein determination

All protein concentrations were determined by the method of Lowry *et al.* (1951). Cell-free extract, 20µl, was added to 180µl distilled water in 1ml alkaline copper solution (100 parts 2% sodium carbonate in 0.1N sodium hydroxide, 1 part 2% ammonium tartrate, 1 part 1% copper sulphate). After 10 min at room temperature, 0.1ml Folin's Ciocalteau reagent (BDH) diluted 1:1 with distilled water, was added and the mixture agitated immediately with a 'vortex' and left at room temperature for 30 min.

The developed blue colour was read at 670nm on a Pye-Unicam SP8-100 spectrophotometer. The blank was 1ml alkaline copper solution, 200µl water and 100µl Folin's reagent. Bovine serum albumin serial dilutions were used as the standard. Protein concentrations were generally in the range, 1-3.5mg/ml.

3. Thiourea Uptake Assays

Cells were grown as described in Chapter II.2.a., harvested through nylon net, washed with -C-N minimal medium at 25°C, lightly blotted, and 1g resuspended in 30ml -C-N minimal medium at 25°C, in a 100ml conical flask. This flask was placed in a shaking water bath at 25°C. To start the reaction, 20ml -C-N medium with ^{12}C-thiourea and

^{14}C-thiourea was added. The final concentrations in 50ml were 200µM ^{12}C-thiourea and 2.5µCi ^{14}C thiourea. At times 0, 2, 4, 6, 8, 10 minutes, 5ml aliquots were withdrawn, filtered by suction, and washed twice with 10ml distilled water. The cells were scraped off the filter paper, weighed and placed in 5ml scintillation fluid and the radioactivity measured in a Searle Delta 300 scintillation counter.

Once it had been established that uptake was linear for at least 10 min and that the value of the 10 min measurement was reliable, the above procedure was modified. It was considered that measuring the total uptake during 10 min in duplicate for each flask of cells would provide an even more accurate measurement of uptake than testing one sample every two minutes. In addition, measuring the total uptake during 10 min would be less time-consuming and laborious and would allow the use of less ^{14}C-thiourea.

The modified procedure, carried out in duplicate for each flask of cells, was as follows:

Approximately 0.1g cells was resuspended in 9ml -C-N minimal medium at 25^{o}C in a 25ml conical flask. To start the assay, 1ml -C-N minimal medium containing ^{12}C- and ^{14}C-thiourea to final concentrations as above, was added. After 10 min shaking incubation at 25^{o}C, the contents of the flask were filtered and washed and the cells were weighed and placed in scintillation fluid as above.

Scintillation fluid:

toluene	1000ml
triton-X-100	500ml
2,5,-diphenyloxazole	3.0g
5-phenyloxazolyl benzene	0.3g

Thiourea uptake activity is expressed in pmoles thiourea taken up/min/mg wet weight cells. The ratio of wet to dry weight was a constant. Samples of cells from growth flasks were filtered by suction, washed, filtered and weighed exactly as were the cells used for uptake measurement. The weighed cells were then placed in a 37^{o}C incubator for 48h, to dry. The dried cells were weighed. The ratio of wet to dry weight was always close to 10.

4. Statistics

Most urease and thiourea uptake activities in this thesis are the average of at least three independent determinations. The standard deviation of these determinations (not standard deviation of the mean) is given in brackets after the averaged value.

5. Routine Plate Tests

a. Growth test for ability to use urea as a sole source of nitrogen

Solid -N minimal medium with vitamin solution and 3mM urea.

b. Growth test for resistance to thiourea

Solid minimal medium with vitamin solution and 5mM thiourea.

c. Indicator-dye test for urease activity

Conidia were inoculated (usually 10 strains/ plate) into solid minimal medium supplemented with vitamin solution, and incubated at 37°C for 18h. The petri dish was then flooded with a 6ml solution of urea and phenol-red (4 parts 1M urea, 1 part 0.04% phenol red in distilled water) and incubated for 30 min. The solution was tipped off and the petri dish returned to the incubator for a further hour or until the staining pattern was clear. A negative and a positive control strain were inoculated into each dish.

6. Strains

A biotin auxotroph, biA1, known to be translocation-free was used throughout as wild-type unless a yellow wild-type parent was required for a cross. The yellow wild-type requires p-aminobenzoic acid and pyridoxin. Both wild-type strains were obtained from Mr. E. Forbes, Institute of Genetics, the University of Glasgow. A master strain, M.S.E. (Glasgow number 94) carrying a marker on each chromosome, was used for assigning loci to linkage groups.

The following strains were supplied by Professor J.A. Pateman or Dr. H.N. Arst:

gdhA1 was isolated on the basis of sensitivity to high concentrations of ammonia. It carries a mutation in the structural gene for NADP-glutamate dehydrogenase and lacks that activity. gdhA1 is ammonia derepressed (Kinghorn and Pateman, 1973, 1975b).

meaA8 is resistant to high concentrations of methylammonia and is ammonia derepressed (Arst and Cove, 1969).

tamA^{r}105 and tamA^{r}119 were isolated on the basis of their simultaneous resistance to thiourea, aspartic hydroxymate and methylammonia. They are partially ammonia repressed (Kinghorn and Pateman, 1975a).

areA^{r}550 was isolated by J.R. Kinghorn and J.A. Pateman and is a fully repressed areA allele (Arst and Cove, 1973).

ureA1 is resistant to thiourea and has defective urea and thiourea transport (Dunn and Pateman 1972).

ureB2, B3, B6, ureC1, C5, C7, C8, ureD4 and D9

were isolated on the basis of their failure to grow on purines as a sole source of nitrogen by a replica plating technique following treatment of wild-type with U.V. or diethylsulphate (Darlington, 1966).

ureE3 (now renamed ureB12) was isolated by J.R. Kinghorn and J.A. Pateman. It grows about half as well as wild-type on urea.

All other ure strains were isolated in the course of the present work.

7. Isolation of Thiourea Resistant Mutants

a. Spontaneous mutations

Wild-type conidia were inoculated (about 16 inocula per petri dish) into solid minimal medium containing vitamin solution and thiourea in the range 1-5mM, and incubated for 2-3 days or until resistant colonies appeared on the plates.

b. Induced mutations

The following procedure was carried out several times and variations were tried with most of the conditions. Each condition, such as temperature of incubation, is therefore given as the range in which the condition was varied.

Conidia were scraped from a 10-14 day-old wild-type slope and suspended in 20ml 0.067M Na_2HPO_4-KH_2PO_4 buffer pH7 and counted with a haemocytometer. The conidial suspension (1-40 x 10^6 conidia/ml) was divided equally between two 25ml conical flasks, one for treatment, one as a control. Diepoxyoctane 10-100mM was added to one of the flasks. The flasks were then placed in a shaking water

bath at 20-37°C and incubated for 45 min - 6h.

The suspensions were then transferred to sterile capped tubes and spun down in a bench centrifuge and the buffer decanted. The cells were resuspended in 10ml distilled water and spun down again. This washing procedure was repeated three times. The cells were then resuspended in 10ml distilled water and 10µl withdrawn from each tube for serial dilutions which were plated on complete medium to find how many conidia had survived the mutagenic treatment.

The 10ml treated suspension was divided between 10 flasks of cooled molten medium (200ml minimal medium, vitamin solution and 5mM thiourea per flask) and eight plates were poured from each flask.

Part of the untreated sample was also plated out in thiourea medium to find by how much DEO increased the mutation rate. 1ml of the untreated suspension was added to each of two flasks and poured as above.

The embedded cells were incubated at 37°C for a few days, until colonies appeared on the plates.

8. Isolation of Revertants

Nitrosoguanidine (NTG) was used as the mutagen in all reversion experiments.

Conidia were scraped from a 10-14 day-old slope, suspended in 10ml distilled water with a few drops of tween-80 solution, and filtered through non-absorbent cotton wool. This heavy conidial suspension was spun down in a bench centrifuge, the water decanted and the pellet of cells was resuspended in 10ml of Gomories buffer

(Chapter II.3.c) in which 2.5mg NTG had been dissolved in a 25ml conical flask. The cells were then incubated for 10 min in a shaking water bath at 37°C.

The conidia were spun down in a bench centrifuge, the treatment solution poured off, and the cells resuspended in distilled water. The cells were again spun down and the cells resuspended in fresh distilled water. After this washing procedure had been repeated three times, the cells were resuspended in 10ml distilled water and a sample withdrawn to be plated, after dilution, on solid complete medium to find how many conidia had survived the mutagenic treatment.

The 10ml conidial suspension was added to a flask containing 200ml cooled molten -N minimal medium with vitamins and 3mM urea, and this was divided between 10 petri dishes. The embedded cells were incubated at 37°C for 3-4 days or until colonies appeared on the plates.

9. Crosses

All crosses were set up between strains carrying different colour and vitamin markers. About three loopfuls of conidia from each parent strain were floated onto the surface of about 10ml liquid complete medium in a universal container, mixed together gently, and incubated at 37°C for 24h until a firm mycelial mat had formed. This mat was picked out with sterile forceps, washed by shaking in 10ml sterile distilled water in a universal, and dried by pressing between two sterile filter papers. The dried mat was transferred to a petri dish filled up with solid minimal medium (with no vitamins) and incubated at

37°C for 24h. The plate was then sealed with tape and incubated at 37°C for another 10-14 days until cleistothecia (tiny black balls containing spores) were produced.

Cleistothecia were picked from the plate with a dissecting needle and rolled on 2.5% agar to remove attached conidia and cell debris.

a. Analysis of a small number (100) of progeny

The cleaned cleistothecium was squashed in 1ml of sterile distilled water in a test tube and the tube was agitated vigorously to release the ascospores. This ascospore suspension was stored at 4°C.

A loopful of the ascospore suspension was streaked onto complete medium. Cleistothecia may contain ascospores of only one parent strain ('selfed' cleistothecia) or may contain ascospores resulting from a cross of both parent strains. A crossed cleistothecium is easily recognised as progeny of both parental colours are present.

An ascospore suspension from a crossed cleistothecium was diluted and spread onto complete medium plates and incubated at 37°C for two days. Discrete colonies were randomly picked from these plates and 26 inoculated into solid complete medium in a glass petri dish, and incubated for 3 days. The colonies on this 'master plate' (four master plates for 100 progeny) were then replicated onto various solid selective media with a wire and perspex replicator, to growth test for the appropriate markers.

b. Velvet replica plating

A 1ml ascospore suspension was obtained as above, and diluted by various amounts. Samples of these dilutions were spread on complete medium containing 0.08% sodium

deoxycholate, which causes small compact colonies, incubated for 2 days and the number of colonies on each plate counted. From these counts the amount of ascospore suspension which would result in about 200 colonies/plate was calculated and complete medium and deoxycholate plates (about 30) spread accordingly. Those plates were then incubated for 2-3 days.

The colonies on these plates were replicated onto appropriate selective media by the velvet replicating technique of Mackintosh and Pritchard (1963).

c. Detection of intragenic recombination

A cleaned cleistothecium was squashed in a drop of sterile distilled water on a sterile glass coverslip (on the 2.5% agar plate). The coverslip was then transferred to one plate containing solid -N minimal medium with vitamins, 3mM urea and 0.08% sodium deoxycholate. The ascospore drop was washed off with 0.2-0.3ml sterile distilled water and the ascospores were spread evenly over the surface of the medium. Just before spreading, a loopful of the ascospore solution was taken and spread onto complete medium to determine whether the cleistothecium was crossed.

When crosses were very poor and produced very small cleistothecia, about 30-50 were collected, squashed together on the coverslip, and plated on urea medium as above.

If there were any recombinants, they were easily distinguished after 3 days incubation at 37^{o}C.

d. Measurement of intragenic recombination

A cleaned cleistothecium was squashed in 1ml

distilled water in a test tube. After vigorous agitation a 10µl sample was withdrawn and after dilution (1:100 was usually suitable) was plated onto solid complete medium plates and incubated at 37°C to find the total number of viable spores. The remainder of the suspension was spread over 3 plates containing solid -N minimal medium with vitamins, deoxycholate and 3mM urea. The test tube was washed out with 0.2ml sterile distilled water which was then also distributed between the 3 plates. These plates were incubated at 37°C for 2-3 days until recombinants appeared.

10. Production of Diploids

a. Formation of heterokaryons

Mycelial mats were formed as described for the Crosses. The washed and dried mats were torn into small pieces and plated onto solid minimal medium (without vitamins). After a few days incubation at 37°C some sectors of mixed conidial colour grew out from the piece of mat. The hyphal tips growing out from these regions were cut out and replated on fresh minimal medium. This procedure was repeated until a stable heterokaryon was established, recognised by its equal mix of conidial colours.

b. Selection of diploids

Conidia were scraped from the heterokaryon and suspended in sterile distilled water. The conidial suspension was added to a flask of molten minimal medium and plated out. On incubation at 37°C only diploid colonies could grow well and were recognised by their pale green conidia. These colonies were purified by streaking on

minimal medium and examined with a microscope. Diploid conidia are bigger than haploid conidia.

Diploids were either used for complementation tests or for haploidisation.

c. Haploidisation of diploids

Diploid conidia were inoculated (9 inocula per plate) into solid complete medium containing p-fluoro-phenylalanine (0.0125%). This substance greatly increases the yield of haploid sectors (Lhoas, 1961). After 5-7 days incubation at 37°C the haploid sectors were picked off and streaked onto solid complete medium and incubated to obtain single pure colonies. A master plate was then made by inoculating the discrete haploid colonies into complete medium, 26 inocula per plate. After 3 days incubation the colonies on the master plate were growth tested by replicating them onto the various appropriate media with a perspex and wire replicator.

The strain used for making diploids for assigning loci to chromosomes was M.S.E. This strain carried the following markers on each chromosome:

I	yA2	yellow conidial colour
II	w3	white conidial colour, epistatic to yellow
III	galA1	unable to utilize galactose as carbon source
IV	pyroA4	pyridoxin auxotroph
V	facA303	unable to utilize acetate as carbon source
VI	sB3	thiosulphate auxotroph
VII	nicB8	nicotinic acid auxotroph
VIII	riboB2	riboflavin auxotroph

11. Heterokaryon Complementation Tests

These tests were only set up between strains carrying different colour and vitamin markers. Conidia from each of the parent strains were inoculated into the same point in solid -N minimal medium with 3mM urea and no vitamins. Controls, between strains known to be at different loci, were set up on each plate. The petri dish was incubated at 37°C for 4-5 days or until the control heterokaryons were established.

CHAPTER III

ASSAYING UREASE ACTIVITY AND SOME PROPERTIES OF ASPERGILLUS UREASE

Table 1: Comparison of urease activities measured by the Muftic and Conductimetric methods : urease activity after growth on various nitrogen sources

	Urease Activities (nmoles urea hydrolysed/min/mg)	
Nitrogen Source	Muftic Assay	Conductimetric Assay
10mM L-alanine	364 (32)	641 (100)
10mM L-arginine	441 (52)	660 (89)
10mM nitrate	220 (34)	441 (59)
5mM ammonium tartrate	78 (18)	254 (29)

Strain: biA1, wild-type.

Growth medium: -N minimal medium, vitamin solution, nitrogen sources as shown.

Muftic determinations at 30°C.

Conductimetric determination at 37°C.

Standard deviations are given in brackets.

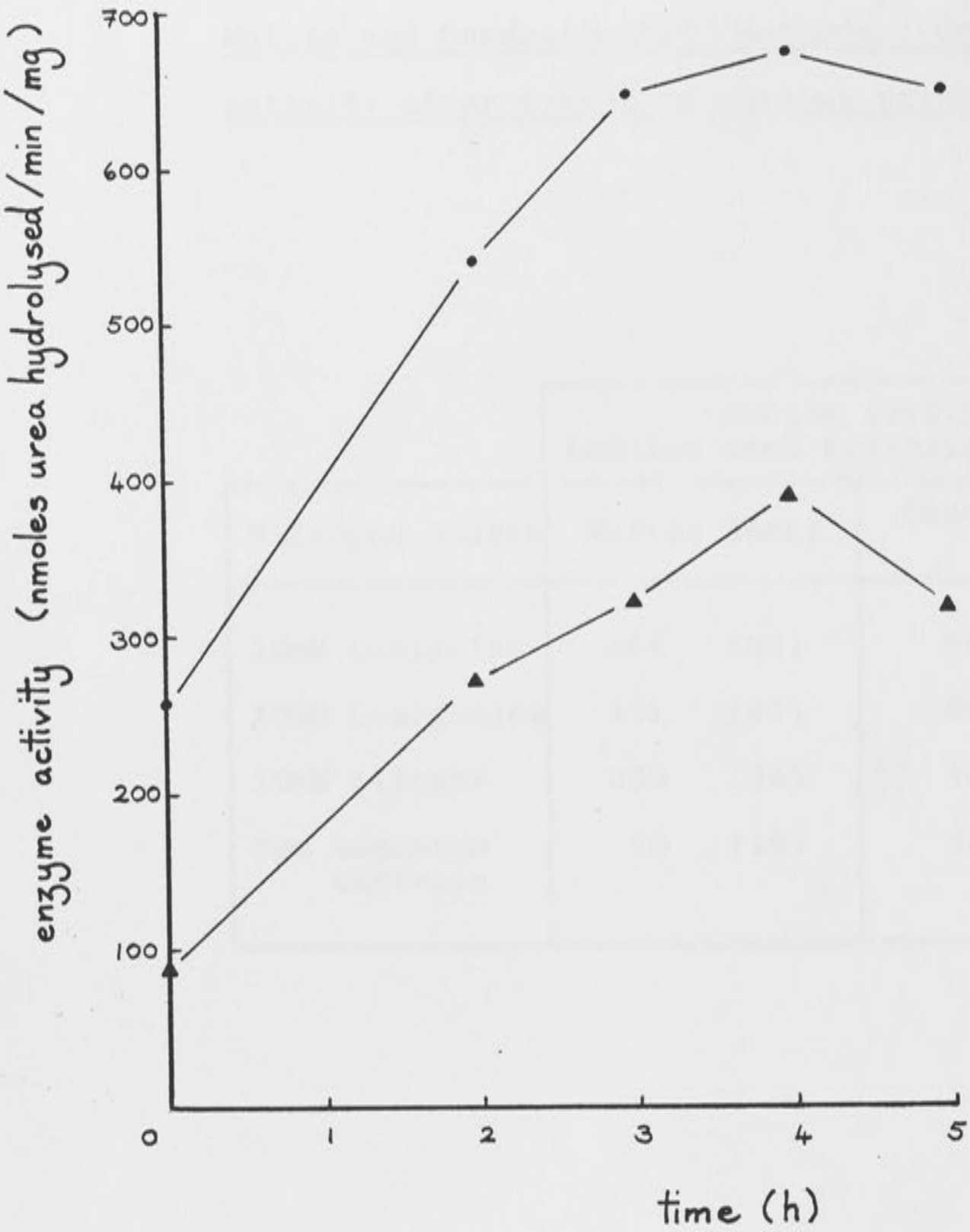


Figure 1: Comparison of urease activities measured by the Muftic and Conductimetric methods : urease activities after various times of nitrogen starvation

Strain: biA1, wild-type

Growth medium: -N minimal medium, vitamin solution, 5mM ammonium tartrate.

Treatment medium: -N minimal medium, vitamin solution.

Symbols: • Conductimetric urease activities (37°)
▲ Muftic urease activities (30°)

ASSAYING UREASE ACTIVITY AND SOME PROPERTIES OF ASPERGILLUS UREASE

1. Urease activity was assayed by two methods:

a. Muftic Method of Ammonia Determination

Initially, urease activity was assayed by measuring the ammonia produced from the hydrolysis of urea by the method of Muftic (1964). This is a colourimetric assay.

This method proved fairly satisfactory for measuring the urease activity of wild-type under various conditions but was insensitive and irreproducible for the very low activities of ure mutant strains. The Muftic method was largely abandoned for the potentially much cleaner and more sensitive system described below. Some wild-type activities measured with the Muftic method are given in Table 1 and in Figure 1 to show that they are in reasonable relative agreement with those measured by the conductimetric method.

b. Conductimetric assay

An assay for urease activity in crude extracts was developed using a direct recording conductimetric method. The conductimetric apparatus was designed and built in the Cell Biology Department of the University of Glasgow by Dr. A.J. Lawrence and is described in Chapter II.2.d.

The system measures the change in the conductance of a solution as urea is hydrolysed by urease. Though conductimetry can be used to measure the activity of many enzymes, the urease reaction gives one of the most sensitive

of all conductimetric assays (Lawrence and Moores, 1972). The properties and reaction of purified commercial jack bean urease have been studied conductimetrically by Hanss and Rey (1971), and by Lawrence and Moores (1972). Urease in crude extracts has not previously been studied by conductimetry.

The sensitivity of the method depends on the ratio of total conductance change to initial conductance. The assay buffer should therefore be present at low concentration and have low conductance. After trying the buffers, bicine, tricine, hepes and tris-HCl, urease was routinely assayed in 10mM tricine. The routine assay mixture and procedure is described in Chapter II.2.d. Under the routine conditions the assay was linear for at least fifteen minutes.

c. Specificity of the conductimetric assay

Assaying enzymes in crude extracts by conductimetry is a non-specific method. The assay system cannot discriminate between different pathways and reactions. It is therefore necessary to justify the assumption that the activities measured here are those of a urease. The activities of particular enzymes may sometimes be identified by the use of specific inhibitors but no such inhibitor is known for urease.

The activity of a crude extract measured here by conductimetry is dependent on the addition of urea and is proportional to that measured by the Muftic method of ammonia determination. It is reasonable to suppose that it is the degradation of urea which is causing the changes in conductance. Further, the activity appears to be necessary for

in vivo degradation of urea as it is very low in ure strains which are specifically unable to utilize urea as a nitrogen source (Chapter VIII.1).

In yeast, urea is degraded by an avidin sensitive, ATP dependent activity (Roon and Levenberg, 1968), a multienzyme complex of urea carboxylase and allophanate hydrolase (Whitney and Cooper, 1972, 1973). This does not appear to be the activity measured here, however, as the Aspergillus reaction, like that of jack bean urease, is not energy-requiring. The Aspergillus activity shares some other properties with jack bean urease:

(i) Both activities have a similar affinity for urea. Jack bean urease has a Km for urea of 2.5mM in 4.5mM citrate buffer pH 6.5 (Hanss and Rey, 1971) and in 10mM Tris, ph 8.0 (Lawrence and Moores, 1972) (presumably these measurements were made under optimum conditions). As reported in a later section of this chapter, the Aspergillus Km for urea in 10mM tricine pH 7.5 was 1.8 (0.3)mM.

(ii) Jack bean urease has a large temperature coefficient (Reithel, 1971). The Aspergillus activity also increases considerably with increasing temperature as reported in a later section of this chapter.

(iii) The enzyme which degrades urea in Aspergillus has recently been purified and is polymeric, with only one type of subunit (E.H. Creaser, personal communication). Jack bean urease is also believed to be a polymeric enzyme with one type of subunit (Staples and Reithel, 1976; Fishbein *et al.*, 1977; Dixon *et al.*, 1980d).

(iv) Jack bean urease contains nickel ions (Dixon *et al.*, 1975). In Aspergillus, the addition of nickel causes an

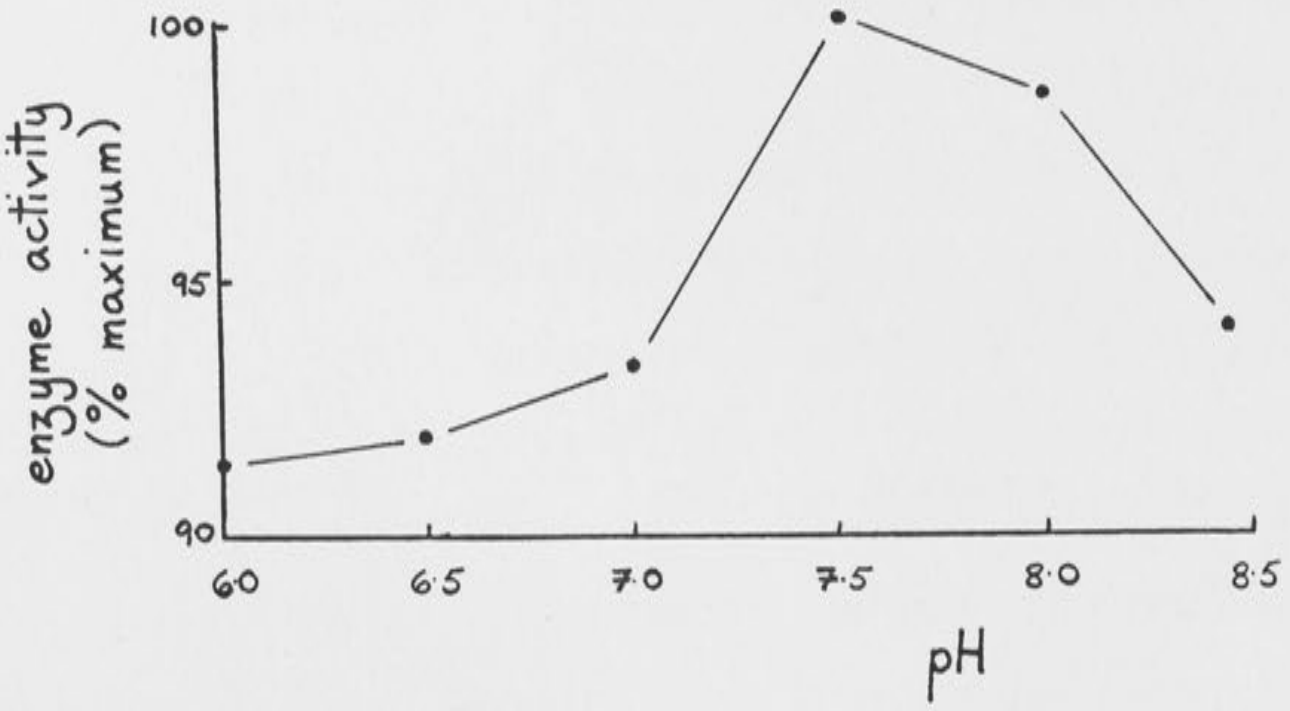


Figure 2: Effect of pH on wild-type urease activity

Strain: biA1

Growth medium: -N minimal medium, vitamin solution, 10mM L-alanine.

Assay: Conductimetric

Points are the average of six independent determinations.

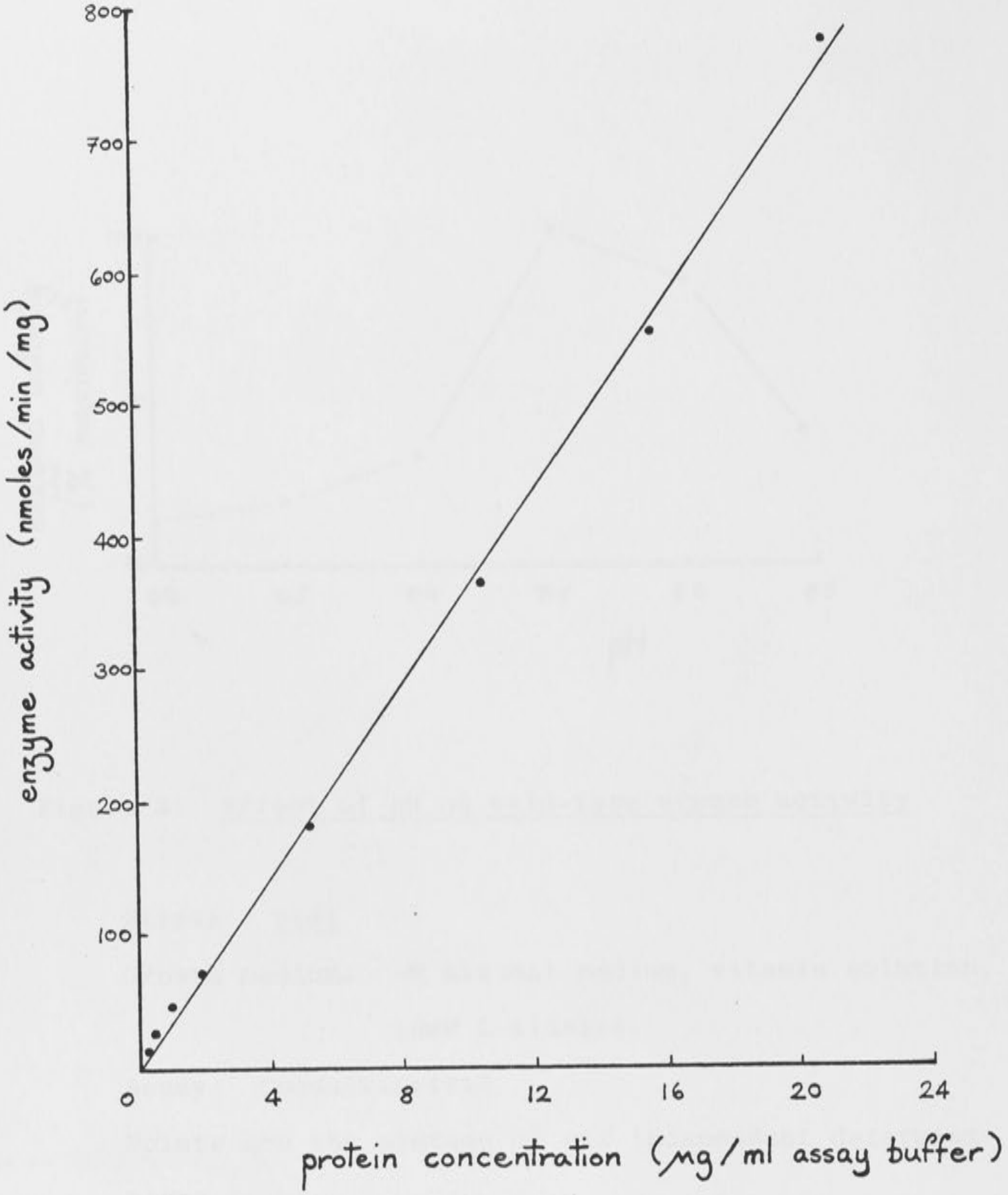


Figure 3: Effect of protein concentration on urease activity

Strain: biA1, wild-type

Growth medium: -N minimal medium, vitamin solution, 10mM urea.

Treatment medium: -N minimal medium, vitamin solution

Substrate concentration: 20mM urea

Assay: Conductimetric

increase in the conductimetrically measured activity of a urea non-utilising strain, ureD9, (Chapter VIII.4c.,d.).

Therefore it is likely that the activity measured here by conductimetry is that of a urease (E.C. 3.5.15).

All urease activities in this thesis, other than those in Table 1 and in Figure 1, were assayed by the conductimetric method.

2. Some Properties of Aspergillus Urease

Wild-type cells were grown up under various conditions as described in the legends of Figures 2-7, crude extracts were prepared and some of the properties of Aspergillus urease were investigated.

a. Effect of pH

The pH of the assay buffer, 10mM tricine, was varied within its buffering range, pH 6-8.4. The optimum pH was 7.5 but the reaction was not very sensitive to pH, retaining >90% of the optimum activity throughout the buffering range of tricine (Figure 2).

b. Effect of enzyme concentration

With excess substrate present, urease activity was proportional to protein concentration (Figure 3).

The assay could detect and accurately measure the activity of very low enzyme concentrations. Urease activity did not appear to decrease through a destabilising effect of protein dilution.

c. Effect of substrate concentration

The concentration of urea was varied in the range 0.5-20mM, with fixed enzyme concentration. The urease

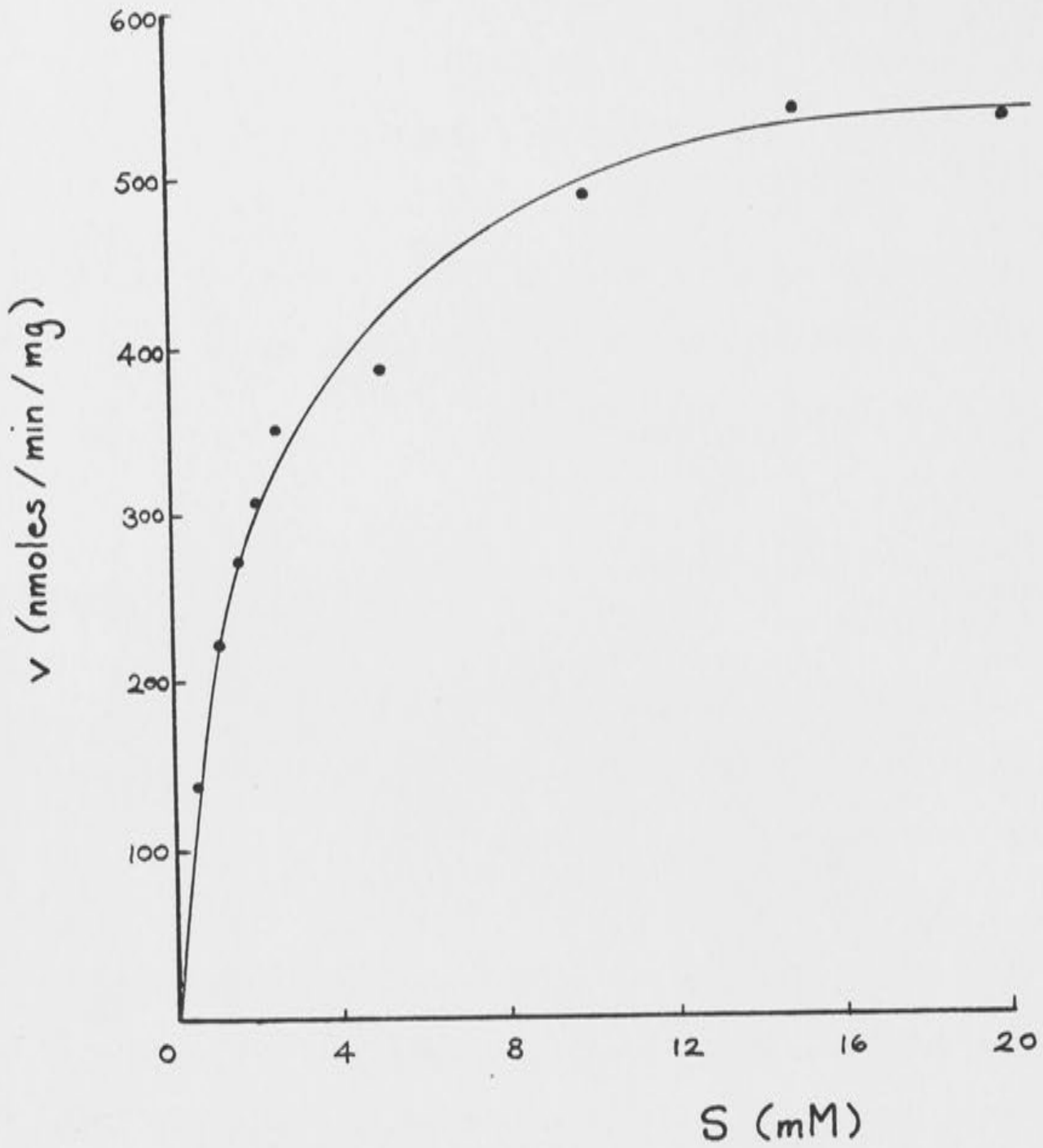
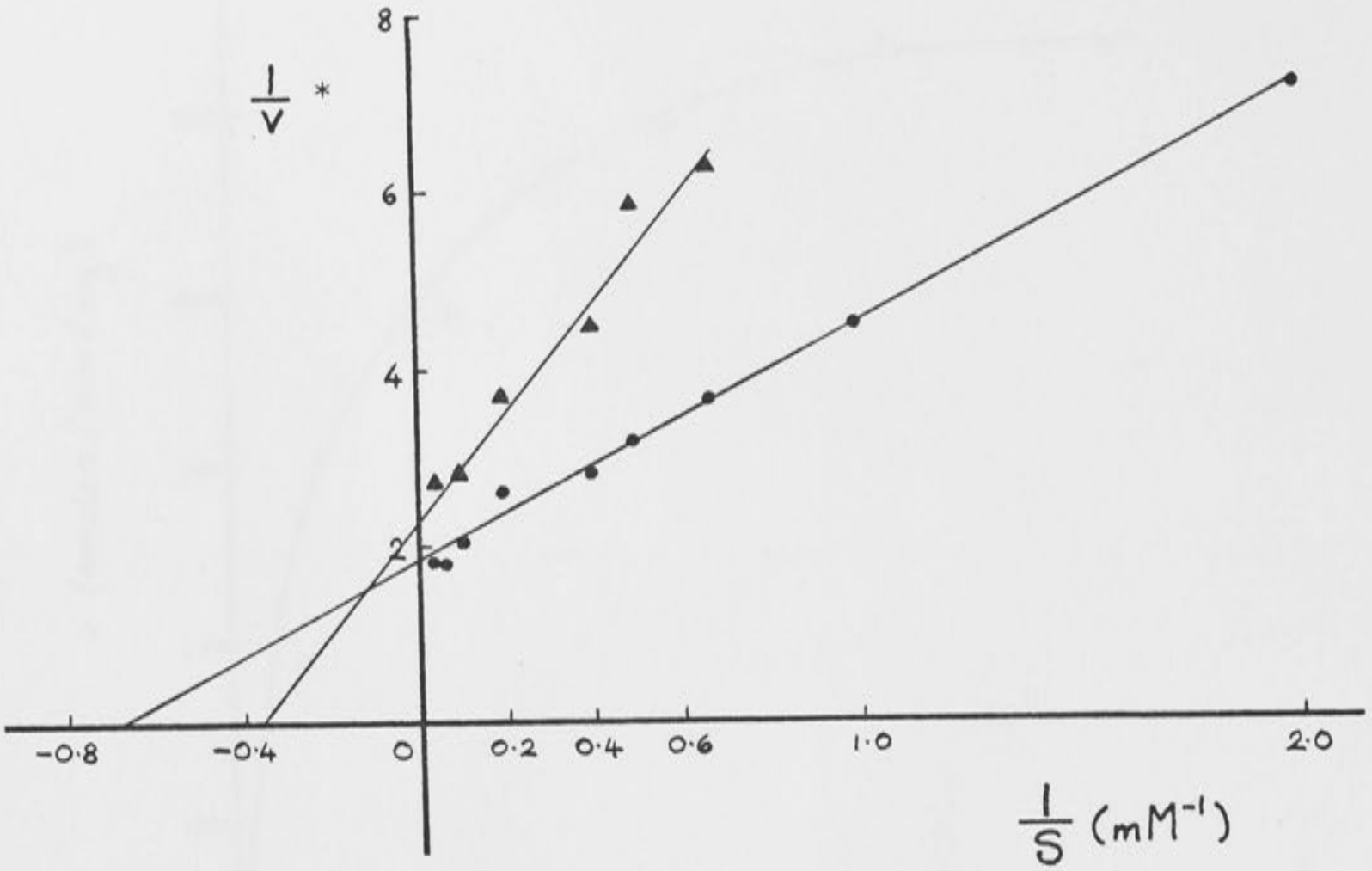


Figure 4: Effect of substrate concentration on urease activity

Strain: biA1, wild-type

Growth medium: -N minimal medium, vitamin solution, 10mM L-glutamate

Assay: Conductimetric



* ((nmoles/min/mg x 10^{-3})$^{-1}$)

Figure 5: Effects of substrate concentration and presence of hydroxyurea on urease activity : Lineweaver-Burk double reciprocal plots

Strain: biA1, wild-type

Growth medium: -N minimal medium, vitamin solution, 10mM L-glutamate

Assay: Conductimetric

Symbols and Km:
- • no hydroxyurea in assay system, Km is 1.5mM
- ▲ 0.1mM hydroxyurea in assay system, Km is 2.6mM

The intercepts of the axes were calculated by linear regression analysis.

activities obtained were plotted against substrate concentration and showed the typical varying order Michaelis-Menten curve (Figure 4).

d. Km for urea

The Km for urea in 10mM tricine pH 7.5 was 1.8mM, standard deviation 0.3mM. This Km value is the average of six independent determinations. Wild-type was grown on -N minimal medium with 10mM L-alanine or L-glutamate as the nitrogen source and with vitamin solution. After a crude extract had been prepared, urease activity was assayed with varying substrate concentration in the range 0.5-20mM. The Km value was determined from a Lineweaver-Burk double reciprocal plot. One such determination is shown in Figure 5.

e. Effect of hydroxyurea on the Km for urea

Jack bean urease can hydrolyse hydroxyurea (Fishbein *et al.*, 1965). This could not be demonstrated for Aspergillus urease but the presence of hydroxyurea in the assay mixture did inhibit the hydrolysis of urea. After preliminary trials to determine a suitable concentration, 0.1mM hydroxyurea was added to the reaction mixture and the system allowed to equilibrate before the addition of urea at various concentrations. The presence of the hydroxyurea altered the Km for urea. The Km for urea almost doubled in the presence of 0.1mM hydroxyurea, while the change in Vmax was comparatively small (Figure 5). The characteristics of a competitive inhibitor, an inhibitor which competes for the catalytic site, are an unaltered Vmax and an increased Km (Mahler and Cordes, 1971). It might be that hydroxyurea is a competitive inhibitor of

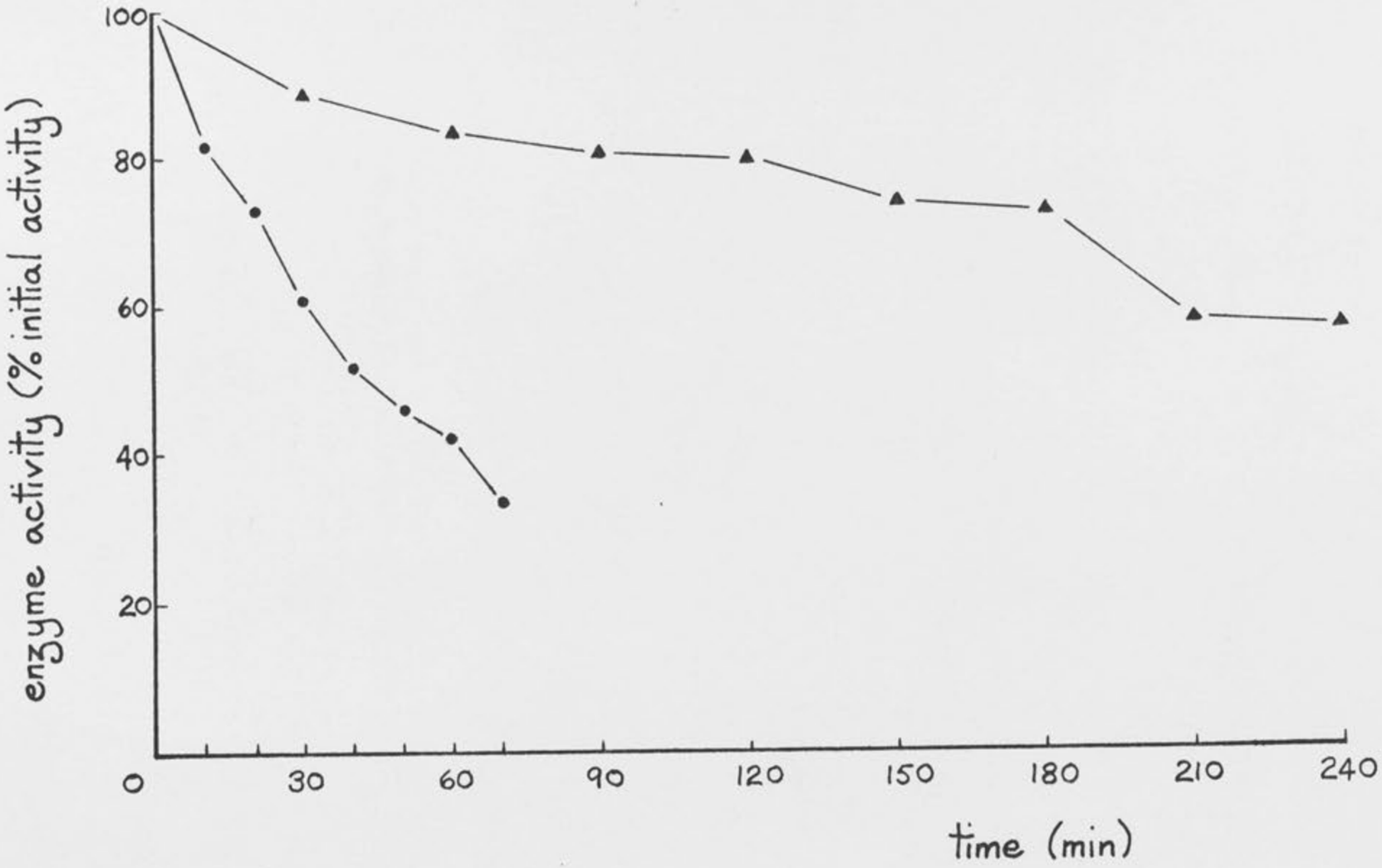


Figure 6: Loss of urease activity with time of incubation at 65 and 75°C

Strain: biA1, wild-type Assay: Conductimetric

Growth medium: -N minimal medium, vitamin solution, 10mM L-alanine

Symbols: ▲ extract incubated at 65°C, average of 6 independent determinations
• extract incubated at 75°C, average of 3 independent determinations

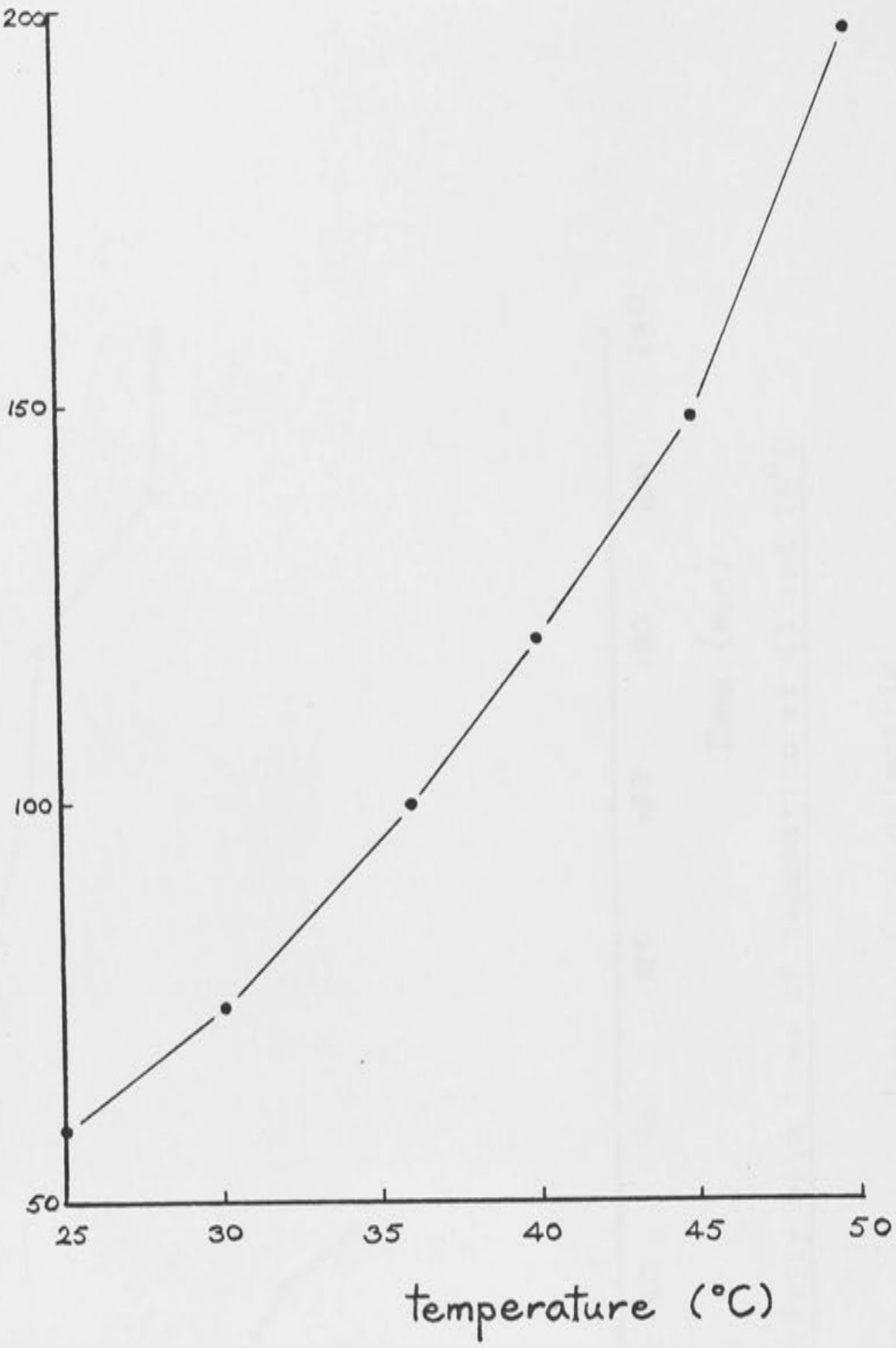


Figure 7: Effect of temperature on urease activity

Strain: biA1, wild-type

Growth medium: -N minimal medium, vitamin solution, 5mM ammonium tartrate.

Treatment medium: -N minimal medium, vitamin solution.

Assay: Conductimetric

Points are the average of two determinations.

Aspergillus urease and that the measured alteration in Vmax is not significant. Further studies with other concentrations of hydroxyurea present in the assay would be required to verify this.

f. Heat stability

Wild-type extracts were incubated in covered test-tubes in a shaking water-bath at 65°C and at 75°C. Samples were withdrawn at intervals and assayed (Figure 6). The extracts retained >40% of their initial activity after 1h at 75°C and >50% of their initial activity after 4h at 65°C.

g. Temperature sensitivity

Urease activity increased with temperature from 25-50°C (Figure 7). The activity at 50°C was approximately twice that at 37°C. It might be possible to increase urease activity further by assaying at even higher temperatures but this was not attempted.

CHAPTER IV

THE REGULATION OF UREASE ACTIVITY

Table 2: Urease activity after growth on various nitrogen sources

Nitrogen Source	Urease Activity nmoles/min/mg
ammonium tartrate	148 (28) * 254 (29)
L-glutamine	226 (47) 236 (40)
L-proline	311 (126) 414 (88)
nitrate	287 (63) 441 (59)
L-arginine	354 (25) 660 (89)
L-ornithine	- 570
L-alanine	334 (82) 641 (100)
L-glutamate	438 (7) 602 (87)
urea	419 (32) 555 (70)

Strain: biA1, wild-type.

Growth medium: -N minimal medium, vitamin solution, 10mM nitrogen source as shown, (5mM ammonium tartrate).

* The explanation for there being two values is given in Section 1.a of this chapter.

The bracketed figures are standard deviations.

THE REGULATION OF UREASE ACTIVITY

All urease activities in this chapter were assayed by the conductimetric method.

1. Nitrogen Regulation

Many enzyme systems in Aspergillus are subject to nitrogen regulation (Chapter I.3.b). It is not known whether this control is exerted at the level of protein synthesis or at the level of enzyme inhibition or inactivation or at both levels. The cellular effector of nitrogen regulation has not been identified though ammonia and L-glutamine have each been suggested for this role.

a. Urease activity after growth on various nitrogen sources

Wild-type cells were grown up on various nitrogen sources with glucose as the carbon source and cell-free extracts were assayed for urease activity (Table 2). The results in Table 2 were collected in two stages. By the time of the second stage of assaying, probably largely due to some alterations which were made to the conductivity apparatus, it had become possible to detect and measure a wider range of activities. Thus two values are shown for urease activity after growth on each nitrogen source. The pattern of urease activities after growth on the various nitrogen sources observed during the second stage of assaying was largely similar to the pattern observed during the first stage of assaying. Only the higher activity of each pair, the number written below the other in Table 2, is referred to here as the higher activities are directly

comparable to nearly all other urease activities presented in this work.

Growth on L-arginine, L-ornithine, L-alanine, L-glutamate and urea resulted in the highest urease activities, from 550-660nmoles/min/mg. The lowest activities occurred after growth on L-glutamine and on ammonium tartrate, about 250nmoles/min/mg. Growth on nitrate and on L-proline resulted in intermediate levels of activity.

The lower activities after growth on L-glutamine and on ammonia suggest that urease is subject to some nitrogen control. However, in comparison to a number of systems in Aspergillus in which growth on ammonia causes minimal levels of activity (Pateman and Kinghorn, 1977, review), the urease activity measured here after growth on ammonium tartrate is rather high. Aspergillus can use tartrate as a weak carbon source (McCullough *et al.*, 1977) and as this might somehow have been alleviating the effect of ammonia, wild-type cells were assayed after growth on ammonium chloride. As would have been the case with other inorganic ammonium salts, this was not a very satisfactory alternative as the pH of the medium dropped to about 3 during growth. Under these conditions the urease activity was 165nmoles/min/mg, not strikingly lower than the activity after growth on ammonium tartrate.

b. Urease activities of repressed strains

The strains, $tamA^r119$, $tamA^r105$ and $areA^r550$ and wild-type were assayed after growth on ammonium tartrate and after transfer from ammonium tartrate medium to nitrogen-free medium. These strains were also assayed

Table 3: Urease activities of repressed mutant strains

Strain	Growth Conditions *	Treatment, transfer for 4h to:	Urease Activity nmoles/min/mg
biA1	L-glutamine	-	236 (40)
	ammonium tartrate	-	254 (29)
		-N MM †	671 (83)
	urea	-	555 (70)
		-N MM	719 (77)
tamA^{r}119	L-glutamine	-	90 (5)
	ammonium tartrate	-	177 (15)
		-N MM	661
	urea	-	401 (76)
		-N MM	676 (133)
tamA^{r}105	L-glutamine	-	132 (29)
	ammonium tartrate	-	136 (2)
		-N MM	690
	urea	-	393 (53)
		-N MM	583 (91)
areA^{r}550	L-glutamine	-	72 (7)
	ammonium tartrate	-	68 (6)
		-N MM	90
	urea	-	133 (33)
		-N MM	133 (8)

* Growth medium: -N minimal medium, vitamin solution, 10mM L-glutamine or urea, 5mM ammonium tartrate.

† -N MM: -N minimal medium, vitamin solution.

The bracketed values are standard deviations.

before and after transfer from urea to nitrogen-free medium (Table 3).

Transfer from ammonium tartrate to nitrogen-free medium resulted in a 2-fold increase in the urease activity of wild-type. Transfer from urea also caused an increase in wild-type activity to a slightly higher level than transfer from ammonia.

The usually fully repressed strain, areA^{r}550, in comparison to wild-type, had low but easily detectable activities after growth on ammonium tartrate and on urea. These activities did not increase much, if at all, after transfer to nitrogen-free medium.

The partially repressed strains, tamA^{r}119 and tamA^{r}105 had lower urease activities than wild-type after growth on ammonium tartrate but had derepressed activities like wild-type after transfer to nitrogen-free medium. After growth on urea, the tamA^r strains had about 70% of wild-type activity. Their activities increased after transfer to nitrogen-free medium. The tamA^r strains generally have about 25% of the wild-type activities of nitrogen controlled systems, even after transfer to nitrogen-free medium (Kinghorn and Pateman, 1975a; Pateman and Kinghorn, 1977). With respect to urease activity, it seems that compared to wild-type, the tamA^r strains are more sensitive to nitrogen regulation in the presence of a nitrogen source but that in the absence of a nitrogen source these strains can achieve full wild-type-like activity.

c. Activities of derepressed mutants

The strains, meaA8 and gdhA1 are ammonia

Table 4: Urease activity of derepressed mutant strains

Strain	Growth Conditions *	Treatment, transfer for 4h to: †	Urease activity (nmoles/min/mg)
biA1	L-glutamate	-	602 (87)
		ammonium tartrate	277 (62)
	L-glutamine	-	236 (40)
meaA8	L-glutamate	-	613 (47)
		ammonium tartrate	631 (39)
	L-glutamine	-	274 (60)
gdhA1	L-glutamate	-	570 (9)
		ammonium tartrate	513 (41)
	L-glutamine	-	164 (22)

* Growth medium: -N minimal medium with vitamin solution and 10mM nitrogen source as shown.

† Treatment medium: -N minimal medium with vitamin solution and 5mM ammonium tartrate.

Standard deviations are shown in brackets.

derepressed for a number of nitrogen controlled systems. Wild-type, meaA8 and gdhA1 were assayed after growth on L-glutamate and transferred from L-glutamate to ammonium tartrate (Table 4). This transfer was necessary as gdhA1 grows very poorly with ammonium tartrate as the nitrogen source. The activity of wild-type decreased by about 60% after transfer to ammonium tartrate. The activities of meaA8 and gdhA1 did not decrease.

d. Effect of L-glutamine on repressed and derepressed mutants

As growth on L-glutamine and on ammonium tartrate produced similarly low wild-type urease activities (Table 2), the repressed and derepressed mutants were also assayed after growth on L-glutamine (Tables 3 and 4).

The repressed mutants, tamA^{r}119, tamA^{r}105 and areA^{r}550 had low activities after growth on L-glutamine, similar to their activities after growth on ammonium tartrate.

The derepressed mutants, meaA8 and gdhA1 had low activities, like wild-type, after growth on L-glutamine.

As growth on L-glutamine but not transfer to ammonium tartrate resulted in low activities of meaA8 and gdhA1, it is unlikely that L-glutamine has its effect through the catabolic production of ammonia. Rather, this suggests that L-glutamine may be closer to the real effector of urease activity than ammonia, and that ammonia may have its effect through the production of L-glutamine.

e. Effect of cycloheximide added on transfer from L-glutamine or ammonia

Wild-type cells were grown up on ammonium

Table 5: Effect of cycloheximide added after transfer to and from L-glutamine and ammonium tartrate: urease activities

Growth Conditions	Treatment, transfer for 4h to: †	Urease Activity nmoles/min/mg
urea	-	555 (70)
	-N MM + cychmd	465 (61)
	ammonium tartrate	243 (37)
	ammonium + cychmd	288 (48)
	L-glutamine	306 (56)
	L-glutamine + cychmd	315 (49)
ammonium tartrate	-	254 (29)
	-N MM	671 (83)
	-N MM + cychmd	265
L-glutamine	-	236 (40)
	-N MM	602 (52)
	-N MM + cychmd	244 (34)

Strain: biA1, wild-type.

* Growth medium: -N minimal medium, vitamin solution, 10mM urea or L-glutamine, or 5mM ammonium tartrate.

† Treatment: -N minimal medium (-N MM) with vitamin solution, and where applicable: cycloheximide (cychmd) 10 µg/ml; L-glutamine, 10mM; ammonium tartrate, 5mM.

Standard deviations shown in brackets.

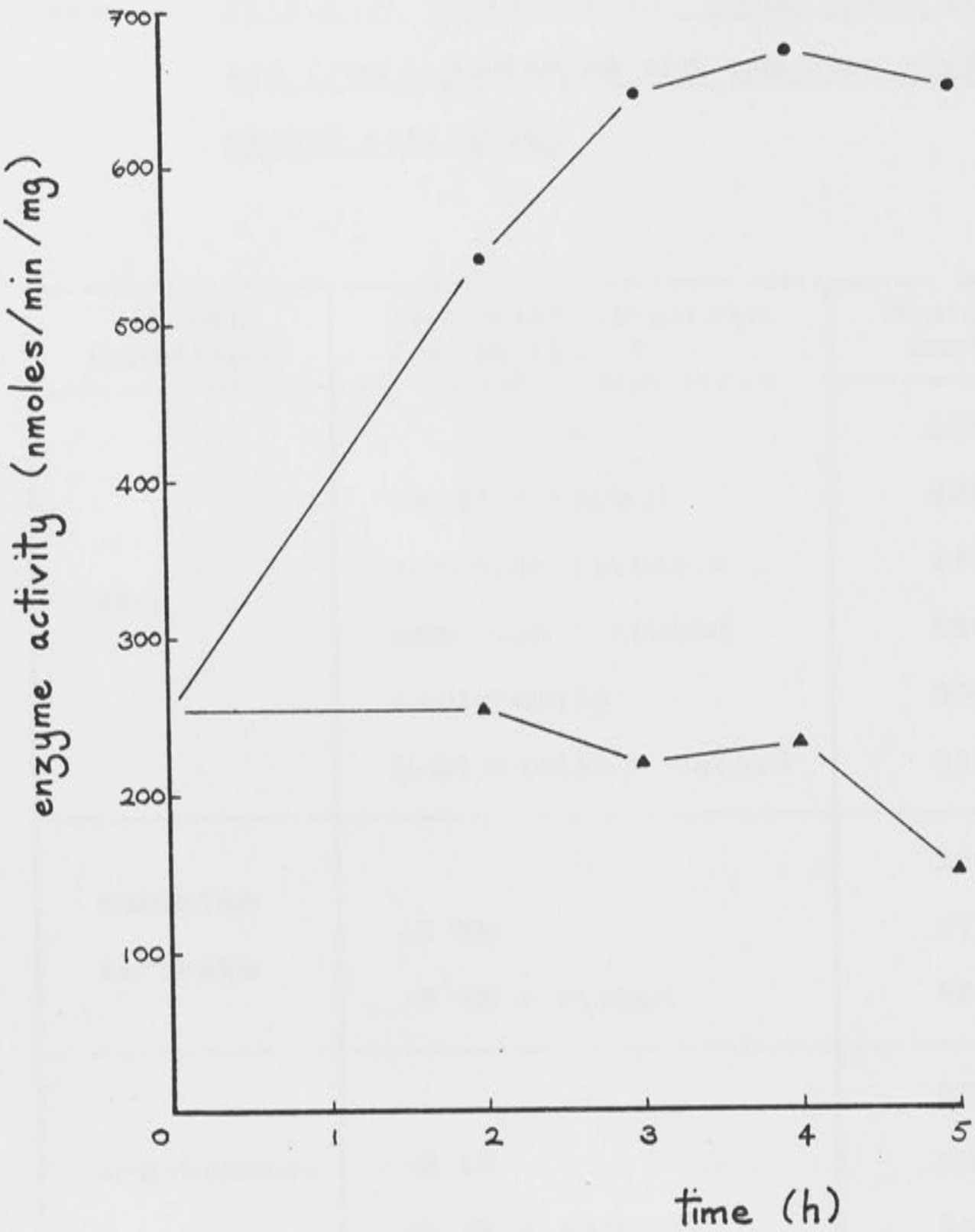


Figure 8: Urease activity after various times of nitrogen starvation in the presence and absence of cycloheximide

Strain: biA1, wild-type

Growth medium: -N minimal medium, vitamin solution, 5mM ammonium tartrate.

Treatment medium: -N minimal medium, vitamin solution, and where applicable, cycloheximide (10 µg/ml).

Symbols: • nitrogen starvation
▲ nitrogen starvation with cycloheximide

tartrate and on L-glutamine, transferred to nitrogen-free medium in the presence and absence of cycloheximide and assayed for urease activity (Table 5). Cycloheximide inhibits protein synthesis in fungi (Siegel and Sisler, 1963).

The rise in urease activity with time of transfer from ammonium tartrate to nitrogen-free medium, with and without cycloheximide is shown in Figure 8.

The presence of cycloheximide prevented the increase in urease activity after transfer from L-glutamine or ammonia to nitrogen-free medium, implying that protein synthesis is required for the rise.

f. Effect of cycloheximide added on transfer to L-glutamine or ammonia

Wild-type cells were grown up on urea, which resulted in high urease activity, and transferred to medium containing L-glutamine or ammonium tartrate as the source of nitrogen, with and without cycloheximide (Table 5). Wild-type cells were also grown up on urea medium and transferred to nitrogen-free medium with cycloheximide to find by how much urease activity decreased when maintenance synthesis was prevented.

Urease activity decreased by about 50-60% after transfer to L-glutamine or ammonium tartrate, both in the absence and presence of cycloheximide. Transfer to nitrogen-free medium with cycloheximide resulted in a decrease in activity of about 15%. The decreases after transfer to L-glutamine and ammonium tartrate were therefore greater than the fall which could be attributed to turnover. As these decreases occurred both in the absence

Table 6: Effect of incubation of extract with L-glutamine or ammonium tartrate : urease activity

Substance incubated with extract	urease activity (nmoles/min/mg) time of incubation (min) 30	90	135	% initial activity remaining at 135 min
L-glutamine	557	495	454	79
ammonium tartrate	545	473	495	86
-	547	516	475	82

Extract: biA1 grown on -N minimal medium, vitamin solution, 10mM urea.

Incubation: 37°C, 2mM L-glutamine, 1mM ammonium tartrate.

Initial activity: 578nmoles/min/mg.

and presence of cycloheximide, it is unlikely that they were caused by a newly synthesised protease.

g. Incubation of extracts with L-glutamine or ammonium tartrate

As transfer from urea to L-glutamine or ammonia resulted in a decrease which was not dependent on protein synthesis, L-glutamine and ammonium tartrate were added to cell-free extracts and the effect on urease activity was studied:

(i) Wild-type was grown up with ammonium tartrate as the nitrogen source and transferred to nitrogen-free medium. This treatment produced high urease activity. Extract was incubated in the glass cells of the conductivity apparatus in tricine buffer with 2.5 and 5mM ammonium tartrate and 10 and 20mM L-glutamine, at 37°C. After the system had equilibrated, substrate, 20mM urea, was added. The presence of 2.5mM ammonium tartrate reduced the urease activity by about 5% and 5mM ammonium tartrate reduced activity by about 25%. The presence of L-glutamine had no effect on urease activity.

(ii) Wild-type cells were grown on urea and a cell-free extract prepared. The extract, undiluted with assay buffer, was incubated in a 37°C incubator with 1mM ammonium tartrate or 2mM L-glutamine. A portion of the extract was incubated by itself. Samples were withdrawn and assayed at various times up to 135 min (Table 6). These incubations did not result in any decrease in urease activity other than the slight drop attributable to heat.

Table 7: Urease activities with potential inducers

Strain	Growth Conditions	Treatment, transfer for 4h to:	Urease activity (nmoles/min/mg)
biA1	ammonium tartrate	urate	491 (83)
		allantoin	359 (34)
		-N MM	671 (83)
	urea	-	555 (70)
meaA8	urea	-	515 (60)

Growth medium: -N minimal medium, vitamin solution, 5mM ammonium tartrate or 10mM urea.

Treatment medium: -N minimal medium, vitamin solution with no nitrogen source or sodium urate, 0.25mg/ml, or 0.5mM allantoin.

Standard deviations are shown in brackets.

2. Investigation of Potential Urease Inducers

The most likely inducer of urease would be its substrate, urea, or one of the intermediates of the purine degradation pathway. Uric acid appears to induce xanthine dehydrogenase I, urate oxidase, allantoinase and allantoicase. The latter two activities are also and additively induced by allantoin (Scazzocchio and Darlington, 1968; Scazzocchio and Gorton, 1977).

Wild-type cells were grown up on ammonium tartrate and transferred to sodium urate, allantoin or nitrogen-free medium (Table 7). Transfer to nitrogen-free medium produced considerably higher urease activity than did transfer to urate or to allantoin.

After growth on urea, wild-type cells did not have higher activity than after growth on L-alanine, L-arginine or L-glutamate (Table 2). Urease activity after growth on urea appeared to be slightly lower than after transfer to nitrogen-free medium from either ammonium tartrate or urea (Table 3).

These results indicate that there is no obvious induction by urea, urate or allantoin. If production of ammonia from urea were obscuring an induction effect, it would be reasonable to expect that the ammonia derepressed strain, meaA8 would have higher activity than wild-type, after growth on urea. However, the activities of meaA8 and of wild-type after growth on urea are very similar (Table 7).

The possibility of urease being subject to endogenous induction is discussed in Chapter X.2.

3. Effect of the Carbon Status on Urease Activity

a. Urease activity after growth on various carbon and nitrogen sources

Cell-free extracts of wild-type cells were assayed after growth on various nitrogen sources with each of the carbon sources, D-glucose, D-glycerol, sodium acetate and L-arabinose (Table 8). The results with D-glucose as the carbon source have been presented in Table 2 but are included in Table 8 for ease of comparison. Two values are presented in Table 8 for urease activity after growth on the various carbon and nitrogen sources. The reason for this was given in Section 1.a. with reference to Table 1. For each pair of values, the value written below the other in Table 8 is directly comparable to nearly all other activities in this work.

The carbon sources were selected for their differing effects on carbon metabolism (McCullough *et al.*, 1977; Arst and Bailey, 1977). D-glucose is a good carbon source for Aspergillus, with respect to growth yield, providing intermediates for glycolysis, the TCA cycle and pentose phosphate pathway. D-glycerol enters the glycolytic pathway through conversion to dihydroxyacetone-phosphate and subsequently pyruvate, and provides acetyl-CoA and oxaloacetate for the TCA cycle. L-arabinose is converted to xyulose-5-phosphate and causes increased activity of some pentose phosphate pathway enzymes and increased production of NADPH (Dunn-Coleman, 1977). Through subsequent conversion to pyruvate, L-arabinose also provides oxaloacetate and acetyl-CoA for the TCA cycle. Acetate is converted to acetyl-CoA and causes elevation of

Table 8: Urease activity after growth on various carbon sources and various nitrogen sources

urease activity (nmoles/min/mg)				
nitrogen source	carbon source			
	D-glucose	D-glycerol	acetate	L-arabinose
ammonium tartrate	148 (28) 254 (29)*	132 (15) 145 (48)	65 (9) 155 (12)	124 (26) 172 (64)
L-glutamine	226 (47) 236 (40)	94 (50) 82 (26)	80 (18) 173 (12)	78 (2) 85 (22)
L-proline	311 (126) 414 (88)	74 (17) 24 (10)	256 (86) 253 (109)	65 (29) 36 (1)
nitrate	287 (63) 441 (59)	237 (108) 312 (99)	249 (17) 386 (44)	293 (42) 342 (72)
L-arginine	354 (25) 660 (89)	327 460 (91)	301 (30) 412 (114)	320 (52) 349 (10)
L-ornithine	- 570	- 307	- 460	- 399
L-alanine	334 (82) 641 (100)	213 (45) 381	337 (98) 392	339 (87) 506
L-glutamate	438 (7) 602 (87)	224 (74) 409 (111)	308 (86) 458 (130)	288 (37) 469 (72)
urea	419 (32) 555 (70)	186 (38) 287 (76)	404 (110) 542 (52)	212 (76) 207 (17)

Strain: biA1, wild-type.

Growth medium: -C-N minimal medium, vitamin solution, 50mM carbon source, 10mM nitrogen source (5mM ammonium tartrate).

* The explanation for there being two values is given in Sections 1.a. and 3.a. of this chapter.

The bracketed numbers are standard deviations.

Table 9: Effect of carbon starvation and carbon and nitrogen starvation on urease activity

	urease activity (nmoles/min/mg)				
	Growth Conditions	Treatment: transfer from urea + glucose for 4h to			
Strain	Urea + glucose	Urea, -C	Urea, -C + cychmd	-C, -N	-C, -N + cychmd
biA1	555 (70)	288 (64)	556 (108)	428 (107)	467 (42)
meaA8	515 (60)	261 (29)	406 (90)	347 (96)	460 (101)
tamA^{r}119	401 (76)	180 (26)	474 (27)	265 (80)	450 (90)
tamA^{r}105	393 (53)	189 (7)	520 (17)	207 (80)	453 (76)
areA^{r}550	133 (33)	71 (11)	109 (17)	41 (14)	96 (6)

Growth medium: -C-N minimal medium, vitamin solution, 50mM D-glucose, 10mM urea.

Treatment media: -C-N minimal medium, vitamins, 10mM urea where applicable, 10µg/ml cycloheximide (cychmd) where applicable.

Standard deviations are given in brackets.

the enzyme activities of the TCA cycle.

Carbon catabolite repression appears to occur in Aspergillus (Chapter I.2.e). D-glucose and acetate are repressing carbon sources while D-glycerol and L-arabinose are derepressing. Since L-arabinose, D-glycerol and acetate all provide intermediates for the TCA cycle, the cellular basis of this distinction is unclear.

With every nitrogen source (Table 8), the urease activity was highest after growth on D-glucose as the carbon source. There is no overall pattern with the other three carbon sources. The order of the levels of activity after growth on D-glycerol, acetate and L-arabinose varied with the nitrogen source, though the three tended to result in similar activities. There were two exceptions to this tendency. With L-proline or urea as the nitrogen source, the urease activities were considerably lower after growth on D-glycerol and L-arabinose than after growth on D-glucose and acetate as the carbon source.

b. Effect of carbon, and of carbon and nitrogen, starvation

The strains, meaA8, tamA^{r}119, tamA^{r}105, areA^{r}550 and wild-type were grown up on -C-N minimal medium with urea and glucose as the nitrogen and carbon sources. The cells were then transferred to medium with urea and no carbon source, with and without cycloheximide, or to medium lacking both a carbon and a nitrogen source, with and without cycloheximide. The urease activities which resulted from these treatments are shown in Table 9.

The activities of the mutant strains largely followed the wild-type pattern after transfer from urea to

the various treatment media. Transfer to medium lacking only a carbon source decreased the activities of all the strains by about 50%. This decrease was largely prevented by the presence of cycloheximide implying that the decrease was mediated by protein synthesis and was not due to the cessation of maintenance synthesis.

Transfer to medium lacking both a carbon and a nitrogen source also resulted in a decrease in the activities of all strains. The decreases in the activities of biA1 and meaA8 did not appear to be so large as the decreases after transfer to medium lacking only a carbon source. To find out whether or not this was a statistically significant result, the replicated determinations made after each of the following pairs of growth and treatment conditions were treated with a two sample t-test and with a Fisher-Behrens d-test:

(i) growth on urea and glucose - growth on urea and glucose followed by transfer to medium lacking glucose;

(ii) growth on urea and glucose - growth on urea and glucose followed by transfer to medium lacking urea and glucose;

(iii) growth on urea and glucose followed by transfer to medium lacking glucose - growth on urea and glucose followed by transfer to medium lacking urea and glucose.

Both tests gave the same trends of probabilities. The results obtained with the t-test are quoted here. For biA1 the probability that there was no difference in the means in (i) was <0.1%, in (ii) was <2%, and in (iii) was >5%, <10%. For meaA8 the probability that there was no difference in the means in (i) and (ii) was <0.1% and in

(iii) was >5%, <10%. Therefore it is unlikely that transfer to medium lacking both a carbon and a nitrogen source really resulted in a lesser decrease in the urease activities of biA1 and meaA8 than did transfer to medium lacking only a carbon source.

The fall in the activity of all strains after transfer to medium lacking both a carbon and a nitrogen source was largely prevented by cycloheximide. Thus the decreases in urease activity after transfer to carbon- and nitrogen-free medium appeared to require protein synthesis, as did the decreases after transfer to carbon-free medium.

It appears that shortage of carbon results in a decrease in urease activity and that this decrease requires protein synthesis.

CHAPTER V

REGULATION OF THIOUREA UPTAKE ACTIVITY

THE REGULATION OF THIOUREA UPTAKE ACTIVITY

The following is a summary of the work of Dunn and Pateman (1972) and their unpublished results. Their preliminary characterisation of the thiourea uptake system forms the basis for the study of the regulation of the system reported in this chapter.

Thiourea is a toxic analogue of urea. Urease does not degrade thiourea and the basis of the toxicity is unknown. The vast majority of strains isolated for resistance to thiourea map at one locus, ureA. These strains grow less well than wild-type on concentrations of urea below 5mM, but achieve full wild-type growth in the presence of higher urea concentrations.

The ureA strains show decreased uptake of urea and thiourea, compared to wild-type. The activities of the ammonia and amino acid uptake systems, however, are normal in ureA strains and ureA strains show normal sensitivity to toxic analogues other than thiourea. Thiourea and urea each inhibit the uptake of the other. These facts suggest that thiourea and urea are taken up by the same transport system and that this system is defective in ureA strains.

The strain, ureA1, has a higher Km for thiourea than does wild-type. This plus the specific decrease in thiourea and urea uptake in ureA strains, suggests that ureA may be a structural gene for the urea and thiourea transport system.

The transport system appears to be energy requiring as the addition of any of the energy inhibitors

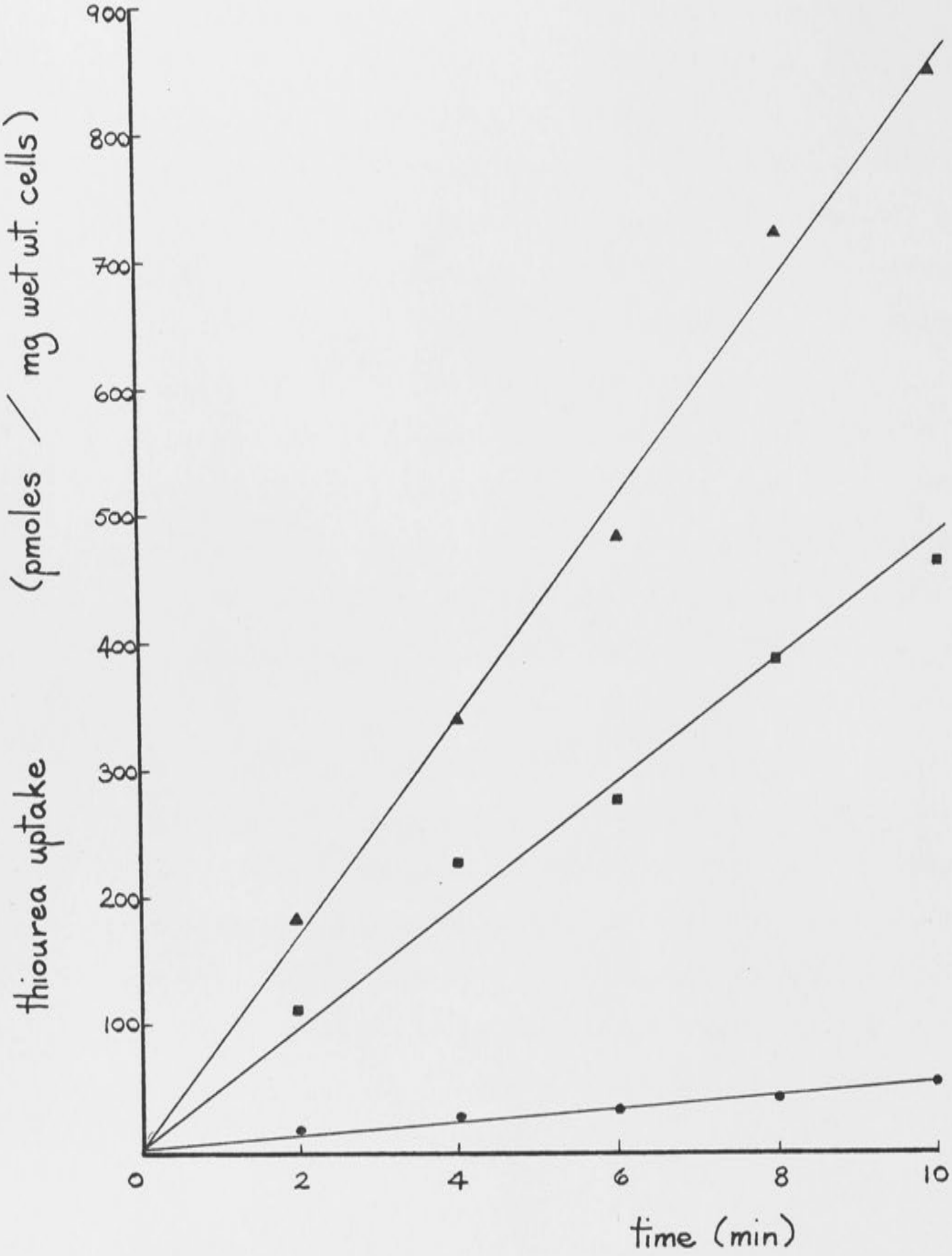


Figure 9: Thiourea uptake with time of assay

Strain: biA1, wild-type

Growth medium: -N minimal medium, vitamin solution, 10mM L-alanine or L-proline or 5mM ammonium tartrate.

Symbols: • cells grown on ammonium tartrate
▲ cells grown on L-alanine
■ cells grown on L-proline

Table 10: Thiourea uptake activities after growth on various nitrogen sources

Nitrogen Source	Thiourea uptake activity ρmoles/min/mg	
ammonium tartrate	7	(1)
L-glutamine	31	(14)
L-alanine	88	(10)
L-proline	51	(5)
L-glutamate	66	(4)
nitrate	43	(20)
urea	91	(22)

Strain: biA1, wild-type.

Growth medium: -N minimal medium with vitamin solution, 10mM nitrogen source as shown, (5mM ammonium tartrate).

Standard deviations shown in brackets.

potassium cyanide, sodium azide and mercuric chloride greatly reduces the level of wild-type urea uptake. The system is substrate concentration dependent and saturable.

It is likely that the physiological role of the urea transport system is scavenging low concentrations of urea when nitrogen is in short supply. At higher extracellular urea concentrations, sufficient urea for normal growth can enter the cell by diffusion.

Thiourea uptake activities were assayed as described in Chaper II.3. Uptake was linear for at least 10 min, as shown in Figure 9.

1. Nitrogen Control of Uptake Activity

a. Uptake activity after growth on various nitrogen sources

Wild-type cells were grown up on various nitrogen sources, with glucose as the carbon source, and assayed for thiourea uptake activity (Table 10). Growth on urea and on L-alanine resulted in the highest activities. Cells grown on ammonium tartrate had the lowest activity. The uptake activity after growth on L-glutamine, though lower than the activities after growth on L-proline, nitrate and L-glutamate, was 4-5-fold higher than that after growth on ammonium tartrate.

b. Uptake activities of repressed mutants

The strains, $tamA^r119$, $tamA^r105$, $areA^r550$ and wild-type were assayed for thiourea uptake after growth on L-glutamine, ammonium tartrate and urea and after transfer from each of these nitrogen sources to nitrogen-free

Table 11: Thiourea uptake activities of repressed mutants

Growth Conditions	Treatment, transfer for 4h to	thiourea uptake activities (pmoles/min/mg) of the strain			
		biA1	tamA^{r}119	tamA^{r}105	areA^{r}550
ammonium tartrate	-	7 (1)	7 (2)	7 (2)	5 (1)
	-N MM	106 (23)	90 (19)	53 (5)	27 (5)
L-glutamine	-	31 (14)	7 (2)	6 (1)	11 (1)
	-N MM	98 (19)	145 (26)	92 (5)	31 (6)
urea	-	91 (22)	21 (1)	13 (2)	17 (4)
	-N MM	147 (17)	163 (48)	101 (12)	41 (11)

Growth medium: -N minimal medium with vitamin solution, 10mM L-glutamine or urea, or 5mM ammonium tartrate.

Treatment medium: -N minimal medium (-N MM) with vitamin solution.

Standard deviations are shown in brackets.

Table 12: Thiourea uptake activities of derepressed mutants

strain	growth conditions	treatment, transfer for 4h to	thiourea uptake activities (pmoles/min/mg)
biA1	ammonium tartrate	-	7 (1)
	L-glutamine	-	31 (14)
	L-glutamate	-	66 (4)
meaA8	ammonium tartrate	-	104 (25)
	L-glutamine	-	17 (2)
gdhA1	L-glutamate	-	90 (10)
		ammonium tartrate	96 (11) *
		L-glutamine	27 (2)
	L-glutamine	-	11 (2)

Growth medium: -N minimal medium with vitamin solution and 10mM L-glutamate or L-glutamine or 5mM ammonium tartrate.

Treatment medium: -N minimal medium with vitamin solution and 10mM L-glutamine or 5mM ammonium tartrate.

Standard deviations shown in brackets.

* biA1 was not assayed after transfer from L-glutamate to ammonium tartrate but transfer from urea to ammonium tartrate resulted in a large decrease in activity.

medium (Table 11). The tamA^r strains and areA^{r}550 had very low activities after growth on ammonium tartrate and L-glutamine. After growth on urea all three strains had about 20% of the wild-type activity. The activities of the tamA^r strains and wild-type increased considerably after all transfers to nitrogen-free medium. The activities of areA^{r}550 also increased but to a lesser extent.

It seems that when a nitrogen source is present the tamA^r strains are more sensitive to nitrogen regulation than is wild-type but that in the absence of a nitrogen source these strains can achieve wild-type levels of thiourea uptake activity. This does not agree with the results of Kinghorn and Pateman (1975a), who found that the thiourea uptake activity of tamA^{r}119 did not increase after transfer, for 3h, from medium containing L-alanine to nitrogen-free medium. These authors also observed that in growth tests on solid medium, tamA^{r}119 was much more resistant to thiourea when L-alanine was the nitrogen source than when nitrate was the nitrogen source. This is rather surprising as in the present work wild-type thiourea uptake was considerably higher after growth with L-alanine as the nitrogen source than with nitrate as the nitrogen source. It may be that L-alanine has some particular effect on tamA^{r}119 and that transfer to nitrogen-free medium for 3h did not allow the strain to overcome this.

c. Uptake activities of derepressed mutants (Table 12)

The strain, meaA8 was assayed after growth on ammonium tartrate. Unlike wild-type, meaA8 had high uptake activity after growth on ammonium tartrate. The strain, gdhA1 grows poorly on ammonium tartrate and was

Table 13: Effect of cycloheximide added after transfer to and from L-glutamine and ammonium tartrate : thiourea uptake activities

Growth Conditions *	Treatment, transfer for 4h to †	Thiourea uptake activity pmoles/min/mg
urea	-	91 (22)
	-N MM + cychmd	48 (17)
	ammonium tartrate	7 (1)
	ammonium + cychmd	15 (1)
	L-glutamine	47 (3)
	L-glutamine + cychmd	20 (3)
ammonium tartrate	-	7 (1)
	-N MM	106 (23)
	-N MM + cychmd	6 (0)
L-glutamine	-	31 (14)
	-N MM	98 (19)
	-N MM + cychmd	16 (4)

Strain: biA1, wild-type.

* Growth medium: -N minimal medium, vitamin solution, 10mM urea or L-glutamine, or 5mM ammonium tartrate.

† Treatment: -N minimal medium (-N MM) with vitamin solution and where applicable: 10µg/ml cycloheximide (cychmd); 10mM L-glutamine; 5mM ammonium tartrate.

Standard deviations are shown in brackets.

therefore grown up on L-glutamate and transferred to ammonium tartrate. The uptake activity of gdhA1 was similarly high before and after the transfer. Wild-type was not assayed after transfer from L-glutamate to ammonium tartrate but transfer from urea to ammonium tartrate resulted in low uptake activity.

The two strains, meaA8 and gdhA1 were also assayed after growth on L-glutamine. Though both strains had high activities after growth on ammonia, both meaA8 and gdhA1 had low activities after growth on L-glutamine. This suggests that although wild-type uptake activity was higher after growth on L-glutamine than after growth on ammonium tartrate, L-glutamine is closer to the effector of uptake activity than is ammonia.

d. Effect of cycloheximide added on transfer from L-glutamine or ammonia

To establish whether protein synthesis was required for the increase in uptake activity after transfer to nitrogen-free medium (Table 11), wild-type cells were grown up on L-glutamine and on ammonium tartrate and transferred to nitrogen-free medium in the presence and absence of cycloheximide (Table 13). The addition of cycloheximide at the time of transfer prevented the increases in activity, suggesting that protein synthesis is required for the increase.

e. Effect of cycloheximide added on transfer to L-glutamine or ammonia

Wild-type cells were grown up on urea which produced high uptake activity (Table 10), and transferred to medium containing L-glutamine or ammonium tartrate as the

Table 14: Inhibition of thiourea uptake assay

Inhibitor	Thiourea uptake activities (ρmoles/min/mg) with inhibitor present in assay system at concentrations:		
	20μM	200μM	2mM
L-glutamine	62	35	25
NH_4^+	72	13	9

Strain: biA1, wild-type.

Growth medium: -N minimal medium, vitamin solution, 10mM urea.

Uptake activity in absence of inhibitor was 91 (22) ρmoles/min/mg.

sole source of nitrogen, with and without cycloheximide (Table 13). Cells were also grown up on urea and transferred to nitrogen-free medium with cycloheximide, to estimate by how much uptake activity might decrease through lack of maintenance synthesis.

Uptake activity decreased after transfer to both ammonium tartrate and L-glutamine and the decreases occurred both in the absence and presence of cycloheximide. After transfer to ammonium tartrate, the activity decreased by about 93% without cycloheximide and slightly less when cycloheximide was present. Transfer to L-glutamine resulted in a decrease in uptake activity of about 50% without cycloheximide, and in a decrease of about 80% in the presence of cycloheximide. The activity of cells transferred to nitrogen-free medium with cycloheximide decreased by about 47%. Transfers to ammonium tartrate with and without cycloheximide and to L-glutamine with cycloheximide therefore appear to have resulted in greater decreases in activity than could be attributed to turnover of the uptake system. As the decreases after transfer to L-glutamine and to ammonia do not appear to be dependent on protein synthesis it is unlikely that they are mediated by a newly synthesised protease.

f. Inhibition of uptake activity

Wild-type cells were grown up on urea which resulted in high uptake activity. Cells were incubated in the assay system with various concentrations of L-glutamine or ammonium tartrate for approximately 2 min before the addition of thiourea (Table 14). Both L-glutamine and ammonium tartrate inhibited uptake. The

Table 15: Effect of carbon starvation and carbon and nitrogen starvation on thiourea uptake activity

	thiourea uptake activity (ρmoles/min/mg)				
	growth conditions	treatment: transfer from urea + glucose for 4h to			
Strain	urea + glucose	urea, -C	urea, -C + cychmd	-C, -N	-C, -N + cychmd
biA1	91 (22)	5 (1)	6 (1)	8 (3)	58 (20)
meaA8	80 (24)	4 (1)	5 (2)	9 (3)	62 (12)
tamA^{r}119	21 (1)	4 (2)	4 (1)	7 (2)	17 (1)
tamA^{r}105	13 (2)	5 (0)	4 (1)	10 (2)	6 (0)
areA^{r}550	17 (4)	19 (3)	13 (1)	16 (4)	14 (4)

Growth medium: -C-N minimal medium, vitamin solution, 50mM D-glucose, 10mM urea.

Treatment media: -C-N minimal medium with vitamin solution and, where applicable, 10mM urea, 10μg/ml cycloheximide (cychmd).

Standard deviations are shown in brackets.

amount of inhibition increased with the concentration of L-glutamine or ammonia. Inhibition by ammonium tartrate was stronger than inhibition by L-glutamine.

2. Effect of Carbon Starvation on Uptake Activity

Wild-type, meaA8, tamA^{r}119, tamA^{r}105 and areA^{r}550 were grown up on medium containing urea as the source of nitrogen and D-glucose as the carbon source. The cells were either harvested and assayed directly or assayed after transfer to medium lacking D-glucose or lacking both urea and D-glucose, with and without cycloheximide (Table 15).

Transfer to medium without a carbon source resulted in a decrease in the activity of all strains except areA^{r}550. The decrease was not prevented by cycloheximide implying that it was not mediated by protein synthesis. Possibly this decrease largely resulted from the fall in energy accompanying carbon starvation.

After transfer to medium lacking both urea and D-glucose, the activities of all strains, except areA^{r}550, decreased by almost as much as after transfer to medium lacking only D-glucose. The decrease in activity after transfer to carbon- and nitrogen-free medium, unlike the decrease after transfer to carbon-free medium, appeared to be largely dependent on protein synthesis.

To find out whether this apparent difference in protein synthesis dependence was statistically significant, the replicate determinations made after each of the following pairs of growth and treatment conditions were treated with a two-sample t-test and with a Fisher-Behrens d-test:
(i) growth on urea and D-glucose - growth on urea and

D-glucose followed by transfer to medium lacking urea and D-glucose.

(ii) growth on urea and D-glucose - growth on urea and D-glucose followed by transfer to medium lacking urea and D-glucose with cycloheximide.

(iii) growth on urea and D-glucose followed by transfer to medium lacking D-glucose and urea - growth on urea and D-glucose followed by transfer to medium lacking D-glucose and urea with cycloheximide.

The tests were carried out on the results for wild-type and for meaA8. The two-sample t-test and the Fisher-Behrens d-test gave the same trend of probabilities. The probabilities obtained with the two-sample t-test are quoted here. For biA1 the probability that there is no difference in the means in (i) and in (iii) is <0.1% and in (ii) is >2%, <5%. For meaA8 the probability that there is no difference between the means in (i) and in (iii) is <0.1% and in (ii) is >5%, <10%.

It appears therefore that the decreases in uptake activity after transfer to carbon-free medium and after transfer to carbon- and nitrogen-free medium have different causes. A possible explanation for this is given in Chapter X.

CHAPTER VI

EFFECT OF L-HISTIDINE ON UREASE AND THIOUREA UPTAKE ACTIVITIES

EFFECT OF L-HISTIDINE ON UREASE AND THIOUREA UPTAKE ACTIVITIES

When present in solid medium, L-histidine, which is itself a very poor nitrogen source, prevents the utilization of many other nitrogen sources. In addition, urease activity after growth on ammonium tartrate and after transfer from ammonium tartrate to uric acid, is decreased when L-histidine is present in the medium (Polkinghorne and Hynes, 1975). This effect on urease activity, and the effect of L-histidine on uptake activity, were investigated.

1. Effect of L-Histidine on Urease Activity

a. L-histidine as the nitrogen source with various carbon sources

Wild-type cells were assayed after growth on 10mM L-histidine with each of the carbon sources, D-glucose, D-glycerol, acetate and L-arabinose. All four growth conditions resulted in very low urease activities, <30nmoles/min/mg.

b. L-histidine with various nitrogen and various carbon sources

Wild-type cells were grown on 10mM L-histidine together with one of the following nitrogen sources, urea, L-glutamate, L-proline or ammonium tartrate and with one of the following carbon sources, D-glucose, D-glycerol, acetate or L-arabinose. The urease activities under these conditions in the absence of L-histidine are reported in Chapter IV, Table 8. In the presence of L-histidine, the

Table 16: Effect of L-histidine on urease activity of nitrogen regulation mutants

Strain	Urease activities (nmoles/min/mg) after growth on urea ± L-histidine	
	-L-histidine	+L-histidine
biA1	555 (70)	15 (7)
meaA8	515 (60)	19 (8)
tamA^{r}119	401 (76)	14 (4)
tamA^{r}105	393 (53)	19

Growth medium: -N minimal medium with vitamin solution, 10mM urea ± 10mM L-histidine.

Standard deviations are shown in brackets.

urease activities after growth under all sixteen conditions were reduced to the very low level observed with L-histidine as the sole nitrogen source, <30nmoles/min/mg. The presence of L-histidine in the growth medium also reduced the growth yield on urea and L-glutamate but growth on L-proline and on ammonium tartrate was largely unaffected.

The effect of L-histidine on urease activity appears to occur irrespective of the carbon or nitrogen status of the cell.

c. Effect of L-histidine on nitrogen regulation mutants

The strains, meaA8, tamA^{r}119, tamA^{r}105, areA^{r}550 and wild-type were grown on -N minimal medium with urea and with and without L-histidine (Table 16). The strain, areA^{r}550 could hardly grow in the presence of L-histidine and could not be assayed. The urease activity of all the other strains was drastically reduced after growth in the presence of L-histidine.

d. L-histidine inhibition of urease

(i) Wild-type was grown up on medium containing ammonium tartrate as the nitrogen source and glucose as the carbon source, transferred to nitrogen-free medium and cell-free extracts prepared. These extracts had high urease activity. Extract was incubated with 2mM L-histidine in the glass cells of the conductivity apparatus, under the routine assay conditions for about 2 min before the addition of urea. Samples of partially purified and highly purified Aspergillus urease (kindly supplied by Dr. E.H. Creaser), were also incubated in this way. In all three cases, the incubation with 2mM L-histidine had no

Table 17: Thiourea uptake activities after growth on various nitrogen sources with and without L-histidine

Nitrogen source	Thiourea uptake activity (ρmoles/min/mg) after growth ± L-histidine	
	-L-histidine	+L-histidine
urea	91 (22)	40 (22)
ammonium tartrate	7 (1)	6 (1)
L-proline	51 (5)	53 (9)
L-glutamate	66 (4)	74

Growth medium: -N minimal medium with vitamin solution with 10mM nitrogen source as shown (5mM ammonium tartrate), ± 10mM L-histidine.

Standard deviations shown in brackets.

effect on the urease activity. Incubation of crude extract with 10mM L-histidine decreased the activity by about 33%. This suggests that inhibition by L-histidine cannot account for the very low activities measured after growth in the presence of L-histidine as it seems unlikely that the intracellular concentration of L-histidine would greatly exceed 10mM.

(ii) Wild-type cells were grown on urea which resulted in high urease activity. Crude extract of the cells, undiluted with assay buffer, was incubated in a 37°C incubator with 2mM L-histidine for 135 min. Samples were withdrawn and assayed at intervals. This incubation did not cause a L-histidine-specific decrease in urease activity. This suggests that L-histidine does not have its effect on urease activity by binding to urease and causing ligand-induced conformational changes.

2. Effect of L-Histidine on Thiourea Uptake Activity

a. Effect of L-histidine with various nitrogen sources

Wild-type cells were grown up on -N minimal medium with L-histidine and one of the following, urea, L-proline, L-glutamate, or ammonium tartrate, and assayed for thiourea uptake activity (Table 17). Cells grown on urea with L-histidine present in the medium had about half the uptake activity of cells grown on urea alone. Cells grown on L-proline, ammonium tartrate or L-glutamate had the same activity after growth in the presence and absence of L-histidine.

Table 18: Effect of L-histidine on thiourea uptake activity of nitrogen control mutants

Strain	Thiourea uptake activity (pmoles/min/mg) after growth ± L-histidine	
	-L-histidine	+L-histidine
biA1	91 (22)	40 (22)
meaA8	80 (24)	31 (8)
tamA^{r}119	21 (1)	41 (15)
tamA^{r}105	13 (2)	24 (9)

Growth medium: -N minimal medium with vitamin solution, 10mM urea ± 10mM L-histidine.

Standard deviations are shown in brackets.

b. Uptake activity of nitrogen regulation mutants in the presence of L-histidine

The strains, biA1, meaA8, tamA^{r}119 and tamA^{r}105 were grown up on urea in the presence and absence of L-histidine and assayed for thiourea uptake activity (Table 18). The strain areA^{r}550 hardly grew at all in the presence of L-histidine and could not be assayed. After growth in the presence of L-histidine, the activities of biA1 and meaA8 were decreased to about 35-40% of the levels after growth on urea without L-histidine.

The activities of the tamA^r strains which were low after growth on urea without L-histidine, were not decreased further after growth on urea with L-histidine present in the medium. The results actually suggest that the activities of tamA^{r}119 and tamA^{r}105 increased after growth in the presence of L-histidine.

The replicate determinations of uptake activity after growth of each of the four strains, biA1, meaA8, tamA^{r}119 and tamA^{r}105 on urea with and without L-histidine, were treated with a two sample t-test and the Fisher-Behrens d-test. Both tests gave the same trend in probabilities. The results obtained with the t-test are quoted here. For biA1 the probability that there was no difference between the means after the growth with and without L-histidine is <0.2%, for meaA8 is <0.1%, for tamA^{r}119 is >2% <5% and for tamA^{r}105 is >1% <2%.

The presence of L-histidine during growth on urea does appear to cause a real decrease in the uptake activities of meaA8 and biA1. The apparent increases in the uptake activities of the tamA^r strains are less

statistically significant.

c. L-histidine inhibition of uptake activity

Concentrations of 20 and 200μM L-histidine were added to the uptake assay system containing wild-type cells grown on urea, about 2 min before the addition of thiourea. Growth on urea produces an uptake activity of about 91ρmoles/min/mg. This was reduced to 20 and 9ρmoles/min/mg when L-histidine was present at 20 and 200μM respectively.

L-histidine appears to strongly inhibit uptake activity when present externally.

CHAPTER VII

GENETIC CHARACTERISATION OF THE URE LOCI

GENETIC CHARACTERISATION OF THE URE LOCI

Aspergillus strains unable to grow on urea as a sole source of nitrogen were first isolated in the mid-1960s during an investigation of the purine degradation pathway. Urease is the final enzyme in this pathway (Scazzocchio and Darlington, 1968). A.J. Darlington (1966, Ph.D thesis, Cambridge) showed by heterokaryon complementation tests that the mutations in the urea non-utilizing strains fell into three complementation groups and these loci were named *uX*, *uY* and *uZ*. These loci were subsequently renamed *ureB*, *ureC* and *ureD*, respectively, in accordance with the nomenclature proposed by Clutterbuck (1973).

Darlington (1966), by haploidisation of diploids of *ureB2*, *ureB3*, *ureC5* and *ureD4* with Master Strain F, assigned the loci to chromosomes VIII, VII and VIII, respectively. Darlington showed by meiotic mapping that *ureB* and *ureD* are not closely linked.

Another strain, which grows more poorly on urea than wild-type but considerably better than the original *ure* strains has also been isolated. This strain was assigned to a new *ure* locus and called *ureE3* (Kinghorn and Pateman, 1977).

A further *ure* locus is also known. The vast majority of strains showing resistance to the toxic urea analogue, thiourea, map at one locus *ureA* (Dunn and Pateman, 1972, and unpublished results). It is probable that *ureA* is a structural gene for the urea uptake system (the reasons for thinking this are given in the introduction

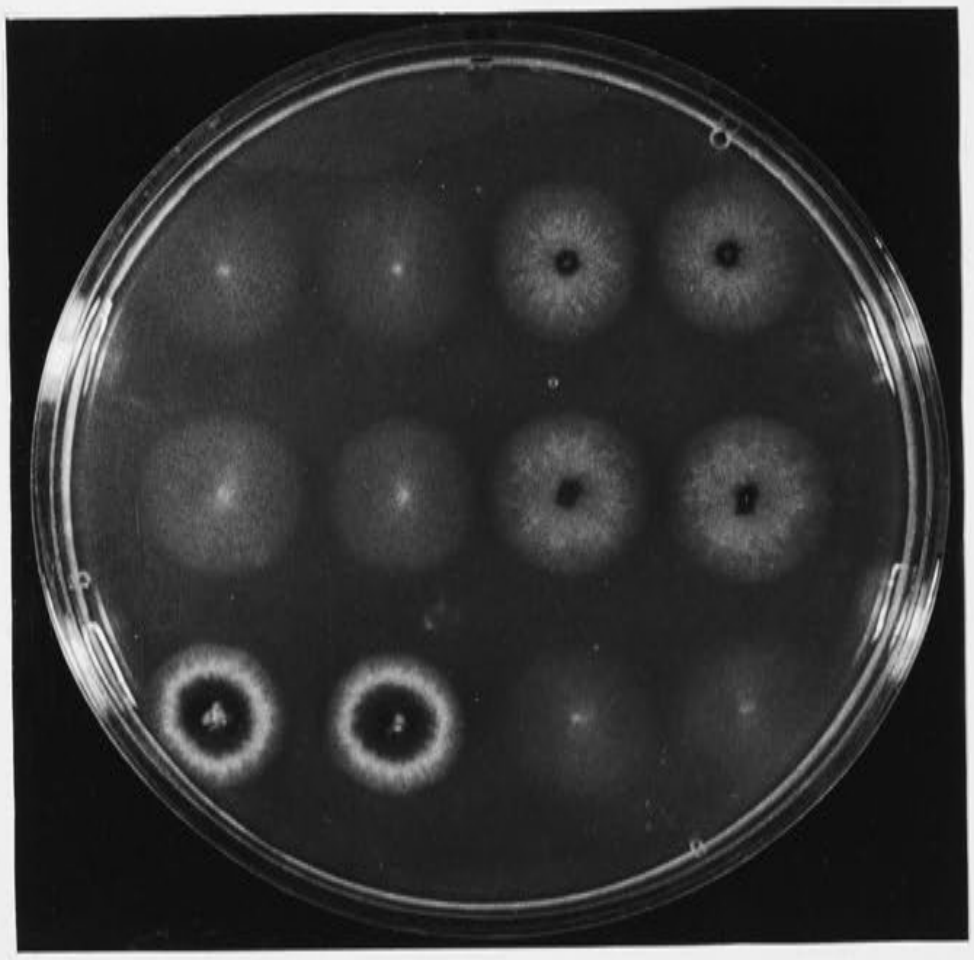


Plate 1: Growth of ure mutants with urea as the nitrogen source

Growth medium: solid -N minimal medium, vitamin solution, 3mM urea

Strains inoculated into the medium in duplicate in the following arrangement:

ureB2	ureA1
ureC5	ureB12
biA1	ureD9

Photographed after 48h incubation at 37°C.

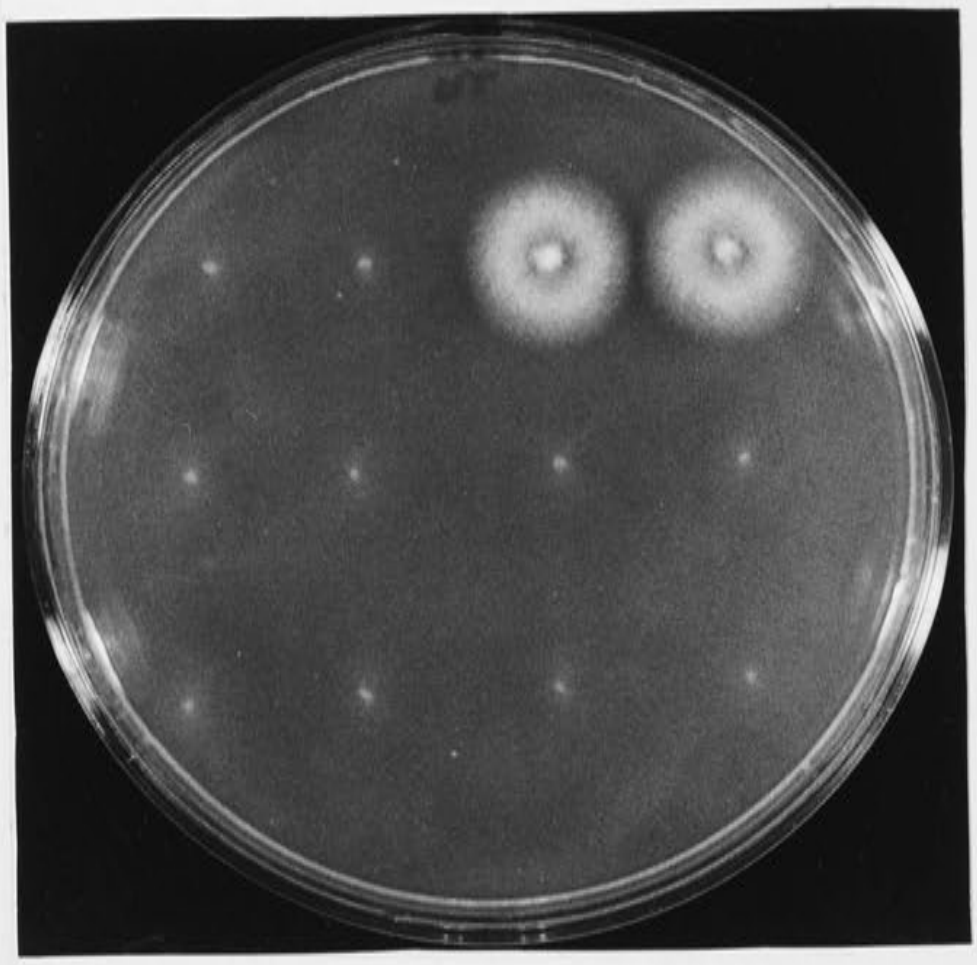


Plate 2: Growth of ure mutants in the presence of thiourea

Growth medium: solid minimal medium (10mM nitrate is the nitrogen source), vitamin solution, 5mM thiourea.

Strains inoculated into the medium in duplicate in the following arrangement:

ureB2	ureA1
ureC5	ureB12
biA1	ureD9

Photographed after 48h incubation at 37°C.

to Chapter VIII). Strains carrying a mutation in ureA grow about half as well as wild-type with 3mM urea as the sole source of nitrogen.

The preliminary results of Pateman and Kinghorn (1977), indicate that ureA and ureB are closely linked.

The eleven ure strains available at the onset of the present work are listed in Chapter II.6. A considerable length of time had passed since any extensive genetic work had been done with the ure strains and much of the more recent work had been of a preliminary nature. In addition, the chromosome assignment of ureC and ureD in a review (Pateman and Kinghorn, 1977) and, the assignment of ureC on the standard Aspergillus linkage map (obtainable from A.J. Clutterbuck, Institute of Genetics, the University of Glasgow) contradict the results of Darlington (1966).

It seemed advisable to repeat and extend the genetic characterisation of the ure strains.

The growth with 3mM urea as the sole source of nitrogen of a strain representing the effect of mutation at one of each of the ure loci is shown in Plate 1. These strains can all grow well like wild-type with nitrate as the sole source of nitrogen. Plate 2 shows the growth of the representative strains with nitrate as the sole source of nitrogen with 5mM thiourea in the medium.

1. Chromosome Assignment of ure Loci

A diploid with a master strain (M.S.E.), bearing a marker on each of the eight chromosomes, was established for each of the strains, ureA1, ureB2, ureC5, C7, ureD4

Table 19: Chromosome assignment of ureA1 and ureB2

Chromosome	marker allele	Haploidised Diploid of ureA1/MSE			Haploidised Diploid of ureB2/MSE	
		ure$^+$	ure$^-$		ure$^+$	ure$^-$
I	bi$^+$	8	8		1	13
	bi$^-$	2	2		1	9
II	w$^+$	4	5		0	14
	w$^-$	6	5		2	8
III	gal$^+$	6	5		1	13
	gal$^-$	4	5		1	9
IV	pyro$^+$	5	4		1	12
	pyro$^-$	5	6		1	10
V	fac$^+$	7	5		0	3
	fac$^-$	3	5		2	19
VI	sb$^+$	6	9		1	13
	sb$^-$	4	1		1	9
VII	nic$^+$	6	4		1	12
	nic$^-$	4	6		1	10
VIII	ribo$^+$	0	10		0	22
	ribo$^-$	10	0		2	0
ure$^-$ marker assigned to		VIII			VIII	

The figures are the number of haploidisation progeny which carried the markers shown.

Table 20: Chromosome assignment of ureC5 and ureC7

Chromosome	marker allele	Haploidised Diploid of ureC5/MSE			Haploidised Diploid of ureC7/MSE	
		ure$^+$	ure$^-$		ure$^+$	ure$^-$
I	bi$^+$	21	12		22	13
	bi$^-$	3	2		8	14
II	w$^+$	7	8		13	14
	w$^-$	17	6		17	13
III	gal$^+$	11	10		19	13
	gal$^-$	13	4		11	14
IV	pyro$^+$	8	8		14	17
	pyro$^-$	16	6		16	10
V	fac$^+$	11	4		11	12
	fac$^-$	13	10		19	15
VI	sb$^+$	14	9		20	18
	sb$^-$	10	5		10	9
VII	nic$^+$	0	14		0	27
	nic$^-$	24	0		30	0
VIII	ribo$^+$	15	11		8	11
	ribo$^-$	9	3		22	16
ure$^-$ marker assigned to		VII			VII	

The figures are the number of haploidisation progeny which carried the markers shown.

Table 21: Chromosome assignment of ureD4 and ureD9

Chromosome	marker allele	Haploidised Diploid of ureD4/MSE			Haploidised Diploid of ureD9/MSE	
		ure$^+$	ure$^-$		ure$^+$	ure$^-$
I	bi$^+$	18	17		17	13
	bi$^-$	13	3		5	3
II	w$^+$	19	12		11	7
	w$^-$	12	8		11	9
III	gal$^+$	18	6		0	16
	gal$^-$	13	14		22	0
IV	pyro$^+$	18	10		11	9
	pryo$^-$	13	10		11	7
V	fac$^+$	17	10		8	11
	fac$^-$	14	10		14	5
VI	sb$^+$	18	12		11	11
	sb$^-$	13	8		11	5
VII	nic$^+$	19	10		10	7
	nic$^-$	12	10		12	9
VIII	ribo$^+$	0	10		0	16
	ribo$^-$	31	0		22	0
ure$^-$ marker assigned to		VIII			III or VIII	

The figures are the number of haploidisation progeny which carried the markers shown.

and ureD9. The principle of the assignment technique, Chapter II.10.a.,b.,c., is that on subsequent haploidisation, the ure locus is assigned to a chromosome on the basis of its failure to segregate independently with the master strain marker on that chromosome (Forbes, 1959).

The haploidisation progeny were replicated onto solid-N minimal medium with all necessary supplements and 3mM urea, and onto the seven appropriate selective media for testing for the chromosome markers (the eighth marker is conidial colour). Each of the ure strains tested was a biotin auxotroph. This auxotrophy was used as the marker for chromosome I, rather than an M.S.E. marker. The haploidisation results are shown in Tables 19, 20 and 21. The loci, ureA, ureB and ureD appear to be on chromosome VIII, and ureC on chromosome VII. This agrees with the results of Darlington (1966), for ureB, ureC and ureD.

The haploidisation data revealed that ureD9 carried a III-VIII translocation. The possibility therefore arose that the failure of ureD4 and ureD9 to complement (Darlington, 1966) might have resulted from an effect of the translocation. The two ureD strains, ureD4 and ureD9 were crossed together to establish whether they really were allelic. It was expected that the translocation would further decrease the normally fairly rare occurrence of intragenic recombination but would not completely prevent it, and that if a sufficiently high number of progeny were examined, a few recombinants would be detected.

The technique used for the progeny analysis is

described in Chapter II.9.d. The ascospores from seven crossed cleistothecia (two from one cross, five from a second cross) were separately plated out on solid-N minimal medium with vitamins, sodium deoxycholate which restricts colonial growth, and 3mM urea, to select for wild-type recombinants. A diluted sample of each ascospore suspension was spread on complete medium to provide a good estimate of the total number of viable progeny examined.

Wild-type recombinants were recovered amongst the progeny of three of the cleistothecia. In total, seven wild-type recombinants were recovered amongst about 470,000 progeny from the seven cleistothecia. This represents a recombination frequency of 0.003%.

As it is possible that about half of the total number of recombination events which produced "ureD4$^+$, ureD9$^+$" would have been undetectable as the haploid meiotic product would have lacked part of chromosome III or VIII, a presumably lethal effect, it might be more reasonable to consider the frequency as 0.006%.

Intragenic recombination frequencies are known which are as high as 0.3% (Hynes, 1978; J.A. Pateman, personal communication). Even if the translocation were decreasing the frequency of intragenic recombination by as much as fifty-fold, the figure of 0.006% would be compatable with the two mutations being allelic. It seems likely that ureD4 and ureD9 do both carry a mutation in one locus.

2. Diploid Complementation of ure Strains

To confirm that there were five loci known to be

Table 22: Diploid complementation of ure strains

Strain	Growth on 3mM urea *
biA1	+
ureA1	(+)
ureB2	-
'ureE3'	(+)
ureC5	-
ureD9	-
MSE	+
ureB2/ureA1	+
ureB2/'ureE3'	(+)
ureB2/ureC5	+
ureB2/ureD9	+
ureB2/MSE	+
'ureE3'/MSE	+

* Medium: solid -N minimal medium, 3mM urea, vitamin solution.

Growth: + good growth like wild-type
(+) about half wild-type growth
- no growth

concerned with urea utilization, diploids between ureB2 and a representative of each of the other four loci were established by the method described in Chapter II.10.a.,b. The strains were then tested for diploid complementation on -N minimal medium with 3mM urea (Table 22).

All the combinations in the diploids complemented, except ureB2 and ureE3. Both these strains were recessive in a diploid with the master strain, M.S.E. It appeared that ureB2 and ureE3 might be allelic.

A cross was set up between ureB6 and ureE3 (results in Chapter 9 confirm that ureB2 and ureB6 are allelic). The progeny from a crossed cleisthothecium were analysed by plating known amounts of ascospore suspension on -N minimal medium with vitamins, sodium deoxycholate and 3mM urea. To provide a good estimate of the total number of progeny plated on the urea medium, a diluted sample of the ascospore suspension was plated onto complete medium. The strain, ureB6 could hardly grow on the urea and deoxycholate medium though ureE3 grew a little better. It was confirmed that wild-type recombinants could be easily distinguished by plating out samples of ascospore suspension from 'selfed' yellow wild-type and 'selfed' green wild-type cleistothecia with a sample of the ascospore suspension from the ureB6 x ureE3 crossed cleistothecium.

No wild-type recombinants were found amongst the, approximately, 1500 progeny plated on urea medium. As ureE3 did not complement with ureB2 or yield recombinants when crossed to ureB6, it seems likely that 'ureE3' actually carries a mutation in ureB. The strain was

renamed ureB12.

3. Confirmation of Gene Designation of ure Strains

As most of the ure strains had been in existence for several years and as mutation at three different loci produced the same phenotype for growth on urea, it was necessary to confirm that the strains had not been mixed up and that the gene designations were correct.

ureC strains: The two ureC strains which had not been used for haploidisation and chromosome assignment were crossed to ureC5, which had been haploidised. Analysis of 100 progeny per cross confirmed that all the strains labelled 'ureC' were ureC$^-$ alleles.

ureD strains: Analysis of the crossed progeny of ureD4 and ureD9, the only two ureD strains, is described above. They do appear to be allelic and correctly labelled.

ureB strains: Crosses between ureB strains is a subject of Chapter IX. These crosses confirmed that the ureB strains were also correctly labelled.

4. Linkage of ureA and ureB

Crosses between ureA1 and ureB2 or ureB6 were set up. In addition, ureB2 or ureB6 was crossed to some newly isolated thiourea resistant strains, putative ureA alleles (Chapter II.7.a., Chapter IX.1). The crosses were analysed by the method described in Chapter II.9.a.

The cross progeny were tested for growth on solid -N minimal medium with vitamins and 3mM urea, and on minimal medium with vitamins and 5mM thiourea. Wild-type

Table 23: Recombination frequency of ureA and ureB

Parent Strains	Number of progeny examined	Number of wild-type recombinants	Number of double mutant recombinants	Recombination frequency %
ureA1 x ureB2	104	0	0	<0.96
	103	0	1	0.97
	100	1	0	1.0
	86	0	1	1.16
ureA1 x ureB6	104	0	0	<0.96
ureA1368 x ureB2	101	0	0	<0.99
ureA1502 x ureB2	101	1	0	0.99
ureA1117 x ureB6	103	2	0	1.94
ureA1ureB2 x ++	102	no recombinants		<0.98

The analysis of these crosses is described in Section 4 of this chapter.

could only grow on the urea medium, double ureA ureB mutants could grow only on the thiourea medium, ureA mutants could grow on both media and ureB strains could grow on neither medium. The results of these crosses are shown in Table 23.

The recombination frequencies from the nine crosses were in the range 0-2% indicating that the two loci were closely linked. The close linkage of the newly isolated thiourea resistant strains to the ureB locus confirmed that they most likely carried a mutation in ureA.

Further results for the linkage of ureA and ureB are given in Chapter IX.2.a.

5. Summary

In a diploid the two strains, ureA1 and ureB2 complemented for growth on 3mM urea. The two loci showed close linkage and were assigned to chromosome VIII.

A strain carrying a mutation in ureC complemented with ureB2 and the ureC locus was assigned to chromosome VII.

The strain, ureD9 complemented with ureB2 and the ureD locus was assigned to chromosome VIII. It has previously been shown, Darlington (1966), that ureD is not closely linked to ureB.

The locus assignment of ureC and ureD in the review by Pateman and Kinghorn (1977) is the wrong way round and the locus marked ureC on chromosome VIII of the standard Aspergillus linkage map should read ureD.

Four loci are known which are involved in urea utilization in Aspergillus.

CHAPTER VIII

BIOCHEMICAL PROPERTIES OF URE STRAINS

BIOCHEMICAL PROPERTIES OF URE STRAINS

Four loci, ureA, ureB, ureC and ureD are known which are involved in urea utilization in Aspergillus. A role has previously been proposed for the ureA locus (Dunn and Pateman, 1972, and unpublished results):

It would be expected that mutation in a structural gene for the urea and thiourea transport system would confer resistance to thiourea and that strains selected for thiourea resistance would include at least some structural gene mutants. The mutations in all but two of about ninety strains isolated for resistance to thiourea mapped at one locus, ureA. The two exceptions were areA mutants. Strains carrying a mutation in ureA have defective urea and thiourea uptake activity and ureA1 has a higher Km for urea than does wild-type. Therefore it seems likely that ureA is a structural gene for the urea transport system.

No role has previously been suggested for any of the other three loci. The urease and thiourea uptake activities and some biochemical properties of ure strains were investigated. The strains, ureB3, ureC5 and ureD4 have previously been assayed for urease activity (Scazzocchio and Darlington, 1968). These authors used the Muftic method for ammonia determination (Muftic, 1964) and did not detect any activity in the three strains.

1. Urease and Thiourea Uptake Activities of ure Mutants

Wild-type, ureA1, all ureC and ureD strains and seven ureB strains were grown up with ammonium tartrate as

Table 24: Urease and thiourea uptake activities of some ure strains

Strain	urease activity nmoles/min/mg	thiourea uptake activity ρmoles/min/mg
biA1	671 (83)	106 (23)
ureA1	623 (68)	13 (3)
ureB2	<10	88 (8)
ureB3	21 (4)	126 (10)
ureB6	<10	36 (6)
ureB20	<10 *	68 (5)
ureB21	ND *	68 (6)
ureB22	ND *	75 (14)
ureB23	<10	63 (5)
ureC1	<10	51 (5)
ureC5	15 (3)	124 (21)
ureC7	<10	37 (9)
ureC8	<10	36 (6)
ureD4	<10	73 (15)
ureD9	11 (2)	160 (19)

Growth medium: -N minimal with vitamin solution and 5mM ammonium tartrate.

Treatment: transfer for 4h to, -N minimal medium with vitamin solution.

Standard deviations shown in brackets.

* These results were not replicated. N.D. = not detectable.

the nitrogen source, transferred to nitrogen-free medium and assayed for urease and thiourea uptake activities (Table 24). This treatment produced high activities of both systems in wild-type.

The conductimetric urease assay system can accurately measure activities of 10nmoles/min/mg or higher. Even below 10nmoles/min/mg it is possible to make a distinction between detectable and undetectable activity.

The strain, ureA1, had wild-type urease activity and about 12% of the wild-type uptake activity. All the other ure strains had very low urease activities. The ureB, ureC and ureD strains showed a 2-3-fold variation in the levels of their uptake activities. This variation occurred between alleles at each locus.

The lowered uptake activities, compared to wild-type, of most of the ure strains may be caused by inhibition of transport through the intracellular accumulation of urea. The three strains having the highest urease activities, ureB3, ureC5 and ureD9, also had the highest uptake activities. Though the difference in the urease activities of these strains and the other ure mutants is only in the order of a few nmoles/min/mg, it may be sufficient to considerably reduce the intracellular urea concentration.

2. Properties of ureB2 Revertants

The introduction of a second mutation into a gene which already contains a mutation causing decreased activity of a particular enzyme, may partially restore that activity. If when compared to the wild-type activity, this restored activity shows different properties such as

an altered Km or heat stability, it is likely that the gene codes for part or all of the enzyme.

The possibility of ureB being a structural gene for urease was investigated.

a. Isolation of revertants

The strain, ureB2 cannot grow on urea as a sole source of nitrogen. Conidia of ureB2 were treated with NTG (Chapter II.8) and embedded in solid medium with 3mM urea as the nitrogen source. Fifty colonies were isolated and assayed for urease activity after growth with L-alanine as the nitrogen source.

There was a considerable background of poorly growing mycelium after mutagenesis. As the colonies had not been purified before assay, many of the fifty colonies had <10% wild-type activity and were probably a mixture of revertants and the background. Those which had ≥10% wild-type urease activity were purified by streaking on urea medium and assayed again. The eleven strains which again had ≥10% of the wild-type activity were then examined for differences in the characteristics of their urease activity compared to wild-type.

b. Growth test for temperature sensitivity

The eleven revertants, ureB2 and wild-type were tested for growth on solid medium with 3mM urea as the nitrogen source, at 25°C and 37°C. None of the strains showed temperature sensitivity of growth. All the revertants could grow as well as wild-type at both temperatures.

c. Km measurements

The Km for urea of each of four revertants was determined after growth with L-glutamate as the nitrogen

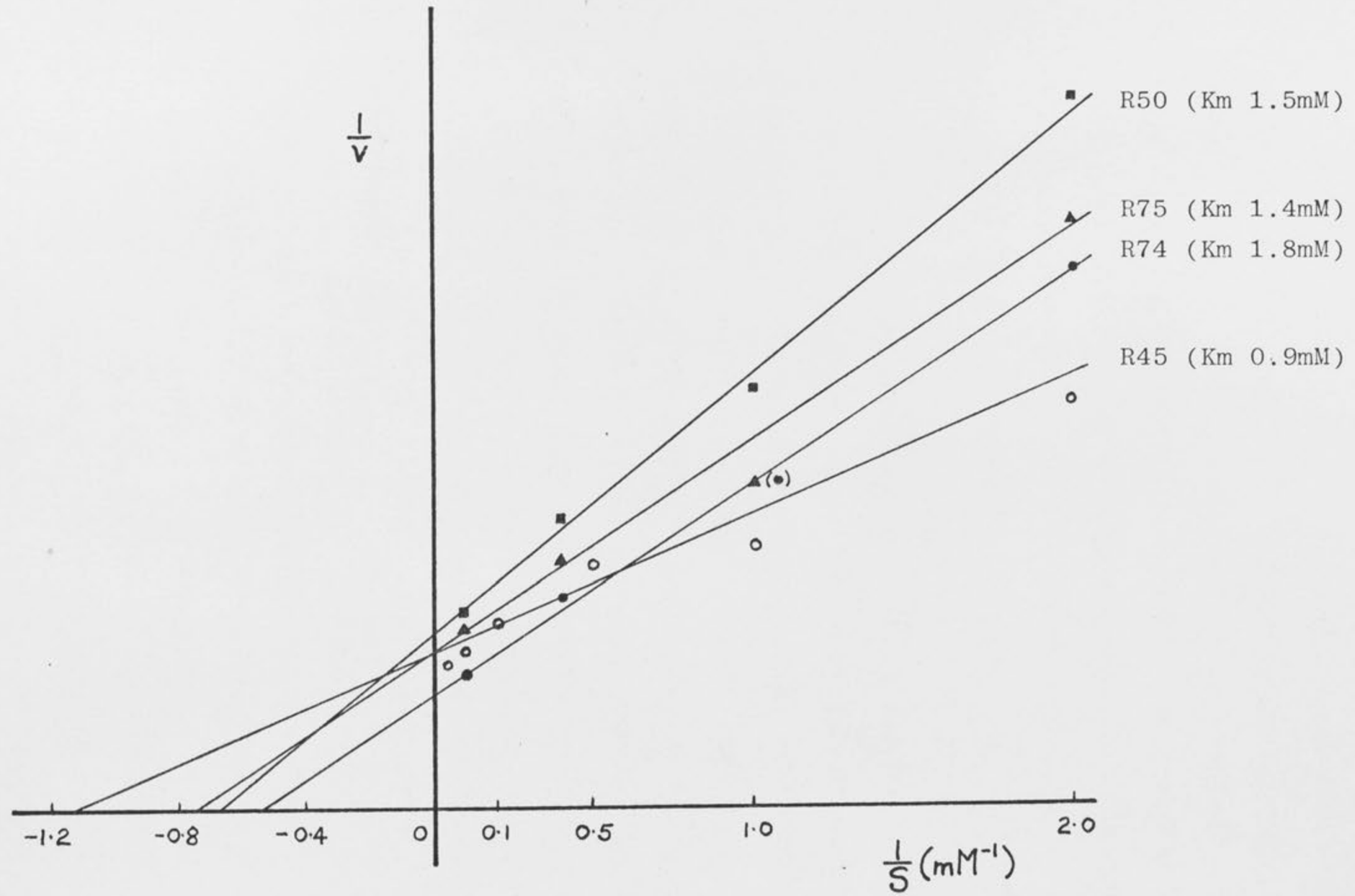


Figure 10: Km determinations for four ureB2 revertant strains: Lineweaver-Burk double reciprocal plots

Growth medium: -N minimal medium, vitamin solution, 10mM L-glutamate. v was measured in arbitrary units.

The intercepts of the axes were calculated by linear regression analysis.

Table 25: Urease activities of ureB2 revertants before and after incubation at 65°C

Strain	% wild-type urease activity before incubation	% initial activity remaining after 75 min at 65°C
biA1	100	87
ureB2	<10	100
R17	52	93
R45	15	41
R46	14	63
R50	76	96
R64	15	98
R66	13	60
R67	54	100
R72	10	100
R74	63	78
R75	64	90
R79	90	76

The growth and incubation conditions are described in the text.

Table 26: Heat stability of R45 and wild-type

Strain	% of initial urease activity remaining after 75 min incubation at 65°				
	Determination				Average
	1	2	3	4	
biA1	87	62	88	74	78
R45	41	28	43	19	33

The growth conditions and incubation are described in the text.

source (Figure 10). The Km value is independent of the units in which activity is measured. The activities, velocities, were not measured in nmoles/min/mg but as gradients from the chart of the chart recorder connected to the conductivity apparatus. The velocities in Figure 10 therefore have no meaningful units. The Km determination for three of the revertants were in the range 1.4-1.8mM, very close to the wild-type value, 1.8(0.3)mM (Chapter III.2.d.). The Km of the fourth revertant tested, R45, was slightly lower, 0.9mM.

d. Heat stability

Wild-type Aspergillus urease appeared to be very heat stable (Chapter III.2.f.). The eleven revertants and biA1 were grown up with L-glutamate as the nitrogen source and assayed before and after 75 min incubation in sealed test-tubes in a shaking water-bath at 65°C (Table 25).

The urease activity of one revertant, R45, appeared to be considerably less heat stable than wild-type. This revertant and wild-type were grown up on L-glutamate and assayed before and after incubation at 65°C a further three times. The results of the four independent determinations of activity are shown in Table 26. The wild-type enzyme retained an average of 78% of its original activity (this is in close agreement with the values in Chapter III, Figure 6, for 60-90 min incubation at 65°C which were determined in a separate series of experiments). The revertant, R45, retained an average of only 33% of its original activity.

The statistical significance of this difference in heat stability was calculated by applying a matched

pairs t-test to the results. The probability of the observed difference in the heat stability of wild-type and R45 having occurred by chance is 0.12%.

e. Cross of R45 with wild-type

The revertant strain, R45 was crossed to the yellow wild-type strain (Chapter II.6). The progeny of the cross were spread onto complete medium, incubated and then velvet replica plated (Chapter II.9.b.) onto solid medium with 3mM urea as the nitrogen source.

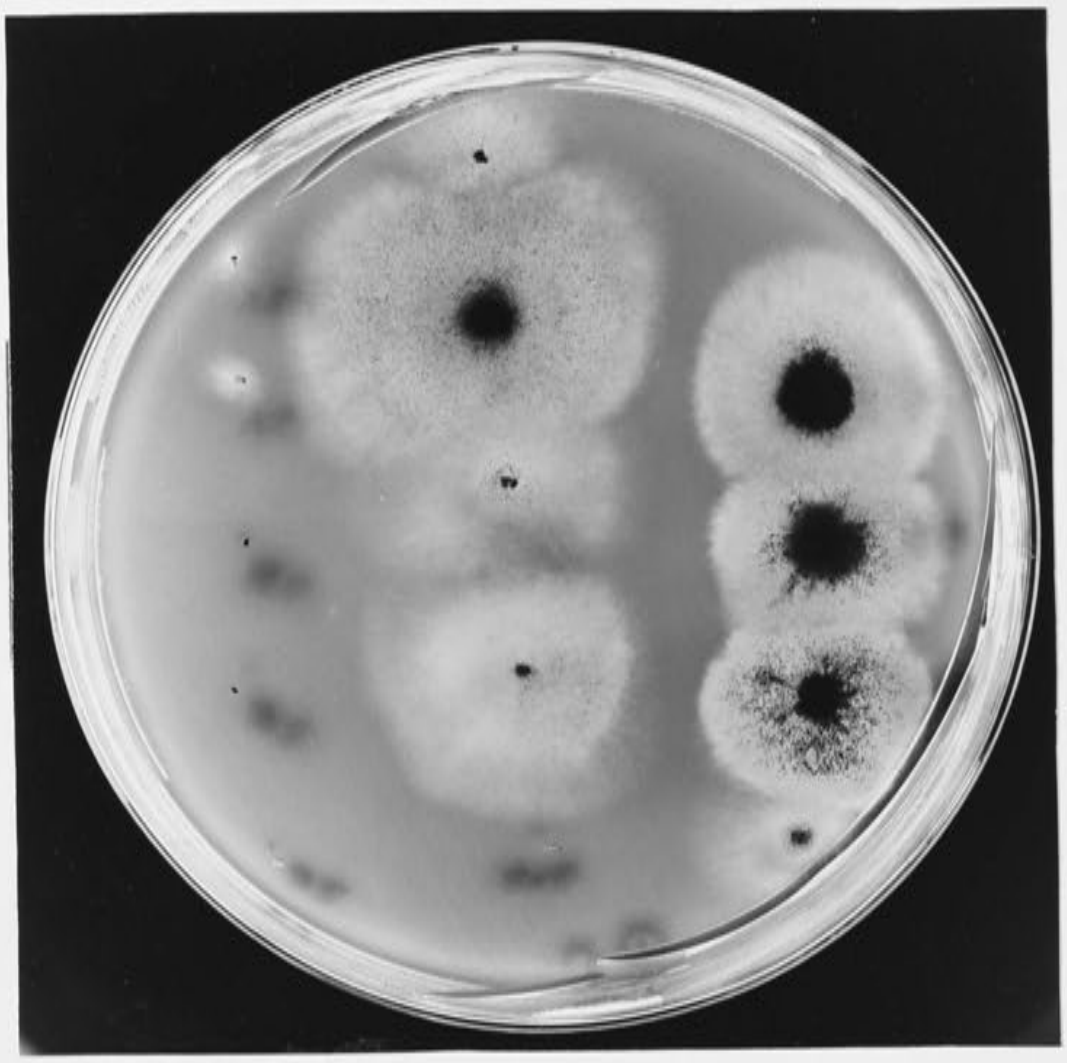
All 5804 progeny examined could grow with 3mM urea as the nitrogen source. This indicates that the mutation which caused the reversion is <0.03 map units from the original ureB2 mutation. The reversion mutation is therefore most likely to be within ureB.

As the urease activity of the revertant, R45, appears to be less heat stable than that of wild-type and as the reversion mutation is probably within ureB, it is likely that ureB is a structural gene for urease.

3. Complementation of ureB Alleles

Though there is some debate about the number of subunits in the active jack bean urease species most commonly prepared it appears to be generally agreed that jack bean urease is a polymeric enzyme with one type of subunit (Staples and Reithel, 1976; Fishbein *et al.*, 1977; Dixon *et al.*, 1980,d.). It seemed reasonable to expect that the Aspergillus urease might also contain only one type of subunit.

Strains carrying mutations in the structural gene of a polymeric enzyme with one type of subunit, may



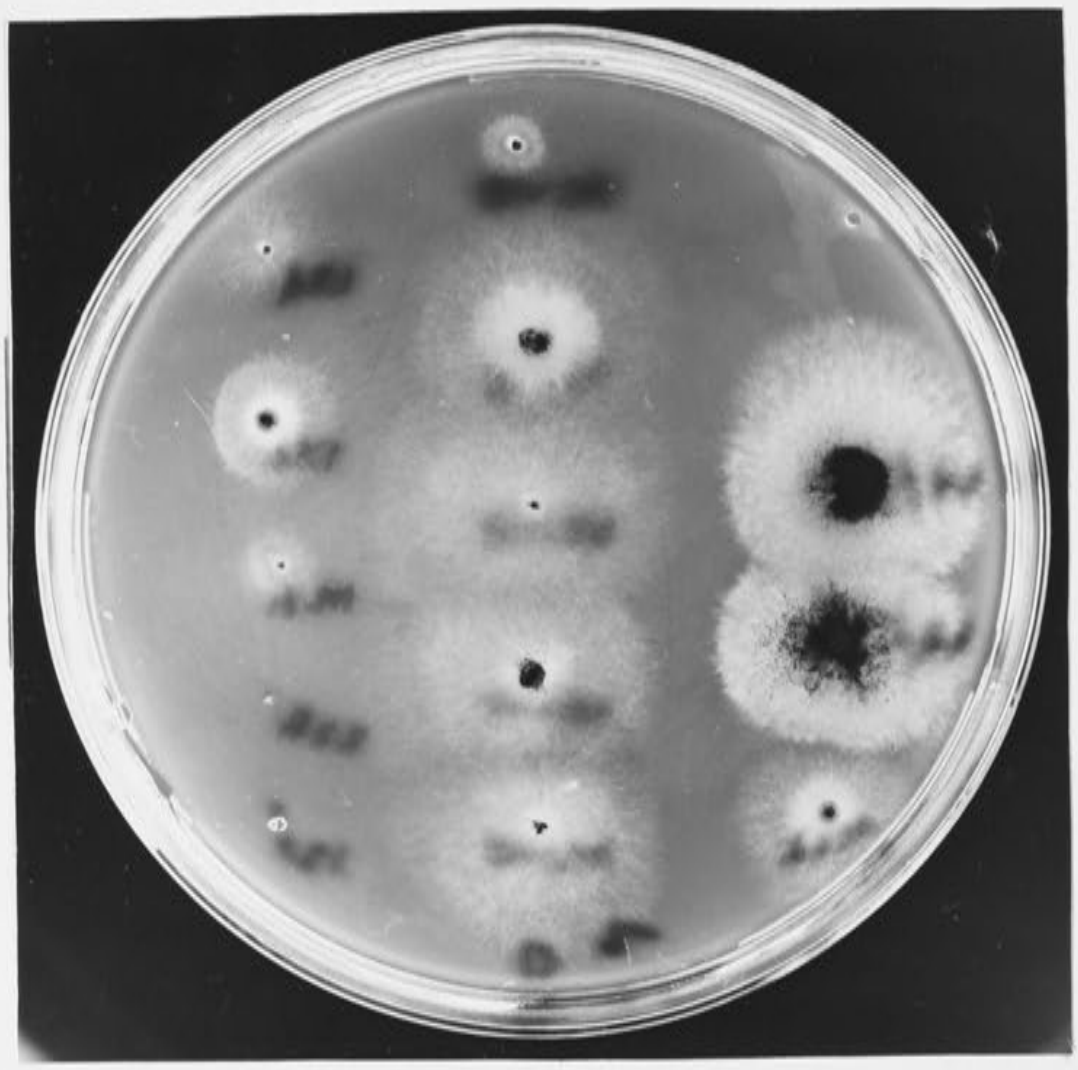


Plate 3: Heterokaryon complementation tests between some ureB alleles

Plate 3: Heterokaryon complementation tests between ureB alleles

Growth medium: solid -N minimal medium, 3mM urea.

Top petri dish: strains inoculated in following arrangement:

ureB31	ureB6/ureB34	
ureB6 (yellow)	ureB6/ureB3	ureD9/ureB3
ureB6 (green)	ureB31/ureB3	ureD9/ureC5
ureB3	ureB34/ureB3	ureC5/ureB3
ureB2	*	ureB34/ureB34 (yellow/green)

Lower petri dish: strains inoculated in following arrangement:

ureB29	ureB23/ureB34	
ureB33	ureB21/ureB26	ureD9/ureB3
ureB34	ureB21/ureB29	ureD9/ureC5
ureB23	ureB21/ureB33	ureB26
ureB21	ureB21/ureB34	

*one of the strains for this test was unintentionally omitted.

Photographed after 4-5 days incubation at 37°C.

sometimes complement, restoring partial activity (Fincham and Pateman, 1957; Zabin and Villarejo, 1975, review). As ureB is likely to be a structural gene for urease, complementation tests were set up between ureB alleles.

One hundred and thirty-five of the possible pairwise combinations of non-leaky ureB strains were tested for heterokaryotic, intragenic complementation (Chapter II.11.). The solid medium contained 3mM urea as the nitrogen source and did not contain vitamin solution. Each strain of every pair tested carried a different mutation resulting in separate vitamin requirements. Thus heterokaryon formation was forced both by the vitamin requirements and the inability of each parent strain to utilize urea.

Two of these strains, ureB3 and ureB6 possibly do complement (Plate 3). It is difficult to be more positive as ureB3, though non-leaky in a 48h growth test, did grow a little by itself on 3mM urea in the course of the 4-5 day incubation required for the intragenic complementation test. This was observed when two ureB3 strains which had different vitamin requirements were inoculated into the same point in the urea medium. It was found (Table 24) that ureB3 had slightly higher urease activity than the other ureB strains assayed.

The amount of growth achieved by ureB3 by itself did, however, appear to be less than that resulting from mixed inocula of ureB3 and ureB6. The possibility of making a diploid of these two strains and assaying it for urease activity was considered. As a falsely negative result could have arisen through instability of the

Table 27: Growth of ure strains with urea as the nitrogen source, with and without nickel sulphate

	Growth with 3mM urea as nitrogen source with nickel sulphate in the concentrations, (mM):					
Strain	0	0.01	0.05	0.1	0.5	1.0
biA1	+++	+++	+++	+++	+	-
ureA1	+	+	+	+	(+)	-
ureB2	-	-	-	-	-	-
ureC5	-	-	-	-	-	-
ureD4	-	-	-	-	-	-
ureD9	-	(+)	(+)	+	(+)	-

Growth medium: solid -N minimal medium, vitamin solution, 3mM urea, nickel sulphate as shown.

Scoring:
+++ good wild-type growth
+ about half wild-type growth
(+) slight growth
- no growth

Scored after 48h incubation at 37^{o}C.

resultant protein, this did not seem worthwhile.

Thus amongst the pairs of alleles tested only one pair, if any, showed intragenic complementation.

4. Effect of Nickel on Urea Utilization of ureD9

Though the crystallisation of urease (Sumner, 1926) contributed to the principle that enzymes need not contain metal ions, it seems that urease is, in fact, a nickel metalloenzyme (Dixon *et al.*, 1975).

This suggested the possibility that some of the ure loci might have a role concerning nickel.

a. Growth tests with nickel

All the available ure alleles at each locus were tested for growth on solid medium containing 3mM urea as the nitrogen source with nickel sulphate in the range 0.01-1mM. The growth of strains representing the alleles of each of the ure loci is shown in Table 27.

One strain, ureD9 responded, growing about half as well as wild-type in the presence of 0.1mM nickel sulphate. Higher concentrations did not further increase the growth of ureD9, but rather, had a toxic effect on all the strains. The other ureD strain, ureD4, did not respond to the presence of nickel sulphate.

All the available ure strains were tested for improved growth on urea in the presence of other metal salts, copper sulphate, manganese sulphate and nickel acetate, in the concentration range 0.1-10mM. Copper sulphate and nickel acetate are lethal when present in the medium at 1mM. Manganese sulphate has a strong toxic effect when present at 10mM.

Table 28: Effect of nickel acetate, copper sulphate and manganese sulphate on the growth of ureD mutants on urea

	Growth with 3mM urea as nitrogen source with metal salts in the concentrations (mM) shown below									
	nickel acetate			copper sulphate			manganese sulphate			
Strain	0.01	0.1	1.0	0.01	0.1	1.0	0.01	0.1	1.0	10.0
biA1	+++	+++	-	+++	+++	-	+++	+++	+++	(+)
ureD4	-	-	-	-	-	-	-	-	-	-
ureD9	(+)	+	-	-	-	-	-	-	-	-

Growth medium: solid -N minimal medium, vitamin solution, 3mM urea, metal salts as shown.

Scoring: +++ good wild-type growth

\+ about half wild-type growth

(+) slight growth

\- no growth

Scored after 48h incubation at 37°C.

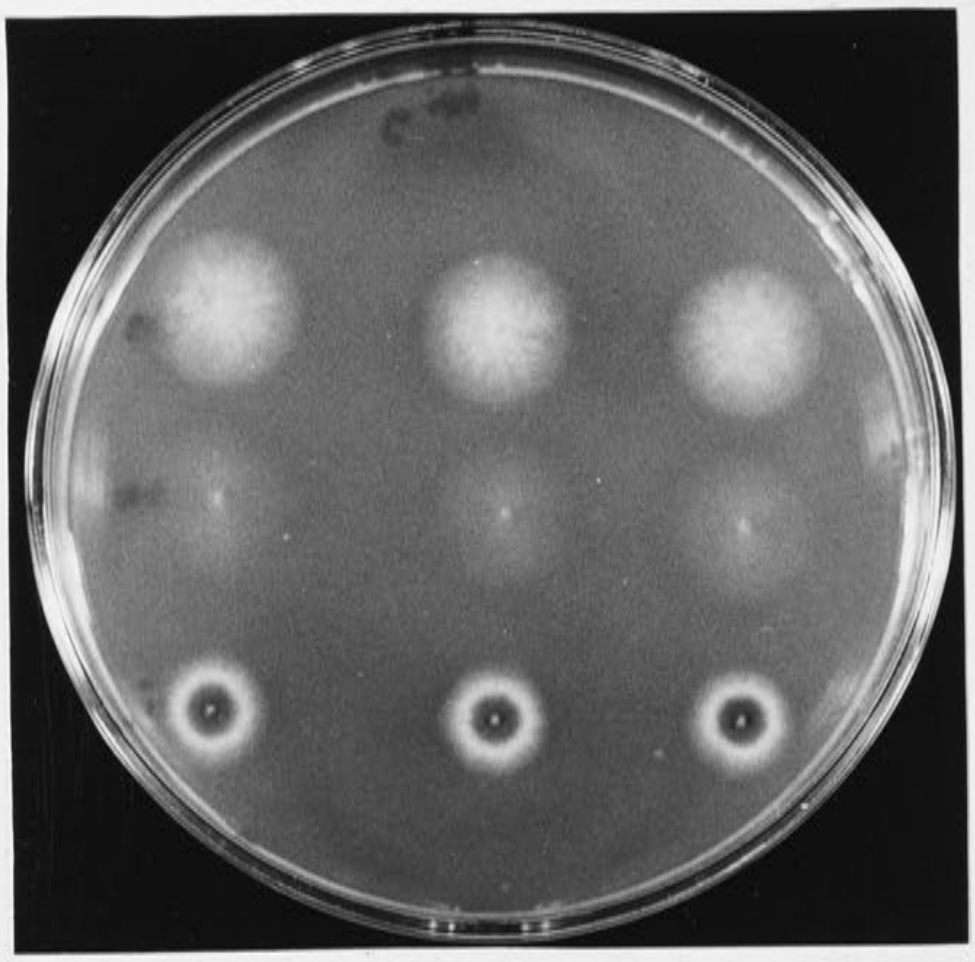


Plate 4: Growth of ureD strains and wild-type with urea as nitrogen source, with and without nickel

Plate 4: Growth of ureD strains and wild-type with urea as nitrogen source, with and without nickel

Growth medium: solid -N minimal medium, vitamin solution, 3mM urea ± 0.1mM nickel acetate.

Lower petri dish without nickel.

Strains inoculated in triplicate: ureD9 (yellow)
ureD4 (green)
biA1 (green)

Photographed after 48h incubation at 37°C.

Only ureD9 responded to the presence of a metal salt and this response was specific to nickel. The growth of ureD4, ureD9 and wild-type in the presence of metal salts is shown in Table 28. The optimum concentration for growth of ureD9 on urea with nickel acetate is 0.1mM, the same optimum concentration as nickel sulphate.

The ure strains were also tested for improved growth on 5mM L-arginine in the presence of nickel. The urease deficient mutants grow poorly on this nitrogen source which is converted by arginase to urea and L-ornithine. Addition of 0.1mM nickel sulphate again improved the growth of ureD9.

The ureB strains isolated in the course of the present work were also tested for improved growth with 3mM urea as the nitrogen source in the presence of 0.1mM nickel acetate. None of these strains responded to the presence of nickel.

The growth of biA1, ureD9 and ureD4 with 3mM urea as the nitrogen source, with and without 0.1mM nickel acetate in the medium is shown in Plate 4.

b. Effect of nickel on urease activity *in vitro*

The strains, ureD4 and ureD9 were grown up with ammonium tartrate as the nitrogen source and transferred to nitrogen-free medium. This treatment produced high urease activity in wild-type. Cell-free extracts were incubated in assay buffer in the glass cells of the conductivity apparatus at 37^{o}C for 2-3 min with 0.2-2mM nickel acetate.

These incubations had no effect on the urease activities of ureD4 and ureD9.

Table 29: Urease activity of some ure strains after growth on varous nitrogen sources with and without nickel acetate

		Urease activity (nmoles/min/mg) after growth ± nickel acetate, 0.1mM	
Strain	growth conditions	- nickel	+ nickel
biA1	L-alanine	334 (82)	315 (34)
ureB2	"	11	<8
ureB6	"	<8	<8
ureC1	"	<8	9
ureC5	"	11	12
ureC7	"	11	10
ureC8	"	13	13
ureD4	"	<8	<8
ureD9	"	<8	18 (4)
"	L-glutamine	<8	17
"	L-proline	<8	13
"	nitrate	<8	20
biA1	"	287 (63)	272 (53)

Growth medium: -N minimal medium with vitamin solution and 10mM nitrogen source as shown, ± nickel acetate, 0.1mM.

These activities are not directly comparable to the majority of activities in this work. At the time of these assays, 8nmoles/min/mg was considered to be the lowest detectable activity.

c. Addition of nickel to the growth flasks

As was found with solid medium, the addition of nickel acetate to liquid medium had a toxic effect on cell growth. The maximum concentration which could be satisfactorily added to the growth flasks was 0.1mM. This concentration decreased the growth weight of cells by more than half but did not alter the wild-type urease activity of the cell-free extract.

The presence of nickel ions in the extracts did not interfere with the conductimetric assay. The urease activity of a wild-type extract mixed with 0.1M nickel acetate was altered only by dilution.

Several ure strains were grown with L-alanine as the nitrogen source with and without 0.1mM nickel acetate and assayed (Table 29). Growth in the presence of nickel increased the activity of ureD9 but had no effect on that of ureD4 or the other ure strains. The strain, ureD9, was also assayed after growth with either L-proline, nitrate or L-glutamine as the nitrogen source, with and without 0.1mM nickel acetate (Table 29). After growth on all three nitrogen sources the urease activity of ureD9 was higher with nickel acetate present in the growth medium.

d. Addition of nickel on transfer

To find out whether the addition of more nickel would further increase the activity of ureD9 while avoiding the problem of toxicity decreasing growth weight, nickel acetate was added after growth on medium with ammonium tartrate as the nitrogen source, at the time of transfer to nitrogen-free medium. Nitrogen starvation resulted in high wild-type urease activity.

Table 30: Urease activity of ureD9 after transfer to medium with and without nickel acetate

	Urease activity (nmoles/min/mg) of ureD9 after transfer to medium with nickel acetate at the concentrations (mM):			
	0	0.1	0.5	1.0
	11 (2)	24 (4)	29	31
Number of determinations	7	4	1	1

Growth medium: -N minimal medium with vitamin solution and 5mM ammonium tartrate.

Treatment: transfer to -N minimal medium with vitamin solution and nickel acetate as shown.

Standard deviations shown in brackets.

Table 31: Urease activity of some ure strains transferred to medium with and without nickel acetate

Strain	Urease activity (nmoles/min/mg) after transfer to medium ± nickel	
	- nickel	+ nickel
ureB3	21 (4)	14
ureB12	32	30
ureB23	<10	<10
ureB24	<10	<10
ureB26	<10	<10
ureB27	<10	<10
ureB28	<10	10
ureB29	<10	<10
ureB31	10	<10
ureB33	<10	<10
ureB34	10	<10
ureD4	<10	<10
biA1	671 (83)	643 (51)

Growth medium: -N minimal medium with vitamin solution, 5mM ammonium tartrate.

Treatment: transfer for 4h to -N minimal medium with vitamin solution ± 1mM nickel acetate.

Standard deviations shown in brackets.

Nickel acetate, 0.1, 0.5 and 1mM, was added to the transfer flasks. The addition of nickel on transfer was as effective in increasing the activity of ureD9 as the addition of nickel during growth (Table 30). The addition of concentrations higher than 0.1mM did not, however, further increase the urease activity of ureD9 : addition of 0.5 and 1mM nickel acetate resulted in the same increase in the activity of ureD9 as did 0.1mM. Addition of 1mM nickel acetate on transfer did not increase the activity of ureD4. Some of the ureB strains isolated in the course of the present work, and two of the original strains which had not been assayed when nickel was added during growth, were assayed after growth on ammonium tartrate followed by transfer to nitrogen-free medium with and without 1mM nickel acetate (Table 31). The addition of nickel acetate on transfer had no effect on the activity of any of these strains.

The increase in the activity of ureD9 after transfer to nitrogen-free medium with nickel acetate was also demonstrated at 50°C. Assaying at 50°C effectively expands the activity scale 2-fold (Chapter III.2.g.) and amplified the nickel-mediated increase in the activity of ureD9. At 50°C the urease activity of ureD9 was 21nmoles/min/mg after transfer without nickel and 46nmoles/min/mg after transfer to medium containing nickel.

The response of ureD9 to the presence of nickel ions suggests that the ureD locus has some role concerning nickel and urease.

Part of this work has been published in the Journal of General Microbiology, (Mackay and Pateman, 1980).

CHAPTER IX

ATTEMPTS TOWARDS MAPPING THE URE A URE B REGION

ATTEMPTS TOWARDS MAPPING THE URE A URE B REGION

The loci, ureA and ureB are closely linked with a recombination frequency of less than 2% (Chapter VII.4). It is likely that ureA is a structural gene for the urea transport system (Dunn and Pateman, unpublished results, see introductions to Chapters III and VIII) and that ureB is a structural gene for urease (Chapter VIII.2.d.,e.).

Clustering of genes with related functions is uncommon in eukaryotes and it is of interest to know how the structural genes are organised in relation to control regions. It is useful to have a fine structure genetic map of the region on which to base such an investigation. The mapping of a collection of small overlapping deletions is the best way of achieving this. Mapping with deletions avoids the need to calculate recombination frequencies which are subject to error through marker effects and gene conversion (Fincham *et al.*, 1979).

Once a number of mutant strains has been isolated a map of the region may be gradually built up by crossing the strains together pairwise and establishing which pairs yield recombinants. If no recombinants are recovered from a cross, it is possible that one parent strain contains a deletion and the other parent strain contains either an overlapping deletion or a point mutation, or smaller deletion, which lies within the region deleted in the first parent. A deletion is genetically defined as a mutation which cannot recombine with two other mutations which can recombine with each other. As the crossing programme progresses it should become apparent which strains carry

deletions and possible to order the deleted segments linearly as a map.

A search for strains carrying deletions in the ureA ureB region was undertaken. The vast majority of strains isolated by resistance to the toxic urea analogue, thiourea, carry a mutation in one locus, ureA (Dunn and Pateman, unpublished). It was expected that if a large number of thiourea resistant strains was isolated, a small percentage of the mutations would be deletions and that some of these would run into ureB. A deletion affecting both genes would result in the strain showing both thiourea resistance and lack of urease activity.

1. Spontaneous Mutations

Wild-type conidia were inoculated into solid minimal medium (in which nitrate is the nitrogen source) containing 1-5mM thiourea. Wild-type cannot grow on minimal medium in the presence of thiourea at these concentrtions. During 2-3 days incubation, colonies, apparently thiourea resistant, arose at high frequency. Within the range 1-5mM, the concentration of thiourea did not appear to affect the number of colonies arising. To confirm that the colonies really were thiourea resistant, conidia from seventy-five of the colonies were streaked onto complete medium and incubated. A single discrete colony resulting from each of the original 75 colonies was then tested for growth on minimal medium in the presence of 1 and 5mM thiourea. Each colony could grow in the presence of both concentrations. The colonies arising on the thiourea medium therefore appeared to be strongly thiourea

resistant and pure. A large number of these strains was isolated.

Each thiourea resistant strain was tested for urease activity with an indicator-dye plate test (Chapter II.5.c.). Conidia were picked from the centre of each thiourea resistant colony and inoculated into solid minimal medium, incubated for 20h and the petri dish then flooded with a solution of urea and phenol red. This test distinguishes colonies with and without urease activity by forming red haloes around colonies able to produce ammonia from urea.

The strains, ureB2, B12, ureC5, ureD9, ureA1 and biA1 were tested as a control for the dye test. Red haloes formed around biA1 and ureA1 but not around the other ure strains, which have very low urease activity. The ureB2 revertants (Chapter VII.2.a.) were also tested as these strains have a range of urease activities. The test of the revertants indicated that any strain with up to 15% of wild-type urease activity would appear to lack urease activity with the dye test.

Diffusion of the dye was not a problem. The haloes remained discrete for several hours. It seems unlikely that any strain lacking urease activity could be missed using this test.

All the 2031 spontaneous thiourea resistant strains tested with the indicator test had urease activity.

2. DEO Induced Mutations

Diepoxyoctane (DEO) causes a high frequency of multi-site deletions in Neurospora crassa (Ong and

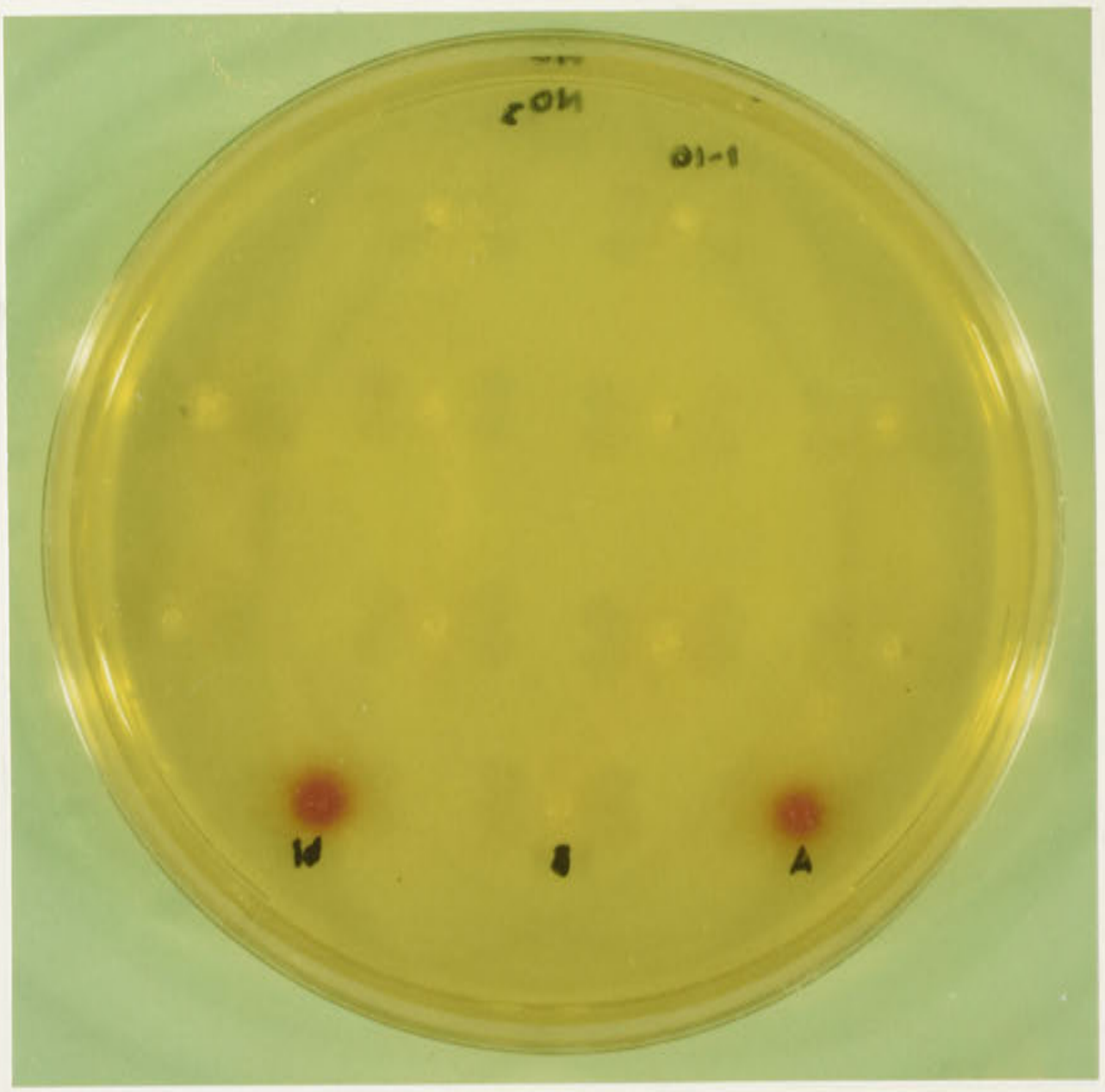


Plate 5: Indicator dye test for urease activity

The procedure for this test is described in Chapter II.5.c.

The strains inoculated into the medium are:

10 of the strains isolated following DEO treatment, (Section 2.a. of this chapter)

3 control strains,

biA1 ureB2 ureA1

de Serres, 1975).

Wild-type conidia were treated with DEO then embedded in minimal medium with 5mM thiourea and incubated (Chapter II.7.b.). The resulting mutants were tested for urease activity with the indicator dye test.

a. Isolation of some strains which lacked urease activity

One mutation run yielded sixteen strains which lacked urease activity. These strains were tested for growth on urea and for resistance to thiourea. The strains were then streaked on complete medium to obtain single colonies and retested. The indicator dye test for urease activity of some of these strains is shown in Plate 5. The purified strains were also tested for heterokaryotic complementation on 3mM urea with ureB2, ureC5 and ureD9. These growth and complementation tests revealed that seven of the 16 strains carried a mutation in ureB and nine appeared to have a mutation in both ureA and ureB.

The nine double phenotypes were crossed to wild-type to establish whether single ureA and ureB strains could be recovered. The progeny from these crosses were spread on complete medium, incubated, and then velvet replicated (Chapter II.9.b.) onto -N minimal medium with 3mM urea and onto minimal medium with 5mM thiourea. After incubation the progeny were examined for growth on these two media. In each case, $ureA^{-}$ $ureB^{+}$ and $ureA^{+}$ $ureB^{-}$ colonies were found amongst the progeny. None of the nine strains, therefore, contained a deletion running through ureA and ureB.

To confirm that ureA and ureB really were closely linked as indicated by the results in Chapter VII, the

Table 32: Outcrossing of ureA ureB strains

Strain crossed to wild-type	Total progeny analysed	Number of recombinants		Recombination frequency %
		$ureA^-$ $ureB^+$	$ureA^+$ $ureB^-$	
ureA2501 ureB27	4707	12	14	0.57
ureA2502 ureB28	7287	15	28	0.59
ureA2503 ureB29	6232	13	22	0.56
ureA2505 ureB31	6259	18	22	0.64
ureA2506 ureB32	6999	16	8	0.34
ureA2507 ureB33	7395	16	22	0.51
ureA2508 ureB34	7109	15	7	0.31
Total	45,988	105	123	0.50

recombination frequency of ureA and ureB was calculated from seven of these crosses (Table 32). The total progeny from the seven crosses, some 45,988, combine to give a recombination frequency of 0.50%. Thus the two genes are indeed very closely linked.

b. Use of adenine to promote the appearance of ure strains

Seven of the mutants lacking urease activity which had arisen on thiourea medium were ureB strains. This suggested that in ure strains the build-up of urea resulting from purine break-down might confer some resistance to thiourea by inhibiting the urea transport system. It seemed likely that this effect could be enhanced by adding adenine to the selective thiourea medium in which the DEO-treated conidia were embedded.

Some ure strains and wild-type were tested for growth on minimal medium plus thiourea and adenine. These growth tests showed that the addition of adenine did increase the resistance of ureB, ureC and ureD strains to thiourea.

Though this seemed a reasonable way of promoting the appearance of ureB and ureA ureB strains following DEO treatment, it was unsuccessful in practice. No strains lacking urease activity arose when adenine was added to the selective medium.

c. Use of urea analogues for selecting ure strains

Some urea analogues were tested for a toxic effect on wild-type growth. An analogue which is not toxic itself but from which urease could produce a toxic substance should provide a means of selecting for ure strains with

either defective transport or defective urease activity, or both.

(i) Allylurea

Wild-type and several ure strains were tested for growth on -N minimal medium with 10mM nitrate or 5mM ammonium tartrate as the nitrogen source, with 5mM allylurea. All the strains grew normally on both nitrogen sources in the presence of allylurea. Allylurea had no toxic effect.

(ii) Hydroxyurea

Jack bean urease can hydrolyse hydroxyurea (Fishbein *et al.*, 1965). One of the products is hydroxylamine and this substance is very toxic to Aspergillus (Drainas, 1977). However, the presence of 5mM hydroxyurea had no effect on the growth of wild-type on solid medium with 10mM alanine or nitrate as the nitrogen source. Hydrolysis of hydroxyurea by Aspergillus urease could not be demonstrated in a conductimetric assay (Chapter III.2.e.).

(iii) Selinourea

With the exception of ureB12 which has more urease activity than other ureB strains, ure strains are resistant to selinourea and wild-type is sensitive. Resistance appears to be conferred through the strain having either defective transport or very low urease activity.

DEO-treated conidia were embedded in minimal medium with 0.2mM selinourea.

Resistant strains appeared after 2-3 days incubation and 507 strains were tested for urease activity with the dye test. All had urease activity. Thirty were tested for growth on minimal medium with selinourea and with

thiourea. The thirty strains were more resistant to selinourea than wild-type but were less resistant than the ure strains. Surprisingly, considering ureA is resistant to both selinourea and thiourea, all thirty strains were sensitive to thiourea. Four of the strains were grown up in liquid -N minimal medium with L-glutamate as the nitrogen source and assayed for urease activity. All four had wild-type urease activity.

It seems likely that the locus here concerned with selinourea resistance has no real connection with urea utilization but perhaps may be involved in sulphur metabolism.

d. Summary of mutation runs with DEO

In total, eighteen mutation runs were carried out with DEO. Various concentrations of DEO and times and temperatures of incubation were tried, including those successfully employed in the making of deletions in Neurospora (Ong and de Serres, 1975) and in Aspergillus (Hynes, 1978). By plating out control conidia on most occasions (Chapter II.7.b.) it was possible to calculate the increase in the mutation rate of DEO-treated conidia and to confirm that DEO was acting as a mutagen. The increase in the mutation rate was generally between 2-10-fold.

Amongst the 1937 thiourea resistant colonies isolated following DEO treatment only the 16 already discussed lacked urease activity. No deletion was detected running through both ureA and ureB.

It is not understood why no deletions involving both of these closely linked loci were found. A possible

Table 33: Reversion frequency of ureB strains

Strain	Reversion frequency of strains surviving treatment with NTG
ureB2	$2/10^7$
ureB6	$1/10^7$
ureB3	$2/10^8$
ureB20	$1/10^7$
ureB22	$1/10^7$
ureB23	$4/10^7$
ureB24	$3/10^7$
ureB26	$3/10^7$
ureB28	$3/10^7$
ureB29	$3/10^7$
ureB33	$1/10^8$
ureB34	$2/10^8$

explanation is discussed in Chapter X.

3. Reversion of ureB Strains

Though no deletions were detected running through both ureA and ureB, it was possible that some of the ureB strains might have resulted from a small deletion within ureB. The three original non-leaky ureB strains, ureB2, ureB3 and ureB6 and eight of the DEO-induced ureB strains, were tested for the ability to revert on treatment with NTG (Chapter II.8.).

A point mutation, or a frameshift mutation caused by the insertion or deletion of a few bases, may sometimes be repaired by subsequent mutation. The second mutation might directly repair the first defect or might compensate for it. A small deletion of four bases, for example, might be compensated for by the subsequent insertion of one base as this would restore the correct reading frame. Provided the mutations have not occurred in a region coding for an essential part of the enzyme such as the catalytic site, the enzyme may be able to tolerate some substitutions or the loss of one or two amino acids: reversion of the original mutation may occur.

Larger deletions, however, which result in the loss of several amino acids or more, cannot be repaired by further mutation. By subjecting strains carrying mutations to further mutagenic treatment and determining their ability to revert, it is possible to establish which, if any, of the strains are likely to carry a deletion.

The results of the reversion experiments are shown in Table 33.

Due to the large numbers of conidia involved in reversion experiments and the rare occurrence of reversion, a stage is reached at which it is difficult to distinguish the occurrence of reversion from unavoidable rare contamination by wild-type. Most of the ureB strains tested had a reversion rate of 1-3 revertants per 10^7 conidia surviving mutagenic treatment. The three strains, ureB3, ureB33 and ureB34 had a lower rate of reversion of 1-2 revertants per 10^8 surviving conidia. It seemed likely that the apparent revertants from these three strains were in fact very rare wild-type contaminants and that the three strains possibly carried deletions in ureB.

4. Crosses Between ureB Strains

While mutation runs were still in progress towards isolating more strains, a crossing programme was begun to map the existing ureB strains, including the three potential deletions.

This crossing programme proved unexpectedly difficult. Most crosses between ureB alleles were infertile and produced either no cleistothecia or small unhealthy cleistothecia which contained very few ascospores. To increase the number of progeny examined, the ascospores from 30-50 cleistothecia were plated out together. This 'bulking' of cleistothecia still did not provide very high numbers of progeny; less than may usually be obtained from one large healthy cleistothecium. 'Bulking' of cleistothecia suffers from the disadvantage that it is not possible to know how many of the cleistothecia were crossed as the progeny will include the products of 'selfed'

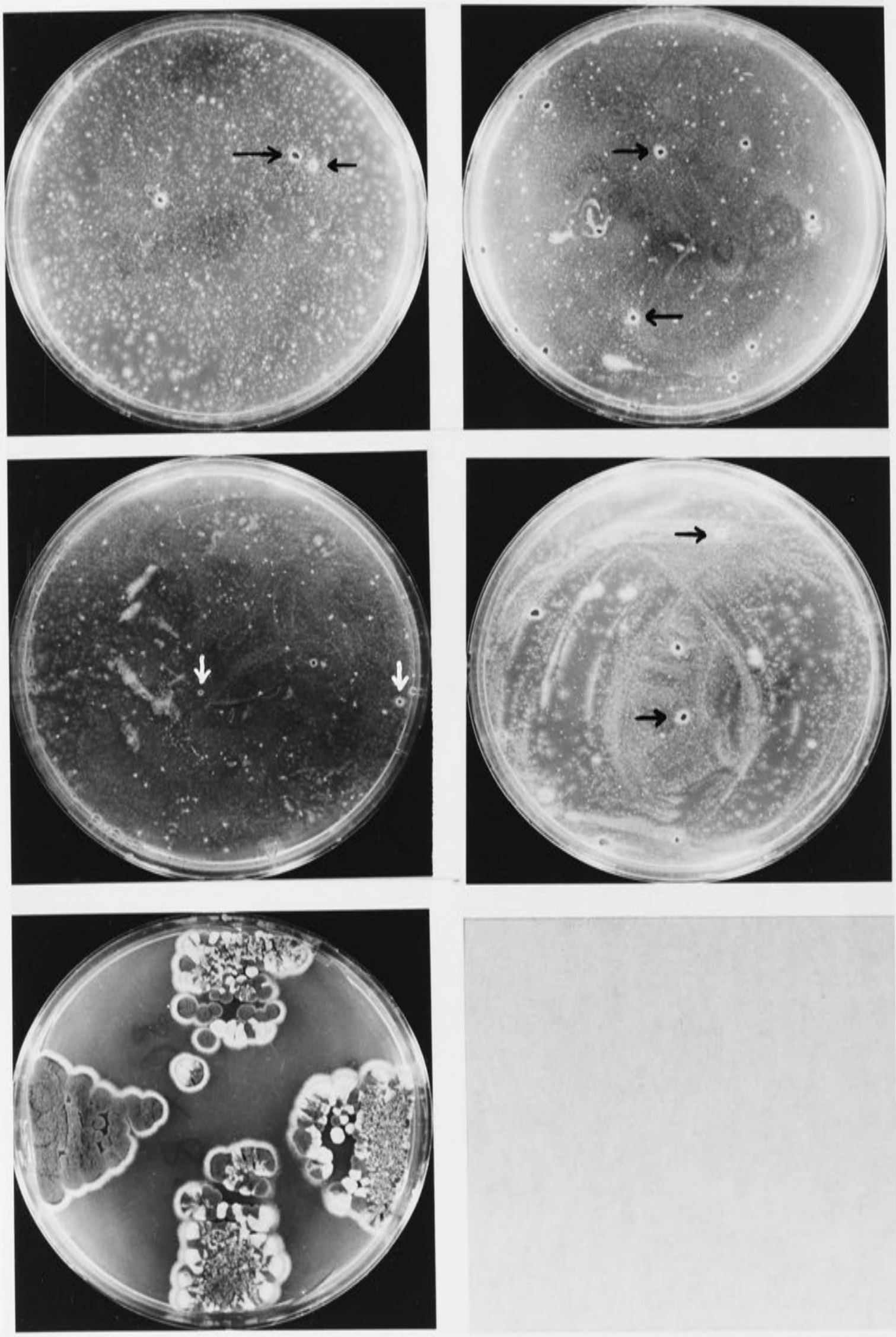


Plate 6: Cross of ureB6 x ureB3, detection of wild-type recombinants

Plate 6: <u>Cross of ureB6 x ureB3, detection of wild-type recombinants</u>

c	d
b	e
a	

Petri dish a: Growth medium: solid complete medium with sodium deoxycholate.
Colonies: sample of the ascospore suspension of each of the four cleistothecia plated in b,c,d,e.

Petri dishes b,c,d,e: Growth medium: solid -N minimal medium with vitamin solution, 3mM urea, sodium deoxycholate.
Colonies: total spores from one of four cleistothecia.

Black arrows indicate typical wild-type recombinants in dishes c,d,e. Petri dish b corresponds with the 'selfed' cleistothecium in petri dish a. The white arrows indicate pieces of cleistothecium 'shell' supporting local growth. Though it is not very clear in the photo, this 'debris-supported' growth is easily distinguished from that of wild-type recombinants.

Photographed after 3 days incubation at 37^{o}C.

cleistothecia of both parental types.

The plating system for the detection of recombinants was itself very promising. Ascospores were spread on solid -N minimal medium with 3mM urea and with sodium deoxycholate which restricts colonial growth. The background on this urea medium was very faint and on the rare occasions that wild-type recombinants were recovered from a cross, the recombinants were very easily recognised (Plate 6).

Several attempts were made to improve the yield of ascospores from the crosses. Crosses are usually set up on solid minimal medium in which nitrate is the nitrogen source and glucose is the carbon source. The petri dishes are taped up as shortage of oxygen promotes cleistothecium formation.

(i) To make the conditions as anaerobic as possible the petri dishes were filled practically to the rim with solid medium.

(ii) Some other good nitrogen sources were tried as alternatives to the traditional nitrate. These nitrogen sources were ammonium tartrate, urea, casamino acids and L-glutamate. Some crosses were also set up on L-glutamate as sole carbon and nitrogen source.

(iii) The ureB strains resulting from DEO treatment might have contained other mutations not directly related to urea utilization which affected fertility. Crosses were therefore usually set up between strains which had already been through a cross to wild-type. Thus in many instances at least, mutations affecting fertility should have been lost and the resultant backcrossed ureB strains should

Table 34: Crosses yielding recombinants

Strain in cross to ureB6	Total progeny* plated on 3mM urea $x10^{-3}$	Number of ureB+ recombinants	Recombination frequency %
ureB3 ureB22 ureB27 ureB29 ureB31	RECOMBINANTS RECOVERED BUT FREQUENCY NOT CALCULATED		
ureB2	386	8	0.0047
ureB20	221	13	0.0118
ureB24	75	17	0.0453
ureB25	26	4	0.0308
ureB28	58	3	0.0103
ureB31	113	13	0.0230
ureB33	79	6	0.0152
ureB34	101	4	0.0079

* Calculated by plating diluted ascospore suspension on complete medium.

Some of the numbers shown are 'pooled' from the separate platings of 2 or 3 cleistothecia.

have shown improved fertility.

None of these measures improved cleistothecium formation.

In total, at least 450 crosses were set up and about 120 of the ureB combinations were tried. Only crosses in which one of the parents was the strain ureB6 ever produced large healthy cleistothecia. Large cleistothecia usually contain a high number of spores and tend to contain crossed progeny more often than small cleistothecia. Thirteen crosses involving ureB6 were analysed and in each case wild-type recombinants were recovered amongst the progeny (Table 34). The recombination frequency in eight of these crosses was calculated and ranged from 0.0047-0.0453%.

The failure of the majority of ureB strains to produce large cleistothecia when crossed to each other is not understood. Nor is it obvious why ureB6 should alone be able to promote healthy cleistothecia when crossed to other ureB strains.

CHAPTER X

DISCUSSION

DISCUSSION

The following is a discussion of the results reported in Chapters III-IX.

1. Conductimetric Assay

Conductimetry proved to be a good means of assaying urease activity in crude extracts. Once the optimum conditions for assaying urease had been established, the assay was quick, each determination in duplicate took less than ten minutes, and the system was quite easy to use. The best attribute of the conductimetric assay is probably its great sensitivity - it proved to be reproducible even for very low activities. The urease activities of *ure* mutant strains could not have been reliably determined using the assay based on the Muftic method for ammonia determination.

Lawrence (1971) lists dehydratases, decarboxylases, esterases, kinases, phosphatases, sulphatases and amidases as enzyme groups suited to a conductimetric type of assay. Though the action of urease provides one of the most sensitive assays, it is likely that a conductimetric assay would prove to be an extremely useful system for measuring the activities of many other enzymes in crude extracts.

2. Apparent Lack of Induction of Urease Activity

Scazzocchio and Darlington (1968) observed a small rise in wild-type urease activity when cells were transferred from nitrate medium to medium containing uric

acid as the sole source of nitrogen but concluded that urease showed no marked induction. The present work supports their conclusion. Growth on urea or transfer from ammonium tartrate to urate or to allantoin medium, the latter two substances being responsible for the induction of other enzymes in the purine degradation pathway, did not produce higher urease activity than did growth on several other nitrogen sources or transfer to nitrogen-free medium.

The induction of the enzymes of the purine degradation pathway in Neurospora crassa follows a similar pattern to that in Aspergillus, (Reinert and Marzluf, 1975). In Neurospora too, urease does not appear to be induced.

Though urease is not induced noticeably during growth on any nitrogen source tested, it might be that urease is subject to induction by intracellularly produced urea. Urea is produced intracellularly through purine breakdown and arginine catabolism. If the urea resulting from purine breakdown were inducing urease it would be expected that a strain carrying a mutation which blocked the purine degradation pathway would have lowered urease activity. This is not the case. Strains lacking either xanthine dehydrogenase, urate oxidase, allantoinase or allantoicase activity do not have lower urease activity than wild-type during growth on nitrate (Scazzocchio and Darlington, 1968). During growth on nitrate, arginase activity, which is subject to arginine induction and nitrogen regulation, is very low (Bartnik *et al.*, 1977). Thus it seems unlikely that much urea would be produced

from arginine catabolism during growth on nitrate, though urease activity is quite high. Since arginase and the purine degradation enzymes (other than urease) are subject to strong nitrogen regulation, it seems unlikely that there would be any significant production of urea by these enzymes during growth on ammonia although there is significant urease present.

Though it is difficult to envisage endogenous induction by urea produced from the two most obvious sources, purine degradation and arginine catabolism, the possibility that urease is subject to induction by intracellularly produced urea cannot be excluded.

3. Nitrogen Regulation of Urease Activity

a. Extent of nitrogen regulation

Urease does not appear to be subject to such strong nitrogen regulation as some systems in Aspergillus, such as nitrate reduction, which have only basal levels of activity after growth on ammonia (Pateman and Kinghorn, 1977, review). However, as the urease activity of wild-type after growth on either ammonium tartrate or L-glutamine was about 60% lower than after transfer to nitrogen-free medium, there does appear to be some nitrogen regulation of urease activity.

b. The effector of nitrogen regulation

The term, 'effector', is used here to describe the physiological signal which results in nitrogen regulation of an activity. The effector might, for example, be

a high intracellular concentration of L-glutamine or ammonia or might be the amount of one of these substances bound to glutamine synthetase. As used here, 'effector' is not intended to carry any implication about the level of activity control at which nitrogen regulation acts.

When wild-type cells were grown up on a range of nitrogen sources and assayed for urease activity, growth on ammonium tartrate and on L-glutamine resulted in the lowest levels of activity. The activities after growth on either of these two nitrogen sources were very similar. The strains, meaA8 and gdhA1, which are ammonia derepressed for a number of systems in Aspergillus, were assayed for urease activity after growth on L-glutamine and after transfer from L-glutamate to ammonium tartrate.

There are two ways of determining the effect of a nitrogen source on a particular activity. Cells can be grown up with the nitrogen source in the growth medium, or, the cells can be grown up with some other nitrogen source and then transferred to medium containing the nitrogen source of interest. For example, to determine the wild-type level of urease activity with ammonium tartrate as the nitrogen source, wild-type cells may be grown up with ammonium tartrate as the nitrogen source. Alternatively, the cells may be grown up with another nitrogen source such as urea or L-glutamate, and then transferred to medium containing ammonium tartrate as the nitrogen source. The transfer method is particularly useful when a strain grows poorly with the nitrogen source of interest.

The strain, gdhA1 grows poorly with ammonium tartrate as the nitrogen source. Thus to determine the

effect of ammonium tartrate as the nitrogen source on the urease activities of gdhA1 and meaA8, these strains were grown up with L-glutamate as the nitrogen source and transferred to medium containing ammonium tartrate. To determine the effect of L-glutamine as the nitrogen source on the urease activities of gdhA1 and meaA8 it was not necessary to use the transfer method. Both meaA8 and gdhA1 grow well with L-glutamine as the nitrogen source.

Unlike that of wild-type, the urease activities of gdhA1 and meaA8 did not decrease after transfer from L-glutamate to ammonium tartrate. After growth on L-glutamine, however, wild-type, meaA8 and gdhA1 all had low urease activities. As the urease activites of meaA8 and gdhA1 did not decrease when these strains were transferred to medium containing ammonium tartrate, it is unlikely that the low activities of meaA8 and gdhA1 after growth on L-glutamine resulted from the catabolic production of ammonia from L-glutamine. Rather, this suggests that L-glutamine is closer to the effector of urease activity than is ammonia and that ammonia caused a low level of activity through the production of L-glutamine.

That growth on L-glutamate, which is converted to L-glutamine by glutamine synthetase, did not result in lowered urease activity is not inconsistent with this interpretation. Pateman (1969) observed that cells grown on a high concentration of L-glutamate (100mM) have high glutamine synthetase activity but that if a low concentration of a second nitrogen source, nitrate or ammonia (5mM) is added to the growth medium, glutamine synthetase activity is low. It was also observed that growth on

L-glutamate results in very low NAD-glutamate dehydrogenase activity, the only direct means of obtaining ammonia from L-glutamate. Pateman therefore proposed that glutamine synthetase is repressed by L-glutamine and that the high glutamine synthetase activity in L-glutamate-grown cells was not caused by glutamate induction but by L-glutamine derepression, on the basis that the level of ammonia in these cells would severely limit the synthesis of L-glutamine from L-glutamate.

The work of Cook and Antony (1978) supports the proposal of Pateman (1969) that cells grown on L-glutamate have a low intracellular concentration of L-glutamine. These authors report that while growth of cells on ammonia for 16-20h causes about a 7-fold increase in the intracellular L-glutamine concentration, growth on L-glutamate does not result in an increase.

The high urease activity of gdhA1 after transfer to ammonium tartrate medium also indicates that the low urease activity of wild-type cells grown on ammonia may be caused by the production of L-glutamine. gdhA1 has an obvious defect in the production of L-glutamine, as the strain carries a mutation in the structural gene for NADP-L-glutamate dehydrogenase and lacks that activity (Kinghorn and Pateman, 1973, 1975). It would therefore not be surprising that gdhA1 should be derepressed in the presence of ammonia if L-glutamine is closer to the real effector of nitrogen regulation of urease activity and if ammonia has its effect through the production of L-glutamine.

It is less clear why meaA8 should not accumulate

a high intracellular concentration of L-glutamine during growth on ammonia. It has been proposed (Arst and Page, 1973) that in addition to conferring resistance to high concentrations of methylammonia and decreased activity of the ammonia uptake system, mutation at the meaA locus results in defective bulk transport of ammonia. This suggests the possibility that the intracellular ammonia concentration in these cells is not high enough for the synthesis of sufficient L-glutamine to cause urease activity to be decreased. However, measurements of the intracellular ammonia concentrations of wild-type and meaA8 (Pateman *et al.*, 1973) under various growth conditions did not reveal any significant difference between the two strains. The basis of the ammonia derepression of meaA8 remains obscure.

c. Mode of nitrogen regulation

The increase in urease activity of wild-type cells transferred from L-glutamine or ammonium tartrate to nitrogen-free medium was prevented by cycloheximide and thus probably requires protein synthesis. A possible explanation is that L-glutamine, either present as the nitrogen source or produced during growth on ammonia, partially represses the synthesis of urease. Another possible explanation is that urease is constantly synthesised in the presence of L-glutamine but is inactivated either irreversibly or in such a way that protein synthesis is required to reverse the inactivation.

After growth on urea, transfer to ammonium tartrate or to L-glutamine resulted in a similar decrease in urease activity. The extent of this decrease was the same

both in the absence and presence of cycloheximide. This suggested that the decrease in activity after transfer to ammonia or to L-glutamine might result from urease synthesis being stopped. It was therefore necessary to find out the extent to which cessation of urease synthesis and protein turnover could decrease urease activity during the time of transfer, four hours. This was estimated by growing cells up with urea as the nitrogen source and transferring them to nitrogen-free medium with cycloheximide. This treatment indicated that cessation of maintenance synthesis and turnover resulted in a decrease in activity of only about 16%. Transfer from urea to ammonium tartrate or L-glutamine resulted in decreases of about 50%.

There is a possible objection to having used nitrogen starvation with cycloheximide to estimate the extent to which cessation of synthesis and turnover might decrease urease activity. It might be that urease activity would initially rise in response to nitrogen starvation before the cycloheximide became effective. This would result in the decrease in urease activity due to turnover appearing to be smaller than it really was. It is unlikely, however, that this occurred. Transfer of cells to carbon-free, and to carbon- and nitrogen-free medium, resulted in a decrease in urease activity which was largely prevented by the presence of cycloheximide. In the presence of cycloheximide these treatments did not result in a greater decrease in urease activity than did transfer to nitrogen-free medium plus cycloheximide.

It appears, therefore, that transfer to ammonium tartrate or to L-glutamine results in a decrease in urease

activity which cannot be solely accounted for by cessation of urease synthesis and turnover. As the decrease occurs both in the absence and presence of cycloheximide it appears to be independent of protein synthesis and therefore probably does not result from the action of a newly synthesized protease.

L-glutamine may partially repress urease synthesis but it seems that inhibition or inactivation of urease also occurs. While it appears that ammonia largely has its effect on urease activity through the production of L-glutamine it might be that ammonia which is a product of the urease reaction has itself an inhibition effect. The presence of 5mM ammonium tartrate (10mM NH_4^+) in the urease assay system, added prior to the addition of urea decreased the *in vitro* urease activity by about 25%. After growth on 5mM ammonium tartrate, the concentration used in growth medium in the present work, the intracellular ammonium concentration in wild-type is about 0.2mM (Pateman *et al.*, 1973). This suggests that inhibition by ammonia *in vivo* would be very slight. The addition of 20mM L-glutamine had no effect on *in vitro* urease activity.

The possibility that either ammonia or L-glutamine might decrease urease activity by tight binding to the urease subunits, as proposed to explain the inactivation of glutamine synthetase in C.utilis (Switzer, 1977), seems unlikely. Incubation of extract at 37^{o} for up to 135 min with L-glutamine or ammonium tartrate had no effect on *in vitro* urease activity. That ammonia per se probably has little direct effect on urease activity is also indicated by the high activity of gdhA1 after transfer

to ammonia medium. As gdhA1 has very low NADP-L-glutamate dehydrogenase activity and as this is the main route for ammonia assimilation it would be expected that after transfer to ammonia medium the intracellular ammonia concentration would be very high. Pateman *et al.* (1973) have shown that this is indeed the case. After transfer to ammonia medium gdhA1 has a 5-10-fold higher intracellular ammonia concentration than does wild-type. It is difficult to reconcile this with a strong inhibiting or inactivating role for ammonia.

d. Summary

Ammonia itself appears to have little, if any, effect on urease activity. It is likely that the low urease activities of wild-type, observed after growth on, or after transfer to, ammonium tartrate result from the production of a high intracellular concentration of L-glutamine.

A high intracellular concentration of L-glutamine appears to result in an inactivation of urease. There is no evidence to suggest that the inactivation involves a direct interaction between L-glutamine and urease - incubation of cell-free extract with L-glutamine did not affect urease activity. The decrease in urease activity after transfer to medium containing L-glutamine appears to be independent of protein synthesis. If the inactivation results from the action of a specific protease it seems that L-glutamine must mediate an activation of the protease rather than its synthesis.

The occurrence of inactivation makes it difficult to judge to what extent, if any, L-glutamine causes

urease synthesis to be repressed.

4. Regulation of Uptake Activity

Thiourea uptake activity varied with the nitrogen source and appeared to be under strong nitrogen regulation.

a. The effector of nitrogen regulation

Growth on ammonium tartrate resulted in lower thiourea uptake activity in wild-type than did growth on L-glutamine. However, the uptake activities of meaA8 after growth on ammonia and of gdhA1 after transfer from L-glutamate to ammonia, were high. Growth of meaA8 on L-glutamine and either growth on, or transfer to, L-glutamine of gdhA1 resulted in low uptake activities. These latter observations suggest that, as appears to be the case with urease activity, L-glutamine is closer to the real effector of uptake activity than is ammonia.

Unlike the situation with urease activity, growth with L-glutamate as the nitrogen source appeared to result in lower uptake activity than did growth with urea or L-alanine. This may not be a significant result because the uptake activity of gdhA1 after growth on L-glutamate was as high as wild-type uptake activity after growth on urea or L-alanine. If, however, the wild-type result is significant and growth on L-glutamate does result in a decrease in uptake activity compared to growth on urea or L-alanine, this would not be incompatible with the urease results. Uptake activity appears to be more sensitive to nitrogen regulation than is urease activity. It might be therefore, that while growth on L-glutamate

produces insufficient L-glutamine to affect urease activity, sufficient L-glutamine is produced to cause a decrease in uptake activity.

If L-glutamine is, or is closer to, the real effector of uptake activity than is ammonia, it is a little surprising that growth on ammonia should have caused a lower level of wild-type uptake activity than did L-glutamine. It might be that growth on ammonia results in a higher intracellular concentration of L-glutamine than growth on L-glutamine itself and, or, that ammonia, in addition to having an effect on uptake activity through the production of L-glutamine may cause some degree of inhibition.

b. Mode of nitrogen regulation

Transfer of cells from ammonium tartrate or L-glutamine to nitrogen-free medium resulted in an increase in uptake activity. This increase was prevented by cycloheximide and therefore appeared to require protein synthesis. This suggests that L-glutamine, either present as the nitrogen source or produced during growth on ammonia, may partially repress the synthesis of the uptake system. Alternatively, the uptake system may be constantly synthesised and inactivated either irreversibly or in such a way that protein synthesis is required to reverse the inactivation.

When wild-type cells were transferred from medium containing urea as the nitrogen source to medium containing either L-glutamine or ammonium tartrate, uptake activity decreased. The decrease after transfer to ammonium tartrate was greater than that after transfer to

L-glutamine. Decreases in uptake activity after transfer to either ammonia or L-glutamine occurred both in the absence and presence of cycloheximide.

To find out by how much uptake activity decreased through cessation of synthesis of the uptake system and protein turnover, cells were grown up with urea as the nitrogen source and transferred to nitrogen-free medium with cycloheximide. There is a possible objection to having used a nitrogen starvation treatment to estimate this effect. As discussed above with reference to urease, it might be that as nitrogen starvation produces high uptake activity the activity would rise before the cycloheximide became effective. This would result in the decrease in uptake activity due to cessation of synthesis of the system and turnover appearing smaller than it really was. It is unlikely, however, that this occurred. Transfer of cells to carbon- and nitrogen-free medium resulted in a large decrease in activity in the absence of cycloheximide. In the presence of cycloheximide this treatment resulted in a similar decrease, 36%, to that measured after nitrogen starvation in the presence of cycloheximide, 47%.

After transfer to ammonium tartrate in the presence of cycloheximide uptake activity decreased by about 85%, and in the absence of cycloheximide the decrease was about 90%. Since the presence of cycloheximide made little difference to the uptake activity it might be that after transfer to ammonium tartrate protein synthesis was stopped through the production of L-glutamine from ammonia. As the decrease in activity in the presence and absence of

cycloheximide was greater than the decrease attributable to cessation of protein synthesis and turnover it seems that inhibition or inactivation also occurred.

Transfer to L-glutamine resulted in a decrease in uptake activity of about 48%. In the presence of cycloheximide the decrease in activity was about 80%. This suggests that after transfer to L-glutamine the primary effect of L-glutamine is one of inhibition or inactivation. This is not inconsistent with the suggestion that after transfer to ammonia protein synthesis might have been stopped through the production of L-glutamine. It might be that:

(i) The intracellular concentration of L-glutamine is higher after transfer to ammonia than after transfer to L-glutamine itself. Possibly the rate of L-glutamine transport limits the intracellular L-glutamine concentration.

(ii) L-glutamine has its effect in nitrogen regulation through complexing with glutamine synthetase. The L-glutamine produced by glutamine synthetase after transfer to ammonia would therefore be more closely associated with glutamine synthetase than the L-glutamine taken up from the external environment.

These suggestions plus the possibility that ammonia itself may inhibit or inactivate uptake might also account for transfer to ammonia resulting in a greater decrease in uptake activity than transfer to L-glutamine.

The possibility of L-glutamine or ammonia inhibiting uptake activity was tested by adding various concentrations of these substances to the uptake assay

system. Both L-glutamine and ammonia did appear to strongly inhibit uptake activity when present in the medium in the assay system. The presence of ammonia in the system resulted in a greater decrease in activity than did the same concentration of L-glutamine.

As the increase in uptake activity following transfer from L-glutamine or ammonia to nitrogen-free medium is protein synthesis dependent, it is rather unlikely that inhibition is the only effect of L-glutamine and ammonia. However, ammonia being the stronger inhibitor may at least in part account for growth on, or transfer to, ammonia, resulting in a lower level of uptake activity than growth on, or transfer to, L-glutamine.

There are two results, however, which do not appear to be consistent with an inhibition role for ammonia - the uptake activity of meaA8 after growth with ammonia as the nitrogen source was as high as that of wild-type transferred from ammonia to nitrogen-free medium; the uptake activity of gdhA1 did not decrease after transfer from L-glutamate to ammonium tartrate. While these results, together with the activities of meaA8 and gdhA1 after growth on L-glutamine, indicate that L-glutamine is closer to the effector of nitrogen regulation than is ammonia, it is suprising that ammonia did not decrease the activities of these strains by inhibition.

It is particularly difficult to interpret this since these mutant strains, meaA8 and gdhA1, are not fully understood. The basis of the ammonia derepression of meaA8 is unclear and though the primary gene defect of gdhA1 is known, the basis for some of the properties of

gdhA1 are not understood. For example, high external concentrations of ammonia have a more toxic effect on the growth of gdhA1 than on the growth of wild-type.

The thiourea uptake activity of meaA8 after growth on ammonia and the activity of gdhA1 after transfer to ammonia, are both high compared to wild-type. These activities of meaA8 and gdhA1, however, are only about 75% of the uptake activities of wild-type and meaA8 after transfer from urea to nitrogen-free medium. With ammonia as the nitrogen source, therefore, something prevents the activities of meaA8 and gdhA1 from reaching the highest possible levels. It seems reasonable to suppose that this might be ammonia inhibition.

Even if this is the case, however, it is difficult to understand why the activity of gdhA1 did not decrease at all after transfer from L-glutamate to ammonium tartrate. It seems that during growth on L-glutamate some factor, or factors, keeps the level of gdhA1 activity at about 75% of the highest wild-type activities observed. These factors might be the accumulation of sufficient L-glutamine to have some effect on uptake activity and perhaps there might be some inhibition by the ammonia produced from the hydrolysis of intracellularly produced urea. It might be that on subsequent transfer to ammonia, the effect of ammonia inhibition is no greater than the cumulative effects of L-glutamine and intracellularly produced ammonia.

5. Role of L-glutamine in Other Fungal Systems

In Aspergillus, Hynes (1974) reported that the

activities of acetamidase, benzamidase, formamidase, histidase and extracellular protease were repressed by L-glutamine and L-glutamate in addition to ammonia and that growth on L-glutamine resulted in a low level of urate oxidase activity. A strain carrying a mutation in gdhA was ammonia derepressed for the three amidases, histidase and extracellular protease but was sensitive to repression by L-glutamine and L-glutamate. The effect of L-glutamine was generally stronger than that of L-glutamate.

Cook and Antony (1978, 1980) measured the intracellular concentrations of ammonia and L-glutamine in Aspergillus cells grown under various conditions and concluded that the activities of the ammonia and acidic amino acid uptake systems reflected the intracellular concentration of L-glutamine, not ammonia. While a high intracellular concentration of L-glutamine was probably responsible for repressing the synthesis of both systems, the preformed ammonia transport system was sensitive to inhibition by intracellular L-glutamine and L-asparagine and the preformed acidic amino acid uptake system was inhibited by intracellular ammonia. These authors suggest that L-glutamine and glutamine synthetase may have a more important role in the regulation of some aspects of nitrogen metabolism than has previously been supposed.

In Aspergillus it has been proposed that glutamine represses the synthesis of glutamine synthetase (Pateman, 1969) and in the yeast, Candida utilis this enzyme appears to be regulated by glutamine, not ammonia (Ferguson and Sims, 1974).

Dantzig *et al.* (1978) propose that a nitrogen

metabolite other than ammonia is responsible for nitrogen repression of nitrate reductase in Neurospora crassa. Further studies by Premakumar *et al.* (1979) indicate that L-glutamine, not ammonia, prevents induction by nitrate. This lack of induction was not due to an inhibition of nitrate uptake, and appeared to be mediated by L-glutamine itself, not a metabolite of L-glutamine, as histidine, pyrimidine, and arginine requiring auxotrophs were all affected.

Dunn-Coleman *et al.* (1979) have also studied the regulation of nitrate reductase in Neurospora crassa. L-glutamine but not ammonia could repress nitrate reductase in a strain with defective glutamine synthetase activity. These authors present evidence that under nitrate reductase repressing conditions (growth on ammonium nitrate) glutamine synthetase is predominantly in an octameric form, while during growth on the inducer nitrate, glutamine synthetase is distributed about equally between the tetrameric and octameric forms. The authors suggest that the octameric form may repress the synthesis of the product of the nit-2 locus. This locus appears to be analogous to the areA locus in Aspergillus.

In addition, therefore, to urease and the urea uptake system in Aspergillus, results from several other systems both in Aspergillus and in other fungi indicate that L-glutamine may be, or may be closer to the real effector of nitrogen regulation than is ammonia. As has been noted by some of the authors above, this suggests a possible involvement of glutamine synthetase in fungal nitrogen regulation, analogous to the situation in enteric

bacteria.

6. Effect of the Carbon Source on Urease Activity

Irrespective of the nitrogen source, urease activity was higher with D-glucose as the carbon source than with D-glycerol, acetate, or L-arabinose. With L-proline or urea as the nitrogen source, the urease activities after growth with D-glucose and acetate, catabolite repressing carbon sources, were higher than after growth with D-glycerol or L-arabinose, catabolite derepressing carbon sources. After transfer of wild-type cells to carbon-free medium from medium containing urea and D-glucose, urease activity approximately halved. As this decrease was prevented by cycloheximide it appeared to be dependent on protein synthesis.

It is rather surprising that urease activity is influenced by the carbon status of the cell since its activity has no obvious connection with carbon metabolism and the urease reaction is not energy-requiring. The protein synthesis dependent decrease in urease activity after transfer to carbon-free medium might result from the activity of a specific protease whose synthesis is carbon catabolite repressed. Alternatively it may be that urease is unspecifically inhibited or inactivated through newly synthesised enzymes causing changes in the intracellular environment, perhaps resulting in the accumulation of some inhibiting metabolite.

7. Effect of Carbon Starvation on Uptake Activity

When cells grown on urea and glucose were

transferred to carbon-free medium, with urea as the nitrogen source, the uptake activity fell by about 95%. This fall in activity was not affected by the presence of cycloheximide and thus was probably not dependent on protein synthesis. The fall in activity might therefore have resulted at least in part from the decrease in available energy within the cell accompanying carbon starvation.

The activity of cells transferred to medium lacking both a carbon and nitrogen source fell by about the same amount as did that of cells transferred to medium lacking only a carbon source. The decrease in uptake activity after transfer to medium lacking both a carbon and a nitrogen source, however, appeared to be largely dependent on protein synthesis - the activity of cells transferred to medium without a carbon and a nitrogen source but with cycloheximide fell by only about 36%. This is similar to the amount by which uptake activity decreased after transfer from medium containing urea and glucose to nitrogen-free medium with cycloheximide, 47%. These decreases, 36% and 47%, probably reflect the effect of cessation of synthesis of the uptake system and protein turnover on uptake activity.

The difference between the measurements made after transfer to carbon- and nitrogen-free medium in the absence and presence of cycloheximide is statistically significant at the 01% level. There therefore appears to be a genuine difference in the protein synthesis requirements for the decreases in uptake activity measured after transfer to carbon-free medium with urea as the nitrogen source, and after transfer to carbon- and nitrogen-free medium.

It might be that after transfer to carbon-free medium with urea as the nitrogen source, inhibition of the uptake system by ammonia produced from urea by urease obscured the effect of carbon starvation. The activity measured is close to the limit of resolution of the assay. It might be that the protein synthesis dependent fall in uptake activity observed after transfer to carbon- and nitrogen-free medium more accurately indicates the effect of carbon starvation on uptake activity.

8. The Effect of L-Histidine

a. Effect of L-histidine on urease activity

The urease activity of wild-type cells grown on L-histidine, a poor nitrogen source, was extremely low. When wild-type was grown on L-histidine together with a second nitrogen source, ammonium tartrate, L-glutamate, L-proline or urea, the urease activities were reduced to the very low level observed after growth on L-histidine as the sole nitrogen source. This occurred whether the carbon source was catabolite repressing, D-glucose or acetate, or catabolite derepressing, D-glycerol or L-arabinose. The histidine effect therefore appeared to occur regardless of the second nitrogen source and the carbon source. The urease activities of the strains meaA8, tamA^{r}119 and tamA^{r}105 were, like that of wild-type, greatly reduced after growth on urea plus L-histidine compared to the activities measured after growth on urea as the sole source of nitrogen.

In addition to its effect on urease activity the presence of L-histidine in the growth medium reduced the

growth weight of cells on urea and L-glutamate, but growth on L-proline and ammonium tartrate was largely unaffected. These results for the growth of cells in liquid medium concur with the observations of Polkinghorne and Hynes (1975) for growth on solid medium. In Neurospora a histidine effect was observed during an investigation of the control of nitrate reductase (Premakumar *et al.*, 1979). In this organism the presence of 10mM L-histidine in the growth medium decreased the growth weight of cells on ammonia by about half. It was also observed that the presence of L-cysteine completely prevented growth on ammonia medium, though none of the other 18 amino acids tested had this type of effect. Of the twenty amino acids tested, only the presence of L-histidine, L-cysteine or L-glutamine prevented the induction of nitrate reductase by nitrate. The authors concluded that the effects of L-histidine and L-cysteine were non-specific.

In Aspergillus it has been shown that it is probably L-histidine itself and not a related metabolite which is responsible for decreasing the utilization of other nitrogen sources (Polkinhorne and Hynes, 1975). Also, though L-histidine appears to have various effects, it does not affect all enzyme systems. L-histidine does not prevent the induction of urate oxidase by urate.

The drastic effect on urease activity suggests that L-histidine may prevent urease synthesis. Polkinghorne and Hynes (1975) noted that cycloheximide prevented the rise in urease activity when cells were transferred from medium with L-histidine to medium lacking L-histidine. However, L-histidine may not prevent urease synthesis

(E.H. Creaser, personal communication). After cells were grown on L-proline with and without L-histidine it was in both cases possible to obtain a protein band on a gradient polyacrylamide gel which appeared to correspond to urease. The band from the cells grown on L-proline alone stained for urease activity, but the protein band from the cells grown on L-proline plus L-histidine was not catalytically active. While it might be that a second protein runs with urease and that this second protein was the catalytically inactive band, this result suggests that the effect of L-histidine may be one of inactivation.

In the present work L-histidine did not appear to cause sufficient inhibition of urease *in vitro* to account for the very low activities measured after growth of cells in medium containing L-histidine. Incubation of a wild-type extract for up to 135 min with 2mM L-histidine did not cause any decrease in urease activity. It might be that in order to have its full effect, L-histidine must be present during urease synthesis.

It is interesting that both the enzymes on which L-histidine has been observed to have a rather severe effect, urease and nitrate reductase, are metalloenzymes. As urate oxidase is probably also a metalloenzyme, however, L-histidine clearly does not affect all enzymes with metal cofactors.

b. Effect of L-histidine on uptake activity

The presence of L-histidine in the growth medium did not affect the thiourea uptake activities of cells grown on L-proline, ammonium tartrate or L-glutamate compared to that with these nitrogen sources without L-histidine

in the medium. The presence of L-histidine did not decrease the already very low uptake activity which results from growth with ammonium tartrate as a nitrogen source. The uptake activities of wild-type and of meaA8 grown on urea plus L-histidine were about half of those measured after growth of these strains with urea as the sole source of nitrogen. The uptake activities of the tamA^{r} strains which are low after growth on urea were not decreased further by the presence of L-histidine in the urea growth medium.

External L-histidine inhibits the activity of the acidic amino acid permease as measured by the uptake of ^{14}C-L-glutamate (Robinson *et al.*, 1973; Polkinghorne and Hynes, 1975). Thiourea uptake of wild-type cells was also strongly inhibited by external L-histidine. An extracellular concentration of 20μM L-histidine in the uptake assay caused a lower level of uptake than that measured after growth on urea with 10mM L-histidine.

This situation is a little puzzling. The fact that the presence of L-histidine during growth on urea decreased uptake while uptake after growth on L-proline and on L-glutamate was unaffected suggests that the decrease in uptake activity after growth on urea plus L-histidine was caused, not directly by a L-histidine effect on uptake, but rather, through the L-histidine effect on urease activity. As L-histidine would cause a severe decrease in urease activity, the decrease in uptake activity might result through inhibition of the uptake system by the urea accumulated within the cell. However, external L-histidine strongly inhibits uptake. This implies that either:

(i) though L-histidine inhibits while present externally it does not inhibit internally; or

(ii) L-histidine does inhibit uptake internally but during growth on L-proline or L-glutamate, the amount of L-histidine taken up by the cell is decreased such that sufficient L-histidine is present within the cell for its effect on urease activity but this is insufficient to inhibit thiourea uptake.

9. Properties of ure Mutants

The ure mutants are deficient in the utilization of urea and have no other known metabolic abnormality. Genetic analysis of the ure strains indicated that a deficiency affecting only urea utilization could result from mutation in any one of four loci.

It was previously shown that amongst ninety strains isolated for resistance to thiourea, all but two mapped at the ureA locus. The two which did not mapped at the areA locus (Dunn and Pateman, unpublished). In the present work, a further twelve thiourea resistant strains were shown by their close linkage to ureB to probably carry a mutation in ureA.

It would be expected that amongst mutations conferring resistance to thiourea, a frequent class would be mutations in structural genes for the protein components of the urea uptake system. Since the great majority of mutations conferring resistance to thiourea map at one locus, ureA, it is likely that ureA is a structural gene for a protein component of the urea transport system. Further, since only one common class of thiourea resistant mutants

has been found, it is probable that only a single specific protein is necessary for urea transport. It appears, therefore, that ureA is the structural gene for the urea-specific transport protein.

The enzyme responsible for urea degradation in Aspergillus appears to be a urease (E.C.3.5.1.5.), as discussed in Chapter III.1.b. It is likely that jack bean urease is a hexamer with a single type of subunit (Fishbein, 1977; Dixon *et al.*, 1980d). Aspergillus urease has recently been purified and is also a hexamer, with a single type of subunit (E.H. Creaser, personal communication). Mutations in either ureB, ureC or ureD result in deficient urease activity.

Since urease is not subject to strong nitrogen regulation - urease activity is quite high even after growth on ammonia or glutamine - it may be that urease is not just a catabolic enzyme. Intracellular urea accumulation may have a toxic effect on the cell and some urease activity might therefore be essential. However, such a toxic effect is not apparent during, at least, vegetative growth. ureB, ureC and ureD strains have very low urease activity but look healthy and grow like wild-type on nitrogen sources other than urea. Since mutation in a single gene can result in inability to utilize urea as a nitrogen source, other enzymes cannot be responsible for degrading endogenous urea in these strains. Further, thiourea uptake assays with ureB, ureC and ureD strains (Table 24) indicate that it is likely that they do accumulate urea as those strains with the lowest urease activities also have lowered uptake activity, presumably

reflecting urea inhibition of the uptake system. Thus no toxic effect is apparent in strains which do accumulate urea. In addition, wild-type and ure strains are unharmed by external urea concentrations > 100 mM. This was previously specifically investigated. Had the ure mutants been especially sensitive to urea, sensitivity to high urea concentrations could have been used as a selection procedure for obtaining ureaseless mutants.

In Aspergillus, three loci appear to each have a role concerning urease activity. In Neurospora, four loci are known, mutation in any of which may result in deficient urease activity and inability to utilize urea as a sole source of nitrogen (Kolmark, 1969; Haysman and Branch Howe, 1971). Revertants of strains carrying a mutation at one of each of the four loci have been isolated. The urease activity of at least one of the revertants at each locus was less heat stable than that of wild-type, suggesting that each locus was a structural gene for urease (Benson and Branch Howe, 1978). In addition, intragenic complementation was observed between alleles of each of the four loci. Benson and Branch Howe (1978) suggest that each of the four loci represents a structural gene for a subunit of urease. It seems reasonable to suppose, however, that since Aspergillus urease and jack bean urease contain only one type of subunit, that Neurospora urease is also homomeric.

If this is the case it is extremely unlikely that all four loci code for urease subunits as it would not be expected that mutation in only one could result in undetectable urease activity and inability to utilize urea as the sole source of nitrogen.

Jack bean urease contains nickel ions (Dixon *et al.*, 1975). It seems that there are two nickel ions per subunit of the hexamer, that nickel is essential for enzymatic activity and that the nickel ions are at the active site (Dixon *et al.*, 1980 a,b,c). In the present work, the addition of nickel ions increased the urease activity and improved the growth on solid urea medium of the strain, ureD9 which was otherwise unable to utilize urea. This suggested that of the ure loci, at least ureD might have some role concerning nickel and urease.

The situation with urease in Aspergillus, of having more than one gene apparently specifying only one enzyme activity is not uncommon in fungi. Perhaps the best example in Aspergillus is that of nitrate reductase. At least eighteen genes can mutate resulting in a decrease in the utilization of nitrate. It appears that several of these loci, the cnx genes, are involved in various ways in the synthesis of a molybdenum cofactor essential for the activity of both nitrate reductase and xanthine dehydrogenases I and II. The charactertistics of the ureD9 mutant with respect to nickel and urease activity are analogous to those of the cnxE mutants with respect to molybdenum and the activities of nitrate reductase and xanthine dehydrogenase.

Mutations at the cnxE locus result in loss of the

activities of nitrate reductase and xanthine dehydrogenase. The addition of molybdenum to the growth medium results in a significant increase in the two enzyme activities and in growth on nitrate and purines of the cnxE mutants. Although only this one class of cnx mutant actually responds to the addition of molybdenum there are another five, possibly six, cnx loci, cnxA, B, C, F, G and H, all of which appear to be involved in the synthesis or function of the cofactor. The loci, cnxA, B and C are closely linked and the other four loci are scattered about the genome.

The evidence suggests that the cofactor common to nitrate reductase, and xanthine dehydrogenases I and II consists of a polypeptide component coded for by the cnxH gene and a molybdenum-ligand group coded for by at least cnxE and F. Nothing is so far known of the roles of the products of cnxA, B, C and G in the synthesis of the cofactor (Pateman and Kinghorn, 1977, review; Cove, 1979, review).

In analogy with the cnx mutants it seems likely that ureD is responsible for the production or incorporation of a nickel cofactor essential for urease activity. Though the ureB and ureC mutant strains failed to respond to the addition of nickel, in analogy with most of the cnx mutants and their failure to respond to the addition of molybdenum, the possibility of one of these loci having some role concerning nickel cannot be excluded. It seems reasonable to suppose that either ureB or ureC is the structural gene for the core, or main, polypeptide of urease. However, since four loci are known in Neurospora, which each appear to have a role concerning urease activity, it is possible that in Aspergillus there are also at least four loci involved and

that the structural gene for the core polypeptide, the major urease subunit, has not yet been discovered.

From the present evidence, ureB seems the most likely of the ure loci to be the structural gene for the core polypeptide. The urease activity of a ureB2 strain carrying a reversion mutation within ureB was less heat stable than that of wild-type. This suggests that ureB may be a structural gene for the core polypeptide of urease. However, the decreased heat stability of the revertant is by no means certain evidence for this. The heat stability studies basically indicate that the ureB product functions in specifying a component of the enzyme. The decreased heat stability of the revertant does not necessarily imply that the ureB product is part of the enzyme. If, for example, ureB coded for an enzyme involved in the synthesis of a ligand-nickel cofactor, an imperfectly synthesised cofactor might result in decreased heat stability.

Only one rather doubtful example of intragenic complementation was observed amongst all the pairs of ureB alleles tested. As Aspergillus urease is a homomeric hexamer (E.H. Creaser, personal communication), it seems a likely system in which to find intragenic complementation and the doubtful result may well be true. Even if there were no intragenic complementation between alleles at the ureB locus it would not detract from the possibility of ureB being a structural gene for urease. Though intragenic complementation seems to occur in most allelic series it probably does not occur in all (Fincham *et al.*, 1979). In any case, the definite existence of intragenic

complementation would not be strong evidence for ureB coding for the core polypeptide. In the cnx situation, intragenic complementation has been observed between alleles of the cnxH locus, which is believed to code for the molybdenum carrier protein.

The largest intragenic recombination frequency observed between alleles of ureB was 0.045%. This is quite low in comparison to, for example, the structural gene for acetamidase in which recombination frequencies of 0.3% are known (Hynes, 1978). However, it is impossible to judge how much of ureB is represented by the observed intragenic recombination frequencies.

The close linkage of ureA and ureB suggests that ureB might be the structural gene for the core polypeptide. Presumably the linkage has some significance. It might reflect coordinate regulation of the uptake system and urease (this possibility is discussed in a later section). If there were coordinate expression it seems reasonable to suppose that it would be the synthesis of the core polypeptide of urease that would be regulated in step with the uptake system. Expression of the genes for the synthesis or incorporation of the nickel cofactor might be constitutive as are the cnx genes (Cove, 1979).

Alternatively, the reason for the close linkage may be evolutionary. A urea uptake system would be of no use to the organism without urease. The two genes might therefore be inherited as a unit. This type of explanation has been suggested for the close linkage of niaD and niiA, the structural genes for nitrate and nitrite reductases (Cove, 1979).

A possible overall interpretation of the results presented here is that ureA is the structural gene for the urea specific transport protein; ureB is the structural gene for the core polypeptide of urease; ureD is involved in the synthesis or incorporation of a nickel cofactor analogous to cnxE; ureC is also involved in the synthesis or incorporation of the nickel cofactor analogous to the cnx loci which do not respond to the addition of molybdenum.

To further investigate the roles of the ure loci, it would be desirable to isolate more ureaseless mutants both to increase the number of ureC and ureD alleles, and to obtain alleles of any other ure locus which might exist. It is unfortunate that no successful means of selecting for ureaseless mutants was found. Study of the properties of strains carrying a reversion mutation in ureC or ureD would show whether these loci function in specifying a component of urease. If urease is subject to endogenous induction it might be that ureC specifies a positive-acting regulatory protein. It might therefore be possible to isolate ureC revertants with higher urease activity than wild-type.

Immunological screening of mutants for urease protein is not at present possible because no antibodies are available (Chapter X.13). If it were possible to clone the ureB gene, sequencing of urease and the gene would demonstrate whether or not ureB is the structural gene for urease. This is not at present feasible (Chapter X.13).

10. Infertility of Crosses Between ureB Alleles

When ureB alleles were crossed together only those crosses in which ureB6 was one of the parents produced large healthy cleistothecia - most crosses between pairs of ureB alleles were infertile. Crosses between ureB strains and wild-type and between a ureB strain and a strain carrying a mutation in a second ure locus showed the normal fertility. It is difficult to interpret the failure of the ureB alleles to cross with each other. If urease had some role concerning reproduction the infertile crosses might be explicable. It might be that urea interferes with some stage of sexual reproduction and that in crosses involving a ureB allele and a strain carrying a mutation in a second ure locus there is sufficient urease activity through complementation to prevent this. The ureB strain which did cross well, ureB6, does not have any more urease activity *in vitro* than the other ureB alleles. However, this strain was also one of the two which complemented intragenically and it might be that ureB6 complements with other ureB alleles at least sufficiently to prevent urea interfering with reproduction. Thus, though urea has no apparent toxic effect during vegetative growth, some urease activity may be necessary for sufficient sexual reproduction.

Infertility of crosses between alleles has been previously reported. Only some combinations of alleles at both the adE and adF loci of Aspergillus were fertile (Pritchard, 1955; Calef, 1957).

11. Failure to Isolate ureA ureB Deletions

The genes for nitrate and nitrite reductases are closely linked with a recombination frequency of 2% (Cove, 1979, review). One percent of spontaneous chlorate resistant mutants result from a deletion running through both genes (Tomsett and Cove, 1979). It is therefore surprising, especially as ureA and ureB appear to be more closely linked than niaD and niiA, having a recombination frequency of 0.5%, that none of the 2031 spontaneous thiourea resistant mutants tested resulted from a deletion which extended into ureB.

It is also surprising that no ureA ureB deletion resulted from treatment with DEO. This mutagen has been successfully employed in the production of deletions in the amdS region in Aspergillus (Hynes, 1978). In Neurospora, in the ad-3 region, as high as 42% of mutations induced by DEO were multilocus deletions (Ong and de Serres, 1975). The amount by which DEO increased the mutation rate in the present work was estimated by comparing the number of thiourea resistant mutants arising spontaneously with those arising from DEO-treated cells, after each mutation experiment. The DEO-induced increase in the mutation rate was usually 2-10-fold. Taking the lower estimate of 2-fold, by analogy with the situation with the ad-3 region in Neurospora, 407 of the 1937 thiourea resistant mutants isolated after DEO treatment should have resulted from a multilocus deletion.

The most likely explanation of the lack of ureA ureB deletions is that there is a region between the two genes with an essential function. The lowest

recombination frequency measured for ureA and ureB was 0.31%. The largest intragenic recombination frequency observed between ureB alleles was 0.045%. While it is impossible to judge how much of ureB this frequency represents, it might be that there is sufficient space for another gene between the two ure loci.

Should another gene exist between the two ure loci it might have a function essential to the organism such as an involvement in cell division. Alternatively the gene product might only be essential for growth on nitrate or on glucose as in all experiments for the isolation of thiourea resistant mutations these served as the nitrogen and carbon sources. As so many genes are already known which have a role in nitrate utilization the existence of another is perhaps unlikely.

12. Comparison of the Control of Urease and Urea Uptake Activities

The activities of urease and the urea uptake system appear to be controlled largely by the same metabolic factors:

(i) The activities of both urease and urea uptake largely follow the same pattern after growth on various nitrogen sources with glucose as the carbon source. The level of uptake activity with the various nitrogen sources might, therefore, directly reflect the amount of urease activity. The lower urease activities might result in an accumulation of intracellular urea. This does not appear to be the case, however. The high uptake activities of some of the ure strains indicates that even a very low level of urease

activity is sufficient to prevent inhibition by intracellular urea.

(ii) For both systems, the effector of nitrogen regulation appears to be, or to be closer to, L-glutamine than ammonia.

(iii) The increases in the activities of both systems after transfer to nitrogen-free medium require protein synthesis implying that when the intracellular concentration of L-glutamine is high the synthesis of both systems might be reduced or both systems inactivated either irreversibly or in some way which requires protein synthesis to reverse the inactivation. Alternatively, one system might be inactivated and the synthesis of the other reduced.

(iv) It seems likely that L-histidine has a more direct effect on urease activity than it has on uptake activity. The uptake activities after growth on L-proline or L-glutamate were not affected by the presence of L-histidine in the growth medium, though urease activity was extremely low. This is not inconsistent with the proposal that the uptake activities of ure strains which have very low urease activity were decreased through the accumulation of urea produced intracellularly - the enzymes of the direct purine degradation pathway are subject to nitrogen regulation (Scazzocchio and Darlington, 1968) and the uptake activities of the ure strains were assayed after transfer to nitrogen-free conditions. It seems likely that more urea would be produced under these conditions than during growth on L-proline or L-glutamate. If the effect of L-histidine on urease and nitrate reductase did involve an

interaction with their metal cofactors, the lack of inhibition by intracellularly produced urea after growth of cells in the presence of L-histidine might be further explained. As the molybdenum cofactor is common to both nitrate reductase and xanthine dehydrogenase the latter enzyme might also be affected and the production of urea by the pruine degradation pathway greatly reduced.

(v) Urease appears to be subject to protein synthesis independent inactivation after transfer to medium containing L-glutamine or ammonia. Uptake activity is also decreased by some protein synthesis independent means under these conditions but this may be inhibition.

(vi) The activities of both systems decrease during carbon starvation and the decreases appear to require protein synthesis. Possibly both systems are inactivated by a newly synthesised protease.

Inactivation appears to be important in the regulation of both systems. This contributes further to the observation of Switzer (1977) that inactivation is a generally important and perhaps underestimated level of control in microorganisms. However, the occurrence of inactivation makes it more difficult to judge to what extent the synthesis of urease and the uptake system is regulated, or to attempt to evaluate the likelihood of coordinate expression of the two linked genes, ureA and ureB. If the failure to isolate deletions of this region does, as suggested, indicate the existence of a probably unrelated gene between ureA and ureB it is perhaps unlikely that ureA and ureB would be coordinately expressed.

Only one ureA strain was assayed for urease and

uptake activities but several ureB strains were assayed for both. Considerable variation was observed in the uptake activities of these strains and this appeared to correspond to the amount of urease activity. However, this variation also occurred between alleles at the ureC and ureD loci. The variation in the activities of the ureB strains did not, therefore, appear to indicate polarity but rather that the lower urease activities resulted in lower uptake through the accumulation of intracellular urea.

13. Limitations for Further Investigation

Urea utilization in Aspergillus does not at present seem such a good system for investigating gene regulation at the molecular level as it first appeared for the following reasons:

Since attempts to isolate deletions in the ureA ureB region were unsuccessful and since ureB strains do not cross well, a map of the region could not be produced. If the failure to isolate deletions does as suggested indicate the existence of an unrelated gene between ureA and ureB the regulation of ureA and ureB may not be as interesting as was first supposed. Certainly it seems unlikely that their regulation would be based on any operon-like structure. No suitable deletion strain was found which could be used to isolate a probe for ureB. Attempts (by others) to identify urease specific mRNA by growing up wild-type and one of the ureB strains which had a low reversion frequency, and fractionating their mRNA on gels, was unsuccessful.

Aspergillus urease proved to be a much more difficult enzyme to purify than anticipated. The cell makes very little urease protein and some of its properties are different to those of other ureases (E.H. Creaser, personal communication). Since Aspergillus makes very little urease protein, there may also be very little urease mRNA. To further investigate the control of urease activity, it would be desirable to follow mRNA production under various growth conditions. However, since the cell may not make much urease and since urease levels do not vary over a wide range under various growth conditions, this would probably be difficult. Though it had been previously found that urease activity was not subject to such strong nitrogen regulation as other systems, it was thought that once a clean efficient assay was developed, it might be found that growth on ammonia or glutamine did result in very low levels of urease activity. This did not turn out to be the case, and, further, it is unlikely that the extent of the variations in urease activity that do exist result solely from control at the level of protein synthesis.

Investigation of the metabolic regulation of urease activity did not indicate a means of specifically increasing or decreasing urease activity which could be successfully used to isolate a probe in place of a deletion strain. Attempts (by others) to identify urease mRNA by growing wild-type with glutamate and with glutamate plus histidine were unsuccessful.

When injected with crude extract or even partially purified Aspergillus urease, rabbits did not make

antibodies against urease. Urease-deficient mutants cannot therefore be screened for urease protein using antibodies.

It is doubtful if further work with this system is worthwhile until means of making Aspergillus produce more urease and of isolating a urease probe are available. Unfortunately no urease gene is at present available from another organism. Most fungal cloning work has been done with the S. cerevisiae genes but this organism does not have urease. In any case, since Aspergillus urease has different properties to other ureases, a gene from another source might not be sufficiently homologous to be useful. Even if cloning of ureA and ureB were successful the infertility of ureB crosses would continue to limit a thorough investigation of the genetics of the system.

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