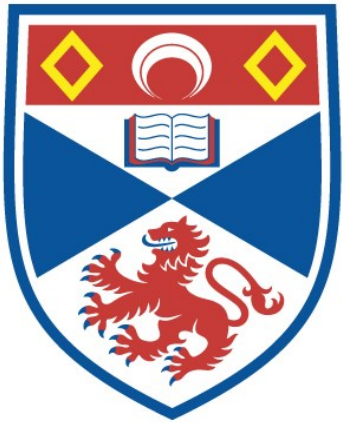
STUDIES ON AMMONIUM ASSIMILATION BY 'SACCHAROMYCES CEREVISIAE'

Andrew John Racher

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Studies on Ammonium Assimilation by *Saccharomyces cerevisiae*

Andrew John Racher

Submitted for the Degree of Doctor of Philosophy,

University of St. Andrews, March, 1988.

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Abstract

Saccharomyces cerevisiae can assimilate ammonium by NADP-GDH or by GS-GOGAT. The aim of this project was to improve the efficiency of ammonium assimilation, and therefore substrate utilisation, of *S. cerevisiae* by elimination of the energy inefficient pathway (GS-GOGAT).

GOGAT$^-$ mutants were isolated from a GDH$^-$ parent strain by their inability to use ammonium as sole nitrogen source. Two structural gene mutants were identified, one in each of the two structural genes encoding GOGAT.

Constructs with different combinations of GDH$^-$ and GOGAT$^-$ mutations and corresponding wild type alleles were made, and their growth studied in medium supplemented with different levels of ammonium. The growth properties (as final culture density and growth rate) of GOGAT$^-$ and GOGAT$^+$ strains transformed with the *GDH1* gene, and grown with excess ammonium were very similar. It was concluded that, under the conditions used in this study, the loss of GOGAT does not improve the growth properties of the strain. Non-transformed constructs were grown with excess and limiting ammonium. Growth properties of the GDH$^-$ and GOGAT$^-$ strains suggest that GS-GOGAT functions in ammonium assimilation at very low ammonium levels. This conclusion needs further investigation because the GDH$^+$ GOGAT$^-$ construct had lower NADP-GDH activity than the wild type.

The physiology of ammonium assimilation by two industrial strains was compared to that of a laboratory wild type at different ammonium concentrations using shake-flask culture. All three strains possessed the three activities in MM+20mM NH_4^+, and the profiles of appearance/disappearance of activity were very similar. At lower ammonium concentrations, important differences between the strains became apparent. It is unclear if it is due to simple strain heterogeneity or represents significant differences between industrial and laboratory strains. On the basis of the enzyme data, GS-GOGAT appears to be important in ammonium assimilation by DCL1 at limiting concentrations.

Contents

Acknowledgements

Although the declaration at the front of this work states "this thesis has been composed by myself, that it is a record of my own work", during the course of every Ph.D. contributions are made by other people. I would therefore like to take this opportunity of acknowledging and thanking those people who have helped me.

Thanks must go primarily to my supervisors Jim Kinghorn and Diana Wright for their encouragement and interest over the last three years. If they had not chosen me for the studentship in their laboratories, none of this would have happened. I appreciate their critical reading of the manuscript.

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The *Schiz. pombe* transformations were done by Alan Livingston. I would like to thank Alan for his help during my first few months in St. Andrews, plus the introduction to the cultural high spots of St. Andrews.

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Finally, I would like to thank my parents for their help and understanding over the last eight years.

List of Abbreviations

ADH	Alcohol Dehydrogenase
BSA	Bovine Serum Albumin
CLP	Chromosome Length Polymorphism
CoA	Coenzyme A
DEO	1,2,7,8-diepoxyoctane
dNTP	Deoxynucleotide Triphosphate
DON	6-diazo-5-oxo-L-norleucine
ds	Double Stranded
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic Acid
EMS	Ethanemethanesulphonate
EtBr	Ethidium Bromide
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
GAP	General Amino Acid Permease
GDH	Glutamate Dehydrogenase
GOGAT	Glutamate Synthase
GS	Glutamine Synthase
GSα	Tetrameric Form of *N. crassa* GS
GSβ	Octameric Form of *N. crassa* GS
kb	Kilobase
MES	2-(*N*-morphilino)ethanesulphonate
MM	Minimal Medium
M_r	Relative Molecular Weight
NCR	Nitrogen Catabolite Repression
NPD	Non-parental Ditype

PD	Parental Ditype
PEG	Polyethylene Glycol
PMSF	Phenylmethylsulphonylflouride
SCI	Single Colony Isolate
SPADY	Small Particle Active Dried Yeast
ss	Single Stranded
TCA	Tricarboxylic Acid
t_d	Doubling Time
ts	Temperature Sensitive
YC	Yeast Complete

Chapter 1

Introduction

1.1 Background

Microorganisms are capable of using atmospheric nitrogen, nitrate and ammonium as sources of inorganic nitrogen. Although yeasts are reported to lack the ability to fix dinitrogen, some have been shown to utilise nitrate and all are reported to use ammonium (references cited in Burn *et al.* 1974). Nitrate assimilation has been demonstrated in species of the yeasts *Candida*, *Hansenula*, *Rhodotorula* and *Sporobolomyces* but not in *Saccharomyces cerevisiae*. Consequently this discussion will be limited to ammonium assimilation by yeast, although, as some aspects of ammonium assimilation are better understood in other microorganisms, reference will be made to these other systems where necessary.

In its normal habitat of decaying vegetable matter *S. cerevisiae* encounters a variety of compounds able to serve as a nitrogen source. These range from simple compounds such as amino acids and pyrimidines to complex nucleic acids and proteins, although its metabolic versatility is not as great as that of the filamentous fungi or aerobic yeasts. In response to such a heterogenous environment *S. cerevisiae* has evolved a complex system of degradative enzymes and control mechanisms. These degradative pathways can be divided on the basis of their end-products: several systems, such as those degrading allantoin or asparagine, generate ammonium as the final product while the remaining ones e.g. those for arginine or proline catabolism, generate glutamate (see Cooper 1982a).

The common intermediate in the microbial assimilation of inorganic nitrogen into organic compounds is ammonium. Ammonium is generated from dinitrogen by the reduction of the latter to nitrate and then ammonium. The primary products of ammonium assimilation in *S. cerevisiae* are glutamate and glutamine, which are themselves major nitrogen donors in biosynthetic reactions (see Jones and Fink 1982). These considerations emphasise the central position of ammonium and glutamate in the nitrogen metabolism of *S. cerevisiae*. Glutamate is synthesised by the amination of 2-oxoglutarate, which is an intermediate of the tricarboxylic acid (TCA) cycle. This demonstrates that the enzymes which catalyse the interconversion of ammonium and glutamate are at the interface of carbon and nitrogen metabolism.

S. cerevisiae is propagated commercially on either cane or beet molasses as the carbon source. The nitrogenous compounds in molasses cannot be relied upon to provide an adequate source of nitrogen since only some of them are assimilated. In practice most of the required nitrogen is supplied as added ammonia, either as the liquid or its salts. Consequently, the pathways of ammonium assimilation are of central importance in the production of yeast biomass.

The enzymes of the ammonium assimilatory pathways have been identified, as have some of the factors affecting their regulation. However, there are still considerable gaps in the understanding of the enzymes and pathways e.g. which pathway is physiologically more important, and under what growth conditions. Such basic knowledge is important to anyone propagating yeast for biomass. The data reported in this study improve the understanding of the function of the glutamine synthase-glutamate synthase (GS-GOGAT) pathway and nitrogen metabolism of industrial yeast strains during a commercial propagation.

1.2 Ammonium Assimilation

1.2.1 Tracer studies, using $^{15}NH_4^+$, of ammonium assimilation by *Candida utilis* (Sims and Folkes 1964) have shown that the primary products are glutamate and glutamine. These workers concluded that, although the rate of synthesis of glutamate and glutamine was insufficient to meet the total demand for nitrogen, it met the demand for amino nitrogen for amino acid biosynthesis and that the other amino acids were derived from glutamate and glutamine.

Studies similar to those with *C.utilis* have been done with *S. cerevisiae* (Jones *et al* 1969). If the findings of Sims and Folkes (1964) are applicable to *S.cerevisiae i.e.* the amino acids in the metabolite pools participate in many transamination reactions, then a random distribution of label in the amino acid pools could be expected. This was observed. In tracer studies it is not possible for a product to be labelled to a greater degree than its precursor. So it is significant that using ^{15}N-labelled alanine, ammonium and glutamate, that cellular glutamate had the highest level of labelling. These workers concluded that the other amino acids are synthesised by the transaminase-catalysed transfer of the amino group of glutamate to a 2-oxo acid. Additionally, this work showed that the primary product of ammonium assimilation is glutamate. The high level of ammonium observed was probably an artefact of sample preparation for amino acid analysis. It is interpreted as showing that a high proportion of the observed glutamate was derived from cellular glutamine. The conclusion can be restated as "the primary products of ammonium assimilation by *S. cerevisiae* are glutamate and glutamine".

1.2.2 Ammonia transport has been studied in *S. cerevisiae* using radioactive methylamine, an analogue of ammonia. Roon and co-workers did preliminary biochemical and genetical studies of methylamine uptake by strain X2180-1A (Roon *et al.* 1975).

Methylamine uptake is pH dependent with maximal activity at pH6.0-6.5. The pK_a of methylamine is 10.6 but the culture pH is 4.5-6.5. It suggests that the molecular species which is transported, or initially bound is methylammonium. At the culture pH ammonia exists as the quaternary ion.

These workers found that methylammonium uptake is dependent upon the presence of an energy source in the medium. Egbosimba and Slaughter (1987) demonstrated that the ammonium permease activity in strain NCYC 1108 is modulated by the nature of the energy source. The uptake rate was greater with glucose as the carbon source compared to maltose. Roon *et al.* (1975) measured the internal and external methylammonium concentrations, and showed that the transport system could concentrate methylammonium about 1000-fold. Methylammonium uptake is saturable, with a V_{max} of 17nmol/min per gramme cells (dry weight) and an apparent K_m of 0.22mM. This data shows that methylammonium uptake is mediated by an active transport system.

Ammonium is a good competitive inhibitor of methylammonium uptake. Although most common amino acids cause only slight inhibition of permease activity, arginine and asparagine, which are degraded to ammonium, are inhibitors. These workers obtained methylammonium-resistant mutants, which define the *amt* locus and exhibit reduced ability to transport ammonium and methylammonium. Revertants of *amt* simultaneously recovered the ability to transport both compounds. On the basis of this data it was concluded that both compounds share a common transport system in *S. cerevisiae* X2180-1A.

Ammonium uptake by strain Σ1278b has been studied (Dubois and Grenson 1979). Lineweaver-Burk plots of methylammonium uptake, over the concentration range 0.01-2.0mM, exhibited an abrupt transition between apparently linear sections at about 0.1mM methylammonium. Each of the activities could be lost separately as the result of unlinked mutations. The low affinity, high capacity activity (V_{max} for methylammonium of 50nmol/min/mg protein, K_m of 2mM) is lost by the *mep1* mutation, which also confers methylammonium resistance. The *mep2* mutation causes the loss of the high affinity, low capacity system (V_{max} of 20nmol/min/mg protein, K_m of 0.25mM). Double mutants grow very slowly at low ammonium concentrations, although the single mutants are only slightly affected.

Roon and co-workers showed that methylammonium uptake is subject to nitrogen catabolite repression (NCR). NCR is the phenomenon whereby *S. cerevisiae*, or other microorganisms, can preferentially degrade good nitrogen sources (e.g. ammonium or glutamine) before poor ones (e.g. allantoin or proline). Arginine, asparagine and glutamine were found to repress permease activity by 60-80% (Roon *et al.* 1975). Both components of the uptake system in Σ1278b are subject to NCR (Dubois and Grenson 1979). In a mutant with a temperature-sensitive (*ts*) GS grown at the restrictive temperature, repression was lost for *MEP2* only, indicating that glutamine is the effector for only one of the two components of the uptake system. On the basis of this evidence it was concluded that ammonium/methylammonium uptake is mediated by two functionally distinct systems.

The *mep1 mep2* mutant grows almost normally at high ammonium concentrations and can still slowly assimilate methylammonium. From this, the existence of a third , low affinity ammonium permease was postulated. Additional evidence was provided by the *npr-1* mutation. This mutant exhibited increased resistance to methylammonium and modification of a Lineweaver-Burk plot of methylammonium uptake at high concentrations. Also in a *npr-1* mutant the general amino acid permease (GAP) was undetectable, and the activities of the proline plus ureidosuccinate permeases were reduced.

As the *mep1* locus conferred resistance to methylammonium, Dubois and Grenson postulated that this locus corresponded to the *amt* locus identified by Roon and co-workers. Cooper (1982b) has suggested, on the basis of the K_m values, that the *amt* locus is possibly allelic to *MEP2* and not *MEP1*. Cooper also observed that the methylammonium concentrations tested by Roon and co-workers (Roon *et al.* 1975) covered the range where biphasic behaviour was seen (Dubois and Grenson 1979). As biphasic activity was not observed, Cooper (1982b) postulated that X2180-1A lacked the *MEP1* encoded function.

1.2.3 As has been discussed in section 1.2.1 the primary products of ammonium assimilation are glutamate and glutamine. The interconversion of ammonium and glutamate is mediated by two glutamate dehydrogenases (GDHs). One, the NADPH-dependent GDH (E.C. 1.4.1.4) synthesises glutamate from ammonium, 2-oxoglutarate and NADPH. The anabolic role of this enzyme has been inferred from the elevated activity found in ammonium grown cells (Thomulka and Moat 1972, Roon and Even 1973). The NAD-GDH (E.C. 1.4.1.2) degrades glutamate to ammonium, 2-oxoglutarate plus NADH, and is present at higher levels in glutamate compared to ammonium grown cells (Bernhardt *et al.* 1965, 1966, Roon and Even 1973, Burn *et al.* 1974). From this evidence it has been concluded that NAD-GDH has a catabolic function. Glutamine is synthesised from ammonium and glutamate in an ATP-dependent reaction catalysed by glutamine synthase (E.C. 6.3.1.2). Mutants deficient in GS activity require glutamine (Dubois and Grenson 1974) suggesting that this is the sole route for glutamine biosynthesis in *S. cerevisiae*.

The intracellular ammonium concentration of *Aerobacter aerogenes* grown in ammonium-limited chemostat culture is less than 0.5mM, below the K_m for ammonium (3-4mM) of the *Aer. aerogenes* GDH. This observation led to the discovery of a novel pathway for glutamate biosynthesis with a low K_m for ammonium. Glutamate is synthesised by coupling GS with glutamate synthase, which catalyses the NADPH-dependent transfer of the amido group of glutamine to 2-oxoglutarate (Tempest *et al.* 1970). Subsequently GOGAT activity has been detected in a number of microorganisms including *S. cerevisiae* (Roon *et al.* 1974. E.C. 1.4.1.13), The yeast enzyme differs from the bacterial ones in that its cofactor is NADH and not NADPH. Although Roon and co-workers detected GOGAT activity in *S. cerevisiae*, its function is unclear because its activity is low even in cultures grown with poor nitrogen sources.

The pathways of ammonium assimilation in *S. cerevisiae* are shown diagrammatically in Fig. 1.

In this study, the phrase "enzymes of ammonium assimilation" is used as a general collective term for GS, GOGAT NADP-and NAD-GDH.

1.3 Biochemistry of the Enzymes of Ammonium Assimilation

In this section the biochemistry of the enzymes of ammonium assimilation will be discussed. However, as only a few studies have been done with the *S. cerevisiae* enzymes, reference will be made to other systems where more information is available.

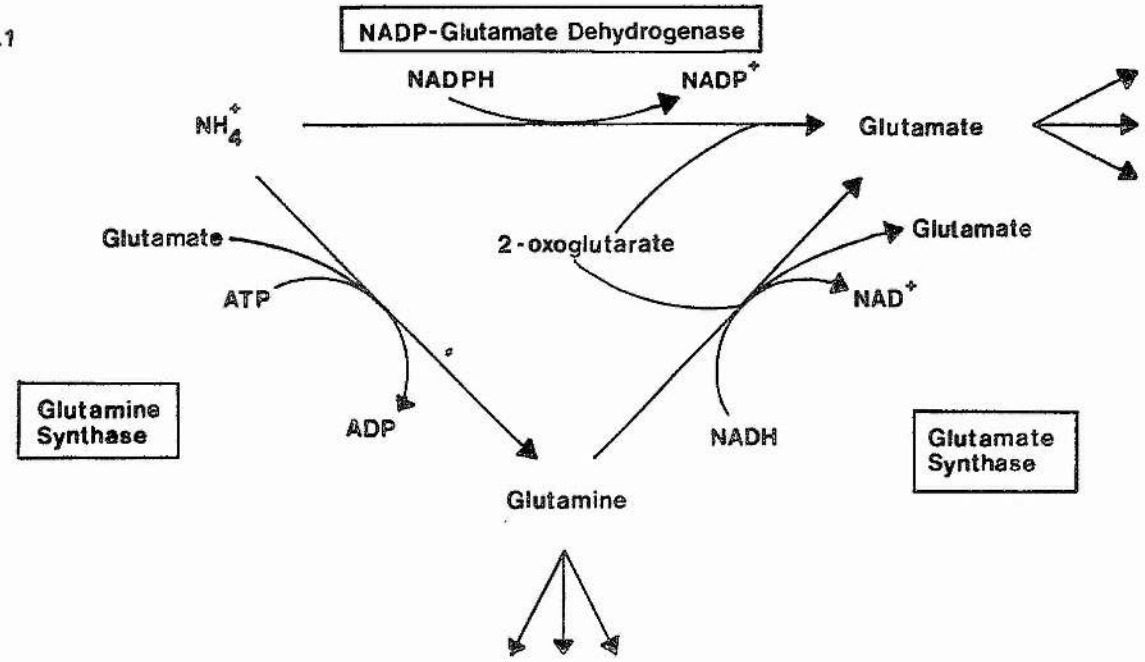
1.3.1 Glutamate dehydrogenases can be classified on the basis of their cofactor specificity. The three classes are: (i) the homotetrameric NAD-GDHs; (ii) homohexameric NADP-GDHs ; and (iii) the homohexameric GDHs of dual cofactor specifity found in vertebrate liver (references cited in McPherson and Wootton 1983, Miller and Brenchley 1984). *S. cerevisiae* possesses both NAD- and NADP-GDH.

The *S. cerevisiae* NADP-GDH has been purified by a number of workers and shown to be a homomeric protein composed of subunits of M_r 54000 (Grisolia *et al.* 1964, Mazon and Hemmings 1979). The measured subunit size is in agreement with the value (49559) calculated from the nucleotide sequence (Moye *et al.* 1985). This is similar to the subunit M_r of about 48000 for other microbial NADP-GDHs (McPherson and Wootton 1983, Kinnaird and Fincham 1983, Miller and Brenchley 1984, and references cited therein). The native *S. cerevisiae* enzyme has a M_r of 280000, which indicates that the yeast enzyme is also a hexamer (reference cited in Roon *et al.* 1974).

The *S. cerevisiae* NAD-GDH has been purified to homogeneity by a number of workers, although there is disagreement in the reported sizes. Bernhardt *et al.* (1966) reported a M_r of 315000, which is markedly lower than the value of 455000 for the native enzyme, with a subunit M_r of 100000-117000, reported elsewhere (Hemmings 1980a, Uno *et al.* 1084). The reason for this discrepancy is unclear. From this size data it has been concluded that the *S. cerevisiae* enzyme is a tetramer of identical subunits. This makes it similar to the NAD-GDHs of *C. utilis* (Hemmings 1980b) and *Neurospora crassa* (Haberland *et al.* 1980, and references cited therein) which are tetramers (M_r 460000) of identical subunits (M_r 116000).

There is little data in the literature on the biochemistry of the NAD- and NADP-GDH of *S. cerevisiae*. The best studied enzymes are from the bovine liver and *N. crassa*. GDHs vary widely in kinetic characteristics, metabolic function and molecular properties especially between animal and non-animal enzymes (reviewed in Goldin and Freiden 1971). For example, vertebrate enzymes can use either NADPH or NADH whereas the microbial ones are specific for only one cofactor. Based on the limited

Fig.1



Pathways of Ammonium Assimilation in S. cerevisiae

data available to these authors, they concluded that non-animal enzymes, in contrast to those from animal sources, do not polymerise to higher M_r aggregates. Due to these fundamental differences, only the microbial enzymes will be discussed.

Grisolia *et al.* (1964) examined the pH optima of the *S. cerevisiae* NADP-GDH. They found that, for NADPH oxidation, the pH optimum was 8.0 compared to pH7.6 for NADP reduction. This is much higher than the pH optima observed for *Schizosaccharomyces* spp. (Johnson and Brown 1974). The NADP-GDHs from the *Schizosaccharomyces* species *pombe*, *malidevorans* and *versatilis* plus *Saccharomycodes ludwigii* were fully active at pH 6.5, although the optimum pH for *Schiz. octosporus* was pH7.0.

The *N. crassa* NADP-GDH is similar to the *S. cerevisiae* one *i.e.* maximum activity is observed at a high pH. It is inactive at pH values below 7.2, fully active above pH 8.0, and partially active at intermediate pH values (West *et al.* 1967). These workers observed a complex relationship between pH and the presence of NADPH and/or 2-oxoglutarate. If purified NADP-GDH was pre-incubated at pH7.2-7.4 at increasing 2-oxoglutarate concentrations, a sigmoidal relationship was observed between activity and 2-oxoglutarate concentration. This sigmoidal relationship could be due to 2-oxoglutarate acting as both substrate and activator. At pH7.8 a closer approximation to normal Michalis-Menten kinetics was observed *i.e.* an increase in pH activated the enzyme. However, at this pH, if NADPH was included in the pre-incubation mixture a sigmoidal relationship was again observed between activity and the 2-oxoglutarate concentration. It is apparent that NADPH was modifying the effect of the activator 2-oxoglutarate.

It was concluded that 2-oxoglutarate functions in a co-operative manner to shift the equilibrium between the active and inactive states of the enzyme towards the active form. The inactive form is favoured by NADPH, unless 2-oxoglutarate is present, when a potentially active quaternary complex is formed. Stabilisation of the inactive form by protons at pH values below 7.0 is the process which limits the pH range of the enzyme activity on the low pH side of the optimum pH.

The data presented by Fincham and co-workers (West *et al.* 1967, and references cited therein) show that the *N. crassa* NADP-GDH is an allosteric enzyme. The physiological significance of this is unclear.

Similar studies have not been done with the *S. cerevisiae* NADP-GDH. The sigmoidal relationship between activity and activator concentration is due to conformational changes in adjacent monomers. Like the *N. crassa* enzyme, the *S. cerevisiae* NADP-GDH is also a homohexamer. Additionally, there is considerable homology in the amino acid sequence of the substrate binding domains of the *N. crassa*

and *S. cerevisiae* enzymes (see below). So it is possible that the yeast enzyme also exhibits allosteric properties.

Allosteric behaviour has also been demonstrated in NAD-GDH (LéJohn 1967). *Thiobacillus novellus* possesses NAD- and NADP-GDH, which both catalyse the reversible formation of 2-oxoglutarate from glutamate. LéJohn showed that AMP altered the K_m values for NAD-GDH, but not the NADP-GDH, for its substrates to favour glutamate degradation. In the absence of AMP, the NAD-GDH exhibited a sigmoidal relationship between the NAD^+ or glutamate concentration in the pre-incubation mixture and activity. In the presence of AMP, a closer approximation to the Michalis-Menten relationship was observed. It was concluded that AMP was an allosteric effector activating the degradative function of the *T. novellus* NAD-GDH. LéJohn postulated that the ATP/AMP ratio may be critical in the regulation of NAD-GDH. Similar studies have not been reported with *S. cerevisiae*.

The enzyme kinetics of NADP-GDH, isolated from a number of microorganisms, have been investigated. There is considerable similarity among the microbial NADP-GDHs in their affinities for both NADPH and 2-oxoglutarate. This probably reflects the marked similarity of the substrate binding domains (see below). For NADPH, the K_m was 0.02mM for the *Aspergillus nidulans* enzyme oxoglutarate was measured in *S. cerevisiae* (Grisolia *et al.* 1964, Grenson *et al.* 1974) and various species of *Schizosaccharomyces* plus *Saccharomycodes ludwigii* (Johnson and Brown 1974) at 1mM. The affinity of the *A. nidulans* (Kinghorn and Pateman 1975) and *Schiz. octosporus* (Johnson and Brown 1974) enzymes were lower, K_m values of 3.4 and 8mM respectively were measured.

Considerable variation in the affinity of NADP-GDH for ammonium, depending upon the source of the enzyme, has been observed. Additionally, there is disagreement in the K_m values reported by different workers for enzyme from the same source. For the *A. nidulans*, (Kinghorn and Pateman 1975), *Aer. aerogenes* (Tempest *et al.* 1970), *E. coli* and *Klebsiella aerogenes* (cited in Senior 1975) NADP-GDHs, the K_m for ammonium is 1.5-4mM. The affinity for ammonium of the various *Schizosaccharomyces* species studied (Johnson and Brown 1974) is much lower, the K_m values were measured at 15-25mM. The K_m of the *S. cerevisiae* enzyme has been measured at 1mM (unpublished data cited in Roon *et al.* 1974), 2mM (Grenson *et al.* 1974) and 11.1mM (Grisolia *et al.* 1964). Similar results have been observed with the *N. crassa* NADP-GDH. The K_m has been measured at 0.7, 1.1 and 10mM (references cited in Wootton 1983).

Wootton and co-workers (Wootton 1983, and references cited therein) have studied the effect of ammonium on the activity of *N. crassa* NADP-GDH. Wootton (1983) showed that a Lineweaver-Burk plot of activity and ammonium concentration was biphasic, with a transition point between

approximately linear regions at about 2mM. The effects of ammonium are independent of the position of equilibrium between the active and inactive states of the *N. crassa* enzyme. It was concluded that ammonium probably acts as both substrate and activator.

Wootton concluded that the ammonium mediated activation of NADP-GDH at concentrations above 5mM implied that the affinity for ammonium as a substrate is reflected by K_m values only when ammonium concentrations below 5mM are used (Wootton 1983). In this concentration range activation does not cause marked deviation from linearity. The K_m for ammonium of the *N. crassa* NADP-GDH was determined at different pH values and 2-oxoglutarate concentrations over the ammonium concentration range 0.1-2mM (Wootton 1983). These showed that the K_m for ammonium was 0.5-1.1mM; with infinite 2-oxoglutarate, the K_m was calculated to be 1.1mM.

Wootton examined the published values for the K_m for ammonium. He concluded that the widely differing K_m values, mostly greater than 5mM, were probably due to similar biphasic interactions with ammonium, as only concentrations greater than 5mM were used. The strong conservation of both primary polypeptide structure and enzymological properties (Goldin and Freiden 1971) between NADP-GDH from prokaryotes and eukaryotes sources supports this hypothesis.

The enzyme kinetics of the yeast NAD-GDH activity have been investigated. One of the mechanisms which regulates yeast NAD-GDH activity is phosphorylation. It has been shown that the phosphorylated form is inactive, and that dephosphorylation restores activity. The restoration of activity is accompanied by a marked increase in affinity for the substrate glutamate, the K_m for NAD^+ is subject to only slight variation by the degree of phosphorylation.

The phosphorylated *S. cerevisiae* enzyme has K_m values of 1.1 and 95mM for NAD^+ and glutamate respectively, while the dephosphoGDH has K_m values of 1.2mM for NAD^+ and 10mM for glutamate (Uno *et al.* 1984). Hemmings (1980b) showed that the kinetics of the *C. utilis* enzyme were similar to those of the *S. cerevisiae* one. The K_m values for NAD^+ were 0.86mM for the dephosphoGDH and 1.08mM for dephosphoGDH. The inactive, phosphorylated NAD-GDH has a K_m for glutamate of 128mM. The dephosphoGDH has a markedly higher affinity, the K_m was measured at 20mM. There are few reports on the biochemistry of the yeast NAD-GDH in the literature.

Amino acid sequences have been obtained for the NADP-GDH from prokaryotes, higher and lower eukaryotes either directly or deduced from the nucleotide sequence. Sequence comparison has shown that there is strong sequence conservation, especially in the amino terminal region of the polypeptide (Wootton *et al.* 1973, Juillard and Smith 1979, Mattaj *et al.* 1982, McPherson and Wootton 1983, Moye Et al. 1985, Gurr *et al.* 1986).

The monomer of the bovine NADP-GDH is a polypeptide of 501 amino acid residues, the chicken polypeptide 504 residues and the human one 500. It has been shown that there are 24 differences between the bovine and human GDHs, 27 between the bovine and chicken but 41 differences between the chicken and human. These differences appear to be randomly distributed (Juillard and Smith 1979). Comparison of the deduced amino acid sequence of the *E. coli* NADP-GDH with the amino acid sequence of the *N. crassa* enzyme shows 253 identical residues of 428 compared [59% homology] (McPherson and Wootton 1983). The *S. cerevisiae* gene shows 60% homology to the *N. crassa* one (64% at the protein level), while the deduced amino acid sequence shows 51% homology with the *E. coli* enzyme and 24% with the bovine one (Nagasu and Hall 1985).

The regions of strong homology are non-uniformly distributed along the polypeptide. Greatest homology is in the N-terminal region, amino acids 57 to 175 of the 447 residue *E. coli*, which shows 94/119 amino acid identities with the *N. crassa* enzyme [79%] (Mattaj *et al.* 1982). The bovine GDH is less homologous in this region; it shows 28% and 31% homology with the corresponding regions of, respectively, the *E. coli* and *N. crassa* enzymes (Mattaj *et al.* 1982). Comparison of the nucleotide sequences of the 5′ regions of the *A. nidulans* and *N. crassa* genes has shown a high degree of homology (Gurr *et al.* 1986). The deduced amino acid sequence between residues 60 and 111 of the 453 residue *S. cerevisiae* polypeptide exhibits 90% homology with the corresponding region of the *N. crassa* enzyme and 80% with the *E. coli* one (Nagasu and Hall 1985).

The *N. crassa* NAD-GDH shows homologies of 17, 18 and 25% with the highly conserved regions of the *E. coli* and *N. crassa* NADP-GDHs and the bovine GDH respectively (Mattaj *et al.* 1982). Homologies are largely restricted to regions around functional residues (cited in Austen *et al.* 1980)

Reaction of the bovine GDH with either glyoxal or pyridoxal phosphate leads to the inhibition of 2-oxoglutarate binding (Rasched *et al.* 1974, and references cited therein). Glyoxal reacts with the Lys-27 residue and prevents substrate binding, although allosteric properties remain unaffected. Pyridoxal phosphate reacts with the Lys-126 residue, although glyoxal does not react with this residue Pyridoxal phosphate is the prosthetic group of all transaminases. These enzymes form Schiff-base intermediates with their substrates. In the absence of substrate, the aldehyde group of pyridoxal phosphate forms a Schiff-base with the ε-amino group of a specific lysyl residue of the active centre (Stryer 1981). It is believed that 2-oxoglutarate forms a Schiff-base aduct with Lys-126 during catalysis. Although the two lysyl residues are separated by 99 amino acids in the polypeptide, they are both involved in substrate binding. It would appear that they are positioned close together in the tertiary structure of the active centre. The Lys-126 is conserved in *S. cerevisiae* (position 138) and all other available sequences (Wootton 1973, Moye *et al.* 1985 and references therein). However, the Lys-27 residue is not observed in the *A. nidulans*, *E. coli*, *N. crassa* or *S. cerevisiae* amino acid sequences.

Sequence comparison of the bovine GDH and *N. crassa* NADP-GDH with other dehydrogenases suggested that each GDH possessed two cofactor binding domains. Direct binding studies demonstrated that cofactor bound at only one site on the *N. crassa* enzyme (Wootton 1974). However, not withstanding the 64% homology of the *N. crassa* and *S. cerevisiae* NADP-GDHs, only one cofactor binding domain has been predicted for the yeast enzyme (Moye *et al.* 1985). This domain correlates with the C-terminal binding domain predicted for *N. crassa.*

Haberland and Smith with colleagues have made predictions about the structure of the *N. crassa* NAD-GDH (Haberland *et al.* 1980, Austen *et al.* 1980). Separation of tryptic digests of the enzyme revealed the presence of two major fragments. This suggested that the tertiary structure of the subunit is organised into two major domains. Comparison of the sequence of peptide T-B (M_r 63100), representing the C-terminal domain, with animal and NADPH-specific GDHs showed that homology was limited to regions around reactive residues. A cofactor binding domain has been predicted in this fragment. As peptide T-B contains all the regions of homology, it would appear that the C-terminal domain is the portion of the subunit involved in GDH function. Peptide T-A (M_r 47300), the N-terminal domain, exhibited no obvious homology with other GDHs. The function of this domain is unclear.

Tryptic cleavage of the *C. utilis* NAD-GDH generated two fragments of M_r 64500 and 48000, suggesting that the subunits of this enzyme are also composed of two domains (Hemmings 1980b). Analysis of the fragments from ^{32}P-labelled phosphoGDH showed that only the 48000 fragment contained radioactivity. Digestion of phosphoGDH with trypsin promoted a rapid increase in activity, although prolonged incubation caused a subsequent loss of activity. As has been stated above, yeast NAD-GDH is regulated by the degree of phosphorylation. Hemmings has suggested that the smaller tryptic fragment is a regulatory domain. Reactivation by limited proteolysis suggests that the 48000 fragment domain interacts with the 64500 one, and, when it is phosphorylated, results in enzyme inactivation. Limited proteolysis possibly decreases the interaction between the two domains, due to a conformational change, causing a relief of inhibition of the active centre.

Regulation by phosphorylation has not been demonstrated with the *N. crassa* NAD-GDH (cited in Haberland *et al.* 1980). Although the *S. cerevisiae* NAD-GDH is known to be regulated by phosphorylation (Uno *et al.* 1984), the effect of limited proteolysis on the activity of phosphoGDH has not been reported .

Amino acid and nucleotide sequence comparisons show a high degree of homology in NADP-GDHs from a variety of sources, and lower homology with either the dual specificity or NAD-specific GDHs. These comparisons support the idea of three separate classes of GDH suggested by the observed

cofactor specificity and enzyme structure. Additionally, that as a family, GDHs are highly conserved among a range of classes of organisms. The high degree of homology observed among the NADP-GDHs, especially in the substrate binding domains, suggests that there is both structural and functional conservation within this class. This is probably also true for the NAD-specific and dual specificity GDHs.

1.3.2 The two GDHs found in *S. cerevisiae* have been located in the soluble phase of the cytosol (Perlman and Mahler 1970). There are no reports in the literature on the location of GS or GOGAT in the yeast cell, although Brown *et al.* (1973) has suggested that the *Schiz. pombe* GOGAT is a cytoplasmic enzyme.

1.3.3 There are a large number of reports in the literature on the biochemistry of GS isolated from a number of organisms. It would appear that, unlike the microbial NADP-GDHs, there are considerable differences in the structural and functional properties of GS from Gram-negative bacteria and the fungi.

The bacterial enzyme is a dodecameric protein (M_r 620000) of identical subunits (M_r 52000), although the subunits from different sources are not identical. The three-dimensional structure of the *Salmonella typhimurium* GS has been elucidated by X-ray crystallography (Almassy *et al.* 1986). This model, which places the active centres at the subunit interfaces, suggests a mechanism for the ATP-mediated transfer of an amido group to glutamate to generate glutamine. Additionally, the model suggests how the enzyme activity is regulated in response to covalent modification and feedback inhibition.

Several reviews of the biochemistry and regulation of the bacterial GS have been published (Magasanik *et al.* 1974, Tyler 1978, Magasanik 1982). The bacterial GS is regulated by three different mechanisms: (i) alteration of the position of equilibrium between active and inactive states in response to variation in the concentration of the divalent cations Mg^{2+} and Mn^{2+} ; (ii) feedback inhibition by end-products of glutamine metabolism; and (iii) covalent modification of each subunit by the reversible adenylation of a specific tyrosyl residue. The unadenylated enzyme is fully active, and activity decreases with increasing adenylation. The adenylated form is more sensitive to feedback inhibition than the unadenylated one. The degree of adenylation is modulated by a regulatory protein and the levels of Mg^{2+} , Mn^{2+} , ATP, glutamine and 2-oxoglutarate plus other metabolites.

The affinity of GS for ammonium is much higher than that of NADP-GDH, unadenylated *E. coli* GS has a K_m of 0.2mM (cited in Senior 1975) while that of *Aer. aerogenes* is below 1mM (Tempest *et al.* 1970). The catalytic co-operativity and subunit interactions of the *E. coli* GS have been studied (Wedler *et al.* 1982). These workers chemically inhibited GS (equivalent to the inactivation resulting from increased adenylation), and then characterised the enzyme in terms of the remaining (active) subunits. It was observed that there was a two-fold increase in the K_m for glutamate in 90% inactivated GS

compared to fully active enzyme. The decrease in affinity was more dramatic for ammonium, about five-fold. Conversely, as the degree of inactivation increased, the K_m for ATP decreased. Lineweaver-Burk plots of activity and ammonium concentration show a biphasic relationship, which is more accentuated at lower glutamate concentrations and with higher degrees of inactivation. It would appear that, like the NADP-GDHs, GS is an allosteric enzyme subject to substrate activation by ammonium.

Adenylation seems to cause loss of activity by decreasing the affinity of the active centre for substrate. The three-dimensional model of the *Sal. typhimurium* GS suggests that this could be due to restriction of the motion of the two domains forming the active centre or access to it (Almassy *et al.* 1986).

The structure and regulation, plus the biochemical characteristics these confer, of the bacterial GSs are different to the fungal enzymes. Consequently, the bacterial GSs will not be considered further except in the discussion of the physiology of ammonium assimilation.

GS activity can be assayed by two different procedures: the synthase assay measures the physiologically important function *i.e.* the amidation of glutamate. The alternative, the transferase assay, measures the ability of GS to function as a general amidotransferase. Synthase, but not transferase, activity is modulated by the degree of adenylation. The number of adenylated subunits is modulated by the growth conditions, and is reflected in the ratio of the two activities. A sudden increase in the availability of ammonium to *E. coli* causes a loss of synthase, but not transferase, activity and a decrease in the synthase:transferase ratio (see Ferguson and Sims 1974b).

The inactivation of *C. utilis* and *N. crassa* GS by variation of the growth conditions has been examined. Ferguson and Sims (1974b) showed that, for *C. utilis*, there was no change in the synthase:transferase activity ratio during the rapid inactivation of GS (50% loss in about 20 minutes) caused by the addition of ammonium to a glutamate grown culture. The rapid restoration of GS activity on removal of ammonium was due to *de novo* protein synthesis. The synthase:transferase ratio was found to be independent of the growth conditions.

Partially purified GS is not inhibited by glutamine.

Sims and co-workers have concluded that the inactivation of the *C. utilis enzyme* is not due to feedback inhibition or changes in affinity for its substrates. The inactivation of the yeast enzyme is due to a change in the degree of aggregation. In glutamate grown cells GS exists as an octomer, but the rapid inactivation occurs concomitantly with the appearance of a tetrameric form (Sims *et al.* 1974).

The best characterised fungal GS is the *N. crassa* one (Mora and co-workers). GS purified from glutamate cultures is an octameric protein of identical subunits (reviewed in Marzluf 1981). Mora and co-workers (Vichido *et al.* 1978) have shown that, when shifted from glutamate to glutamine, there was

a slow loss of activity (50% inactivation in about 150 minutes). Initially this loss was faster than the dilution of activity expected by growth, although after about 30 minutes the decrease in activity corresponded with the theoretical dilution rate. The synthase:transferase ratio remained constant, and immumnoprecipitation of enzyme activity showed that changes in activity corresponded with variation in enzyme concentration. The enzyme remained as an octomer during the shift.

It would appear that the *C. utilis* and *N. crassa* enzymes are fundamentally different to the bacterial GSs. Both fungal enzymes are octameric, whereas the bacterial ones are dodecameric. The activity of bacterial GS is regulated by feedback inhibition and covalent modification, which is not observed with the two fungal enzymes.

GS from *C. utilis* and *N. crassa* are different. The *C. utilis* enzyme is rapidly inactivated, which results in the appearance of a tetrameric form. In contrast, the *N. crassa* GS activity is not modulated by inactivation but by growth dilution. In both microorganisms, the subsequent increase in activity is due to *de novo* protein synthesis.

Mora and co-workers have shown that two species of the *N. crassa* GS exist. They showed that the *N. crassa* enzyme is formed from two monomers, α and β. The α subunit is slightly larger than the β one, but they are believed to be different gene products with no product/precursor relationship (Marzluf 1981). Davila *et al.* (1980) showed that the predominant form in ammonium-limited cultures is a tetramer of mainly α subunits. In cultures grown with excess ammonium the predominant form is an octomer of β subunits. Intermediate oligomers are composed of both α and β subunits. Presumably the various forms of the enzyme have different functions.

GS from *S. cerevisiae* has been purified to homogeneity (Mitchell and Magasanik 1983). The enzyme was shown, by SDS-gel electrophoresis, to be a homomeric protein composed of subunits of M_r 43000, which is comparable to the *C. utilis* and *N. crassa* subunit M_rs. The size of native *S. cerevisiae* GS, estimated by gel filtration chromatography, is 470000, indicating that there are apparently 10-11 subunits per multimer. Other eukaryotic GSs studied are octomers, which would give a predicted M_r of 340000 for the *S. cerevisiae* enzyme. The *S. cerevisiae* GS was almost completely resolved from a protein of M_r 370000, so the difference between observed and predicted M_rs is too large to be explained by asymmetry of the enzyme structure. These workers postulated that the *S. cerevisiae* enzyme is structurally similar to the dodecameric bacterial enzymes.

Other workers have purified the *S. cerevisiae* GS, but from a different strain. They observed a native enzyme M_r of 630000, with a subunit M_r of 61000 (reference cited in Mitchell and Magasanik 1983). The two different preparations exhibited markedly different pH optima. Therefore it is possible that *S. cerevisiae* exhibits a polymorphism with respect to the GS subunit.

It has been shown that the addition of glutamine to glutamate grown *S. cerevisiae* causes a rapid loss of synthase activity [50% inactivation in about 30 minutes] (Mitchell and Magasanik 1984c). However, unlike *C. utilis*, there is a marked decrease in the synthase:transferase ratio. The inactivation is not associated with an alteration of the degree of aggregation, although it is accompanied by a cessation of subunit synthesis.

Mitchell and Magasanik (1983) showed that the increase in GS activity observed on transfer from glutamine to glutamate supplemented medium was due to *de novo* protein synthesis. In this experimental system, synthase and transferase activity increased in parallel. However, if glutamine was added to glutamate grown cells and then removed, removal of glutamine resulted in reactivation of the GS. Initially the synthase activity increased more rapidly than the transferase activity, but after about 10 minutes increased in parallel (Mitchell and Magasanik 1984c). Therefore it was concluded that the inactivation of the *S. cerevisiae* GS is reversible. This has also been observed by Legrain *et al.* (1982).

The *S. cerevisiae* GS is a multimeric enzyme of 10-12 subunits. Its activity is regulated by reversible inactivation. In these respects, *S. cerevisiae* GS is more similar to the bacterial enzymes than to either the *C. utilis* or *N. crassa* ones. However, the incorporation of labelled adenine or phosphate into the *S. cerevisiae* GS has not been demonstrated (unpublished data cited in Mitchell and Magasanik 1984c).

1.3.4 Since its initial discovery in *Aer. aerogenes*, GOGAT activity has been demonstrated in both eukaryotic and prokaryotic microorganisms. The best studied enzymes, with respect to their biochemistry, are probably those from the Gram-negative bacteria.

The affinity of GOGAT for its substrates glutamine and 2-oxoglutarate has been reported for a number of microorganisms. The K_m for 2-oxoglutarate varies over a 290-fold range: the value measured for *Bacillus megaterium* (cited in Desphande and Kane 1980) and *E. coli* (Miller and Stadtman 1972) is about 0.008mM; for *Aer. aerogenes* it is 0.3-2mM (Tempest *et al.* 1970, Trotta *et al.* 1974); 0.04-1.0mM for *S. cerevisiae* (Roon *et al.* 1974, Masters and Meister 1982); and 0.9mM for *Schiz. pombe* (Brown *et al.* 1973). In contrast, the K_m for glutamine varies over a seven- fold range. The value is about 0.25-0.3mM for *B. megaterium* (cited in Desphande and Kane 1980), *E. coli* (Miller and Stadtman 1972), *S. cerevisiae* (Roon *et al.* 1974, Masters and Meister 1982), and *Schiz. pombe* (Brown et al. 1973), although for *Aer. aerogenes* it is 0.3-1.8mM (Tempest *et al.* 1970, Trotta *et al.*1974). Miller and Stadtman (1972) observed a hyperbolic relationship in plots of activity against substrate concentration suggesting that the *E. coli* GOGAT is not an allosteric enzyme.

The pH optima of these enzymes are very similar at about pH7.4-7.6 (Tempest *et al.* 1970, Miller and Stadtman 1972), although for *B. megaterium* and *S. cerevisiae*, the optima are slightly broader (Roon *et*

al. 1974, cited in Desphande and Kane 1980). However, the optimum pH for the *Schiz. pombe* GOGAT is markedly more acidic, pH6.4 (Brown *et al.* 1973).

There is a major difference between GOGAT from bacteria and eukaryotic microorganisms. The bacterial enzymes use only NADPH as cofactor, while GOGAT from fungal and yeast sources use only NADH.

Considerable size heterogeneity has been observed in GOGAT isolated from these different microorganisms. The *N. crassa* enzyme is formed from a polypeptide of M_r 200000 (Hummelt and Mora 1980b), but it is unclear if the native enzyme is a multimer. The other microbial GOGATs characterised are heteromers.

Most GOGATs that have been studied are formed from two subunit species of markedly different sizes. In *Aer. aerogenes, E. coli* and *K. aerogenes* the small subunit has a M_r of 51000-53000. In contrast, the large subunit shows a marked size heterogeneity: for *Aer. aerogenes* and *K. aerogenes* it has a M_r of 175000 but in *E. coli* it is 135000 (Miller and Stadtman 1972, Trotta *et al.* 1974, Tyler 1981). The subunit sizes of the *E. coli* GOGAT have been confirmed by polypeptide synthesis in minicells containing the cloned genes (Lozoya *et al.* 1980). The enzyme from *B. megaterium* is a heteromeric protein, and it is implied from the genetics that the *B. subtilis* enzyme is also a heteromer (Desphande and Kane 1980, and references cited therein).The dimer formed from the non-identical subunits found in *K. aerogenes* is fully functional (Tyler 1981). In contrast, GOGAT from *E. coli* has a M_r of 800000, which implies that the native enzyme consists of four dimeric units.

The *S. cerevisiae* enzyme is also a heteromer composed of two non-identical subunits, but the subunits are larger than the corresponding ones from the bacterial enzymes. The subunit M_rs are 169000 and 61000 (Masters and Meister 1982). However, there is disagreement in the literature about the M_r of native *S. cerevisiae* GOGAT. Masters and Meister obtained a M_r of 265000 by gel filtration chromatography. This was confirmed by cross-linking studies where polypeptides of M_r 265000, 169000 and 61000 were observed, which corresponded to the native enzyme and its two subunits. In contrast, Roon *et al.* (1974) observed that, in gel filtration studies, GOGAT eluted ahead of the NAD-GDH (M_r 455000). From these studies a M_r of greater than 455000 was indicated for the *S. cerevisiae* GOGAT. Comparison of the two M_rs would indicate that, possibly, the *S. cerevisiae* enzyme contains two dimeric units per native enzyme. The reason for this difference in the reported M_rs is unclear.

Purified GOGAT from *Aer. aerogenes* and *E. coli* has been biochemically characterised by several workers (Miller and Stadtman 1972, Trotta *et al.* 1974, Mantsala *et al.* 1976a, b, Geary and Meister 1977). Both enzymes are heteromers and contain non-haem iron, flavin (as flavin adenine dinucleotide [FAD] and flavin mononucleotide [FMN]) and sulphur. The involvement of flavin in the glutamine-

dependent activity is suggested by the observation that deflavo enzyme has lost this activity. This was confirmed by the demonstration that non-enzymatically reduced flavoprotein could serve as an electron donor in the glutamine-dependent transamidation. On the basis of the absorbance changes, it was shown that the FAD is involved in catalysis and suggested that FMN is catalytically inactive.

Inhibitors which specifically inhibit the glutamine-dependent activity have been shown to bind to a cysteine residue in the large subunit. It implies that the active centre, and therefore the flavin, is located in the large subunit. The location of the flavin has been confirmed for the *Aer. aerogenes* and *E. coli* enzymes. Glutamine amidotransferases exhibit a glutaminase activity which reflects the binding of glutamine and hydrolysis of the amido group independently of the amination reaction. Both *Aer. aerogenes* and *E. coli* GOGAT possess glutaminase activity. Inhibition of the glutaminase function can be prevented by glutamine which shows that the glutaminase activity is part of the normal function of GOGAT.

Miller and Stadtman (1972) have proposed that the GOGAT catalysed reaction is the sum of two partial reactions:

$$H^+ + NAD(P)H + E.flavin \longrightarrow E.flavin.H_2 + NAD(P)^+$$

$$E.flavin.H_2 + 2\text{-oxoglutarate} + glutamine \longrightarrow 2\ glutamate + E.flavin$$

Sum: $NAD(P)H + H^+ + 2\text{-oxoglutarate} + glutamine \longrightarrow 2\ glutamate + NAD(P)^+$

These workers observed no reduction of cofactor in the presence of glutamate. This suggests that the reaction is essentially irreversible. The forward reaction, however, is inhibited by glutamate and other amino acids derived from glutamate, suggesting that the enzyme is subject to end-product inhibition.

Both the *Aer. aerogenes* and *E. coli* enzymes have been shown to have an ammonium-dependent activity (2-7% of the glutamine-dependent one) which is typical of most amidotransferases. The ammonium-dependent activity has been localised to the small subunit and is increased in the deflavo enzyme. It suggests that this activity is masked by the normal functioning of GOGAT. The physiological and evolutionary significance of this activity is not obvious. It is unclear whether interaction between the two subunits is required for full activity.

The heteromeric nature of the *S. cerevisiae* enzyme plus the similarity of the kinetics and pH profile, suggests at least a degree of homology between the yeast and bacterial enzymes.

1.4 Genetics of the Enzymes of the Ammonium Assimilation Pathways and their involvement in Nitrogen Catabolite Repression

1.4.1 There is considerable difference in the interest shown in cloning the structural genes encoding the various enzymes of ammonium assimilation. This reflects the bias of the biochemical and genetic studies.

The gene encoding the NADP-GDH subunit (designated *am*, *gdhA* or *gdh1* depending upon the organism) has been cloned from a number of microoganisms including the bacteria *E. coli* (Covarrubias *et al.* 1980, Windass *et al.* 1980, Mattaj *et al.* 1982) and *Sal. typhimurium* (Miller and Brenchley 1984), the fungi *A. nidulans* (Gurr *et al.* 1986) and *N. crassa* (Kinnaird *et al.* 1982), and the yeast *S. cerevisiae* (Moye *et al.* 1985, Nagasu and Hall 1985). The nucleotide sequences have been determined for most of the cloned genes. The sequences show a high degree of homology (see section 1.3.1). There are no reports of the cloning of genes encoding the NAD-GDH.

In *S. cerevisiae* highly expressed genes exhibit a very biased codon usage. For example, in some of the yeast glycolytic genes over 90% of the amino acids are encoded by the 25 codons corresponding to the major isoaccepting species of tRNA. The *S. cerevisiae GDH1* uses this group of tRNAs to specify 84% of its amino acids. It is predicted from this data that the *GDH1* mRNA comprises a high proportion of the cellular mRNA. This is supported by the finding that NADP-GDH comprises 0.5% of the soluble protein of *S. cerevisiae* (Moye *et al.* 1985, Nagasu and Hall 1985, and references cited therein).

GDH1 is the homologue of the *N. crassa am* and *A. nidulans gdhA*. Although there is a high degree of homology in both the amino acid and nucleotide sequences, the gene structures are different. The *A. nidulans* and *N. crassa* genes contain introns (Kinnaird and Fincham 1983, S.J. Gurr, pers. comm.), but none were found in the *S. cerevisiae* (Moye *et al.* 1985, Nagasu and Hall 1985). The introns in the *N. crassa am* interrupt highly conserved sequences between the *E. coli* and *N. crassa* (Kinnaird and Fincham 1983).

GLNA, which encodes the GS subunit, has been cloned from *E. coli* (Covarrubias *et al.* 1980) and *Sal. typhimurium* (references cited in Wootton and McPherson 1983). Partial sequence data has been reported for the coding and regulatory sequences of these two genes (Wootton and McPherson 1983). Complete sequence data is available for *GLNA* from a species of the cyanobacterium *Anabaena*. Comparison of the *Anabaena* sequence with the available *E. coli* data reveals strong homology (references cited in Wootton and McPherson 1983). The GS gene of *S. cerevisiae*, *GLN1*, has been cloned although no sequence data has been reported (Gonzalez *et al.* 1985a).

There is only one report in the literature of the cloning of GOGAT from enteric bacteria, fungi or yeast. In *E. coli* loss of GOGAT activity results from a mutation at the *GLTB* locus. GOGAT has been cloned from *E. coli* by complementation of *gltB* (Covarrubias *et al.* 1980). Lozoya *et al.* (1980) showed that the complementing plasmid carried both genes encoding the polypeptides forming the *E. coli* GOGAT. As the plasmid carries an insert of 8.5kb, it implies that the two genes are closely linked. Garciarrubio *et al.* (1983) confirmed this and showed that the gene arrangement is: 5′- large subunit - intergenic region (0.2kb) - small subunit - 3′. As subclones of each gene can direct the synthesis of full-size products, it appears that each gene has its own promoter. Since there is no known function for either of the isolated subunits, it would be expected that equimolar amounts of each subunit would be synthesised. These workers proposed that the simplest model is that if both genes form part of a single operon, which would allow coordinated regulation of synthesis.

1.4.2.1 Several workers have isolated NADP-GDH structural gene mutations in *A. nidulans*, *N. crassa* and *S. cerevisiae*. The NADP-GDH mutations from these three species have all been found to be pleiotropic. As the *S. cerevisiae gdh1* mutants have been extensively studied, the phenotypes of the *A. nidulans* and *N. crassa* will only be briefly discussed.

Various workers have isolated and studied *gdhA* mutants of *A. nidulans* (Kinghorn and Pateman 1973, 1975, Arst and MacDonald 1973). *gdhA* strains have a decreased growth rate on ammonium and grow very poorly on inorganic nitrogen sources, but like the wild type on amino acid supplemented media. These workers also observed that the *gdhA* mutation caused loss of ammonium repression of glutamate, methylammonium, thiourea and urea uptake, plus nitrate reductase and xanthine dehydrogenase activities. NAD-GDH activity is altered in *gdhA* mutants compared to the wild type. The growth properties of the *N. crassa am* mutants are similar to those of *A. nidulans gdhA* ones. (Arst and MacDonald 1973). Kinsey (1977) found that *am* mutants have a derepressed neutral amino acid permease. Other workers (e.g. Hummelt and Mora 1980b, Dunn-Coleman *et al.* 1981) have shown that GS, GOGAT and NAD-GDH activities are altered in *am* mutants. The loss of ammonium repression in these mutants has caused speculation that NADP-GDH, either directly or by its involvement in ammonium metabolism, functions as a regulatory element of NCR.

The pleiotropic nature of the *S. cerevisiae gdh1* and its involvement in NCR has been extensively studied by Wiame and co-workers. The biochemical and genetic data have been recently reviewed (Cooper 1982a, Wiame *et al.* 1985). Biochemical models attempting to link the enzyme regulation to the presence of different quality nitrogen sources have become increasingly more complex and controversial. In view of this, and as a critical analysis of NCR in *S. cerevisiae* is beyond the scope of this work, only a summary of the genetic data and current understanding of NCR will be presented.

gdh1 mutants of *S. cerevisiae* have been isolated by selection for strains which had lost the ammonium repression of GAP (Grenson and Hou 1972) or by the slow growth on ammonium (Grenson *et al.* 1974). *gdh1* has been identified as the structural gene for NADP-GDH on the basis of altered affinities for substrate and increased thermolability of mutant enzymes (Grenson *et al.* 1974). Subsequently a fragment which complements *gdh1* has been cloned either by heterologous probing (Nagasu and Hall 1985) or complementation of the *E. coli gdhA* (Moye *et al.* 1985). The deduced amino acid sequence is highly homologous to the *N. crassa* NADP-GDH amino acid sequence (see section 1.3.1). The biosynthetic function of NADP-GDH is shown by *gdh1* mutants. The *gdh1* mutation, which abolishes NADP-GDH activity, causes a generation time on ammonium supplemented minimal medium of 240 minutes compared to 120 minutes for the wild type. Addition of glutamate causes restoration of wild type growth rates. As *gdh1* mutants still grow on ammonium as sole nitrogen source, it suggests that there is a second route for ammonium assimilation in *S. cerevisiae*. It has been shown that the major products of ammonium assimilation are glutamate and glutamine (Sims and Folkes 1964, Jones *et al.* 1969), which suggests that any alternative pathway involves these compounds.

The intracellular ammonium concentration of the *gdh1* mutant 4324c was six-fold higher than the wild type, showing that ammonium uptake is not limiting growth. The 2-oxoglutarate concentration was not significantly altered compared to the wild type (Grenson and Hou 1972). Arginase, allantoinase, GAP, NAD-GDH and urea amidolyase are all subject to ammonium repression. In a *gdh1* mutant grown in ammonium supplemented medium NAD-GDH, but not the other three enzymes or GAP, is still subject to ammonium repression (Grenson and Hou 1972, Dubois *et al.* 1973). A second mutant with altered NADP-GDH activity has been isolated. The *gdhCR* mutation causes derepression of NAD-GDH and GS plus repression of NADP-GDH in ammonium grown cells (Grenson *et al.* 1974). This mutation is unlinked to both *gdh1* and *ama*. *gdhCR* causes the release of NCR of arginase and allantoinase plus glutamine repression of GS. Currently GDHCR is believed to be a general regulatory element in the metabolism of nitrogenous compounds and have a more general function than NADP-GDH (Wiame *et al.* 1985).

1.4.2.2 Mutations at the *GLN1* locus cause loss of GS activity and tight glutamine auxotrophy, suggesting that GS is the only route for glutamine biosynthesis in *S. cerevisiae* (Dubois and Grenson 1974). Subsequently it has been shown that *GLN1* is the structural gene for GS (Mitchell 1985). Wiame and colleagues (Dubois *et al.* 1977) showed that *gln1* has no regulatory function *per se*. However, in combination with *gcnR*, *gln1* causes the loss of the NCR of NAD-GDH. In addition to being regulated by *GDH1*, NCR of allantoinase and urea amidolyase is also mediated by *gln1 gcnR*. The relationship between *GDHCR* and *GCNR* is unclear, although *GCNR* has been shown to limit glutamine transport (unpublished data in Wiame *et al.* 1985).

GS is regulated by several different control systems. The nature of the nitrogen source modulates activity over a 150-fold range. Activity is repressed in glutamine grown cells but fully derepressed in glutamate grown ones (Dubois and Grenson 1974). GS (plus NAD-GDH but not NADP-GDH) is subject to general amino acid control - this cross-pathway regulatory system couples derepression of enzymes in multiple amino acid biosynthetic pathways to starvation for any one of a number of different amino acids (reviewed in Hinnebusch 1986). The derepression of GS has been observed upon purine and pyrimidine starvation (Mitchell and Magasanik 1984c).

Mutants with altered regulation of GS by amino acid, glutamine, purine and pyrimidine starvation have been isolated and partially characterised (Mitchell and Magasanik 1984a, c). These workers isolated mutants at the *GLN3* locus. *gln3* has a pleiotropic effect; it prevents complete derepression of GS and represses NAD-GDH. By the use of two-dimensional gel electrophoresis, the coordinate regulation of GS, NAD-GDH and a family of four unknown proteins by *GLN3* was demonstrated. *GLN3* appears to regulate GS and NAD-GDH in the opposite direction to *GDHCR*.

Mitchell and Magasanik (1984c) have assigned *GLN3* to the glutamine control circuit. This is based on the observation that *gln3* eliminates most of the range of expression of GS activity, which correlates well with the relative degree of regulation caused by glutamine. Derivatives of glutamine cause complete repression of GS in glutamine-limited *gln3* mutants, indicating that the residual GS activity is modulated by the levels of these metabolites. The "missing" activity is modulated by glutamine starvation interacting with the *GLN3* product.

Although the general control of amino acid biosynthesis is mediated through the *GCN4* product, *GLN3* specifically amplifies the response of GS suggesting that the proteins interact (Mitchell and Magasanik 1984c). These workers concluded, on the basis of the hyper-derepression of GS in a *GLN3 gln1-7* mutant during the general control response, that the glutamine pool is depleted during the general control response. Additionally, although the *GCN4* and *GLN3* elements act independently, that the physiological consequences of the general control response derepress GS by activating the *GLN3* system.

Mitchell and Magasanik argue that the derepression of GS activity observed on pyrimidine starvation is achieved by the *URA2* carbomyl-phosphate synthase depleting the intracellular glutamine pool on relief of feedback inhibition. The reduced pool stimulates the *GLN3* system. It is believed that purine starvation modulates GS independently of either the general control or *GLN3* mechanisms, because GS activity is derepressed in a *gln3 gcn4* mutant upon purine starvation. No regulatory elements have been identified in this circuit.

1.4.2.3 Strains mutant in the locus encoding the NAD-GDH (*GDHB*) have been isolated in *A. nidulans* (Kinghorn and Pateman 1973, 1976) but not *N. crassa* and *S. cerevisiae*. These workers showed that *gdhB* mutants could use both organic and inorganic nitrogen compounds as sole nitrogen source, although they could not use glutamate as sole carbon source. It is unlikely that this phenotype would be observed in *S. cerevisiae*, because *Saccharomyces* spp. can only use organic nitrogen compounds as nitrogen, and not carbon, sources (Wiame *et al.* 1985). The regulation of nitrate reductase and xanthine dehydrogenase activity plus glutamate and thiourea uptake were not affected in the *gdhB* mutant. This would suggest that NAD-GDH is not a regulatory element modulating nitrogen metabolism.

Middlehoven and co-workers (Middlehoven *et al.* 1978) have isolated mutants of *S. cerevisiae* lacking NAD-GDH activity. The mutants were isolated by their inability to use glutamate as sole nitrogen source, although its use as sole carbon source was not tested. However, these workers concluded that the lack of NAD-GDH activity was due to loss of the ability to induce NAD-GDH and not a structural gene defect.

1.4.2.4 GOGAT structural gene mutants have been isolated from the *B. megaterium* (Elmerich and Aubert 1971), *B. subtilis* (Desphande and Kane 1980), *E. coli* (Berberich 1972), *K. aerogenes* (Brenchley *et al.* 1973) and *Sal. typhimurium* (Dendinger *et al.* 1980), and the filamentous fungus *N. crassa* (Hummelt and Mora 1980b, Dunn-Coleman *et al.* 1981, Romero and Davila 1986).

The bacterial GOGAT is known to be heteromeric, although the *N. crassa* enzyme is composed of a single subunit species. The loss of GOGAT activity in *B. subtilis* has been shown to be caused by one of two unlinked mutations, *gltA* and *gltB*, presumably encoding the two different subunits (Desphande and Kane 1980). The loss of GOGAT activity in *E. coli* is due to mutations at the *gltB* locus, despite the fact that the native enzyme is a multimer of non-identical subunits. Subsequently it has been shown that the genes encoding the two subunits are tightly linked and are probably part of a single operon (see section 1.4.1). In contrast, the subunit of the *N. crassa* enzyme has been shown to be encoded by a single gene, *en(am)2* (Romero and Davila 1986). No mutations affecting the regulation of GOGAT have been reported.

The GDH$^-$ mutants of the Gram-negative bacteria and *N. crassa* are leaky glutamate auxotrophs. The presence of *glt* or *en(am)2* in a GDH$^-$ strain results in tight glutamate auxotrophy, which shows that ammonium assimilation by GOGAT is responsible for the residual growth of GDH$^-$ mutants on ammonium. Studies of a *GDH glt* strain of *K. aerogenes* have shown that this strain cannot use ammonium at concentrations below 1mM, although normal growth is observed at higher concentrations (Brenchley *et al.* 1973). In contrast, wild type growth of a *E. coli GDHA gltB* construct is observed at 0.1mM NH_4^+ (Pahel *et al.* 1978) The *K. aerogenes* NADP-GDH is repressed at low ammonium

concentrations, and the repressed enzyme level is insufficient to compensate for the lack of GOGAT activity. The *E. coli* NADP-GDH is not repressed at limiting ammonium concentrations, and so ammonium assimilation can occur through this pathway even at very low concentrations (Brenchley *et al.* 1973, Senior 1975). *GDH glt* constructs of *E. coli* (Pahel *et al.* 1978), *K. aerogenes* (Brenchley *et al.* 1973) and *Sal. typhimurium* (Dendinger *et al.* 1980) cannot grow on nitrogen compounds e.g. arginine, which are assimilated slowly *i.e.* generate a low intracellular ammonium concentration.

On the basis of the phenotype of *GDH glt* constructs, it has been concluded that, in these species, GOGAT is involved in the assimilation of ammonium when the nutrient is present at a limiting concentration.

1.4.3 As has been discussed in the previous section, some enzymes and products of ammonium assimilation are possibly effectors of NCR. In this section a summary of the current understanding of NCR and the role of these effectors will be presented.

It has been observed that there are three classes of enzyme subject to NCR (Dubois *et al.* 1977): (i) enzymes e.g. arginase subject to ammonium repression, which is relieved in *gdh1* mutants; (ii) those enzymes e.g. NAD-GDH subject to glutamine repression which is relieved in *gln1 gcnR* strains; and (iii) enzymes e.g. allantoinase and urea amidolyase which are repressed by both ammonium and glutamine, which is relieved in *gdh1* or *gln1 gcnR* mutants. This indicates that there are two types of regulatory element involved in NCR, a genetic one and a metabolic one (ammonium or glutamine, or their derivatives).

It is important for the understanding of the following discussion, to note that the addition of glutamate or glutamine to a *gdh1* mutant does not restore NCR.

Wiame and co-workers have studied the NCR of the enzymes of arginine catabolism (reviewed in Wiame *et al.* 1985). Arginase in a *gln1* strain is subject to normal NCR by either ammonium or glutamine. In chemostat culture, limited for glutamine, a *gln1* strain is derepressed for NCR sensitive enzymes including arginase and NAD-GDH. The addition of ammonium to this culture restores arginase to the level observed in the wild type grown with excess ammonium. This suggests that NCR of arginase is not mediated by conversion of ammonium to glutamine, but by ammonium directly. Although NAD-GDH activity is repressed by ammonium, it is not derepressed in a *gdh1* mutant, but NCR is relieved by glutamate. In a *gln1* strain, grown as described above, NAD-GDH activity is completely insensitive to ammonium. This showed that ammonium must be converted to glutamine before repression of NAD-GDH by ammonium can be affected. It also suggested the existence of two regulatory circuits, one ammonium and the other glutamine dependent (Dubois *et al.* 1977).

The role of NADP-GDH in mediating NCR has been investigated in derivatives of Σ1278b which have lost the ability to induce urea amidolyase [*durM*] (Wiame *et al.* 1985). Wild type strains grown in glutamate medium derepress this enzyme 15-fold compared to ammonium grown cells and about six-fold less than urea grown ones. *durM* strains grown with glutamate or urea, or wild type grown with glutamate, derepress urea amidolyase to the same degree. In absence of induction, and being non-inducible, this is solely the result of release from NCR. The *gdh1* mutation alone, or in combination with *durM*, promotes enzyme synthesis even in the most repressive medium (ammonium plus glutamine). Wild type and *durM* strains exhibit almost complete repression of urea amidolyase. Although the data does not prove or disprove the hypothesis that the ammonium effect is mediated by NADP-GDH, Wiame and co-workers concluded that the ammonium-NADP-GDH regulatory hypothesis remained an attractive one for Σ1278b (Wiame *et al.* 1985).

It has been shown that the *gdhCR* mutation has a derepressive effect on all enzymes sensitive to NCR (Wiame *et al.* 1985). NAD-GDH has been shown to be insensitive to *gdh1* and regulated by the glutamine circuit: the converse is true for arginase. The NCR of allantoinase and urea amidolyase is mediated by both regulatory circuits. Consequently it was concluded that *GDHCR* is a common element of both ammonium-NADP-GDH and glutamine circuits.

The data show that GS and NADP-GDH are important elements of NCR of *S. cerevisiae*. Although it has been shown that *gln1* has no regulatory function *per se* (Dubois *et al.* 1977), GS is an important element in NCR because it is the only route for glutamine biosynthesis in *S. cerevisiae* (Dubois and Grenson 1974). The *GDH1* product NADP-GDH has been considered as a regulatory element mediating the ammonium and 2-oxoglutarate signals. This hypothesis is based upon the result that addition of glutamate to a *gdh1* mutant, which compensates for the main catalytic defect of this mutation, does not restore the ammonium effect. The validity of this hypothesis has not been proved or disproved.

Currently GDHCR is viewed as the central repressor exerting negative control on all elements subject to NCR (Wiame *et al.* 1985). GDHCR is thought to be activated by either glutamine or NADP-GDH, mediating the ammonium and 2-oxoglutarate signals (see section 1.5.3). Wiame and colleagues believe that this will generate distinct repressor elements capable of interacting with different receptors. This activated element is postulated to act at a level, as yet unknown, prior to the formation of functional proteins.

1.5 The Physiology of Ammonium Assimilation

The assimilation of ammonium has been studied in a large number of microorganisms, both prokaryotes and eukaryotes. Extensive studies have been done in *E. coli*, *K. aerogenes* and *N. crassa*.

As the physiology of ammonium assimilation is best understood in these microorganisms, the discussion in this section will be biased towards them and to *S. cerevisiae*.

1.5.1 Kinetic data suggested that NADP-GDH, because of its high K_m for ammonium (greater than 2mM), could not assimilate ammonium in cells grown at low ammonium concentrations. The high affinity of GS for ammonium (K_m below 1mM), when coupled with GOGAT (K_m for glutamine of 0.3mM) has been proposed as the route for ammonium assimilation under these conditions. An alternative explanation is that an increase in NADP-GDH activity compensates for the low substrate affinity of this enzyme.

Chemostat studies of *Aer. aerogenes* and *K. aerogenes* have shown that NADP-GDH activity was reduced under conditions of ammonium limitation compared to those of ammonium excess. However, in ammonium-limited cultures GS and GOGAT activities were elevated compared to ammonium-excess cultures (Tempest *et al.* 1970, Brenchley *et al.* 1973, Senior 1975). It was concluded that GS-GOGAT was the major pathway for ammonium assimilation at limiting concentrations, and NADP-GDH was used when ammonium was present in excess. This is in agreement with the prediction based on the kinetic data. Brenchley *et al.* (1973) have confirmed this for *K. aerogenes*. *GDH glt* mutants were found to be unable to grow at ammonium concentrations below 1mM, although *gdh* strains could grow with excess or limiting ammonium. This showed that GOGAT is involved in the synthesis of glutamate at concentrations too low for NADP-GDH to function, confirming the role of GS-GOGAT suggested by the kinetic and enzyme data.

As ammonium assimilation proceeds via different pathways depending upon the ammonium concentration, it suggests that regulatory systems have evolved to regulate GS and NADP-GDH activities. In *K. aerogenes* it has been observed that a high level of GS activity is associated with low levels of NADP-GDH, with the converse being true (Brenchley *et al.* 1973, Senior 1975). Brenchley *et al.* (1973) suggested that the decrease in NADP-GDH activity was a consequence of an increase in GS activity. This was based on the observation that *gln* mutants produced high levels of NADP-GDH in ammonium-limited culture, yet mutants constitutive for GS production were repressed for NADP-GDH synthesis even in medium with excess ammonium. It is unclear how GS regulates NADP-GDH, although the GS molecule itself has been implicated as a regulatory element for the transcription of genes involved in nitrogen metabolism (Tyler 1978).

Similar chemostat studies to those described above have been done with various species of yeast. It has been observed (Brown *et al.* 1973, Johnson and Brown 1974) that, in *Schiz. pombe* and *malidevorans* plus *Saccharomycodes ludwigii*, GOGAT activity was not influenced by the ammonium concentration. NADP-GDH activity was unaltered in the two *Schizosaccharomyces* species, although it increased two-

fold in *Saccharomycodes ludwigii* grown with excess compared to limiting ammonium. In *Schiz. pombe*, GS activity was detectable in cultures grown with limiting but not excess ammonium. For *Saccharomycodes ludwigii*, GS activity of ammonium-limited cultures was about twice that of ammonium-excess ones. On the basis of the enzyme kinetics (section 1.3.1) and relative enzyme levels, these workers concluded that the three yeasts assimilate limiting ammonium by the GS-GOGAT pathway.

Alternative patterns of regulation of the enzymes of ammonium assimilation have been found, which suggests that the relative contribution to ammonium assimilation is different in other microorganisms. Miller and Stadtman (1972) found that NADP-GDH and GOGAT activities in *E. coli* grown with 4 and 20mM NH_4^+ were very similar, although GS activity was markedly lower in the 20mM NH_4^+ culture. Senior (1975) studied ammonium-limited chemostat cultures of *E. coli*. At low specific growth rates, where no ammonium was detectable in the medium, GS was unadenylated and its activity high. It remained so at all growth rates tested. As the specific growth rate increased, GS activity decreased slightly. However, as the maximum specific growth rate was approached, ammonium could be detected in the medium, and GS became repressed. In contrast, NADP-GDH activity increased as the specific growth rate increased - the rise in activity reached a plateau at the point where GS was repressed. GOGAT activity remained relatively constant at a low level as the specific growth rate increased, but at the point where GS was repressed, it exhibited a rapid increase in activity. This result contrasts with the situation in *K. aerogenes* where a reciprocal relationship between GS and NADP-GDH was observed.

From this data it would appear that, when subject to ammonium limitation, *E. coli* synthesises elevated levels of GS which is maintained in its most active form *i.e.* unadenylated. In ammonium-limited cultures it is assumed that the intracellular metabolite pools are depleted, so feedback inhibition of GS is probably unimportant. As the external ammonium concentration is increased, GS activity decreases and NADP-GDH activity increases.

Although both *E. coli* and *K. aerogenes* exhibit high GS activities in ammonium-limited growth, they assimilate ammonium by different routes. *K. aerogenes* maintains complete repression of NADP-GDH until ammonium is present in the medium when GS is repressed. *E. coli* synthesises NADP-GDH in response to the increased availability of ammonium. As has been argued above, the kinetic data suggest that GS-GOGAT should function at limiting ammonium, and NADP-GDH at excess levels. Genetic and chemostat studies confirm this proposal for *K. aerogenes*. The chemostat studies suggest that the *E. coli* NADP-GDH, despite its low affinity for ammonium, has a biosynthetic role even in ammonium-limited cultures. This is confirmed by the phenotype of a *GDHA gltB* mutant which can grow at ammonium concentrations down to 0.1mM (Pahel *et al.* 1978).

1.5.2 Mora and co-workers (Quinto *et al.* 1977, Vichido *et al.* 1978, Davila *et al.* 1980, Hummelt and Mora 1980a, b, Lara *et al.* 1982, Lomnitz *et al.* 1987) have studied the physiology of ammonium assimilation by *N. crassa*. These workers have found that ammonium assimilation by this organism involves four enzymes: NADP-GDH, GOGAT and the two isozymes of GS. Cultures of wild type *N. crassa* grown with either excess or limiting ammonium have high levels of NADP-GDH activity, although growth with limiting ammonium causes an increase in GS and GOGAT activity. It has been shown that the predominant form of GS in ammonium-limited cultures is a tetramer of αsubunits (GSα) whereas in ammonium-excess or glutamate -limited ones it is an octomer of βsubunits (GSβ). The change in the predominant form of GS is effected at the level of transcription. The presence of two isozymes of GS under different conditions suggests that they are involved in different assimilatory pathways.

It has been shown that a GOGAT$^-$ mutant grows as well as the wild type in excess ammonium, suggesting that GOGAT is not important for ammonium assimilation under these conditions. The GS mutant grows equally as well as the GS$^-$ GOGAT$^-$ one, although the growth is markedly worse than the wild type. It would appear that GSβ is important for ammonium assimilation under these conditions. The growth of a GDH$^-$ strain is very poor, while a GDH$^-$ GSβ^- one grew only very slightly. This indicates that NADP-GDH has a very important function in ammonium assimilation by *N. crassa* under conditions of ammonium excess.

It was found that the intracellular ammonium concentration in GSβ^- GOGAT$^-$, GSβ^- or GOGAT$^-$ mutants grown with excess ammonium was at the wild type level. However, a marked increase was observed in the GDH strain, and the level in the GSβ^- GDH$^-$ mutant was much higher than that of the GDH$^-$ strain.

On the basis of this data, Mora and co-workers concluded that ammonium assimilation by *N. crassa* grown in excess ammonium was mediated by NADP-GDH and GS .

Examination of ammonium-limited cultures showed that, in the wild type, GOGAT and NADP-GDH levels were unchanged although GS activity had increased. In GDH$^-$ and GOGAT$^-$ strains, both remaining enzymes exhibited increased activity. No accumulation of ammonium was observed in the wild type, GSβ^-, GOGAT$^-$ or GSβ^- GOGAT$^-$ strains, unlike the GDH$^-$ mutant where it had. This suggests that a major route of ammonium assimilation by *N. crassa* in ammonium-limited cultures is by the NADP-GDH.

In the GSβ^- mutant, the GOGAT activity was unchanged although NADP-GDH activity had increased. NADP-GDH activity also increased in the GSβ^- GOGAT$^-$ mutant. Glutamate accumulated in the GSβ^- mutant. This probably occurs because the low activity of GSα cannot metabolise the glutamate,

synthesised by the NADP-GDH, sufficiently quickly to prevent its accumulation. The GSβ^-GOGAT$^-$ mutant transiently accumulated glutamate, and also showed a steady increase in the glutamine pool. This is probably due to the high residual GSα activity in this mutant. Although GSα is the predominant form of GS under these conditions, *gln-1b* eliminates most of the measured GS activity.

It would appear that *N. crassa* uses both NADP-GDH and GS-GOGAT to assimilate ammonium present at limiting concentrations. The observation that there is ammonium accumulation in GDH$^-$ but not GSβ^- mutants suggests that, under both conditions, NADP-GDH is the major route for ammonium assimilation. As *gln-1* mutants are glutamine auxotrophs, it suggests that the function of GS is to synthesise glutamine. If so, then what is the primary function of GOGAT, if it is not to synthesise glutamate at limiting ammonium concentrations?

Mora and co-workers (Lomnitz *et al.* 1987) have proposed that the primary function of GOGAT in *N. crassa* is the recycling of organic nitrogen from glutamine to glutamate. The large glutamate pool observed in ammonium-limited wild type *N. crassa* may ensure substrate availability for the efficient operation of reversible transamination reactions. The GOGAT$^-$ strain, grown under both conditions, exhibits a low ratio of glutamate to glutamine. Therefore GOGAT could function to recycle glutamine to glutamate, thereby maintaining the correct glutamate:glutamine ratio. This would ensure the correct distribution of nitrogen by the various transamidation and transamination reactions.

Recent studies (Kusnan *et al.* 1987) have suggested that the pattern of ammonium assimilation by *A. nidulans* is very similar to that of *N. crassa.* Earlier workers had shown that *gdhA* and *gdhA gdhB* mutants of *A. nidulans* grow on ammonium as sole nitrogen source, although more slowly than the wild type (Kinghorn and Pateman 1973, 1976). This suggested that NADP-GDH is an important route for ammonium assimilation plus the existence of an alternative assimilatory pathway.

Kusnan and co-workers observed that GS, NAD- and NADP-GDH activities in ammonium-limited cultures were derepressed two-three-fold compared to ammonium-excess culture, although GOGAT activity was not increased markedly. For both conditions, NADP-GDH activity was very much higher than that of GS. Azaserine, which inhibits GOGAT but not GDH, caused a 60% decrease in the glutamate pool when added to either ammonium-excess or -limited cultures. Complete depletion of the glutamate pool was not observed. These workers concluded that the loss of glutamate was due to the inhibition of the GS-GOGAT pathway, and that the residual glutamate was synthesised by NADP-GDH. It would appear that *A. nidulans* uses NADP-GDH and GS-GOGAT to assimilate ammonium irrespective of the external concentration.

1.5.3 Thomulka and Moat (1972) studied ammonium assimilation by *S. cerevisiae.* They observed that NADP-GDH had a markedly higher activity than either GS or NAD-GDH in ammonium supplemented

cultures. GS activity was minimal during the propagation, even though the ammonium concentration was limiting (4mM). From this observation it was suggested that GS has a relatively minor role in ammonium assimilation by *S. cerevisiae*. NADP-GDH activity was greatest when the culture was in the transition between lag and exponential growth phases *i.e.* when there was a very large demand for synthetic activity. It was concluded that in *S. cerevisiae* the primary route for ammonium assimilation is by the NADP-GDH. The demonstration that glutamate is a primary product of ammonium assimilation (Jones *et al.* 1969) supports this conclusion.

Burn *et al.* (1974) examined NADP-GDH activity in *S. cerevisiae* grown with 4 or 20mM NH_4^+. NADP-GDH activity was found to be higher in ammonium-limited cultures compared to ammonium-excess ones, although NAD-GDH was not derepressed. These workers concluded that NADP-GDH is the primary route for ammonium assimilation in cultures with excess or limiting ammonium. They argued that the increase in NADP-GDH activity compensated for its low affinity for ammonium. This contrasts with the data obtained by other workers (Roon and Even 1973, Bogonez *et al.* 1985). Roon and Even found that at external ammonium concentrations greater than 1mM, NADP-GDH activity was constant at a high level, but at lower concentrations the activity decreased three-fold. The data suggest that the primary route for ammonium assimilation at external ammonium concentrations above 1mM is by NADP-GDH. At lower concentrations, the route for assimilation is unclear as no data were reported for GS or GOGAT activities. The reason for the difference in results from the two groups is unclear.

These three groups reported minimal NAD-GDH activity in ammonium medium. Activity was derepressed in glutamate medium, confirming the catabolic function assigned to this enzyme (Bernhardt *et al.* 1965, 1966)

Bogonez *et al.* (1985) studied the regulation of the *S. cerevisiae* NADP-GDH by ammonium over a wider range of external concentrations than Roon and Even (1973) (720 *cf.* 5mM NH_4^+). Bogonez and colleagues noticed a rapid increase in activity as the external ammonium concentration was raised to about 10mM, maximal activity was observed at 24mM although at higher concentrations the activity was decreased. The intracellular 2-oxoglutarate concentration varied only slightly. However, the ammonium and glutamate pools expanded as the external ammonium concentration increased, although a constant size was observed at 80-160mM NH_4^+ . Variation in the intracellular ammonium concentration, achieved either by growth with acetate or adjusting the medium pH, was found to alter the NADP-GDH activity. Consequently these workers suggested that the intracellular ammonium concentration acted as a negative regulatory element to modulate NADP-GDH activity.

Calculation of the theoretical flux through NADP-GDH at the different ammonium concentrations showed that the rate of glutamate synthesis was constant. These workers suggested that NADP-GDH

activity varied in response to changing ammonium levels in order to maintain a constant 2-oxoglutarate pool. Consequently it would appear that the maintenance of a minimal level of 2-oxoglutarate is of central importance to the cell, and that NADP-GDH, ammonium and glutamate levels are modulated to achieve this.

The effect of altered 2-oxoglutarate levels on NADP-GDH activity has been investigated (Gonzalez *et al.* 1985b). It was found that an aconitase mutant (*i.e.* deficient in 2-oxoglutarate synthesis) accumulated ammonium in ammonium medium. NADP-GDH activities in wild type and the aconitase mutant grown with ammonium + glutamate were comparable. The mutant had greater than wild type NADP-GDH activity in glutamate medium, although the converse was true for ammonium medium. These results were interpreted as showing that the low NADP-GDH activity in the ammonium grown mutant was because the lack of 2-oxoglutarate (or other TCA cycle intermediate) prevented the derepression/induction of NADP-GDH. The 2-oxoglutarate generated by the degradation of glutamate is assumed to be sufficient to induce NADP-GDH. The extent of the induction of NADP-GDH in mutants grown with glutamate is less than that in ammonium grown wild type, probably because glutamate represses NADP-GDH. The observation that NADP-GDH activity in glutamate grown wild type cultures was lower than in ammonium ones supports this view.

Evidence from chemostat studies supports the view of 2-oxoglutarate as a positive regulatory element. In ammonium-excess glucose-limited cultures, it was observed that as the intracellular glutamate and 2-oxoglutarate levels increased NADP-GDH activity also increased. Earlier work had suggested that glutamate represses NADP-GDH (Roon and Even 1973, Gonzalez *et al.* 1985b). The increase in NADP-GDH was attributed to an increase in 2-oxoglutarate.

It has been suggested that glyoxylate modulates the functioning of the TCA cycle by inhibiting citrate synthase. As glyoxylate is not metabolised to 2-oxoglutarate, glyoxylate grown cells should be depleted for 2-oxoglutarate. The lack of 2-oxoglutarate and increased ammonium pool should cause a decrease in NADP-GDH activity and, therefore, ammonium assimilation. However, analysis of glyoxylate grown *S. cerevisiae* showed an accumulation of both ammonium and 2-oxoglutarate (Gonzalez *et al.* 1987). The increase was proposed to be a consequence of the inhibition of NADP-GDH by glyoxylate (glyoxylate has been shown to inhibit NADP-GDH *in vitro*). It was suggested that this inhibition provides a mechanism which compensates for the decreased carbon flux, due to inhibition of citrate synthase, and maintains a constant 2-oxoglutarate pool. This mechanism could be important when the yeast uses a poor carbon source e.g. acetate, as under these conditions the synthesis of 2-oxoglutarate is reduced.

Acetate grown cells have a high intracellular ammonium level, which represses NADP-GDH. The metabolism of acetate generates a low level of 2-oxoglutarate, consequently a low level of NADP-GDH activity is required to maintain the pool size. It has been reported that the enzymes which participate in glyoxylate biosynthesis are induced in acetate grown cells. The synthesis of glyoxylate further inhibits NADP-GDH functioning. This indicates the complex manner in which nitrogen and carbon metabolism interact to maintain a constant 2-oxoglutarate pool.

Addition of glyoxylate to ammonium grown cells caused a rapid depletion of the glutamate pool and a transient decrease in the glutamine pool. The alanine and glycine pools increased. This suggested to Gonzalez and co-workers that high intracellular concentrations of 2-oxo acids cause channeling of glutamate and glutamine towards transamination pathways. The decreased glutamine pool would derepress GS and increase ammonium assimilation through GS-GOGAT. It should be noted that GOGAT is inhibited by some 2-oxo acids (Masters and Meister 1982). The total ammonium assimilated was not decreased in glyoxylate grown cultures, even though NADP-GDH was decreased. This implies the functioning of an alternative pathway *i.e.* GS-GOGAT. It would appear that glyoxylate effects the coordinate regulation of carbon and nitrogen metabolism by modulating the activity of both the TCA cycle and NADP-GDH.

From this work it would appear that NADP-GDH is regulated in opposite directions by its two substrates. Ammonium acts in a negative manner by repressing NADP-GDH: 2-oxoglutarate in a positive manner by inducing the enzyme. Bogonez *et al.* (1985) showed that ammonium acts at the level of transcription. They found that the rate of NADP-GDH degradation was the same in ammonium-accumulating cultures as in controls, although synthesis is severely inhibited. A loss of enzyme is probably achieved by dilution due to growth over several generations *i.e.* the response is slow compared to GS or NAD-GDH which lose about half their activity in 30 minutes (see section 1.3). This suggests the existence of other fine control mechanisms to achieve a rapid response. It has been shown that the *N. crassa* NADP-GDH is an allosteric enzyme subject to substrate activation by both ammonium and 2-oxoglutarate (see section 1.3.1). It is possible, given the considerable homology between the yeast and fungal enzymes, that the *S. cerevisiae* enzyme also exhibits allosteric properties. So one of the factors mediating short term control of the *S. cerevisiae* NADP-GDH could be substrate activation.

It has been shown that GS is the only enzyme which can synthesise glutamine in *S. cerevisiae* (Dubois and Grenson 1974, Mitchell 1985). However, there is no published data on how its activity is regulated by the ammonium concentration. Dubois and Grenson showed that GS is derepressed in glutamate grown cells. Although glutamate is considered a poor nitrogen source it is unclear how this relates to

growth with limiting ammonium. Thomulka and Moat (1972) demonstrated that GS activity was minimal in cells grown with 4mM NH_4^+ , and markedly lower than the NADP-GDH activity.

Roon *et al.* (1974) detected GOGAT activity in *S. cerevisiae*. They found that changes in GOGAT activity paralleled the changes in the NADP-GDH. Highest activity was observed in cultures with excess ammonium, and lowest in nitrogen starved cultures. An intermediate level was observed in glutamate cultures. Growth with 1mM NH_4^+ , or with poor nitrogen sources, caused a 20-30% decrease in activity compared to the ammonium-excess culture. However, NADP-GDH activity was ten-fold higher even under these conditions.

On the basis of the relative enzyme activities (NADP-GDH activity is ten-fold higher than GS-GOGAT levels) it would appear that NADP-GDH is the primary route for ammonium assimilation by *S. cerevisiae*. Examination of enzyme levels reported in the literature for different growth conditions suggests that the GS-GOGAT pathway makes, at most, only a minor contribution to glutamate biosynthesis, even in ammonium-limited cultures. This raises the question of what is the primary function of GOGAT in *S. cerevisiae*? It may be that it is involved in glutamine recycling. The slow growth of *gdh1*, and the inability of *gdh1 ama* (which lacks GOGAT activity) to grow, on ammonium as sole nitrogen source, suggests an assimilatory role for GOGAT. Wiame *et al.* (1985) have proposed that this is a consequence of the *gdh1* mutation and does not occur in the wild type. They argue that the *MEP2* function could concentrate ammonium sufficiently to enable NADP-GDH to function. Gonzalez *et al.* (1985) have suggested that GS-GOGAT functions in ammonium assimilation in *S. cerevisiae* grown with poor carbon sources. As GOGAT structural gene mutants of *S. cerevisiae* were not available, studies to investigate these possibilities have not been done.

In conclusion, there is a considerable difference in the understanding of ammonium assimilation by various microorganisms. Currently, *S. cerevisiae* is relatively poorly understood. It would appear that the primary route in *S. cerevisiae* is by the NADP-GDH, with little contribution (even at limiting concentrations) from the GS-GOGAT pathway. In this respect it is different from other microorganisms where GS appears to make a major contribution to ammonium assimilation, at least at limiting concentrations. The significance of the different patterns of ammonium assimilation is unclear. Further work is required to improve the understanding of ammonium assimilation by *S. cerevisiae*.

1.6 Industrial Yeast Strains

Laboratory and industrial strains of *S. cerevisiae* exhibit marked differences. There is considerable variation in cell size between industrial and laboratory strains. The industrial ones are much larger, which causes the formation of larger colonies on solid media. Certain characteristics have been bred into industrial strains, largely by trial-and-error due to the (probably) complex polygenic nature of the

traits. Bakers' yeasts have been bred for the ability to ferment complex sugars in dough with the concomitant production of large quantities of CO_2. The ideal bakers yeast will have a short, but consistent, fermentation time with a high initial fermentation rate. An additional property is a high yield of yeast biomass per unit substrate during yeast propagation. In contrast, brewers' yeasts are bred for high alcohol production and tolerance, and the production of essential flavours. Other desirable traits are the ability to use the majority of the sugars in the wort, and easy separation from the medium. Haploid derivatives of commercial strains have usually lost some of these characteristics or do not perform as well as the parent (reviewed in Johnston and Oberman 1979). Different characteristics e.g. fertility and heterothallism, have been selected for in laboratory strains. The different breeding programmes have resulted in marked dissimilarity between the two types of yeast strain.

Genetic studies of *S. cerevisiae* have also shown considerable heterogeneity between different commercial strains and with laboratory ones. Various workers have examined the chromosome complement of a range of *S. cerevisiae* strains by orthogonal field alteration or pulsed field gradient electrophoresis. These techniques separate intact yeast chromosomses.

Johnston and Mortimer (1986) compared the chromosome complement of industrial and laboratory *S. cerevisiae* strains. These workers observed minor chromosome length polymorphisms (CLPs) among six of the seven laboratory strains examined. The majority of the thirteen baking, brewing, and distillery strains examined, exhibited unique banding patterns after electrophoresis. Heterogeneity in band intensities was also observed in these gels. This is consistent with the proposition (based on DNA measurements) that commercial strains exhibit both aneu- and polyploidy (Johnston and Oberman 1979). DNA measurements suggest that the ploidy level of bakers' and brewers' yeasts range from two to six.

von Wettstein (1986) reported a preliminary analysis of the molecular genetics of brewers' and distillers' yeasts. It was reported that a production lager strain from the Carlsberg brewery was amphiploid *i.e.* a permanent hybrid with respect to chromosome structure and gene organisation. A distillery yeast was found to be tetrasomic for at least seven chromosomes. Although the linkage maps are conserved, nucleotide sequence polymorphisms among the four copies of a chromosome were found to be common. This data shows the existence of considerable genetic heterogeneity between the different commercial strains and within a single strain, and with laboratory strains.

The strains of *S. cerevisiae* used in modern yeast genetics were constructed by interbreeding strains of *S. cerevisiae*, *S. uvarum* and *S. chodati*, some of which came from an industrial source. As has been described above, commercial yeast strains exhibit considerable heterogeneity; consequently variation would be expected in the wild type isolates of different workers. The existence of CLPs among

laboratory strains has been described (Johnston and Mortimer 1986, and references therein). This heterogeneity is reflected at the biochemical level. For example, the existence of two ammonium permease functions has been reported for Σ1278b compared to the one of X2180-1A (Roon *et al.* 1975, Dubois and Grenson 1979). Some strains have been found to secrete an extracellular asparaginase which is not present in other strains (see Cooper 1982a), while the existence of two isozymes of the GS subunit has been postulated (Mitchell and Magasanik 1983). Laboratory strains exhibit less genetic variation than industrial strains, yet there is biochemical dissimilarity between the different wild type isolates. Consequently, considerable biochemical heterogeneity between industrial strains would be expected, especially as they have been bred for certain traits, depending upon their use.

Biochemical characterisation of industrial strains has been dictated by the nature of the product. Brewers' and distillers' yeasts are used to produce potable alcohol, which has certain flavour/taste characteristics. Bakers' yeasts have been selected for the ability to ferment complex sugars in bread doughs. Consequently biochemical studies have been biased towards fermentation and related processes in these environments. Such studies are often commercially sensitive and are not published. Although studies of nitrogen metabolism by industrial strains have been reported, they have generally been concerned with amino acid absorption from wort (e.g. Jones *et al.* 1969). Consequently, little is known about ammonium metabolism during commercial propagations.

1.7 Aims

Fig. 1 shows the routes used by *S. cerevisiae* to incorporate ammonium into amino and amido nitrogen. There are two alternative routes: (i) via NADP-GDH; and (ii) by the GS-GOGAT (under certain conditions). Examination of the energy requirement of each route shows that NADP-GDH requires 1mole NADPH to synthesise 1mole of glutamate from 1mole ammonium. The GS-GOGAT pathway is more energy expensive, it requires the equivalent of one NADPH plus one ATP. The higher energy requirement for the GS-GOGAT pathway is a potential source of inefficiency in the conversion of substrate into biomass. If the efficiency of substrate conversion could be improved, it might be reflected in improved biomass yields. This has been demonstrated for the bacterium *Methylophilus methylotrophus*, used in the ICI process for the production of single cell protein (Windass *et al.* 1980).

M. methylotrophus possesses only the GS-GOGAT pathway. A GOGAT-mutant of *M. methylotrophus* was transformed with the *E. coli GDHA* gene. The recombinant strain was found to transform more substrate into biomass than the parental one. The underlying aim of the work reported below was to determine if *S. cerevisiae* would benefit from similar manipulations to those described for *M. methylotrophus*. The main product of Distillers Company (Yeast) Ltd. is yeast biomass, for both the

baking and distilling industries. So yeasts with improved growth rates and/or biomass yields are of considerable interest to the collaborating body.

This part of the project can be divided into three phases. Firstly, isolation and characterisation of GOGAT mutants in *S. cerevisiae*. Although structural gene mutants have been isolated in a number of microorganisms, none had been reported in *S. cerevisiae*. Additionally, such mutants would allow investigation of the function of GOGAT in *S. cerevisiae*. It was not necessary to isolate *gdh1* mutants as these were already available (see section 1.4.2.1).

Secondly, cloning of the *S. cerevisiae GDH1* gene. This was of interest *per se* because no yeast *GDH* genes had previously been cloned. Additionally, it was proposed to investigate the effect of elevated NADP-GDH levels on biomass production.

Thirdly, the construction of strains with different combinations of the GDH- and GOGAT- mutations, and the cloned gene. It was intended to investigate the growth characteristics (*i.e.* biomass yield and growth rate) of these constructs in shake-flask culture, and therefore determine whether *S. cerevisiae* would benefit from similar manipulations as *M. methylotrophus*. The growth of the constructs at different ammonium concentrations would also allow the study of the contribution of the two pathways to ammonium assimilation. Such basic knowledge is important to anyone propagating yeast for biomass.

As has been stated in the previous section, there are few comparative biochemical studies on industrial yeast strains and little is known about ammonium assimilation in these strains. The effect of a GOGAT- mutation on the growth characteristics of different constructs was studied in a laboratory model. In order to extrapolate from this model to the industrial system, an improved understanding of the physiology of ammonium assimilation by industrial yeast strains is required. Ammonium assimilation by two DC(Y)L production strains was compared with that of a laboratory wild type using shake-flask culture. Due to the lack of data about the physiology of nitrogen metabolism in industrial processes, ammonium assimilation by the two industrial strains was studied in simulated commercial propagations.

Chapter 2

Materials and Methods

2.1 Strains

The strains of *S. cerevisiae* used in this study are listed in Tables 1 and 2. *Schiz. pombe* strains are given in Table 3.

S. cerevisiae 4324c carries the *gdh1-1* allele and is isogenic to the wild type strain 1278b (Grenson and Hou 1972). Strain 4324c is the parent used in the mutagenesis experiments described below. To ensure a standard genetic background in the complementation and growth tests, the original GLU$^-$ mutants were backcrossed to a derivative of 4324c, AR2. Suitably marked derivatives (the series AR5 to AR43) were isolated from the resulting diploids by mass spore isolation. NADP-GDH and GOGAT were assayed in these strains to confirm the phenotype. It was not possible to isolate derivatives of strains 4-5, 4-8 and 4-17. The reasons for this are not clear but all the GLU$^-$ mutants show weak mating activity and are poor sporulators.

Strains ART10-1a to 1d and ART10-2a to 2d are progeny of the cross AR2 x 4-10. They were isolated by tetrad dissection and each set of four strains represents the contents of a single ascus.

AR44 was obtained by tetrad dissection of asci from the cross AR3 x 4-10. The function of this cross was to generate GDH$^+$ GOGAT$^-$ strains for growth tests. Phenotypically such strains will be GLU$^+$ *i.e.* indistinguishable from GDH$^+$ GOGAT$^-$ and GDH$^-$ GOGAT$^+$ strains. GLU$^+$ strains were obtained from the cross and NADP-GDH plus GOGAT activities measured. AR44 was shown, on the basis of its enzyme activities, to be GDH$^+$ GOGAT$^-$.

DCL1 and DCL2 are production strains used by DC(Y)L. DCL1 is used to produce compressed wet yeast, while DCL2 is used for small particle active dried yeast (SPADY) production. These strains are prototrophic and are thought to be aneuploid, since they contain approximately 3.5 times as much DNA as that present in haploid laboratory strains (D. Wright, pers. comm.).

The *E. coli* strains used are listed in Table 3. BJ5183 was used in the cloning studies, while JM103 was used to propagate the phage M13.

Table 1.

Strain	Genotype	Source
Σ1278b	∝	J-M. Wiame
4324c	∝ *gdh1-1*	"
BC55	*a leu2-3,-112 gdh1-6 per1-30*	B.D. Hall
SAY1	*leu2-3,-112 gdh1-1 can1*	J.R. Kinghorn
AH216	*a leu2-3,-112 his3-11,-15 pho3 pho5*	"
216/4324c	*a leu2-3,-112*	AH216 x 4324c
AR1	∝ *leu2-3,-112 gdh1-1*	216/4324c x 4324c
AR2	*a leu2-3,-112 gdh1-1*	"
AR3	*a leu2-3,-112*	"
AR4	∝ *leu2-3,-112*	"
DS278	*a lys2 CUP1*	DC(Y)L
DS280	∝ *lys2 CUP1 mal*	"
DCL1	Unknown	"
DCL2	Unknown	"
BC55(pCYG4)	BC55 transformed with plasmid plasmid pCYG4	This study
BC55(YEp13)	Transformed with YEp13	"
BC55(p8)	Transformed with p8	"
BC55(p9)	Transformed with p9	"
BC55(p11)	Transformed with p11	"
BC55(P18)	Transformed with p18	"
AR2(pCYG4)	AR2 transformed with pCYG4	"

Saccharomyces cerevisiae strains used in this study.

Table 2.

Strain	Phenotype	Derivation
3-25, 3-26, 3-34	α glu^-	DEO mutagenesis
4-1, 4-2, 4-3, 4-5, 4-6, 4-8, 4-9, 4-10[1], 4-11 4-17, 4-18, 4-20 4-21, 4-22, 4-23 4-25[2]	α glu^-	EMS mutagenesis
AR5	a leu^- glu^-	AR2 x 4-9
AR42	a glu^-	"
AR5(pCYG4)	AR5 transformed with pCYG4	This study
AR8	a glu^-	AR2 x 4-1
AR9	α glu^-	"
AR11	α glu^-	AR2 x 4-2
AR12	a leu^- glu^-	"
AR13	α glu^-	AR2 x 4-3
AR15	a glu^-	"
AR18	a glu^-	AR2 x 4-6
AR21	α glu^-	AR2 x 4-11
AR22	a glu^-	"
AR23	a glu^-	AR2 x 4-18
AR28	a leu^- glu^-	AR2 x 4-20
AR29	α glu^-	AR2 x 4-21
AR31	a leu^- glu^-	"
AR32	α glu^-	AR2 x 4-22
AR34	a glu^-	"
AR37	a glu^-	AR2 x 4-23
AR39	α glu^-	"
AR40[2]	α glu^-	AR2 x 4-25
AR43[2]	a glu^-	AR2 x AR40
AR44[1]	GLU^+	AR3 x 4-10
ART10-1a	LEU^+ GLU^+	Spores from a
ART10-1b[1]	leu^- glu^-	single tetrad
ART10-1c	leu^- GLU^+	of the cross
ART10-1d[1]	a LEU^+ glu^-	AR2 x 4-10
ART10-2a	leu^- GLU^+	As ART10-1
ART10-2b[1]	α LEU^+ glu^-	
ART10-2c	LEU^+ GLU^+	
ART10-2d	LEU^+ GLU^+	

S. cerevisiae strains generated in this study. The strain used in the mutagenesis experiments was 4324c. 1 - data presented in Chapter 5 and 6 have allowed an allele number (got1-10) to be assigned to the mutation carried by this strain; 2 - the mutation has been designated got2-25.

Table 3.

Strain	Genotype	Source
(a)		
972	Wild type	A. Livingston
I67	*leu1-32 gdhI67*	"
I67 (pRac1)	I67 transformed with pRac1	"
(b)		
BJ5183	*F⁻ endA sbcB recBC galK met⁻ str⁺ thi-1 hsdR* (r_K^- m_K^+)	S.J. Gurr
JM103	*(lac, pro) supE thi strA endA sbcB15 lacI hsdR4 traD36 proAB*	"

Other strains used: (a) Schizosaccharomyces pombe; (b) Escherichia coli.

2.2 Plasmids and Gene Libraries

The *S. cerevisiae GDH1* gene was obtained from Dr. B.D. Hall (Nagasu and Hall 1985). The clone consists of a 3.0kb DNA fragment inserted into the *Eco*RI site of YEp13. This fragment complements the *gdh1* mutation and results in higher than wild type NADP-GDH activity.

Initially the *N. crassa am* gene was used as a probe in the heterologous hybridisation experiments, and was a gift from Dr. S.J. Gurr. It consisted of a 2.7kb *Bam*HI fragment encoding the entire *am* gene. Later experiments used a fragment of *A. nidulans gdhA* (obtained from Dr. S.J. Gurr). The probe consisted of the 0.4kb *Hin*dIII-*Bgl*II fragment inserted into M13mp9.

The *S. cerevisiae* gene library was provided by Dr. K. Nasmyth. It was a partial *Sau*3A digest of genomic yeast DNA into the *Bam*HI site of YEp13. The average insert size was 6.3kb.

The *Schiz. pombe* library was obtained from Dr. P.Nurse via Dr. P. Fantes. Total *Schiz. pombe* DNA was partially restricted with *Sau*3A and ligated into the *Bam*HI site of pDB248. The average insert size was 5kb.

2.3 Media

The yeast minimal medium (MM) used in this study was (g/l) Difco yeast nitrogen base without amino acids or $(NH_4)_2SO_4$ 1.7 and glucose 20. As necessary glucose was replaced by either potassium acetate (20g/l) or glycerol (30g/l) as the carbon source (Dickenson *et al.* 1986). MM was supplemented with (final concentration, mM) NH_4Cl 0.2, 2 or 20, aspartate 10, copper sulphate 0.5, glutamate 10, glutamine 10, D-histidine 1, L-histidine 10, leucine 1, 2-oxoglutarate 1, proline 5, serine 5, tryptophan 10, urea 5 or allantoin 0.1% w/v as required.

The complex medium for yeast growth was normally yeast complete (YC) which contained (g/l) yeast extract 5 and glucose 20. The tetrad analyses were done using YEPD as the complex medium. YEPD was (g/l) yeast extract 10, Bacto-peptone 20 and glucose 20 (Sherman *et al.* 1983).

The pre-sporulation medium was (g/l) yeast extract 8, Bacto-peptone 3 and glucose 100 (Sherman *et al.* 1983). Sporulation medium was (g/l) yeast extract 1, glucose 0.5 and potassium acetate 10 (Sherman *et al.* 1983).

For yeast transformation by the protoplasting method, cells were grown in YC containing only 10g/l glucose. The protoplasts were regenerated in MM containing 1.2M sorbitol plus the appropriate amino acid supplements.

E. coli was cultured in Luria (L-) broth. This was (g/l) tryptone 10, NaCl 5 and yeast extract 5, pH adjusted to 7.2. Antibiotic supplements were (μg/ml): ampicillin 75, chloramphenicol 170 or tetracycline 15 or 25.

Solid media were prepared by the addition of 15g/l agar, although YEPD used as the support for tetrad dissection was solidified with 30g/l agar.

2.4 Growth Conditions

Studies on the enzymology of the industrial strains were done using cells grown in either shake-flask culture or in simulated industrial propagations.

For the shake-flask studies, starter cultures of DCL1, DCL2 and Σ1278b were grown overnight in 5ml YC. The starter cultures were harvested in a MSE bench-top centrifuge, washed once with water and inoculated into MM + glutamate (300ml in 1l non-baffled flask, 30°C, 150rpm in a New Brunswick orbital shaker). The cultures were grown overnight to early stationary phase, when they were harvested by centrifugation (5 minutes, 20°C, 2660xg). The cells were washed once with water and ,if necessary, split into aliquots. Each aliquot was inoculated into fresh, pre-warmed MM + 20mM NH_4^+ to an initial A_{610} of about 0.55. Growth conditions were as for the overnight cultures except that, after the desired time, the cells were harvested (5 minutes, 4°C, 2660xg). The cells were washed with cold (4°C) water and centrifuged in a MSE bench-top centrifuge. Cell pellets were stored at -20°C until required.

Investigation of the response of the industrial strains and Σ1278b to different nitrogen sources was done using cells grown under similar conditions. However, nitrogen was supplied as either 0.2 or 2mM NH_4^+, or glutamate. The cultures were grown for 6h and then harvested. Cells for physiochemical studies were grown in the same way except that they were switched to 20mM NH_4^+.

Simulated industrial propagations were done in a 2l fed-batch operated fermentor exactly resembling an industrial propagation with respect to nutrient feed rates. The inoculum was ex-factory seed, stage D5. The carbon source was molasses, and nitrogen was supplied as $(NH_4)_2SO_4$. Exact details of the growth conditions are commercially sensitive and are not presented. Due to the differences in the product, the nutrient feed parameters are different for DCL1 and DCL2. 20ml samples were taken at 1h intervals during the propagation, as well as from the seed and yeast cream. The samples had to be washed twice to remove the molasses contamination before the pellets were frozen at -20 C.

Studies on the enzymology of the GLU- mutants were done with ammonium grown cells. Starter cultures of the mutants were grown overnight in 5ml YC, harvested, and washed as before. The starter cultures were inoculated into MM + glutamate and grown overnight (100ml in 250ml non-baffled flask, grown at 30°C in a reciprocally shaking water bath - 80rpm, stroke-length 4cm). The cultures were

harvested (5 minutes, 20°C, 2660xg), washed once with water and inoculated into 100ml MM + 20mM NH_4^+. As the GLU$^-$ mutants cannot use ammonium as nitrogen source, the need for sufficient cells for enzyme studies required that all the overnight culture was inoculated into the fresh medium. The cultures were grown for 6h (conditions as before), and harvested by centrifugation (5 minutes, 4°C, 2660xg). The cells were washed once with cold water, pelleted in a bench-top centrifuge and the cell pellet stored at -20°C until required.

Growth curves were done using the same experimental conditions except that the initial cell density was A_{610} 0.55. Absorbance readings were taken every 2h over a 12h period.

The permissive temperature for both liquid and plate cultures of a *ts*-phenotype was 19°C, otherwise the growth conditions were as normal. The restrictive temperature was 30°C. Cells for the physiochemical studies were grown in 300ml cultures at 19°C, although other culture conditions were the same as described for the industrial strains. Measurement of the reversion frequency of various mutants were done at 19°C. Phenotypic characterisation was done at 30°C.

If yeast cells were required for transformation with DNA, the growth conditions depended upon the protocol to be used. For transformation by the protoplasting method, the recipient strain was inoculated into YC (100ml in 250ml non-baffled flask, 30°C, in a reciprocally shaking water bath - 80rpm, stroke length 4cm) and grown overnight. 5ml of overnight culture was inoculated into fresh YC and growth allowed to continue (conditions as before). The culture was harvested when the cell density was 1-$2x10^7$/ml - approximately 4-5h after inoculation (initial cell density was about $3.5x10^6$/ml). Cells to be transformed by the alkali cation method were grown as follows. A single colony was inoculated into YC (50ml in 250ml non-baffled flask, other conditions as in section 2.6.1). The culture was grown overnight and harvested by centrifugation (5 minutes, 20°C, 2660xg) when A_{610} 0.8 *i.e.* late exponential growth phase.

E. coli starter cultures were grown overnight in 5ml L-broth with vigorous agitation. Rapid, small scale isolation of plasmid DNA was done from 1ml aliquots of such cultures. If larger quantities of plasmid DNA were required, the starter culture was inoculated into 100ml fresh L-broth (100ml in 250ml non-baffled flasks, 200rpm in an orbital shaker). The culture was allowed to grow for 3.5-4h when chloramphenicol was added. Growth was continued for a further 4-18h. The cells were harvested by centrifugation (15 minutes, 4°C, 6810xg).

To prepare *E. coli* cells competent for transformation with DNA, 1ml of the starter culture was inoculated into 100ml pre-warmed L-broth. The culture was grown with vigorous shaking for 2-4h to A_{550} 0.5 when the cells were harvested (as above).

For the preparation of single-stranded (ss) M13 DNA templates, the overnight culture was diluted 1:100. 5ml aliquots were dispensed and inoculated with phage from a single M13 plaque. The infected culture was grown for 5.5-6h with vigorous shaking when the culture was harvested (10 minutes, 20°C, 18200xg). The supernatent was removed with a pasteur pipette: to ensure that there was no carry over of cells, not all the supernatent was taken.

The appropriate antibiotic was added to the L-broth as required. Solid media was prepared by the addition of 15g/l agar. *E. coli* was cultured at 37°C.

2.5 Genetical Procedures

Standard genetic techniques (described in Sherman *et al.* 1983) were used in the study of the yeast GLU- mutants.

2.5.1 To construct the various strains required in this study, strains with the required genetic markers and opposite mating type were mixed together on a YC plate. The mating mixture was incubated for 3-6h, when cells were picked and streaked onto MM + 20mM NH_4^+. The culture was incubated until single colonies appeared along the line of the streak. Single colonies were picked and used for subsequent work. Growth on MM + NH_4^+ allows selection for diploids because the haploid parents cannot use ammonium as sole nitrogen source. In the diploid strains, all mutant alleles, excepting *gdh1-1*, exist in a heterozygous condition. A homozygous *gdh1-1* diploid can utilise ammonium as its sole nitrogen source. The presence of at least one wild type allele of each gene allows growth of diploids on MM + NH_4^+. The alleles used for diploid selection during strain construction were *leu2-3,-112* and the mutation causing the GLU- phenotype. It was decided not to introduce flanking auxotrophic markers into the GDH- GOGAT- strains because of the central position of these enzymes in nitrogen metabolism and the uncertainty about the effect of introducing an amino acid auxotrophy into such a strain.

The GLU+/GLU- diploids were sporulated by streaking a single colony onto pre-sporulation medium and incubating for 2 days. Subsequently cells were transferred to sporulation medium for 5-7 days. Haploid progeny were isolated by mass spore isolation. Strains with the desired phenotype were detected by replicating the colonies to suitable test media. Microscopic examination of sporulating GLU+/GLU- diploids showed that the asci took longer than normal to mature (5 - 7 days), and that the sporulation frequency was low.

2.5.2 The mass spore isolation procedure was based on that of Sherman *et al.* (1983). Sporulated cells were picked off the sporulation medium from a region of high cell density and resuspended in 0.2ml 1:40 diluted glusalase (a commercial preparation of glusalase was diluted with water prior to use). The

cells were incubated at 30°C for 1h in a shaking water bath. 0.1ml sterile glass beads (0.5mm diameter) were added, and incubation continued with vigorous shaking for 1h. 1ml water was added and the cell suspension vortexed on a whirlimix for 1 minute. The majority of vegetative cells can be killed by treating the suspension with diethyl ether. 1ml diethyl ether was added to the suspension, and mixed by vortexing for 1 minute. The aqueous phase was removed and transferred to a fresh tube. A 10µl aliquot was rapidly diluted 1:100 into YC. A suitable dilution series was made (usually 10^{-2}, 10^{-4} and 10^{-6}) and 100µl aliquots spread onto YC. The plates were incubated for 48h, when single colonies were visible.

2.5.3 Segregation of the GLU- phenotype was studied by tetrad analysis. A sporulated GLU+/GLU- diploid culture was resuspended in 90µl 0.1M citrate-phosphate buffer (pH5.5). 10µl undiluted glusalase was added and the spore suspension incubated for 15-30 minutes at 30°C without shaking. The tetrads were taken for dissection when they had attained the characteristic diamond shape.

The agar slab used as the support for the tetrad dissections was prepared as follows: 10ml YEPD (3% agar) was melted, immediately poured into a 90mm diameter petri dish and dried in a laminar flow cabinet for 10 minutes. An agar slab was cut from the plate and mounted on a double-size microscope slide. The dimensions of the slab were such that it fitted into the dissection chamber mounted on the microscope. 10µl of the glusalase-treated spore suspension was dribbled down one edge of the slab, and excess moisture allowed to evaporate.

The dissecting needle was prepared from a 25µl glass micropipette. The micropipette was heated in a small flame and pulled into a fine tube about 15µm diameter: needles, about 5mm long, were cut from it. Each needle was stuck to the end of a second 25µl micropipette with canada balsam, and the supported needle mounted on a Leitz micro-manipulator.

The needle was used to pick up and transfer both asci and ascospores. Disruption of the asci, and separation of joined ascospores, was done by rubbing each ascus across the agar surface with the needle tip or gently tapping the bench so that the needle flicked the ascus. By a combination of both techniques asci could be dissected and the spores arranged in a defined pattern on the agar slab. Colonies were usually visible at the site of inoculation after 24h incubation.

2.5.4 The ability of a strain to utilise a compound as a carbon or nitrogen source was tested with either streak or patch plates. For streak plates, the strain being tested was streaked directly onto medium containing the test compound. Growth was scored as the appearance of single colonies along the line of inoculation. With patch plates, the strains under investigation were inoculated at defined points on a YC plate. The master plate was replicaplated to medium supplemented with the test compound. A positive

result was the appearance of a patch, comparable in density to the master plate, at the corresponding position on the test plate.

The mating-type tests were done by cross-stamping. Test strains of the same mating-type were streaked onto YC along a series of parallel lines. The cultures were incubated so that there was good growth along the line of inoculation. The plate was pressed onto a velvet pad. A second plate, inoculated with strains of the opposite, or unknown mating-type, was pressed onto the pad, so that the two sets of lines were at 90° to one another. A fresh YC plate was pressed onto the pad, removed, and then incubated for 3-6h so that mating could occur. The cross-stamped YC plate was replicaplated to suitable test medium. Growth was scored at the points where the lines intersected after 48 and 72h incubation.

2.5.5 Reversion of the GLU^- mutants was measured by determining the frequency at which colonies capable of growing on MM + NH_4^+ arise. The GLU^- mutants were inoculated into MM + glutamate and incubated at 19°C for 5 days. Aliquots were plated onto MM + 20mM NH_4^+ and growth scored after 4 days incubation at 19°C. The cell density was measured by spreading 100µl aliquots of suitably diluted culture onto YC and scoring the number of colonies.

2.6 Transformation Procedures

Two different yeast transformation protocols were tried because the procedure used initially (transformation by protoplasting) gave low transformation frequencies.

2.6.1 Protoplasts were generated and transformed using a modification of the method of Sherman *et al.* (1983). Cells were grown as described in section 2.4. After washing with 10ml 1.2M sorbitol, 20mM citrate-phosphate buffer (pH5.6), 40mM EDTA and 150mM 2-mercaptoethanol, the cells were pelleted in a bench-top centrifuge. The cells were resuspended in 5ml 1.2M sorbitol, 50mM citrate-phosphate buffer (pH5.6), 30mM 2-mercaptoethanol containing 25mg NovoZym 234, and incubated at 30°C with occasional, gentle shaking. Protoplasting was assayed by diluting 10µl cell suspension into a drop of water, and examining the suspension microscopically for cell lysis. Cell wall digestion was stopped when 10-20% lysis was observed.

The protoplasts were washed twice with 20ml 1.2M sorbitol, 10mM Tris-Cl (pH7.6), and resuspended in 1ml 1.2M sorbitol, 10mM Tris-Cl (pH7.6), 10mM $CaCl_2$. 0.1ml aliquots of the protoplast suspension were dispensed into microfuge tubes. DNA (0.1-5µg in 1-10µl) was added to each tube and mixed with the cells by gentle inversion of the tube. The tubes were kept at room temperature for 20 minutes. 1ml Tris-Cl (pH7.6), 10mM $CaCl_2$, 20% w/v PEG 4000 was added to each tube, mixed by inversion and the incubation continued for another 20 minutes. The protoplasts were pelleted in a microfuge and resuspended in 0.2ml 10mM Tris-Cl (pH7.6), 10mM $CaCl_2$, 1.2M sorbitol, 0.5g/l yeast extract. The

protoplast suspensions were incubated at 30°C for 45 minutes. As the strains used in these experiments were *leu2* mutants, the recovery solution contained 5µg/ml leucine.

Aliquots of 100µl of the protoplast suspension were dispensed into 10ml sorbitol + MM agar maintained at 55°C, before being poured onto sorbitol + MM plates. Both the top agar and the plates were supplemented with the appropriate amino acids. The plates were incubated for 2-4 days.

2.6.2 Transformation of intact cells treated with alkali cations was done by a modification of the method of Ito *et al.* (1983). Cells were grown as described in section 2.4. The cells were washed twice with 10ml TE buffer (10mM Tris-Cl pH7.6, 0.1mM EDTA), and resuspended in 0.1M lithium acetate in TE buffer at a final density of $2x10^8$/ml. After incubation at 30°C for 1h with agitation, 0.3ml aliquots were dispensed into microfuge tubes.

Plasmid DNA (0.1-10µg in 10µl) and 0.7ml 50% w/v PEG 4000 were successively added to the cells. The tubes were inverted three times and incubated statically at 30°C for 1h. The cells were heat-shocked by immersing the tubes in water at 42°C for 5 minutes. After cooling to room temperature, 0.1ml aliquots were plated directly on MM supplemented with the appropriate amino acids to select for transformants.

2.6.3 *E.coli* cells competent for transformation were prepared by the method of Morrison (1979). Cells were grown as detailed in section 2.4. The cell pellet was resuspended in 25ml ice-cold 0.1M $MgCl_2$. The cells were sedimented (15 minutes, 4°C, 6810xg) and resuspended in 25ml ice-cold 0.1m $CaCl_2$. The cell suspension was kept on ice for 20 minutes and then at 4°C for 24h (Maniatis *et al.* 1982). The $CaCl_2$-treated cells were harvested as before and resuspended in 5ml 0.1M $CaCl_2$, 14% glycerol. The suspension was dispensed in 0.2ml aliquots and frozen at -70°C until needed.

Competent cells were transformed as follows. A tube of frozen cells was thawed in an ice-water bath and dispensed in 0.1ml aliquots. DNA was added (0.1µg in 10µl) and the cells kept on ice for 30 minutes. The tubes were placed in a 42°C water bath for 2 minutes, and then 1ml L-broth added. The transformed cells were incubated at 37°C for 1h before aliquots were plated on L-agar. Transformants were selected by supplementing the medium with the appropriate antibiotic. If tetracycline was used as the selective agent, it was added to a final concentration of 15µg/ml.

2.7 Mutagenesis and Mutant Selection

Two mutagens were used in this study: 1,2,7,8-diepoxyoctane (DEO) and ethanemethanesulphonate (EMS). Although both mutagens are alkylating agents causing point mutations, DEO is also reported to cause multi-site mutations in *A. nidulans* (Hynes 1979).

Cells were harvested from an overnight culture of 4324c by centrifugation (5 minutes, 20°C, 2660xg) - all subsequent centrifugation steps were done in an MSE bench-top centrifuge. The cells were washed once with water and resuspended to a final density of 10^7/ml. If the cells were to be exposed to DEO, they were resuspended in water: 0.1M sodium phosphate (pH7.0) was used for EMS mutagenesis. 5ml aliquots were incubated at 30°C for 10 minutes prior to the addition of the mutagen.

DEO was added at 10µl/ml and incubation continued, with agitation, at 30°C. It was found that the DEO kill rate was not reproducible, so samples were taken after 5, 15, 30 and 45 minutes exposure. The cells were washed three times with 20ml water to remove the mutagen, and resuspended in 5ml YC. The culture was allowed to recover by overnight incubation at 30°C.

To determine the kill frequency, aliquots were taken at time T_0 and at the appropriate time points, serially diluted and spread on YC plates. DEO-mutagenised cultures which had a 75-95% loss of viability were chosen for further study. The mutation frequency was measured by the appearance of cycloheximide resistant colonies, due to mutation of *CYH2* to *cyh2* (Struhl 1983). Aliquots of cells were plated on YC supplemented with 40µM cycloheximide, and the number of colonies appearing after 48h incubation scored.

Mutagenesis using EMS was done using a modification of the method of Sherman *et al.* (1983). 0.7ml cell suspension was mixed with 1ml 0.1M sodium phosphate (pH7.0), 50µl EMS added and dispersed by briefly vortexing the suspension. The cells were incubated at 30°C with shaking for 1h. The reaction was quenched by diluting the mixture into 20ml 5% w/v sodium thiosulphate. The cells were harvested by centrifugation, washed three times with water before being resuspended in 10ml YC. The culture was allowed to recover by incubating it at 30°C for 48h. The kill rate under these conditions was about 50%.

The mutation frequency obtained using DEO was low, so the culture was enriched for mutants using nystatin (Snow 1966). Cells from a culture which had been allowed to recover after mutagenesis were pelleted, washed once with water and resuspended in an equal volume of MM. The culture was incubated overnight at 30°C with shaking. The cell suspension was diluted into fresh MM to a final A_{610} 0.2-0.3. Ammonium, to 20mM, was added and incubation continued. Growth was followed spectrophotometrically. When the culture had gone through at least one generation, the culture was harvested by centrifugation and resuspended in MM + 20mM NH_4^+ at 10^7cells/ml. Nystatin was added to a final concentration of 10µg/ml and incubation continued for 5.5h. The cells were washed three times with water and resuspended in 100ml YC. The culture was allowed to recover by overnight incubation. Enrichment was done on a given culture up to three times.

Mutant selection was done as follows. The culture was spread on YC medium at 200-250 colonies per plate. After 48h incubation, the YC master plates were replicaplated onto MM + glutamate and MM + 0.2, 2 or 20mM NH_4^+. After overnight incubation, the plates were screened for glutamate auxotrophs *i.e.* colonies which grew on glutamate but not ammonium supplemented medium. The putative GLU- mutants were picked from the master plate and patched onto fresh YC before being rescreened to confirm the phenotype.

2.8 Assay Procedures

2.8.1 Cell extracts for enzyme studies were prepared by mechanical disruption with glass beads. The cell pellet was resuspended in the appropriate extraction buffer at 1g wet weight cells:5ml ice-cold buffer, and allowed to thaw. Two methods were used to disrupt the cells. The cell suspension was transferred to a chilled 3cm diameter boiling tube containing 1ml glass beads (0.5mm diammeter) per 5ml buffer. The cell-glass bead mixture was vortexed for 3 minutes on a whirlimix set at maximum power. Alternatively, the cells were broken open in a Braun Homogeniser. The suspension was transferred to the chilled homogeniser cell containing glass beads (ratio as above). The cell-glass bead suspension was shaken at the maximum power output for two 90s bursts, maintained on ice for 120s between bursts. The two methods were of comparable efficiency as judged by the yield of soluble protein per gramme cells. Once the pellet had thawed, the cells and cell extracts were stored on ice. The cell debris was removed by centrifugation (15 minutes, 4°C, 23220xg) and the supernatent taken.

2.8.2 Both the NAD- and NADP-GDHs were assayed spectrophotometrically at 30°C (Doherty 1970). The extraction buffer (0.1M potassium phosphate pH7.5, 5mM EDTA, 0.2% v/v 2-mercaptoethanol, 0.1mM phenylmethylsulphonylfluoride [PMSF]) was the same for both enzymes. The reaction mixture for the NADP-GDH assay contained cell extract (up to 200µg protein), 0.1ml 3.6mg/ml NADPH, 0.1ml 0.1M 2-oxoglutarate (pH7.8), 0.15ml 1.0M NH_4Cl and 0.1M potassium phosphate buffer (pH7.8) to 3.0ml. The NAD-GDH assay had cell extract, 0.1ml 3.6mg/ml NADH, 0.1ml 0.1M 2-oxoglutarate (pH7.0), 0.15ml 1.0M NH_4Cl and 0.1M potassium phosphate (pH7.0) to 3.0ml.

The reaction was initiated by the addition of the NH_4Cl and followed by recording the absorbance change at 340nm in a Pye Unicam SP6-550 or SP1800 spectrophotometer fitted with a chart recorder and water-jacketed cell carriage. The reaction rate was corrected by subtracting the rate of cofactor oxidation observed in the absence of NH_4Cl.

For GOGAT, NAD- and NADP-GDH, 1 unit (U) of activity was defined as 1µmol of cofactor consumed per minute. The definition for GS was 1µmol γ-glutamylhydroxamate formed per minute. Specific activities were expressed as units per mg protein, abbreviated to U/mg.

2.8.3 GOGAT was assayed by following the oxidation of NADH (Roon *et al.* 1974). The extraction buffer was that used for GDH. The reaction mixture contained cell extract (up to 0.5mg protein), 0.1ml 0.3M 2-oxoglutarate (pH7.8), 0.1ml 0.3M glutamine and 0.1M potassium phosphate buffer (pH7.8) to 3.0ml. The reaction was initiated by the addition of glutamine and followed as described in section 2.8.2. The rate was corrected by subtracting the value observed in the absence of glutamine.

The normal assay temperature was 30°C, but for the thermolability studies of mutant GOGAT 19°C was used. Two parameters were investigated: stability with respect to time at a fixed temperature; and constant time at different temperatures. Time effects were studied by pre-incubating 200µg protein in a water bath held at 32°C for increasing time periods, before residual activity was assayed at 19°C. The effect of temperature was investigated by heating cell extract (200µg protein) in a water bath held at different temperatures for 10 minutes: GOGAT activity was then assayed at 19°C.

The pH profiles of the mutant enzymes were derived by assaying 200µg protein (in a volume of 200-300µl) in a reaction system containing buffer at the desired pH. The buffer was 0.1M potassium phosphate - 10mM Tris-Cl. The cell extract was not pre-incubated in the buffer prior to being assayed.

The effect of the inhibitor 6-diazo-5-oxo-L-norleucine (DON) on GOGAT activity was studied by incorporating DON in the reaction mixture The concentrations used are given in the figure and table legends. The reaction mixture contained 100µg protein but otherwise was as normal. In some experiments, cell extract (about 200µg protein) + buffer was pre-incubated with DON at 30°C for 10 minutes prior to being assayed. This latter system was used to study the inhibition of NAD-GDH by DON.

One experiment examined the effect of dialysis on GOGAT activity in cell extracts from GLU- mutants. 2ml cell extract was dialysed overnight at 4°C against three changes of extraction buffer (1000-fold excess). The non-dialysed control was kept at 4°C.

2.8.3 GS was assayed by the synthase assay (Mitchell and Magasanik 1983). The extraction buffer was 5mM 2-(*N*-morphlino)-ethanesulphonate (MES) (pH6.3), 0.4mM $MnCl_2$, 0.1mM PMSF. The reaction mixture contained cell extract (up to 50µg protein), 37.5µl each of 10mM EGTA, 1.066M $MgSO_4$, 1.066M glutamate and 0.266M hydroxylamine-HCl (pH6.3), 75µl MES (pH6.3) and water to 325µl. After a 10 minute pre-incubation at 30°C, 50ul 0.1M disodium ATP was added and incubation continued for 30 minutes. The reaction was stopped by the addition of 1ml stop-mix (55g $FeCl_3.6H_2O$, 20g trichloroacetic acid and 21ml concentrated HCl per litre). The precipitated protein was pelleted by centrifugation in a microfuge for 1 minute, and the supernatent taken. It was found that this modification gave a more stable reading. The absorbance of the supernatent was read at 540nm against

a blank, where 50µl water was added instead of ATP. The yield of product (γ-glutamylhydroxamate) was obtained from a standard curve of A540 *vs.* µmol γ-glutamylhydroxamate.

2.8.4 The concentration of soluble protein was determined by a colorimetric method (Bradford 1976). The reagent was 100mg Coomassie Brilliant Blue G in 55ml 95% ethanol, 100ml concentrated phosphoric acid, 500ml 0.2M potassium phosphate buffer (pH7.5) and water to 1l. The reagent was filtered twice through Whatman No. 1 filter paper under gravity.

A standard curve was prepared against bovine serum albumin (BSA) fraction V. To 10-100µg BSA, in up to 0.1ml and made up to 0.1ml with water, 5ml reagent was added and mixed by vortexing. The absorbance was read at 595nm, 10-60 minutes after mixing against a blank of water plus reagent. Each point on the standard curve was done in triplicate. The sample (in a final volume of 0.1ml) was treated as above, but only duplicates were measured. The protein concentration was determined from the standard curve.

2.9 Plasmid and M13 DNA Isolation

2.9.1 Rapid, small scale isolation of plasmid DNA from *E. coli* was done by the alkaline lysis method (Maniatis *et al.* 1982). Cells were grown as described in section 2.4. The cell pellet was resuspended in 0.1ml ice-cold solution I (50mM glucose, 10mM EDTA, 25mM Tris-Cl pH8.0, 5mg/ml lysozyme) and maintained at room temperature. After 5 minutes incubation, 0.2ml solution II (0.2M NaOH, 1% w/v SDS) was added and mixed by rapid inversion. The lysis mixture was kept on ice for 5 minutes, and then 150µl ice-cold solution III (pH4.8) (60ml potassium acetate, 11.5ml glacial acetic acid, 28.5ml water) were added. The mixture was gently vortexed and then stored on ice for 5 minutes. The cell debris was pelleted by centrifugation in a microfuge for 5 minutes. An equal volume of phenol/chloroform was added to the supernatent. In this study phenol was saturated with 10mM Tris-Cl (pH8.0), 1mM EDTA (TE); chloroform was 24:1 chloroform:isoamyl alcohol. After mixing by vortexing, the aqueous and organic phases were separated by centrifugation in a microfuge for 2 minutes. The aqueous phase was taken and two volumes ice-cold ethanol added. After vortexing, the mixture was kept at room temperature for 2 minutes. The DNA was pelleted by centrifugation in a microfuge for 5 minutes. The supernatent was removed by aspiration and the pellet washed with 1ml 70% ethanol. After recentrifugation, the supernatent was removed and the pellet dried *in vacuo*. The pellet was resuspended in 20µl TE containing 20µg/ml DNase-free pancreatic RNase.

2.9.2 Large scale preparation of *E. coli* plasmid DNA was done using a similar method (Maniatis *et al.* 1982). The cells were cultivated as described in section 2.4. The cell pellet was re-suspended in 2ml solution I and kept at room temperature. After 5 minutes incubation, 4ml solution II was added and the lysis mixture kept on ice for 10 minutes. 3ml ice-cold solution III was added and the incubation

continued for a further 10 minutes. Cell debris was removed by centrifugation (20 minutes, 4°C, 11700xg). The supernatent was taken and filtered through gauze. Cold (-20°C) isopropanol (0.6 volumes) was added, mixed by inversion and kept on ice for 15 minutes. The DNA was recovered by centrifugation (15 minutes, 4°C, 12000xg). The supernatent was discarded and the pellet washed once with 10ml 70% ethanol. After recentrifugation, the supernatent was discarded and the pellet dried *in vacuo*.

The plasmid DNA was further purified by isopynic centrifugation over a CsCl-ethidium bromide (EtBr) gradient. The DNA pellet was dissolved in a total volume of 2.0ml TE. CsCl was added at 1.1g/ml and dissolved by gentle mixing. EtBr (20mg/ml) was added at 20µl/ml TE. To remove residual protein and high M_r RNA which might interfere with gradient formation, the DNA-CsCl-EtBr solution was spun for 2 minutes in a microfuge and the supernatent taken. The supernatent was centrifuged to equilibrium in a Beckman TL-100 ultracentrifuge using a TLA 100.2 rotor (16h, 18°C, 80000rpm).

The super-coiled plasmid DNA was recovered from the gradient and the EtBr removed by extraction with CsCl-saturated isopropanol. CsCl was removed by overnight dialysis at 4°C against three changes of TE. The DNA was concentrated by precipitation with ethanol - 2 volumes were added and the DNA-alcohol mixture kept at -20°C overnight. The DNA was recovered by centrifugation (15 minutes, 4°C, 12000xg). The pellet was dried *in vacuo* and resuspended in a suitable volume of TE.

2.9.3 Preparation of M13 ss-DNA template for probe synthesis by the sequencing reaction has been described previously (Anon. 1982). M13 was grown as detailed in section 2.4. Phage was precipitated by the addition of 0.2ml 20% w/v PEG 6000, 2.5M NaCl per ml supernatent. The solutions were mixed by shaking, and kept on ice for 30 minutes. The phage suspension was centrifuged (15 minutes, 4°C, 7500xg) and the supernatent discarded. The pellet was recentrifuged for 2 minutes and all remaining traces of the PEG/NaCl solution were removed by aspiration. The phage were resuspended in 100µl TE and 50µl phenol added. After mixing, the suspension was stood at ambient temperature for 15 minutes. The phases were separated by centrifugation (5 minutes, 4°C, 11700xg). The aqueous phase was taken and extracted with an equal volume of chloroform. This was done twice. The DNA was precipitated from the aqueous phase by the addition of 0.1 volumes 3M sodium acetate (pH5.2) and 2.5 volumes ethanol, and stored overnight at -20°C. The DNA was sedimented by centrifugation (15 minutes, 4°C, 12000xg), washed once with 70% ethanol, repelleted and dried *in vacuo*. The DNA pellet was finally dissolved in an appropriate volume of TE.

2.10 Restriction Endonuclease Digestion

DNA was restricted with a variety of enzymes using the conditions recommended by the manufacturer. The buffer systems used were those described by Maniatis *et al.* (1982).

2.11 Agarose Gel Electrophoresis and Southern Transfer

2.11.1 Restricted DNA was separated by agarose gel electrophoresis (Maniatis *et al.* 1982). Agarose was used at a concentration of 0.8-1.5% w/v and the electric field strength was 1V/cm. The running buffer was TBE (89mM Tris-borate, 89mM boric acid, 2mM EDTA, pH8.0). DNA was visualised after electrophoresis by staining the gel with EtBr (0.5μg/ml) and illuminating it with uv-light.

2.11.2 The DNA was denatured and neutralised *in situ*, and transferred to Pall Biodyne A nylon membranes according to the manufacturer's recommendations. After electrophoresis, the DNA was denatured by immersing the gel in 150ml denaturing solution (2.5M NaCl, 0.5M NaOH) for 30 minutes with gentle agitation. The gel was neutralised by washing it with 150ml 3M sodium acetate (pH5.5). DNA was transferred from the gel to the membrane by the capillary movement of 20x SSC (SSC: 0.15M NaCl, 15mM sodium citrate, pH7.0) through the gel. Transfer was allowed to proceed for at least 12h. After briefly rinsing the membrane with 2x SSC, it was baked at 80°C for 1h.

2.12 Preparation of DNA Probes and Hybridisation Procedures

^{32}P-labelled DNA probes were prepared either by nick-translation of double-stranded (ds) DNA (Rigby *et al.* 1977), or synthesis of the complementary strand against ss-M13 templates (Hu and Messing 1982).

2.12.1 ds-DNA probes were prepared as follows (S.J. Gurr, unpublished). A low melting point agarose gel was prepared in TBE, and pre-run at the running voltage. Restricted DNA was separated on the gel and visualised by staining with EtBr (0.2μg/ml) for 10 minutes in TBE. The bands of interest were excised from the gel and the gel fragment melted by heating at 65°C. The molten agarose was cooled to 37°C and an equal volume of pre-warmed (37°C) phenol added. The agarose was extracted by gentle mixing at ambient temperature for 10 minutes. The phases were separated by centrifugation (5 minutes, 20°C, 11700xg). The aqueous phase was taken and re-extracted with phenol, the mixture was kept on ice for 10 minutes. The phases were separated by centrifugation and the aqueous phase extracted with chloroform. After separation by centrifugation, the DNA was precipitated from the aqueous phase with sodium acetate (pH7.0), final concentration 0.3M, and 2 volumes ice-cold 96% ethanol. The DNA suspension was kept over-night at -20°C and then sedimented by centrifugation (15 minutes, 4°C, 12000xg). The DNA pellet was washed once with 70% ethanol as described above and the pellet dried *in vacuo*. The pellet was resuspended in a suitable volume of TE.

∝ ^{32}P-dNTPs were incorporated into ds-DNA by the 5′ to 3′ polymerase activity of DNA polymerase I (Rigby *et al.* 1977). The reaction mixture contained unlabelled dNTPs to a final concentration of 0.5mM, pancreatic DNase I to 2μg/ml, 1-2μg DNA, 50μCi ∝^{32}P-dATP or -CTP, 10μl nick translation

buffer (0.5M Tris-Cl pH7.2, 0.1M $MgSO_4$, 0.1mM dithiothretol), 5 units DNA polymerase I and water to 100µl. The reaction was allowed to proceed for 2h at 15°C, when it was terminated by the addition of 10µl Orange G (1mg/ml in TE). Separation of labelled DNA from unincorporated α^{32}P-dNTPs was done by gel filtration chromatography. The reaction mixture was applied to a Sephadex G-50 column (15cm x 0.7cm diameter) and eluted off the column with 10mM Tris-Cl (pH8.0), 0.1M NaCl, 1mM EDTA. Ten-drop fractions were collected and monitored for radioactivity. The labelled DNA eluted from the column before the fractions containing unincorporated label. The labelled DNA was pooled and boiled for 10 minutes before use as a probe in hybridisation.

2.12.2 α^{32}P-dNTP labelled DNA probes prepared from ss-M13 templates were made using a modification of the method of Hu and Messing (A. Jefferys, unpublished). The reaction contained about 400ng template DNA (heated at 60°C for 5 minutes prior to use), 1µl nick translation buffer, 2µl 17mer primer (2µg/ml) and water to 10µl. The primer was annealed to the template by incubation at 60°C for 30 minutes. After cooling to 37°C, unlabelled dNTPs were added to a final concentration of 40µM. 50µCi α^{32}P-dNTP, 6µl TE, 3.5 units Klenow fragment of DNA polymerase I and water to 30µl were added. Incubation was continued at 37°C for 15 minutes. The unlabelled form of the α^{32}P-dNTP was added (2.5µl of a 0.5mM solution) and incubation continued for 15 minutes.

The insert was cleaved from the vector by digestion with restriction endonucleases. The choice of enzyme depends upon the vector and insert, but the enzyme of choice cleaves 5′ to the insert in the polylinker. The enzymes used to cleave the fragment of the *A. nidulans GDHA* gene from M13 mp9 were *Eco*RI and *Hin*dIII. 10 units of each enzyme, 4µl enzyme buffer and 1.2µl 0.1M spermidine were added and the reaction continued for 20 minutes. The reaction was stopped by the addition of 5.2µl 1.5M NaOH, 0.1M EDTA and tracking dye (2.5ml 1% bromophenol, 20g Ficoll and water to 50ml).

The unincorporated label, insert and vector were separated by electrophoresis on a small 2% w/v low melting point agarose gel. The gel was run at 100V until the dye band was half-way down the gel. The DNA bands were visualised by autoradiography at room temperature - the gel was exposed to Fuji RX(Safety) X-ray film.The insert band was cut out of the gel and transferred to a 1.5ml microfuge tube. The probe was denatured by heating the agarose slice at 65°C for 10 minutes.

2.12.3 Pre-hybridisation of the Biodyne filters and the hybridisation of probes to them were according to the manufacturer's recommendations.

Hybridisation solution contained (final concentration): 5x Denhardt's [100x Denhardt's: 2% w/v Ficoll (M_r 400000), 2% w/v polyvinylpyrrolidine (M_r 360000), 2% w/v BSA], 5x SSPE (SSPE: 0.18M NaCl, 10mM NaH_2PO_4, 1mM EDTA, pH7.7) and 0.2% w/v SDS. Salmon sperm DNA was denatured by sonication (Maniatis *et al.* 1982). The filter was pre-hybridised by incubating it with hybridisation

solution (4ml/100cm^2 membrane) and salmon sperm DNA (final concentration 100μg/ml) for 1h with agitation at 65°C. To hybridise the probe to the filter, the hybridisation solution was replaced with fresh solution, and denatured probe plus salmon sperm DNA added. Hybridisation was done at 65°C for 12-16h with agitation.

After hybridisation, the filter was briefly immersed in wash buffer and then washed three times with fresh buffer. The buffer was 5mM NaH_2PO_4, 1mM EDTA, 0.2% w/v SDS, pH7.0. Wash buffer was added at 250ml/100cm^2 membrane, and each wash was for 30 minutes at ambient temperature (18-22°C) with vigorous agitation. The filters were air dried and exposed to autoradiography (minimum of 12h exposure to Fuji RX(Safety) X-ray film at -70°C).

2.13 Library Screening

GDH1 clones were revealed in *S. cerevisiae* and *Schiz. pombe* gene libraries by colony hybridisation (S.J. Gurr, unpublished). The screening of the libraries was done in two stages. First, a high density screening to select positive areas. These areas were subsequently excised, diluted and plated at a low density for further screening which enabled single colonies to be selected.

E. coli transformed with a gene library was spread on L-agar supplemented with the appropriate antibiotic at 500 colonies per plate. The plates were incubated until the colonies were approximately 1mm diameter. Impressions of these plates were made using Whatman hardened ashless 541 paper. The plates were overlaid with circles of 541 paper moistened with L-broth, and incubated at 37°C for 2h. The colonies were lysed, their DNA denatured and bound to the filters by washing, twice for 5 minutes with agitation, with successively 0.5M NaOH, 0.5M Tris-Cl (ph7.4) and 2x SSC. The filters were briefly washed with 96% ethanol and air dried.

The filters were pre-hybridised at 52°C by incubation in 10ml hybridisation per filter for 1h. The buffer was 50% formamide, 5x SSC, 100μg salmon sperm DNA. The probe was hybridised to the filters in fresh hybridisation buffer held at 52°C for 12-16h with agitation. After hybridisation, the filters were washed four times in 0.5x SSC at 38°C and air dried. The filters were exposed to autoradiography (72h exposure at -70°C using Fuji RX(Safety) X-ray film). Positive areas were picked from the master plates. The procedure for the low density screen was as described above, except the colony density was such that single colonies could easily be selected after screening.

2.14 Chemicals

Allantoin, all antibiotics and amino acids, ATP, NADH, NADPH, hydroxylamine, glusalase, lysozyme, pancreatic RNase A, 2-oxoglutarate, EMS, PMSF, DON, salmon sperm DNA, Coomassie Brilliant Blue G and Orange G were from Sigma Chemical Company Ltd., Poole. DEO was obtained

from Aldrich Chemical Company Ltd., Gillingham, Dorset. Unlabelled dNTPs were from Boehringer Gmbh., Mannheim, FRG. NovoZym 234 was supplied by Novo Industri A/S Bagsvaerd, Denmark. ^{32}P-dNTPs were purchased from Amersham International plc or New England Nuclear. Restriction endonucleases and DNA-modifying enzymes were from BRL-Gibco or NBL. All other chemicals were of the highest quality available. γ-glutamylhydroxamate was a gift from Dr. J. Ip.

Results and Discussion

Chapter 3

Cloning of the Yeast GDH1 Gene

3.1 Two approaches were used in the experiments to clone the *S. cerevisiae GDH1* gene. These were complementation of a *gdh1* mutation or heterologous probing of a *S. cerevisiae* gene library using a previously cloned NADP-GDH gene.Grenson *et al.* (1970) demonstrated that the GAP of *S. cerevisiae* is inhibited by ammonium. However, ammonium inhibition of GAP is relieved in a *gdh1* mutant (Grenson and Hou 1972), resulting in sensitivity to D-amino acids in the presence of ammonium and the inability to use allantoin (Grenson *et al.* 1974, and references cited therein). Strains transformed with the *GDH1* gene can be identified by screening transformants of a *gdh1* mutant for colonies which grow normally on MM supplemented with either allantoin or D-histidine + NH_4^+.Comparison of the amino acid sequence of NADP-GDH from a number of prokaryotes and lower eukaryotes has shown that there is a strong sequence homology, especially in the amino-terminal region of the polypeptide (see section 1.3.1). This observation can be exploited, and a previously cloned NADP-GDH gene used to identify homologous sequences in other organisms (see Miller and Brenchley 1984, Nagasu and Hall 1985, Gurr *et al.* 1986).

3.1.1 To isolate the *S. cerevisiae GDH1* gene by complementation, strain SAY1 was transformed with CsCl-purified plasmid DNA from the Nasmyth gene library. Transformants were selected by using the vector-encoded *LEU2* gene to complement the chromosomal marker *leu2-3 ,-112*. Clones concomitantly transformed with the *GDH1* were screened for by replicaplating the LEU+ transformants onto MM + allantoin, and examining the replicates for colonies exhibiting normal growth.

Transformation of protoplasts (Sherman *et al.* 1983) and of alkali-treated cells (Ito *et al.* 1983) were both tried. However, it was not possible to achieve transformation frequencies greater than 10 transformants per µg DNA with either protocol. This observation was also made using strain BC55. The basis of the refractory nature of these strains to transformation is unclear. Approximately 500 transformants were screened for GDH+ transformants. None were obtained. Due to the difficulties encountered with the transformation of *gdh1* strains, and the availability of suitable probes, it was decided to attempt to clone the *S. cerevisiae GDH1* by heterologous probing.

Approximately 5500 colonies from the Nasmyth gene library were screened with the *N. crassa am gene*. Cross-hybridisation was observed with 16 colonies, although the signal strengths varied. The recombinant plasmids giving the strongest signals were designated p8, p9, p11 and p18, and were taken for further study.

3.1.2 CsCl-purified DNA of plasmids p8, p9, p11 and p18 plus the vector YEp13 were restricted with *Eco*RI. A Southern blot of this material was probed with the *N. crassa am* gene (Fig. 2). The probe hybridised to a single *Eco*RI fragment in each of recombinant plasmids (positions 1d-4d) - this fragment contains all or part of the insert (Fig. 3). No hybridisation was observed with vector alone (position 5). It can be concluded that the probe cross-hybridises to an insert sequence which is homologous to the *N. crassa am* gene, and that the plasmids are not false positives due to probe-vector hybridisation

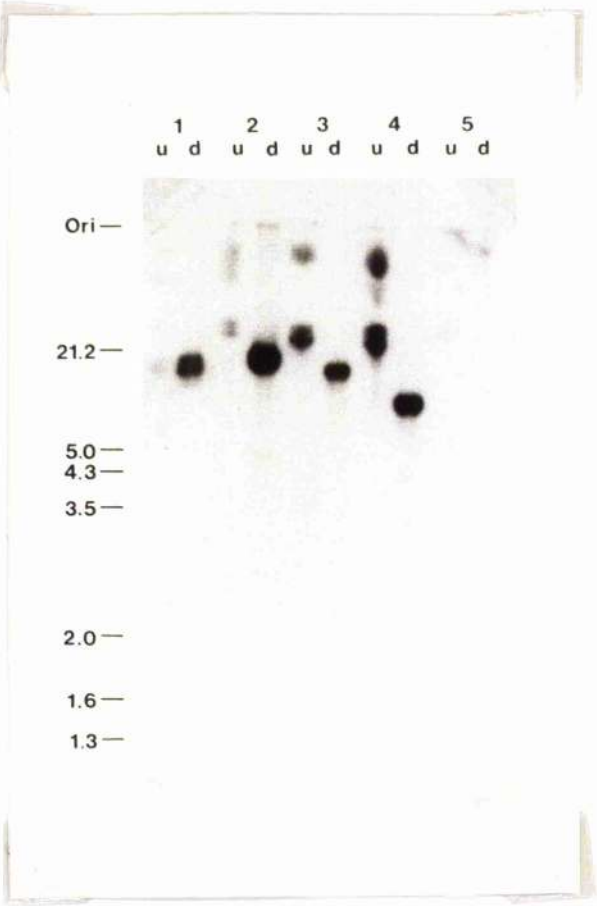
3.1.3 Although the inserts carried by the recombinant plasmids contain sequences which cross-hybridise with the *N. crassa am* gene, this is itself not sufficient evidence for it to be concluded that the entire *GDH1* has been cloned. The simplest test is to determine if the recombinant plasmids can complement a *gdh1* mutation. Strain BC55 was transformed with the four plasmids, the vector YEp13 and also the *S. cerevisiae GDH1* plasmid, obtained from Dr. B.D. Hall (Nagasu and Hall 1985). The growth characteristics and NADP-GDH activities of the various transformants were determined, and are presented in Tables 4 and 5.

BC55(YEp13) grew on MM + NH_4^+ because, although it carries the *gdh1-6* allele, glutamate was synthesised by the GS-GOGAT pathway. So the growth observed for the other transformants on ammonium was due either to the functioning of this alternative pathway or because the recombinant plasmid encoded a functional NADP-GDH. As was described in section 3.1.1, restoration of the ability of a *gdh1* strain to grow on allantoin is a diagnostic test for the presence of a cloned *GDH1* gene. BC55(pCYG4) can utilise allantoin as sole nitrogen: BC55(YEp13) cannot. None of the putative *GDH1* plasmids obtained in this study gave rise to transformants capable of growing on MM + allantoin.

NADP-GDH activity was measured in the recombinant strains (Table 5). Strains BC55 and BC55(YEp13) have no detectable NADP-GDH activity, compared to 1.4U/mg for the wild type Σ1278b. Thus the presence of the vector does not cause restoration of wild type enzyme activities. Enzyme activity is elevated 10-fold in BC55(pCYG4) compared to Σ1278b. As YEp13 is a 2μ-based plasmid, it is present in multiple copies in the cell, so overproduction of the enzyme was expected. Strains transformed with plasmids p8, p9, p11 and p18 have enzyme activities comparable to that observed with vector alone. These recombinant plasmids do not restore wild type NADP-GDH activity, let alone the elevated level observed with pCYG4.

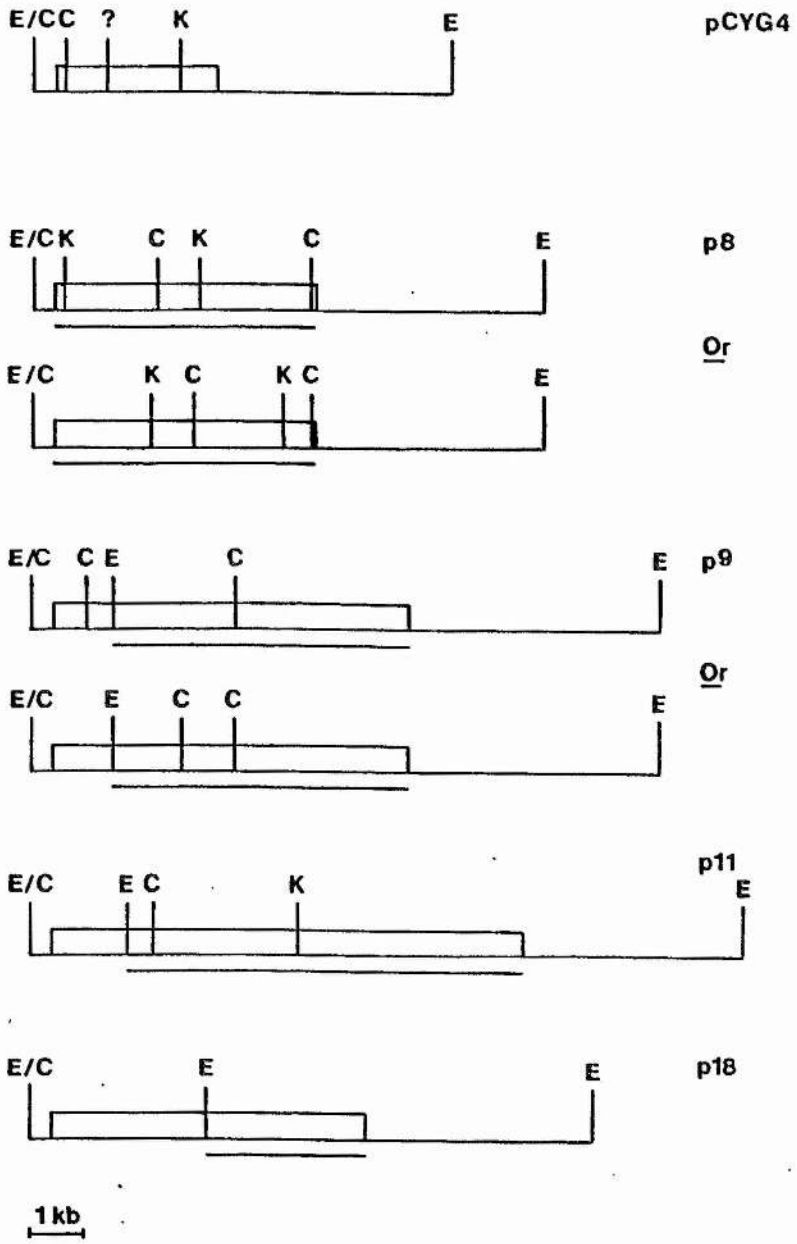
The data presented in Tables 4 and 5 show that, although p8, p9, p11 and p18 may possess a sequence which cross-hybridises with the *N. crassa am* gene, that sequence does not specify a functional NADP-GDH.

Fig. 2



Southern blot of the four plasmids carrying the putative S. cerevisiae GDH1 gene. Plasmid DNA was restricted with EcoRI, and a Southern blot of this material probed with the N. crassa am gene. 1 - p8; 2 - p9; 3 - p11; 4 - p18; 5 - YEp13; u - undigested DNA; d - digested. The figures down the left-hand edge are size markers (kb) from a HindIII/EcoRI digest of λ DNA.

Fig. 3



Partial restriction map of the four plasmids carrying the putative S. cerevisiae GDH1 gene, plus pCYG4 for comparison. It was not possible to orientate some of the fragments in p8 and p9, so both the two most probable forms are shown. The regions of the insert which cross-hybridise to the N. crassa am probe (Fig. 2) are indicated by the horizontal line under each plasmid. The additional KpnI or ClaI site observed in pCYG4 is indicated. C - ClaI; E - EcoRI; K - KpnI; ? - ClaI or KpnI; ▭ - genomic insert; ⎵ - YEp13 sequences.

Table 4.

Strain	MM supplemented with:				
	10mM Glu 1mM Leu	1mM Leu 2mM NH_4^+	10mM Glu 2mM NH_4^+	2mM NH_4^+	0.1%w/v allantoin
BC55(pCYG4)	++	++	++	++	++
BC55(YEp13)	++	++	++	++	-
BC55(p8)	++	++	++	++	-
BC55(p9)	++	++	++	++	-
BC55(p11)	++	++	++	++	-
BC55(p18)	++	++	++	++	-

Growth tests of S. cerevisiae gdh1 mutants transformed with the putative GDH1 plasmids. Each strain was streaked onto appropriately supplemented MM and incubated for 48h, when growth was scored. (++) appearance of large (>1mm) colonies along line of streak; (+) small colonies (<0.5mm) visible along streak; (-) no growth.

Table 5.

Strain	NADP-GDH activity (U/mg)
Σ1278b	1.4
BC55	0.0
BC55(pCYG4)	14.1
BC55(YEP13)	0.0
BC55(p8)	0.01
BC55(p9)	0.01
BC55(p11)	0.01
BC55(p18)	0.0

NADP-GDH activity in the recombinant S. cerevisiae strains. S. cerevisiae BC55 transformants were grown in MM + 2mM NH_4^+ and assayed for NADP-GDH activity. Each extract was assayed twice; the values presented are the means of duplicate experiments.

3.1.4 There are several possible explanations of why the putative *GDH1* plasmids do not complement a *gdh1* mutation. To investigate the possibility that either the insert was too small to encode a functional product or only a part of the gene had been cloned, partial restriction maps of the recombinant plasmids were made. The restriction endonucleases *KpnI* and *ClaI* were used because it was known from the nucleotide sequence of the *S. cerevisiae* gene that the entire coding sequence, plus putative control regions, were carried by a 2.4kb *Kpn*I-*Cla*I fragment (Moye *et al.* 1985, Nagasu and Hall 1985). The results are presented in Figs. 3 and 4.

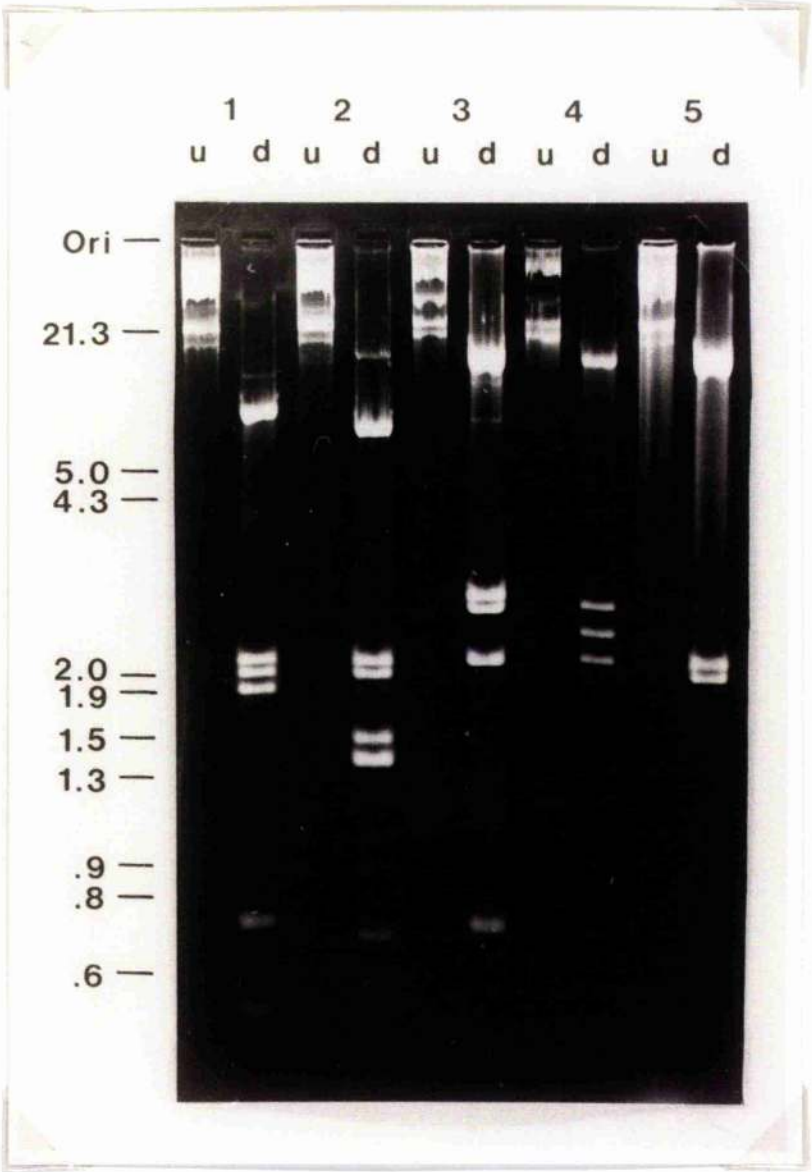
A 2.4kb fragment was not detected in a *KpnI-ClaI* digest of pCYG4. Instead 1.8kb and 0.6kb fragments were observed (Fig. 4). The 1.8kb fragment hybridises with the *am* probe (data not shown). The nature of this additional site was not investigated further. Examination of the sequence obtained by Moye *et al.* (1985) does not reveal an additional *Kpn*I or *Cla*I site in the coding sequence. Therefore, this result may be due to a mutation which had occurred after the sequence had been determined. As the two published sequences do not have additional sites, it will be assumed in the discussion below that the entire coding sequence is carried on a 2.4kb fragment.

The *S. cerevisiae* NADP-GDH subunit has a M_r 54000 (Mazon and Hemmings 1979), so the minimum coding sequence is ca.1.4kb. The insert sizes are 4.9, 6.7, 8.8 and 5.9kb for p8, p9, p11 and p18 respectively. Therefore, the observed lack of enzyme activity with the putative *GDH1* plasmids is not because the cloned genomic fragment is too small to encode the complete subunit.

With the three enzymes used to make the partial restriction map of the putative *GDH1* plasmids, it was not possible to order unequivocally some of the *Cla*I-*Cla*I or *Kpn*I-*Cla*I fragments generated from p8 and p9. Consequently the two alternative forms of p8 and p9 are shown in Fig. 3. The inserts of plasmids p8 and p11 both contain a *Kpn*I-*Cla*I fragment of approximately 2.4kb. This fragment lies within the *Eco*RI fragment which cross-hybridises with the *am* gene (Figs. 2 and 3). However, the restriction enzyme cleavage sites flanking this 2.4kb fragment do not show any obvious similarity either to one another, or to the map of Moye *et al.* (1985). The partial restriction maps of p9 and p18 do not show any similarity with the other putative *GDH1* plasmids, nor with the published data (Moye *et al.* 1985). From this data it would appear that plasmids p9 and p18 do not contain sequences encoding part of the *S. cerevisiae* NADP-GDH. The situation with p8 and p11 is less clear.

3.1.5 All four plasmids selected from the library screen carry insert sequences which cross-hybridise to the *N. crassa am* gene. None of these recombinant plasmids complement the *gdh1-6* mutation. However, with the possible exception of p8 and p11, the putative *GDH1* plasmids do not show any obvious physical similarity with pCYG4.

Fig. 4



KpnI/ClaI digest of the plasmids carrying the putative S. cerevisiae GDH1 gene, plus pCYG4. 1 - pCYG4; 2 - p8; 3 - p9; 4 - p11; 5 - p18; u - undigested DNA; d - digested. The figures down the left-hand edge are size markers (kb) obtained from a HindIII-EcoRI digest of DNA. Ori - origin.

The inability to complement a *gdh1* mutation could be because the insert does not carry a complete *GDH1* gene. As has been shown in section 3.1.5 all four plasmids carry a genomic insert of sufficient size to code for a functional NADP-GDH. This does not exclude the possibility that only part of the gene has been cloned as it lies in either extreme 3′ or 5′ regions of the insert. Since the 2.4kb *Kpn*I-*Cla*I fragment in both p8 and p11 lie completely within the insert, this possibility is unlikely. The sequences surrounding these fragments do not show obvious similarity to the published data (Moye *et al.* 1985). This heterogeneity may have arisen from fragment rearrangement during the construction of the gene library. However, this does not explain why p8 and p11 do not express a functional NADP-GDH. It is unlikely that a processed *GDH1* pseudogene has been cloned because *S. cerevisiae* carries few, if any, processed pseudogenes (Fink 1987). The reason for the lack of complementation by p8 and p11 remains unclear.

A hypothesis which explains the lack of similarity observed in the restriction maps of p9 and p18 is that another dehydrogenase gene was identified by the probe. Moye *et al.* (1985) have identified a region of the *S. cerevisiae* NADP-GDH amino acid sequence which exhibits homology with the cofactor binding domains of the yeast alcohol dehydrogenase (ADH) and glyceraldehyde-3-phosphate dehydrogenase. These workers also observed similar homology with the horse liver ADH and the lobster glyceraldehyde-3-phosphate dehydrogenase. Two cofactor binding domains have been identified in the *N. crassa* NADP-GDH (section 1.3.1). The entire *N. crassa am* gene was used as a probe. It is therefore possible that the nucleotide sequence coding for the cofactor binding domain cross-hybridised with the equivalent sequence of a genomic fragment encoding an undefined dehydrogenase.

These possibilities were not investigated further. The attempted cloning of the *S. cerevisiae GDH1* was not continued, because, early in this study the DNA sequence and *GDH1* clone were received from Dr. B.D. Hall (Nagasu and Hall 1985).

3.2.1 Due to the interest of this laboratory in fungal GDHs, plus the availability of suitable DNA probes, it was decided to attempt to clone the *GDH1* gene from *Schiz. pombe*. Comparison of the deduced amino acid sequences of the *A. nidulans* and *N. crassa* enzymes with the deduced *S. cerevisiae* sequence showed that the *A. nidulans* enzyme was the more homologous (69% *cf.* 61% of 96 residues examined). As the *A. nidulans* gene was available in a suitable vector, it was decided to use this gene as the probe to clone the *Schiz. pombe* gene.

The 0.4kb *Hin*dIII-*Bgl*II fragment of the *A. nidulans GDHA* (Gurr *et al.* 1986) was used to probe 9600 colonies of a *Schiz. pombe* gene library. A positive signal was obtained from a single colony. The recombinant plasmid was designated pRac1.

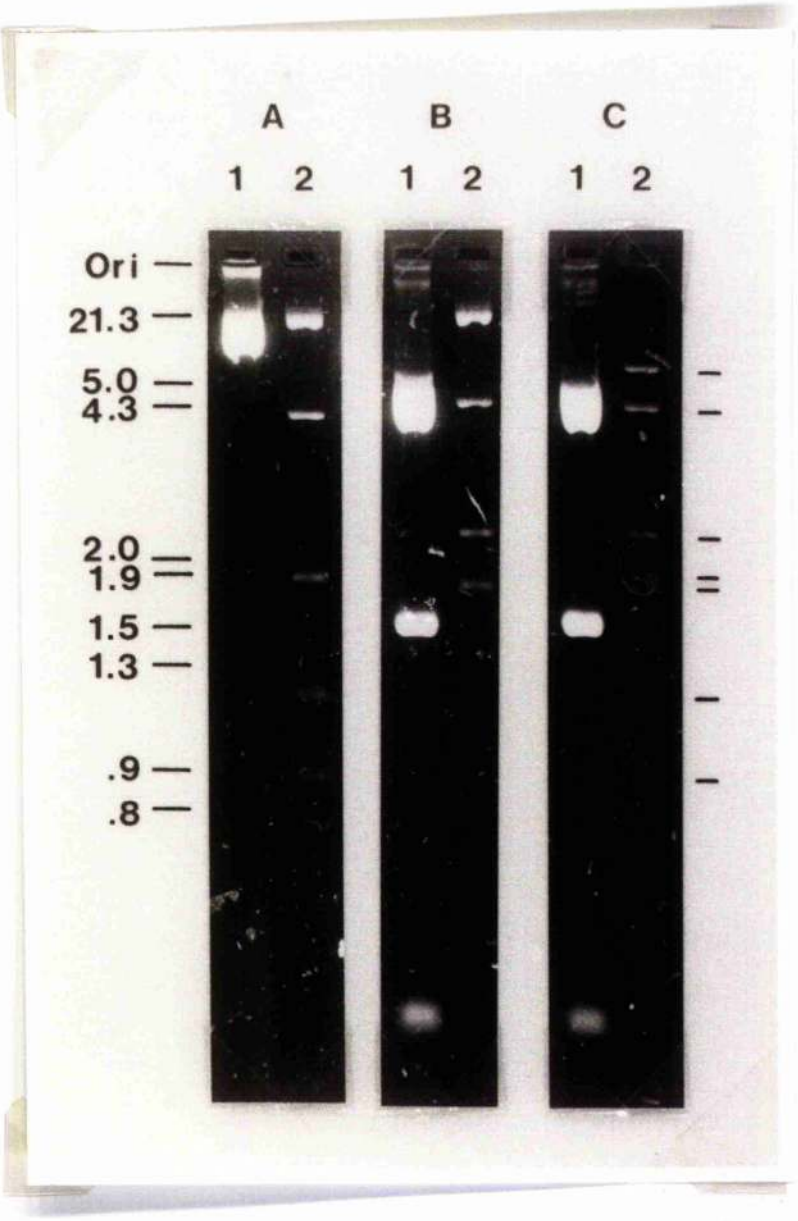
3.2.2 A partial restriction map of the insert was prepared using the enzymes *Hin*dIII and *Eco*RI (Figs. 5 and 6). The insert was found to possess two *Hin*dIII restriction sites (giving fragments of 0.9, 1.2 and 1.9kb) which could not be ordered with this combination of enzymes (indicated by the stippled region in Fig. 6). If the *Schiz. pombe* enzyme is similar to other microbial NADP-GDHs and has a subunit M_r of about 48000, then ca.1.2kb DNA would be required to encode the entire polypeptide. The insert was estimated at 9.6kb, and so contains sufficient sequence information to code for the complete subunit.

As with the *S. cerevisiae* plasmids, demonstration of homology with the probe is not sufficient evidence in itself to conclude that a given gene has been cloned. A functional demonstration is also required. *Schiz. pombe leu1 gdh167* (a tight glutamate auxotroph due to inactivation of the NADP-GDH) was transformed (Beach *et al.* 1982) with pRac1. LEU+ transformants, which were simultaneously glutamate-independent,were obtained at a high frequency (A. Livingston, unpublished).

The specific activity of NADP-GDH in a pRac1 transformant was measured (Table 6). The transformant has slightly elevated enzyme activity compared to the wild type, although the level was markedly higher than that of the untransformed strain. The vector pDB248 is present at only three copies per cell in *Schiz. pombe* (P. Nurse, unpublished). So the ten-fold increase in activity observed with the cloned *S. cerevisiae* gene was not expected. If only part of the gene had been cloned, the gene product would possibly not have been fully functional. Additionally, the large insert may decrease the copy number of the plasmid. These factors could explain why the enzyme activity measured in the transformant was only 1.1x wild type. On the basis of these two functional tests (*i.e.* complementation of a *gdh167* mutation and restoration of wild type enzyme activity), it can be concluded that pRac1 carries the *Schiz. pombe GDH1*.

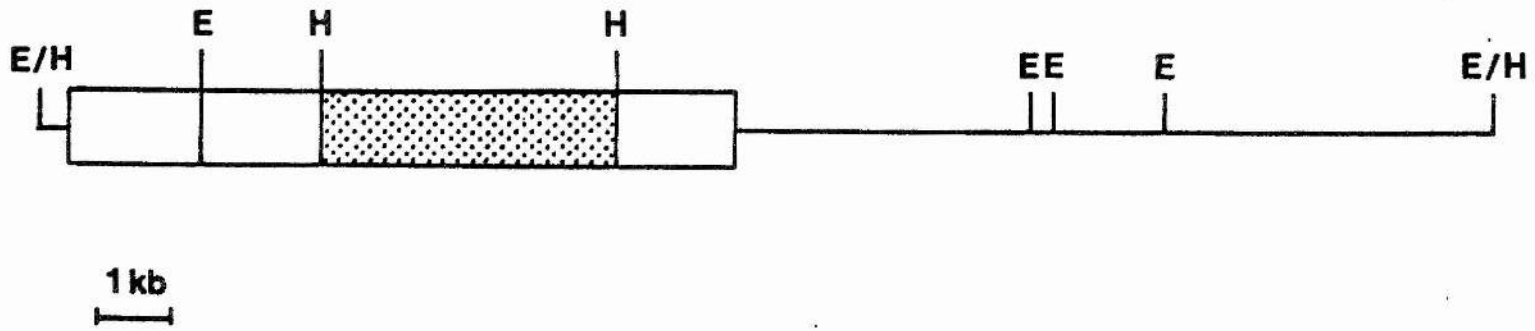
The *E. coli* strain bearing pRac1 was stored as an agar stab at ambient temperature. However, it was not possible to reisolate pRac1 from the stab at a later date. Consequently, it was decided not to pursue this work further.

Fig. 5



Agarose gel electrophoresis of undigested pRac1 (tracks 1) or fragments produced by restriction endonuclease digestion of pRac1 (tracks 2). pRac1 DNA was restricted with HindIII (A), EcoRI (B) or both enzymes (C). The bars beside track C2 show the position of fragments (visible in the original photograph) produced from a HindIII/EcoRI digest of pRac1. The figures along the left-hand edge are size markers (kb) obtained from a HindIII/EcoRI digest of λ DNA. Ori - origin.

Fig. 6



A partial restriction map of pRac1 was made using the endonucleases EcoRI (E) and HindIII (H). The insert was found to possess two HindIII sites (giving fragments of 0.9, 1.2 and 1.9kb) which cannot be ordered with this combination of enzymes. This is indicated by the stippled region. □ - genomic insert; ⎵ - pDB248.

Table 6.

Strain	NADP-GDH activity (mU/mg)
972	105.1
I67	14.6
I67(pRac1)	113.1

NADP-GDH activity of the Schiz. pombe recombinants. Schiz. pombe transformants were cultured and assayed as described for S. cerevisiae except that the assay pH was reduced to pH6.5. Each extract was assayed in duplicate, and the values reported are the means of three independent experiments.

Chapter 4

Studies on Ammonium Assimilation by Industrial and Laboratory Yeast Strains

4.1 Laboratory wild type strains of *S. cerevisiae* are derived from a variety of industrial sources and are known to exhibit marked heterogeneity in both regulatory mechanisms and enzyme complement. It seems probable that industrial strains will show similar variation.

The two industrial strains used in this study are production strains obtained from DC(Y)L. DCL1 is grown for compressed wet yeast production while DCL2 is for SPADY production. DCL1 was of interest because wet yeast is the high volume product of DC(Y)L, DCL2 was studied as its nitrogen feed profile has interesting features. Nitrogen feed profiles are markedly different for the two propagations. So, it would be expected that any differences between industrial strains would be accentuated because of the selective pressure exerted by the two different growth regimes.

It was decided to investigate the behaviour of the enzymes of ammonium assimilation in DCL1 and DCL2 grown in both shake-flask culture and simulated industrial propagations. Shake-flask culture allowed investigation of the physiology of these strains under controlled conditions, which was not possible in the industrial propagations. A well characterised laboratory haploid strain, 1278b, was also investigated in shake-flask culture.

Although there is a large body of information in the literature regarding the behaviour of the enzymes of ammonium assimilation, the growth conditions used in studies comparable to those reported below were different - aeration of the culture (Thomulka and Moat 1972) or lower growth temperature (Roon *et al.* 1974). Consequently, no direct comparison of the results from the industrial strains to a laboratory strain could be made. Data from a laboratory strain had to be obtained before a comparison could be attempted. A marked heterogeneity is also apparent in the results reported in the literature. There is no report in the literature describing the changes in activity of GS, GOGAT, NAD- and NADP-GDH in a single strain grown under the same culture conditions.

Σ1278b is the ancestor of the strain used to generate GOGAT$^-$ mutants and those used in the growth tests reported below. Information about changes in the enzymes of ammonium assimilation in both the industrial and laboratory strains allows an assessment to be made of the validity of any extrapolations from the laboratory model .

Data about the behaviour of the enzymes of ammonium assimilation is of interest for several reasons: (i) as the aim of the project is to determine if *S. cerevisiae* could benefit from similar manipulations as *M. methylophilus*, then GOGAT activity has to be demonstrated in commercial propagations: (ii) there

are no reports in the literature of how the enzymes of ammonium assimilation behave in industrial strains in a commercial propagation; and (iii) the collaborating body wanted information about the biochemistry of their propagations.

4.2 In order to demonstrate that the project was feasible, it was first necessary to demonstrate GOGAT activity in DCL1 and DCL2. GOGAT can be assayed spectrophotometrically by measuring the rate of oxidation of NADH. The reaction components of the GOGAT assay are very similar to those for the NAD-GDH assay, the only difference being that the former requires glutamine and the latter ammonium. Since cell extracts and the glutamine solution may not be free of ammonium plus the fact that GOGAT is only detectable at low levels in *S. cerevisiae*, it cannot be said unequivocally that the activity measured is GOGAT and not NAD-GDH. To demonstrate that the assay system used in this study was specific for GOGAT, DON was used to inhibit GOGAT activity. DON is a glutamine analogue known to competitively inhibit glutamine binding, as it binds to the active centre of the enzyme (Hartman 1963, 1968, and see section 1.3.4). Therefore, DON should inhibit GOGAT but not NAD-GDH. This approach has been used to show that *K. aerogenes* possesses GOGAT (Brenchley *et al.* 1973).

Approximately 200µg soluble protein was pre-incubated at 30°C for 10 minutes with or without DON (1mM final concentration). GOGAT was assayed as normal. To demonstrate the specificity of the inhibitor, NAD-GDH activity was assayed in cell extract prepared from Σ1278b and treated as for the GOGAT assay. The results are presented in Table 7.

The Σ1278b NAD-GDH loses 7% of its activity after incubation with DON. This compares with a loss of 95% of GOGAT activity under the same conditions. GOGAT from DCL1 and DCL2 lost 88% and 98% respectively of their uninhibited activities.

The data for the enzymes from Σ1278b show that while NAD-GDH was subject to limited inhibition there was a marked loss of GOGAT activity. This demonstrated that the reaction system used to assay GOGAT is specific and does not measure NAD-GDH. When cell extracts of DCL1 and DCL2 were assayed using the same reaction system, they showed a degree of inhibition comparable to that observed with the Σ1278b GOGAT. It can be concluded that both DCL1 and DCL2 possess GOGAT. Although this experiment demonstrates that the industrial strains possess GOGAT activity it does not follow that GOGAT functions during an industrial propagation.

4.3 Physiological studies with a variety of microorganisms suggest that for a large number of species, GS-GOGAT makes a major contribution to ammonium assimilation when ammonium is present at limiting concentrations. This is reflected in the higher affinity of GS for ammonium, compared to that of NADP-GDH. In *S. cerevisiae* the primary route of ammonium assimilation is, apparently, via the

Table 7.

Strain	Enzyme	-DON (% activity remaining)	+DON (% activity remaining)
Σ1278b	NAD-GDH	100.0	93.2
	GOGAT	100.0	4.7
DCL1	GOGAT	100.0	12.1
DCL2	GOGAT	100.0	1.6

Inhibitor studies of GOGAT and NAD-GDH. Cell extract (about 200µg protein) plus buffer were incubated in the presence (+) or absence (-) of the inhibitor DON, added to a final concentration of 1mM, at 30°C. After 10 minutes incubation, GOGAT and NAD-GDH activity were assayed. The results are expressed as the percentage of the activity observed in the absence of DON. The values are means of two independent experiments.

NADP-GDH, irrespective of the ammonium concentration. Industrial strains of *S. cerevisiae* are poorly characterised with respect to both the regulation of the enzymes of ammonium assimilation and the enzymes themselves. As part of the study of DCL1 and DCL2, it was decided to examine the kinetic properties of these enzymes.

K_m values were determined for GS, GOGAT and NADP-GDH from DCL1, DCL2 and Σ1278b. Σ1278b was examined to enable a complete comparison with the industrial strains to be made as there is considerable variation in the published K_m values for some *S. cerevisiae* enzymes (see section 1.3).

The data for K_m determinations was generated by measuring the reaction rate at different substrate concentrations, with the non-varying substrate(s) held at a constant, saturating level. K_ms were calculated by computer using the Hymic program of Barlow (1983). This program fits a line to the data using the method of least squares, and was primarily written for values of rate and substrate concentration obtained in enzyme reactions. The K_m values are presented in Table 8.

Wootton (1983) has presented data suggesting that ammonium is both a substrate and an activator of the *N. crassa* NADP-GDH. He concluded that the affinity for ammonium as a substrate is reflected by K_m values only when ammonium concentrations of 5mM or less are used. In this concentration range, activation does not cause a marked deviation from linearity in a Lineweaver-Burk plot. In this study, a concentration range of 0.1-100mM was used. However, six of the concentration values used were 5mM or less, the other values were 10, 50 and 100mM. Wootton showed that the deviation from linearity is increased at lower 2-oxoglutarate concentrations, below about 2.5mM. In this study, 2-oxoglutarate was used at 3.3mM. Therefore, it is highly probable that the K_m values for ammonium reported in Table 8 reflect the affinity of the three NADP-GDHs for ammonium as a substrate and not an activator.

The GS assay used in this study (Mitchell and Magasanik 1983) uses hydroxylamine instead of the physiological substrate ammonium. This may result in underestimation of the affinity of GS for ammonium. A comparison of the chemical formulae of ammonia and hydroxylamine (NH_3 *cf.* NH_2OH) reveals that a hydrogen atom has been replaced by a hydroxyl group, with the concomitant increase in "bulk" of the substrate. Since the shape of the substrate has altered, it may not bind as tightly to enzyme as the physiological one. The K_m for hydroxylamine may therefore be greater than the K_m for ammonium.

The K_m values for GS, GOGAT and NADP-GDH assayed in extracts of Σ1278b are comparable to those reported in the literature (Grisolia *et al.* 1964, Grenson *et al.* 1974, Roon *et al.* 1974, Masters and Meister 1982, Mitchell and Magasanik 1983). The affinity of the Σ1278b GS for hydroxylamine is three-times greater than that of NADP-GDH for ammonium. *S. cerevisiae* would appear to be similar to other microorganisms studied, in that its GS has a higher affinity than NADP-GDH. Both GOGAT and

Table 8.

Enzyme	Substrate	Strain		
		Σ1278b	DCL1	DCL2
NADP-GDH	2-oxoglutarate	0.84	1.02	0.78
	ammonium	2.60	3.45	2.70
GOGAT	2-oxoglutarate	0.12	1.11	0.42
	glutamine	1.40	0.73	1.24
GS	glutamate	6.64	6.44	3.56
	hydroxylamine	0.78	0.94	0.59
	ATP	2.67	6.48	4.65

K_ms (mM) for GS, NAD- and NADP-GDH for strains Σ1278b , DCL1 and DCL2. The data were generated by the varying the concentration of one substrate at a constant, saturating level of the other substrates, with a fixed amount of protein in each reaction mixture. K_ms were calculated using a computer program which fitted a line to the data by the method of least squares. K_ms for NADP-GDH are the mean values of three or more independent experiments - each point on the curve was obtained from duplicate assays. Other K_ms are the means of duplicate experiments.

NADP-GDH bind 2-oxoglutarate more tightly than glutamine or ammonium respectively. This contrasts with GS, where the affinity for glutamate and ATP is less than that for hydroxylamine.

The K_m values were compared between the three strains. There was no marked difference for both of the K_ms of NADP-GDH from the various strains. The K_m values of GOGAT show differences among the strains. The affinity of the Σ1278b enzyme for 2-oxoglutarate is ten-fold greater than that of DCL2, and four-fold greater than that of DCL1. The K_m for glutamine of Σ1278b and DCL2 GOGAT are comparable, although the Σ1278b K_m is twice that of the DCL1 value. The affinities of GS from the strains for hydroxylamine are similar, and are three-four-fold greater than those of the NADP-GDHs for ammonium. Both Σ1278b and DCL1 GS have similar K_m values for glutamate, although the affinity of the DCL2 enzyme is twice that of the Σ1278b one. The K_m values for ATP vary between 2.7 and 6.5mM.

Comparison of the K_m values for ammonium of GS and NADP-GDH reveals that GS has about a four-fold higher affinity than NADP-GDH (0.8 *cf.* 2.9mM) - the actual values depending upon the strain. The physiological significance of this difference will depend upon the intracellular ammonium concentrations existing under different growth conditions. Metabolite concentrations in cells grown with different ammonium concentrations were not determined in this study. The data available in the literature are for cells grown with excess ammonium (20mM or more). The cellular ammonium concentration for *S. cerevisiae* grown in MM supplemented with either 20 or 50mM ammonium is 8mM (Saez and Lagunas 1976, cited in Messenguy *et al.* 1976), which is a saturating level for both GS and NADP-GDH. Saez and Lagunas measured the intracellular concentration of 2-oxoglutarate in *S. cerevisiae* at 0.5mM, ATP was about 1mM and glutamate 15mM. There is no data available on the cellular concentration of glutamine. These metabolite concentrations, excepting the glutamate one, are not saturating for any of the three enzymes. Consequently maximal activity will not be observed for GS, GOGAT and NADP-GDH, even though ammonium is at a saturating level, in *S. cerevisiae* grown in MM + 20mM NH_4^+.

Based on the K_m values of the different enzymes and metabolite concentrations, it would appear that modulation of pre-existing enzyme activities could be achieved by fluctuation in the levels of TCA cycle intermediates and not ammonium. This is in agreement with genetic and physiological studies on how intermediates of the TCA cycle regulate NADP-GDH activity (see section 1.5.3). If it is assumed that the metabolite levels in shake-flask cultures of the industrial strains are comparable to those in wild type laboratory strains, then it can be postulated that GS, GOGAT and NADP-GDH are not maximally active in DCL1 or DCL2 in MM + 20mM NH_4^+. It is futile to speculate on how the different K_m values influence enzyme activity and ammonium assimilation in cells grown in medium supplemented with limiting ammonium, because there is no data on metabolite levels in such cultures.

Although wild type and industrial strains exhibit differences in their enzyme make-up and in the regulation of specific enzymes, this is probably a reflection of gross genetic differences between strains. It does not follow that the physical properties of the different gene products will show marked variation because the coding sequences probably do not show marked heterogeneity. However, such heterogeneity may exist e.g. it is possible that the GS subunit exhibits heterogeneity. Comparison of K_m values from the three strains used in this study shows that, as expected, the enzymes exhibit more similarities than differences. The most marked difference lies in the K_m of GOGAT for 2-oxoglutarate which varies over a ten-fold range. The other variations observed between strains are unlikely to be significant.

4.4.1 Studies on the behaviour of the enzymes of ammonium assimilation were done in shake-flask culture because it allowed the use of defined growth conditions. It was hoped to relate the results from these studies to the data from the industrial propagations. This study examined the behaviour of the enzymes of ammonium assimilation, but, as they are modulated by the nitrogen source, it was essential to use the same nitrogen source as in commercial propagations. Industrially, nitrogen is supplied as ammonium: therefore ammonium was used in the laboratory system. It was proposed to study the industrial strains during the course of a commercial propagation, so the the shake-flask cultures were grown for a period comparable to the industrial propagation. In the final D_6 stage of the propagation where most biomass is synthesised, the culture goes through only a limited number of generations, so a high initial cell density was used in the laboratory studies.

Characterisation of the GOGAT mutants, and studies of constructs made with these mutations, involved the use of glutamate auxotrophs. To obtain sufficient material for enzyme studies, these strains had to be grown in MM + glutamate. It was hoped to apply the information from this work to the industrial strains, which were grown in MM + NH_4^+. Consequently a compromise had to be made: all strains were grown in MM + glutamate prior to switching to MM + NH_4^+. There was an inherent problem with this experimental design, the switch of nitrogen sources causes changes in the cell physiology e.g. repression of GS and NAD-GDH. Such physiological changes are probably secondary to other changes which occur in this experimental system, even on transfer of cells to fresh medium of the same composition. The overnight culture was in early stationary phase. The physiology of stationary phase cells is different to those in exponential growth e.g. an increase in protein degradation is observed during the transition from exponential to stationary phase. Therefore, the cells will have to adapt to fresh medium. In this experimental system, this is probably more important than changes due to switching of the nitrogen source.

It was decided to extend the comparative studies by examining the behaviour of the enzymes of ammonium assimilation under different growth conditions. The time-course experiment used

ammonium at a non-limiting concentration (20mM). The growth parameters of commercial propagations are such that ammonium is limiting for at least part of the culture. There is selective pressure for the retention of the ability to assimilate low levels of ammonium. Although there are no reports in the literature that laboratory strains of *S. cerevisiae* assimilate limiting ammonium through the GS-GOGAT pathway, this ability may be retained in production strains. The three strains were grown in limiting ammonium (0.2 and 2mM), and enzyme activities determined. Two concentrations were used to determine whether the ammonium concentration of the medium modulated enzyme levels, because Roon and Even (1973) had shown that ammonium levels below 1mM had profound effects on NAD- and NADP-GDH activities in strain X2180-1A.

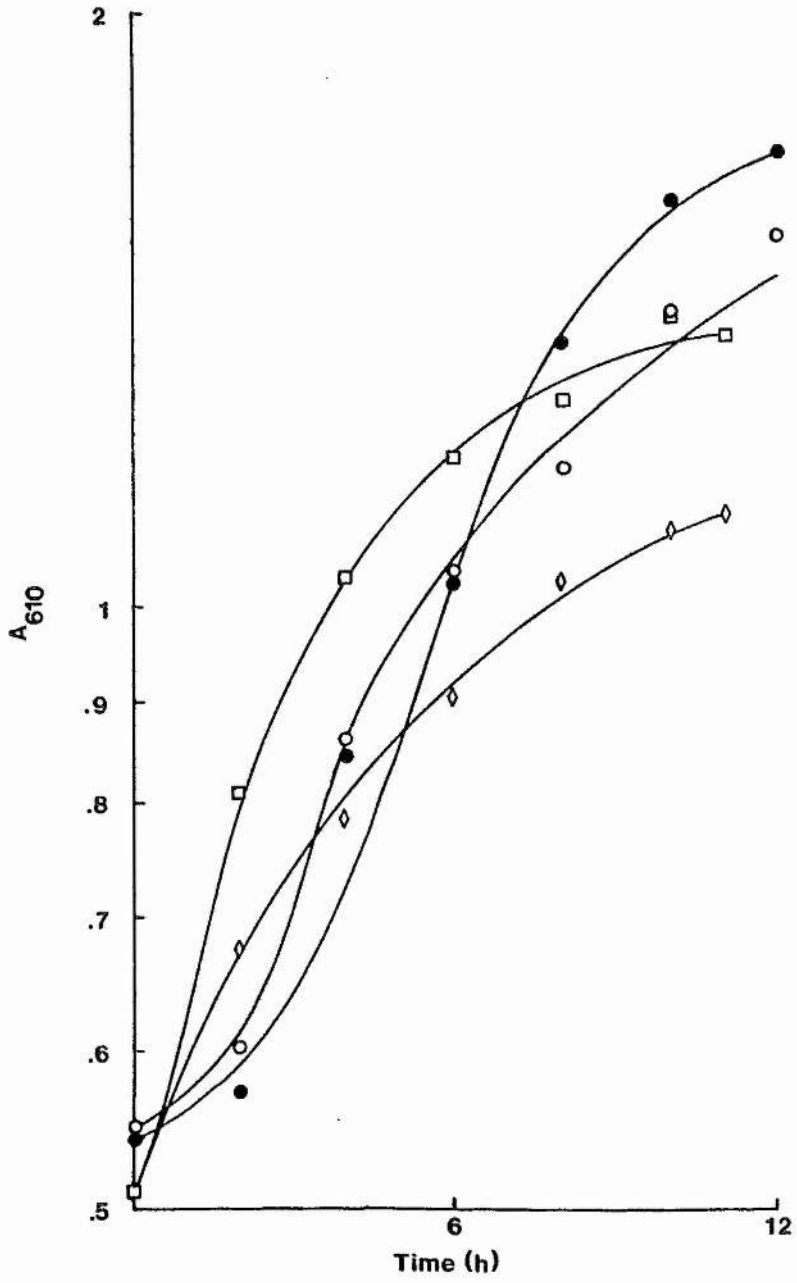
GS and NAD-GDH activities are repressed by ammonium but derepressed by glutamate. Enzyme activities were compared in ammonium and glutamate grown cells to demonstrate any differences in the regulation of GS and NAD-GDH.

Growth curves were prepared for each growth condition. The experiment was done to reveal differences in the ability to use a poor nitrogen source (glutamate) and ammonium, at limiting and non-limiting concentrations. It is possible that differences observed in the enzyme studies arose because the samples were taken at different points on the growth curve. This possibility could be investigated by examination of the growth curves for each condition.

4.4.2 Growth curves were obtained for the same conditions used in the enzyme studies. Glutamate grown cells were inoculated into MM + 20mM NH_4^+ at an initial A_{610} of 0.55 and grown, with shaking, at 30°C. Absorbance readings at 610nm were taken at 2h intervals over a 12h period. The data were plotted graphically and representative curves are presented in Figs. 7, 8 and 9. The doubling-time (t_d) of each culture during exponential growth was measured from these figures and are reported in Table 9.

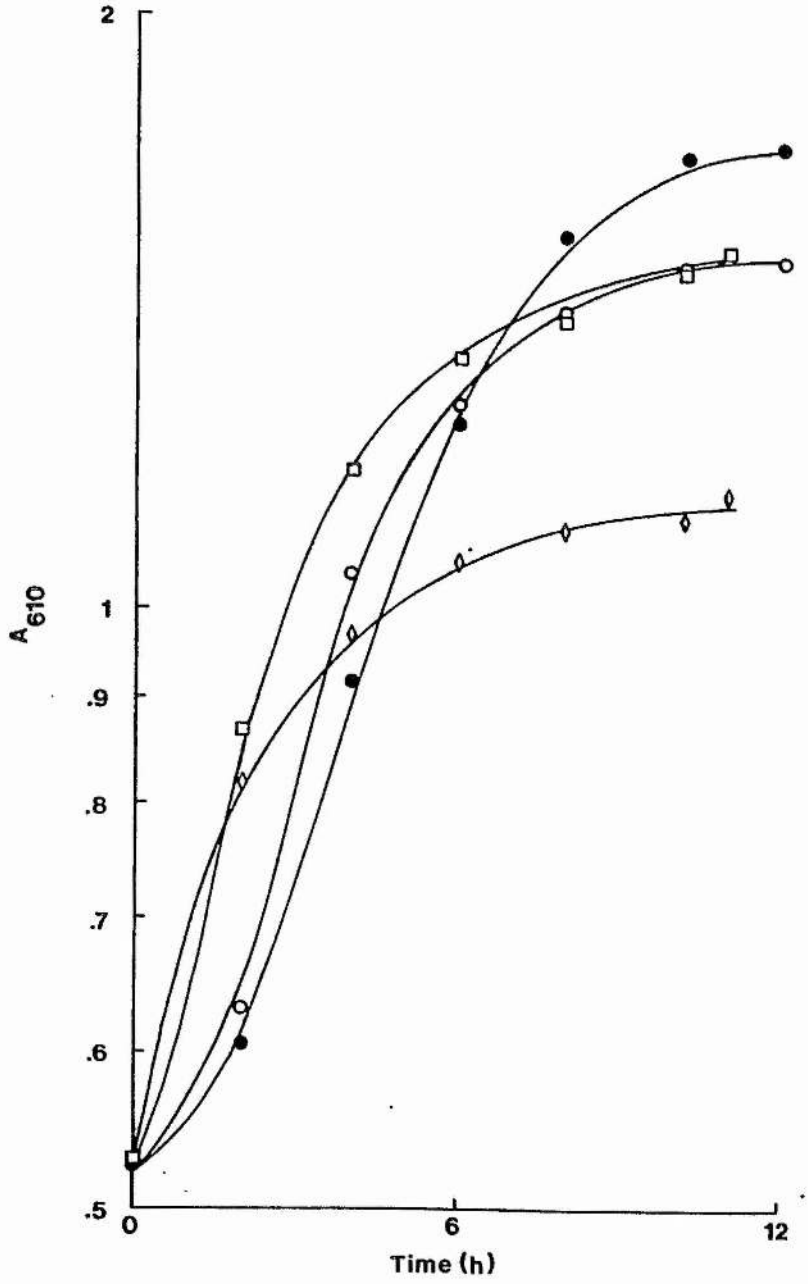
Examination of the data in Figs. 7, 8 and 9 shows that, on the basis of the final absorbance reading, 10mM glutamate was the best nitrogen source. At the end of the experiment the absorbance readings for all three strains were very similar (about 1.8). The data shows that 2 and 20mM NH_4^+ were comparable in their ability to support growth. The final A_{610} values for DCL1 and DCL2 grown at these two ammonium concentrations (plus 20mM NH_4^+ grown Σ1278b) were all about 1.6, which is markedly lower than glutamate grown cultures. The final A_{610} reading for Σ1278b grown with 2mM NH_4^+ was about 1.45, a lot lower than the 20mM NH_4^+ value. Growth on 0.2mM NH_4^+ was very poor for all the strains. The final A_{610} value was only about 1.15, markedly lower than even the 2mM NH_4^+ cultures.

Fig. 7



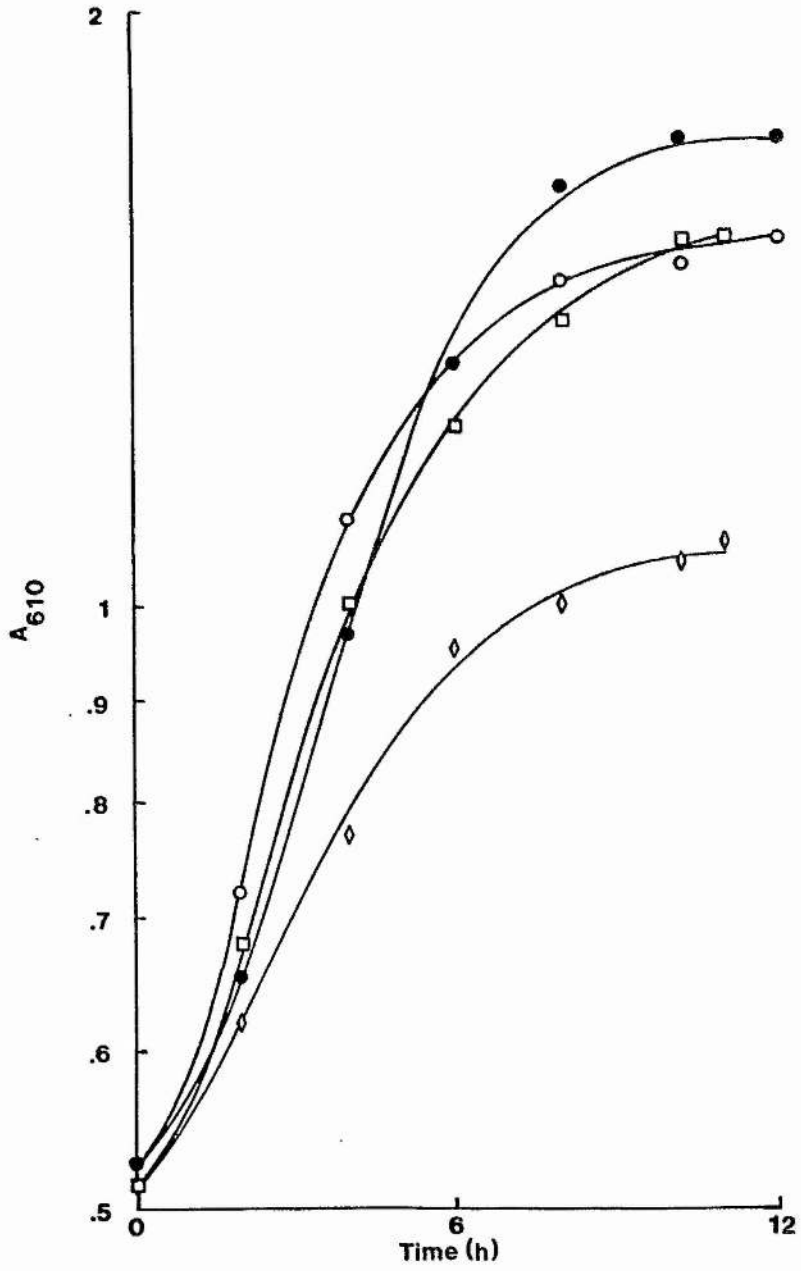
Growth curves of Σ1278b in shake-flask culture. Glutamate grown stationary phase culture was used to inoculate fresh MM (300ml in a 1l non-baffled flask, 150rpm, 30°C) supplemented with various nitrogen sources to an initial $A_{610} \sim 0.5$. Growth was followed by measuring the change in A_{610}. Nitrogen was supplied as ammonium at 0.2 (◊), 2 (□) or 20mM (○), or as 10mM glutamate (●). The experiments were done twice and representative curves are shown.

Fig. 8



Growth curves for DCL1 grown with different nitrogen sources. Experimental conditions and symbols are as described in Fig. 7. Representative results from two independent experiments are shown.

Fig. 9



Growth curves of DCL2 grown in MM supplemented with different nitrogen sources. Growth conditions and symbols are as for Fig. 7. Two independent experiments were done and representative curves are shown.

Table 9.

N-source + concentration (mM)		Strains		
		Σ1278b	DCL1	DCL2
Ammonium	20	156	144	153
	2	198	186	150
	0.2	285	324	153
Glutamate	10	209	184	205

Doubling times (minutes) of Σ1278b, DCL1 and DCL2 grown in MM supplemented with glutamate or various concentrations of ammonium. Values were obtained from the growth curves presented in Figs. 7-9.

The growth profiles show that the three strains are very similar in their ability to grow in different ammonium concentrations and on glutamate. Examination of the t_d values (Table 9) reveals that there are considerable differences between the strains. The three strains have similar t_ds when grown on 20mM NH_4^+ (144-156 minutes) and are comparable to published values (Cooper 1982a). Both Σ1278b and DCL1 show a marked increase in t_d as the ammonium concentration decreases. For 2mM the values are comparable (198 and 186minutes for Σ1278b and DCL1 respectively). Σ1278b and DCL1 grow much slowly still (285 and 324 minutes respectively) on 0.2mM NH_4^+. Growth on limiting ammonium accentuates the differences in growth characteristics of the two strains. DCL2 is very different from both Σ1278b and DCL1. At all three test concentrations the t_ds were very similar (150-153 minutes), and comparable to the rate observed for the other two strains on 20mM NH_4^+,indicating that DCL2 can grow well in ammonium-limited culture.

For a nutrient limited culture (in this system, ammonium-limited), the concentration of nutrient in the culture medium limits the rate of cell synthesis. The rate of nutrient uptake into the cell is a function of the concentration of the limiting nutrient. At low nutrient concentrations this results in less nutrient available to the cell per unit time, and, for example, decreases the rate of enzyme synthesis (by limiting the rate of amino acid biosynthesis). As there are fewer enzyme molecules in the cell, the flux through the various metabolic pathways is reduced. Consequently, there is a decrease in the rate of synthesis of other cellular components and t_d increases. The data presented in Table 9 shows that this hypothesis is true for Σ1278b and DCL1. However, the t_d of DCL2 is independent of the ammonium concentration. It would appear that DCL2 is able to absorb ammonium present at 0.2mM in the medium just as rapidly as if it was present at 20mM.

A possible explanation for the result with DCL2 is that DCL2 has a high affinity, high capacity ammonium permease, not present in Σ1278b or DCL1. Dubois and Grenson (1979) have identified two ammonium permeases in *S. cerevisiae* Σ1278b. These workers, using a biochemical technique, have presented evidence that there may be a third, and possibly more, permease of unknown characteristics in Σ1278b. Therefore, it has been suggested in the literature that *S. cerevisiae* may possess additional ammonium permeases. There is a precedent in the literature for variation in the number of ammonium permeases in different strains. Dubois and Grenson presented evidence for two permeases in Σ1278b but Roon *et al.* (1975) only detected one permease in X2180-1A.

If DCL2 has an additional high affinity, high capacity ammonium permease, then an elevated intracellular ammonium concentration would be expected with enzyme levels comparable to those observed in either 2 or 20mM NH_4^+ grown cells. These changes in enzyme levels were not observed (Table 10). However, if DCL2 does possess a high affinity, high capacity permease, then ammonium would be rapidly depleted from

the medium. Consequently the cells would enter retardation phase earlier and enzyme levels would decrease. This could explain the results shown in Table 10.

Although glutamate was the best nitrogen source tested in these studies, as judged by the final A_{610} values, this was not confirmed when the t_ds were examined. The t_ds of Σ1278b and DCL1 (209 and 184 minutes respectively) are comparable to the values observed with 2mM NH_4^+ although there is a marked difference in final A_{610} readings, especially for Σ1278b. DCL2 grows more slowly on glutamate than on ammonium (t_ds of 205 *cf.* 150-153 minutes). Published data for a variety of strains (Cooper 1982a) show that for a given nitrogen source a range of t_ds are observed, and, of the five strains examined, four of them grow faster on ammonium than on glutamate. The exception was Σ1278b, with t_ds of 148 and 136 minutes on ammonium and glutamate respectively. Dubois *et al.* (1974) reported t_ds for Σ1278b of 120 and 150 minutes respectively. The reason for this marked difference between the published data and the values reported in this study is unclear, although it may be partly explained by differences in culture conditions. The variations in published t_ds for the same strain on the same nitrogen source (plus those in enzyme K_m values) highlight the need for the use of a control strain in these comparative studies.

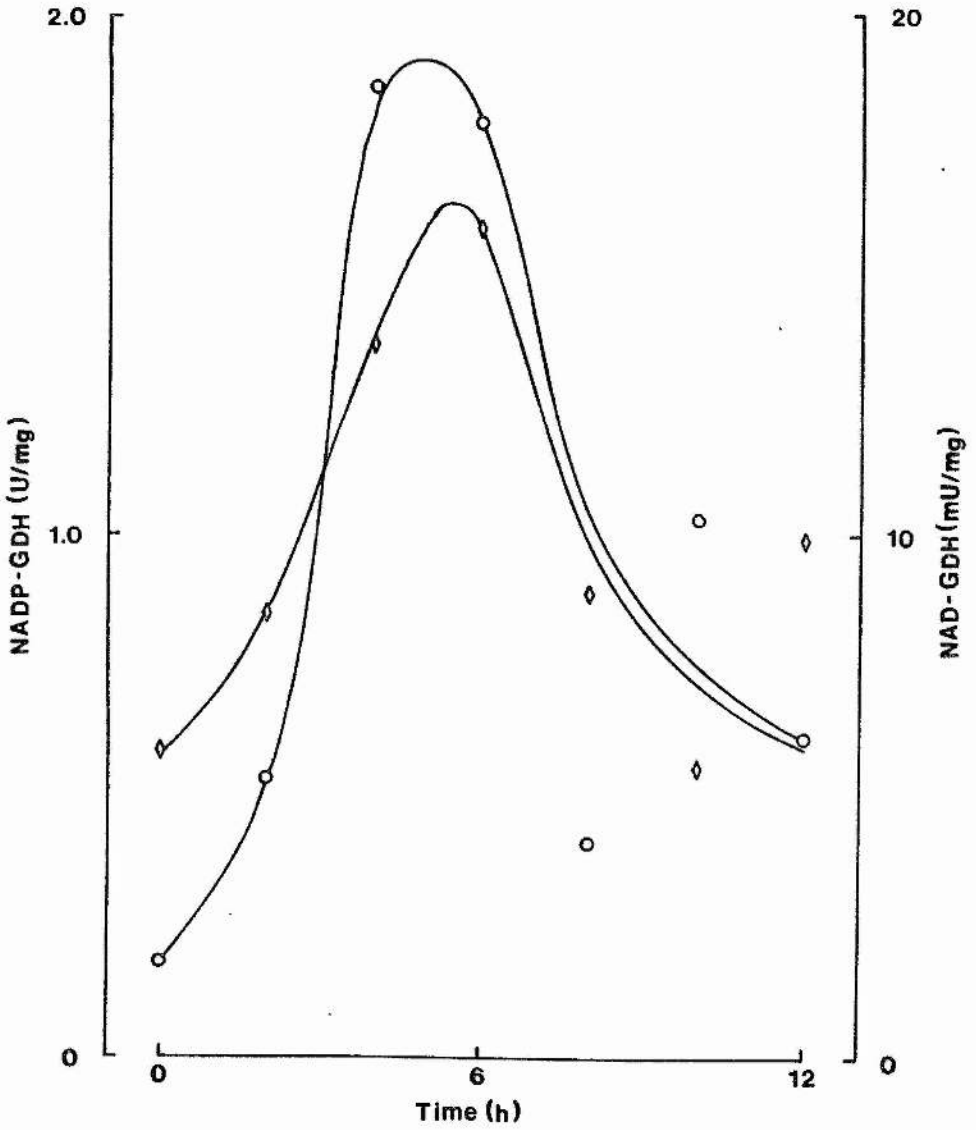
4.4.3 Changes in the activities of GS, GOGAT, NAD- and NADP-GDH in shake-flask cultures of Σ1278b, DCL1 and DCL2 were followed over a 12h period. The results are presented in Figs. 10-15.

With the exception of the DCL1 NAD-GDH, all the enzymes from the three strains show an increase in activity. The NAD-GDH from DCL1 exhibited a decrease in activity to a constant level. After reaching a maximum value, nearly all the other enzymes decreased in activity back to the level observed at the start of the culture. The exception was GOGAT from DCL2, which had a final activity intermediate between the initial and maximal activities.

Strain Σ1278b showed a six-fold increase in GS activity during exponential growth to a maximum level (about 73mU/mg) at the mid-point of the exponential growth phase (3h). GS activity was lost during late exponential/early retardation phases. GOGAT and NAD-GDH exhibited highest activity at 6h: NADP-GDH peaked at about 5.5h, which was the middle of the retardation phase. The maximum activities were approximately 44, 19 and 1650mU/mg respectively, which were increases of about eleven-, ten- and three-fold over the initial level, The activities of all three enzymes increased during exponential growth and then decreased during the retardation phase.

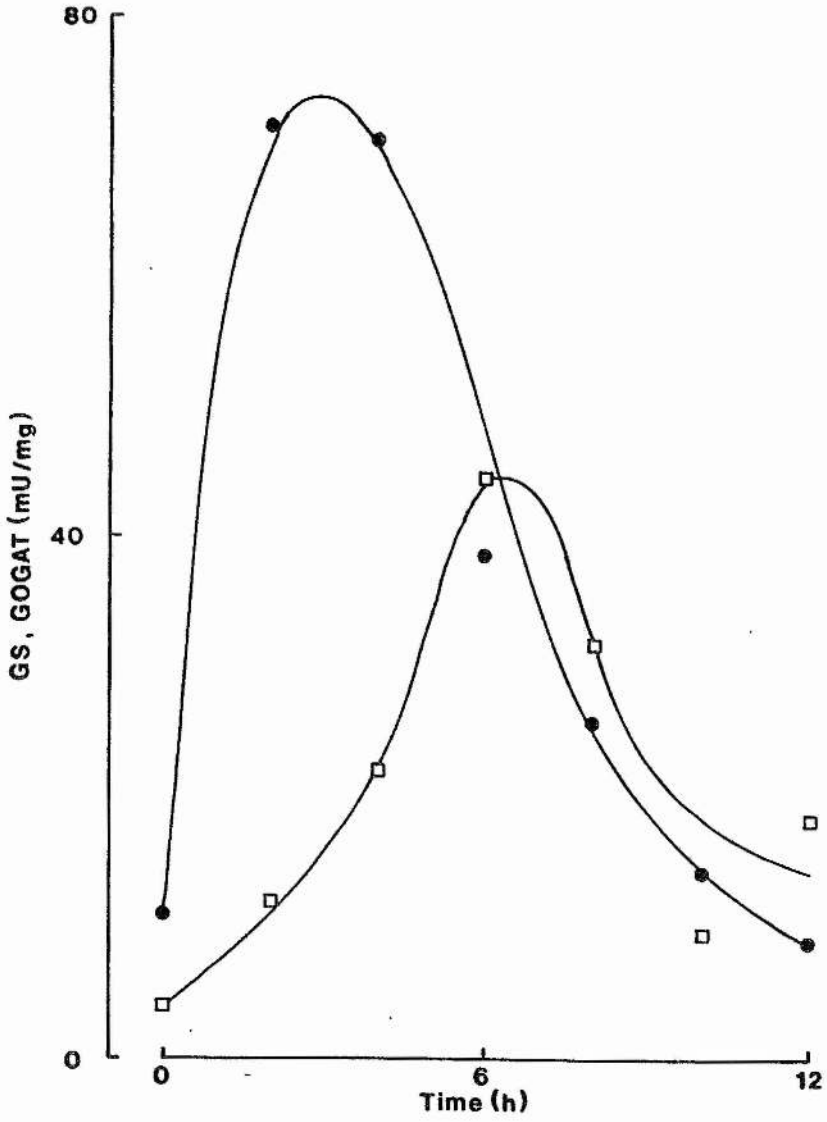
Examination of Figs. 10-13 shows that DCL1 was different to the laboratory wild type Σ1278b. The most marked difference was the behaviour of NAD-GDH. The NAD-GDH activity in Σ1278b rose to a peak before declining, but the activity in DCL1 decreased during the first 3-4h of the culture to a constant level (about 9mU/mg). The maximum activities observed in both strains, although they

Fig. 10



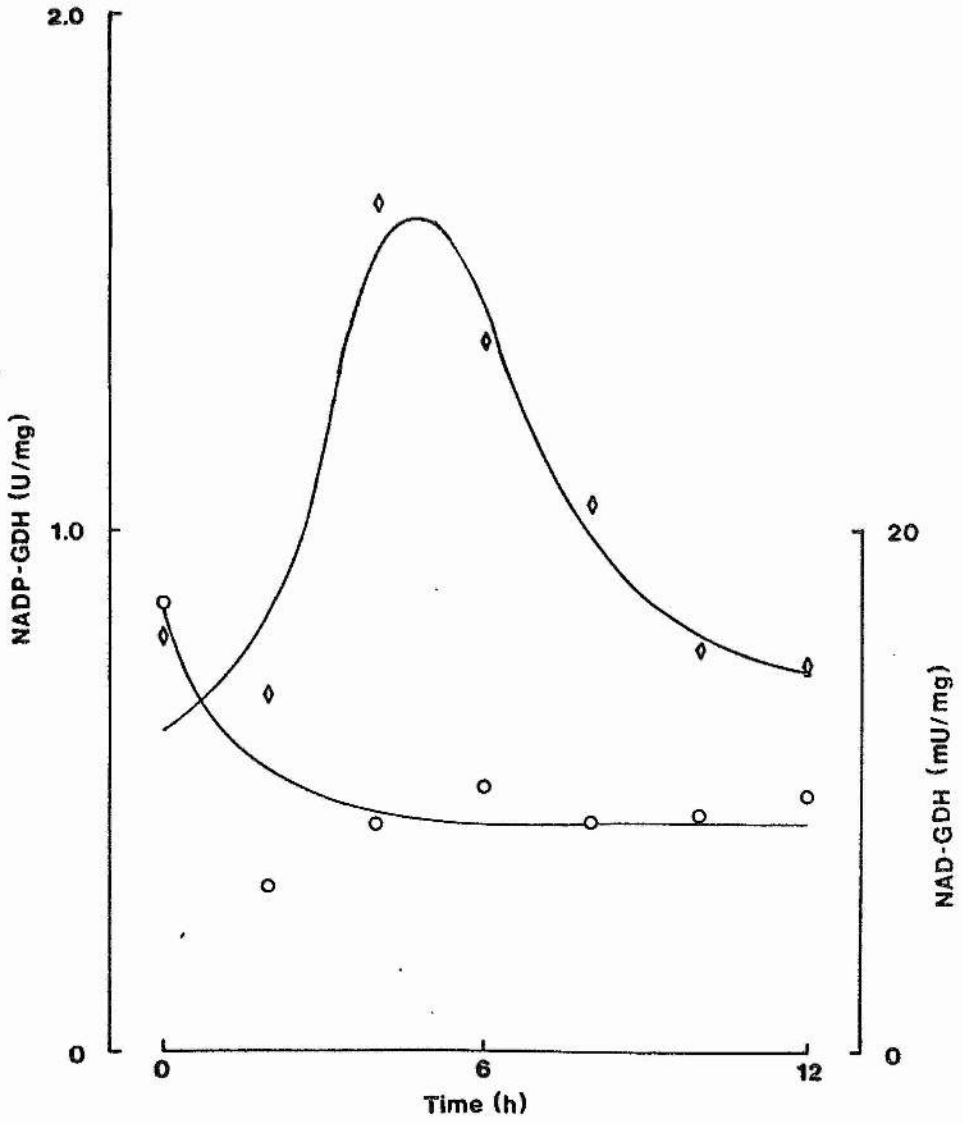
NADP-GDH (◊) and NAD-GDH (o) activities in cultures of Σ1278b grown in MM + 20mM NH_4^+ (other growth conditions as in Fig. 7). Cells were taken at the points shown and assayed for NAD- and NADP-GDH activities. Duplicate assays were done on each extract and the results presented are the means of at least two independent experiments.

Fig. 11



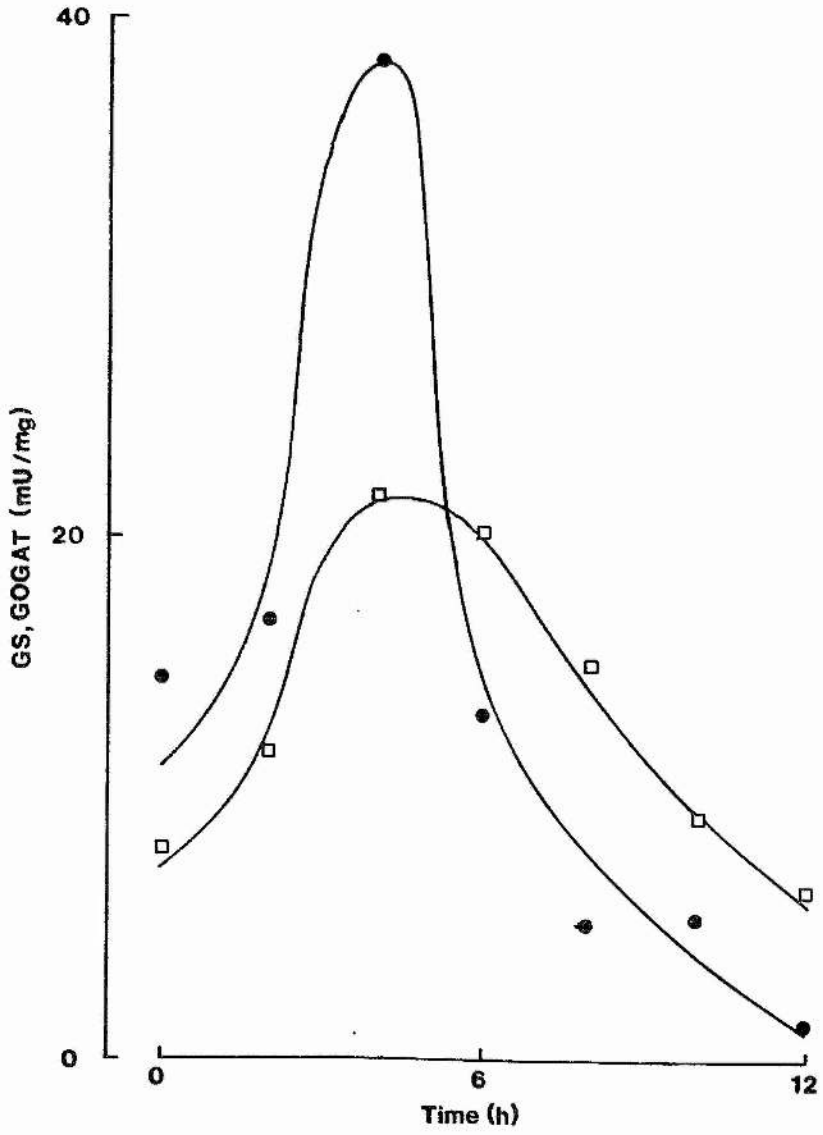
Enzyme profiles of Σ1278b grown in MM + 20mM NH_4^+ (other growth conditions as in Fig. 7). Cells, taken at 2h intervals, were assayed for GS (●) and GOGAT (□). The data are the average of two independent experiments: duplicate assays were done on each extract.

Fig. 12



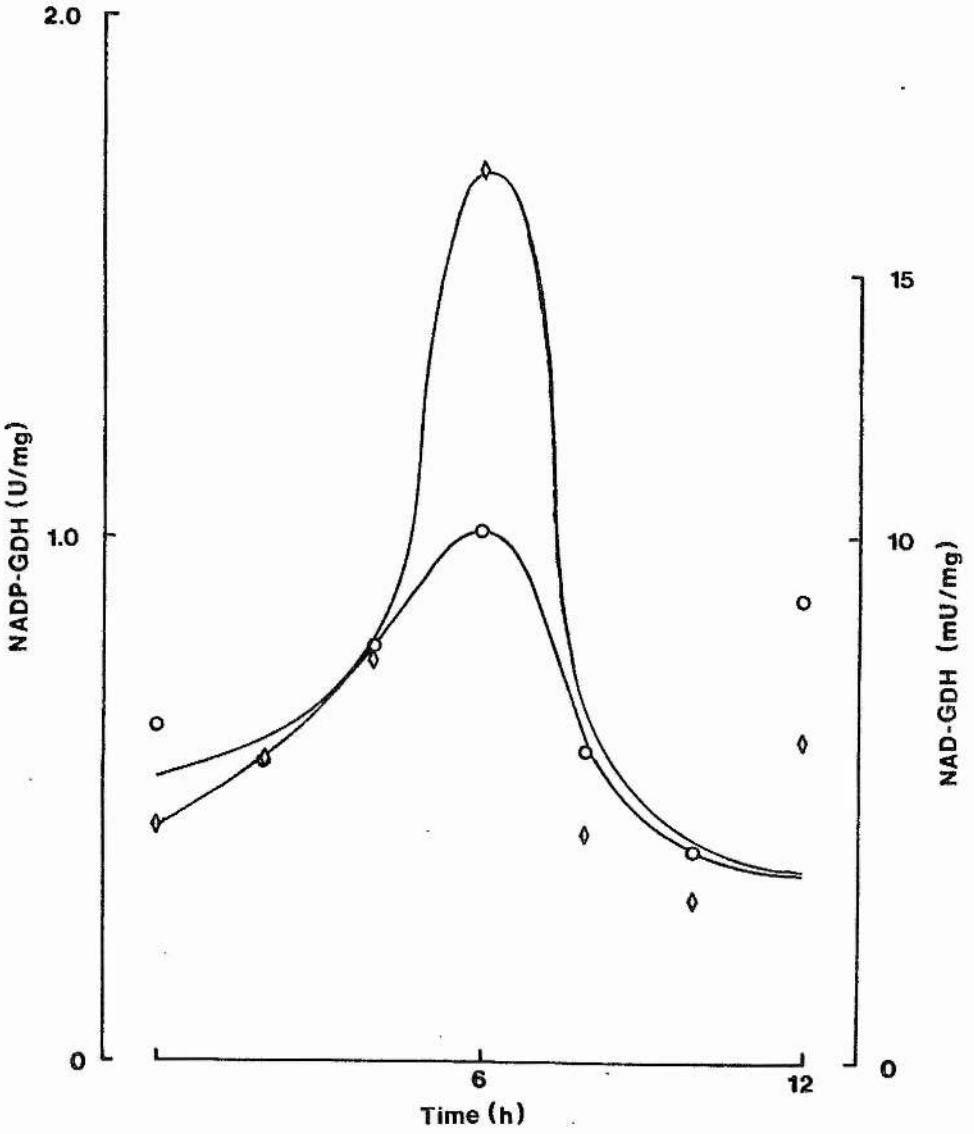
NAD-GDH (○) and NADP-GDH (□) activities in DCL1 grown in MM + 20mM NH_4^+, other growth conditions as in Fig. 7. The results shown are the average of at least two independent experiments with duplicate assays done on each extract.

Fig. 13



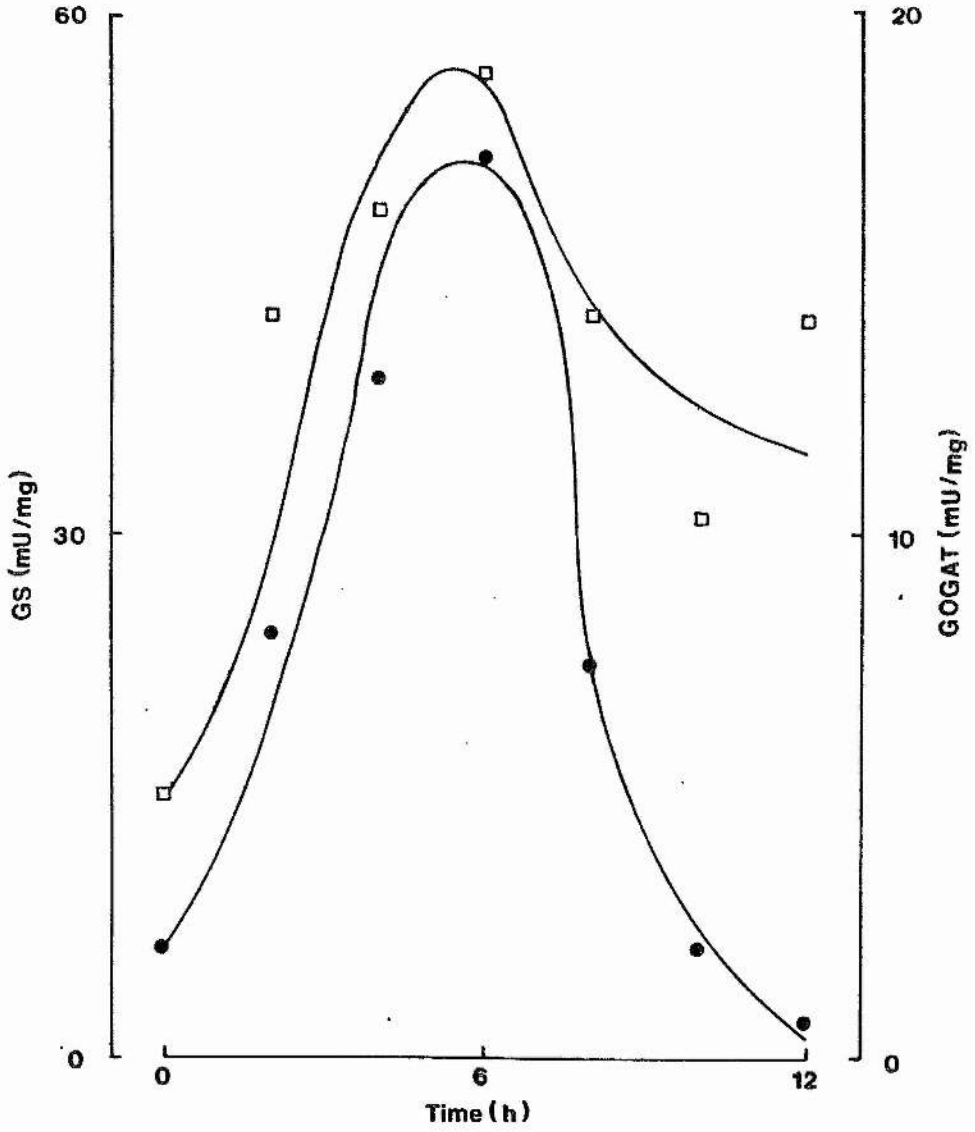
GS (●) and GOGAT (□) activities in cultures of DCL1 grown in MM + 20mM NH_4^+ (other growth conditions as in Fig. 7). The data are the means of two or more experiments, with duplicate assays done on each extract.

Fig. 14



Enzyme profile of DCL2 grown in MM + 20mM NH_4^+ (other growth conditions as in Fig. 7). Cells taken at 2h intervals were assayed for NAD-GDH (O) and NADP-GDH (◊). The results are the average of at least two independent experiments with each extract assayed twice.

Fig. 15



GS (●) and GOGAT (□) activity profiles of DCL2 grown in MM + 20mM NH_4^+ (other growth conditions as in Fig. 7). Extracts were assayed twice, and the data presented are the means of at least two independent experiments.

appeared at different times, were similar (about 18 mU/mg). The second difference was that maximum GS activity in DCL1 was coincident with the maxima for GOGAT and NADP-GDH activities. Highest activities occurred at the middle of the retardation phase (about 5h). At this point GOGAT activity was 22 mU/mg, about 50% of the value for Σ1278b. Maximal NADP-GDH activities in Σ1278b and DCL1 were comparable (about 1.6U/mg). The highest GS activity measured in DCL1 (38.3mU/mg) was about 50% of the activity in Σ1278b. GS, GOGAT, and NADP-GDH behaved in a similar manner to the Σ1278b enzymes. Enzyme activity increased during exponential growth, and was then lost during the retardation phase.

The enzymes from DCL2 (Figs. 14 and 15) are unlike those from the other two strains in that they all reach maximal activity at the same point in the growth curve *i.e.* the middle of the retardation phase (6h). GOGAT activity, unlike the three other enzymes in DCL2, plus GOGAT in Σ1278b and DCL1, did not subsequently decrease back to the level measured at the start of the culture. It remained at an intermediate level (about 12mU/mg) relative to the initial (5mU/mg) and highest (about 19mU/mg) activities. The maximal activity observed was less than 50% of that for Σ1278b but comparable to the figure for DCL1. As with the Σ1278b enzyme, activity increased during exponential growth before declining during the retardation phase.

The highest GS activity observed in DCL2 was about 52mU/mg (an increase by a factor of 9 relative to the initial level) which is lower than the figure for Σ1278b (about 72mU/mg) but higher than for DCL1. Unlike GS in Σ1278b and DCL1, activity increased during lag, exponential and early retardation phases before declining in late retardation phase. Highest NAD-GDH activity observed in DCL2 was about 10mU/mg compared to 6.4mU/mg at the start of the culture. Maximal activities in Σ1278b and DCL1 were higher (about 17mU/mg). Like 1278b, NAD-GDH activity in DCL2 appeared during exponential growth and then declined in the retardation phase. Maximum NADP-GDH activity measured was 1.7U/mg (a fourfold increase over the initial level) compared to 1.6U/mg for Σ1278b and DCL1. However, unlike these other two strains, NADP-GDH activity in DCL2 appeared and was lost very rapidly (over a 4h period it increased from 0.7U/mg to 1.7U/mg before decreasing to 0.45U/mg). NADP-GDH activity appeared during late exponential phase and was lost during late retardation phase.

The studies reported above show that both DCL1 AND DCL2 possess all four enzyme activities when cultured in MM + NH_4^+. Although it had previously been demonstrated that both industrial strains have GOGAT activity (section 4.2), the studies reported in this section show that GOGAT activity increased during exponential growth when biomass was being synthesised. The data show that the manipulations described in the Introduction are potentially capable of improving the biomass yield of *S. cerevisiae*.

The main route for ammonium assimilation in *S. cerevisiae* is through NADP-GDH, which is reflected in the high activity of this enzyme relative to the other enzymes of ammonium assimilation. The maximal NADP-GDH activities observed in DCL1 and DCL2 are comparable to Σ1278b and are markedly higher than the GS activities. This indicates that the primary route of ammonium assimilation by DCL1 and DCL2 (at least for the growth conditions used in this experiment) is via the NADP-GDH.

The data presented in Figs. 10-15 show that there are strong similarities in the behaviour of GS, GOGAT, NAD- and NADP-GDH activities between the two industrial strains and the control laboratory wild type. In the experimental system used in this study, enzyme activities increased during exponential growth to maximum levels at mid-retardation phase, before decreasing in late retardation phase to levels comparable to those at the start of the culture. The major differences between the strains are: (i) in DCL1, NAD-GDH decreases to a constant level instead of increasing; (ii) the Σ1278b GS peaks in mid-exponential phase compared to mid-retardation phase for the other enzymes; and (iii) GOGAT from DCL2 does not decrease to the same extent as the other enzymes. Additionally, the data showed that the maximal GS, GOGAT and NAD-GDH activities of Σ1278b were generally higher (by a factor of 2) than the activities for DCL1 and DCL2. Also, the increase in enzyme activity during growth was generally greater for Σ1278b (about nine-fold) than for DCL1 and DCL2 (factors of about 2 and 4 respectively).

Other workers have done similar experiments to those described above, using slightly different growth conditions, with other strains. Thomulka and Moat (1972), using an adenine auxotroph, found that although NAD- and NADP-GDH activities increased to maxima, GS activity declined to a constant level. NAD-GDH reached its highest level in early exponential phase before declining, while NADP-GDH increased slightly during lag phase to remain constant at a high level. Roon *et al.* (1974) using the wild type strain X2180-1A observed that both GOGAT and NADP-GDH increased (by factors of 2 and 3 respectively) during growth in MM + NH_4^+. GOGAT activity remained relatively constant while NADP-GDH showed a slight decrease over the remainder of the culture. Roon and Even (1973), using conditions very similar to those used in this study, found that NAD-GDH activity in X2180-1A exhibited a constant decrease on transfer from MM + glutamate to MM + NH_4^+.

The literature shows considerable differences in the behaviour of the enzymes of ammonium assimilation among different strains. It is therefore unclear whether the differences observed between Σ1278b, DCL1 and DCL2 are simply due to strain variations or whether they are significant differences between industrial and laboratory strains. At this point, with the lack of information about the detailed biochemistry and genetics of these strains, it would be futile to speculate about the reasons for the observed strain variability. The strong similarities observed between the industrial and laboratory

strains in this study do suggest that one can successfully extrapolate from the laboratory to the industrial strains.

4.4.4 To investigate how ammonium concentration and growth on a poor nitrogen source modulates the activity of the enzymes of ammonium assimilation in the industrial strains, they were grown in MM supplemented with either 0.2 or 2mM NH_4^+, or 10mM glutamate. The strains were grown as for the experiments described above , except that, after switching to appropriately supplemented MM, they were harvested after 6h incubation. Cell extracts were prepared and assayed for all four activities. Cells were harvested at 6h because the data reported in Figs. 10-15 had shown that maximum enzyme activity was, generally, observed at about 6h. The results for these experiments are reported in Table 10.

Examination of the data in Figs. 7-15 shows that the position in the growth cycle affects the enzyme activity detected in the cell. Inspection of the growth curves (Figs. 7-9) shows that, for ammonium grown cultures (at all three concentrations) of the three strains, the 6h timepoint occurs in the middle of the retardation phase. For glutamate grown Σ1278b the 6h timepoint lies in mid/late exponential growth; for DCL1 it is in the early part of the retardation phase, yet for DCL2 it occurs at the end of the exponential growth phase. Consequently, any differences observed from comparison of ammonium grown cells are unlikely to arise from variations in the point in the growth cycle at which samples were taken. However, for the glutamate grown cells, variability in the point at which samples were taken may give rise to differences which cannot, currently, be distinguished from those due to strain variability and to the different nitrogen source.

The data in Table 10 show that, for a given enzyme, the response to the various nitrogen sources was not the same in the three strains.

GS activity in Σ1278b was highest in 20mM NH_4^+ grown cells, lowest in 2mM NH_4^+ grown ones, with an intermediate level measured in the 0.2mM NH_4^+ culture. A similar response to ammonium concentration was observed with strain DCL2. For these two strains there is no obvious relationship between GS activity and ammonium concentration. However, for DCL1 there is an inverse relationship: as the external ammonium concentration decreases, GS activity increases.

Growth with glutamate as sole nitrogen source causes derepression of GS and an increase in GS activity compared to ammonium grown cells (Dubois and Grenson 1974). This was observed with all three strains used in this study. The degree of derepression observed (by comparison of the glutamate and 20mM NH_4^+ values) was three- and five-fold for Σ1278b and DCL2 respectively, which is comparable to the value obtained from the data of Dubois and Grenson (1974). GS activity was 17-fold higher in glutamate grown cells of DCL1 compared to 20mM NH_4^+ grown ones. The increase in the degree of

Table 10.

Strain	N-source + concentration (mM)		Enzyme Activities			
			GS[1]	GOGAT[1]	NAD-GDH[1]	NADP-GDH[2]
Σ1278b	Ammonium	0.2	17.5	22.4	10.3	1.133
		2	9.2	20.2	11.2	1.427
		20	34.2	44.2	17.9	1.589
	Glutamate	10	153.0	90.2	187.0	2.167
DCL1	Ammonium	0.2	74.0	9.7	30.2	0.280
		2	45.9	11.9	70.1	0.398
		20	13.2	20.2	10.1	1.357
	Glutamate	10	216.0	85.1	513.0	0.926
DCL2	Ammonium	0.2	36.2	12.0	33.0	0.655
		2	20.1	17.9	11.4	0.977
		20	18.8	10.1	10.1	1.698
	Glutamate	10	181.0	39.0	152.0	0.631

Enzyme activities of the three strains grown with various nitrogen sources. Cells were grown as described in the legend to Fig. 7, except that the culture was harvested after 6h. Cell extracts were assayed for GS, GOGAT, NAD- and NADP-GDH. The data are the means of a minimum of three independent experiments: duplicate assays were done on each extract. 1 - mU/mg; 2 - U/mg.

derepression was due to at least two factors: (i) a high ammonium concentration represses GS in DCL1 more severely than in Σ1278b and DCL2; and (ii) the GS activity measured in DCL1 was greater than that in the other two strains.

The effect of ammonium concentration on GS activity has been examined in *N. crassa* (Lara *et al.* 1982, Lomnitz *et al.* 1987) and *A. nidulans* (Kusnan *et al.* 1987) plus *Saccharomycodes ludwigii* and various species of *Schizosaccharomyces* (Brown *et al.* 1973, Johnson and Brown 1974). These workers found that growth on limiting ammonium led to derepression of GS. Similar studies have not been reported for *S. cerevisiae*. DCL1 responded to a decrease in ammonium concentration as was expected from the studies with other microorganisms, yet Σ1278b and DCL2 did not. The reasons for these differences are unclear.

In ammonium supplemented cultures it was found that, for all three strains studied, GOGAT activity was highest in 20mM NH_4^+ grown cells and generally decreased as the medium ammonium concentration was lowered. This is in agreement with observations reported by Roon *et al.* (1974). The activities measured in the 0.2mM NH_4^+ cultures were still about 50% of the maximal levels detected. It would appear that the *S. cerevisiae* GOGAT is weakly regulated by the level of ammonium in the medium. In this respect it is similar to the other microbial eukaryotes which have been studied.

Growth of *S. cerevisiae* with glutamate as sole nitrogen source causes derepression of GOGAT activity - an increase in activity by a factor, relative to the 20mM NH_4^+ value, of 2 for Σ1278b and DCL1, but 4 for DCL2. This contrasts to the results obtained by Roon *et al.* (1974). These workers, using strain X2180-1A, found that GOGAT activity in glutamate grown cells was about 50% of the level in ammonium grown ones. The experimental conditions used in that study were very similar to those used here. Therefore, it would appear that , in *S. cerevisiae*, there are differences between strains in the regulation of GOGAT by nitrogen source.

A priori it would be expected, as GOGAT forms part of the same pathway as GS, that if GS activity was high then GOGAT levels would be elevated. However, it would appear from the data presented in Table 10 that GOGAT activity is not linked to the level of GS. Examination of the data for ammonium grown cells shows that, while GS activity varies over a wide range, GOGAT levels remain comparatively constant. Why GS activity varies independently of the GOGAT level in ammonium grown cells is unclear.

Growth on glutamate causes derepression of both GS and GOGAT. But GOGAT synthesises glutamate from glutamine. Therefore, increased synthesis by elevated levels of GOGAT in glutamate grown cells would establish a futile cycle of glutamate synthesis/degradation, but this may be needed to maintain the correct ratio of glutamate and glutamine (by removing the glutamine synthesised by GS activity) to

ensure the correct distribution of nitrogen by the transamidation and transamination systems (see sections 1.5.2 and 1.5.3). The reason for this response is unclear. The lack of any relationship between GS and GOGAT levels in ammonium grown cells suggests that some factor, other than GS or glutamine (which is the product of GS activity) may also be modulating GOGAT activity.

The relationship between NAD-GDH activity and ammonium concentration was different in each of the three strains examined. In Σ1278b the highest NAD-GDH activity was detected in 20mM NH_4^+ grown cells; this value (18mU/mg) is about 50% higher than the levels measured in 0.2 and 2mM NH_4^+ grown cultures. The opposite pattern was found in DCL2. The highest enzyme level (33mU/mg) was measured in 0.2mM NH_4^+ cultures and was about three-fold greater than the level in 2 or 20mM NH_4^+ grown ones. Maximum activity (70mU/mg) was detected in 2mM grown cells, the lowest (about 10mU/mg) in the 20mM NH_4^+ cultures yet an intermediate level (30mU/mg) was measured in 0.2mM NH_4^+ grown cells.

Similar experiments to those described in this study have been reported in the literature. Burn *et al.* (1974) did not detect NAD-GDH activity in cultures where ammonium was supplied at either limiting (4mM) or non-limiting (30mM) levels. Roon and Even (1974) observed a decrease in activity from 120 to 20mU/mg as the ammonium concentration was increased from zero to 1mM. For ammonium concentrations greater than 1mM, NAD-GDH was present at a constant level. Comparable results were found with DCL2, but not Σ1278b or DCL1. The reasons for this difference are unclear.

Growth in MM + glutamate derepressed NAD-GDH in all three strains examined. The activity measured in DCL1 (513mU/mg) was about three-fold higher than that in Σ1278b (187mU/mg) and DCL2 (152mU/mg). The degree of derepression (relative to the 20mM value) was also greatest in this strain (factors of 11, 51, and 15 for Σ1278b, DCL1 and DCL2 respectively). It has been shown (Bernhardt *et al.* 1965, 1966, Roon and Even 1973, Burn *et al.* 1974) that NAD-GDH, in a variety of strains of *S. cerevisiae*, was derepressed in glutamate grown cells and repressed in ammonium grown ones. From these results it has been concluded that the NAD-GDH has a catabolic function degrading glutamate to ammonium plus 2-oxoglutarate. Similar behaviour was observed with the two industrial strains, and it can be concluded that NAD-GDH has a catabolic function in DCL1 and DCL2.

In the three strains investigated, NADP-GDH activity increased as the ammonium concentration went from 0.2 to 20mM. However, the extent of the modulation was not the same in each of the three strains. The enzyme level in Σ1278b exhibited only limited regulation by ammonium level, it increased from 1.13 to 1.59U/mg. In contrast, NADP-GDH in DCL1 and DCL2 (but especially the former) showed marked changes when the ammonium level increased. In DCL1, the level went from 0.28 to 1.36U/mg; and for DCL2, 0.66 to 1.70U/mg.

Similar studies have been reported in the literature (Roon and Even 1973, Burn *et al.* 1974, Bogonez *et al.* 1985). Roon and Even, using strain X2180-1A, showed that as the external ammonium concentration was increased from zero to 1mM, NADP-GDH activity went from 0.06 to 0.18U/mg. At concentrations greater than 1mM (the highest level tested was 5mM), the enzyme level remained constant at 0.18U/mg. Bogonez *et al.* used a diploid strain and found that NADP-GDH activity increased (from 1.8U/mg at about 2mM NH_4^+) as the ammonium concentration was raised. Maximum activity (3 U/mg) was at 48mM NH_4^+, but at higher concentrations the activity decreased markedly (about 2.3U/mg at 100mM NH_4^+). Burn *et al.* found that NADP-GDH activity was 50-100% higher in cells grown with 4mM compared to 30mM NH_4^+. As can be seen from the literature, the response of the *S. cerevisiae* NADP-GDH to ammonium depends upon the strain being investigated. This was observed with the strains in this study.

Both DCL1 and DCL2 showed lower NADP-GDH levels in glutamate grown cells compared to the 20mM NH_4^+ cultures. The degree of repression was greater in DCL1 than in DCL2. In contrast, Σ1278b exhibited elevated levels of NADP-GDH in glutamate grown cells. NADP-GDH is considered to be an anabolic enzyme because it shows increased levels in ammonium grown cells and repressed levels in glutamate cultures (Roon and Even 1973, Roon *et al.* 1974). However, Burn *et al.* (1974) found that in the strains used in their study NADP-GDH activity in glutamate grown cells was markedly higher than in the 30mM NH_4^+ culture. In one of the two strains tested, the activity in the glutamate cultures was greater than in the 4mM NH_4^+ grown cells (in which NADP-GDH was derepressed), and , for the other strain, the activities were comparable. The literature shows that the response of NADP-GDH to different nitrogen sources is strain dependent. This is in agreement with the results of this study. The reasons for this strain variability are currently unclear.

In *S. cerevisiae*, NADP-GDH is apparently the main route for ammonium assimilation and glutamate biosynthesis while GS is the only system for glutamine biosynthesis (Dubois and Grenson 1974). *A priori* it would be expected that GS and NADP-GDH levels would be highest when the t_d was lowest, because the demand for glutamate and glutamine as precursors for protein synthesis would be greatest, and enzyme levels be lowest when the t_d was highest.

In Σ1278b and DCL1 the NADP-GDH behaved as expected. However, the response of GS to change in t_d was more complex. As expected, Σ1278b showed highest GS activity with the lowest t_d, but there was no correlation for the other strains. In contrast, for DCL1, highest GS activity was observed with highest t_d, and lowest enzyme activity with lowest t_d. DCL2 showed no correlation between NADP-GDH or GS activity and t_d, because t_d did not vary in these studies.

It would appear that this simple hypothesis is valid for a number of systems. However, because the hypothesis does not hold true for all systems, it would appear that other, unknown, factors affect and modulate enzyme levels.

NADP-GDH had the highest activity of the four enzymes assayed at each of the ammonium concentrations tested. The Σ1278b enzyme did not exhibit marked modulation by the external ammonium level, increasing from 1.13 to 1.59U/mg, with a maximum GS activity of 34mU/mg. DCL2 showed a greater degree of modulation in NADP-GDH levels (from 0.66 to 1.70U/mg, with a maximal GS activity of 52mU/mg). For both Σ1278b and DCL2, maximum GS and NADP-GDH activities occur at high ammonium concentrations. Therefore, NADP-GDH is probably the main route of ammonium assimilation at all external concentrations in both these strains, with GS-GOGAT making only a minor contribution.

In DCL1, NADP-GDH activity is modulated over a five-fold range (0.28 to 1.36U/mg). However, unlike Σ1278b and DCL2, maximum GS activity (74mU/mg) occurs when the NADP-GDH level is lowest, and when the external ammonium concentration was the lowest tested. Both *N. crassa* and *Schiz. pombe* exhibit elevated GS activity at limiting ammonium levels, although NADP-GDH activity is not repressed. The kinetics of NADP-GDH suggest that it cannot function at low ammonium concentrations. Consequently, it is believed that these microorganisms use GS-GOGAT to assimilate ammonium at limiting concentrations (see sections 1.3.1 and 1.4). On the basis of data in Tables 8 and 10, and in comparison with other systems, it would appear that DCL1 probably uses NADP-GDH for ammonium assimilation when the concentration is high but at low levels of ammonium, GS-GOGAT makes a major contribution to ammonium assimilation. Further studies with DCL1 are needed before the validity of this hypothesis can be confirmed.

4.5 As part of the comparative studies of the industrial strains, DCL1 and DCL2 were examined during a simulated industrial propagation. These propagations were done in a 2l-fed batch system with growth parameters (molasses and nitrogen feed rates, growth rate etc.) exactly resembling those of a full scale commercial propagation. The cultures were grown in the 2l-fermentor facility at DC(Y)L, Menstrie. The growth parameters used in such propagations are not reported in this study because of their commercially sensitive nature. However, the growth parameters for DCL1 are markedly different from those for DCL2. Additionally, because DC(Y)L also consider the enzyme data obtained from these studies to be potentially commercially sensitive, data for only a single point in the propagation are presented for each strain. The data presented in Table 11 were chosen because, at this point in the propagations, the growth rates were similar and representative of the growth rate profile, and the points are in the middle of nitrogen feed profile.

Table 11.

Enzyme	Strain	
	DCL1	DCL2
GS[1]	35.4	24.1
GOGAT[1]	14.1	17.2
NAD-GDH[1]	1.0	55.1
NADP-GDH[2]	0.460	0.903

Enzyme activities of DCL1 and DCL2 grown in 2l-fed batch simulated industrial propagation. Samples were taken every 2h and assayed for GS, GOGAT, NAD- and NADP-GDH. The data in this table are from single time points during the propagation: each value is the mean of duplicate assays. 1 - mU/mg; 2 - U/mg.

Studies were not done with Σ1278b because, in this system, haploid strains go into glucose repression and do not grow (D.M. Wright, pers. comm.).

The data presented in Table 11 are for single propagations of DCL1 and DCL2 because factors, over which there was no control, prevented the growth of duplicate cultures. The value of averaged data from commercial propagations is unclear as it is difficult to reproduce batch processes in a commercial environment. For example, molasses are obtained from a variety of sources as the by-product of the sugar industry. However, as molasses are bought at a fixed fermentable sugar concentration, the sugar concentration has to be adjusted prior to purchase. Differences in how this is achieved, over which the yeast manufacturer has no control, may affect growth. Additionally, the quality of the molasses decays on storage.

No control was possible over the inocula. The inocula used in the 2l-propagations were ex-factory seed from the D5 stage, harvested and stored as wet yeast at 4°C overnight prior to use. Commercially, *S. cerevisiae* is not grown as an axenic culture. Usually, it contains various microbial contaminants. The most common are lactic acid bacteria, generally of the genera *Lactobacillus* and *Leuconostoc*. The total bacterial count is usually 10^4 to 10^9 cells per gramme wet yeast (Reed 1982). Although these microorganisms have no effect in normal baking practices, this study investigated the biochemistry of ammonium assimilation by an industrial strain of *S. cerevisiae*. It is unclear how the microbial contaminants affect the observed pattern of changes in enzyme activity, but it is probably minor, considering the vast excess of yeast to contaminant.

Only limited control over the harvesting of the cultures was possible. These factors introduce a degree of uncertainty over the reproducibility of the results between experiments.

It can be seen that, in both DCL1 and DCL2, NADP-GDH had the highest activity of the four enzymes although it was more active in DCL2 than in DCL1. This was true during the whole propagation. However, the pattern of change in the NADP-GDH level was different in the two strains. The general trend in DCL1 was a steady loss of activity during the propagation, irrespective of changes in the growth rate. In contrast, activity in DCL2 increased coordinately with the growth rate but, after a decrease in the growth rate, the NADP-GDH activity remained relatively constant before decreasing.

Both strains have GS and GOGAT activities. GS levels in DCL1 and DCL2 varied over a large range of activities, and fluctuations occurred over a relatively short period of time. In DCL2, highest activity coincided with maximal growth rate *i.e.* greatest demand for glutamine. For DCL1, GOGAT activity fluctuated over a large range. Again, the changes occurred over a relatively small time span. The trend in DCL2 was for GOGAT activity to increase during the propagation.

At the point in the DCL1 culture where the data in Table 11 were taken, NAD-GDH activity in DCL1 was negligible. In this strain, NAD-GDH activity decreased at the start of the culture followed by an increase at the end of the propagation. Minimal NAD-GDH activity was coincident with maximal growth rate. The regulation of NAD-GDH in DCL2 is different. Highest NAD-GDH activity coincided with significant changes in nitrogen feed profile.

It has been found that both DCL1 and DCL2 possess GS, GOGAT and NADP-GDH activities during industrial propagations. However, NADP-GDH activity was higher than that of the other ammonium assimilatory enzyme GS. The NADP-GDH level in DCL1 was lower than that measured in DCL2: additionally, GS activity in DCL1 was higher compared to that in DCL2. It is probable, on the basis of the high NADP-GDH activity, that the main assimilatory pathway in both strains is by the NADP-GDH. However, the reduced, and decreasing, NADP-GDH activity in DCL1 plus the elevated GS activity at the end of the propagation suggest that GS-GOGAT may make a significant contribution to ammonium assimilation by DCL1 during the industrial propagation. The functioning of this pathway in DCL1 at low ammonium concentrations is suggested by data from the shake-flask cultures (section 4.4.4). At the end of the propagation, DCL2 exhibited a marked fall in NADP-GDH activity while GS increased and GOGAT was at a maximal level. It is possible that during this phase of the propagation, GS-GOGAT could be making a significant contribution to ammonium assimilation by DCL2. It may be that both DCL1 and DCL2 could benefit from the manipulations described for *M. methylotrophus* (Windass *et al.* 1980).

The shake-flask studies demonstrated that, although there are differences between DCL1 and DCL2 (e.g. behaviour of GS and NAD-GDH in ammonium grown cells, effect of ammonium concentration on t_d), generally these strains are very similar. However, enzyme profiles of simulated industrial propagations are very dissimilar for the two strains, and are also different to the profiles observed in the shake-flask cultures.

The data show that, in DCL1 and DCL2, both GS and NADP-GDH levels were lower in the simulated commercial cultures compared to those in shake-flask culture. In contrast, GOGAT and NAD-GDH activities were present at elevated levels in the simulated industrial propagations. Enzymes in the shake-flask cultures exhibited smooth changes in activity, but in the 2l-fed batch cultures the enzymes showed a rapid fluctuation in activity. In simulated commercial cultures of DCL2, GS, NAD- and NADP-GDH activity appears to be influenced by changes in the growth rate and ammonium feed profile during the course of the propagation. However, in DCL1 only GS and NAD-GDH activities appear to correlate with changes in these two growth parameters.

From the data presented in this study, it is likely that the differences observed between DCL1 and DCL2, and between shake-flask and fed-batch culture, are due to the markedly different growth parameters used in the different culture systems. These differences make it difficult to answer the question "are extrapolations from shake-flask to simulated industrial propagation valid?". To the extent that, in both systems, all four enzyme activities are present and at comparable levels, extrapolations are valid.

4.6 The studies reported in this chapter have characterised two industrial strains of *S. cerevisiae* with respect to the behaviour of the enzymes of ammonium assimilation in a variety of experimental conditions, including simulated industrial propagations. These studies have also allowed a comparison to be made with a standard laboratory wild type.

The experimental data have shown that DCL1 and DCL2 are very similar to Σ1278b. However, differences do exist between the industrial strains and Σ1278b, and also between DCL1 and DCL2. What has become apparent during this study, is the heterogeneity of the response of the enzymes of ammonium assimilation in the different laboratory strains used in the studies reported in the literature.

Inhibition studies using DON demonstrated that all three strains used in this study possess GOGAT activity, and also that DON is a specific inhibitor of GOGAT in the assay system used in this study. The shake-flask studies demonstrated that GS, GOGAT, NAD- and NADP-GDH are expressed during the exponential growth of DCL1 and DCL2. The data from these experiments show that the industrial strains are similar to the laboratory one although there are, apparently, important differences.

The data presented in this chapter show that the greatest similarity between the three strains is in 20mM NH_4^+ grown cultures. Therefore, it would appear that extrapolation from the laboratory to the industrial system is valid provided 20mM NH_4^+ grown cells are used. As has been previously stated, the question of extrapolation from the laboratory to the industrial system is unclear. The growth parameters are too different, and have too profound effect on the enzyme profiles, for extrapolations to be made with a high degree of confidence.

There are apparently two important differences between the industrial strains and the laboratory one. Firstly, it has been suggested that DCL2 has a high affinity, high capacity ammonium permease function. This has been discussed above. The second difference is that DCL1 apparently uses GS-GOGAT to assimilate ammonium at limiting concentrations. The data in Table 10 shows that both GS and GOGAT activities are elevated in ammonium-limited cells and NADP-GDH is markedly lowered in comparison to cells grown with excess ammonium. Table 8 shows that the affinity of the DCL1 GS for ammonium (as hydroxylamine) is markedly higher than that of NADP-GDH which has the highest K_m of the strains studied. This suggests that the NADP-GDH cannot function at low ammonium

concentrations. The elevated GS-GOGAT activity, and its higher affinity for ammonium, suggests that assimilation of limiting levels of ammonium in DCL1 is by the GS-GOGAT pathway, or makes a significant contribution to ammonium assimilation. However, without data on the intracellular ammonium concentration in *S. cerevisiae* grown at different ammonium concentrations, the validity of the statement about the functioning of NADP-GDH in cells grown with limiting ammonium is unknown.

The industrial propagations showed that all four enzyme activities are present in DCL1 and DCL2 grown in simulated industrial propagation. The data suggest that GS-GOGAT functioning may be more important in such propagations of DCL1 than in DCL2, because the NADP-GDH activity was lower in DCL1 than DCL2. This result is in agreement with the shake-flask studies. If so, then DCL1 may benefit from inactivation of GOGAT in a GDH^+ background.

Chapter 5

Isolation and Characterisation of Glutamate Synthase Mutants of *S. cerevisiae*

5.1 As was discussed in the Introduction, one of the aims of this work was to examine the growth characteristics of a GDH^+ $GOGAT^-$ strain. Since there were no reports of *S. cerevisiae* GOGAT mutants in the literature, these mutants had to be generated during the course of this study. Subsequently, other workers have independently isolated GOGAT mutants in *S. cerevisiae* [J. Mora, pers. comm., E. Dubois, pers. comm. and unpublished data cited in Wiame *et al*. (1985)].

In *S. cerevisiae* the primary route of ammonium assimilation is via the NADP-GDH and the main product is glutamate. However, *gdh1* strains can still grow on MM + NH_4^+ (*i.e*. they are leaky glutamate auxotrophs), although at a reduced rate compared to a *GDH1* strain (Grenson *et al*. 1974). These workers postulated that this ability was due to NAD-GDH activity, but the demonstration of GOGAT activity in *S. cerevisiae* (Roon *et al*. 1974) provided an alternative explanation. Fig. 1 shows that by coupling GS with GOGAT, glutamate can be synthesised from ammonium. *A priori*, loss of GOGAT activity in a *gdh1* strain should result in the inability to use ammonium as sole nitrogen source and, consequently, tight glutamate auxotrophy.

5.2 The parent strain, 4324c, used in the mutagenesis experiments carries the *gdh1-1* allele. As has been discussed in section 5.1, 4324c is a leaky glutamate auxotroph. Tight glutamate auxotrophs were selected by replicaplating YEPD master plates of mutagenised 4324C onto MM supplemented with either ammonium or glutamate. The replicaplates were screened for colonies which grew on MM + glutamate but not MM + NH_4^+. Similar screening protocols have been successfully used with a number of prokaryotes (Elmerich and Aubert 1971, Berberich 1972, Brenchley *et al*. 1973, Dendinger *et al*. 1980, Desphande and Kane 1980) and the fungus *N. crassa* (Hummelt and Mora 1980b, Dunn-Coleman *et al*. 1981, Romero and Davila 1986).

If GS-GOGAT does function to assimilate ammonium in *S. cerevisiae*, but only at low concentrations, mutations in GOGAT might be identifiable only under such conditions even in a *gdh1* background. Therefore, ammonium was tried at 0.2mM in addition to 2 and 20mM. However, Σ1278b colonies grew very poorly on 0.2mM NH_4^+ and could not be distinguished from non-growing colonies. Consequently, only ammonium concentrations of 2 and 20mM were used in the mutant isolation experiments.

Initially the mutagen DEO was used because it is reported to cause a large number of multi-site mutations, in addition to point mutations, in *A. nidulans* (Hynes 1979). It has been successfully used in *S. cerevisiae* to generate *gln1* mutants (Mitchell 1985). The experimental conditions for DEO mutagenesis used in this study gave a frequency for mutation at the *CYH2* locus (as judged by the appearance of CYH^r colonies) of 6.3×10^{-7}, which was an increase of greater than four-fold over the spontaneous rate ($<1.7 \times 10^{-7}$).

DEO-mutagenised cultures, either enriched with nystatin or not, were screened for GLU$^-$ colonies. Approximately 50000 colonies were screened, of which 85% were from nystatin-enriched cultures. A total of 430 putative GLU$^-$ mutants were initially detected. After, rescreening, the number was reduced to about 20. The data did not conclusively demonstrate that these mutants lacked GOGAT activity or had markedly altered growth characteristics on MM + NH_4^+. Data for the best mutants generated by DEO mutagenesis (strains 3-25, 3-26, 3-34) are presented below.

None of the 430 mutants were tight glutamate auxotrophs. This inability to generate GLU$^-$ mutants using DEO was surprising and the reason remains unclear. Consequently it was decided to change the mutagen used in this study. EMS was chosen because it is known to give high frequencies of auxotrophs in *S. cerevisiae*, even in unenriched cultures (Fink 1970). Using the conditions described in Materials and Methods, EMS gave a mutation frequency at the *CYH2* locus of 2.1×10^{-6} (three-fold higher than that with DEO): 8% of the population were auxotrophs. The culture was not enriched with nystatin. Approximately 11000 colonies were screened , of which about 1000 were auxotrophs. The general auxotroph population contained 84 putative GLU$^-$ mutants. Rescreening reduced this number to 16 (designated 4-1 to 4-25) and the studies reported below were done using these mutants.

5.3.1 Sections 5.1 and 5.2 describe the isolation of GLU$^-$ mutants from a *gdh1-1* parent and why these mutants should be GOGAT$^-$. However, this is not the only mutation which could generate GLU$^-$ mutants in this system. In *S. cerevisiae* glutamate is primarily synthesised by the amination of 2-oxoglutarate. Therefore, GLU$^-$ mutants can arise either by a mutation in the amination system or in the synthesis of 2-oxoglutarate. 2-oxoglutarate is synthesised by the citrate synthase (*GLU3*), aconitase (*GLU1* and *GLU2*), isocitrate dehydrogenase reaction sequence of the TCA cycle. Burand *et al.* (1975) have shown that mutations in *GLU3* result in glutamate auxotrophy, while Ogur and co-workers (Ogur *et al.* 1964, 1965) have done the same with *GLU1* and *GLU2*. It was therefore necessary to demonstrate that the 16 GLU$^-$ mutants which had been isolated had altered GOGAT activities.

The putative GOGAT mutants were grown overnight in MM + glutamate before being switched to MM + 20mM NH_4^+ for a further 6h. The cultures were assayed for GS, GOGAT, NAD- and NADP-GDH activities. The data is presented in Table 12.

All the mutant strains have reduced GOGAT activities with respect to the levels measured in both the parent and, excepting 3-26, the wild type strain. The DEO-generated mutants still have greater than 66% of the parental activity remaining. In contrast, 10/16 of the EMS-generated mutants had less than 10% parental activity, 2/16 less than 20%, while the remaining four mutants had 20-55% of the parental activity remaining. The mutants generated with EMS have markedly altered GOGAT activities. It would appear that in these strains, the mutation probably lies within the GOGAT structural genes. However, for the DEO-generated mutants the situation is less clear. None of these mutants, especially 3-26, show a marked loss of GOGAT activity. They are probably not mutant in GOGAT.

The loss of GOGAT activity has a pleiotropic effect. The data in Table 12 shows that this loss affects other enzymes in ammonium assimilation. Strain 4324c, which carries the *gdh1-1* allele, has markedly higher GS activity and elevated GOGAT activity compared to the wild type. Presumably this simply reflects the fact that GS-GOGAT is the only route for glutamate biosynthesis in a *gdh1* strain and that enzyme levels have to be increased to meet the demand for glutamate. GS activities of the mutants are all reduced below the level of both the parental and, with one exception, the wild type strains. There is no obvious relationship between GS activity and the residual level of GOGAT in a given mutant strain. A similar observation has been made with *Sal. typhimurium*, where it was postulated that the accumulation of glutamine (caused by the blockage in the GS-GOGAT pathway due to inactivation of GOGAT) represses the synthesis of GS (Dendinger *et al*. 1980). It is conceivable that the same situation is being observed here. In strain 4324c, NAD-GDH activity is elevated four-five-fold compared to the wild type (93.9 *cf.* 17.9mU/mg), while the $GOGAT^-$ mutants have two-three-fold higher levels. There is no apparent relationship between the NAD-GDH activity and either the GS or GOGAT level. Why NAD-GDH should be affected by the GOGAT mutation is unclear.

5.3.2 The ability of the $GOGAT^-$ mutants to use a variety of nitrogenous compounds as sole nitrogen source was tested. Derivatives of the original mutants were streaked onto MM supplemented with the various test compounds and scored for growth after 72h incubation at 30°C. The results are presented in Table 13. Fig. 16 shows the growth of ART10-1d, derived from 4-10, on four different nitrogen sources.

The data shows that the parental and wild type strains both have the same growth characteristics on all compounds tested, except with serine where the wild type grew better than the *gdh1* strain. Both strains grew well with all the growth conditions tested.

Table 12.

Strain	Enzyme Activities			
	GS mU/mg	NAD-GDH mU/mg	GOGAT mU/mg	NADP-GDH U/mg
Σ1278b	34.2	17.9	44.2	1.589
4324c	79.7	93.9	54.2	0.004
3-25			36.4	
3-26			48.9	
3-34			37.9	
4-1	25.9	30.7	<0.1	0.001
4-2	12.0	42.4	<0.1	0.020
4-3	13.6	35.2	<0.1	0.010
4-5	17.6	55.7	0.7	0.003
4-6			9.6	
4-8			18.7	
4-9			1.0	
4-10	24.5	72.8	6.7	0.005
4-11			12.9	
4-17			<0.1	
4-18			2.3	
4-20	12.3	52.6	2.5	0.005
4-21	15.0	27.6	3.8	0.014
4-22			<0.1	
4-23			30.0	
4-25	52.6	75.2	25.1	0.005
AR44		39.0	17.2	0.109

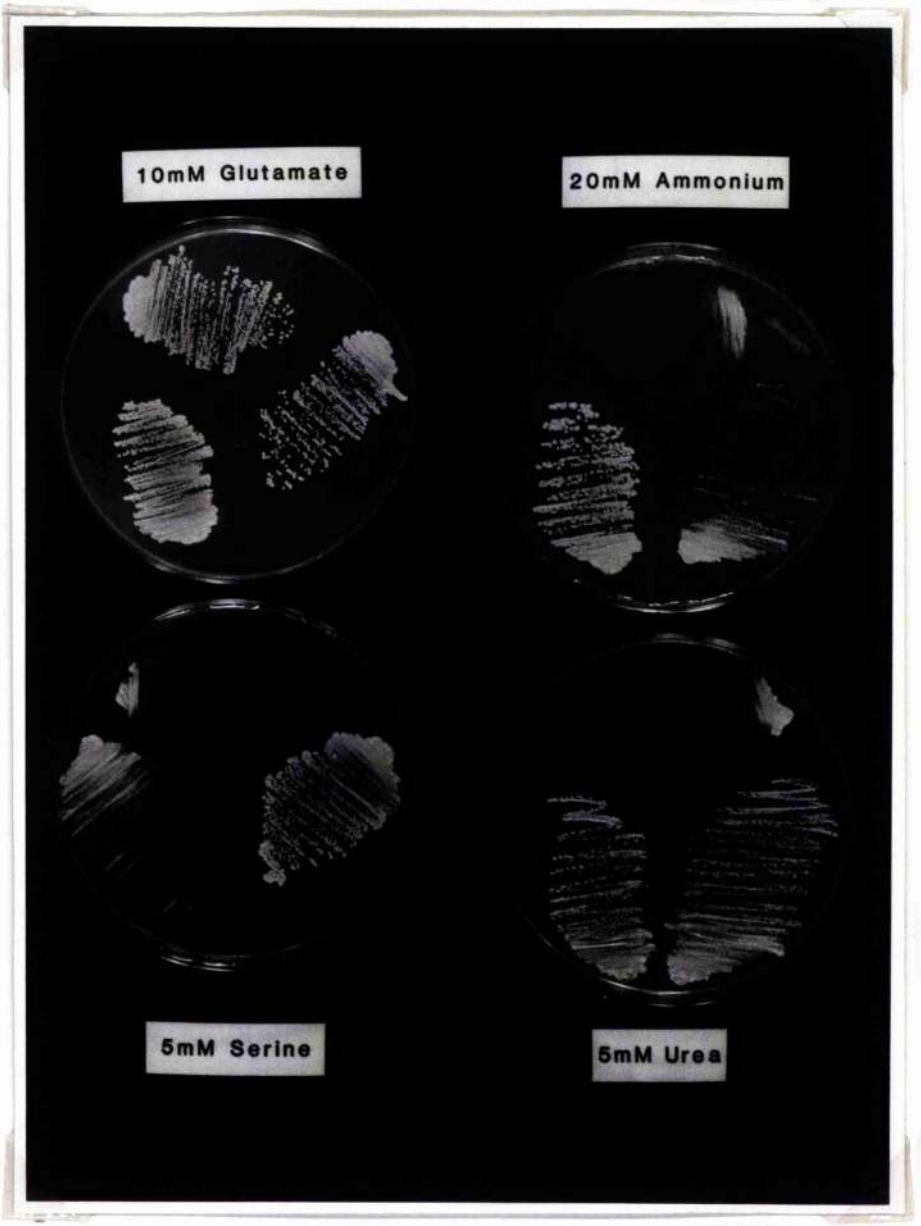
Enzyme activities of the putative GOGAT$^-$ mutants of S. cerevisiae. Overnight cultures of glutamate grown cells were switched to MM + 20mM NH_4^+ and incubated for a further 6h. The values shown for each enzyme are the means of at least two independent experiments, with duplicate assays done for each experiment.

Table 13.

Strain	MM supplemented with (mM):											
	Glu	NH_4^+	NH_4^+	NH_4^+	Gln	Pro	Urea	Asp	Ala	Arg	Ser	2-oxoglutarate + NH_4^+
	10	0.2	2	20	10	5	5	10	10	5	5	1 + 20
Σ1278b	++	+	++	++	++	++	++	++	++	++	++	++
4324c	++	+	++	++	++	++	++	++	++	++	+	++
3-25	++	(+)	(+)	++								
3-26	++	(+)	(+)	++								
3-34	++	(+)	(+)	++								
AR9	++	-	-	-	++	-	-	++	++	++	-	
AR11	++	-	-	-	++	+	-	++	++	++	-	
AR13	++	-	-	-	++	+	-	++	++	++	-	
4-5	+	-	-	-	(+)	-	-	++	+	-	-	
4-8	++	+	+	(+)	++	-	+	+	+	+	-	
AR42	++	-	-	-	+	++	-	++	++	++	-	
ART10-1d	++	-	-	-	++	++	-	++	++	++	-	-
AR21	++	-	-	-	++	-	-	++	+	+	-	
4-17	+	-	-	-	+	-	-	++	+	-	-	
AR23	++	-	-	-	+	-	-	++	++	-	-	
AR29	+	-	-	-	(+)	-	-	+	-	-	-	
AR39	+	-	-	-	++	(+)	(+)	++	++	+	-	
AR40	++	-	-	-	++	++	(+)	++	++	++	-	
AR44	++			++								

Data from growth tests of the putative GOGAT$^-$ mutants. Derivatives of the original mutants were streaked onto MM supplemented with the various test compounds, and the plates incubated for 72h at 30°C before being scored. ++ appearance of large colonies along line of streak: + small colonies visible along streak: - no growth visible: (+) microcolonies visible. These semi-quantative units are shown in Fig. 16 where, on 5mM serine, Σ1278b was scored as ++, 4324c +, and ART10-1d -.

Fig. 16



Growth of Σ1278b, 4324c and ART10-1d on four different nitrogen sources. Each strain was streaked onto MM supplemented with the nitrogen sources shown, and incubated at 30°C for 72h. (strains, starting from the top and proceeding clockwise: ART10-1d, 4324c and Σ1278b - except for 5mM serine: ART10-1d, Σ1278b and 4324c.

The growth on MM + glutamate of all the mutant strains tested (excepting 4-5, 4-17 and AR39) was comparable to the wild type. This was expected because the mutants were selected as glutamate auxotrophs. Why strains 4-5, 4-17 and AR39 grew poorly on 10mM glutamate is unclear. It is unlikely to be due to defects in glutamate uptake because it is mediated by three different permeases in *S. cerevisiae* (Darte and Grenson 1975).

Both the DEO and EMS generated mutants were selected on the basis of growth on glutamate but not ammonium. This was initially done by replicating single colonies and then patches. In the experiment reported in Table 13, a more sensitive test was used: mutants were streaked onto MM and the appearance of single colonies along the streak examined. With this experimental design, the growth of strains 3-25, 3-26 and 3-34 on 20mM NH_4^+ was comparable to that of Σ1278b. The mutants grew poorly at the other ammonium concentrations tested. The data supports the conclusion, made from the data in Table 12, that 3-25, 3-26 and 3-34 are not GOGAT$^-$. The reduced GOGAT activities are probably the consequence of an unknown non-specific mutation.

In contrast, the EMS generated mutants did not grow at any of the three ammonium concentrations tested (excepting 4-8, which grew poorly). The predicted phenotype of a GOGAT$^-$ mutant correlates well with the loss of GOGAT activity. The GOGAT$^-$ mutants have elevated NAD-GDH activity compared to Σ1278b, and strains 4-10 and 4-25 have levels comparable to 4324c, but do not grow on ammonium as sole nitrogen source. Therefore, it can be concluded that it is GOGAT and not NAD-GDH which enables a *gdh1* strain to grow on ammonium. Consequently, it can also be concluded that GS-GOGAT does function in ammonium assimilation by *S. cerevisiae*. The contribution of this pathway in a *GDH1* strain cannot be elucidated from the data reported in Tables 12 and 13.

Strain 4-8 grew on MM + NH_4^+, albeit poorly, which correlates well with the high residual GOGAT activity (35% parental activity). Therefore it would appear that this level is sufficient to support growth on ammonium. However, strains 4-23 and 4-25 have 55 and 46% parental activity, but exhibit no growth on ammonium. An alternative explanation is needed to explain the residual growth of 4-8 on ammonium.

An alternative explanation of why these strains cannot grow on MM + NH_4^+ is because they cannot absorb ammonium. Inducer exclusion prevents induction of GOGAT. Transport of ammonium in Σ1278b has been shown to be mediated by at least two permeases which can be lost separately as the result of two genetically unlinked mutations, *mep1* and *mep2* (Dubois and Grenson 1979). These workers showed that the presence of either *mep1* or *mep2* did not have a marked effect on generation time. Simultaneous loss of both functions markedly increased the time on 1 and 4mM but not 20mM NH_4^+. The multiplicity of ammonium permeases makes it highly improbable that all such systems have

been inactivated simultaneously. The mutants isolated in this study did not have the phenotype of a *mep1 mep2* strain. Additionally, low external ammonium concentrations only reduced the GOGAT activity in Σ1278b by 50% (Table 10) where as in 5/16 mutants the activity was reduced by 99.8%. Therefore the possibility that these strains are mutant in an ammonium permease function can be excluded.

The ability of alanine, arginine, aspartate, glutamine and proline to repair the glutamate auxotrophy was tested. All the strains tested grew on aspartate and glutamine, the majority with growth comparable to the wild type. Only AR29 grew poorly on both these two amino acids. The growth of the majority of the GOGAT$^-$ strains tested on alanine was comparable to Σ1278b, the remaining mutants (excepting AR29) grew poorly. AR29 did not grow on alanine. Four of the strains tested could not use arginine as sole nitrogen source, and six could not use proline. The four strains unable to grow on arginine could not use proline, yet all six strains grew on aspartate. There is no obvious relationship between the GOGAT activity in each of the mutant strains and their ability to utilise these five amino acids. Only AR29 grew poorly on all the nitrogen sources tested. The GOGAT activity in 4-21, from which AR29 was derived, was 3.8mU/mg yet AR42, derived from 4-9 which had an activity of 1.0mU/mg, grew well on all but one of the amino acids tested.

S. cerevisiae possesses two glutaminase activities (Soberon and Gonzalez 1987). Glutamine, absorbed by one of three uptake systems (references cited in Wiame *et al.* 1985), is degraded by glutaminase activity to glutamate plus ammonium which repairs the cellular demand for glutamate. It is unclear why some GOGAT$^-$ mutants grew better than others on glutamine. This difference is unlikely to be due to variation in the ability to absorb glutamine.

Although glutamate is generated by glutaminase-mediated deamination of glutamine, glutamine synthesis by GS activity uses glutamate as the receptor molecule. Therefore, glutamate generated by a glutaminase would be used in the synthesis of glutamine, with no net yield of glutamate although one ATP molecule would have been used. This explains why a GS-glutaminase pathway does not allow a GDH$^-$ GOGAT$^-$ strain to grow on ammonium as sole nitrogen source.

The degradation of aspartate and alanine in *S. cerevisiae* is probably via a transaminase. It has been shown (Woodward and Cirillo 1977, and references cited therein) that in nitrogen limited cultures of *S. cerevisiae*, hydrophobic amino acids are accumulated and undergo transamination. The amino group is transferred to 2-oxoglutarate to generate glutamate. However, Woodward and Cirillo did not detect alanine transaminase activity in their assay system. Jones *et al.* (1969), investigating the fate of ^{15}N-labelled alanine, presented data showing that the most abundantly labelled amino acid was glutamate. This work appears to suggest that in *S. cerevisiae* alanine is degraded (either directly or indirectly) by

the same mechanism as other hydrophobic amino acids *i.e.* by transamination to generate glutamate plus a 2-oxo acid.

Aspartate is also degraded by transamination. Aspartate aminotransferase transfers the amino group to 2-oxoglutarate to generate oxaloacetate plus glutamate. Lamminmaki and Pierce (1969) demonstrated aspartate aminotransferase activity in *S. cerevisiae* during fermentation.

Arginine is degraded, via proline, to glutamate (reviewed in Cooper 1982a). This explains why the GOGAT$^-$ mutants grew with arginine, alanine, aspartate or proline as sole nitrogen source. As has been stated above, all the mutants tested were able to grow on aspartate and only AR29 could not use alanine, but not all of them could use arginine or proline. The reason for this variation in the ability to utilise arginine or proline as sole nitrogen source is unclear. It might be due to secondary mutations in arginine or proline catabolism. It should be noted that if the strain could not utilise proline, it did not follow that the strain could not use arginine. This is unlikely to be due to the inability of the mutants to absorb the amino acid from the medium because all four amino acids can be transported by the GAP and also by a specific permease (reviewed in Cooper 1982b).

The glutamate generated by these mechanisms can repair the glutamate auxotrophy.

Only strain 4-8 exhibited marked growth on urea, two strains grew very poorly while the remaining strains did not show any growth after 72h incubation. None of strains tested grew on serine as sole nitrogen source: no growth was observed after 72h incubation. Urea is degraded by urea carboxylase and allophanate hydrolyase to ammonium plus CO_2 (reviewed in Cooper 1982a). The pathway of serine degradation is unknown in *S. cerevisiae*, but serine can be degraded by serine dehydratase to pyruvate plus ammonium (Stryer 1981). As the GOGAT$^-$ mutants cannot assimilate ammonium (they also carry the *gdh1-1* allele), they cannot synthesise glutamate. As the cellular demand for glutamate cannot be satisfied by serine or urea, GOGAT$^-$ mutants do not grow.

The alternative explanation is that of inducer exclusion. The presence of two urea uptake systems in *S. cerevisiae* (Cooper and Sumradra 1975) makes it unlikely that a single mutation resulted in the loss of of both uptake systems. Serine is transported into the cell by the GAP, although there is no evidence for a specific permease, one probably exists (threonine is transported by both the GAP and the threonine permease) (Cooper 1982b, Wiame *et al.* 1985). Therefore, the lack of growth of the GOGAT$^-$ mutants on serine or urea is probably due to their inability to assimilate ammonium.

Histidine, methionine and tryptophan were also tested as sole nitrogen source. However, Σ1278b would not grow under these conditions.

As was discussed in section 5.3.1, glutamate auxotrophy can arise by mutations in several genes apart from in the GOGAT genes. The data presented in Table 12 show that all the GLU$^-$ mutants have reduced GOGAT activities. However, it is still possible that this a consequence of reduced intracellular 2-oxoglutarate concentrations. To test this possibility, strain ART10-1d was grown on MM + NH_4^+ + 2-oxoglutarate. MM contains glucose, so 2-oxoglutarate functions as a growth supplement and not as the carbon source. Both Σ1278b and 4324c grew on this test medium, but ART10-1d did not. It would appear that 2-oxoglutarate does not repair the glutamate auxotrophy of this strain. This data supports conclusion that, in ART10-1d, the mutation lies in the GOGAT genes and is not the result of a non-specific mutation.

There is no data in the literature regarding the absorption of 2-oxoglutarate. Therefore, an alternative explanation, which cannot be excluded is that 2-oxoglutarate is not absorbed by *S. cerevisiae*. Consequently, there is no receptor present in the cell for glutamate biosynthesis. Σ1278b and 4324c grew because they could use glucose as a precursor for 2-oxoglutarate biosynthesis.

To exclude the possibility that the lack of growth was due to glucose repression of a 2-oxoglutarate uptake system, MM in which glucose had been replaced by 2% potassium acetate (pH5.5) as carbon source was tested. No growth of Σ1278b was observed on this medium. It is unclear why this was so, unless Σ1278b lacks a 2-oxoglutarate uptake system. Therefore, the results with ART10-1d still do not exclude the possibility that lack of growth is due to the inability to synthesise 2-oxoglutarate and not the inability to produce a functional GOGAT. The work reported below confirms the latter.

5.3.3 It was hoped to use the GOGAT$^-$ mutants in experiments to clone the GOGAT structural genes and to investigate the growth properties of a GDH$^+$ GOGAT$^-$ strain. As both these experimental systems will exert strong selective pressure for the reversion of the mutation causing glutamate auxotrophy, it is essential to use a strain which reverts at a low frequency. Reversion frequencies were measured for 12/16 GOGAT$^-$ mutants. Such data also give limited information about the nature of the mutation: deletion mutations are non-revertable.

Measurement of the reversion frequency was done in conjunction with investigation of the *ts*-phenotype of the mutants (section 5.4.1). This is why the experimental conditions described were chosen. Aliquots of stationary phase cells grown in MM + glutamate at 19°C were plated onto MM + 20mM NH_4^+ and incubated at 19°C for 4 days. Loss of the glutamate auxotrophy could have been due to two different events: (i) reversion of the mutation causing the loss of GOGAT activity; or (ii) reversion of *gdh1-1*. The reversion frequency of the *gdh1-1* allele was determined by plating aliquots of 4324c onto MM + 20mM NH_4^+ + D-histidine (see section 3.1.1). The results are presented in Table 14.

Table 14.

Strain	No. of revertants	No. of cells screened ($x10^6$)	Reversion frequency ($x10^{-8}$)
4324c	1	55.0	1.8
4-1	0	58.0	<1.7
4-2	4	133.3	3.0
4-3	0	93.0	<1.1
4-5	0	73.0	<1.4
4-9	14	69.5	20.1
4-10	0	79.0	<1.3
4-11	0	5.9	<16.9
4-17	0	73.3	<1.4
4-18	0	121.5	<0.8
4-20	1	26.8	3.7
4-21	0	43.5	<2.3
4-22	0	77.0	<1.3

Spontaneous reversion frequencies of the different GOGAT$^-$ mutants. Aliquots of glutamate grown stationary phase cultures of each mutant were plated on to MM + NH_4^+. The plates were incubated at 19°C for 6 days before being screened. The reversion frequency of the *gdh1-1*locus was determined by plating 4324c onto MM + NH_4^+ + D-histidine.

The data shows that *gdh1-1* reverts at a frequency of $1.8x10^{-8}$. Revertants were obtained for only 3/12 of the mutants tested. The reversion frequencies of 4-2 and 4-20 were comparable to that of the parental strain but 4-9 reverted eleven-times more frequently. The reversion frequencies of the majority of the other strains tested were comparable to or less than that of *gdh1-1*. Although no revertants were obtained from these strains, the number of cells screened was similar, or, for the majority of the strains examined, greater than the number for 4324c. The maximal reversion frequency for 4-11 was eight-fold higher than that for 4324c, but ten-fold fewer cells had been examined.

It would be futile to speculate about whether any of the mutations are non-revertable because insufficient cell numbers have been examined. The reversion frequencies are low enough, for all the strains examined, so that reversion of the mutation causing loss of GOGAT activity is not a problem in cloning or growth experiments. It is unclear why some mutants carrying two mutations, whose reversion would independently cause loss of glutamate auxotrophy, should revert less frequently than the parental strain.

5.4.1 There are several explanations of why the GLU^- mutants described above have reduced GOGAT activity. Firstly, the mutation could lie within the coding sequence of the GOGAT genes. These sequence alterations could result in changes in the primary structure of the polypeptide(s) and subsequent loss/decrease in catalytic activity. Secondly, lesions could lie in regulatory regions which modulate gene expression either at the level of transcription or translation. Such mutations would cause a decrease in the number of enzyme molecules but not in the activity of each molecule. Thirdly, a non-specific mutation results in altered levels of an effector e.g. a TCA cycle intermediate which modulates the activity of the enzyme, although the number of enzyme molecules and the activity *per se* per molecule remains unaltered. Additional explanations of the reduced GOGAT activities are possible. To determine if any of the GLU^- mutants isolated carry mutations which lie within the GOGAT coding regions, physiochemical properties (e.g. stability to heat or pH, sensitivity to inhibitor) of GOGAT from the mutant strains was examined.

5.4.2.1 The approach first tried was an investigation of the thermolability of the mutant GOGAT enzymes. The mutants were initially screened for a *ts*-phenotype. The mutants were inoculated into MM + glutamate and incubated at 19°C for 5 days. Appropriately diluted aliquots of each culture (to give about 100 colonies per plate) were spread onto MM + NH_4^+ at 2 or 20mM, and incubated at 19 or 30°C for up to 6 days.

Single colonies of Σ1278b and 4324c were observed after about 40h growth at 30°C, but at 19°C colonies appeared at 3-4 days. It was found that only strain 4-25 grew on MM + NH_4^+ under these conditions. At 19°C single colonies were visible after about 4 days incubation, but at 30°C no growth

was apparent even after 6 days incubation. As has been stated above, the ability to grow on MM + NH_4^+ could be due to reversion of the *gdh1-1* allele. This possibility was excluded by plating the cells onto MM + NH_4^+ + D-histidine. No growth of strain 4-25 on this test medium was apparent, even after 6 days incubation at 19°C. It would appear that, on the basis of these plate tests, the mutation causing the loss of GOGAT in 4-25 confers a *ts*-phenotype on this strain. This is shown in Fig. 17. The same experiment using strain 4-9 is shown in Fig. 18. Strain 4-9 did not exhibit a *ts*-phenotype. The putative *ts*-mutant was taken for further study.

5.4.2.2 The approach used to identify whether the mutation in 4-25 lies within the GOGAT structural gene exploits the *ts*-phenotype of the mutant. If a particular gene specifies a polypeptide which is structurally part of the GOGAT molecule (the *S. cerevisiae* enzyme is a heteromeric dimer), *ts*-mutations at such a locus should make functional enzyme when the cells are grown at the permissive temperature (19°C). However, this enzyme should be more heat labile than the wild type enzyme. If the gene in question specifies a product not structurally part of the GOGAT molecule, the GOGAT moiety in such a mutant should have the same thermostability as the wild type enzyme.

To test this possibility, 4-25 was grown at both permissive and restrictive (30°C) temperatures. GOGAT was assayed in extracts prepared from each culture: cells grown at the permissive temperature were assayed at 19°C, and ones grown at the restrictive temperature at 30°C. The results are presented in Table 15.

The data show that GOGAT activity in 4-25 grown at 30°C is 46% of the activity measured in 4324c. At 19°C, the GOGAT level in 4-25 is 86% of the parental activity. This shows that GOGAT in strain 4-25 is more stable at 19 than 30°C, as would be expected of a *ts*-mutant. The 4-25 enzyme has an altered thermolability relative to the wild type. Therefore it suggests that the mutation in 4-25 lies within the GOGAT structural genes.

Although the percentage figure for 4-9 increases from 2 to 8, the measured activities increased from 1 to 3mU/mg. The increase in absolute activity is not marked and probably reflects sampling errors. The decrease in parental activity increases this variation. The 30°C figure is the mean of four independent experiments while the 19°C value was obtained from a single experiment. It is therefore improbable that GOGAT in 4-9 has an altered thermolability relative to the wild type.

5.4.2.3 The data presented in Figs. 17 and 18 suggest that strain 4-25 has a *ts*-phenotype and that 4-9 does not. To investigate this further, growth curves of both these strains were made at the permissive and restrictive temperatures.

Fig. 17

The ts-nature of the mutation conferring the glu^- phenotype on strain 4-25 is demonstrated by the ability of this strain to grow on MM + NH_4^+ at 19°C but not at 30°C. 4-25 was streaked onto MM and incubated at 19°C for 4 days or 30°C for 6 days.

Fig. 18a

The non-ts-nature of the mutation carried by strain 4-9 was shown by the inability of this strain to grow on MM + NH_4^+ at both 19°C (a) and 30°C (b). 4-9 was streaked onto MM and incubated at the appropriate temperature for 6 days.

Fig. 18b

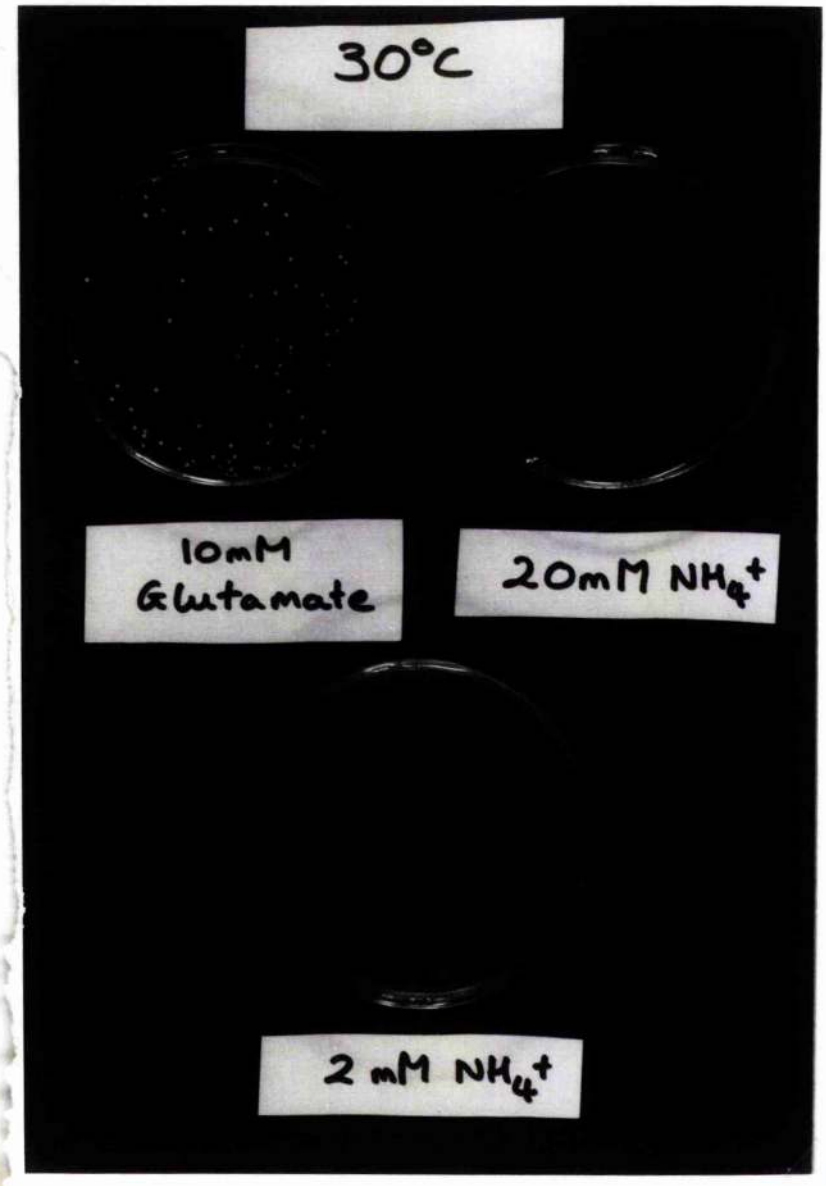


Table 15.

Strain	GOGAT Activity (mU/mg) at (°C): 19		30	
Σ1278b	16.6	(88)	44.2	(82)
4324c	18.8	(100)	54.2	(100)
4-9	3.0	(16)	1.0	(2)
4-25	16.1	(86)	25.1	(46)

GOGAT activities in different mutant strains grown and assayed at either the permissive (19°C) or restrictive (30°C) temperatures. Glutamate grown cells were inoculated into MM + 20mM NH_4^+, and grown grown for 6h before being harvested. GOGAT was assayed at 19°C for the 19°C grown cells, and at 30°C for the 30°C grown ones. Duplicate assays were done on each cell extract and the values presented are the means of at least two experiments (the value for 4-9 grown at 19°C is for a single experiment). The values shown in parenthesis are the percentage of the 4324c activity.

Glutamate grown cells, incubated at either 19 or 30°C were inoculated into MM + 20mM NH_4^+ to an initial A_{610} 0.05 and growth continued at the same temperature. Growth curves are presented in Fig. 19 for the 19°C cultures and in Figs. 25 and 26 for the 30°C ones. Data for the t_ds of the various strains at the two temperatures are shown in Table 25.

The data show that at both temperatures, Σ1278b grew faster than 4324c - the t_d for 4324c was about twice that of Σ1278b. This was expected because of the *gdh1-1* allele in 4324c, which reduces the rate of ammonium assimilation by this strain. The final A_{610} of the Σ1278b cultures was greater than that of 4324c ones. This may be because the 4324c culture had not entered stationary phase 24h after the start of the experiment. Alternatively, it could be because 4324c had to assimilate ammonium by the more energy expensive pathway, and so less energy was available for biomass synthesis. It is not possible to distinguish between the two possibilities from this experimental data.

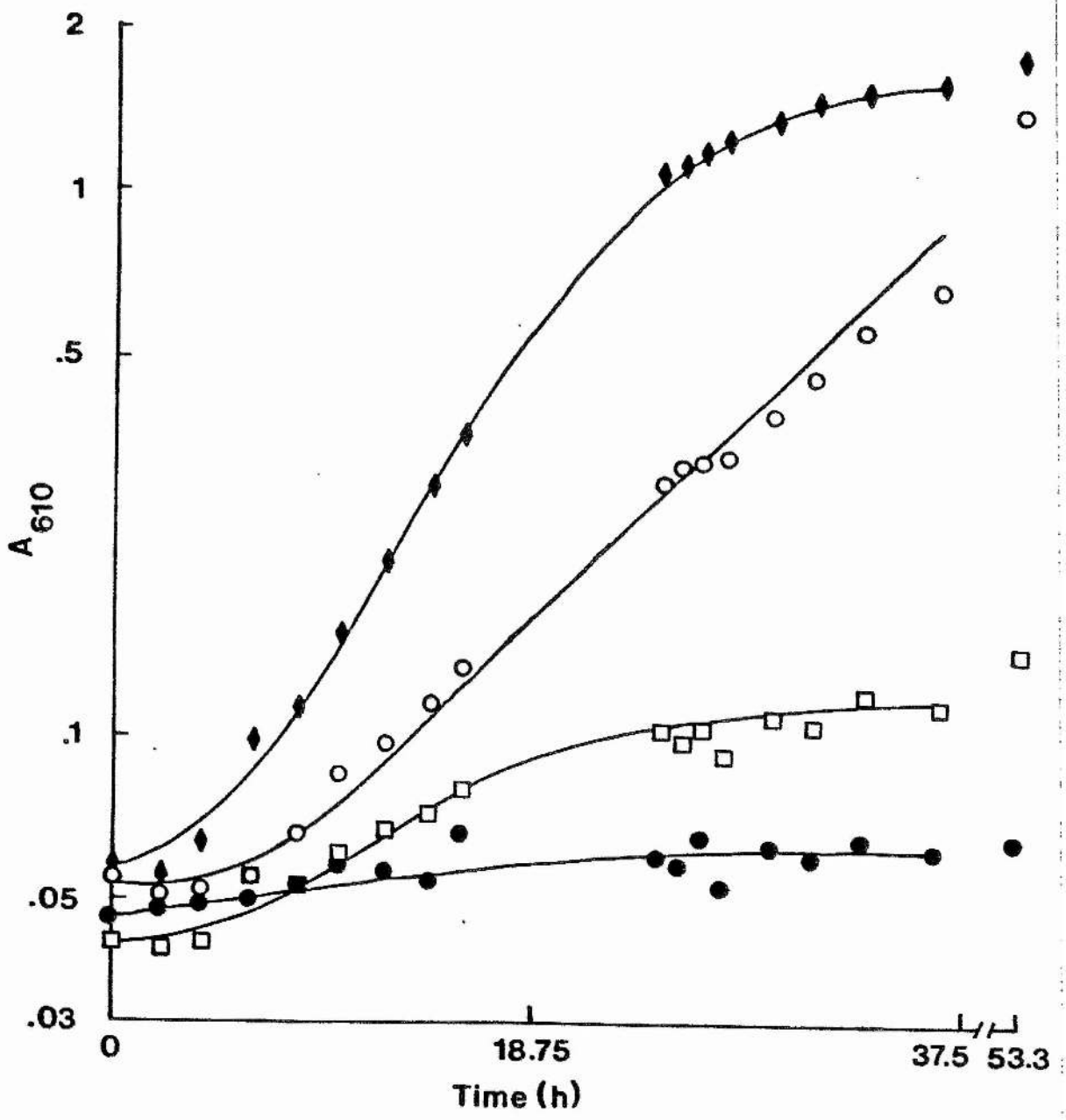
In cultures of 4-9 grown at either temperature, cells went through less than one division before growth ceased. It was expected that 4-9 would not grow in this experimental system because GOGAT was only detectable at very low levels.

At both the permissive and restrictive temperatures 4-25 grew markedly more slowly than the parental strain (t_ds of 173 *cf.* 416 minutes at 30°C, and 450 *cf.* 738 minutes at 19°C). The ratio of the two t_ds was 2.4 at 30°C but 1.6 at 19°C, indicating that 4-25 was growing relatively less slowly than the parental strain at the permissive temperature than it was at the restrictive one. This is what would be expected if the mutation in 4-25 was conferring a *ts*-phenotype on the strain. GOGAT activity in 4-25 at 19°C was comparable to that measured in the parent (16.1 *cf.* 18.8mU/mg). Therefore, it would be expected that the t_ds of 4-25 and 4324c at 19°C would be similar. They were not.

To summarise the data: (i) on the basis of the plate tests, 4-25 has a *ts*-phenotype although it takes longer for colonies of 4-25 to appear than for the parent; (ii) the enzyme studies showed that the mutant enzyme has an increased thermolability relative to the wild type; (iii) GOGAT activities in 4-25 and 4324c are similar at the permissive temperature; and (iv) in liquid culture at the permissive temperature, the growth of 4-25 and 4324c is not comparable.

The enzyme data strongly suggest that GOGAT in 4-25 has an altered thermolability relative to wild type, and, on the basis of the argument advanced in section 5.4.2.2, the mutation probably lies within the coding regions of the GOGAT genes. If the enzyme is functional at the permissive temperature, then growth comparable to the parental strain would be expected. This was observed in the plate test but not in the liquid culture experiment. The reason for the discrepancy in the results for the two growth test systems is unclear. Due to the uncertainty about the *ts*-phenotype of 4-25 it was decided to: (i)

Fig. 19



Growth of the putative ts-glu⁻ mutant 4-25 at the permissive temperature (19°C). The strains were grown in MM + glutamate at 19°C to stationary phase and inoculated into MM + 20mM NH_4^+ (100ml in 250ml non-baffled flask, 19°C, shaking water-bath: 80rpm, 4cm stroke-length) to an initial $A_{610} \sim 0.05$. The growth of the cultures was followed by the change in A_{610}. At least two independent experiments were done and representative curves are shown. (Σ1278b ♦; 4324c ○; 4-9 ●; 4-25 □).

isolate *ts*-revertants of a GOGAT$^-$ strain (section 5.4.2.4); and (ii) examine the enzymes in the other mutants for altered thermolability.

5.4.2.4 Strain 4-9 was chosen for the reversion studies because it had: (i) the highest reversion frequency of the GOGAT$^-$ strains tested; and (ii) the low GOGAT activity of the mutant would make it easy to detect a reversion event at the locus causing the loss of activity.

4-9 was cultured and revertants selected as described in section 2.5.4. A total of 1823 revertants were isolated. They were tested for a *ts*-phenotype by replication onto duplicate MM + 20mM NH_4^+ plates, and incubating the plates at either 19 or 30°C. Three colonies were detected which appeared to grow at 19 but not 30°C. Rescreening of the putative *ts*-revertants, by streaking the strains on MM + 20mM NH_4^+ for the appearance of single colonies, reduced this number to one. This revertant was grown and assayed for GOGAT at the permissive temperature. The activity in this strain was measured at 1.2mU/mg. The data show that there was no marked difference in the GOGAT activities of the original mutant and the revertant. Therefore, the putative *ts*-phenotype of the revertant was not due to reversion of the mutation causing the loss of GOGAT activity.

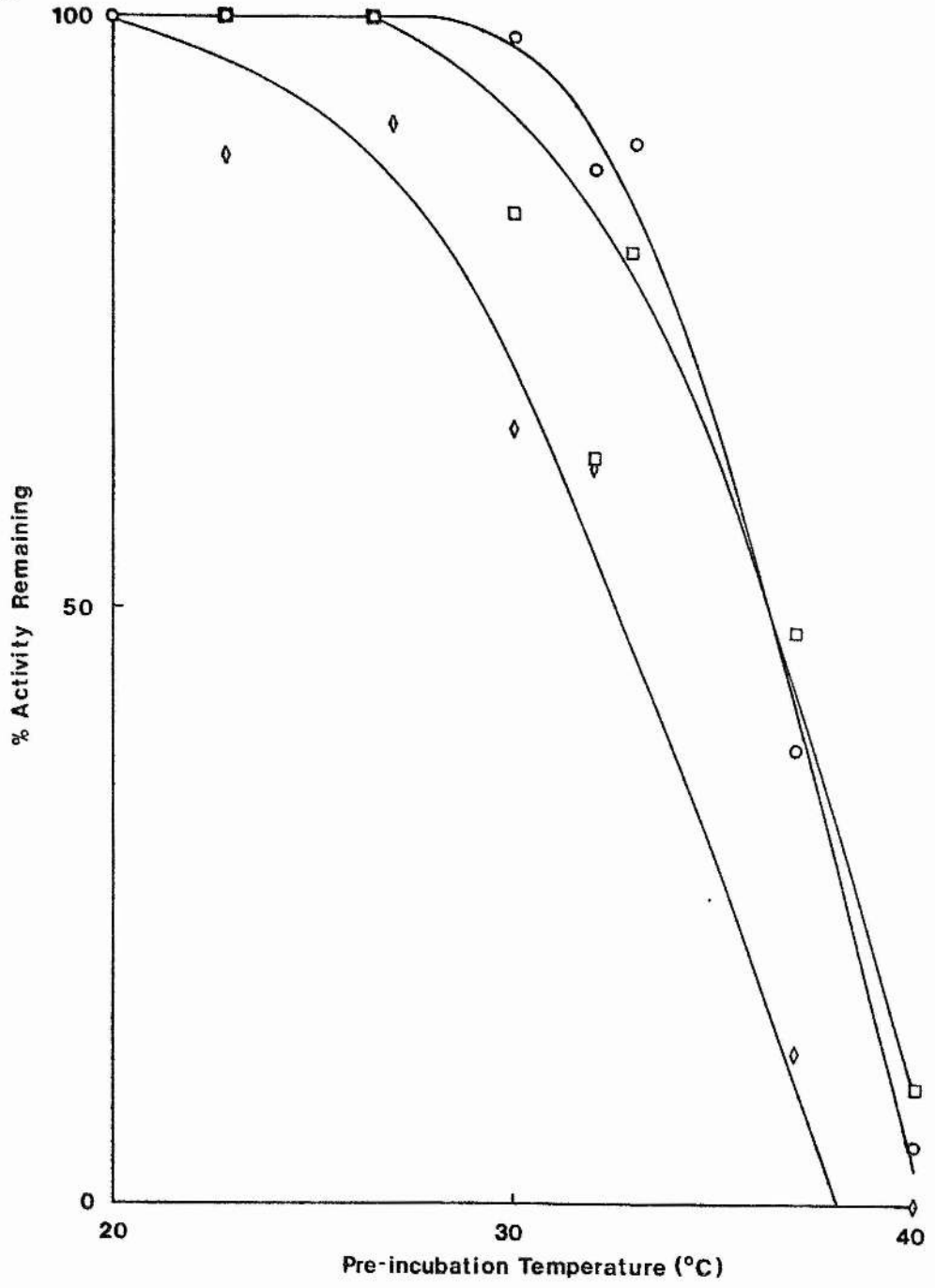
At this point in the study, GOGAT in strain 4-10 was shown to have an altered thermolability. For this reason the search for a *ts*-revertent was stopped.

5.4.3.1 Although the mutation may not generate a *ts*-gene product which is fully functional at the permissive temperature or inactive at the restrictive one, the product may still have an increased thermolability relative to the wild type. The mutant enzymes were screened for altered thermostabilities. GOGAT in strains 4-8 and 4-10 was tested. The enzyme in 4-8 did not have an altered thermostability (data not shown) and work with this strain was stopped. However, the enzyme in 4-10 had an increased thermolability. Studies were continued with the enzyme from 4-25.

Two approaches were used to investigate the thermostability of the mutant enzymes. Firstly, cell extract was pre-incubated for 10 minutes at various temperatures before being assayed at 19°C to study the effect of temperature on enzyme stability. Secondly, time effects were studied by pre-incubating cell extract at 32°C for various time periods before assaying residual activity at 19°C. The data is presented in graphical form in Figs. 20 and 21.

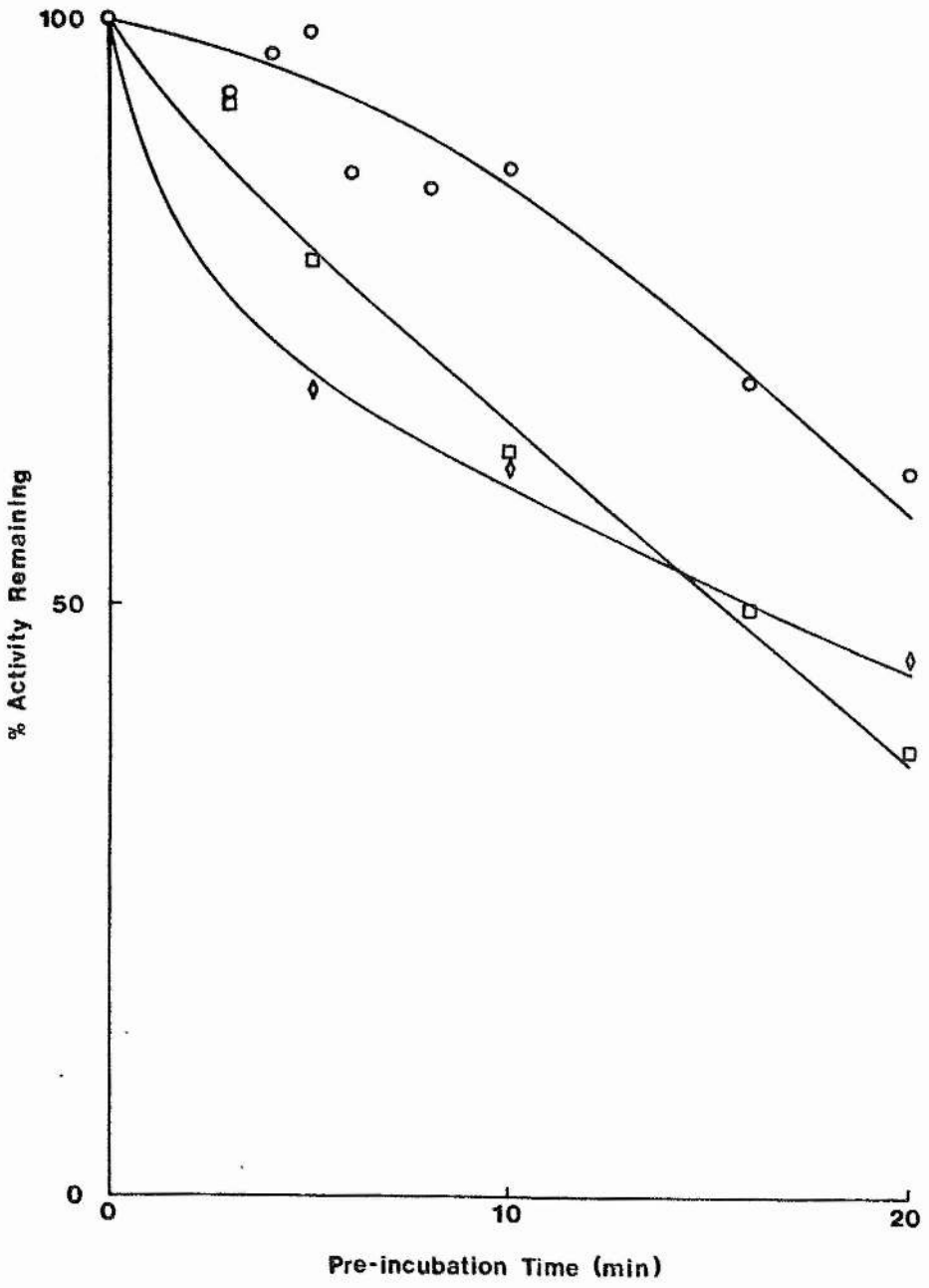
The data in Fig. 20 shows that the wild type enzyme was very stable. As the pre-incubation temperature was increased from 20 to 30°C less than 5% of the activity was lost. But as the temperature was increased from 30 to 40°C, about 85% of the activity was lost. Fifty-percent inactivation occurred at a pre-incubation temperature of 36.4°C (all values given for 50% inactivation were obtained from the curves shown in Figs. 20, 21 and 23). The profile of the 4-25 GOGAT is very similar to that of the wild

Fig. 20



Thermostability of wild type and mutant GOGAT with respect to temperature. Constant amounts of protein (200µg) were pre-incubated for 10 minutes at the temperature shown and residual GOGAT activity assayed at 19°C. Duplicate assays were done at each pre-incubation temperature: the data shown are the means of two independent experiments. (Σ1278b O; 4-10 ◊; 4-25 □)

Fig. 21



Thermostability of mutant and wild type GOGAT with respect to time at a fixed temperature. 200µg protein was pre-incubated at 32°C for the times shown and residual activity assayed at 19°C. Duplicate assays were done for each time period: the data are the means of, respectively, three, four and two independent experiments for Σ1278b, 4-10 and 4-25. Symbols as in Fig. 20.

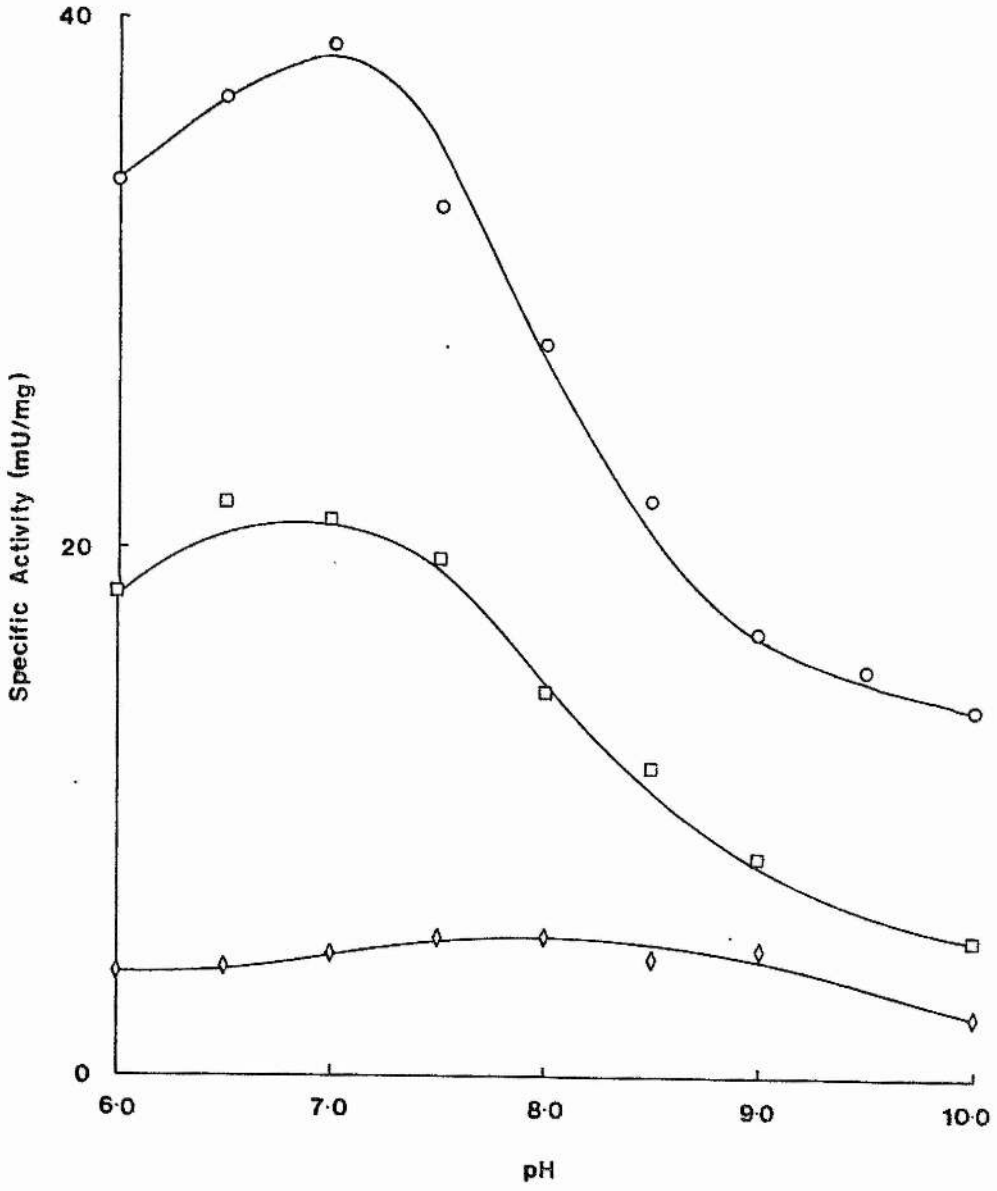
type - 50% inactivation was also at 36.4°C. The profile for the 4-10 enzyme is markedly different from that of the wild type. As the pre-incubation temperature was increased from 20 to 30°C, about 30% of the initial activity was lost. The remaining activity was lost as the temperature was increased to 38°C. Fifty-percent inactivation occurred at 32.6°C. This is markedly lower than the value for the wild type enzyme.

For the studies on the effect of time on enzyme stability, 32°C was chosen as the pre-incubation temperature because the data in Fig. 20 had shown that differences in enzyme stability were greatest at this point. Fig. 21 shows that the profiles of loss of enzyme activity with time were different for each of the enzymes studied. The wild type enzyme is markedly more stable than either of the mutant enzymes studied. About 15% of its activity was lost as the pre-incubation period increased to 10 minutes, but as this period went from 10 to 20 minutes a further 25% of the activity was lost. In contrast, the 4-25 enzyme showed a linear decrease in activity up to 20 minutes pre-incubation. About 30% of the GOGAT in the cell extract of 4-10 was inactivated by 5 minutes pre-incubation, with only a further 20% loss of activity as the pre-incubation period increased by a further 15 minutes. For the Σ1278b enzyme, 50% inactivation occurred after 27.9 minutes pre-incubation, the figures for 4-10 and 4-25 were 16.5 and 15.4 minutes respectively.

The data presented in this section show that both mutant enzymes have an increased thermolability compared to the wild type. If only the data in Fig. 20 had been considered, it would have been concluded that the enzymes in strains Σ1278b and 4-25 have comparable thermostabilities. But, by extending the studies, it has been shown that the 4-25 enzyme is different from that in Σ1278b. The increased thermolabilities of GOGAT from the mutants suggest that one or both of the polypeptides which constitute the *S. cerevisiae* enzyme are altered in these strains *i.e.* the mutations lie within the coding regions. Although heat stability is a physical property of the enzyme, this experiment does not completely exclude the possibility that a prosthetic group, and not a constituent polypeptide, has become more labile. Therefore, further studies of the physiochemical properties of the mutant enzymes were made.

5.4.3.2 In an argument analogous to that advanced in section 5.4.2.2, a mutation in the GOGAT coding regions which results in, for example, the replacement in the polypeptide of a basic amino acid residue by an acidic one, may result in a decrease in activity. Such a mutation may also result in a change in optimum pH for enzyme activity. The effect of pH on the activity of GOGAT from Σ1278b, 4-10 and 4-25 was investigated. GOGAT activity was assayed at various pH values using a Tris-phosphate buffer system. The results are shown in Fig. 22.

- Fig. 22



pH profile of GOGAT activity from Σ1278b and the glu⁻ mutants 4-10 plus 4-25. 200µg protein was assayed at the pH shown using a Tris-phosphate buffer system adjusted to the desired pH. Duplicate assays were done at each pH: the data for Σ1278b and 4-10 are the means of three independent experiments; for 4-25, two experiments were done. Symbols as in Fig. 20.

The data show that the profiles of Σ1278b and 4-25 are very similar, both have a broad pH optimum of 6.7-7.2. The pH optimum of 4-10 is much broader than for the wild type and at a more alkaline pH (7.5-8.9). The maximum activities measured in this system (39.0, 21.7 and 5.2mU/mg for Σ1278b, 4-10 and 4-25 respectively) are lower than those reported in Table 12. This probably reflects differences in the buffer system used, the data in Table 12 were obtained using 0.1M phosphate. The choice of buffer does have an effect on GOGAT activity as can be seen by examination of the literature. Roon *et al.* (1974), using 0.1M phosphate, observed a pH optimum of 7.1-7.7: Masters and Meister (1982) found a pH optimum of 7.0-7.5 in a 10mM Tris-Cl system.

The different pH profiles could be due to pH effects on the substrates. This possibility can be excluded because, at a given pH, the only variable is the source of the cell extract.

The altered pH optimum of the 4-10 GOGAT suggests that one, or both, of the polypeptides which form the enzyme are altered. This experiment cannot exclude the possibility that the effect observed in Fig. 22 is due to an altered prosthetic group.

5.4.3.3 The data presented in Figs. 20-22 do not exclude the possibility that the mutation results in an altered prosthetic group. To demonstrate that the mutation caused a modification of one or both of the polypeptides of GOGAT, properties unique to the polypeptide(s) forming the active centre were studied. The properties examined were the kinetic constants of the enzyme (this section) and the effect of an active centre-specific inhibitor on activity (section 5.4.3.5). Such properties are not affected by an alteration of a cofactor or prosthetic group.

The affinity of the enzyme for its substrates (K_m) and the turnover number of the enzyme are both inherent properties of the polypeptides forming the active centre of the enzyme. However, turnover numbers can only be obtained with pure enzyme. As purified enzymes were not available in this study, V_{max} was measured instead. V_{max} is proportional to the rate constant for the dissociation of the enzyme-substrate complex to enzyme plus product *i.e.* to the turnover number, and is a property of the active centre. K_m and V_{max} values were obtained for GOGAT from Σ1278b, 4-10 and 4-25.

The data for the kinetic constant determinations were generated by measuring the reaction rate at different substrate concentrations with the non-varying substrates held at a constant, saturating level. K_m and V_{max} values were calculated using the Hymic program (Barlow 1983). The kinetic constant data for the three strains is presented in Table 16.

For the substrate 2-oxoglutarate, the K_m values measured in Σ1278b, 4-10 and 4-25 were similar. The sample variation for the K_m values was about 25-30% around the mean value (this is typical for K_m determinations

Table 16.

Substrate	Constant	Strain		
		Σ1278b	4-10	4-25
2-oxoglutarate	K_m[1]	0.12	0.16	0.12
	V_{max}[2]	50.9	13.4	42.4
Glutamine	K_m	1.40	0.83	1.01
	V_{max}	52.2	16.2	49.0

Kinetic constants of wild type and mutant GOGAT. The data was generated by measuring the reaction rate at different concentrations of one substrate, with the other substrate concentrations held constant at saturating level with a fixed amount of protein in each mixture. The kinetic constants were calculated by computer using a program which fits a line using the method of least squares. Each point on the line was the mean of duplicate assays. The data in this table are the means of at least two independent experiments. 1 - mM: 2 - mU/mg.

[R. Griffiths, pers. comm.]). The V_{max} value of Σ1278b was greater than that of the enzyme in 4-25, and was markedly higher than that for the 4-10 enzyme.

Both mutant enzymes have a higher affinity for glutamine than the wild type. The K_m of 4-25 is greater than the value for the 4-10 enzyme. The V_{max} values for Σ1278b and 4-25 are similar, but the 4-10 value shows a marked difference.

Although both the mutant enzymes exhibit altered V_{max} values there are two possible explanations for this difference. V_{max} is the product of the turnover number and the enzyme concentration. Therefore, a decrease in V_{max} could be due to a decrease in the number of enzyme molecules or an alteration in the active centre causing a decrease in turnover number. It was not possible to distinguish between these two possibilities.

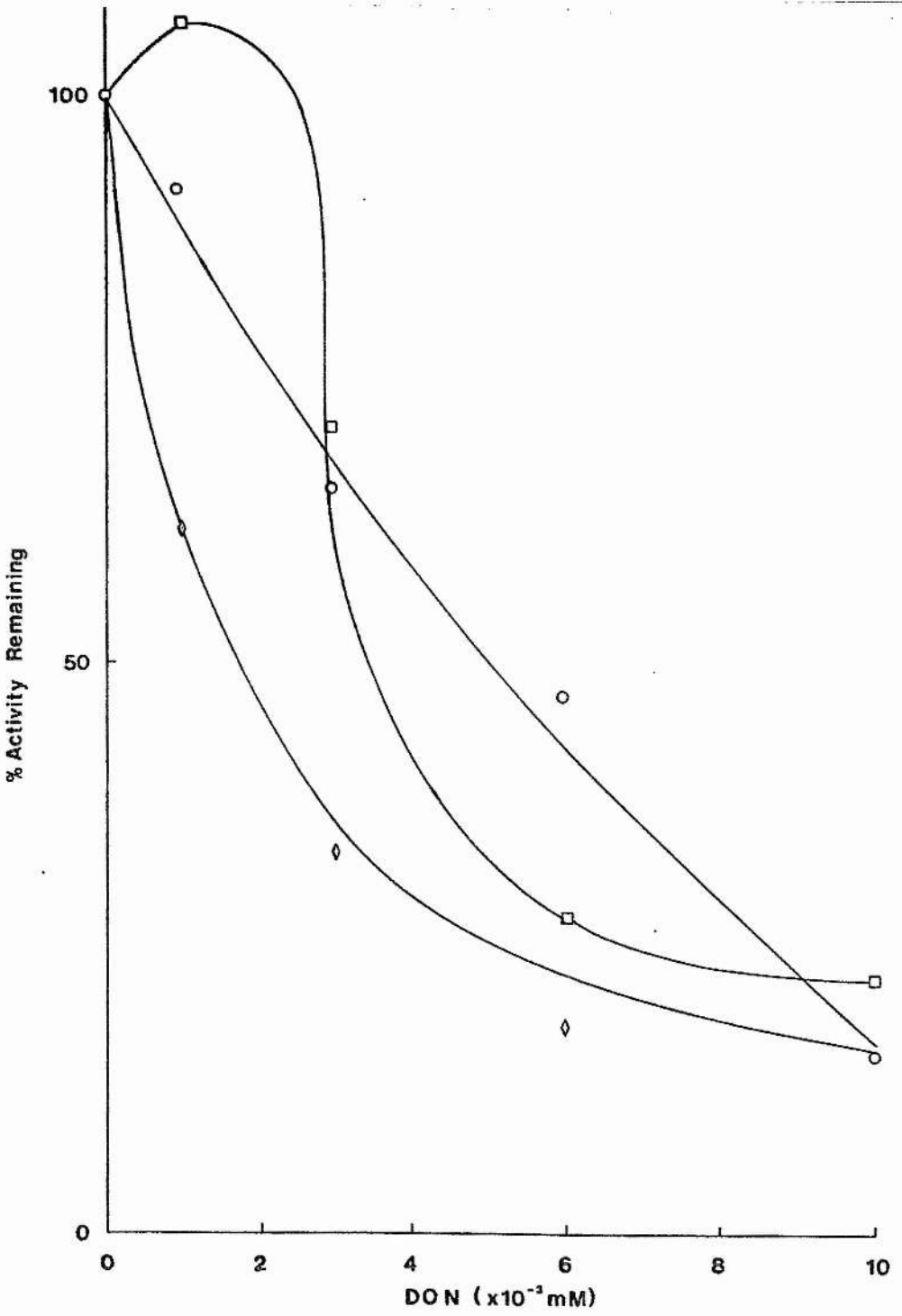
The affinity of both the mutant and wild type enzymes for 2-oxoglutarate are similar, but the mutant enzymes have an increased affinity for glutamine. Why the mutant enzymes should exhibit an increased affinity for glutamine, yet show a loss of activity relative to the wild type, is unclear. The altered affinities of the mutant enzymes for the substrate glutamine is due to an alteration in the polypeptides forming the active centre. The change in the active centre suggests that the decrease in V_{max} observed in Table 16 is probably due to an alteration in turnover number and not enzyme concentration.

5.4.4.4 Hartman (1963, 1968) showed that the glutamine analogue DON competitively inhibited glutamine binding because it bound to the active centre of glutaminase. This is also true for GOGAT (see section 1.3.4). It has been shown (section 4.2) that DON specifically inhibits GOGAT in the assay system used in this study. The effect of DON on GOGAT from the three different strains was investigated. GOGAT activity was assayed in the presence of various concentrations of DON. The results are presented in Fig. 23.

The three enzymes show a marked difference in their response to the inhibitor. Inactivation of the wild type enzyme was approximately linear up to 10μM DON. GOGAT from 4-10 showed an infralinear loss of activity. The response of the 4-25 enzyme to increasing levels of DON was complex. It initially showed an increase in activity before rapidly becoming inactivated. The reasons for this are unclear. Fifty-percent inactivation of the wild type enzyme occurred at a DON concentration of 5.8μM. Both mutant enzymes are more sensitive to the inhibitor than the wild type. For 4-10, 50% inactivation was at 1.7μM DON and 3.4μM for 4-25.

The data in Table 16 show that both the mutant enzymes have an increased affinity for glutamine. DON is an analogue of glutamine which binds to the active centre of GOGAT. The affinity of the 4-10 enzyme for glutamine is greater than that of the 4-25 one, and GOGAT from 4-10 is more sensitive to

Fig. 23



Inhibition of GOGAT activity by the glutamine analogue DON. 100µg protein from wild type and glu⁻ mutant strains was assayed for GOGAT activity in the presence of increasing concentrations of DON. Duplicate assays were done at each DON concentration and the data are the means of three independent experiments. Symbols as in Fig. 20.

the inhibitor. Therefore, the increased sensitivity to the inhibitor is probably a consequence of the increased affinity of the enzyme for the substrate. It is most probably due to an alteration in the polypeptides forming the active centre.

5.4.3.5 Soberon and Gonzalez (1987) showed that glutaminase B of *S. cerevisiae* CN10 was more sensitive to 2-oxo acids than the wild type. Desalting caused an increase in glutaminase activity measured in cell extracts of the mutant, and had a greater effect in extracts of the mutant than with the wild type. Mora *et al*. (1987) observed a similar situation with the *am-132* and *am-132: gly*^{r} mutants of *N. crassa*. GOGAT activity is higher in the double mutant because the level of organic acids, which are inhibitors of GOGAT, is lower than in the *am-132* mutant. To eliminate the possibility that a similar situation *i.e*. the accumulation of an intracellular inhibitor, existed in the mutants studied, cell extracts from 4-9 and 4-10 were dialysed overnight. The data is presented in Table 17.

The data show that dialysis causes a slight increase in the activity measured in extracts of Σ1278b and 4-10, but does not result in restoration of wild type enzyme levels in the 4-10 extract. No increase in activity was observed with strain 4-9. This experiment shows that the loss of enzyme activity in strains 4-9 and 4-10 was not due to the accumulation of a dialysable intracellular effector.

5.4.3.6 The studies reported in this section have shown that the enzyme in strains 4-10 and 4-25 have an increased thermolability compared to the wild type. Additionally, GOGAT from strain 4-10 has an altered pH optimum. Although the data show that GOGAT in the mutant strains is altered, it does not distinguish between the possibilities that this change is due to alterations in the constitutive polypeptides or a prosthetic group. To distinguish between the two possibilities, properties unique to the polypeptides forming the active centre of the enzyme were examined. Both mutant enzymes exhibited an increased affinity for the substrate glutamine, a decreased V_{max} with 2-oxoglutarate and the 4-10 enzyme also had a lower V_{max} with glutamine. GOGAT from the two mutants was more sensitive to an inhibitor which binds to the active centre. The loss of activity in the mutants 4-9 and 4-10 was not due to the accumulation of an intracellular effector.

The data presented suggest very strongly that the mutations in strains 4-10 and 4-25 causing loss of GOGAT activity lie within the sequences encoding the polypeptides of GOGAT. This can only be proved unequivocally by comparison of either amino acid sequences of mutant and wild type enzymes, or the nucleotide sequences of mutant and wild type genes.

Table 17.

Strain	GOGAT Activity (mU/mg) in:	
	Non-dialysed Extract	Dialysed Extract
Σ1278b	24.7	25.4
4-9	0.0	0.0
4-10	8.7	10.6

Effect of dialysis on GOGAT. Cell extracts from each of the strains were dialysed overnight against three changes of buffer. Non-dialysed extract was stored overnight at 4°C.

Chapter 6

Studies on the Genetics of *S. cerevisiae* GOGAT⁻ Mutants

6.1 In this study, 16 mutants with altered GOGAT activity were isolated. Although there is very strong evidence that two of the strains are mutant in the GOGAT structural genes, the nature of the remaining mutants, *i.e.* whether they lie in the coding or regulatory regions, is not known. The biochemical studies reported in Chapter 5 do not indicate whether strains 4-10 and 4-25 are mutant in one or both of the polypeptides which form the functional GOGAT enzyme, or if the two strains are mutant in the same or different polypeptides. To answer these questions a genetic analysis of the various mutants was started.

6.2 Allelism between the different mutants was investigated by testing the ability of the various mutations to complement one another when present in *trans* in a heterozygote. This was done by crossing all the mutants (both *a* and α mating types) in a pairwise fashion and scoring the resultant diploids for the ability to grow on MM + NH_4^+. The data is presented in a complementation matrix (Table 18). A complementation map was constructed from this data and is shown in Fig. 24.

Thee complementation tests were done using mutants which had been backcrossed once to the parental strain (see Table 2). This was not possible with strains 4-5, 4-8 and 4-17 because only LEU⁺ GLU⁺ or leu⁻ GLU⁺ segregants could be isolated from mutant x parent crosses. The reason for this is unclear, but see section 6.3. As there were no suitable segregants available from these three strains, it was only possible to test them, plus 4-20, in one mating type.To make the interpretation of the data easier, the original mutant designations are used in Table 18 and Fig. 24.

The complementation data show that the 16 mutants are related in a complex fashion. This complexity indicates that the mutants are not sisters and have arisen by independent mutations. The *S. cerevisiae* GOGAT is a heteromeric dimer, so it was expected that a minimum of two complementation groups would be found. At least five groups were observed.

Strains 4-10 and 4-25 were found to complement one another. The evidence strongly suggests that both these strains carry structural gene mutations. Therefore it would appear that these mutations lie in the two genes encoding the two polypeptides forming GOGAT. The allele present in 4-10 has been designated *got1-10* and that in 4-25, *got2-25*. From the data presented in this study it is not possible to say which polypeptide is affected by which mutation.

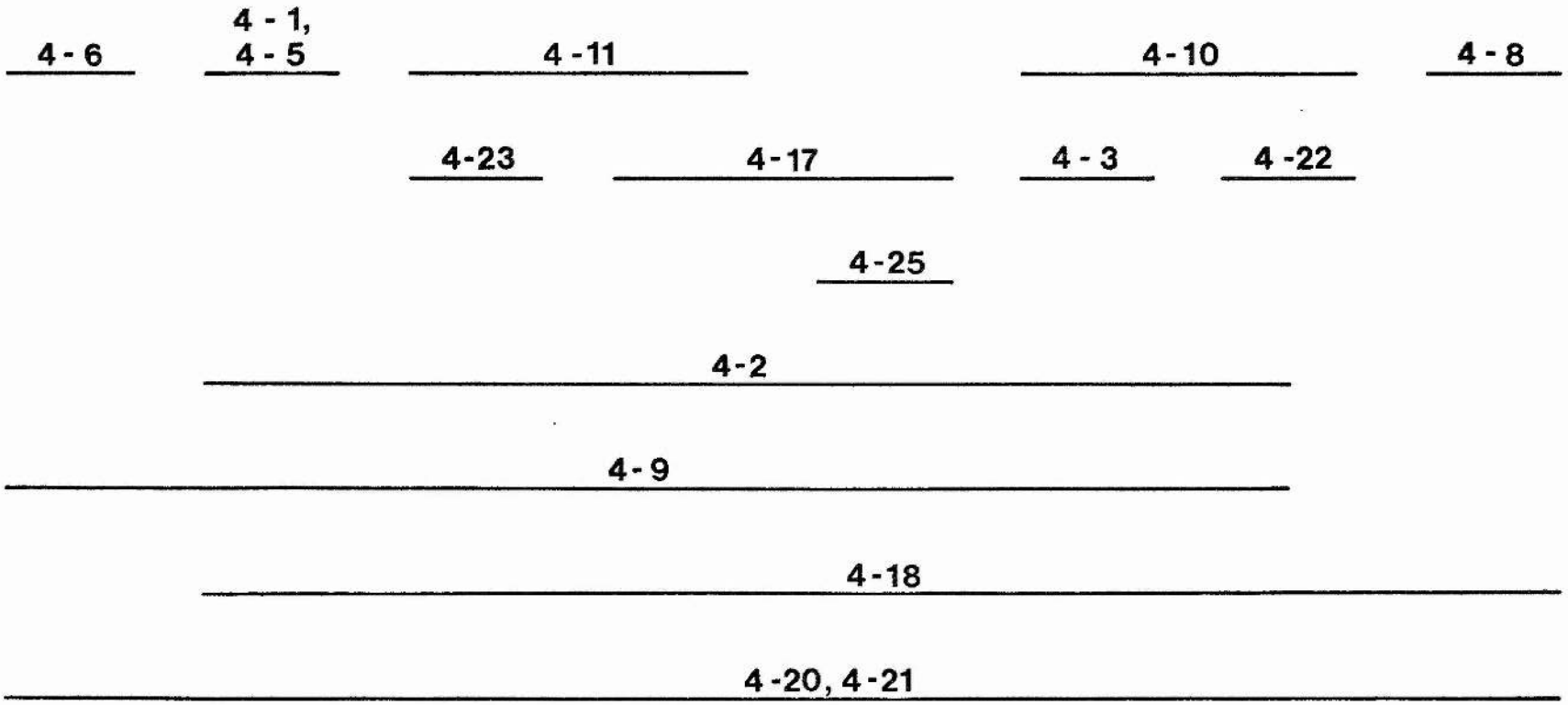
An unexpected result was that the alleles carried by strains 4-22 and 4-25 both complement themselves (Table 18). It is not clear why this was so. There is no reason *a priori* why it should have occurred.

Table 18.

	4-1	4-2	4-3	4-5	4-6	4-8	4-9	4-10	4-11	4-17	4-18	4-20	4-21	4-22	4-23	4-25
4-1	-	-	+	-	+	+	-	+	+	+	-	-	-	+	+	+
4-2		-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
4-3			-	+	+	+	-	-	-	-	-	-	-	+	+	+
4-5					+		-	+	+		-	-	-	+	+	+
4-6					-	+	-	+	+	+	+	-	-	+	+	+
4-8							+	+	+		-	-	-	+	+	+
4-9							-	-	-	-	-	-	-	+	-	-
4-10								-	+	+	-	-	-	-	+	+
4-11									-	-	-	-	-	+	-	+
4-17											-	-	-	+	+	-
4-18											-	-	-	-	-	-
4-20													-	-	-	-
4-21													-	-	-	-
4-22														+	+	+
4-23															-	+
4-25																+

Complementation matrix for the various S. cerevisiae GOGAT$^-$ mutants. All the mutants were crossed in a pairwise fashion, and growth (+) or no growth (-) on MM + 20mM. NH_4^+ scored after 48h incubation at 30°C (it was not possible to get mutants 4-5, 4-8 and 4-17 in both mating types). Each cross was repeated 2-8 times. A map was constructed from this data and is shown in Fig. 24.

Fig. 24 Complementation Map of the GOGAT Mutants



Overlap of horizontal lines indicates no complementation

Two mutations are usually considered to be allelic if, when present in *trans* in the heterozygote, they produce a mutant phenotype and non-allelic if they give a wild type one. However, in this study 16 mutants were investigated. Therefore, to be considered allelic, two (or more) mutations will have to show the same pattern of complementation with all the remaining 14 mutations. Fig. 24 shows that strains 4-20 and 4-21 exhibited the same pattern of complementation and so are probably allelic. 4-1 and 4-5 are probably also alleles of one another. The enzyme profiles of 4-1 plus 4-5 and 4-20 plus 4-21 shown in Table 12 are different. This suggests that although these mutations may be allelic, they arose from independent mutations. Although the remaining strains did not complement each other, the patterns of complementation of each strain suggest that the mutations are non-allelic.

Strains 4-20 and 4-21 did not complement any of the other 14 strains, while 4-2, 4-9 and 4-18 only complemented 4-6 and/or 4-8. This would suggest that: (i) the remaining mutations lie within a discrete region of the genome and can be lost by a single deletion mutation; or (ii) the genes mutated in strains 4-20 etc. specify a regulatory element whose inactivation results in the loss of GOGAT activity. Alternatively both points (i) and (ii) could together explain the results. A trivial explanation of this data is that these five strains were sterile.

The trivial explanation can be discounted because: (i) the complementation tests were done using suitably marked segregants from the cross "mutant x parent" (see Table 2); and (ii) these segregants mated with the mating-type tester strains DS278 and DS280 to give phenotypically wild type diploids.

If the mutations carried by strains 4-2, 4-9, 4-18, 4-20 and 4-21 were deletions it would not be possible to isolate revertants from these strains. The data in Table 14 show that, of these five strains, revertants were isolated from 4-2, 4-9 and 4-20. Revertants were recovered more frequently than would be expected if they arose by reversion of the *gdh1-1* allele alone, eliminating the possibility that all the revertants were GDH^+ $GOGAT^-$. Therefore it would appear that the second hypothesis is possibly the correct one for these three strains. Circumstantial evidence against the first hypothesis is that there are no examples reported for *S. cerevisiae* of genes specifying different polypeptide components of the same enzyme being tightly linked. No revertants were isolated from strains 4-18 and 4-21, although this could be because insufficient cell numbers were screened. Therefore these two mutations could be deletions. This would suggest that, while *got1-10* and *got2-25* are structural gene mutations, the other mutations (or at least those carried in 4-2, 4-9 and 4-20) are in regulatory elements which modulate GOGAT activity.

In summary: the complementation map of the 16 $GOGAT^-$ mutants is very complex. It shows that the mutations carried by 4-10 and 4-25 are in different genes (*GOT1* and *GOT2* respectively). Strains 4-2, 4-9 and 4-20 complement few, if any, of the other 13 mutants but are not deletion mutants. This

suggests that they are regulatory mutations of some sort. As mutations in 4-20 and 4-21 are probably allelic, the mutation in 4-21 is probably also a regulatory mutation.

The function of the genes identified by the ten remaining mutants is unclear. It is unlikely that they are all structural gene mutants because purified *S. cerevisiae* GOGAT is a heteromeric dimer (Masters and Meister 1982) and two structural genes have been identified. This would suggest that the remaining mutants carry mutations in some regulatory element. However, until the non-structural gene mutants have been assayed for aconitase, citrate synthase and isocitrate dehydrogenase activities, it cannot be said unequivocally that the loss of GOGAT activity is not due to the inability to synthesise 2-oxoglutarate. This would imply that GOGAT, like NADP-GDH, is modulated by the level of 2-oxoglutarate.

The map shows that there are apparently a large number of alleles which (on the basis of the above arguments) are regulatory elements. The complementation map suggests that these elements may have overlapping functions. It is futile to speculate further about the nature of these elements because there is insufficient data to make any valid conclusions. Further biochemical studies are needed before the complementation map can be understood.

The enzyme and growth studies using 4-9 were done before the complementation tests, so it was not known that the mutation carried by 4-9 was possibly in a regulatory element and not a structural gene.

6.3 In order to determine the number of loci responsible for the GOGAT$^-$ phenotype, detect gene linkage or chromatid interference, and recognise lethality associated with chromosome aberrations and mutations, tetrad analysis was done on three GOGAT$^-$ mutants. The structural gene mutants 4-10 and 4-25 plus the putative regulatory mutant 4-9 were analysed. Each mutant was crossed to a *GOT* strain and diploids selected. The diploids were sporulated and 12-17 asci were dissected for each cross. The spores were analysed initially for the segregation of the leu$^-$ and glu$^-$ phenotypes by replicating master plates onto suitable diagnostic media.

The diagnostic media were MM supplemented with either NH_4^+, NH_4^+ + leucine, glutamate or glutamate + leucine. LEU$^+$ GLU$^+$ strains will grow on all four test media while leu$^-$ glu$^-$ strains will only grow on MM supplemented with both amino acids. Both LEU$^+$ glu$^-$ and leu$^-$ GLU$^+$ strains will grow on MM + glutamate + leucine, but not MM + NH_4^+. The LEU$^+$ glu$^-$ strains will grow on MM + glutamate but not MM + leucine. The opposite is true for the leu$^-$ GLU$^+$ strains. The segregants were screened twice to ensure that the results were reproducible.

Subsequently it was decided to analyse the spores for the segregation of the mating type. This was done by crossing the segregants with the mating-type tester strains DS278 and DS280. The diploids were scored for a wild type phenotype.

The data are presented in Table 19. Preliminary analysis of the data is shown in Tables 20 and 21.

In all three crosses examined there was an abnormally high frequency of 3:1 LEU+:leu- asci (for 4-10 the frequency was 5/17, AR40 3/15 and AR42 6/12) and 4:0 LEU+ leu- asci (the figures were 2/17 for 4-10 and 7/15 for AR40). Mortimer and Hawthorne (1975) have analysed the segregation pattern of *leu2-1* in 761 asci. They found that only 2.6% of the asci showed a 3:1 segregation ratio and no 4:0 asci were detected. As can be seen by comparison with figures cited from Mortimer and Hawthorne, the segregation pattern of *leu2* in these crosses was unexpected. It would therefore appear that the *leu2* marker was behaving in an aberrant manner.

This result is supported by an observation made during the construction of the strains used in the genetic and growth studies. An excess of LEU+ GLU+ colonies was recovered when diploids from the cross AR2 x mutant were sporulated (Table 22). Additionally, in 13/16 of the crosses, the LEU+ phenotype did not show 2:2 segregation - the exceptions were 4-3, 4-11 and 4-23. Initially, this was thought to be an artefact of the spore disruption technique, either because vegetative cells were not being killed or because the asci were not being disrupted. However, the same result was observed with the tetrad analysis. This would suggest that the result is not an artefact of the dissection technique.

The GLU- marker did not exhibit the segregation pattern (2:2 +:-) expected if the mutation was at a single locus. In 4-10, 8/17 asci did not show a 2:2 segregation of GLU+:glu-: a similar frequency was observed with AR42 (6/12) while for AR40 13/15 asci did not exhibit a 2:2 ratio. If the GLU- phenotype was due to two unlinked mutations (*i.e.* if both polypeptides forming the enzyme had been inactivated) then a ratio of 1:3 GLU+:glu- spores in each ascus would be expected. Asci with this marker combination were only observed at a low frequency (1/17 for 4-10, AR40 0/15 and 3/12). The segregation ratios cannot be explained by postulating that the GLU- phenotype was the result of single or double mutations.

If there was independent assortment of the leu- and glu- phenotypes, then equal numbers of parental (LEU+ glu-, leu- GLU+) and non-parental (LEU+ GLU+, leu- glu-) ditype (PD and NPD) asci would be expected. However, if the two phenotypes were the result of two linked mutations, more PD than NPD asci would be expected. The data is shown in Table 21. Only AR42 shows an excess of PD asci, the other two crosses resulted in an excess of NPD asci. However, in all three crosses the majority of asci were non-PD, non-NPD or non-tetratype. The reason for this is unclear, but consequently no conclusion can be made about the linkage of the mutations causing the leu- and glu- phenotypes.

Table 19.

	Tetrad No.	Marker	Spore A	B	C	D	No. of spores in tetrad with marker combination: LEU GLU	LEU glu	leu GLU	leu glu
(a)	1[1]	LEU	+	−	−	+	1	1	1	1
		GLU	+	−	+	−				
	2	LEU	−	+	−	+	1	1	1	1
		GLU	−	−	+	+				
	3	LEU	+	−	−	+	2		2	
		GLU	+	+	+	+				
	4	LEU	+	−	−	−	1		1	2
		GLU	+	−	−	+				
		Cu^r	+	−	−	−				
		MT	?	∝	∝	a				
	5	LEU	−	+	+	−	1	1		2
		GLU	−	−	+	−				
		Cu^r	−	−	+	−				
		MT	a	∝	?	a				
	6	LEU	−	+	−	+	2			2
		GLU	−	+	−	+				
		Cu^r	−	+	−	+				
		MT	a	?	a	∝				
	7	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
		Cu^r	+	+	+	+				
	8	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
		Cu^r	+/−	+	+	+				
	9[2]	LEU	−	+	+	+	2	1	1	
		GLU	+	−	+	+				
		Cu^r	−	−	+	−				
	10	LEU	−	+	+	+	2	1	1	
		GLU	+	+	+	−				
	11	LEU	+	+	+	−	3			1
		GLU	+	+	+	−				
	12	LEU	−	+	+	+	1	2	1	
		GLU	+	+	−	−				
		Cu^r	−	+	−	−				
		MT	∝	a	a	∝				
	13	LEU	−	+	−	+	2			2
		GLU	−	+	−	+				
		Cu^r	−	−	−	−				
		MT	a	∝	a	∝				
	14	LEU	+	−	+	−	2			2
		GLU	+	−	+	−				
		Cu^r	+	−	−	−				

		MT	?		?	a				
	15	LEU	+	−	+	−	2			2
		GLU	+	−	+	−				
		Cu^r			+					
		MT	∝	a	∝	a				
	16	LEU	−	+	+	+	2	1		1
		GLU	−	−	+	+				
		Cu^r			+	+				
		MT	a	∝	a	∝				
	17	LEU	+	+	−	−	2		1	1
		GLU	+	+	−	+				
		Cu^r		+						
		MT	a	∝	∝	a				
(b)	1	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
		Cu^r	+	+	+	+				
		MT	∝	∝	∝	?				
	2	LEU	+	−	+	+	3			1
		GLU	+	−	+	+				
		Cu^r	+	+	−	+				
		MT		∝	a	∝				
	3	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
		Cu^r	+	+		+				
		MT	?	?	∝	?				
	4	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
	5	LEU	+	−	+	+	3			1
		GLU	+	−	+	+				
		Cu^r	+	+	−	+				
		MT		a		a				
	6	LEU	+	+	−	−	2			2
		GLU	+	+	−	−				
		Cu^r	+	+	−	−				
	7	LEU	+	+	−	−	1	1	2	
		GLU	+	−	+	+				
		Cu^r	+	−	−	−				
		MT	?	∝	∝	?				
	8	LEU	+	+	+	−	3			1
		GLU	+	+	+	−				
		Cu^r	+	+	+	−				
		MT	?	?	a	?				
	9	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
		Cu^r	+	+	+	+				
		MT	a	∝	?	∝				
	10	LEU	+	+	−	−	1	1	1	1
		GLU	−	+	−	+				
		Cu^r	−	+	−	−				
		MT	∝	?	∝	a				
	11	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
		Cu^r	+	+	+	−				
		MT	?	?	?					

	12	LEU	+	+	-	-	2		1	1
		GLU	+	+	+	-				
		Cu^r	-	-	-	-				
		MT	a	?	∝	?				
	13	LEU	+	+	-	-	2	1	1	
		GLU	+	+	+	-				
		Cu^r	+	-	-	-				
		MT	a	?	∝	?				
	14	LEU	+	+	+	+	4			
		GLU	+	+	+	+				
		Cu^r	+	+	+	+				
		MT	?	?	?	?				
	15	LEU	+	+	+	+	3	1		
		GLU	+	-	+	+				
		Cu^r	+	+	-	+				
		MT	?	?	a	?				
(c)	1	LEU	-	-	+	+			2	2
		GLU	+	+	-	-				
		MT	∝	∝	a	a				
	2	LEU	-	-	+	+		2	1	1
		GLU	-	+	-	-				
		MT	∝	∝	a	a				
	3	LEU	+	-	+	+	1	1	1	
		GLU	-	+	+	-				
		Cu^r			+					
		MT	∝	a	∝	a				
	4	LEU	-	+	+	-	1	1		2
		GLU	-	+	-	-				
		Cu^r	-	+	-	-				
		MT	∝	?	a					
	5	LEU	+	+	-	+	2	1	1	
		GLU	-	+	+	+				
		Cu^r	-	-	+	+				
		MT	a	a	∝	∝				
	6	LEU	+	+	+	-	1	2	1	
		GLU	+	-	-	+				
		Cu^r	+		+					
		MT	?	a	a	∝				
	7	LEU	+	-	+	-	1	1	1	1
		GLU	+	-	-	+				
		Cu^r	-	-	-	-				
		MT	a	∝	a	∝				
	8	LEU	+	-	+	+	1	2	1	
		GLU	+	+	-	-				
		Cu^r	+	-	-	-				
		MT	?	∝	∝	a				
	9	LEU	-	+	+	+	3			1
		GLU	-	+	+	+				
		Cu^r	-	+	+	+				
		MT		?	?	?				
	10	LEU	+	-	+	+	2	1	1	
		GLU	+	+	-	+				
		Cu^r	+	-	-	+				
		MT	∝	a		?				

11	LEU	-	-	+	+	1	1	1	1
	GLU	+	-	-	+				
	Cu^r	-	-	-	?				
	MT	a	a	α	?				
12	LEU	+	-	+	-		2	1	1
	GLU	-	-	-	+				
	Cu^r	-	-	-	+				
	MT	α	a	a	α				

The table shows the results of the tetrad analysis of three different GOGAT$^-$ mutants 4-10 (a), AR40 (b) and AR42 (c). Strains 4-10 and AR40 were crossed with strain AR2, and AR42 with AR1. Column 2 indicates the marker which is being tested for (LEU - LEU2; GLU - GOT; Cu^r - CUP1; MT -mating type, a or α). Column 3 shows the phenotypes of each spore (+ growth i.e. presence of wild type allele; - no growth; ? result uncertain as mated with both tester strains). The final column shows the number of spores in an ascus with a given phenotype. 1 - tetrad designated ART10-1; 2 - designated ART10-2.

Table 20.

Cross	No. of spores in ascus with this marker combination				No. of tetrads observed with marker distribution
	LEU GLU	**LEU glu**	**leu GLU**	**leu glu**	
AR2 x 4-10	1	1	1	1	2
	2		2		1
	1		1	2	1
	1	1		2	1
	2			2	3
	4				2
	2	1	1		2
	3			1	1
	1	2	1		1
		2		2	1
	2	1		1	1
	2		1	1	1
AR2 x AR40	1	1	1	1	1
	2			2	1
	4				6
	2	1	1		1
	3			1	3
	1	1	2		1
	2		1	1	1
	3	1			1
AR1 x AR42	1	1	1	1	3
	1	1		2	1
	2	1	1		1
	3			1	1
	1	2	1		3
		2	1	1	2
		2	2		1

Analysis of tetrad data presented in table 19. This shows the different spore combinations and the number of asci with such combinations observed.

Table 21.

Cross	No. of tetrads in the tetrad class:			
	PD	NPD	T	Other
AR2 x 4-10	0	3	2	12
AR2 x AR40	0	1	1	13
AR1 x AR42	1	0	3	8

Analysis of the data presented in Table 20 to show the number of tetrads which were found in each tetrad class. PD parental ditype; NPD non-parental ditype; T tetratype; Other none of other three classes.

Table 22.

Strain	No. of spores with the marker combinations			
	LEU GLU	LEU glu	leu GLU	leu glu
4-1	39	8	18	12
4-2	47	4	27	6
4-3	42	10	27	11
4-5	65	0	25	0
4-6	54	4	15	14
4-8	>500	0	0	0
4-9	45	18	16	12
4-10	>500	0	0	0
4-11	48	4	15	24
4-17	>500	0	0	0
4-20	54	4	19	15
4-21	53	5	14	12
4-22	43	13	23	12
4-23	39	14	22	16
4-25	58	3	18	9

Data for mass spore isolation experiments of the crosses AR2 x mutant. Each colony was scored for the presence of the LEU^+ and GLU^+ markers.

The unexpectedly high number of LEU+ segregants could be due to reversion of the *leu2* marker used in this study. This is very improbable because the marker used does not revert to leucine independence at a detectable frequency. The *leu2-3,-112* allele was constructed by Hinnen and co-workers by introducing two frameshift mutations into *LEU2* and was intended for use in transformation experiments, where low transformation frequencies were expected (cited in Botstein and Davis 1982). Reversion can also be cited to explain the results obtained with the glu- phenotype. The mutation carried by 4-9 reverted with a frequency of $2x10^{-7}$ and *got1-10* at less than $1.3x10^{-8}$ (4324c reverted at a frequency of $1.8x10^{-8}$). These are low enough that reversion of the glu- phenotype should not be a problem. Even if the reversion frequency was high, single colony isolates (SCIs) were used at all stages in the crosses. If a revertent had inadvertently been picked then no leu- and glu- colonies would have been detected.

The excess of LEU+ segregants could be due to the suppression of the *leu2* allele. Although this marker contains two frameshift mutations, frameshift suppressors are known in *S. cerevisiae* and include a class having a wide range of specificity, which are believed to misread several codons (reviewed in Sherman 1982). A frameshift suppressor could have been introduced into the mutant strain during the EMS mutagenesis or from the parent 4324c, which was not detected until the tetrad analysis was done. It is unlikely that the suppressor was introduced by the EMS treatment because the mutants are apparently not sisters. Also two strains have been backcrossed once. It is therefore very improbable that all three mutants would contain the same suppressor, especially when no apparent selection pressure was exerted for its retention. The presence of a wide specificity suppressor can be tested for by crossing the mutants and 4324c to a series of strains carrying different, highly suppressible alleles e.g. *leu2-1* or *leu2-2*, and investigating whether suppression occurred. An additional test for the presence of a suppressor in 4324c is to cross this strain with AR3. A suppressor in 4324c would give a ratio of 3:1 LEU+:leu- segregants while no suppressor would give a ratio of 1:1 +:-. If this cross gives aberrant results, the presence of a suppressor can be further indicated by elimination of the effect of *gdh1-1* by crossing AR3 with Σ1278b. This cross should be done in order to confirm the normal segregation of *leu2*.

The suppressor of *leu2* cannot be carried by AR1, AR2 or AR3 because these strains were selected on the basis of a *leu2* genotype. The presence of a wide specificity suppressor would not explain the high frequency of GLU+ segregants because the original mutants were selected as glu- mutants.

An alternative explanation is that a particular marker combination results in a decrease/loss of spore viability. The data in Table 22 appears to support this. LEU+ GLU+ spores were in excess of other spore types: LEU+ and GLU+ were in excess over leu- and glu-. However, an observation made during the tetrad dissections does not support this. A 100% germination rate was observed with asci from

which four spores were dissected out. This does not eliminate the possibility that a particular marker combination is affecting the ability of a diploid to form four mature spores, although all members of any four spore asci may be viable. It was observed that diploids constructed from these mutants took longer than normal to develop and the sporulation frequency was low.

The phenotypes were scored by replicating master plates, on which the segregants had been patched, onto suitable diagnostic media. It had been observed that the different mutants replicated with various efficiencies *i.e.* for some strains all the patch would be transferred onto the velvet pad, while for others very little material was transferred. Therefore, a trivial explanation of the data in Table 19 is that it was due to the different replicating efficiencies. To eliminate this possibility the segregants were streaked onto the various diagnostic media and growth scored as the appearance of individual colonies. The results from the streak plates (D.M. Wright, unpublished data) and replicaplating experiments agreed very closely. It was concluded that the observed segregation ratios were not an artefact of the technique used to screen the phenotypes.

The excess of LEU+ GLU+ segregants could be an artefact of the analysis technique and result from biochemical/genetical interaction of leu- and glu- phenotypes. The crosses were done by mixing SCIs of each strain on a YC plate and allowing them to mate. Mated cells were streaked onto 20mM NH_4^+. A SCI was taken and streaked onto pre-sporulation medium before being transferred to sporulation medium. The selective step on MM + NH_4^+ selects against the leu- parent and also the glu- parent, which cannot assimilate ammonium due to the GDH- GOGAT- double mutation.

The sporulation medium contained no nitrogen: this provides an additional selective pressure on the diploids. The diploids used in this study have the genotype *got/GOT gdh1/gdh1* and have a reduced capacity to assimilate ammonium. Although Newlon (1979) reported that strains homozygous for *gdh1-6* and *GOT* sporulate normally: other workers have not observed this (I.W. Dawes, pers. comm.). Therefore, in these diploids there may be a strong selective pressure for diploids with an improved ability to assimilate ammonium. NADP-GDH and GOGAT lie at the interface of carbon and nitrogen metabolism, so simultaneous lesions in both genes may be extremely deleterious to a cell in an adverse environment. In such a system only diploids with an amplified level of GOGAT may be able to sporulate.

The excess of GLU+ is possibly a consequence of the postulated elevation of GOGAT levels. This could be achieved by chromosome amplification. The greater than expected frequency of LEU+ GLU+ segregants suggests that the LEU+ and GLU+ phenotypes interact in an unknown manner at either the biochemical or genetical level. It is possible that the excess of LEU+ GLU+ segregants is due to amplification of chromosome III, which carries the *LEU2* gene, coordinately with the amplification of

the chromosome(s) carrying *GOT1* and *GOT2*. Alternatively, *LEU2* and *GOT* could both be on chromosome III. A recombination event could bring them into the pairing phase, with the subsequent selection for the amplification of chromosome III.

Amplification of chromosome III is readily testable as the *MAT* locus is also carried on this chromosome. If the mutant has become disomic for chromosome III, then two of the four spores in the ascus will have two copies of chromosome III and hence the *MAT* locus. Cells with more than one copy of *MAT* are sterile. The hypothesis can be tested by examining the segregation of the *a* and α mating types in these crosses. The segregants were all crossed to the mating type tester strains DS278 and DS280, and diploids selected on MM + NH_4^+ + $CuSO_4$. This selects against the tester strains because they carry a *lys2* marker, and against the segregants because they were believed to have a *cup1* allele. The results are presented in Table 19.

However, it was found that some of the segregants were growing on the Cu^{2+} supplemented plates, although none of the parental strains did, and that this resistance was segregating randomly (Table 19). The results from the mating type tests are unclear because a large number of the segregants tested (38/125) appeared to mate with both tester strains. The presence of Cu^r segregants made scoring of the mating type tests difficult. The data are not sufficient to make any conclusion about the segregation of the mating type locus.

The data show that the genetics of the GOGAT$^-$ mutants isolated in this study are very complex. The data are insufficient for any valid conclusions to be made about the segregation of the GLU$^-$ phenotype. It is not consistent with the mutation causing the GLU$^-$ phenotype either being at one or two loci. From the additional experiment done in this genetic study it has been shown that the problem is not an artefact of the technique used to screen the segregants.

Considerable work is required before these problems *i.e.* excess of LEU$^+$ GLU$^+$ segregants etc., can be resolved. The approach suggested to show that the parental strain does not possess a suppressor of the *leu2* marker and that *leu2* segregates in a normal Mendelian manner has been outlined above. The approach recommended to investigate the segregation of GLU$^-$ would be to cross the original mutants *i.e.* 4-9, 4-10 and 4-25 with a strain carrying only *gdh1-1*. As it is postulated that the selection step on MM + NH_4^+ may select for diploids which produce atypical segregants, selection for diploids should be done in the absence of any selection pressure. This can be done by isolating schmoos from a mating mixture using a dissecting needle, or diploids can be isolated because of their ability to outgrow haploids in complete medium. Then the segregation of the GLU$^-$ phenotype can be tested by tetrad analysis. Normal segregation of chromosome III could be tested by following the segregation of the

mating type. At this stage additional markers should not be examined, so as to avoid potential genetic and/or biochemical interactions, such as those probably observed with *leu2*.

The interaction of GLU- with *leu2 i.e.* whether it is specific for *leu2*, can be examined by analysing tetrads from crosses involving a variety of markers. Non-specific interaction can be examined by using *MAL*, *SUC* or *cdc*: while interaction with amino acid auxotrophies can be tested using, for example, *arg*, *his* or *lys*. Specific interactions can be tested using various *leu2* alleles. These studies should be done with and without selection for diploids. It is not possible to escape the selection exerted at the sporulation stage as the presence of a nitrogen source inhibits sporulation.

It should be noted that the *ama* mutation which results in loss of GOGAT activity (cited in Wiame *et al.* 1985) has been shown to segregate 2:2 *AMA*:*ama* (E. Dubois, pers. comm.). It is not known if *ama* is a regulatory or structural gene mutation.

6.4 The data in Tables 19-21 show that both the GLU+ and LEU+ phenotypes are segregating in an unexpected manner. Additionally, it was known that other workers had observed an unexpected segregation of enzyme activity *i.e.* not 2:2 or 1:3 wild type:mutant activity (J. Mora, pers. comm.). Consequently it was decided to examine the GOGAT activities in the four spores of single tetrads to determine whether the GOGAT activities had altered. As some spores were leu-, leucine was included in the growth medium for all the cultures. It is not known how leucine modulates GOGAT activity, therefore Σ1278b, 4324c and 4-10 were also examined. The results are shown in Table 23.

The data show that GOGAT activity is decreased in Σ1278b grown in MM + NH_4^+ + leucine compared to MM + NH_4^+. Leucine is degraded by transamination to the 2-oxo acid ∝-ketoisocaproate, which is itself degraded to acetyl coenzyme A. Acetyl CoA is an intermediate of the TCA cycle. If acetyl CoA is being fed into the TCA cycle, a build up of organic acid intermediates which are inhibitors of GOGAT (Miller and Stadtman 1972, Mora *et al.* 1987) could occur. This may explain the reduced enzyme activity in Σ1278b. However, GOGAT exhibited an elevated level in 4324c grown in NH_4^+ + leucine compared to NH_4^+ alone. The reason for the opposite response in 4324c is unclear. The enzyme levels measured in 4-10 were comparable in both media.

All eight segregants have the *gdh1-1* allele, therefore the GOGAT activity measured in the GLU+ segregants was expected to be comparable to that in 4324c. This was not observed. Instead the GLU+ segregants (3/5) had an activity comparable to Σ1278b - ART10-1d had higher and ART10-2a lower activity than wild type. Two of the three glu- segregants had higher than mutant activity, only one glu- segregant (ART10-1b) had an activity comparable to 4-10. The reason for this increased activity is unclear.

Table 23.

Strain	Phenotype		GOGAT Activity
	LEU	GLU	(mU/mg)
Σ1278b	+	+	23.7
4324c	+	+	76.7
4-10	+	-	7.1
ART10-1a	+	+	34.3
1b	-	-	8.4
1c	-	+	25.5
1d	+	-	13.0
ART10-2a	-	+	16.5
2b	+	-	12.4
2c	+	+	20.8
2d	+	+	23.9

GOGAT activities in the four spores (a-d)and from single tetrads (ART10-1 and ART10-2) isolated from the cross AR2 x 4-10. Due to the leu^- phenotype, cells were grown overnight in MM + glutamate +leucine before being switched to MM + NH_4^+ + leucine. The data are the means of three independent experiments, duplicate assays were done for each experiment. The values for Σ1278b, 4324c and 4-10 are from single experiments.

The presence of *leu2* in a GLU+ background does not affect GOGAT activity - compare ART10-1c with ART10-2c or 2d.

In tetrad ART10-1, which shows 2:2 segregation of GLU+:glu-, one GLU+ segregant exhibited wild type activity and the other elevated activity. One glu- segregant showed activity comparable to 4-10 and the other elevated activity, although still less than wild type. A similar result has been observed by other workers (J. Mora, pers. comm.). The reason for this result remains unclear. In contrast, ART10-2, which showed 3:1 GLU+:glu- segregation, 2/3 GLU+ segregants exhibited wild type activity. The third GLU+ segregant showed less than wild type activity. The glu- segregant showed elevated activity relative to 4-10. It is unclear why the third GLU+ segregant had lower than wild type GOGAT activity, yet in ART10-1 the second GLU+ spore had elevated GOGAT activity.

The aberrant segregation of enzyme activities is probably related to the unexpected segregation of the GLU+ phenotype. Consequently, before the biochemistry of GOGAT in these tetrads can be understood the genetics of the system will have to be explained.

6.5 The biochemical data has shown that 4-10 and 4-25 carry mutations in the structural genes, while the complementation data indicate that the two mutations are in the two different genes. On the basis of the complementation map and the reversion frequency data, most of the remaining mutants are postulated to carry mutations in regulatory elements. The genetics of the GOGAT- mutants are very complex, which is shown in both the complementation map and linkage data. The reasons for this are unclear, but probably reflect the position of GOGAT at the interface of carbon and nitrogen metabolism plus the postulated regulatory nature of the mutations.

Chapter 7

Studies on the Growth Properties of *S. cerevisiae* GOGAT$^-$ Mutants

7.1 The aim of this project was to determine if a GDH$^+$ GOGAT$^-$ strain or a GDH$^-$ GOGAT$^-$ mutant transformed with *GDH1* on a multi-copy plasmid have improved growth characteristics (better t_d and/or biomass yield) compared to a GDH$^+$ GOGAT$^+$ strain. This was tested by investigating the growth profiles of strains with different combinations of *gdh1-1* and *got1-10* in MM + 20mM NH_4^+. Once a GOGAT$^-$ mutant was obtained it became possible to investigate the contribution GOGAT makes to ammonium assimilation by *S. cerevisiae*. This was done by growing the strains constructed for the previous experiment in medium containing limiting and non-limiting concentrations of ammonium. The data presented below go some way to answering these questions.

7.2 A GDH$^+$ GOGAT$^-$ strain was constructed by crossing AR3 with 4-10. Tetrads were dissected and a LEU$^+$ GLU$^+$ segregant (designated AR44) was taken for further study. NAD- and NADP-GDH plus GOGAT activities were measured in AR44. The data are presented in Tables 12 and 24.

Strain AR44 has a LEU$^+$ GLU$^+$ phenotype (Table 13). The GLU$^+$ phenotype could be due to the presence of either *GDH1* or *GOT1* or both genes. To distinguish between the three possibilities, both enzymes were assayed. The data in Table 12 show that GOGAT activity in AR44 is markedly lower than in Σ1278b and 4324c (17 *cf.* 44 and 54mU/mg respectively) although this is higher than the activity observed in 4-10 (7mU/mg). AR44 would appear to carry the *got1-10* allele. This is confirmed by the data in Table 24, which shows that activities in 4-10 and AR44 are comparable. The data in Table 24 were obtained using a different culture system to that in Table 12 (25ml universals instead of 250ml flasks) which probably caused the differences in measured activity.

NADP-GDH activity in AR44 was 21-fold greater than in 4324c (Table 12), yet was still about 15-fold lower than the level in Σ1278b. This is confirmed by the data in Table 24, although the difference between the activities in Σ1278b and AR44 was less. The reason for the difference in Σ1278b NADP-GDH activity reported in Tables 12 and 24 is uncertain but was probably due to the different culture systems. As *gdh1-1* strains have barely detectable NADP-GDH activity, it would appear that, on the basis of the enzyme activity, AR44 has the genotype *GDH1 got1-10*.

It is unclear why the NADP-GDH level in AR44 is markedly lower than that of Σ1278b. Three possible explanations are outlined below. Firstly, there is a second mutation in 4-10 which regulates NADP-GDH activity. If this was correct then half of the GDH$^+$ segregants would be expected to have wild

Table 24.

Tetrad and spore	Enzyme Activities		Inferred GOGAT Genotype
	NADP-GDH (U/mg)	GOGAT (mU/mg)	
Σ1278b	0.612	19.2	*GOT1*
4-10	0.008	6.1	*got1*
1 A	0.130	10.7	*got1*
B		5.6	*got1*
C		19.2	*GOT1*
D		18.8	*GOT1*
2 A	0.129	18.2	*GOT1*
B	0.086	4.2	*got1*
C[1]	0.119	6.3	*got1*
D	0.241	20.4	*GOT1*
3 A	0.107	7.8	*got1*
B	0.236	25.1	*GOT1*
C	0.192	19.5	*GOT1*
D	0.105	9.5	*got1*
4 A	0.138	19.5	*GOT1*
B	0.175	24.8	*GOT1*
C	0.154	19.5	*GOT1*
D	0.119	7.6	*got1*

NADP-GDH and GOGAT activities plus inferred GOGAT genotype of segregants of the cross AR3 x 4-10. Each segregant was inoculated into 5ml MM + glutamate and grown overnight, with shaking, at 30°C. Washed cells were inoculated into 10ml MM + 20mM NH_4^+ in a 25ml universal, and incubation continued for 6h when the cells were harvested. The enzyme activities are the means two or more experiments, with duplicate assays done for each experiment. 1 - spore 2C was redesignated AR44.

type NADP-GDH activity, but this was not observed. It would appear that this explanation is not correct. Secondly, the result is related to the aberrant segregation of the leu- and glu- phenotypes. No leu- segregants were recovered from the cross (seven tetrads were examined), and all were GLU+. GOGAT and NADP-GDH were measured in four tetrads: the results are shown in Table 24. All the segregants had comparable NADP-GDH activities yet the GOGAT activity appeared to segregate 2:2 wild type:mutant. The reasons for this remain unclear. The genetics of the system will probably have to be better understood before the biochemistry can be explained. Thirdly, it is a genuine result *i.e. gol1-10* represses NADP-GDH activity. This is unlikely because NADP-GDH in GDH+ GOGAT- strains of *Sal. typhimurium* (Dendinger *et al.* 1982) and *N. crassa* (Lomnitz *et al.* 1987) have wild type NADP-GDH activities.

There was insufficient time available to repeat the strain construction and so AR44 had to be used. Strain AR44 was chosen because it had the highest NADP-GDH activity of a *gol1-10* strain, and came from a tetrad showing 2:2 segregation of wild type:mutant GOGAT activity.

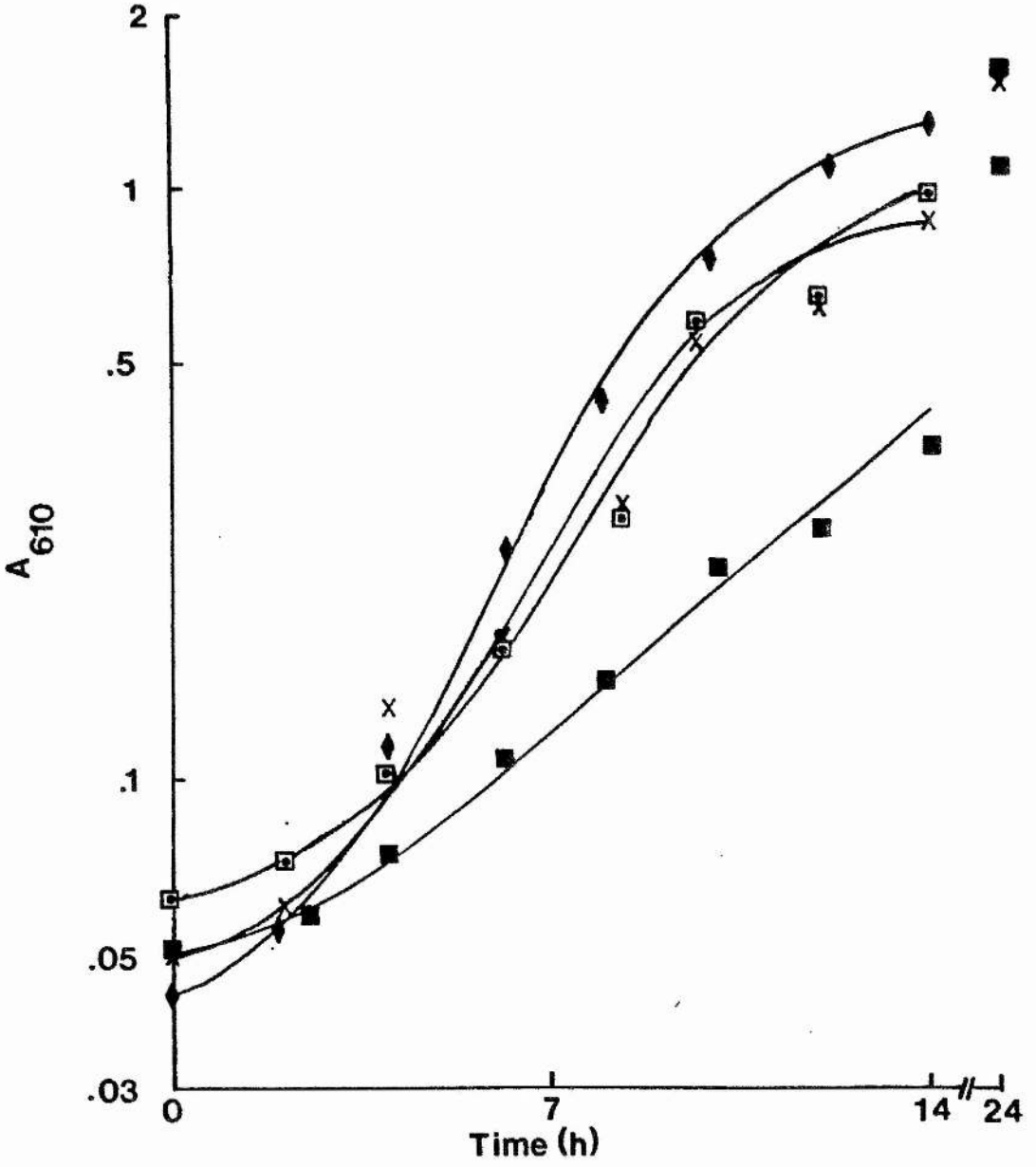
The NAD-GDH activity in AR44 is intermediate to the levels observed in 4324c plus 4-10 and Σ1278b. It is unclear why or how NAD-GDH is modulated by *gol1-10*. The decrease in NAD-GDH probably results from an increase in NADP-GDH activity (Σ1278b *cf.* 4324c) and this is epistatic to the effect of *gol1-10*.

The strains carrying multiple copies of *GDH1* were constructed by transforming AR2 or AR5 with the plasmid pCYG4. AR5 is a derivative of the mutant 4-9. When this transformant was constructed, it was not known which mutants carried mutations in the structural genes. 4-9 was chosen because it had very low GOGAT activity, so any improvement in the growth characteristics would be due to the lack of GOGAT activity. This assumption is still valid, even though it is now postulated that 4-9 is a regulatory mutant.

7.3.1 The effect of *gol1-10* on the growth of a GDH+ strain was studied in liquid culture. The various constructs were grown overnight with shaking at 30°C in MM + glutamate to stationary phase. Washed cells were inoculated into MM + 20mM NH_4^+ to an initial A_{610} 0.05 and incubation continued. Growth of the culture was monitored by following the change in absorbance at 610nm over a 24h period. The results are presented in Figs. 25 and 26. Culture doubling times were obtained from the curves and are shown in Table 25.

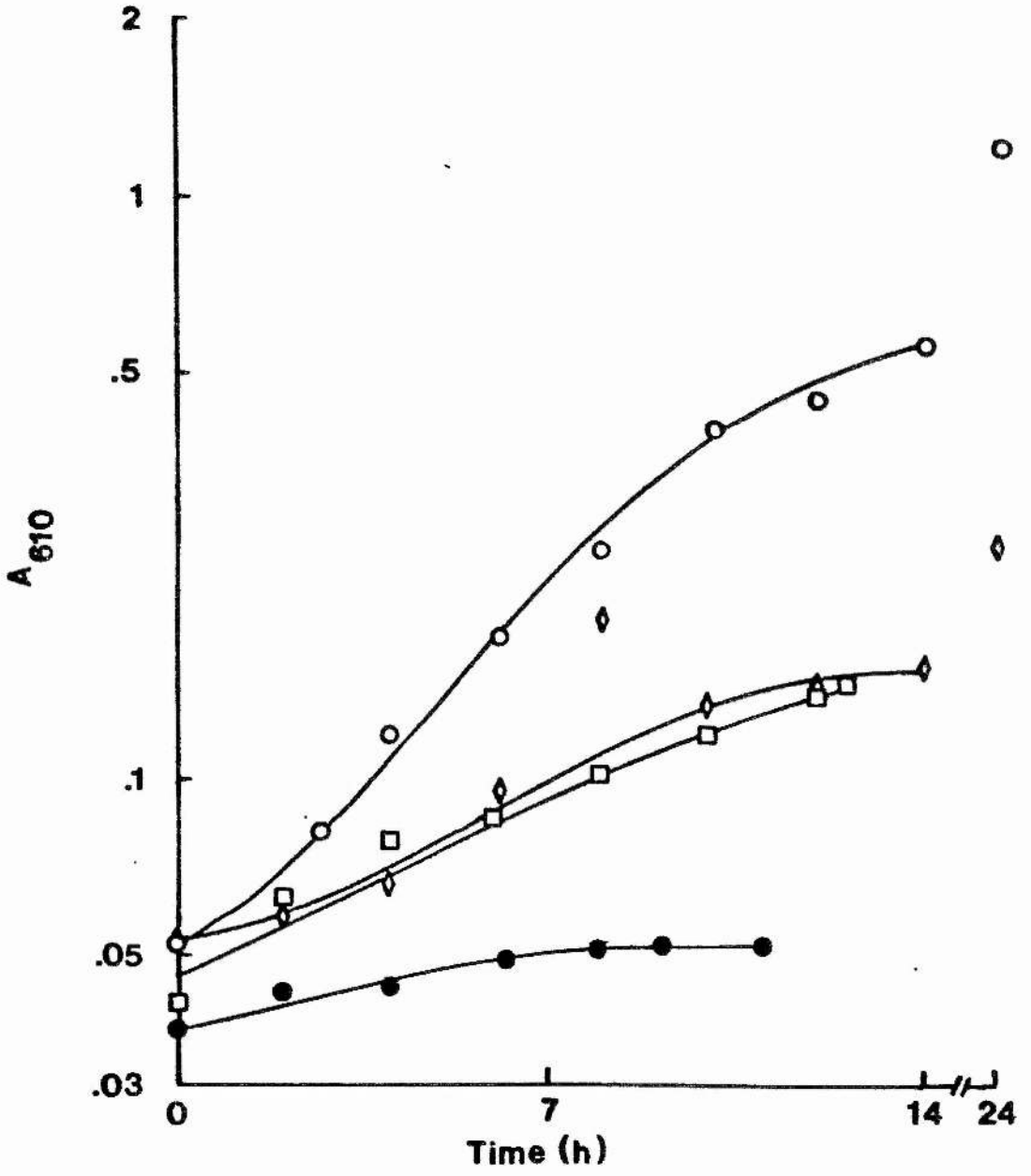
The data show that Σ1278b had the best growth characteristics *i.e.* lowest t_d and highest culture density (final A_{610} value) of the eight strains tested. The GDH- GOGAT+ parent 4324c grew much more slowly than the wild type and had a lower final culture density. At 14h, the A_{610} of the Σ1278b culture was about twice that of the 4324c one, although the difference was less at 24h, it was still marked. The

Fig. 25



Growth curves for wild type and GOGAT$^-$ constructs. Stationary-phase glutamate grown cells were inoculated into MM + 20mM NH_4^+ (100ml in 250ml non-baffled flasks, 30°C, shaking water-bath: 80rpm, 4cm stroke-length) to an initial $A_{610} \sim 0.05$. Growth was measured by following the change in A_{610}. Representative curves are shown from two independent experiments. (Σ1278b ♦; AR2(pCYG4) ⊡; AR5(pCYG4) X; AR44 ■)

Fig. 26



Growth curves for 4324c and different GOGAT$^-$ mutants in MM + 20mM NH_4^+ (other growth conditions as in Fig. 25). Two independent experiments were done and representative curves are shown. (4324c ○; 4-9 ●; ART10-1d ◊; 4-25 □)

Table 25.

Strain	Growth Conditions			
Temp. (°C)	19		30	
NH_4^+ (mM)	20	0.2	2.0	20.0
Σ1278b	239	181	110	94
4324c	450	157	212	173
3-25				165
3-26				204
3-34				196
4-9	-1			-
ART10-1d		275	330	373
4-25	738			416
AR44		275	212	224
AR2 (pCYG4)				118
AR5 (pCYG4)				118

Table 24 shows the t_d (minutes) of different strains used in this study grown under various culture conditions. The values were obtained from Figs. 19, 25-29. 1 - did not complete one cell cycle.

elevated GOGAT activity in 4324c was responsible for its ability to grow on MM + NH_4^+. Therefore, the loss of GOGAT should result in the inability to use ammonium as sole nitrogen source. As expected, the three GDH$^-$ GOGAT$^-$ strains tested showed weak growth. There appeared to be no strong correlation between the residual GOGAT activity and growth rate. Strain 4-9 (1mU/mg) did not complete one cell cycle *i.e.* the culture absorbance did not double, yet ART10-1d (6.7mU/mg) and 4-25 (25.1mU/mg) could grow in MM + NH_4^+. The growth (both t_d and final A_{610}) of these two strains was comparable. At 14 and 24h, the A_{610} values were about 25% of that for 4324c.

The data presented in Table 13 plus Fig. 16 and in Fig. 25 for the GOGAT$^-$ strains are inconsistent. This is probably a consequence of the different test systems used. Measurement of the change in culture absorbance would detect a limited number of culture doublings, but the same number of cell divisions would not generate a single colony which is visible to the naked eye.

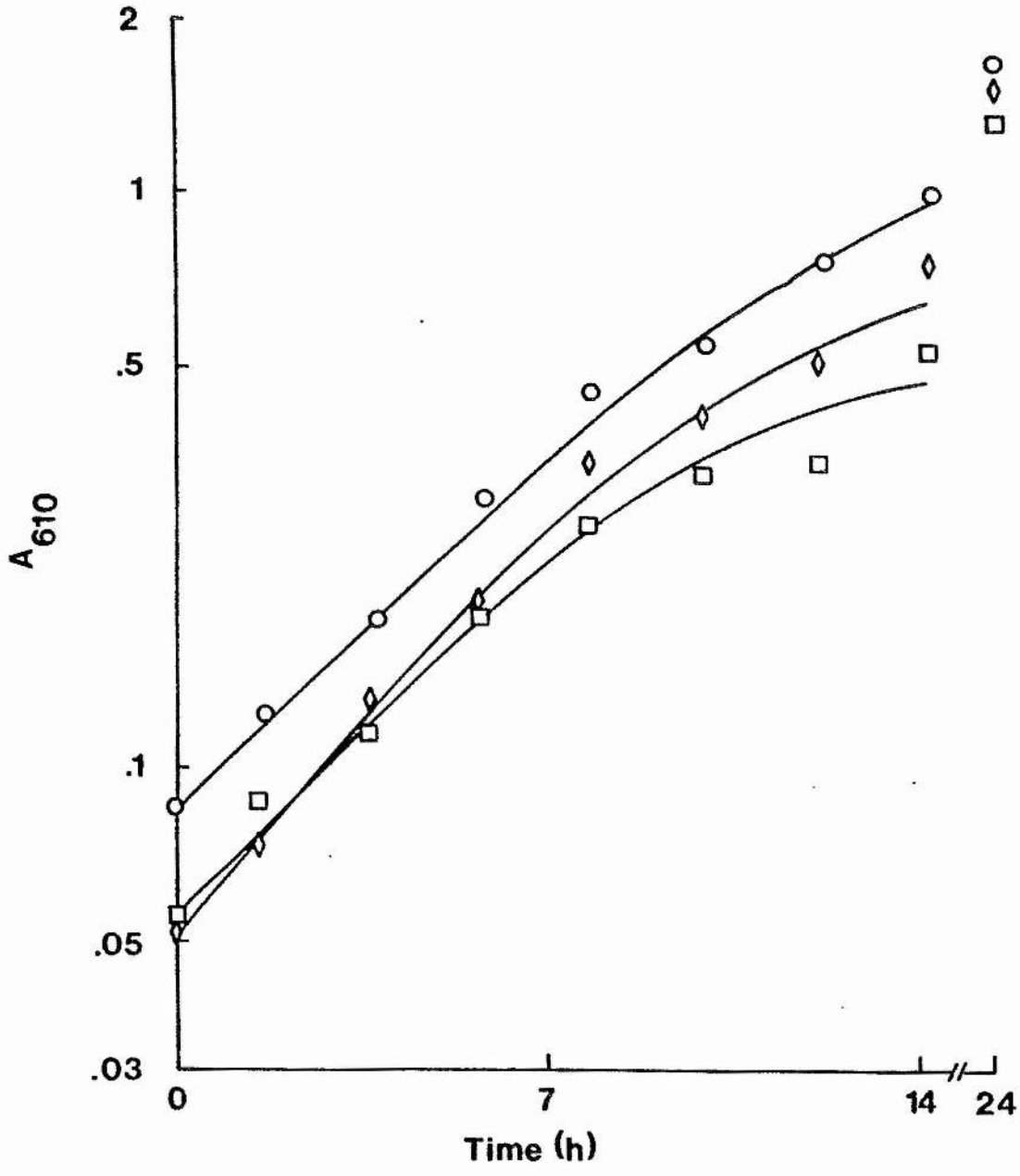
The GDH$^+$ GOGAT$^-$ strain AR44 grew in this test system, although its growth was weaker than for 4324c. After 14h incubation 4324c was in the retardation phase of growth, while AR44 was still in the exponential phase although the A_{610} values were comparable. The final A_{610} measured in this culture was lower than that for 4324c.

The effects of multiple copies of *GDH1* on a GOGAT$^+$ (AR2) and GOGAT$^-$ (AR5) strain were investigated (Fig. 25). The parent of AR5 was 4-9, which does not grow in MM + NH_4^+. So, although the parent strain was postulated to be mutant in a regulatory gene which caused an almost complete loss of activity, the GOGAT$^-$ phenotype could be repaired by *GDH1* (the growth of AR5(pCYG4) *cf.* 4-9). Although the two transformants AR2(pCYG4) and AR5(pCYG4) grew more slowly than the wild type (t_ds of 118 *cf.* 94 minutes), the final A_{610} values were almost identical. The shape of the growth curves for both transformants were very similar, both to each other and to the wild type.

7.3.2 The growth of strains 3-25, 3-26 and 3-34 is shown in Fig 27, and the t_ds in Table 25. The growth curves were similar to the parental strain 4324c. The t_d of the 3-25 culture was comparable to that of 4324c. The putative GOGAT$^-$ mutants 3-26 and 3-34 grew more slowly than 4324c, but about twice as fast as ART10-1d or 4-25.

Comparison of the data in Tables 12 and 25 confirms the initial hypothesis that mutants obtained by mutagenesis with DEO did not carry mutations in the GOGAT structural genes. 3-26 had the highest GOGAT activity of the three strains examined and reported, but had the lowest growth rate. The data in Table 13 show that the three mutants grew on MM + 20mM NH_4^+ but only very poorly on ammonium at limiting concentrations. Comparison of these three bits of data with data from 4-10 and 4-25 suggests that the loss of GOGAT activity was probably due to a non-specific mutation in 3-25, 3-26 and 3-34, and not to a lesion in the GOGAT structural gene.

Fig. 27



Growth curves for the three putative GOGAT$^-$ mutants generated by DEO mutagenesis, in MM + 20mM NH_4^+ (other growth conditions as in Fig. 25). (3-25 □; 3-26 ◊; 3-34 ○)

7.4 The effect of limiting ammonium on the growth of Σ1278b, 4324c, ART10-1d and AR44 was investigated using the experimental system described in the previous section, except that ammonium was used at 0.2 or 2mM. The results are shown in Figs. 28 and 29, the t_d values for each strain were obtained from these curves and are shown in Table 25.

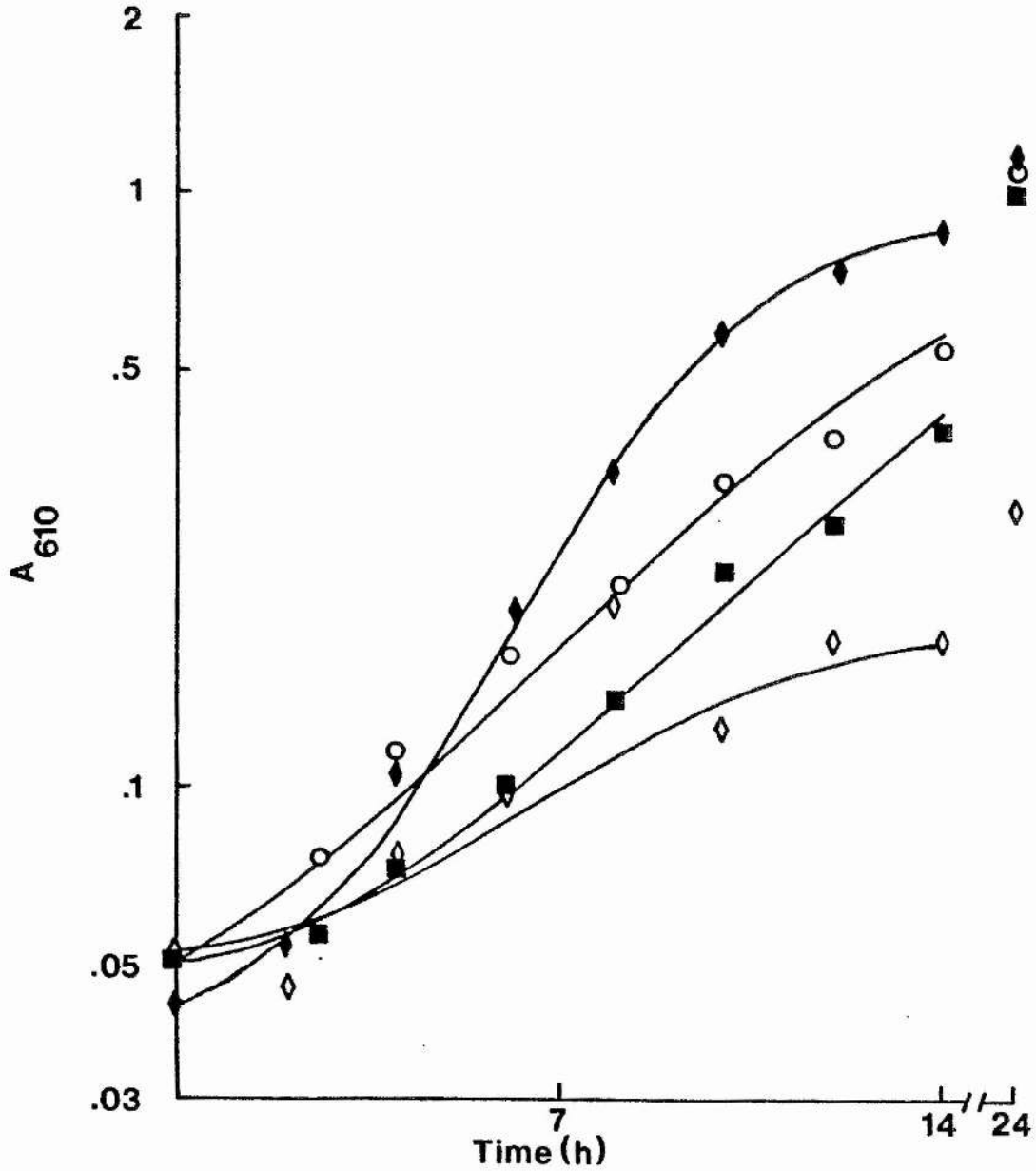
As was expected 20mM NH_4^+ was a better nitrogen source than 0.2 or 2mM NH_4^+, and gave the highest final A_{610} values. Ammonium at 2mM was better than 0.2mM. The difference in final A_{610} values was greater for each strain between 0.2 and 2mM than 2 and 20mM NH_4^+. There was a marked decrease in A_{610} of the Σ1278b culture grown at 2mM compared to 20mM NH_4^+ which was not observed with the other three strains. At 2mM NH_4^+, the A_{610} readings at 24h for Σ1278b, 4324c and AR44 were very similar. This was not observed with 20mM NH_4^+. The absorbance of ART10-1d grown with 2mM NH_4^+ was three-fourfold lower than the values for the other cultures compared to five-eightfold for the 20mM NH_4^+ grown cultures. This was due to the decrease in final A_{610} values for the other strains plus an increase in the value for ART10-1d. In 0.2mM NH_4^+ all strains grew poorly. Strain 4324c was able to grow faster and reached a higher final culture absorbance than the wild type. The culture absorbance values at 24h for ART10-1d and AR44 were almost identical. As the ammonium concentration was decreased from 20 to 0.2mM, the difference in final culture absorbance values became less.

Examination of the t_d values for each condition shows that, while the final A_{610} value for Σ1278b grown on 20 and 2mM NH_4^+ decreased markedly, the change in t_d was not as large (94 *cf.* 110 minutes). When Σ1278b was grown in 0.2mM NH_4^+ the t_d increased to 181 minutes. 4324c showed an increase in t_d (173 to 212 minutes) as the ammonium concentration decreased from 20 to 2mM. However, when the ammonium level was decreased to 0.2mM there was a marked decrease in t_d to 157 minutes. Strain ART10-1d grew faster as the ammonium concentration decreased. The t_d went from 373 to 275 minutes. In contrast, AR44 grew slightly faster in 2mM NH_4^+ than at 20mM. At this concentration, the growth rate of this strain was identical to that of 4324c. The growth rate with 0.2mM NH_4^+ was markedly slower than in 2mM NH_4^+, and was the same as that of ART10-1d.

7.5 The data in Figs. 25 and 26 show that, of the four different *gdh1-1* and *got1-10* constructs tested in MM + 20mM NH_4^+, Σ1278b had the best growth characteristics. As expected, the double mutant grew weakly. The GDH$^-$ GOGAT$^+$ strain had a growth rate only about half that of the wild type and reached a markedly lower final culture absorbance.

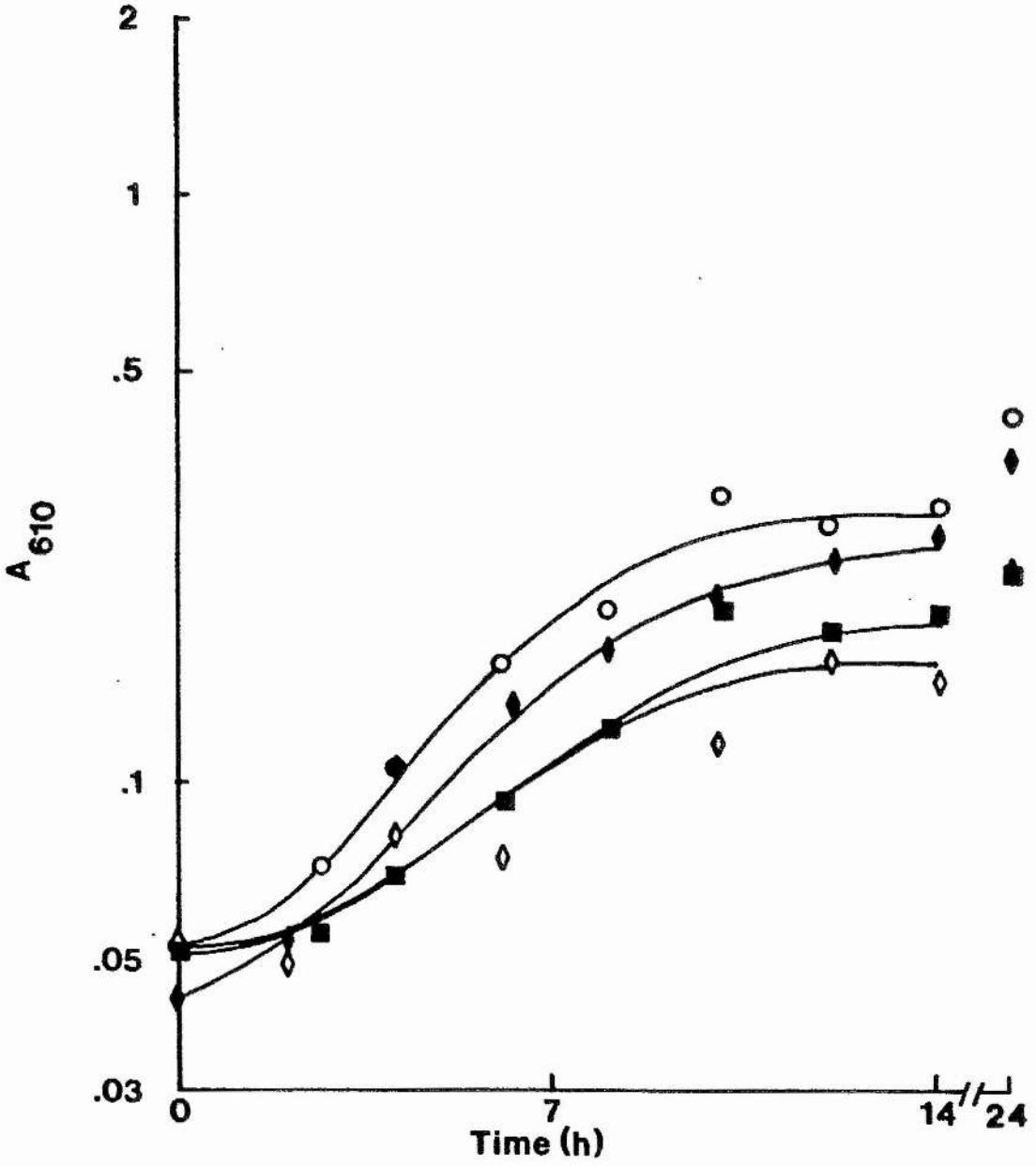
The growth characteristics of the GDH$^+$ GOGAT$^-$ construct AR44 were slightly worse than those of 4324c. This was an unexpected result because NADP-GDH is believed to be the primary route of ammonium assimilation in *S. cerevisiae* and AR44 has NADP-GDH activity. However, the enzyme

Fig. 28



Growth curves for Σ1278b (♦), 4324c (○), ART10-1d (◊) and AR44 (■) in MM + 2mM NH_4^+ (other growth parameters as in Fig. 25). Representative curves are shown from two independent experiments.

Fig. 29



Growth curves for Σ1278b (◊), 4324c (○), ART1D-1d (◊) and AR44 (■) in MM + 0.2mM NH_4^+ (other growth conditions as in Fig. 25). Representative curves are shown from two independent experiments.

level in this strain was about 15-fold lower than in Σ1278b. The reason for this is unclear (see section 7.2). Therefore the reduced growth rate was presumably due to the lower enzyme activity. In 4324c, ammonium is assimilated by GS-GOGAT, but in AR44 ammonium can be assimilated by both GS and NADP-GDH. Although GS activity was not measured in AR44, NADP-GDH activity was higher than the GS activity in 4324c. Ammonium was at a saturating level for both enzymes (see section 4.3) and, assuming that both glutamate and 2-oxoglutarate were at comparable levels in the two strains, then ammonium should be assimilated faster by AR44 than by 4324c. Assuming that ammonium assimilation is the limiting step of biomass biosynthesis in both strains, then AR44 should grow faster. Since both strains carry lesions in the two pathways of ammonium assimilation, this assumption is valid. It is unclear why faster growth of AR44 was not observed.

The GS-GOGAT pathway consumes four NADPH equivalents to synthesise one glutamate molecule from one ammonium ion, while NADP-GDH uses only one NADPH. It has been argued from the data presented in this study that 4324c uses the GS-GOGAT pathway to assimilate ammonium, while NADP-GDH is a major route in AR44. Therefore it would be expected that in AR44 there was more NADPH available for biomass biosynthesis, and consequently a higher final culture absorbance, comparable to Σ1278b, was expected for AR44. The result observed was that the final absorbance readings of AR44 and 4324c were very similar. The reasons for this are unclear. It is unlikely that the culture was still growing, and had not reached the final cell density, because examination of the points on the growth curve indicates that AR44 was out of the exponential growth phase at 24h.

In summary, this experiment shows that a GDH+ GOGAT- construct, which has reduced NADP-GDH activity, does not grow faster than the wild type nor reach a higher final culture density when grown in MM + 20mM NH_4^+.

The data in Fig. 25 show that the growth characteristics of GOGAT+ and GOGAT- strains, both transformed with *GDH1* on a multi-copy plasmid, were very similar. The inactivation of GOGAT in a GDH+ background does not have any marked effect on the growth properties of the strains in this test system *i.e.* the growth rates of both transformants were identical and the final culture density was very similar. This supports the assumption that, in *S. cerevisiae* grown under conditions of ammonium excess, the primary route of ammonium assimilation is by NADP-GDH.

By extrapolation from this system, where *GDH1* was present at multiple copies, to one with only a single chromosomal copy, it can be predicted that no difference would be observed in the growth rate of the wild type and GDH+ GOGAT- construct. This is because the only difference between the two transformants was that one was GOGAT+ and the other GOGAT-. In this test system, the final culture densities of the two transformants were very similar. So it can be predicted that any difference in final

A_{610} observed in the hypothetical test system would also be negligible. The first prediction is supported by a report in the literature. Wiame and co-workers constructed a GDH+ GOGAT- strain and showed that the generation time of this construct in MM + 20mM NH_4^+ was the same as the wild type Σ1278b (unpublished data cited in Wiame *et al.* 1985). These workers did not report the effect on final biomass concentration or the NADP-GDH levels in this construct.

As has been argued in Chapter 5, the greatest similarity between the two industrial strains and the laboratory one is in 20mM NH_4^+ shake-flask culture. Therefore comparisons between the two types of yeast would be most valid under these conditions. As has been stated above, the replacement of GOGAT+ by GOGAT- has no deleterious effects on the cell. Therefore it could be extrapolated that if DCL1 or DCL2 were to be made GOGAT-, it would have no adverse affect on the growth rate, or final culture density. However, this would only be true for 20mM NH_4^+ shake-flask culture. At other ammonium concentrations, or in simulated industrial propagations, the validity of the extrapolations is uncertain. The growth characteristics of the different constructs under these conditions would have to be investigated before a conclusion could be made.

Both transformants grew more slowly than the wild type, but this was due to the stress put on the cell by the presence of multiple plasmid copies in the cell. Studies in *E. coli* (e.g. Padan *et al.* 1983, Remaut *et al.* 1983, Brosius 1984, or Caulcott *et al.* 1985) have shown that high expression of either heterologous or homologous proteins causes a reduction in growth rate, and even cell death. The reasons for the decrease in growth rate are not understood.

Brosius (1984) showed that it was the accumulation of protein and not hybrid mRNAs which was responsible for this decrease. The work of Padan *et al.* (1983) using the *E. coli* lactose permease, and of other workers, indicates that proteins which accumulate in the membrane are particularly toxic.

Presumably competition between chromosomal and plasmid-directed metabolism, either for components of the DNA, RNA or protein synthetic apparatus, or at the level of energy or intermediary metabolism is involved. Miller and Brenchley (1984) investigated the effect of various growth supplements on the growth of *Sal. typhimurium* transformed with *GDHA* on a multicopy plasmid. It was observed that addition of lysine and methionine decreased the t_d. They postulated that elevated NADP-GDH levels depleted the 2-oxoglutarate pool and limited synthesis of succinyl-CoA for lysine and methionine biosynthesis. These, or similar, observations probably explain the decreased growth rate of the transformants compared to the wild type.

The transformants AR2(pCYG4) and AR5(pCYG4) showed that a GDH+ GOGAT- strain had wild type growth in MM + 20mM NH_4^+. However, this was not observed with AR44 and was postulated to be due to the reduced NADP-GDH activity. The difference in the result using AR44 and the data for the

two transformants, plus unpublished data of Wiame and co-workers, suggests that AR44 is atypical of *S. cerevisiae* GDH+ GOGAT- constructs due to its reduced NADP-GDH activity - Wiame and colleagues did not report the NADP-GDH activity in their construct. Therefore the interpretation, and extrapolation, from data obtained using AR44 should be done with caution. However, providing the results are interpreted for a GOGAT- strain with reduced NADP-GDH activity, interpretations will be valid. The work should be repeated using a construct with wild type NADP-GDH activity.

The accepted model for ammonium assimilation by *S. cerevisiae* is that NADP-GDH is the primary route at both limiting and non-limiting concentrations. This model is based on NADP-GDH activity being markedly higher than GS-GOGAT activity and the main product of ammonium assimilation being glutamate (see sections 1.5 and 4.4.4). It is postulated that GS-GOGAT is not involved in ammonium assimilation to a significant degree because of the low levels of enzyme, even in cells grown with limiting ammonium (section 1.5.3). Therefore it can be predicted that Σ1278b should have exhibited the lowest t_d on all three ammonium levels tested in this study. Additionally, AR44 should have grown at a rate comparable to the wild type, or at least 4324c, with ammonium at a non-limiting concentration but more slowly at a limiting one (due to the reduced NADP-GDH activity). ART10-1d should have exhibited very weak growth at all concentrations, while 4324c should have grown more slowly than Σ1278b.

This was not observed. With ammonium at either 2 or 20mM, Σ1278b had the lowest t_d, but at 0.2mM 4324c had the lowest t_d. As has been discussed previously, AR44 did not grow faster than 4324c in MM + 20mM NH_4^+, presumably due to the reduced NADP-GDH activity. However the GDH+ GOGAT- transformant AR5(pCYG4) grew faster than 4324c in MM + 20mM NH_4^+ (118 *cf.* 173 minutes) despite its plasmid burden. The t_ds of AR44 and 4324c in 2mM NH_4^+ were identical, while 4324c grew markedly faster than AR44 in 0.2mM NH_4^+. AR44 grew faster than ART10-1d in 2 and 20mM NH_4^+ but the t_ds were identical in 0.2mM NH_4^+.

4324c has an elevated level of GS-GOGAT activity compared to the wild type when grown with non-limiting ammonium (Table 12). This is also probably true in cells grown with limiting ammonium, although there is no data to support this statement. The *gdh1-1* allele is a mutation in the NADP-GDH structural gene (Grenson *et al.* 1974), so NADP-GDH activity will not be detected in 4324c irrespective of the growth conditions tested. In contrast, Σ1278b has high levels of this enzyme even when grown with limiting ammonium (Table 10). Therefore, the growth of 4324c on MM + 0.2mM NH_4^+ can only be due to GS-GOGAT activity - *gdh1 got* strains cannot use ammonium as sole nitrogen source. The faster growth of 4324c at limiting ammonium concentrations, compared to Σ1278b, is due to the (presumed) elevated GS-GOGAT activities. This would suggest that NADP-GDH functions to

assimilate ammonium at 2 and 20mM (≤1278b grew faster than 4324c) but at 0.2mM, GS-GOGAT is the main assimilatory route (4324c grew faster than ≤1278b).

If this hypothesis is correct then AR44 (GDH+ GOGAT-) should have exhibited similar growth properties to ART10-1d grown with 0.2mM NH_4^+ because neither strain has a functional GS-GOGAT pathway. This was observed (Fig. 29). With 2mM NH_4^+ as the nitrogen source, the t_ds of AR44 and 4324c were identical. This suggests that the reduced level of NADP-GDH activity in AR44 was able to assimilate 0.2mM NH_4^+ ammonium at the same rate as the GS-GOGAT activity in 4324c.

From this data it would appear that, in the strains used in this study, NADP-GDH is the major pathway for ammonium assimilation at concentrations greater than 2mM, but at 0.2mM, GS-GOGAT is a major, if not primary, route. This result is at variance with the data of Wiame and co-workers who observed that a GDH+ GOGAT- strain does not exhibit a decreased growth rate relative to the wild type when grown with limiting ammonium. It is not clear why this should be, but may result from the use of different nitrogen sources. Wiame and colleagues used cytosine to generate limiting levels of ammonium whereas 0.2mM NH_4^+ was used in this study. Further studies using a GDH+ GOGAT- construct which has wild type NADP-GDH activity are needed before this conclusion can be confirmed.

In this study, growth has been measured by following the change in absorbance at 610nm. The absorbance reading is a function of cell density plus the cell shape and size, as all these factors affect light scatter. Therefore, in the studies to examine whether a *got1-10* allele improves the biomass yield of a culture, dry weight or protein concentration would be better measures because they eliminate changes due to cell shape and size. The changes in cell size and shape may explain some of the increase in absorbance observed between 14 and 24h.

Comparison of the t_d data for Σ1278b presented in Tables 9 and 25 show a marked difference in the doubling time at a given ammonium concentration. The data in Table 9 were obtained using 300ml MM in 1l non-baffled flasks inoculated to an initial culture density of A_{610} 0.5. The t_ds reported in Table 25 were obtained using 100ml medium in 250ml non-baffled flasks inoculated to A_{610} 0.05. It is unclear why, when grown with the latter growth conditions, Σ1278b should have grown faster at the same ammonium concentration. The variations probably arose because of differences in the size of the inocula. It can be argued that, with large inocula, the limiting nutrient is rapidly depleted so that cells are only in the exponential growth phase for a limited period which is difficult to detect. Therefore, the values presented in Table 9 are an underestimate of the exponential growth rate as most of the observed growth was in either the lag or retardation phases. The differences in growth parameters mean that data cannot be compared directly between the two tables, but comparisons within a table are valid.

Chapter 8

Conclusion and Further Work

Initially it was decided to clone the *S. cerevisiae GDH1* gene so that the effects of multiple copies of *GDH1* on the growth of a GOGAT- mutant could be investigated. Although clones cross-hybridising with the *N. crassa am* gene were isolated, none of the recombinant plasmids complemented a *gdh1* mutant. This work was halted when it was learnt that other workers had cloned the *S. cerevisiae GDH1* gene (Moye *et al.* 1985, Nagasu and Hall 1985).

GOGAT- mutants were isolated from a *gdh1* strain on the basis of the tight glutamate auxotrophy of the double mutant. Investigation of GOGAT from the two mutants showed that the mutant enzymes had altered physiochemical properties compared to wild type enzyme. These differences are most readily explained by postulating changes in the region of the polypeptide(s) which form the active centre of the enzyme. On the basis of these changes, it was concluded that these mutants probably carry mutations in the GOGAT structural genes. This can only be proven unequivocally by comparison of the nucleotide sequences of wild type and mutant genes, or the amino acid sequences of wild type and mutant enzyme. The complementation data suggest that the two structural gene mutants are mutant in both of the genes encoding the two polypeptides forming the *S. cerevisiae* GOGAT. From the data reported in this study it cannot be determined which polypeptide is encoded by *GOT1* and which by *GOT2*.

The simplest approach to resolving these questions is to use the mutants 4-10 and 4-25 to isolate complementing sequences from a wild type gene library. Sequence data from these fragments can be used to predict the M_r of the gene product specified by the fragment. Comparison with the known M_rs of the polypeptides forming the *S. cerevisiae* GOGAT will indicate which gene encodes which polypeptide.

The genetics of the GOGAT- mutants were found to be complex. The two structural gene mutants were found to be in different complementation groups, but the remaining mutants were found to be in at least five other complementation groups. On the basis of the complementation and reversion frequency data, it was postulated that at least some of the remaining GOGAT- mutants were regulatory mutants. One approach to test this hypothesis is to investigate the effect of gene dosage on GOGAT activity.

It has been observed that enzyme activity is proportional to the number of wild type genes (Nelson and Douglas 1963, references cited in Klar and Halvorson 1976). In contrast, regulatory loci do not show this gene dosage effect (Klar and Halvorson 1976 and references cited therein, Mitchell and Magasanik 1984a). Klar and Halvorson constructed a series of strains with different ploidy (1N-4N) and showed that the *GAL4* gene dosage effect on epimerase activity was only observed in the tetraploid. In contrast Nelson and Douglas observed a definite gene dosage effect for the *GAL1* locus, the structural gene for

galactokinase. Mitchell and Magasanik demonstrated that *GLN3* is a regulatory gene modulating GS activity by showing that GS activity was the same in strains homozygous and heterozygous for *GLN3*.

If this generalisation is accepted as being correct *i.e.* only structural genes show the gene dosage effect, then the structural or regulatory nature of the remaining mutations can be investigated by constructing a series of diploids carrying zero, one or two copies of each mutant allele. It would be expected that if the mutation was in a structural gene, a reduction in the gene product level *in vivo* would lead to a loss of enzyme activity. Examination of GOGAT activity in each diploid should indicate the nature of the mutation.

To exclude the possibility that the loss of GOGAT activity is the consequence of a non-specific mutation resulting in a reduced 2-oxoglutarate pool, aconitase, citrate synthase and isocitrate dehydrogenase should also be assayed in these mutants.

Tetrad analysis showed that both the leu⁻ and glu⁻ phenotypes had aberrant segregation patterns. Therefore, it was not possible to conclude whether the loss of GOGAT activity was the result of a single or double mutation. A number of possible explanations have been suggested to explain these results. For example, selection for spores with increased GOGAT or NADP-GDH activities during sporogenesis may cause chromosome amplification. It is futile to speculate further about the causes of the aberrant segregation patterns reported in Table 19 until the segregation of both leu⁻ and glu⁻ phenotypes in the absence of flanking markers has been investigated. A possible approach to resolving this problem has been suggested in section 6.3.

The data reported in Chapter 4 show that the industrial strains DCL1 and DCL2 exhibited considerable similarity with the laboratory strain Σ1278b. However, it is unclear whether the variations observed are specific to industrial strains because examination of the literature reveals heterogeneity in the response of different strains to similar growth conditions.

One of the major differences observed was that the growth rate of DCL2 did not decrease as the external ammonium concentrations were decreased. On the basis of this result it was postulated that DCL2 has a high affinity, high capacity ammonium permease not present in the other two strains. This can be tested by exploiting the fact that methylammonium is also a substrate of the ammonium permeases. Dubois and Grenson (1979) analysed methylammonium uptake using Lineweaver-Burk plots, and demonstrated the presence of two uptake systems in Σ1278b. A similar approach can be used to investigate the ammonium uptake systems of DCL2. This presupposes that the same result is obtained when DCL2 is grown using the same parameters as used in Chapter 7. The reasons for this are outlined in section 7.5.

The second major difference was observed when cultures grown with limiting ammonium were examined. The results are presented in Table 10. Marked variation was observed in the regulation of GS, GOGAT and NADP-GDH activities. On the basis of increased GS-GOGAT activity and reduced NADP-GDH in DCL1 grown with limiting ammonium plus the greater affinity for ammonium of GS compared to NADP-GDH, it was proposed that this strain uses the GS-GOGAT as the primary or major route of assimilation at limiting concentrations. The growth characteristics of strains carrying different combinations of the alleles *gdh1-1* and *got1-10* suggested that, possibly, Σ1278b also uses this route when grown at limiting ammonium concentrations.

These are controversial conclusions because the accepted view is that NADP-GDH is the primary route for ammonium assimilation in *S. cerevisiae* (e.g. Bogonez *et al.* 1985). Additionally there are questions about the nature of AR44 *i.e.* whether all *S. cerevisiae* GDH+ GOGAT- constructs exhibit reduced NADP-GDH activities. AR44 exhibited the same growth characteristics as a GDH- GOGAT- strain when grown with 0.2mM NH_4^+, and a GDH- GOGAT+ strain grew better than the wild type. This suggests that, if GS-GOGAT is not the primary route of ammonium assimilation by *S. cerevisiae* Σ1278b when grown at limiting concentrations, it makes a major contribution. However, before a definite conclusion can be made about Σ1278b, the experiment will have to be repeated using a GDH+ GOGAT- mutant having wild type NADP-GDH activity. Even if it is shown that GS-GOGAT does not make a significant contribution to the assimilation of limiting ammonium in Σ1278b, DCL1 still requires further investigation.

A possible explanation of the different enzyme levels reported in Table 10 is that each enzyme activity reached a maximum level at a different point in the culture depending upon the ammonium concentration used. Although it may be that similar maximal activities were obtained by a given enzyme at each ammonium concentration, different activities were measured at 6h because enzyme activity had been lost to different extents at this point. To exclude this possibility, the experiments reported in Figs. 10-15 should be repeated using ammonium at 0.2 and 2mM.

As the "Growth Cycle" is the consequence of the interaction of an organism (grown in a closed system) with its environment, it follows that the changes in enzyme activity are ultimately due to environmental changes. But, because a closed system was used in this study, it was not possible to precisely relate the observed enzyme activities with a given ammonium concentration, as the environment was continually changing. This point must be added as a *caveat* to any interpretation of the data in Table 10. However, because the initial ammonium concentration was at a limiting concentration, the maximum enzyme activities are limited by this parameter. Thus the interpretation that DCL1 assimilates limiting ammonium by the GS-GOGAT pathway is probably correct. The best system in which to study the

contribution of each assimilatory pathway is one where precise control of the culture conditions is possible *i.e.* by use of a chemostat.

Although chemostat studies will allow determination of enzyme activities and metabolite pools in ammonium-limited and non-limited cultures, by itself such data can only provide a theoretical answer on the importance of each pathway. The question can only be answered unambiguously by determination of the flux through each pathway for each growth condition. This can be done using tracer studies. The culture can be spiked with $^{15}NH_4^+$, and the distribution of label, with time, between the glutamate and glutamine pools determined. If GS-GOGAT is the main assimilatory route, then the label would accumulate in the glutamine pool before appearing in the glutamate one. In contrast, if NADP-GDH is the primary route then the label would accumulate in the glutamate pool with little, or no, accumulation in the glutamine one. These studies should be done in parallel with studies using the various *gdh1-1* and *got1-10* constructs to eliminate the possibility that the loss of label from the glutamine pool is due to glutaminase activity. Additionally, if this hypothesis is correct, then loss of GOGAT should not affect the flux through NADP-GDH to glutamate in cells grown with excess ammonium, but would prevent accumulation of glutamate in ammonium-limited culture. The prediction is testable in this experimental system.

Mass spectroscopy can be used to assay ^{15}N-glutamate and ^{15}N-glutamine in samples taken from the culture during the course of the experiment. The method of choice is nuclear-magnetic-resonance spectroscopy because it is non-invassive and can be used to continuously monitor the flux.

From the data presented in Table 7, it can be concluded that a mutation causing loss of GOGAT activity does not affect the growth rate of a GDH+ strain (Fig. 25). However, although the final absorbance of the AR2(pCYG4) and AR5(pCYG4) cultures were similar, it does not necessarily follow that the final cell densities were. This is because absorbance is affected by cell size, number of buds and cell density, so the similar final absorbance values may be due to chance. With the data available, it is not possible to say unequivocally that GDH+ GOGAT- and GDH+ GOGAT+ strains achieve the same final culture densities. This awaits a more careful analysis of the growth characteristics of the different constructs, ultimately in a simulated industrial propagation.

The data in Figs. 10-15 shows that the three strains are very similar with respect to the behaviour of GS, GOGAT, NAD- and NADP-GDH, when grown in shake-flask culture with 20mM NH_4^+. However, differences are apparent when the strains are examined at limiting ammonium concentrations. The data reported in section 4.5 show that there are major differences in the enzyme profiles of DCL1 and DCL2 grown in shake-flask and simulated industrial cultures. So, although extrapolations from the laboratory model to the industrial strains grown in shake-flask culture with 20mM NH_4^+ would be valid, the

validity of extrapolation from the laboratory to the industrial growth system is unclear. The only way the validity could be tested is by growing the different *gdh1-1* and *got1-10* constructs in the industrial system. This is difficult for two reasons: (i) haploid strains will not grow in the culture system used by DC(Y)L as they become glucose repressed and do not grow; and (ii) the complex genetics of industrial strains make it difficult to introduce mutant alleles into them, which is compounded by the, apparent, complex genetics of the GOGAT loci.

These problems can be by-passed by using a tetraploid series of each construct *i.e.* a set of strains carrying from zero to four copies of *gdh1-1* and/or *got1-10*. Tetraploid strains can be constructed by classical genetic techniques. Since such strains are polyploid, they will not be subject to glucose repression. Chemostat studies, similar to those described for the haploid constructs, can be used to investigate whether NADP-GDH or GOGAT are present in excess, or if the enzyme levels are closely modulated by the ammonium level as has been observed for the GS of *C. utilis* (Ferguson and Sims 1974). This data will help in the choice of construct to be used in the simulated industrial propagations, in addition to improving the understanding of ammonium assimilation by *S. cerevisiae*. The use of these strains in simulated industrial propagations would allow the effect of a controlled decrease in GOGAT activity on biomass yield to be studied.

Mutants which have decreased GOGAT activity were isolated in this study and have been used to show that loss of GOGAT activity does not affect the growth rate at non-limiting ammonium concentrations. The parameter used to determine the growth of each culture in this study *i.e.* absorbance at 610nm, is affected by factors other than just cell density. Therefore, the conclusion that the mutation causing loss of GOGAT activity does not affect the final biomass concentration is uncertain. Further work, described above, is needed before conclusions about the effect of a decrease in GOGAT activity on the growth characteristics of DCL1 and DCL2 in simulated industrial propagations can be made. Evidence was presented which suggests that GS-GOGAT makes a major contribution to the assimilation of limiting ammonium by both Σ1278b and DCL1. Possible experimental approaches to confirm these hypotheses have been presented. Until these further experiments have been done, uncertainty remains about the validity of the conclusions presented about the importance of GS-GOGAT in ammonium assimilation by *S. cerevisiae*.

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