GLUTAMATE AND GLUTAMINE METABOLISM IN CARROT CELL CULTURE.

by

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To my family, especially Mum and Andrew.

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ABSTRACT.

The objective of the work described in this thesis was to investigate the metabolism of glutamine and glutamate in carrot cell suspension cultures and in particular to focus on the role of the enzyme glutamate dehydrogenase in plant nitrogen metabolism. Experiments using *in vivo* nuclear magnetic resonance spectroscopy, *in vitro* gas chromatography-mass spectrometry and automated $^{15}N/^{13}C$ mass spectrometry have been used, in association with physiological studies, to demonstrate that glutamate dehydrogenase is active in the oxidation of glutamate, but not in the reductive amination of 2-oxoglutarate. These studies of nitrogen metabolism in cell suspension cultures of carrot (*Daucus carota* L. cv Chantenay) have yielded results consistent with the primary assimilation of ammonium occuring via the glutamate synthase pathway. The results show that glutamate dehydrogenase is derepressed under conditions of carbon limitation and are consistent with observations in other plant tissues, particularly roots and senescing organs. The evidence suggests that the function of glutamate dehydrogenase is the oxidation of glutamate, thus ensuring sufficient carbon skeletons for effective functioning of the TCA cycle. This catabolic role for glutamate dehydrogenase implies an important regulatory function in carbon and nitrogen metabolism.

CONTENTS

FIGURES AND TABLES.

Abbreviations:

ANCA, automated $^{15}N/^{13}C$ analyser;
AOA, aminooxyacetic acid;
GABA, γ-aminobutyric acid;
GDH, glutamate dehydrogenase;
GOGAT, glutamate synthase;
GS, glutamine synthetase;
fw, fresh weight;
HFIB, heptafluorbutyryl Isobutyl;
INEPT, Insensitive Nuclei Enhanced by Polarisation Transfer.
MSO, methionine sulphoximine;
MTBSTFA, N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide;
NOE, nuclear Overhauser enhancement;
OPT, o-phthaldialdehyde;
TCA, Tricarboxylic acid cycle;
t.BDMS, tert-butyldimethylsilyl;

CHAPTER 1: INTRODUCTION.

The objective of this research was to investigate nitrogen metabolism in carrot cell suspension cultures and in particular to focus on the role of the enzyme glutamate dehydrogenase (GDH) in this process. Before describing the methods and results of this study it is appropriate to discuss firstly the reasons for using carrot cell suspension culture and secondly to review the current understanding of the role of GDH in nitrogen metabolism in higher plants.

Studies of plant nitrogen metabolism in the whole plant are complicated by a wide range of environmental and physiological factors. The use of cell suspension cultures allows one to isolate a particular group of cells and control the external conditions to a degree not possible in the whole plant. In this study carrot cell suspension cultures were used but other work on nitrogen metabolism has been reported using cell suspension cultures of tobacco (Rhodes et al 1989) and soybean (Shargool and Jain 1987). The carrot cell system is heterotrophic and allows one to investigate primary ammonium assimilation in the absence of photorespiratory nitrogen cycling.

The role of the enzyme GDH has been a matter of debate since the early 1970s when the detection of glutamate synthase led to a rapid if incomplete acceptance of the GS-GOGAT pathway as the major route of ammonium assimilation in higher plants (Miflin and Lea 1977). Studies of primary ammonia assimilation, the photorespiratory nitrogen cycle and secondary ammonia assimilation (that derived from the catabolism of nitrogenous storage and transport compounds) have all yielded results consistent with the consecutive action of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Rhodes et al 1980; Woo et al 1982; Fentem et al 1983; Berger and Fock 1983; Ta et al 1984; Martin et al 1986; Rhodes et al 1986; Sieciechowicz et al 1989). However GDH is present, often at high levels in all higher plants tested but no definite role has been assigned to it. Opinion is divided

between those who suggest that the enzyme operates primarily in the assimilation or reassimilation of ammonia and plays a complementary role to the glutamate synthase cycle (Rhodes et al 1989; Yamaya et al 1986;) and others who argue that the weight of evidence is consistent with a catabolic role for GDH (Thomas 1978). Recent reviews of the role and regulation of GDH include those by Srivastava and Singh (1987), Oaks and Hirel (1985) and Rhodes et al (1989).

The following is an attempt to evaluate the role of GDH based on recent work relating to the general characteristics of the enzyme, including location and regulation of activity; the use of inhibitors and isotope labelling studies; work with isolated mitochondria and genetic studies.

Glutamate dehydrogenase, occurrence, distribution, localization and structure.

GDH is present in almost all organisms, with the apparent exception of mosses of the genus Sphagnum (Meade 1984) and has been detected in all of the higher plant species examined. Two distinct GDH enzymes exist in higher plants a largely NAD-linked mitochondrial enzyme and a predominantly NADP-linked chloroplastic enzyme. In higher plants the mitochondrial form of GDH reacts with both NAD and NADP although the activity with NAD is up to 12 times greater (Pahlick and Joy 1971, Lea and Thurman 1972). The chloroplastic enzyme shows either higher activity with NADP or equal activity with both coenzymes (Leech and Kirk 1968, Lea and Thurman 1972). Algal GDH isoenzymes have also been shown to exhibit dual coenzyme specificity (Jacobi 1967). In contrast the GDH enzymes in fungi and bacteria are generally of a single coenzyme specificity (Sanwal and Lata 1961, Smith et al 1975). Higher fungi possess two enzymes specific for either NAD or NADP while fungi of the lower orders possess only one GDH enzyme which shows absolute specificity for NAD. In higher plants NAD-linked activity has been found in a wide variety of tissues including seeds (Thurman et al 1965), roots (Pahlick and Joy 1971), epicotyls (Davies and Teixeira 1975),

cotyledons (King and Yung Fan Wu 1971), leaves (Barash et al 1973), root nodules (Becana et al 1984) and suspension culture cells (Shargool and Jain 1987). The NADP-linked enzyme has been detected in shoots (Leech and Kirk 1968), and tobacco tissue culture (Washitani and Sato 1977). Various studies using differential centrifugation and density gradient techniques have demonstrated a mitochondrial location for the NAD-linked GDH in higher plants (Davies and Teixeira 1975, Yamaya et al 1984). The mitochondrial enzyme is thought to be loosely bound with the mitochondrial membrane (Yamaya et al 1984). The NADPH dependent GDH isoenzyme of higher plants has been shown to be tightly bound to the chloroplast lamellae (Leech and Kirk 1968). In tobacco tissue culture cells NADP-linked GDH activity is localized in the proplastids (Washitani and Sato 1977). There is some evidence for a separate cytoplasmic NAD-GDH differing in certain kinetic properties and isoenzyme patterns (Kanamori et al 1972). The GDH enzymes of most fungal cells are located in the cytoplasm (Perlman and Mahler 1970). In general both the NADP and NAD-linked enzymes of GDH appear to be hexameric in structure with a molecular weight of 2-3 x 10^5. For example the NAD dependent GDH from soybean cells has a MW of 263,000 with subunits of 41,000 (Jain and Shargool 1989), and the NADP specific GDH of Chlorella sorokiniana has a MW of 290,000 with subunits of 58,000 (Gronestajski et al 1978). However there are reports of NAD linked GDH enzymes which are tetrameric in structure, in for example Chlorella sorokiniana (Gronestajski et al 1978) and Blastocladiella (Le John et al 1969).

Glutamate dehydrogenase characteristics and activity.

Despite the ubiquitous nature of this enzyme and the fact that relatively high levels of the enzyme are found in certain plant tissues the physiological role of GDH in higher plants remains obscure. The thermodynamically favoured direction of the reaction *in vitro* is the production of glutamate and consequently it has been concluded that the enzyme has a role in ammonia assimilation. This view is still common (Srivastava and Singh 1987, Oaks and Hirel 1985, Yamaya et al

1987) despite considerable evidence which makes a physiological role for GDH in ammonia assimilation unlikely (Miflin and Lea 1980; Stewart et al 1980). In general the Km values for ammonium as substrate are high (10-80 mM) (Stewart et al 1980). Despite this it has been argued that the GDH levels are sufficient to account for ammonium assimilation (even with this relatively high Km for ammonia, Loyola-Vargas and Sanchez de Jimenez 1984, Murray and Kennedy 1980 and Murillo and Sanchez de Jimenez 1984). However, even in tissues in which GDH activity is high relative to GS (see eg. Fentem et al 1983; Smirnoff and Stewart 1987) the incorporation of ^{15}N labelled ammonium is almost completely inhibited in the presence of the GS inhibitor, methionine sulphoximine (MSO). Similarly, in soybean cell lines with elevated levels of GDH, grown on high media ammonia concentrations, Jain and Shargool (1987) were unable to demonstrate ammonium assimilation in the presence of MSO. They concluded from this that GDH was playing a role in glutamate degradation. In general, the occurrence of high activity alone is insufficient evidence to ascribe a particular function to GDH and should be supported by additional metabolic evidence. GDH catalyses a reversible reaction and although activities *in vitro* suggest that the aminating action is favoured at physiological pH it cannot be assumed that this reflects the direction of activity *in vivo*.

Observations that GDH levels are higher in root tissues and in the senescing organs of plants have come from many workers (Loyola-Vargas and Sanchez de Jimenez 1984; Becana et al 1984; Groat and Vance 1981, 1982; Murray and Kennedy 1980; Smirnoff and Stewart 1987). The rise in GDH activity during senescence is linked with a corresponding decline in GS/GOGAT activity. Storey and Beevers (1978) showed that although the levels of GS decline sharply in senescing leaves of *Pisum sativum* they remained sufficient to account for any glutamine produced. During senescence the leaf becomes less important as a source of photosynthate but may become a major source of nitrogen as its protein is hydrolysed and the products of hydrolysis are redistributed within the plant (Thomas 1978). Protein

catabolism within the leaves would be expected to produce large pools of glutamate and aspartate but the pools of these amino acids remain constant whereas the pools of their respective amides increase (Storey and Beevers 1978). This implies that glutamate and aspartate are rapidly metabolised as they are released from leaf protein and their nitrogen is transferred to the amide pools and is thus available for transport to other parts of the plant (Thomas 1978). During the later stages of senescence (4-6 days) there was also a large increase in tissue ammonia, apparently at the expense of amino acid nitrogen. The continuing increase in GDH activity at this time may be due to ammonium induction as described by Barash et al (1973), however Thomas (1978) proposed that GDH is active in the catabolism of glutamate and is therefore responsible for the production of this ammonium. Oxidation of glutamate could provide the cell with reduced nucleotides and with carbon for carbon metabolism in circumstances where the chloroplasts are inactivated (Cammaerts and Jacobs 1985). Kar and Feierabend (1984) obtained similar results using wheat leaves and concluded that GDH might represent the main path for the liberation of ammonia from amino acids since no L-amino acid oxidase activity was found in these leaves. When GS was active ammonia released from glutamate would be reassimilated to glutamine but ammonia would accumulate when GS was inactivated either by darkness or inhibitors.

The alternative view is that during senescence GOGAT activity decreases to a level where ammonia assimilation is affected. Under high ammonia, low energy levels there might be a switch from the GS/GOGAT pathway to ammonia assimilation via GDH. Since high ammonia levels are found in senescent leaves GDH would be involved in detoxification of ammonia (Kang and Titus 1980; Lauriere et al 1981; Groat and Vance 1982 and Simpson and Dalling 1981). Cammaerts and Jacobs (1985) suggested that GDH played a dual role in leaf senescence, NADH-GDH being involved in the rapid detoxification of high levels of ammonia and NAD-GDH supplying the cell with energy for GS activity etc. However ^{15}N labelling studies in senescing wheat leaves showed that the amide-N of glutamine was the first labelled

product formed after feeding with [15N]ammonium chloride and that very low levels of glutamate labelling were observed (Berger et al 1985). When cells were fed [15N]glutamate or [15N]glycine the ammonia pool in these leaves was always slightly enriched suggesting that mitochondrial deamination of [15N]glutamate via GDH had occurred. The conclusion from this work is that GDH is active in the deamination of glutamate during senescence and this action is in response to conditions of carbon limitation.

The effect of sugars on GDH has been studied by many workers and there is general agreement that sugars exert a regulatory effect on the enzyme. In general GDH levels rise in response to carbon limitation and this effect is reversed by the addition of various sugars (Oaks et al 1980; Sahulka and Lisa 1980). Sugars appear to play a more central role in the regulation of GDH than ammonia and other nitrogen sources. Nauen and Hartmann (1980) found that floating detached pea shoots in tap water produced 2-8 fold increases in GDH activity. This increase was suppressed by the addition of sugars but ammonia and other metabolites had no effect (Tassi et al 1984). Since supply of sugar increases the energy level and increases GS activity it has been suggested that there is a reciprocal regulation of GS and GDH (Ratajczak et al 1981).

Inhibitor and ^{15}N ^{13}N labelling studies.

Inhibitor studies can be very useful in analysing the flux through certain reactions. In the absence of a specific inhibitor of GDH, work with L-methionine sulphoximine(MSO) and azaserine, the inhibitors of GS and GOGAT respectively, has been central to acceptance of the glutamate synthase pathway of ammonia assimilation. In almost all cases inhibition of GS by MSO prevents ammonia assimilation and when used in conjunction with ^{15}N labelled ammonia stops incorporation into amino acids (Jain and Shargool 1987; Berger and Fock 1983; Rhodes et al 1980; Fentem et al 1983; Martin et al 1986). There have been reports of relatively small levels of incorporation via a MSO insensitive pathway(Schubert et al

1981, Rhodes et al 1989) but these amount to less than 1% of ammonia assimilation. Similar experiments with azaserine or albizzine, inhibitors of the amide transfer reactions indicate that they cause a large inhibition of glutamate synthesis and block the incorporation of ^{15}N labelled nitrate or ammonium into glutamate (Fentem et al 1983; Martin et al 1986). Although the general conclusion from these studies has been that GDH does not play a significant role in ammonia assimilation this interpretation relies on the assumption that MSO does not interfere with the action of GDH. However, Rhodes et al (1989) have suggested that MSO may effect the transport or availability of 2-oxoglutarate and that by limiting this substrate it reduces the activity of GDH. However in the mycorrhizal fungus Cenococcum graniforme, where assimilation is catalysed by glutamate dehydrogenase, MSO inhibits the incorporation of [15N]ammonium into glutamine without inhibiting the incorporation into glutamate (Genetet et al 1984).

Numerous labelling studies using both ^{15}N and ^{13}N have provided additional support for the glutamate synthase pathway as the major route of ammonia assimilation. However labelling studies must be interpreted with caution, studies which show that ^{15}N label appears first as the amide-N of glutamine and subsequently in glutamate prove only that the GS reaction is faster not that it provides the only route for ammonia assimilation.

Tracer studies using ^{13}N and ^{15}N have been used to try to distinguish between the two pathways of ammonia assimilation. These studies have presented strong evidence for the operation of the GS/GOGAT cycle (Shokut et al 1978, Fentem et al 1983). Attempts by Fentem et al (1983) to fit these results to models where ammonia was assimilated solely via GDH were unsuccessful, but models produced by Rhodes et al (1989) are consistent with a role for GDH of up to 30%. These models are based on studies of ammonia assimilation in tobacco cell culture in the absence of inhibitors. However the relative sizes of the glutamate and glutamine pools make it difficult

to distinguish between the simultaneous operation of alternative pathways and Rhodes was unable to substantiate his conclusions using inhibitors of GS and GOGAT. These problems highlight the need for further investigation into the effect of the various inhibitors on GDH activity.

Rhodes et al (1989) has also reported low levels of ammonia assimilation via a MSO insensitive pathway in Lemna minor and the roots of tomato plants, but these represent less than 1% of the total flux in the absence of MSO. Results from ^{13}N labelling studies with Alnus glutinosa (Schubert et al 1981)have been interpreted as indicating low levels of ammonia assimilation via GDH, but since there was some labelling of glutamine this could be explained by an incomplete inhibition of GS. The conclusion from most of the ^{15}N studies has been that ammonia assimilation does not occur via GDH in higher plants. This contrasts strongly with the position in microorganisms where there is no doubt that GDH is active in the assimilation of ammonium (Kanamori et al 1988). In fungi, evidence for an assimilatory role for NADP-GDH comes from studies of [15N]ammonium assimilation in Candida utilis (Sims and Folkes 1964, Ferguson and Sims 1971) and Cenococcum graniforme (Genetet et al 1984). In Cenococcum geophilum studies of ^{15}N incorporation in the presence and absence of MSO have been used to calculate the relative fluxes through the GS and GDH pathways (Martin et al 1988).

Treatment with MSO leads to ammonia accumulation and changes in the relative sizes of amino acid pools. Glutamate, glutamine, alanine and serine decline whereas others, such as proline, valine, leucine and threonine accumulate. Rhodes et al (1986) showed that these amino acids were not newly synthesised and suggested that they are formed from protein turnover. The amino acids which accumulate are those which are relatively poorly catabolised in vivo, the remainder are rapidly catabolised to ammonia.

Failure to demonstrate ammonium assimilation in the presence of MSO coupled with the observed accumulation of ammonia under

these conditions has led to speculations that GDH may be active in the oxidation of glutamate to ammonium and 2-oxoglutarate. The ammonium released by this reaction could be reassimilated into glutamine by GS and transported from the tissues. If the reaction occurred under conditions of carbon starvation the 2-oxoglutarate would provide vital carbon skeletons for continued operation of the TCA cycle.

Mitochondrial glutamate metabolism.

GDH activity is associated with the mitochondria in higher plants (Stewart et al 1980) and several workers have investigated the ability of isolated mitochondria to assimilate ammonia or oxidise glutamate (Hartman and Ehmke 1980, Bergman et al 1981, Yamaya et al 1984, 1986 and Day et al 1988).

Early work in this area suggested that isolated mitochondria could not assimilate ammonia at significant rates to account for a reassimilatory role for GDH (Hartmann and Ehmke 1980; Bergman et al 1981). However Yamaya et al (1984,1986) showed that higher rates of ammonia assimilation were obtained when calcium and NADH levels were optimised. Rates of glutamate production of up to 50 nmolh-1 mg protein were achieved when ammonia was supplied either exogenously or derived from glycine. The mitochondrial preparations showed no appreciable GS activity and produced no glutamine. From these results Yamaya and Oaks concluded that mitochondria could represent a compartment where GDH is isolated from GS and as a result was able to compete for some of the available ammonia. They also suggested that this alternative pathway of ammonia assimilation might explain why leaf ferredoxin dependent GOGAT mutants can survive under non photorespiratory conditions (Somerville and Ogren 1980) An alternative explanation is that a specific root NAD(P)H GOGAT metabolises glutamine in such mutants (Lea et al 1990). Rhodes et al (1989) suggest that such a small rate of mitochondrial ammonium assimilation (less than 1% of the total) would remain undetected in labelling studies with intact tissues unless inhibitors were used.

These results suggest that at the most only a very small proportion of ammonia assimilation may take place via GDH.

Until recently studies of intact mitochondria had failed to show any oxidation of glutamate, but Day et al (1988) demonstrated rapid oxidation of glutamate in mitochondria purified from soybean seedlings. Their results suggest that mitochondria play a role in protein mobilization and amino acid metabolism during seedling growth. Glutamate oxidation would provide a source of ATP from protein degradation for use in biosynthesis within cotyledon cells and in transport of metabolites.

Genetic Evidence.

Compared to GS and GOGAT little work has been done in the area of GDH mutants, but a mitochondrial GDH deficient mutant of Zea mays has been isolated and is being investigated by Rhodes et al (1989). In the absence of MSO, wild type plants have a higher shoot/root ratio and show a significantly higher ammonia assimilation rate than GDH mutants. However if MSO is added to the nutrient media ^{15}N incorporation into amino acids is inhibited in both the wild type and the mutant strain. The results of these experiments show that while GDH may have no role in ammonium assimilation its absence does effect growth. This observation is consistent with the view that GDH has a catabolic function and implies that a deficiency in glutamate catabolism seriously impairs growth. Clearly further characterisation of this mutant is needed to establish if its growth behaviour does in fact relate to the absence of glutamate oxidation.

It is clear from the great majority of published studies that GDH does not play a major role in the assimilation of ammonia in higher plants. One of the difficulties in assigning a definite role to GDH is the tendency to focus on its assimilatory activity and there has been very little investigation of a possible catabolic action for this enzyme. It is possible that GDH occupies a central position in

relation to nitrogen and carbon metabolism, rather than a peripheral role in ammonium assimilation. The present investigation was undertaken to investigate the role of GDH in carrot cell suspension culture.

CHAPTER 2: MATERIALS AND METHODS.

Chemicals.

Common analytical grade inorganic and organic chemicals and HPLC grade solvents were obtained from BDH Chemicals Ltd (Poole). Amino acids, enzyme reagents and inhibitors were obtained from Sigma Chemical Company (London). ^{15}N labelled ammonium chloride, potassium nitrate and L-glutamate were obtained from Cambrian Gases Ltd (Croydon).

Growth conditions of cell suspension cultures.

Suspension cultures of Daucus carota L. cv Chantenay isolated six years previously were maintained on Murashige and Skoog (1962) medium (Flow Labs, Irvine, Scotland), supplemented with 2,4-dichlorophenoxyacetic acid (0.2 mg/L), kinetin (0.1 mg/L) and sucrose (2% w/v). This medium was referred to as MDK. The total nitrogen concentration of the medium was 39.4 mM nitrate and 20.6 mM ammonium (composition of the media is shown in Table 2.1). The cells were grown in 250 ml Erlenmeyer flasks containing 70 ml of medium. The flasks were stoppered with a cap of aluminium foil prior to autoclaving at 121 °C for 20 mins. The cells were subcultured at 14 d intervals by inoculating 7 ml of suspension into 70 ml of fresh medium. The flasks were kept at 25 °C in diffuse fluorescent light on an orbital shaker at 90 rpm. For experiments employing varying nitrogen sources a nitrogen free Murashige and Skoog base was obtained from Flow Labs, which contained no ammonium nitrate or potassium nitrate (N-free media). With the exception of glutamine which was sterilised by millipore filtration, all nitrogen sources were added to the growth medium prior to autoclaving.

Determination of fresh and dry weight of cells.

To determine fresh weight cells were collected on a preweighed Whatmann No 1 filter paper, washed three times with 20 ml

Table 2.1 Carrot cell suspension culture medium.

Constituent	mg/L	Final concentration
Macronutrients		
NH_4NO_3	1650	20.6 mM
KNO_3	1900	18.8 mM
$CaCl_2.2H_2O$	440	3.0 mM
$MgSO_4.7H_2O$	370	1.5 mM
KH_2PO_4	170	1.25 mM
Micronutrients		
KI	0.83	5.0 uM
H_3BO_3	6.3	100 uM
$MnSO_4.4H_2O$	22.3	100 uM
$ZnSO_4.7H_2O$	8.6	30 uM
$Na_2MoO_4.2H_2O$	0.25	1.0 uM
$CuSO_4.5H_2O$	0.025	0.1 uM
$CoSO_4.6H_2O$	0.025	0.1 uM
Na_2EDTA	37.3	100 uM
$FeSO_4.7H_2O$	27.8	100 uM
Additions		
2,4-D*	0.2	
kinetin	0.1	
sucrose	20	

*2,4-dichlorophenoxyacetic acid (2,4-D).

Medium adapted from Murashige and Skoog (1962). The macronutrients and micronutrients were contained in the base medium obtained from Flow Labs.

distilled water, dried under vacuum for 20 s and weighed. Dry weight was determined by collecting cells on preweighed glass fibre disks in the same way and drying overnight in a 80 °C oven.

Enzyme extraction and Assays.

Cells were collected by filtration on Whatman No.1 paper, washed with 25 ml distilled water and weighed. Cells (0.2 g) were ground in liquid nitrogen and extracted in 5 ml buffer. For the GS and GDH assays cells were extracted in the following buffer 25 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM mercaptoethanol, 1 mM reduced glutathione, 10 mM $MgSO_4$, 5 mM glutamate, 2% polyvinylpyrrolidone and 0.01% Triton-X-100. The GOGAT extraction buffer (pH 7.2) contained 100 mM sodium phosphate, 10% v/v glycerol, 5 mM EDTA, 0.1% Triton-X-100, 5 mM mercaptoethanol and 1 mM phenylmethylsulphonylfluoride. The extracts were centrifuged to remove cell debris and the supernatant used for the enzyme and soluble protein assays described below. Enzyme extraction was carried out immediately prior to assay.

Glutamine synthetase **(L-Glutamate: ammonium lygase (ATP) EC 6.3.1.2).**

Glutamine synthetase was assayed using the synthetase assay. 0.25 ml of enzyme extract was incubated in a final volume of 0.75 ml containing 15 umols ATP,20 umols $MgSO_4$, 5 umols hydroxylamine and 60 umols L-glutamate and 37.75 umol Tris (final pH 7.6) for 30 mins at 30 °C. The reaction was initiated by the addition of 0.25 ml enzyme extract. After 30 minutes the reaction was stopped by the addition of 0.75 ml ferric chloride reagent ($FeCl_3$ 26 g/l, trichloroacetic acid 40 g/l, concentrated HCl 80 ml/l). The protein precipitated by the addition of the ferric chloride reagent was removed by centrifugation at 4 °C for 5 mins at 5000 rpm in a bench centrifuge. Glutamyl hydroxymate was used as the standard and the optical density was read at 500 nm using a Beckmann DU-

7 Spectrophotometer. In all cases a blank sample was prepared without ATP.

Glutamate synthase (L-Glutamine: 2-oxoglutarate amido transferase (NADH) EC 2.6.1.53).

Glutamate synthase was assayed by determining the rate of glutamine dependent NADH oxidation in a Beckmann DU-7 Spectrophotometer at 340 nm. The reaction mixture contained, in a final volume of 1 ml, 0.2 ml of enzyme extract, 5 umol glutamine, 5 umol 2-oxoglutarate, 0.08 umol NADH, 5 umol EDTA and 50 umol Tricine. All solutions were adjusted to pH 7.5. The reaction mixture was prewarmed to 30 °C and the reaction initiated by the addition of 2-oxoglutarate. A blank was employed omitting glutamine from the reaction mixture.

Glutamate dehydrogenase (L-Glutamate: NAD Oxido reductase EC 1.4.1.2).

(i) Reductive amination.

This activity was determined by measuring the rate of 2-oxoglutarate-dependent NADH oxidation in a Beckmann DU-7 Spectrophotometer at 340 nm. The reaction mixture contained, in a final volume of 1 ml; 0.05-0.1 ml of enzyme extract, 150 umol NH_4Cl, 1 umol $CaCl_2$, 0.3 umol NADH, 20 umol 2-oxoglutarate and 100 umol Tris buffer. All solutions were adjusted to pH 8.2. The reaction mixture was prewarmed to 30 °C and the reaction initiated by the addition of 2-oxoglutarate. A blank was employed omitting 2-oxoglutarate from the reaction mixture.

(ii) Oxidative deamination

This activity was determined by measuring the rate of glutamate dependent NAD reduction in a Beckmann DU-7 Spectrophotometer at 340 nm. The reaction mixture contained, in a final volume of 1 ml; 0.1 ml of enzyme extract, 50 umol L-glutamate, 1 umol $CaCl_2$, 0.625 umol NAD, and 100 umol Tris

buffer. All solutions were adjusted to pH 9.5. The reaction mixture was prewarmed to 30 °C and the reaction initiated by the addition of L-glutamate. A blank was employed omitting L-glutamate from the reaction mixture.

Initial experiments were carried out to determine the pH optima for this reaction in both the aminating and deaminating directions, and the Km values for ammonium, 2-oxoglutarate and L-glutamate. The pH optima were found to be pH 8.2 for the aminating and pH 9.5 for the deaminating reactions. The Km values are shown in Table 2.2 and are comparable with the range of values described in the literature. The values for ammonium and L-glutamate lie within the range described in the literature. The value for 2-oxoglutarate is slightly high but this may be as a result of using crude extracts for these determinations.

All enzyme assays were linear with respect to length of incubation time and quantity of enzyme assayed. Soluble extractable protein was determined directly using the Bio-Rad protein assay (see below).

Expression of results.

Enzyme activities for GS, GOGAT and GDH are given as nkatals per mg protein.

Determination of total cell protein.

Total cell protein was extracted by alkaline hydrolysis (Dixon 1985). 0.5 g of cells was washed twice in 10 ml methanol and three times with 5% w/v trichloroacetic acid at 0 °C to precipitate the protein (all treatments lasting 5 mins). The washed cells were centrifuged at 5,000 rpm in a bench centrifuge for 5 mins. The pellet was extracted in 3 successive 3 ml aliquots of 0.3 M NaOH for 30 mins at 37 °C. The NaOH extracts were combined, neutralised with 6 M HCL and the protein content determined by the Bradford method (1976) using a Bio-Rad protein assay kit.

Table 2.2. Km values for Glutamate Dehydrogenase from crude extracts of carrot suspension culture cells compared to reported values for higher plants.

Substrate	Km in crude extracts from carrot cells	Range of km values for higher plants*
Ammonium	19 mM	5.2-70 mM
2-oxoglutarate	5 mM	0.62-4 mM
L-glutamate	5 mM	2.5-25 mM
NADH	0.05 mM	0.015-0.12 mM
NAD^+	0.3 mM	0.2-1.2 mM

* References
Stewart and Rhodes (1977); Davies and Teixeira (1975); Pahlick and Joy (1971); Errel et al (1973); King and Yung-Fan Wu (1971); Lea and Thurman (1972).

Bio-Rad protein assay.

2.5 ml of diluted dye reagent was mixed with 0.05 ml of sample and after 30 mins the optical density at 595 nm was measured against a blank containing extraction buffer. A standard curve was prepared using 0.2-1.4 mg/ml bovine albumin.

Extraction of soluble amino acids, ammonium and nitrate from cells.

Cells were collected as described above for fresh weight determination. 0.5 g of cells was extracted in 10 ml methanol and the extract was used for the analysis of intracellular pools of ammonium, nitrate and amino acids. In the case of ^{15}N labelling studies this methanol fraction was also used for GC-MS analysis.

Determination of cell and medium ammonium.

Medium ammonium concentration was determined directly using the colorimetric method described by McCullough (1967). For analysis of ammonium in the medium 200 ul of appropriately diluted sample was assayed. Ammonium chloride solution was used as the standard to calibrate the reagents. Absorbance was read at 625 nm. The ammonium content of the cells was determined using the methanol extract described above. An ammonium chloride in methanol standard was used for calibration.

Determination of cell and medium nitrate.

Nitrate was determined as described by Sloan and Sublett (1966). 0.25 ml of cell methanol fraction or appropriately diluted medium was used in the initial reduction step, to which was added 2 ml of 0.4 M NH_3-NH_4Cl buffer (pH 9.6), 1.75 ml 0.1 M $MgCl_2$ and 0.5 g cadmium. The sample was mixed thoroughly, left to stand for 30 mins and aliquots taken for

nitrite determination. 1 ml of sample was added to 1 ml of 1% sulphonilic acid in 3M HCl and 1 ml 0.02% -naphthyl ethylene diamine dihydrochloric solution. The assay was left at room temperature for 30 mins prior to reading the optical density at 540 nm on a Beckmann Spectrophotometer. Sodium nitrate (0.2-0.6 umol) was used as a standard to test for the efficiency of the reduction and sodium nitrite (5-100 nmols) was used to calibrate the reagents for the nitrite determination.

Determination of medium sucrose.

Medium sucrose concentration was determined using the phenol-sulphuric method described by Dubois et al (1956), using 2 ml of appropriately diluted sample. Sucrose (0-50 ug/ml) was used as a standard to calibrate the reagents. Absorbance was read at 490 nm.

HPLC determination of soluble amino acid pools.

Cell amino acids were determined directly from the methanol extracts using high performance liquid chromatography. The HPLC system (Kontron Instruments, Watford, Herts) consisted of; a Model 420 pump, a Model 425 gradient former, a model 460 autosampler and injector fitted after the injection loop with a stainless steel guard column (2 cm) and column (10 x 0.45 cm) containing Spherisorb 5 um ODS 2 spherical packing (Phase-Sep Ltd, Deeside, UK) and a Milton Roy Fluoro-Monitor 3 fluorescence detector with filters set for o-phthaldialdehyde derivatisation of amino acids. The system was controlled by a Model 450 multitasking data system. The column was maintained at 30 °C by a Model 480 oven controller.

The amino acids were analyzed as o-phthaldialdehyde (OPT) derivatives on a C-18 column using a method adapted from Joseph and Marsden (1986). The OPT stock reagent was prepared as follows; 50 mg OPT was dissolved in 1 ml methanol and made up to 7.5 ml with 0.4 M borate buffer (pH 9.5). For the working reagent 10 ul 2-mercaptoethanol was added to 1.5 ml

OPT stock reagent.
All samples, standards, eluents and derivatising reagents were passed through a 0.5 um FP vericel filter (Gelman Sciences Ltd, Northampton) prior to use. 10 ul of 0.25 mM homoserine and 10 ul sample were derivatised with 60 ul working reagent. After 2 minutes 8 ul derivatised sample was injected. The gradient was produced using two eluents; A, 0.1 M phosphate buffer pH 8.3 with 20 ml/L methanol and 20 ml/L tetrahydrofuran; B, 65% methanol. Eluents were degassed with helium prior to use. Gradient was programmed as follows; 0 to 5 mins, 20% to 35%B; 5 to 27 mins, 35% to 100%B; 27 to 32 mins, 100%B.
Samples were calibrated against standards containing 0.25 nm of Sigma amino acid standard mixture with the addition of 0.25 nm glutamine, γ-aminobutyric acid (GABA), serine and asparagine. All samples contained 0.25 nm of homoserine as an internal standard. A chromatogram showing the separation of amino acids extracted from carrot cells is shown in Figure 2.1 with the corresponding report table (Table 2.3).

ANCA analysis of ^{15}N labelled ammonia and total cell enrichment

Total ^{15}N was determined by the technique of automated $^{15}N/^{13}C$ analysis - mass spectrometry (ANCA-MS) using a Europa Scientific Roboprep- Tracermass system. The technique used is described by Barrie and Lemley (1989). This system was used to analyse total ^{15}N in the cells, insoluble ^{15}N and ^{15}N present as ammonium in the medium. For total nitrogen cells were harvested, washed and dried as described under dry weight determination. Insoluble nitrogen was determined in cells which had been extracted in methanol to remove soluble nitrogen, the cells were then washed in distilled water and dried as described above. The oven dried samples were finely ground (talcum powder quality) before being weighed and sealed into 8 x 5 mm tin capsules for loading onto the autosampler. Triplicate 3-6 mg sample were analysed.

For determination of [^{15}N]ammonium in the medium the ammonium was first purified by cation exchange to remove all amino

Figure 2.1 HPLC chromatogram to show separation of OPT derivatives of amino acids, for peak identification see Table 2.1.

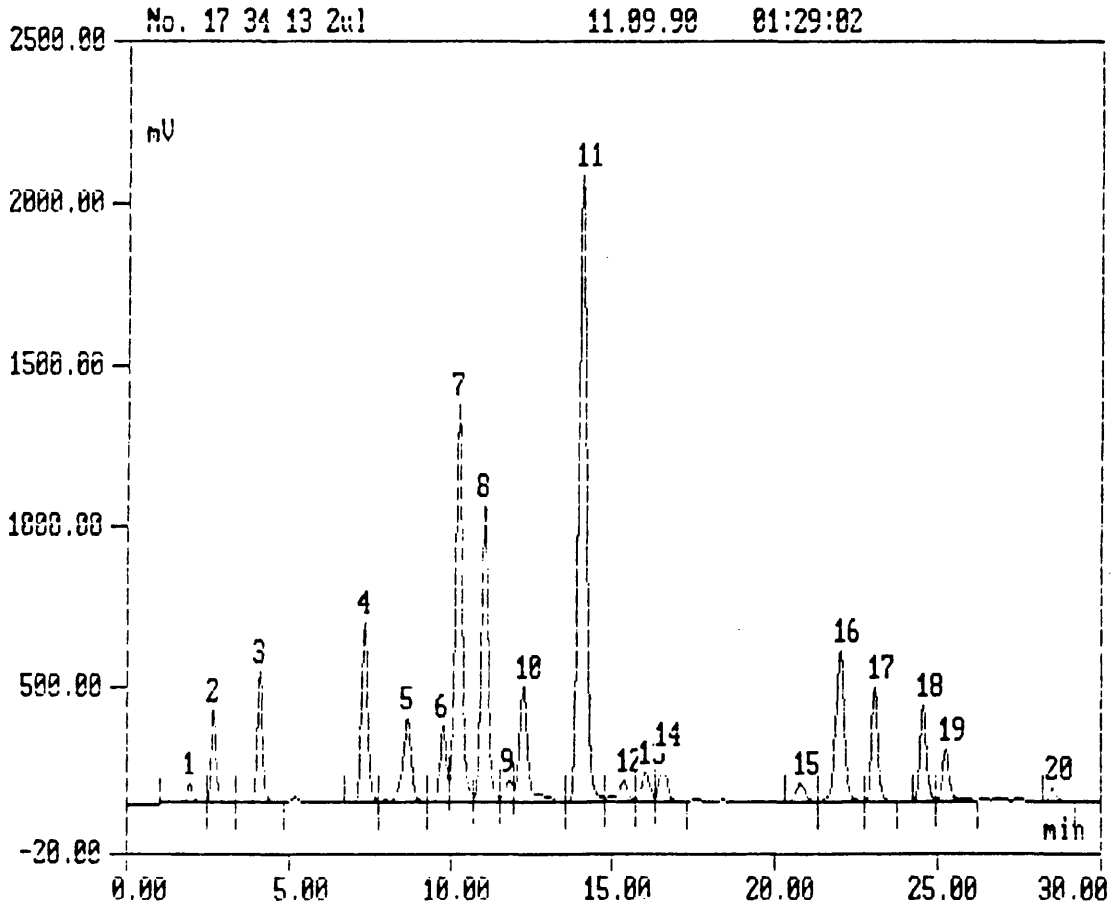


Table 2.3 Report table for HPLC chromatogram shown in Figure 2.1, to show concentrations of amino acids in umols/ml injected.

KONTRON Integrator 3.01 R e s u l t R e p o r t

Chromatogram Identification SR100990:
17 34 13 2ul ..... Chnl.. 1 Date.. 11.09.90 Time.. 01:29:02
Program File.... AA50 .....
Method File..... AA ..... -
Peak Table..... SR1001 ..... Auto-generated
Parameter Table. AA .....
Result File..... .....
Document File... AA .....

No.	Ret.Time min	Type	Name	Area mV*min	Amount nmols	Rel.Area %	Height mV	Nor.Are %
1	1.912	ML	?	11.603	?	0.532	63.60	4.723
2	2.646	M	aspartate	54.704	0.060	2.509	285.61	22.266
3	4.092	M R	glutamate	83.116	0.096	3.813	406.92	33.830
4	7.324	ML	asparagine	133.805	0.161	6.138	560.44	54.461
5	8.666	M	serine	85.793	0.103	3.936	258.00	34.919
6	9.746	M	glutamine	59.277	0.074	2.719	239.46	24.127
7	10.215	M	histidine	327.074	0.371	15.004	1234.0	133.125
8	11.035	M	homoserine-is	245.689	1.009	11.270	923.20	100.000
9	11.831	M	glycine	20.362	0.030	0.934	67.67	8.288
10	12.260	M r	threonine	113.974	0.119	5.228	358.35	46.389
11	14.052	Ml	arginine	584.615	0.715	26.818	1944.2	237.949
12	15.373	M	alanine	25.962	0.032	1.191	66.49	10.567
13	16.058	M	GABA	27.085	0.044	1.242	90.28	11.024
14	16.554	M R	tyrosine	37.990	0.042	1.743	147.56	15.463
15	20.787	ML	?	18.727	?	0.859	56.04	7.622
16	22.006	M	valine	149.608	0.145	6.863	469.25	60.893
17	23.069	M R	phenylalanine	82.965	0.096	3.806	351.86	33.768
18	24.569	ML	isoleucine	69.624	0.073	3.194	295.08	28.338
19	25.256	M R	leucine	39.736	0.042	1.823	158.16	16.173
20	28.543	MLR	lysine	8.232	0.032	0.378	43.26	3.351

acids and to concentrate the sample. The medium was applied to a Bio-Rad 50X-Na+ membrane (Bio Rad Laboratories, Hemel Hempstead, Herts), and after washing with 15 ml sodium acetate (10 mM) to remove amino acids, the ammonium was eluted with 1 ml 0.5M sodium phosphate. The efficiency of the separation of amino acids and ammonium was checked by analysis of the fractions by HPLC. The ammonium fraction was free from amino acid contamination. Samples (40 ul) were absorbed onto Carbosorb and analyzed in triplicate using the small sample mode (Barrie and Lemley 1989). All samples were calibrated against standards containing 15 atom % $[^{15}N]H_4Cl$.

GC-MS analysis of ^{15}N amino acid derivatives.

Cell methanol extracts were taken to dryness by rotary evaporation and redissolved in 2 ml water. The extracts were applied to a Bio-Rex sample preparation disc with AG 50W-X8 cation exchange resin, washed with 5 ml water and amino acids and amides were eluted with 5 ml 6 M NH_4OH. The amino acid fraction was lyophilised and redissolved in 1 ml 50% methanol. 0.5 ml purified extract was taken directly into a silanised glass vial, dried under nitrogen and derivatised with N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA,Pierce Chemical Co) as described by Fortier et al (1986). 50 ul derivatising mixture, MTBSTFA:pyridine:triethylamine (15:15:1 by volume) was added to the dry samples and the vials were heated at 75°C for 30 min. ^{15}N incorporation into each amino acid (including glutamine and asparagine) was then carried out by GC/MS analysis of the tert-butyldimethylsilyl (t.BDMS) derivatives using a VG7070 H mass spectrometer linked to a Finnigan Incos data system. 0.2 to 0.4 ul sample was run on a Pye-Unicam 204 gas chromatograph (GC) with an all glass dropping-needle solid injector and fitted with a 25 m polydimethylsiloxane 0.25 um film thickness fused silica capillary column. Helium was used as carrier gas at a column head pressure of 70 kPa and the oven was temperature programmed from 120°C for 1 min, +4°C /min to 280°C. Mass spectra were acquired using an electron energy 70 eV and the mass range scanned from m/z 750 to 35

every 2 s with a total cycle time of 3 s. Spectra obtained from t.BDMS derivatives generally show an intense ion at $(M-57)^+$ or $(M-159)^+$. ^{15}N incorporation (atom % excess) was calculated after integrating the areas obtained for $(M-57)^+$ for both labelled and unlabelled amino acids.
For example the calculation for glutamate,

$$\text{Atom \% excess } ^{15}N = \frac{\text{Area m/z 433 - Area m/z 432(CF)}}{\text{Area m/z 433 - Area m/z 432(CF) + Area m/z 432}}$$

(CF = correction factor for natural abundance of the isotopic elements ^{13}C, ^{29}Si, ^{15}N).

t.BDMS derivatives allow the determination of the total amount of ^{15}N incorporated into glutamine and asparagine and the proportion of the amides that are singly or doubly labelled. To determine the ^{15}N label in both the amide-N and the amino-N a second derivative was prepared. The remaining 0.5 ml amino acid extract was separated into neutral/basic and acidic amino acids by Dowex 1-acetate ion exchange chromatography (Rhodes et al 1981). The neutral and basic amino acid fraction was lyophilised, redissolved in 50% methanol, dried under nitrogen and derivatised as the heptafluorbutyryl isobutyl (HFIB) esters as described by Rhodes et al (1981). On preparation of these derivatives the amide groups of glutamine and asparagine are lost to form glutamate and aspartate respectively. GC-MS then allows direct determination of the ^{15}N incorporation into the amino-N of glutamine (determined as glutamate). GC/MS of the HFIB derivatives was performed as for t.BDMS derivatives but the mass range scanned was m/z 200 to 400 and the GC was temperature programmed from 120°C for 2 min, +6°C/min to 280°C. The ^{15}N in amino-N of glutamine was calculated after integration of ions m/z 298,299. The ^{15}N label in amide-N of glutamine was then calculated by difference.

total ^{15}N label = single label + (2 x double label)

total amide label = total ^{15}N label - total amino label (calculated from HFIB derivative)

The separation of amino acids obtained by GC-MS is shown in Figure 2.2. The mass spectra of the tBDMS derivative of

Figure 2.2 Reconstructed ion current (RIC) to show seperation of amino acids extracted from carrot cells obtained by GC-MS.

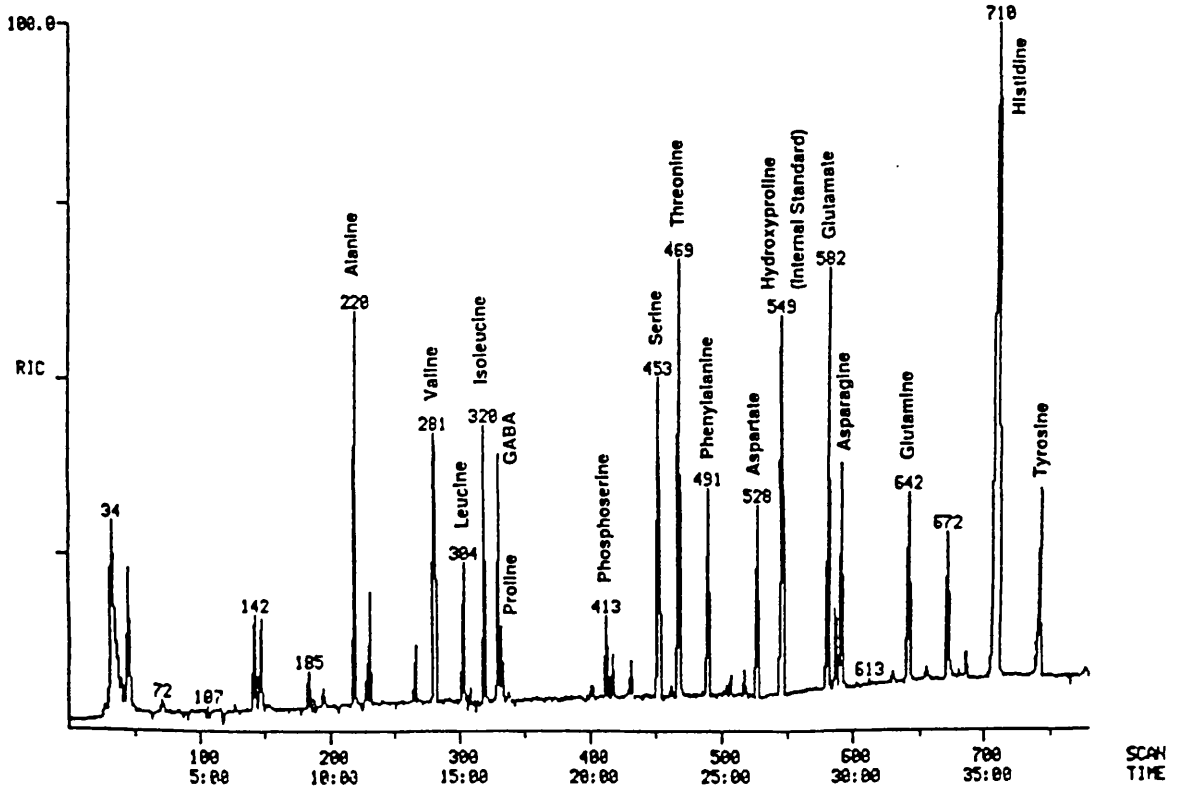


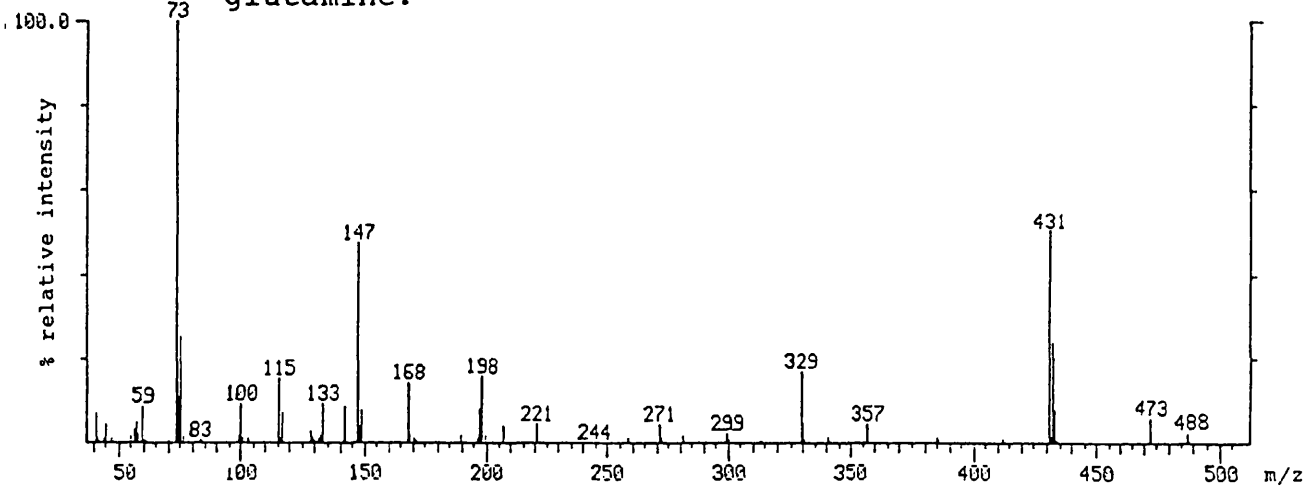
Figure 2.3 Mass spectra of the tBDMS derivative of glutamine from carrot cells grown on [^{15}N] ammonium.

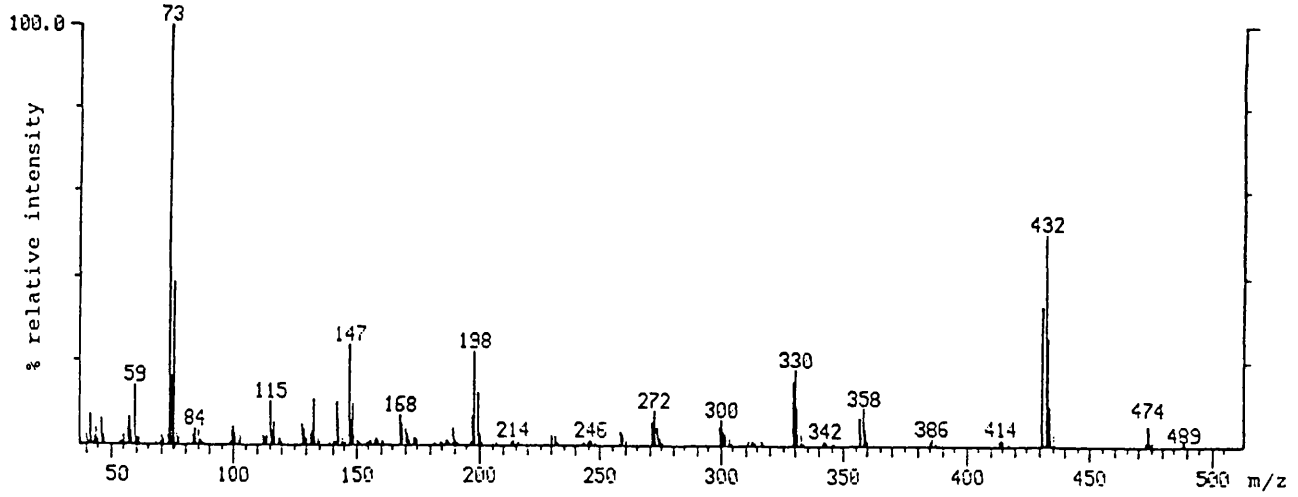
to show,

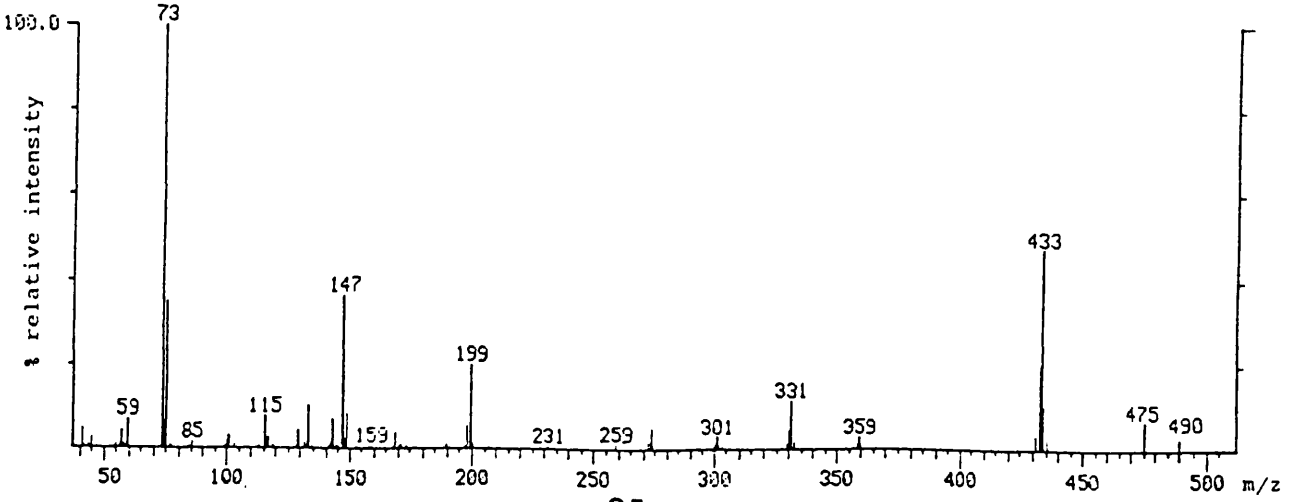
a) glutamine with no ^{15}N label, $^{14}N^{14}N$ glutamine

b) glutamine with predominantly single ^{15}N label, $^{14}N^{15}N$ glutamine

c) glutamine with predominantly double ^{15}N label, $^{15}N^{15}N$ glutamine.







labelled and unlabelled glutamine are shown in Figure 2.3.

Preparation of cells for in vivo NMR.

Carrot suspension cultures were grown in liquid Murashige and Skoog (1962) medium supplemented with 25 g/l sucrose, 0.1 mg/l 2,4-D and 0.1 mg/l zeatin. The cells were subcultured every 14 d by pipetting one volume of carrot culture into 20 volumes fresh medium. The cultures were incubated at 25 °C on an orbital shaker operating at 130 rpm under a 12 h light (1000 lux): 12 h dark regime. Pre-stationary phase cells (11 d) were harvested by gentle filtration through a 40 um nylon mesh. The cells (approximately 12 g fresh weight) were resuspended in nitrogen free medium supplemented with 5 g/L sucrose at pH 5.6 to give a total volume of 25 ml, corresponding to a packed cell volume of 50%. The suspension was transferred to a 20 mm diameter NMR tube and cells were oxygenated with an airlift system operating with an oxygen flow rate of up to 50 ml/min (Fox et al 1989). For experiments performed in a 10 mm diameter NMR tube, approximately 2.5 g fresh weight of cells were resuspended in 6 ml of medium and the suspensions oxygenated using a scaled down version of the airlift system operating at 20 ml/min. The cells were allowed to stabilise in the NMR tube for 3 h before the acquisition of spectra. Enzyme inhibitors were added to the suspending medium in the NMR tube immediately after the transfer of the cells and ^{15}N-labelled glutamate or ammonium chloride were added at the end of the 3 h equilibration period. The initial extracellular concentration was 2 mM for the inhibitors and 20 mM for the labelled glutamate and ammonium chloride.

^{15}N NMR spectroscopy

^{15}N NMR spectra were recorded at 30.42 MHz on a Bruker CXP 300 spectrometer using a 10 or 20 mm diameter broad band frequency probehead. Cell suspensions were oxygenated continuously with the airlift system and the temperature was maintained at 25°C. ^{1}H-coupled ^{15}N NMR spectra were usually accumulated with a 90°

pulse angle, a recycle time of 5 s and a total acquisition time of 1 h for qualitative experiments and a 30° pulse angle, a 20 s recycle time and a 4 h acquisition time for quantitative experiments. ^{1}H-decoupled NMR spectra were usually accumulated with a 90° pulse angle, a recycle time of 2 s, a total acquisition time of 1 h and low power decoupling for 1.75 s prior to acquisition to produce the nuclear Overhauser enhancement (NOE) and high power decoupling for 0.25 s during the acquisition. INEPT (Insensitive Nuclei Enhanced by Polarisation Transfer) spectra were usually accumulated with a 90° pulse angle, a 2.35 s recycle time and an acquisition time of 30 min. Chemical shifts are quoted relative to the resonance at 0 ppm from nitrate.

CHAPTER 3: GENERAL CHARACTERISTICS OF NITROGEN METABOLISM IN CARROT CELL SUSPENSION CULTURE.

Cell suspension culture provides a simplified homogeneous experimental system with which to study nitrogen metabolism in higher plants. By using a heterotrophic cell culture it is possible to tightly control the nutrients available and to alter these with little disruption to the cells. Using this system it was possible to alter the nitrogen available to the cells, provide ^{15}N labelled substrates and also to investigate the effect of sucrose starvation on the nitrogen metabolism of the cells.

The initial experiments performed investigated the nitrogen assimilation of the cells in the standard maintenance media, MDK. This contained 20mM ammonium and 40mM nitrate, levels of nitrogen far in excess of those normally experienced by plant cells. The investigation concerned the uptake of nitrogen from the medium, changes in intracellular nitrogen pools and the specific activities of three enzymes involved in nitrogen metabolism, glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). For full experimental details see Figure 3.1.

The increase in cell fresh weight and the uptake of sucrose from the medium are shown in figures 3.1 and 3.2. When the cells were placed in fresh medium there was a period of conditioning before they start to divide, lasting approximately two days. The period after this was characterised by exponential growth lasting approximately eight days (Fig 3.1). After 11 days growth ceased presumably because the cells had exhausted supplies of certain essential nutrients, and the culture then entered the stationary phase. One of the nutrients which was lacking during this period was sucrose. The data in figure 3.2 shows that less than 1% of the medium sucrose remained by day 11.

Figure 3.1

Changes in fresh weight (g/flask) over 14 day culture period.

Experimental method.

14 flasks containing 35ml of MDK media were innoculated with 7ml (0.8gfw) of suspension culture from a stationary phase (14 d) culture. At daily intervals the contents of one flask were centrifuged to separate the cells from the medium. After determining the fresh weight of the cells, enzyme extractions were performed and the activities of GS, GDH, and GOGAT were determined. Methanol extracts from the cells were analysed for ammonium and soluble amino acid pools. Following methanol extraction the cells were analysed for total extractable protein. The media samples were analysed for ammonia, nitrate and sucrose concentration. All measurements were performed in triplicate.

Figure 3.1

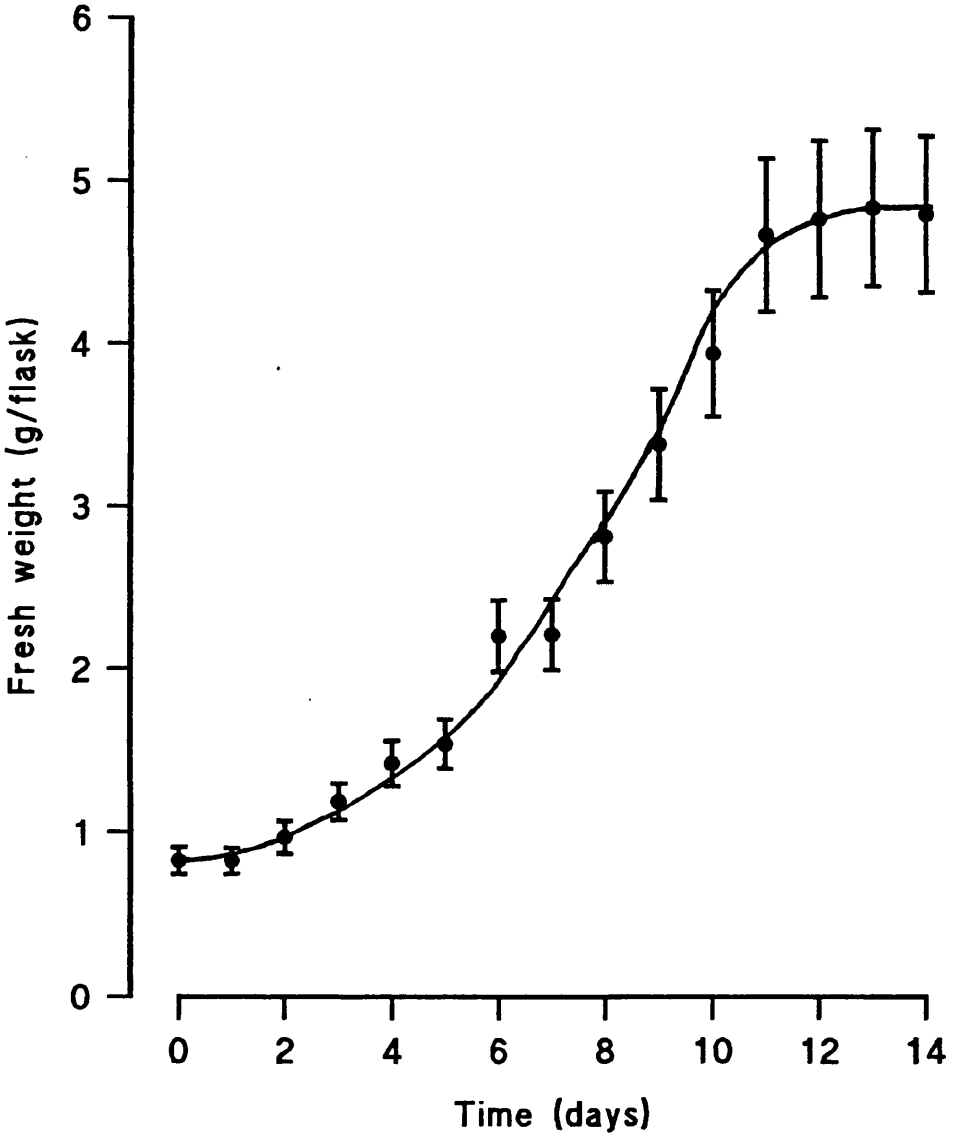
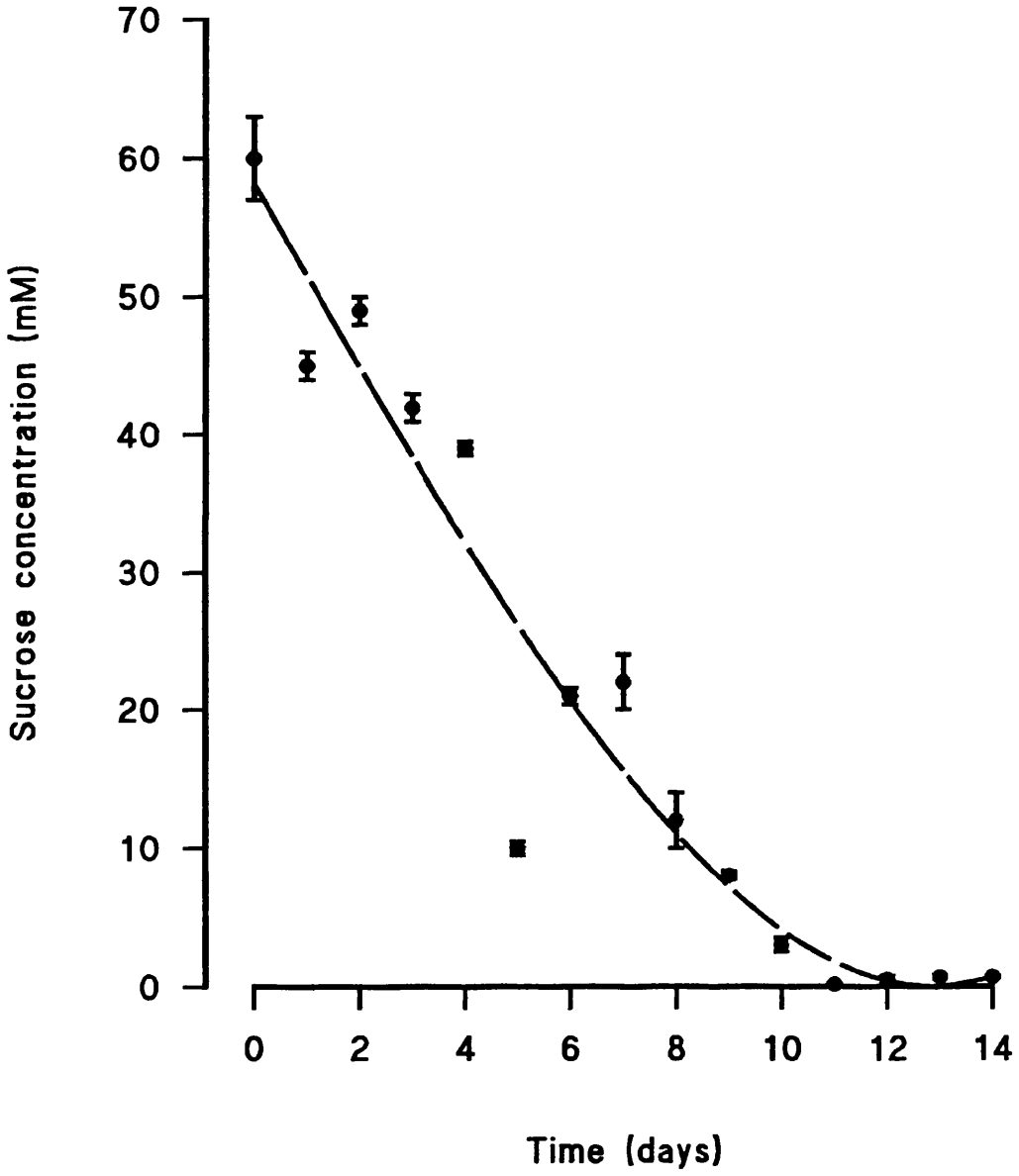


Figure 3.2 Changes in medium sucrose concentration (mM) over 14 day culture period.



The uptake of nitrate and ammonium from the medium is shown in figure 3.3. By the end of the culture period the cells had utilised 49% of the total nitrogen available, (63% of the ammonium and 42% of the nitrate). Since 51% of the original nitrogen was still available on day 14 the cultures were never limited by nitrogen supply. Uptake of ammonium began as soon as the cells were placed in fresh medium, whereas nitrate uptake began after a delay of 24 hours. From day 0 until day 9 ammonium was taken up at a mean rate of 66 umol/gfw/d. During the stationary phase of culture there was a net loss of ammonium from the cells to the medium and between day 11 and day 14 the medium ammonium concentration increased from 3.5mM to 7.5mM (Fig 3.3). By day 11 the cells had utilised 82% of the available ammonium, however the release over the last three days led to a final uptake of 63%. Analysis of the intracellular ammonium concentration (Fig 3.4) showed that the cell ammonium level was high when cells were first placed in fresh medium, declined sharply, and remained at a low level over the first 10 days. From day 11 onwards there was an increase in the cell ammonium concentration from 1.66 umols/gfw to 11.61 umols/gfw. These changes in cell ammonium mirror the increases in medium ammonium.

The uptake of nitrate from the medium (Fig 3.3) was most rapid (62 umol/gfw/d mean value) between day 2 and day 9, during which time the cells took up 29% of the available nitrate. Over the remaining five days nitrate uptake continued at a slower rate (mean value 46 umol/gfw/d). By the end of the culture period 42% of the available nitrate had been utilised.

The changes in the specific activities of GS, GOGAT and GDH over the culture period are shown in figure 3.5. GS and GOGAT activities increased when the cells were placed in fresh medium, remained high during the period of rapid cell growth and fell as the cells entered stationary phase. The activities of these enzymes correlate well with the uptake of ammonium and nitrate from the medium as they were most active during the exponential growth period. Table 3.1 shows that

Figure 3.3 Changes in medium concentration of ammonium and nitrate (mM) over 14 day culture period.

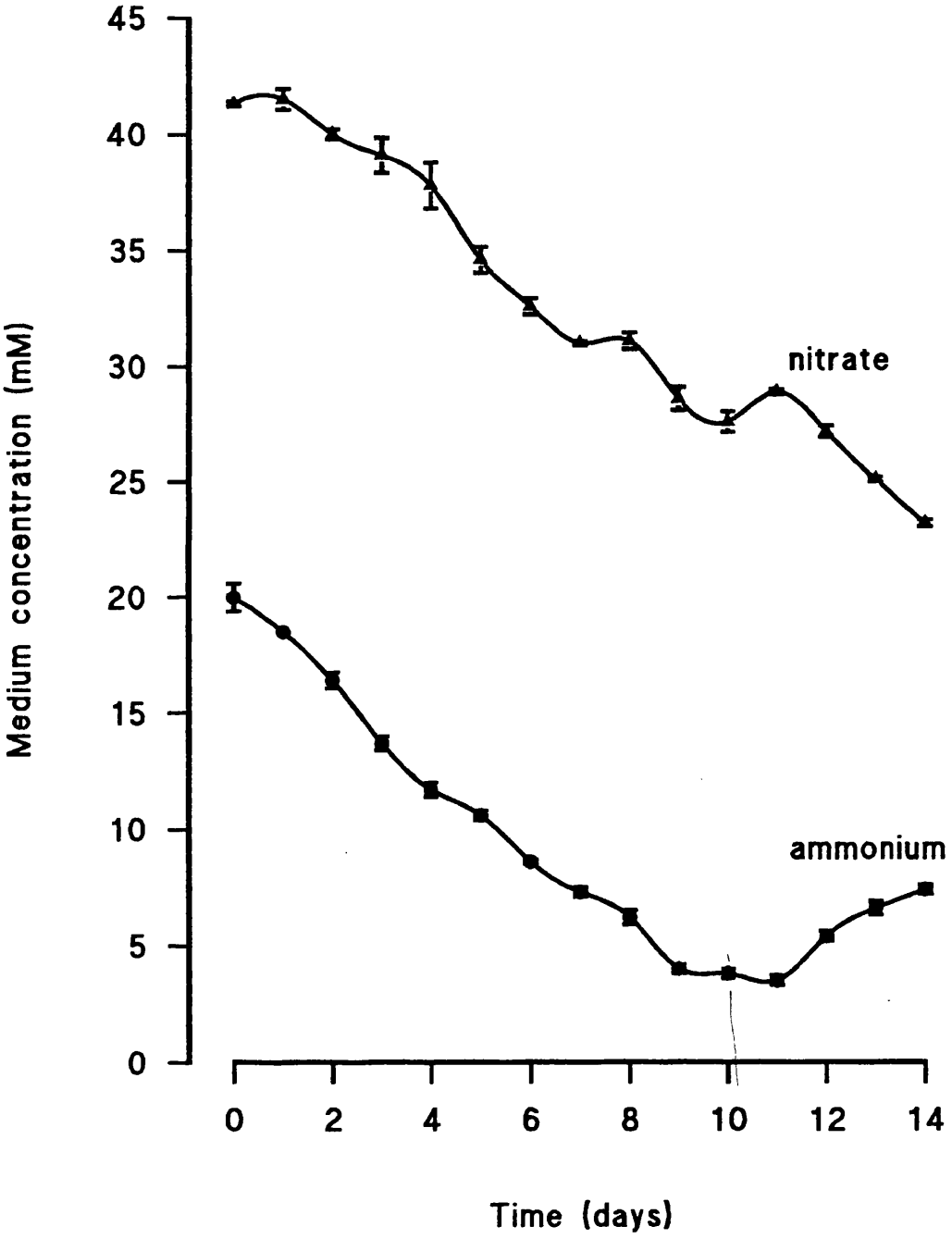


Figure 3.4 Changes in cell ammonium concentration (umols/gfw) over 14 day culture period.

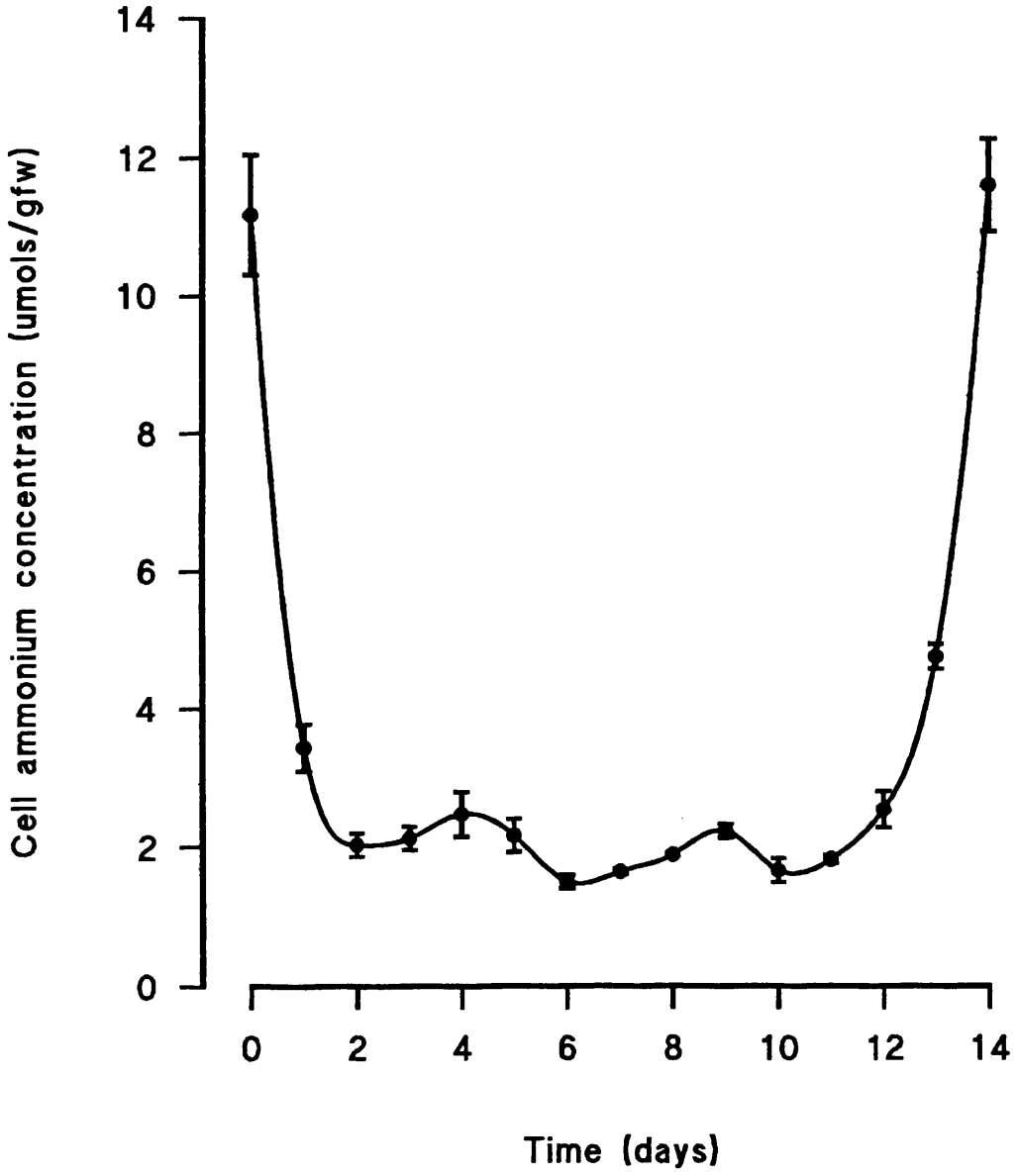


Figure 3.5 Changes in the specific activities of GS, GOGAT and GDH over 14 day culture period. (● GS □ GOGAT ▲ GDH)

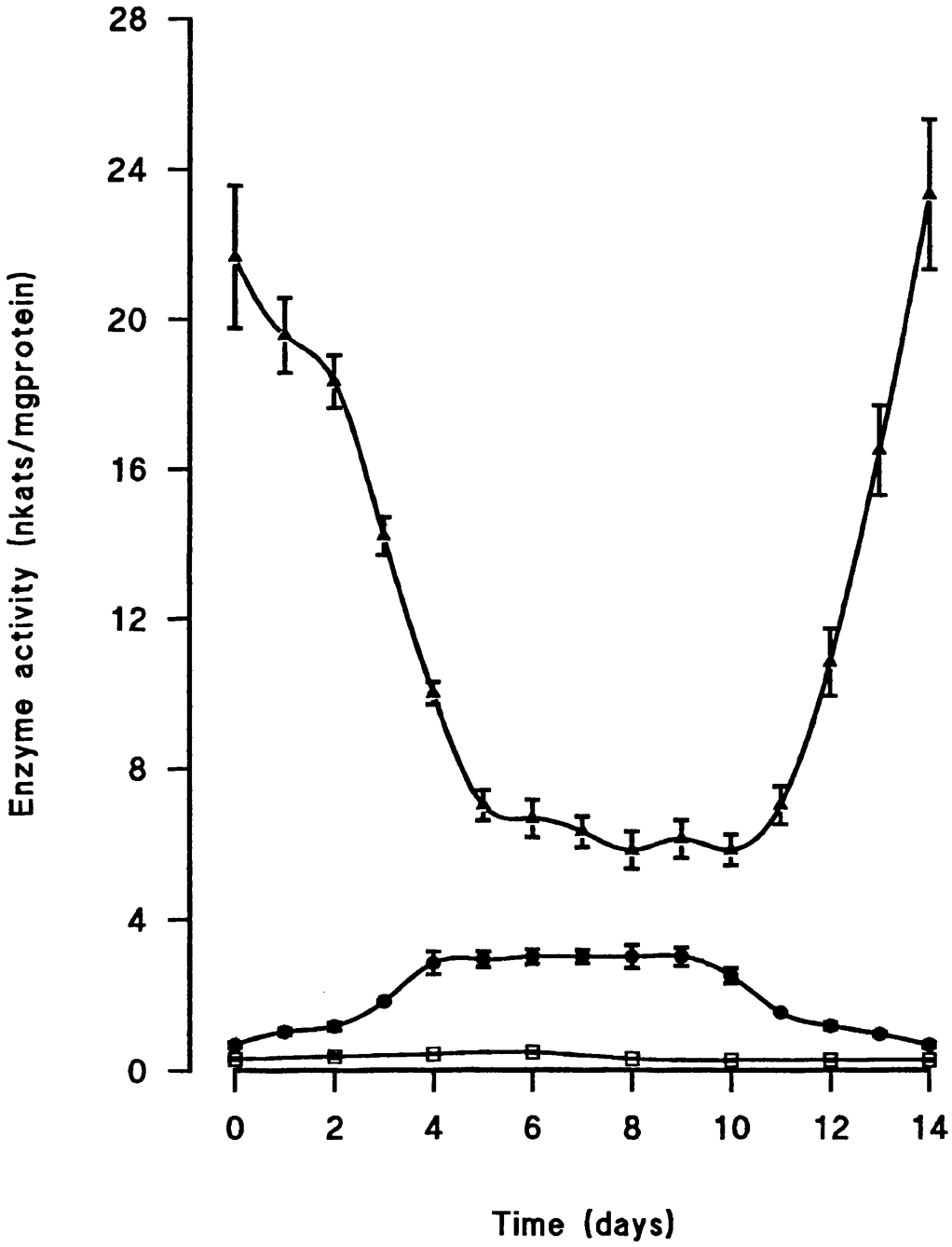


Table 3.1 Potential assimimilation of nitrogen by GS, GOGAT and GDH.

Glutamine synthetase and glutamate synthase.

Days in culture	Cell [NH_4^+] (umols/gfw)	activity (umols/min/gfw)		assimilatory potential (umols/gfw/day)	
		GS	GOGAT	GS	GOGAT
0	11.2	0.18	0.04	259	58
2	2.0	0.29	0.15	418	216
4	2.5	0.77	0.15	1109	216
6	1.5	0.81	0.16	1166	230
8	1.9	0.81	0.08	1166	115
10	1.7	0.65	0.06	936	86
12	2.5	0.29	0.05	418	72
14	11.6	0.18	0.04	259	58

Glutamate dehydrogenase.

Days in culture	Cell [NH_4^+] (umols/gfw)	activity (umols/min/gfw)	assimilatory potential (umols/gfw/day)
0	11.2	5.90	2124
2	2.0	4.51	325
4	2.5	2.70	194
6	1.5	2.07	149
8	1.9	1.44	104
10	1.7	1.51	109
12	2.5	2.63	189
14	11.6	6.44	2318

Nitrogen uptake.

Days in culture	Nitrogen uptake* (umols/gfw/day)
0	-
2	125
4	100
6	80
8	30
10	20
12	<10
14	<10

The assimilatory potential was calculated assuming that both GS and GOGAT were not limited by substrate availability. For GDH, ammonium would certainly limit the rate of assimilation and these figures have been adjusted in accordance with the calculated Km for ammonium. For day 0 and 14 GDH activity is calculated as 25% of its maximum potential and for the remaining days as 5% of maximum. *The nitrogen uptake value is the mean value for nitrate and ammonium uptake over each 2 day interval.

the assimilatory potential of GS and GOGAT is sufficient to account for the observed rates of nitrogen uptake.

The specific activity of GDH shown by these cells is very high. It reaches values ten times higher than those observed in most plant species examined (Stewart and Rhodes, 1978; Pahlick and Joy, 1971). High GDH activity has also been reported in suspension cultures of Soybean grown in medium containing ammonium as the sole nitrogen source (Jain and Shargool 1987). The levels of GDH activity measured in these cells were sufficient to account for ammonium assimilation via the GDH pathway. Using the Km measurements for GDH (Table 2.2 previous chapter) the assimilatory potential for this enzyme was calculated (Table 3.1). From this table it is clear that ammonium assimilation in these cells could occur via either the GDH or GS pathways,and therefore a role for GDH in the assimilation of ammonium can not be ruled out. However the pattern of GDH activity does not correlate with that of nitrogen uptake since GDH levels were highest during the stationary phase of culture when there was very little nitrogen uptake and declined when the cells were placed in fresh medium (Figures 3.3 and 3.5). During the period of rapid ammonium assimilation and cell growth GDH levels were at their lowest. It was also observed that the GDH activity correlates with the concentration of sucrose in the medium (Figures 3.2 and 3.5). GDH activity was highest during the stationary phase of the culture period when the sucrose levels were below 1mM. When the cells were placed in fresh medium containing 60mM sucrose the GDH levels fell. It seems possible that GDH activity responds to the supply of carbon rather than nitrogen. This effect of carbon starvation on GDH activity has been reported by several workers and is discussed above (Chapter 1).

The changes in the cellular amino acid pools over the 14 days of culture are shown in tables 3.2 and 3.3. The data shows the concentration of the amino acids umoles/gfw (Table 3.2) and the proportion that each amino acid contributes to the total soluble pool (percent, Table 3.3). There were large

Table 3.2.

Changes in the soluble amino acid concentration (umols/gfw) over 14 day culture period.

	Concentration (umols/gfw)							
Days	0	1	2	3	4	5	6	7
Amino acid								
Glutamate	1.38	0.91	0.66	0.58	0.73	1.20	1.75	2.31
Glutamine	1.09	2.31	3.43	1.94	1.33	2.01	2.21	2.87
GABA	1.65	1.96	1.52	1.31	1.97	1.88	1.58	1.30
Alanine	0.40	2.93	1.40	1.00	1.46	2.00	2.19	2.39
Arginine	8.38	5.09	2.92	1.59	0.92	0.44	0.25	0.39
Serine	2.75	1.29	0.81	0.54	0.49	0.64	0.69	0.69
Methionine	0.60	0.50	0.29	0.21	0.15	0.17	0.17	0.20
Glycine	0.41	0.33	0.30	0.27	0.31	0.34	0.34	0.33
Valine	4.79	1.74	1.30	0.70	0.60	0.82	0.91	1.06
Aspartate	1.28	0.40	0.26	0.19	0.19	0.29	0.30	0.56
Leucine	1.54	0.71	0.44	0.23	0.23	0.33	0.32	0.32
Isoleucine	2.93	0.96	0.58	0.36	0.33	0.41	0.41	0.36
Threonine	3.25	1.48	1.25	0.82	0.66	0.68	0.72	0.75
Phenylalanine	2.91	0.71	0.21	0.16	0.14	0.16	0.16	0.20
Asparagine	3.63	1.37	1.02	0.67	0.51	0.47	0.48	0.61
Tyrosine	1.35	0.59	0.38	0.24	0.18	0.20	0.19	0.21
Histidine	6.29	3.01	2.83	2.61	2.77	2.80	2.75	2.71
Lysine	0.57	0	0	0	0	0	0	0
Total	45.20	26.29	19.60	13.42	12.97	14.84	15.42	17.26

Table 3.2.cont.

Changes in the soluble amino acid concentration (umols/gfw) over 14 day culture period.

	Concentration (umols/gfw)						
Days	8	9	10	11	12	13	14
Amino acid							
Glutamate	2.17	1.75	1.42	1.4	1.17	0.86	0.78
Glutamine	2.72	2.80	2.43	2.07	1.10	0.67	0.37
GABA	0.87	0.59	0.43	0.54	0.41	0.40	0.48
Alanine	2.10	1.69	1.44	0.72	0.43	0.29	0.27
Arginine	1.94	2.68	4.01	5.15	6.12	6.44	6.14
Serine	0.84	0.85	0.76	0.64	0.66	0.92	1.17
Methionine	0.20	0.20	0.20	0.23	0.25	0.35	0.45
Glycine	0.33	0.32	0.30	0.28	0.27	0.27	0.27
Valine	1.22	1.38	1.39	1.37	1.25	1.30	1.58
Aspartate	0.33	0.33	0.27	0.38	0.42	0.54	0.54
Leucine	0.38	0.43	0.42	0.48	0.41	0.38	0.49
Isoleucine	0.45	0.48	0.43	0.51	0.58	0.66	0.83
Threonine	0.70	0.67	0.59	0.72	0.90	1.07	1.20
Phenylalanine	0.18	0.20	0.19	0.34	0.57	0.86	1.22
Asparagine	0.86	0.89	0.93	1.21	1.37	1.44	1.38
Tyrosine	0.20	0.16	0.17	0.29	0.32	0.38	0.45
Histidine	2.79	2.71	2.30	2.61	3.06	3.34	3.24
Lysine	0	0	0	0	0.26	0.28	0.23
Total	18.28	18.13	17.68	18.94	19.55	20.45	21.09

Table 3.3.

Changes in the soluble amino acid concentration over 14 day culture period (expressed as a percentage of the total pool size).

	Concentration (% of total pool)							
Days	0	1	2	3	4	5	6	7
Amino acid								
Glutamate	3.1	3.5	3.4	4.3	5.6	8.1	11.3	13.3
Glutamine	2.4	8.8	17.5	14.5	10.3	13.5	14.3	16.4
GABA	3.6	7.5	7.8	9.8	15.2	12.7	10.2	7.4
Alanine	0.9	11.1	7.1	7.5	11.3	13.5	14.2	13.6
Arginine	18.5	19.4	14.9	11.8	7.1	3.0	1.6	2.2
Serine	6.1	4.9	4.1	4.0	3.8	4.3	4.5	5.5
Methionine	1.3	1.9	1.5	1.6	1.2	1.1	1.1	1.1
Glycine	0.9	1.3	1.5	2.0	2.4	2.3	2.2	1.9
Valine	10.6	6.6	6.6	5.2	4.6	5.5	5.9	6.0
Aspartate	2.8	1.5	1.3	1.4	1.5	2.0	1.9	3.2
Leucine	3.4	2.7	2.2	1.7	1.8	2.2	2.1	1.8
Isoleucine	6.5	3.7	3.0	2.7	2.5	2.8	2.7	2.1
Threonine	7.2	5.6	6.4	6.1	5.1	4.6	4.7	4.3
Phenylalanine	6.4	2.7	1.1	1.2	1.1	1.1	1.0	1.1
Asparagine	8.0	5.2	5.2	5.0	3.9	3.2	3.1	3.5
Tyrosine	3.0	2.2	1.9	1.8	1.3	1.3	1.3	1.2
Histidine	13.9	11.4	14.5	19.4	21.3	18.8	17.9	15.4
Lysine	1.4	0	0	0	0	0	0	0
Total	100	100	100	100	100	100	100	100

Table 3.3.cont.

Changes in the soluble amino acid concentration over 14 day culture period (expressed as a percentage of the total pool size).

	Concentration (% of total pool)						
Days	8	9	10	11	12	13	14
Amino acid							
Glutamate	11.9	9.7	8.0	7.4	6.0	4.2	3.7
Glutamine	14.9	15.4	13.7	10.9	5.6	3.3	1.8
GABA	4.8	3.3	2.4	2.9	2.1	2.0	2.3
Alanine	11.5	9.3	8.1	3.8	2.2	1.4	1.3
Arginine	10.6	14.8	22.7	27.2	31.3	31.5	29.11
Serine	4.6	4.7	4.3	3.4	3.4	4.5	5.5
Methionine	1.1	1.1	1.1	1.2	1.3	1.7	2.1
Glycine	1.8	1.8	1.7	1.5	1.4	1.3	1.3
Valine	6.7	7.6	7.9	7.2	6.4	6.4	7.5
Aspartate	1.8	1.8	1.5	2.0	2.1	2.6	2.6
Leucine	2.1	2.4	2.4	2.5	2.1	1.9	2.3
Isoleucine	2.5	2.6	2.4	2.7	3.0	3.2	3.9
Threonine	3.8	3.7	3.3	3.8	4.6	5.2	5.7
Phenylalanine	1.0	1.1	1.1	1.8	2.9	4.2	5.8
Asparagine	4.7	4.9	5.3	6.4	7.0	7.0	6.5
Tyrosine	1.0	0.9	1.0	1.5	1.6	1.9	2.1
Histidine	15.2	14.9	13.1	13.8	15.7	16.3	15.3
Lysine	0	0	0	0	1.3	1.4	1.1
Total	100	100	100	100	100	100	100

changes in the total soluble amino acid pool over the 14 days. When the cells were placed in fresh medium the total pool size droped by 71% over the first four days but from day 5 onwards there was a steady increase in the soluble amino acid pool until the end of the culture period (Table 3.2).

The amino acids fall into two main groups, those whose concentration increased when the cells were transferred to fresh medium and those which accumulated during the stationary phase and showed a decrease in pool size when the cells were placed in fresh medium. Glutamine, glutamate, alanine and GABA belong to the first group, all showing substantial increases in pool size over the first seven days of culture; by day 7 they together constitute 50% of the total pool (Table 3.3). The increases in concentration of amino acid over the first day were; glutamine 112%, alanine 638%, GABA 19%. Glutamate levels declined when cells were first transferred to fresh medium but then increased by almost 300% from day 3 to day 7. Glycine and aspartate showed small increases during the mid phase of the culture period, after which glycine concentrations declined but those of aspartate remained high.

The remaining amino acids, which were detected by HPLC, showed a decline in pool size when the cells were placed in fresh medium. The most dramatic decline in pool size was shown by arginine which was the predominant amino acid during the stationary phase (31%) but declined to less than 2% by day 6, The fall in absolute concentration was from 8.4 umols/gfw on d 0 to 0.25 umols/gfw on d 6 a decline of 97%. Histidine also represented a large proportion of the total pool but remained relatively constant as a proportion of the pool, throughout the culture period, fluctuating between 2.65-3.34 umols/gfw or 15 and 21% of the total pool.

The amino acids which increase during the first half of the culture period represent some of the most metabolically active amino acids. Glutamine, glutamate and aspartate are key amino acids in terms of both their role in amino acid metabolism and

protein synthesis. These amino acids would act as precursors in the synthesis of the other amino acids. The initial fall in the concentration of glutamate is probably a reflection of its particular role in the glutamate synthase cycle and as an amino donor for transamination, and initially the demand for glutamate may exceed the rate of synthesis. The fact that the other amino acids do not accumulate during the rapid growth phase implies that synthesis of amino acids and proteins are tightly coupled.

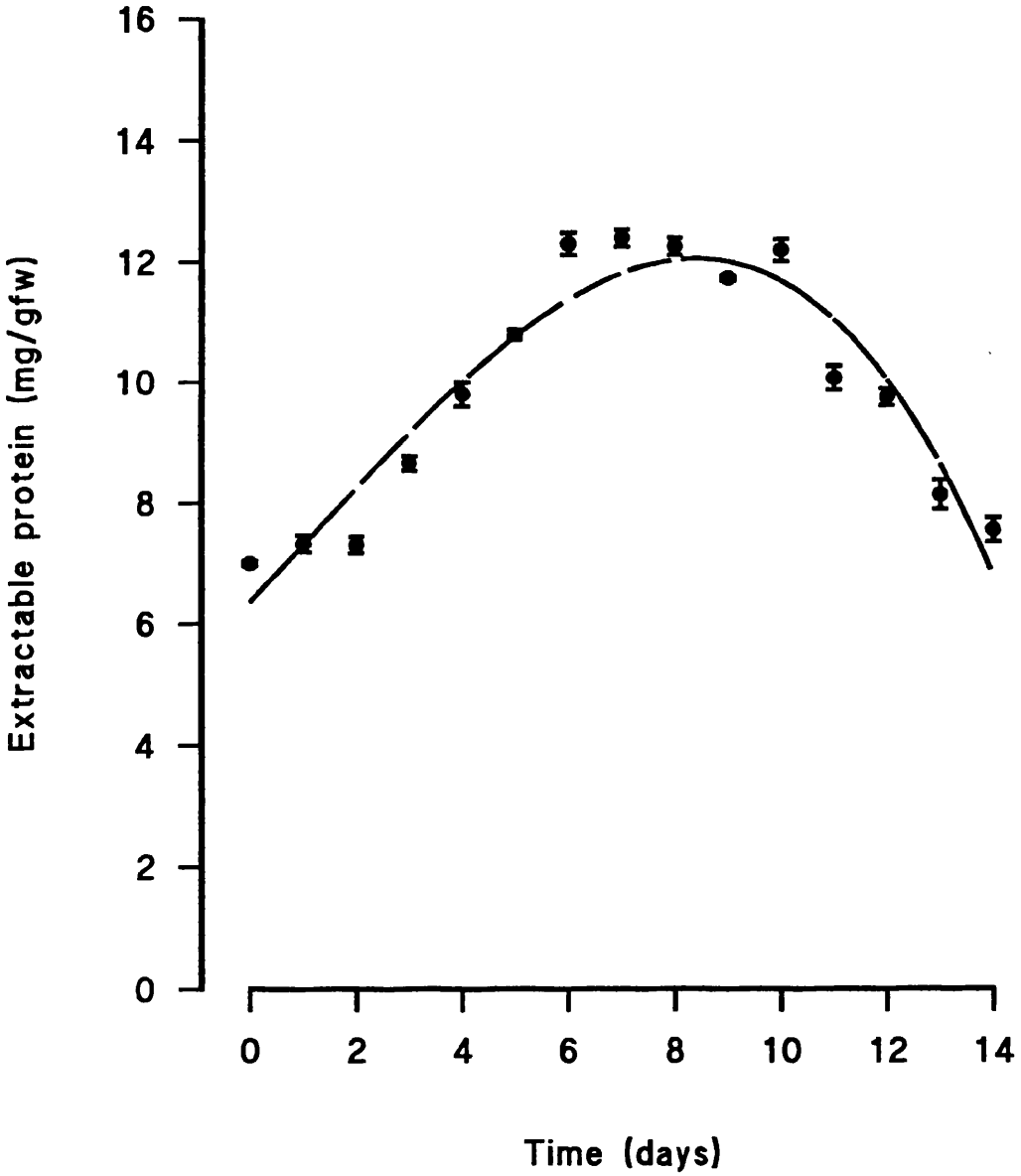
The accumulation of amino acids during the stationary phase occured at a time when protein turnover exceeded new protein synthesis resulting in a net fall in cell protein content (see below). The catabolism of protein would result in the release of the 20 protein amino acids, some of which would be catabolised to ammonium but those which were not easily metabolised would accumulate. When the cells are placed in fresh medium the availability of carbon, in the form of sucrose, permits protein synthesis and consequently amino acid pools decrease. Accumulation of amino acids has been shown to occur in Lemna minor in response to treatment with the GS inhibitor methionine sulphoximine (MSO) (Rhodes 1986). The amino acids which accumulated in Lemna are the same as those which accumulate in these cells. Glutamate, glutamine, alanine, aspartate, asparagine and serine did not accumulate in Lemna and Rhodes (1986) suggested that this was because these amino acids were readily catabolised. Rhodes suggested that the major pathway for catabolism of these amino acids would be the photorespiratory cycle, but this would not operate in these carrot cells. However, the accumulation of ammonium in the medium during stationary phase suggests that some other mechanism of amino acid catabolism exists in the carrot cells. The lack of a photorespiratory pathway may explain why serine accumulates in these cells but not in Lemna.

The high levels of arginine and histidine are probably a consequence of the high concentration of nitrogen in the medium. Both these amino acids have high nitrogen to carbon

ratios compared to the other amino acids and they may be synthesised in order to store the nitrogen in a form which does not involve too much carbon. Arginine is known to be a nitrogen storage compound in seeds (Chou and Splittstoessser 1972), but this role has not been reported for histidine. If these amino acids are involved in storage of nitrogen they would occur mainly as vacuolar pools.

Changes in total extractable protein over the 14 d growth period are shown in figure 3.6. After a 2 day lag following transfer to fresh medium the protein content of the cells increased up to day 6. Levels remained steady between d 6 and d 10 and fell between d 10 and d 14. The increase in protein content was 5 mg/gfw an increase of 71% over the protein content at d 0. The increase in protein reflects the metabolic activity of the cells during this time. As the cells entered stationary phase there was a drop in the protein content of 1 mg/gfw/day. The timing of this change corresponds with that of increases in the soluble amino acid pools and also of the release of ammonium from the cells to the medium. This apparent catabolism of protein occurs at a time when the cells have exhausted their sucrose supply and may provide the cells with carbon skeletons for metabolic reactions. When the cells were placed in fresh medium there was an increase in protein and a corresponding decline in cell and medium ammonium levels as well as in the amino acid pools.

Figure 3.6 Changes in total cell protein (mg/gfw) over 14 day culture period.



Incorporation of ^{15}N labelled ammonium and nitrate into amino acids.

In order to further investigate the assimilation of ammonium and nitrate into amino acids, the uptake and incorporation of ^{15}N labelled ammonium and nitrate was followed over a six day period. Two sets of media containing 5mM ammonium chloride and 10mM potassium nitrate were used with ^{15}N label present in either the ammonium or the nitrate ion (Experimental details are given with Figure 3.7).

The uptake of nitrate and ammonium ions from the medium was similar to that described previously. Ammonium was taken up rapidly and had been completely removed from the medium by d 6, while nitrate uptake was not detected until the second day (Fig 3.7). From d 2 to d 6 ammonium and nitrate were taken up at similar rates. Over the 6 d culture period 155 umols/gfw of ammonium and 150 umols/gfw of nitrate were taken up by the cells.

The incorporation of ^{15}N label into amino acids from the ammonium and nitrate are shown in tables 3.4 and 3.5. ^{15}N label was detected in almost all the amino acids after 24 hours in medium containing [^{15}N]ammonium (Table 3.4). The highest concentration of label was seen in glutamine, (87 atom % excess or 2.8 umols/gfw). Histidine, glutamate, alanine, GABA and glycine were all labelled to at least 30 atom % excess by day 1. Analysis of the labelling of glutamine showed that initially the label was found predominantly in the amide group with the amino group showing a slower accumulation of label. This pattern of labelling suggests that ammonium was being assimilated by GS into the amide group of glutamine and then transferred to glutamate by GOGAT. If ammonium were assimilated by GDH the label would occur mainly in glutamate and would be equally distributed between the amino and amide groups of glutamine. The decline in glutamine label after d 1 was due to the dilution effect of unlabelled nitrate assimilation. As the glutamate pool became labelled a higher

Figure 3.7

Changes in medium ammonium and nitrate concentration (mM) over 6 day culture period.

Experimental method.

A total of 15mM nitrogen was used in these cultures and the samples were taken over a 6 day period. In order to minimise carry over of unlabelled nitrogen, 14ml of innoculating suspension culture was centrifuged aseptically to remove excess media prior to being added to flasks containing 70 ml labelled nitrogen. The flasks contained 5 mM ammonium chloride and 10 mM potassium nitrate with ^{15}N label present either in the ammonium or nitrate ion. At daily intervals one flask from each treatment was harvested. GS and GDH activities were determined and cell extracts were analysed by HPLC and GC-MS to determine amino acid pool size and ^{15}N labelling of amino acids. Media samples were analysed for ammonium and nitrate concentration. The changes in GS and GDH activities were similar to those shown previously for the 14 d cultures.

Figure 3.7

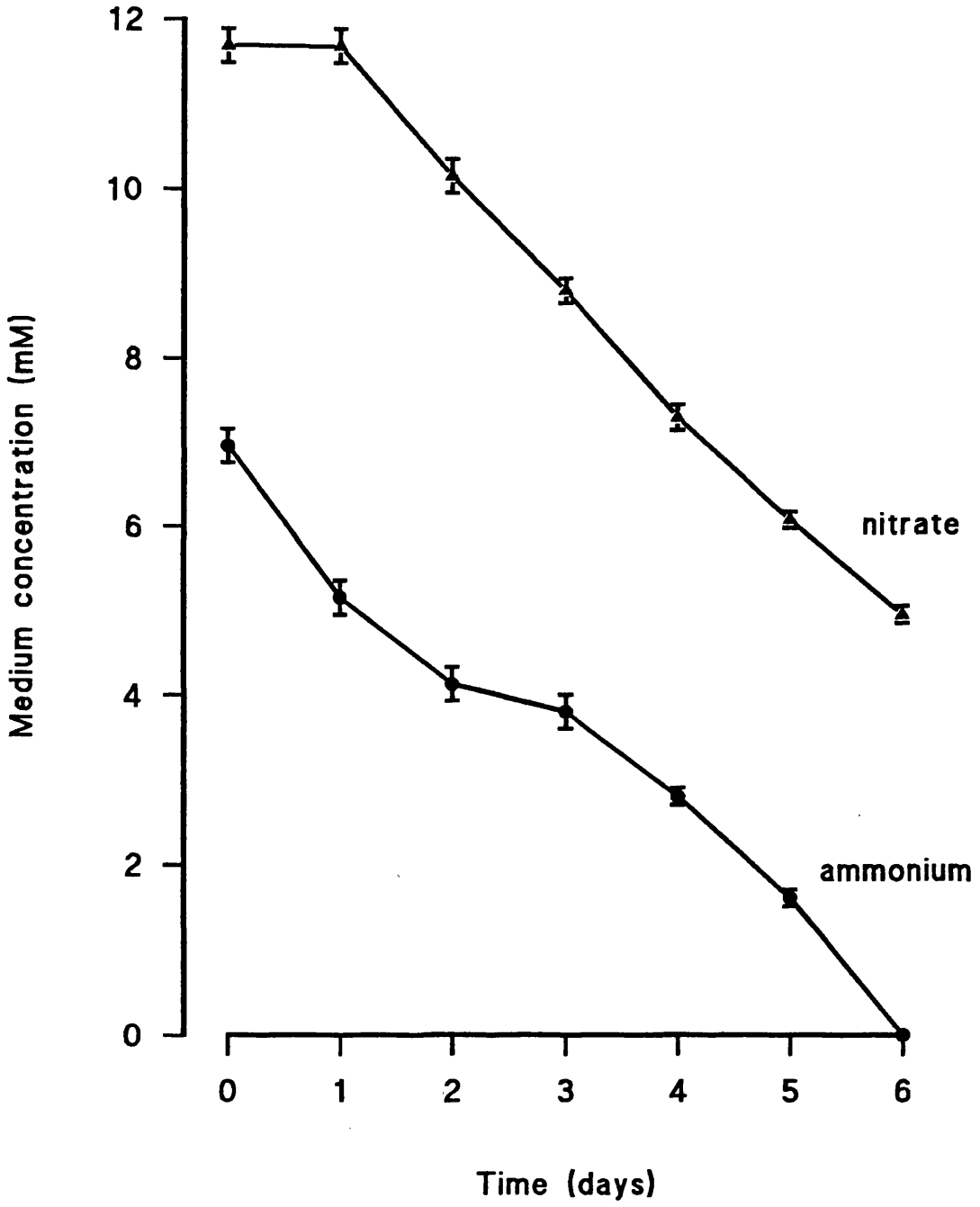

12
10
8
6
4
2
0
Medium concentration (mM)
nitrate
ammonium
0
1
2
3
4
5
6
Time (days)

Table 3.4A.

Incorporation of ^{15}N label into amino acids (atom % excess) during 6 day growth in medium containing [^{15}N] ammonium.

		Incorporation of ^{15}N (atom % excess)					
	Days	1	2	3	4	5	6
Amino acid							
Glutamate		36	43	40	44	43	54
Glutamine	-amino	35	43	29	43	44	33
	-amide	52	68	59	57	54	22
GABA		35	44	43	45	42	25
Alanine		48	53	45	46	44	6
Arginine		3	1	0	0	0	0
Serine		24	32	34	33	40	49
Methionine		14	19	8	2	32	32
Glycine		16	21	27	28	37	20
Valine		17	29	30	35	42	32
Aspartate		19	21	26	36	44	52
Leucine		29	43	37	35	40	24
Isoleucine		20	35	35	51	39	31
Threonine		16	22	28	33	45	59
Phenylalanine		19	19	21	17	27	28
Asparagine	-amino	6	7	7	22	37	30
	-amide	5	8	16	23	38	30
Tyrosine		9	19	29	33	42	67
Proline		34	36	36	45	48	47
Histidine	-total	30	43	58	73	96	97
	-single	7	7	9	15	22	23
	-double	7	9	14	17	22	22
	-triple	3	6	7	8	10	10
Lysine		0	0	0	0	0	0

Table 3.4B

Incorporation of ^{15}N label into amino acids(umols/gfw) during 6 days growth in medium containing [^{15}N] ammonium.

		Incorporation of ^{15}N (umols/gfw)					
	Days	1	2	3	4	5	6
Amino acid							
Glutamate		0.29	0.21	0.22	0.41	0.58	1.15
Glutamine	-amino	1.12	0.74	0.28	0.58	0.86	0.39
	-amide	1.67	1.17	0.58	0.78	1.05	0.26
GABA		0.44	0.86	1.19	1.04	0.76	0.17
Alanine		0.68	0.47	0.31	0.45	0.52	0.06
Arginine		0.14	0.03	0	0	0	0
Serine		0.29	0.23	0.17	0.18	0.28	0.33
Methionine		0.11	0.10	0.03	0.01	0.07	0.05
Glycine		0.05	0.03	0.08	0.08	0.11	0.04
Valine		0.39	0.43	0.25	0.29	0.40	0.27
Aspartate		0.12	0.07	0.06	0.09	0.17	0.23
Leucine		0.23	0.21	0.10	0.10	0.12	0.06
Isoleucine		0.25	0.28	0.15	0.19	0.14	0.10
Threonine		0.30	0.28	0.22	0.20	0.28	0.35
Phenylalanine		0.08	0.09	0.06	0.03	0.06	0.05
Asparagine	-amino	0.12	0.10	0.04	0.08	0.14	0.11
	-amide	0.10	0.10	0.10	0.09	0.15	0.11
Tyrosine		0.07	0.10	0.08	0.07	0.07	0.11
Proline		0.09	0.02	0.05	0.12	0.18	0.31
Histidine		1.16	1.48	1.82	1.95	2.36	2.11
Lysine		0	0	0	0	0	0
Total		7.70	7.00	5.79	6.74	8.30	6.26

Table 3.5A.

Incorporation of ^{15}N label into amino acids (atom % excess) during 6 days growth in medium containing [^{15}N] nitrate.

		Incorporation of ^{15}N (atom % excess)					
	Days	1	2	3	4	5	6
Amino acid							
Glutamate		0	22	28	33	42	49
Glutamine	-amino	0	11	21	28	35	33
	-amide	0	15	18	21	26	44
GABA		0	82	30	33	39	37
Alanine		0	21	32	42	42	51
Arginine		0	1	0	0	0	1
Serine		0	7	15	26	33	33
Methionine		0	0	0	5	28	20
Glycine		0	0	2	19	28	27
Valine		0	8	18	28	34	27
Aspartate		0	3	10	23	37	37
Leucine		0	12	22	29	37	33
Isoleucine		0	8	20	28	35	28
Threonine		0	7	12	22	29	22
Phenylalanine		0	1	9	16	25	15
Asparagine-amino		0	3	1	14	20	11
	-amide	0	0	6	1	23	12
Tyrosine		0	0	0	19	25	15
Proline		0	13	13	24	31	35
Histidine	-total	0	9	13	0	59	0
	-single	0	2	7	0	20	0
	-double	0	2	3	0	12	0
	-triple	0	1	0	0	5	0
Lysine		0	0	0	0	0	0

Table 3.5B.

Incorporation of ^{15}N label into amino acids (umols/gfw) during 6 days growth in medium containing [^{15}N] nitrate.

		Incorporation of ^{15}N (umols/gfw)					
	Days	1	2	3	4	5	6
Amino acid							
Glutamate		0	0.09	0.14	0.32	0.48	1.04
Glutamine	-amino	0	0.17	0.20	0.48	0.64	0.39
	-amide	0	0.22	0.17	0.36	0.47	0.52
GABA		0	1.50	0.79	0.83	1.02	0.25
Alanine		0	0.23	0.24	0.61	0.62	0.53
Arginine		0	0.03	0	0	0	<0.01
Serine		0	0.05	0.08	0.18	0.23	0.22
Methionine		0	0	0	0.02	0.07	0.03
Glycine		0	0	0.01	0.06	0.09	0.06
Valine		0	0.12	0.15	0.25	0.33	0.22
Aspartate		0	0.01	0.02	0.07	0.10	0.16
Leucine		0	0.06	0.06	0.10	0.12	0.08
Isoleucine		0	0.06	0.09	0.11	0.13	0.09
Threonine		0	0.08	0.09	0.15	0.19	0.13
Phenylalanine		0	0.01	0.03	0.04	0.06	0.03
Asparagine	-amino	0	0.04	<0.01	0.06	0.08	0.04
	-amide	0	0	0.03	<0.01	0.09	0.04
Tyrosine		0	0	0	0.04	0.05	0.02
Proline		0	0.01	0.02	0.06	0.12	0.23
Histidine	-total	0	0.31	0.39	0	1.48	0
	-single	0	0.07	0.21	0	0.50	0
	-double	0	0.07	0.09	0	0.30	0
	-triple	0	0.03	0	0	0.13	0
Lysine		0	0	0	0	0	0
Total		0	3.16	2.82	3.75	7.30	4.09

proportion of amino labelling was seen in the glutamine causing a second increase in the labelled pool. Although the data suggests that the majority of assimilation is occurring via the GS/GOGAT pathway, a small role for GDH in ammonium assimilation cannot be ruled out on the basis of this experiment.

The label in glutamate remained steady for the first three days and then showed an increase from d 3 (Table 3.4). The important role of glutamate both within the GS pathway and as an amino donor for transamination means that glutamate would be metabolised quickly over the first few days and label would be transferred to other amino acids. From day 3 onwards there was an increase in the pool of glutamate and a corresponding increase in label.

The amino acids belonging to the aspartate family showed a similar pattern of labelling, asparagine in particular closely reflecting the labelling of aspartate. There was a steady increase in labelling of histidine over the first five days but arginine did not accumulate label to any significant extent.

The dilution effect of unlabelled nitrate assimilation can be seen in most of the amino acids during the second or third days of culture. The incorporation of [^{15}N]nitrate into amino acids (Table 3.5) shows clearly that nitrate assimilation begins to contribute to amino acid synthesis from d 2 onwards. This correlates well with the uptake data (Fig 3.7) which shows there was a lag of one day before nitrate was taken up from the medium and explains the dilution effect observed in the [^{15}N]ammonium treatment (Table 3.4). In the [^{15}N]nitrate treatment most amino acids were labelled by day 2, although the incorporation was lower than that observed during the first days of the [^{15}N]ammonium treatment. The level of incorporation remained low relative to that observed when [^{15}N]ammonium was supplied. This indicated that there was significant incorporation of unlabelled ammonium into these amino acids. The pattern of labelling showed general

increases from day 2 to day 5 but for most amino acids a subsequent decline on day 6. This was probably due to dilution of the pools with amino acids released from protein catabolism. A large proportion of the ^{15}N labelled amino acids must be incorporated directly into protein, since the maximum increase in total amino acid label was 7.7 umol/gfw/d, but the uptake of ^{15}N label from the media occured at rates of up to 130 umols/gfw/d.

The ^{15}N labelling data corresponds with the pattern of nitrate and ammonium uptake from the medium, and it is clear that the cells start to assimilate ammonium as soon as they are placed in fresh medium. The activities of GS and GOGAT increase rapidly over the first days in culture allowing rapid assimilation of ammonium into glutamine, glutamate and other amino acids. The lag in nitrate uptake is reflected in a delay in the assimilation of [^{15}N]nitrate and presumably this reflects a delay in the induction of the enzymes of nitrate assimilation. From the second day of the culture period the two nitrogen sources are utilised at approximately equivalent rates. The uptake of nitrate is also important to balance the uptake of ammonium ions. Ammonium absorption by the cell must be electrically balanced either by the excretion of H^+ into the medium or by the uptake of a negative counter ion. This balancing effect probably accounts for the requirement for nitrate in addition to ammonium in plant tissue culture medium. In other experiments it was found that cells died within 2 days of transfer if grown in medium containing 15 mM ammonium as the sole nitrogen source, (the pH of the medium was reduced from 5.8 to 3.0 over 2 days).

The results from these experiments offer substantial support to the role of the glutamate synthase cycle as the major route of ammonium assimilation in these cells. However, from the results of the ^{15}N labelling experiment alone it is impossible to exclude entirely a small but significant role for GDH in this process. In order to investigate this further the assimilation of [^{15}N]ammonium was followed in the presence and absence of the GS inhibitor methionine sulphoximine (MSO).

CHAPTER 4: AMMONIUM ASSIMILATION IN CARROT CELL CULTURES.

^{15}N labelling studies

From the results described in the previous chapter it seems likely that ammonia assimilation occurs primarily via the glutamate synthase cycle although the high levels of GDH activity could conceivably account for at least some of the ammonia assimilation in these cells. To clarify the situation the incorporation of ^{15}N ammonia in the presence and absence of the GS inhibitor methionine sulphoximine (MSO), was investigated. All experiments were initiated with stationary phase cells to ensure high levels of GDH activity and enable a stringent determination of any assimilatory action by GDH. The incorporation of ^{15}N label into amino acids was determined by GC-MS and *in vivo* NMR spectroscopy.

In vivo NMR spectroscopy.

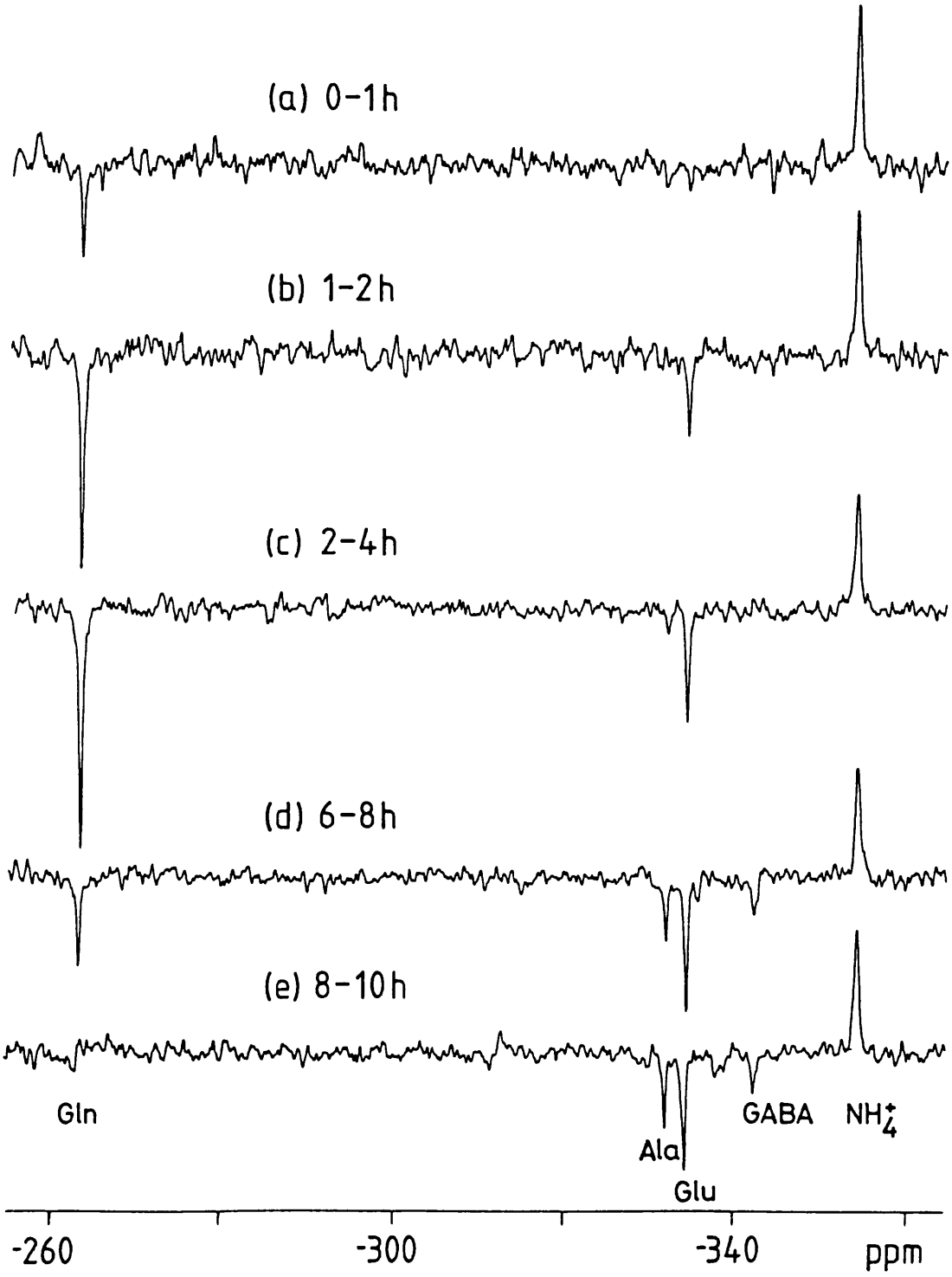
The ^{15}N NMR spectra obtained from *in vivo* NMR of cells in the presence of 10mM $[^{15}N]H_4$ over a 10 hour period are shown in figure 4.1 (a-e). The label from the ammonia appears in the amide-N of glutamine during the first hour (fig 4.1a) and this pool steadily increases over a 6 hour period before declining rapidly. Label is observed in the amino-N of glutamate/glutamine during the second hour (fig 4.1b), increases to a steady state by 4 hours, and remains constant over the remaining 6 hours of the experiment. Alanine label first appears after 2 hours (fig 4.1c) and increases over the remaining 8 hours. GABA is labelled at 6 hours (fig 4.1d) and the level of labelling remains constant until 10 hours. The ammonia label decreases over the first 4 hours (fig 4.1a-c).

The finding that label is first incorporated into the amide of glutamine is consistent with the operation of the glutamate synthase cycle as the route of primary ammonium assimilation in these cells. Similar results have been obtained previously

Figure 4.1

^{1}H-decoupled ^{15}N NMR spectra of 11 d carrot cells following the addition of 20 mM [^{15}N]ammonium chloride. The spectra were obtained in one or two hours over the time intervals indicated. The resonances were assigned on the basis of their chemical shift (Martin, 1985; Thorpe et al, 1989). Several amino acids give an amino resonance at approximately -335 ppm and it was necessary to use the GC/MS data to assign the resonance at -334.8 ppm as predominantly glutamate.

Figure 4.1



by *in vivo* ^{15}N NMR in the ectomycorrhizal fungus *Cenococcum graniforme* (Martin, 1985) and the shoot forming cultures of white spruce buds (Thorpe et al, 1989) as well as in several *in vitro* studies on extracts (Martin et al, 1986; Kanamori et al, 1988; Monselise et al, 1987 and Martin et al, 1988). Insensitive nuclei enhanced by polarisation transfer (INEPT) experiments showed that label appeared in the amide of glutamine within 30 minutes of exposure to [^{15}N]ammonium (Fig 4.2). Labelling of the amino acids did not occur in the presence of MSO, suggesting that GDH does not play a significant role in the assimilation of ammonia.

The assimilation of ^{15}N-labelled ammonium was followed by three different NMR methods; ^{1}H coupled, broad band ^{1}H decoupled with nuclear Overhauser enhancement (NOE) and insensitive nuclei enhanced by polarisation transfer (INEPT) experiments (fig 4.3). The ^{1}H coupled experiment is the simplest method but because the ^{15}N nuclei are bonded to ^{1}H nuclei there is an interaction known as "coupling", which can split the ^{15}N intensity producing more than one signal. Since a high level of background noise is inevitable in *in vivo* studies, this makes the ^{15}N signal difficult to detect. To counter the "coupling" interaction a technique known as ^{1}H decoupling is used in which the ^{15}N signal is not split and is therefore easier to detect. When decoupling is employed there is an enhancement of the signal known as the nuclear Overhauser enhancement (NOE). This NOE factor varies for each individual molecule, depending on the interaction with other molecules. The third technique known as insensitive nuclei enhanced by polarisation transfer (INEPT) is potentially the most sensitive. Here, the ^{1}H nuclei are first excited and by virtue of the coupling between the ^{1}H and ^{15}N nuclei it is possible to transfer the polarisation from the excited ^{1}H nuclei to the ^{15}N nuclei. This results in a greater signal from ^{15}N than is possible using broad band decoupling.

In Figure 4.3 spectra from the same sample have been acquired using each of the three methods described above. The sample contains cells in the process of assimilating [^{15}N]ammonium.

Figure 4.2

Insensitive nuclei enhanced by polarisation transfer (INEPT) NMR spectra of 11 d carrot cells following the addition of 20 mM [^{15}N]ammonium chloride. The spectra were obtained over the time intervals indicated.

Figure 4.2

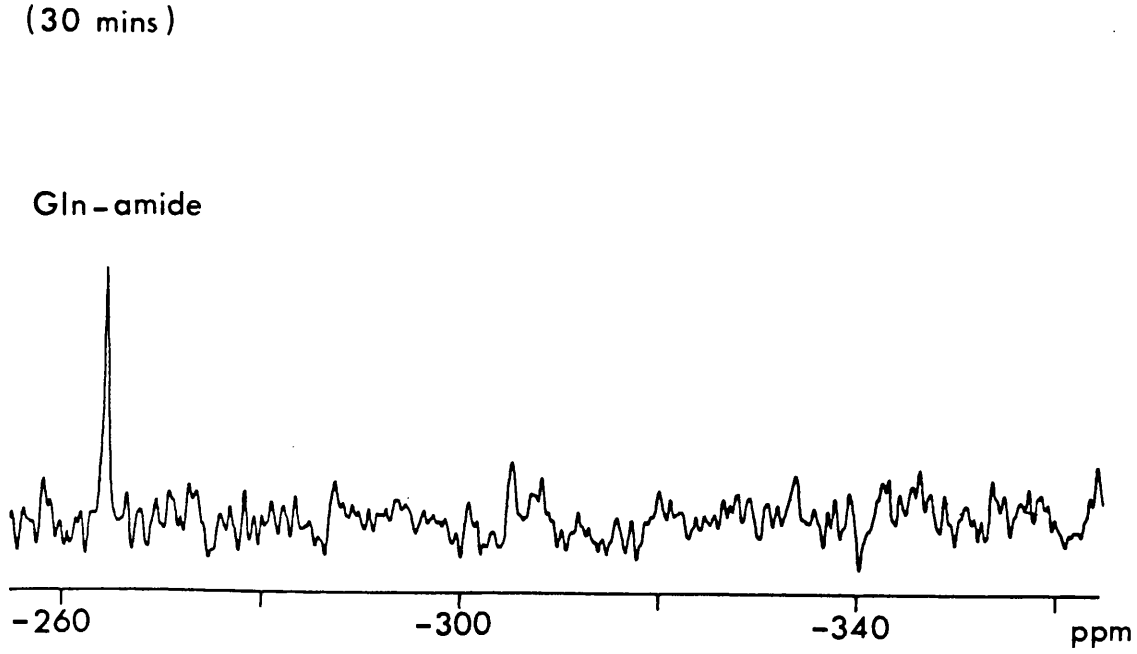
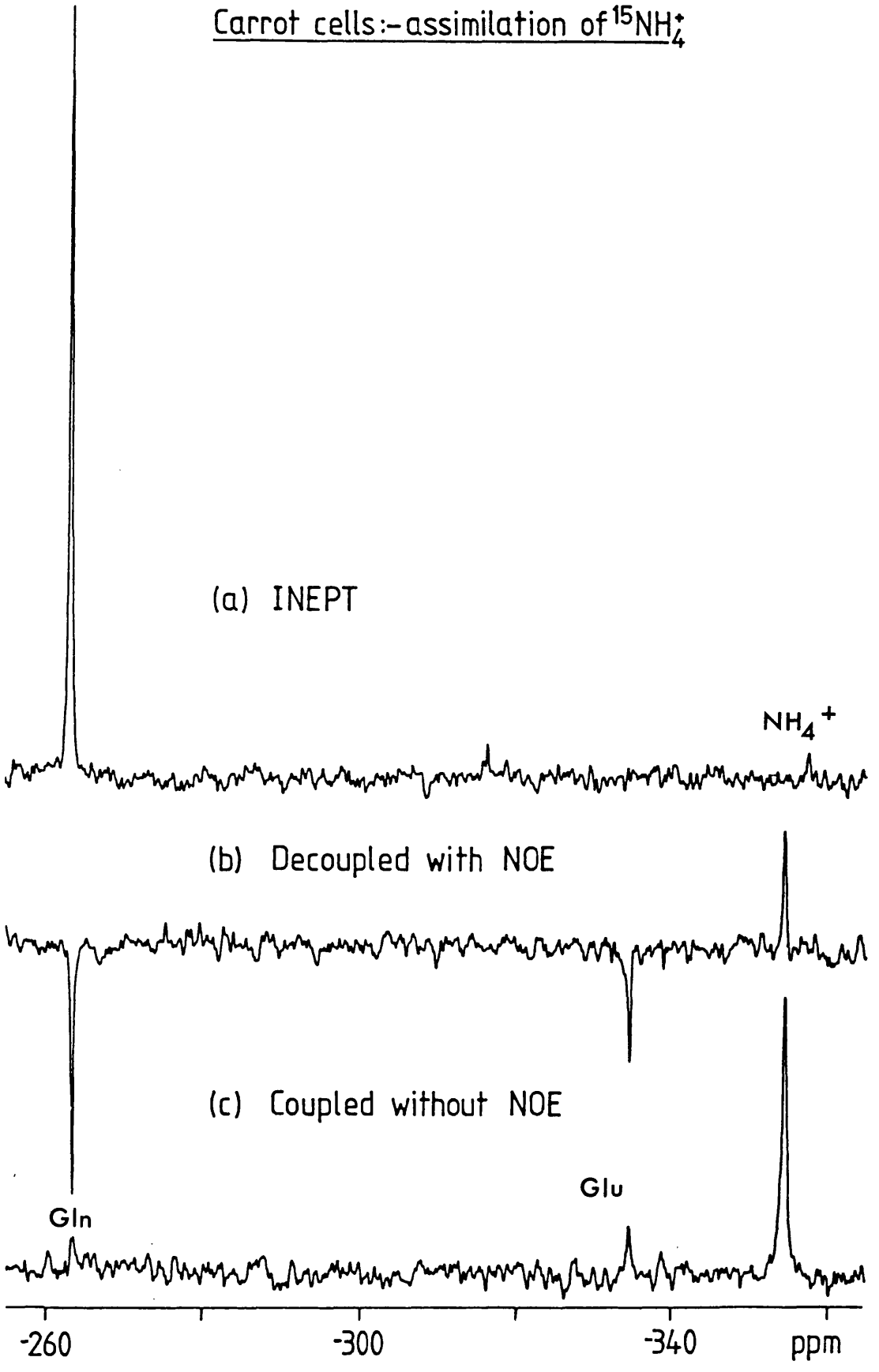


Figure 4.3

NMR spectra showing the assimilation of [^{15}N]ammonium by 11 d carrot cells following the addition of 20 mM [^{15}N]ammonium chloride. The spectra were obtained using three different NMR methods;

a) Insensitive nuclei enhanced by polarisation transfer (INEPT)
b) broad band decoupled with nuclear Overhauser enhancement (NOE)
c) ^{1}H coupled without NOE.

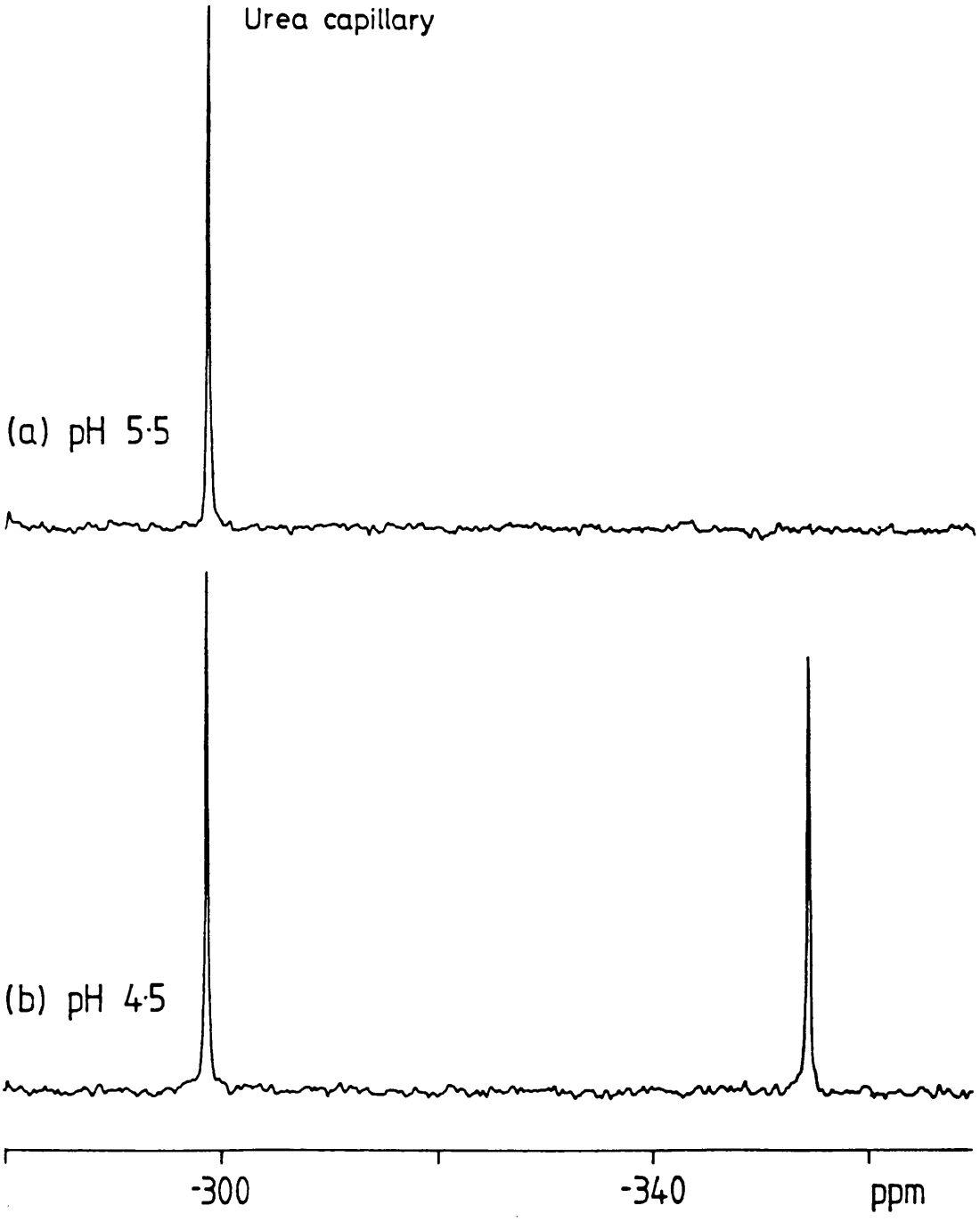
Figure 4.3


Carrot cells:-assimilation of $^{15}NH_4^+$
(a) INEPT
NH_4^+
(b) Decoupled with NOE
(c) Coupled without NOE
Gln
Glu
-260
-300
-340
ppm

Although the INEPT experiment (Fig. 4.3a) is potentially the most sensitive method, the chemical exchange of the NH protons with water can eliminate the expected signal. Amino protons and those of ammonium exchange too quickly at normal physiological pH values for there to be any polarisation transfer, whereas the exchange of amide protons is much slower and therefore the amide-N is readily detected. The effect of pH on the INEPT signal from ^{15}N ammonium is shown in fig 4.4. The result of this is a spectrum where the amide-N is enhanced and easily detected but the amino-N and ammonium are undetectable (Fig 4.3a). Using broad band ^{1}H-decoupling (Fig 4.3b) there is signal enhancement of the amino-N and amide-N resonances (as compared with no decoupling); this is due to the large negative NOE for these resonances (-4). This was not observed with ammonium, as in the presence of cells there was only a small NOE (between 0 and -1), resulting in a reduction of the intensity of the ammonium resonance (Fig 4.3b) when compared with no decoupling (Fig 4.3c). This also explains why the ammonium resonance had a positive signal intensity, whereas the other resonances were negative. In the absence of decoupling (Fig 4.3c) there were no signal enhancements, but there was also no loss of intensity due to unfavourable NOEs. Consequently, the amide-N and amino-N were more difficult to detect, while the ammonium signal was more easily observed. At pH 5.5, the rate of exchange between the NH protons and water is so fast that coupling was not observed in the ammonium and amino signals. From the spectra in figure 4.3 it is clear that there are advantages and disadvantages to each experimental method and the choice of technique depends on the particular investigation. The INEPT experiment is particularly useful for following the kinetics of labelling of amide-N, and enabled rapid detection of ^{15}N incorporation into glutamine amide (Fig 4.2). ^{1}H-decoupling gives most information for monitoring labelling patterns in a pathway, and was used to follow the flux of ^{15}N through several amino acids. However, decoupling would not be used to study ^{15}N labelling into ammonium. The behaviour of the different N groups meant that although it was possible to use NMR to observe the incorporation of [^{15}N]ammonia into living cells,

Figure 4.4.

The effect of pH on the INEPT signal from [^{15}N]ammonium.



this technique did not allow accurate determination of the metabolites. NMR is not a particularly sensitive method for detecting ^{15}N nuclei and detection is generally limited to concentrations above 1mM. This meant that only the more abundant amino acids, glutamate, glutamine, alanine and GABA were detected. For this reason GC-MS was used to quantitatively determine incorporation into all of the amino acids.

Analysis of ^{15}N incorporation by GCMS.

The assimilation of [^{15}N]ammonium was determined over a 24 hour period in the presence and absence of MSO (experimental details are given with figure 4.5). In the control cultures GS activity remained constant over the 24 hour period (Table 4.1). Treatment with MSO produced a steady decline in GS activity and complete inhibition was observed after 5 hours. Over the 24 hour period GDH activity declined by 45% in the control and 30% in the MSO treatment. This was consistent with the decline in GDH activity when cells were placed in fresh media and indicated that MSO did not effect the activity of the enzyme *in vitro*.

Incorporation of ^{15}N label into amino acids.

In the control cells ^{15}N-label was detected in most amino acids after 1 hour and the incorporation of label (nmols/gfw) increased up to 5 hours. Results are given as atom % excess (Table 4.2) and as total ^{15}N concentration, corrected for pool size (umols/gfw Table 4.3). In the control cells label was detected in all of the amino acids analyzed except for histidine and proline (Table 4.2). The labelling data for glutamine and glutamate are particularly interesting. The high ^{15}N incorporation into glutamine (umols/gfw) relative to the low atom % excess is explained by the fact that there is a larger pool of glutamine than glutamate. Over the first 6 h glutamine accounts for between 11-15% of the total amino acid pool, whereas glutamate accounts for between 3-5% (see table 4.4). The glutamine label was found predominantly in

Figure 4.5

Changes in cell and medium ammonium concentration during 24 hour treatment with 2mM ^{15}N ammonium chloride in the presence and absence of 1mM MSO. (■ medium ammonium control treatment, ▼ medium ammonium MSO treatment, □ cell ammonium control treatment, ▽ cell ammonium MSO treatment.)

Experimental Method.

For the ^{15}N labelling studies the following procedure was adopted. Stationary phase cells were used since these exhibited the highest levels of GDH activity. After 12 days growth on MDK media cells were collected aseptically on a 75um mesh sieve, washed, resuspended in nitrogen-free media and returned to the shaker. After 1 hour the cells were centrifuged (1000 rpm) to remove excess media and resuspended in flasks containing 2mM ^{15}N ammonium chloride in the presence and absence of 1mM MSO. The flasks were returned to the shaker and samples removed at 0,1,2,3,4,5,6 and 24 hours. Samples were centrifuged to separate the media and the cells. The media was analyzed for ammonia and nitrate concentration. Cells were collected as for enzyme extraction and the activities of GS and GDH were determined. Cell methanol extracts were analysed for ammonium, nitrate, amino acid and total cell protein content.

Figure4.5

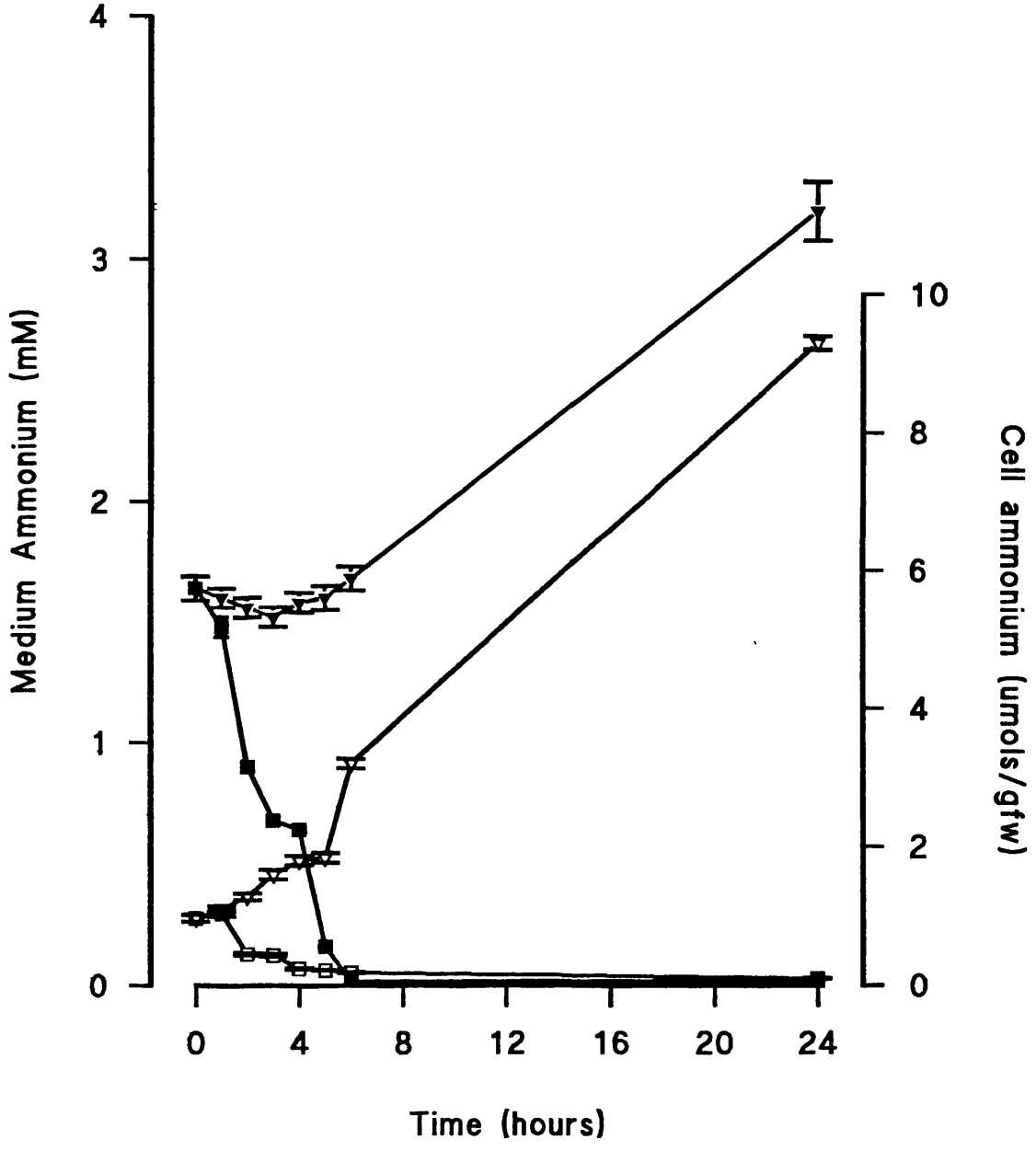

Medium Ammonium (mM)
Cell ammonium (umols/gfw)
Time (hours)
0
1
2
3
4
0
2
4
6
8
10
0
4
8
12
16
20
24

Table 4.1.

Changes in the specific activity of GS and GDH during 24 hour treatment with 2mM ^{15}N ammonium chloride in the presence and absence of 1mM MSO.

Time	Enzyme activity(nkat/mgprotein)			
	GS		GDH	
(h)	control	MSO	control	MSO
0	0.5 ± 0.06	0.5 ± 0.06	18.0 ± 0.7	18.0 ± 0.7
1	0.48 ± 0.05	0.37 ± 0.02	17,7 ± 0.7	16.0 ± 0.6
2	0.47 ± 0.04	0.18 ± 0.01	15.3 ± 0.6	15.5 ± 0.1
3	0.5 ± 0.05	0.18 ± 0.01	16.2 ± 0.6	15.7 ± 0.1
4	0.62 ± 0.04	0.1 ± 0.01	17.8 ± 0.6	17.0 ± 0.1
5	0.62 ± 0.06	0.0007	16.7 ± 0.6	15.2 ± 0.1
6	0.48 ± 0.05	0	14.0 ± 0.5	15.0 ± 0.1
24	0.42 ± 0.04	0	9.8 ± 0.4	12.7 ± 0.1

Table 4.2.

Incorporation of ^{15}N label into amino acids (atom % excess) during 24 hour treatment with 2mM [^{15}N]H_4Cl in the presence and absence of 1 mM MSO.

Control treatment.

		Incorporation of ^{15}N (atom % excess)							
	Hours	0	1	2	3	4	5	6	24
Amino acid									
Glutamate		0	20	23	24	20	24	17	9
Glutamine	-total	0	4	3	4	8	6	3	0
	-amino	0	0	1	1	2	1	1	0
	-amide	0	4	2	3	6	5	2	0
GABA		0	4	9	11	14	16	19	7
Alanine		0	14	17	22	21	24	20	3
Arginine		0	3	4	6	5	5	3	1
Serine		0	8	9	10	11	12	9	2
Methionine		0	4	7	10	13	15	10	4
Glycine		0	0	0	3	5	10	4	0
Valine		0	1	1	4	4	6	3	2
Aspartate		0	11	10	11	10	12	7	4
Leucine		0	3	3	7	10	13	10	3
Isoleucine		0	2	3	5	9	9	9	5
Threonine		0	1	1	2	5	4	1	5
Phenylalanine		0	2	3	6	10	11	10	7
Asparagine	-total	0	2	1	0	0	3	3	2
	-amino	0	1	1	0	0	1	1	1
	-amide	0	1	1	0	0	2	2	1
Tyrosine		0	3	5	3	2	3	3	5
Proline		0	0	0	0	0	0	0	0
Histidine		0	0	0	0	0	0	0	2
Lysine		0	0	0	0	0	0	0	0

Table 4.2 cont.

Incorporation of ^{15}N label into amino acids (atom % excess) during 24 hour treatment with 2mM [^{15}N]H$_4$Cl in the presence and absence of 1 mM MSO.

MSO Treatment.

		Incorporation of ^{15}N (atom % excess)							
	Hours	0	1	2	3	4	5	6	24
Glutamate		0	0	0	0	0	0	0	0
Glutamine	-total	0	0	0	0	0	0	0	0
GABA		0	0	0	0	0	0	0	0
Alanine		0	0	0	0	0	0	0	0
Arginine		0	0	0	0	0	0	0	0
Serine		0	0	0	0	0	0	0	0
Methionine		0	0	0	0	0	0	0	0
Glycine		0	0	0	0	0	0	0	0
Valine		0	0	0	0	0	0	0	0
Aspartate		0	0	0	0	0	0	0	0
Leucine		0	0	0	0	0	0	0	0
Isoleucine		0	0	0	0	0	0	0	0
Threonine		0	0	0	0	0	0	0	0
Phenylalanine		0	0	0	0	0	0	0	0
Asparagine-total		0	0	0	0	0	0	0	0
Tyrosine		0	0	0	0	0	0	0	0
Proline		0	0	0	0	0	0	0	0
Histidine		0	0	0	0	0	0	0	0
Lysine		0	0	0	0	0	0	0	0

Table 4.3.

Incorporation of ^{15}N label into amino acids (umols/gfw) during 24 hour treatment with 2mM [^{15}N]H$_4$Cl.

	Incorporation of ^{15}N (umols/gfw)							
Hours	0	1	2	3	4	5	6	24
Amino acid								
Glutamate	0	0.19	0.18	0.20	0.10	0.16	0.06	0.03
Glutamine								
-total	0	0.11	0.06	0.09	0.12	0.11	0.04	0
-amino	0	0	0.02	0.02	0.03	0.02	0.01	0
-amide	0	0.11	0.04	0.07	0.09	0.09	0.03	0
GABA	0	0.04	0.09	0.12	0.11	0.19	0.21	0.03
Alanine	0	0.08	0.04	0.13	0.08	0.13	0.08	0.01
Arginine	0	0.08	0.09	0.14	0.08	0.10	0.05	0.01
Serine	0	0.06	0.05	0.06	0.05	0.07	0.04	0.01
Methionine	0	0.02	0.03	0.05	0.05	0.07	0.03	0.01
Glycine	0	0	0	0.01	0.02	0.04	0.02	0
Valine	0	0.01	0.01	0.03	0.02	0.04	0.01	0.01
Aspartate	0	0.05	0.04	0.05	0.03	0.04	0.01	0.01
Leucine	0	0.01	0.01	0.02	0.02	0.04	0.03	0.01
Isoleucine	0	0.01	0.01	0.03	0.03	0.04	0.03	0.01
Threonine	0	0.01	0.01	0.02	0.03	0.04	0.01	0.02
Phenylalanine	0	0.01	0.01	0.02	0.02	0.03	0.02	0.02
Asparagine								
-total	0	0.02	0.01	0	0	0.03	0.02	0.01
-amino	0	0.01	0.01	0	0	0.01	0.01	<0.01
-amide	0	0.01	0	0	0	0.02	0.01	<0.01
Tyrosine	0	0.01	0.03	0.01	0.01	0.01	0.01	<0.01
Proline	0	0	0	0	0	0	0	0
Histidine	0	0	0	0	0	0	0	0.06
Lysine	0	0	0	0	0	0	0	0
Total	0	0.71	0.67	0.98	0.77	1.14	0.67	0.26

Table 4.4.

Changes in the soluble amino acid concentration (umols/gfw) during 24 hour treatment with 2mM [^{15}N]H$_4$Cl.

	Concentration (umols/gfw)							
Hours	0	1	2	3	4	5	6	24
Amino acid								
Glutamate	0.72	0.94	0.78	0.85	0.48	0.66	0.36	0.35
Glutamine	3.19	2.66	2.11	2.21	1.48	1.88	1.21	0.31
GABA	2.80	0.90	0.96	1.11	0.77	1.21	1.13	0.35
Alanine	0.53	0.58	0.53	0.61	0.38	0.53	0.41	0.21
Arginine	2.97	2.51	2.12	2.28	1.62	1.99	1.50	0.97
Serine	0.86	0.72	0.58	0.60	0.45	0.57	0.42	0.52
Methionine	0.44	0.37	0.42	0.47	0.35	0.45	0.29	0.28
Glycine	0.56	0.52	0.41	0.37	0.33	0.42	0.37	0.56
Valine	0.95	0.82	0.66	0.70	0.48	0.65	0.48	0.26
Aspartate	0.49	0.47	0.41	0.45	0.29	0.35	0.20	0.13
Leucine	0.49	0.37	0.31	0.34	0.23	0.30	0.25	0.17
Isoleucine	0.64	0.54	0.44	0.49	0.36	0.46	0.35	0.25
Threonine	1.06	0.91	0.80	0.81	0.65	0.85	0.63	0.45
Phenylalanine	0.41	0.33	0.30	0.31	0.21	0.30	0.24	0.32
Asparagine	1.16	0.98	0.84	0.90	0.64	0.83	0.59	0.25
Tyrosine	0.41	0.36	0.53	0.35	0.25	0.32	0.24	0.08
Proline	ND	ND	ND	ND	ND	ND	ND	ND
Histidine	3.71	3.26	3.07	3.29	2.53	3.30	2.45	2.85
Lysine	0	0	0	0	0	0	0	0
Total	21.39	17.24	15.27	16.14	11.50	15.07	11.12	8.31

the amide nitrogen. Although label was present in the amino-N this generally represented only one atom % excess which is almost at the limit of accuracy for this method. The distribution of label suggests that there is a small, metabolically active pool of glutamine in the cytosol and a large, inactive pool in the vacuole. On the other hand , the glutamate labelling pattern suggests that there is a large, metabolically active pool of this amino acid in the cytosol. Glutamate showed a high level of labelling throughout the 24 hours; one possibility to account for this was that some assimilation might be occurring via GDH. However, in the presence of MSO concentrations shown to completely inhibit GS activity, no incorporation of $[^{15}N]H_4$ into amino acids was observed.

Analysis of the amino acid pool sizes (Tables 4.4 and 4.5) show that the total soluble amino acid content declined in both the control (Table 4.4) and the MSO treatment (Table 4.5) over the 24 h period. However the decline in the control was much greater (61%) compared with that in the MSO treatment (10%). In the control there was a general decline in amino acid pools but there were wide variations among the various amino acids. The greatest drop was shown by glutamine (90%) and GABA (87%) and the smallest by serine (40%), histidine (33%), phenylalanine (21%) and glycine which remained constant. In the presence of MSO, on the other hand, half of the amino acid pools actually increased over the 24 hour period but this was masked by the decline in the other amino acids. The pool of glutamine was reduced to zero, GABA declined by 60% and arginine by 24%. The largest increases were shown by glutamate (98%), isoleucine (119%), alanine (134%) and leucine (173%).

ANCA-MS allows the total ^{15}N incorporation into the cells to be measured. After 24 hours the total label in the control cells, was 8.1 umol ^{15}N/gfw. This represents recovery of 84% of the ^{15}N label applied. Of this only 0.26 umols^{15}N/gfw (3.0%) was detected in soluble amino acids and the remaining label was present as insoluble nitrogen. The majority of this

Table 4.5.

Changes in the soluble amino acid concentration (umols/gfw) during 24 hour treatment with 2mM [^{15}N]H$_4$Cl and 1mM MSO.

	Concentration (umols/gfw)							
Hours	0	1	2	3	4	5	6	24
Amino acid								
Glutamate	0.72	0.98	0.90	0.85	0.64	1.00	0.52	1.43
Glutamine	3.19	2.59	2.23	2.00	1.55	1.77	1.05	0
GABA	2.80	1.07	0.95	0.90	0.87	1.07	1.51	1.11
Alanine	0.53	0.56	0.51	0.51	0.44	0.54	0.42	1.24
Arginine	2.97	3.15	3.01	2.95	2.80	3.23	2.51	2.27
Serine	0.86	0.65	0.78	0.77	0.67	0.87	0.64	1.07
Methionine	0.44	0.46	0.47	0.47	0.46	0.47	0.46	0.47
Glycine	0.56	0.46	0.56	0.57	0.54	0.73	0.53	0.62
Valine	0.95	1.00	0.94	0.89	0.89	0.90	0.90	0.89
Aspartate	0.49	0.48	0.45	0.45	0.44	0.52	0.27	0.42
Leucine	0.49	0.50	0.45	0.45	0.41	0.53	0.45	1.34
Isoleucine	0.64	0.70	0.65	0.66	0.64	0.80	0.64	1.40
Threonine	1.05	1.03	1.07	1.08	1.07	1.19	0.90	1.39
Phenylalanine	0.41	0.46	0.44	0.45	0.44	0.54	0.45	0.69
Asparagine	1.16	1.21	1.15	1.17	1.07	1.30	0.93	1.07
Tyrosine	0.41	0.44	0.43	0.43	0.42	0.50	0.43	0.71
Proline	ND	ND	ND	ND	ND	ND	ND	ND
Histidine	3.71	3.87	3.89	3.97	3.78	4.33	3.27	3.31
Lysine	0	0	0	0	0	0	0	0
Total	21.38	19.61	18.88	18.57	17.13	20.29	15.88	19.43

insoluble nitrogen is probably protein and this implies that amino acid synthesis and protein synthesis are tightly coupled and that newly formed amino acids are immediately incorporated into proteins.

Ammonium assimilation and production.

In addition to preventing the incorporation of ^{15}N into amino acids, MSO also affects the concentration of ammonia in the media (Fig. 4.5). In the control the ammonia concentration drops from 1.6mM to zero over 12 hours, an uptake rate of 1.3 umol/h/gfw. Measurements of cell ammonia also show that there is very little residual ammonia in the cells suggesting that this ammonia is assimilated as it is taken up. However, in the presence of MSO there is a net production of ammonium and between 6 and 24 hours ammonium is secreted into the medium at a rate of 0.8 umol/h/gfw. This ammonium could be derived from nitrate, amino acids or protein. Analysis of cell and medium nitrate show that insufficient nitrate is available to account for the ammonium produced. Since the amino acid pools decline in both the control and the MSO treatment (see above) some ammonium might originate from amino acid catabolism. However, the decline in amino acids in the MSO cells is insufficient, and could account for the production of only 0.3 umolN/h/gfw.

A consideration of the changes in cell protein content (see Table 4.6) in both the control and MSO treated cultures shows that total protein per gram cells increases in the control cells, but decreases in the MSO treated cells. If the nitrogen content of protein is taken at 16% (a widely accepted generalisation), it is possible to calculate the nitrogen flux into and out of protein for each treatment as follows; in the control treatment ammonium is utilised at a rate of 1.1 umol/h/gfw and protein increases by 1.38 umol/h/gfw; in the MSO treatment protein is catabolised at a rate of 1.7 umol/h/gfw and ammonium is released into the medium at a rate of 1.14 umol/h/gfw. This strongly suggests that the ammonium released into the medium in the presence of MSO is derived from the catabolism of cellular proteins.

Table 4.6.

Changes in total cell protein during 24 hour treatment with 2mM ^{15}N ammonium chloride in the presence and absence of 1mM MSO.

Time(h)	Total cell protein (mg/gfw)	
	Control	1mM MSO
0	15.5 ± 0.2	15.5 ± 0.2
1	17.8 ± 0.5	15.8 ± 0.3
2	16.7 ± 0.4	15.7 ± 0.2
3	16.2 ± 0.4	14.7 ± 0.2
4	15.6 ± 0.3	10.8 ± 0.1
5	15.5 ± 0.3	11.3 ± 0.1
6	17.8 ± 0.2	13.8 ± 0.2
24	18.4 ± 0.7	11.8 ± 0.4

Effect of 2-oxoglutarate on ammonium assimilation in the presence and absence of MSO.

It has been suggested (Rhodes 1989) that MSO might affect the supply of 2-oxoglutarate to mitochondrial GDH and thereby exert a substrate limitation effect on the enzyme. If this were true it would invalidate conclusions drawn from MSO experiments that GDH was not involved in ammonium assimilation. To investigate this possibility the incorporation of [^{15}N]ammonium into amino acids was followed in the presence and absence of MSO, with the addition of 10 mM 2-oxoglutarate to both the control and MSO treatments. The experiment was carried out over a six hour period and in order to ensure maximum inhibition of GS the cells were incubated with MSO prior to the addition of [^{15}N]ammonium chloride (for full experimental details see Table 4.7).

The addition of 2-oxoglutarate did not have a significant effect on the specific activities of GS and GDH. In both control treatments the activity of GS increased over the 6 hour period (from 0.85 to 1.31 nkats/mgprotein) and in both MSO treatments GS was inhibited by 97% at the end of the preinnoculation period. The activity of GDH showed a slight decline in all treatments over 6 hours; in both control cultures the activity declined by 4% and in the MSO treatments the decline was 13% in the absence of 2-oxoglutarate and 21% in the presence of 2-oxoglutarate.

Table 4.7 shows the effect of 2-oxoglutarate on the medium ammonium concentration. The addition of 2-oxoglutarate has an effect on the utilisation of ammonium by control cells but not on the production of ammonia by the MSO cells. Ammonium uptake increased in the cells supplied with 2-oxoglutarate such that 97% of the ammonium was removed from the medium over 6 h compared with 70% in the control cells. In the MSO treatments ammonium output is the same in the presence or absence of 2-oxoglutarate.

Table 4.7

Changes in medium ammonium concentration during 6 h treatment with 2mM ^{15}N ammonium in the presence and absence of MSO and 2-oxoglutarate.

(A ammonium, AO ammonium + 10mM 2-oxoglutarate, AM ammonium + 1mM MSO, AMO ammonium + 10mM 2-oxoglutarate + 1mM MSO)

	Medium ammonium concentration(mM)							
Treatment	A		AO		AM		AMO	
Time (h)								
0	1.78	±0.01	1.50	±0.01	1.74	±0.01	1.57	±0.01
4	0.93	±0.01	0.35	±0.01	2.14	±0.01	1.78	±0.01
6	0.54	±0.01	0.04		2.22	±0.02	2.26	±0.02

Experimental method.

The 2-oxoglutarate experiment was carried out as described for the ^{15}N labelling experiment above (see Fig 4.5) with minor modifications. In order to ensure complete inhibition of GS at the start of the experiment the cells were preincubated with MSO for 4 hours prior to the addition of label. Once GS was inhibited sterile $[^{15}]NH_4Cl$ was added to each flask to give a final concentration of 2mM ammonium. Four treatments were set up; 2mM ^{15}N ammonium chloride, 2mM ^{15}N ammonium chloride plus 10mM 2-oxoglutarate, 2mM ^{15}N ammonium chloride plus 1mM MSO, 2mM ^{15}N ammonium chloride plus 10mM 2-oxoglutarate plus 1mM MSO. 2-oxoglutarate was added prior to preincubation with MSO. Samples were taken at 0,4 and 6 hours. Samples were centrifuged to separate the media and the cells. The media was analyzed for ammonium concentration. Cells were collected as for enzyme extraction and the activities of GS and GDH were determined. Cell methanol extracts were analysed for amino acids. Total ^{15}N incorporation was determined by ANCA-MS.

The most notable effect of 2-oxoglutarate is on the amino acid pool size and the incorporation of label into amino acids. In the control cells the addition of 2-oxoglutarate produces an increase in the total amino acid pool over 4 h from 11.3 umol/gfw to 15.68 umol/gfw, a 28% increase (Table 4.8). Over the same period the corresponding pool in the cells without 2-oxoglutarate decreases to 9.97 umol/gfw (13% decrease, Table 4.8). This is the typical response of cells in fresh medium. After 4 h the total amino acid pool in the 2-oxoglutarate treated cells is 57% larger than that of the control cells, although the differences in certain amino acids are more pronounced. Comparing the 4 h control treatments the cells supplied with 2-oxoglutarate show large increases in the following amino acid pools; glutamate (260%), alanine (155%), glutamine (54%), asparagine (37%), and serine (52%). Only glycine and valine show a decrease in pool size over this period. The increase in glutamate is interesting because both GDH and GOGAT utilise 2-oxoglutarate as a substrate in the production of this amino acid. Therefore this increase could be as a result of the action of either enzyme.

Increased availability of 2-oxoglutarate might also stimulate TCA cycle activity and this would provide a supply of keto acids for transamination reactions. The increased availability of both glutamate as an amino donor and organic acids explains the increase in those amino acids formed as a result of transamination reactions. It is interesting that GABA, an amino acid formed by the decarboxylation of glutamate is only moderately affected by the addition of 2-oxoglutarate.

In the two MSO treatments there is a small increase in the total amino acid pool size over 6 hours (Table 4.9). In the presence of 2-oxoglutarate there is a slightly larger increase in the pool size, 11% as compared with the MSO treatment alone. This increase in amino acid pools suggests that MSO does not prevent the uptake of 2-oxoglutarate into the cells. The data for the individual amino acids shows that glutamate concentration increases transiently over the six hours (Table

Table 4.8.

Changes in the soluble amino acid concentration (umols/gfw) during 6 hour treatment with 2mM [^{15}N]H$_4$Cl in the presence and absence of 10mM 2-oxoglutarate.

(A ammonium, AO ammonium + 2-oxoglutarate)

	Concentration (umols/gfw)				
Time	0	4 hours		6 hours	
		A	AO	A	AO
Amino acid					
Glutamate	1.372	0.960	3.460	0.730	2.490
Glutamine	1.303	0.760	1.170	0.690	0.940
GABA	0.938	1.010	1.160	1.420	1.220
Alanine	0.602	0.920	2.350	0.670	1.260
Arginine	1.056	0.820	1.030	0.760	0.880
Serine	0.503	0.500	0.760	0.410	0.780
Methionine	0.079	0.100	0.140	0.100	0.140
Glycine	0.168	0.220	0.140	0.210	0.120
Valine	0.701	0.660	0.590	0.480	0.750
Aspartate	0.494	0.250	0.400	0.200	0.540
Leucine	0.257	0.220	0.310	0.230	0.300
Isoleucine	0.365	0.350	0.440	0.350	0.440
Threonine	0.375	0.420	0.420	0.450	0.440
Phenylalanine	0.188	0.190	0.210	0.180	0.200
Asparagine	0.444	0.350	0.480	0.320	0.410
Tyrosine	0.178	0.170	0.190	0.170	0.180
Proline	ND	ND	ND	ND	ND
Histidine	2.261	2.070	2.430	1.920	2.250
Lysine	0	0	0	0	0
Total	11.284	9.970	15.680	9.290	13.340

Table 4.9.

Changes in the soluble amino acid concentration (umols/gfw) during 6 hour treatment with 2mM [^{15}N]H$_4$Cl and 2mM MSO in the presence and absence of 10mM 2-oxoglutarate.

(AM ammonium + MSO, AMO ammonium,MSO + 2-oxoglutarate)

	Concentration (umols/gfw)				
Time	0	4 hours		6 hours	
		AM	AMO	AM	AMO
Amino acid					
Glutamate	1.372	1.240	1.640	1.600	1.421
Glutamine	1.303	0.210	0.210	0.164	0.216
GABA	0.938	0.800	0.820	0.764	0.833
Alanine	0.602	0.620	0.860	0.664	0.686
Arginine	1.056	1.020	0.930	0.936	1.127
Serine	0.503	0.320	0.630	0.736	0.765
Methionine	0.079	0.170	0.130	0.136	0.167
Glycine	0.168	0.130	0.310	0.218	0.265
Valine	0.701	0.880	0.810	1.082	1.264
Aspartate	0.494	0.290	0.200	0.400	0.353
Leucine	0.257	0.650	0.560	0.700	0.843
Isoleucine	0.365	0.730	0.620	0.736	0.902
Threonine	0.375	0.560	0.730	0.809	0.912
Phenylalanine	0.088	0.330	0.0	0.345	0.372
Asparagine	0.444	0.550	0.510	0.573	0.657
Tyrosine	0.178	0.330	0.290	0.327	0.402
Proline	ND	ND	ND	ND	ND
Histidine	2.261	2.160	2.090	2.136	2.490
Lysine	0	0	0	0	0
Total	21.370	11.170	11.640	12.491	13.880

9). The data suggests that although 2-oxoglutarate concentration may be limiting the effectiveness of the glutamate synthase cycle under normal conditions, it does not explain the inability of GDH to assimilate ammonia in these cells.

Analysis of the incorporation of ^{15}N label into amino acids showed that whilst the presence of 2-oxoglutarate increased the incorporation of label into control cells it had no effect on the MSO treatment (Table 4.10). From Table 4.10 it is clear that MSO inhibits incorporation of ^{15}N into amino acids even in the presence of 10mM exogenous 2-oxoglutarate. The control data shows that 2-oxoglutarate increases the level of labelling in all amino acids, particularly at 4 h. Table 4.11 shows the ^{15}N labelling of amino acids as umols^{15}N/gfw, the MSO data was not included in this table because there was no incorporation of ^{15}N in the MSO treatment. Since 2-oxoglutarate increases amino acid concentration in the cells and increases the ^{15}N labelling the combined effect is to increase the total amount of label incorporated into amino acids from 1.5 to 4.73 umol^{15}N/gfw, an increase of 216%.

All of the amino acids show an increase in labelling in the 2-oxoglutarate treatment (Table 4.11), with particularly large increases over the control in the case of glutamate (417%) and alanine (255%), which together account for the majority of the increase in label at 4 h. Large increases in comparison with the control are also seen in asparagine, glutamine and threonine. This labelling data provides further support for the role of the glutamate synthase cycle as the sole route of ammonium assimilation and provides no support for an assimilatory role for GDH. It is clear from this experiment that GDH is not prevented from assimilating ammonia by a lack of 2-oxoglutarate. The control data shows that cells incorporated an increased level of ammonium in the presence of 2-oxoglutarate, indicating that cells can respond to exogenous 2-oxoglutarate. The data suggest that the extra carbon skeletons are stimulating amino acid synthesis in these cells.

Table 4.10.

Incorporation of ^{15}N label into amino acids (atom % excess) after 4 and 6 h treatment with 2mM ammonium chloride in the presence and absence of MSO and 2-oxoglutarate.

Treatments: ammonium (A), ammonium + MSO (AM), ammonium + 2-oxoglutarate (AO), ammonium + MSO + 2-oxoglutarate (AMO). Concentrations: 2 mM [^{15}N]ammonium chloride, 1 mM MSO, 10 mM 2-oxoglutarate.

	Treatments							
	4 hours				6 hours			
Amino acid	A	AO	AM	AMO	A	AO	AM	AMO
Aspartate	21	32	0	0	18	34	0	0
Glutamate	37	53	0	0	38	42	0	0
Asparagine								
-total	5	14	0	0	4	4	0	0
-amino	5	9	0	0	1	4	0	0
-amide	0	5	0	0	3	0	0	0
Serine	19	27	0	0	17	28	0	0
Glutamine								
-total	27	53	0	0	32	47	0	0
-amino	10	19	0	0	7	16	0	0
-amide	17	34	0	0	25	31	0	0
Histidine	0	3	0	0	0	2	0	0
Glycine	0	12	0	0	0	18	0	0
Threonine	5	16	0	0	14	19	0	0
Arginine	0	1	0	0	0	0	0	0
Alanine	36	50	0	0	37	40	0	0
GABA	29	28	0	0	37	27	0	0
Tyrosine	6	4	0	0	0	7	0	0
Methionine	15	21	0	0	16	28	0	0
Valine	4	8	0	0	10	14	0	0
Phenylalanine	12	16	0	0	14	19	0	0
Isoleucine	12	16	0	0	15	21	0	0
Leucine	12	1	0	0	16	23	0	0
Lysine	0	0	0	0	0	0	0	0
Proline	7	13	0	0	0	0	0	0
Ornithine	0	0	0	0	0	0	0	0

Table 4.11

Incorporation of ^{15}N label into amino acids (umols/gfw) during 6 hour treatment with 2mM [^{15}N]H$_4$Cl in the presence and absence of 10mM 2-oxoglutarate.

		Incorporation of ^{15}N (umols/gfw)			
Hours		4 hours		6 hours	
		A	AO	A	AO
Amino acid					
Glutamate		0.355	1.834	0.277	1.046
Glutamine	-total	0.205	0.620	0.221	0.442
	-amino	0.076	0.222	0.049	0.150
	-amide	0.129	0.398	0.172	0.291
GABA		0.293	0.325	0.525	0.329
Alanine		0.331	1.175	0.248	0.504
Arginine		0	0.010	0	0
Serine		0.095	0.205	0.070	0.218
Methionine		0	0	0	0.039
Glycine		0	0.017	0	0.022
Valine		0.026	0.047	0.048	0.105
Aspartate		0.053	0.128	0.036	0.184
Leucine		0.026	0.053	0.037	0.069
Isoleucine		0.042	0.070	0.053	0.092
Threonine		0.021	0.067	0.063	0.084
Phenylalanine		0.023	0.034	0.025	0.038
Asparagine	-total	0.018	0.067	0.013	0.016
	-amino	0.018	0.043	0.003	0.016
	-amide	0	0.024	0.010	0
Tyrosine		0.010	0.008	0	0.013
Proline		ND	ND	ND	ND
Histidine		0	0.073	0	0.045
Total		1.498	4.733	1.615	3.246

The ANCA-MS data for 6 h shows that ^{15}N incorporation is greater in the 2-oxoglutarate-treated cells compared with the control. After 6 hours the control cells had incorporated 7.95 umols^{15}N/gfw and the 2-oxoglutarate cells 9.74 umols^{15}N/gfw, an increase of 22.5%. In the MSO treatments there is no increase in ^{15}N above natural abundance levels in the presence or absence of 2-oxoglutarate.

The treatment with 2-oxoglutarate suggests that glutamate synthase activity may be limited, under normal conditions, by the supply of 2-oxoglutarate. In addition synthesis of other amino acids by transamination appears to be limited either by supplies of glutamate or organic acids. In relation to the role of the enzyme GDH there is no evidence that this enzyme is involved in the assimilation of ammonium even under conditions where cells are supplied with excess 2-oxoglutarate and have a plentiful supply of ammonium.

The results from these experiments show that despite the high levels of GDH present in these cells ammonia assimilation occurs solely via the glutamate synthase cycle and this view is also supported by the relative activities of GS and GDH throughout the cell culture period (see Chapter 3). GS activity is high during the period of rapid nitrogen assimilation and declines during the stationary phase, GDH on the other hand responds to sucrose levels and is lowest during the period of nitrogen assimilation and highest when sucrose is limited. The effect of sucrose concentration on GDH activity is considered in the next chapter.

CHAPTER 5: THE EFFECT OF SUCROSE ON GDH ACTIVITY AND AMINO ACID METABOLISM.

The initial growth experiments suggested a possible link between GDH activity and sucrose concentration. The effect of carbon supply on GDH activity has been studied by many workers and there is general agreement that sugars exert a regulatory effect on this enzyme. In general GDH levels rise in response to carbon limitation and this effect is reversed by the addition of various sugars (Oaks et al 1980, Sahulka and Lisa 1980). Sugars have been shown to play a more central role in the regulation of GDH than ammonia and other nitrogen sources(Nauen and Hartmann 1980, Tassi et al 1984). The link between GDH activity and carbon starvation is therefore well established.

In these cell cultures GDH levels are highest during stationary phase when the cells have exhausted the supply of certain nutrients. Early investigations (Chapter 3) showed that although there was an adequate supply of nitrogen throughout the culture period, sucrose was completely utilised by d 10. The apparent correlation between sucrose concentration and GDH activity was investigated first at an enzymic level and secondly in terms of changes in amino acid metabolism.

The effect of sucrose concentration on the specific activity of GDH.

The first experiment investigated changes in GDH activity in cultures grown in medium containing a range of sucrose concentrations, 0, 1% and 2% w/v, (for full experimental details see Fig 5.1). At the beginning of the experiment the GDH levels were low, typical of a 4 d culture. The effect of the various sucrose treatments on GDH levels is shown in Fig 5.1. In the presence of 1% or 2% sucrose the activity remained low for the first 5 days and then rose towards the end of the culture period. (These cultures were inoculated

Figure 5.1

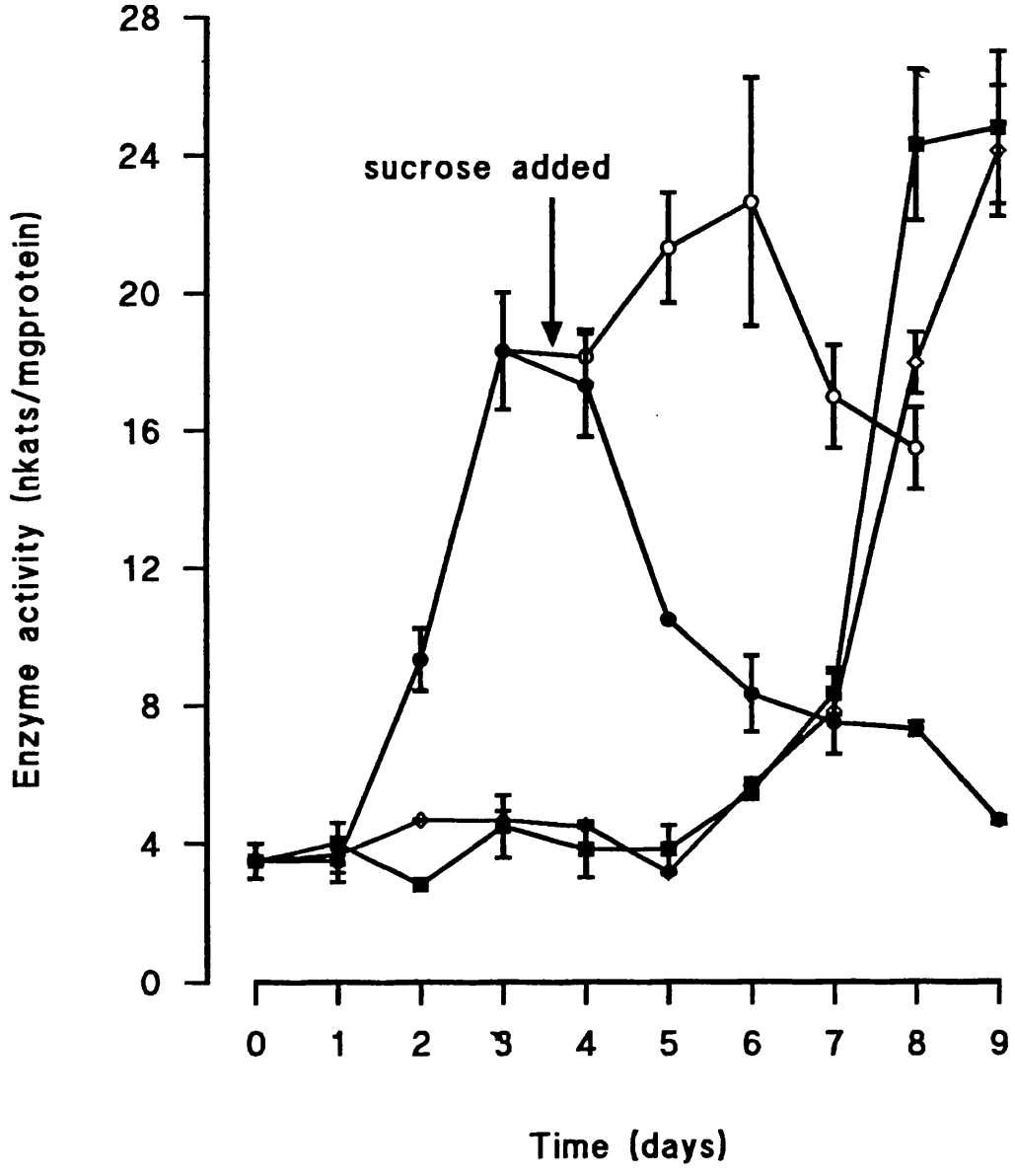
Changes in the specific activity of GDH (nkats/mgprotein) during growth in media containing 0%, 1% and 2% sucrose (w/v).

○ 0% sucrose
● 0% sucrose to which 2% sucrose was added on day 4.
◇ 1% sucrose
■ 2% sucrose

Experimental detail.

Six flasks containing 140 ml MDK media were innoculated with 14 ml of stationary phase culture. After 4 days in fresh medium GDH levels had fallen to 3.5 nkats/mgprotein. Cells were harvested aseptically on a 70 um metal seive, washed with sucrose-free medium and resuspended in sucrose-free medium for 1 h. The medium was then divided into 40 ml aliquots, centrifuged at 1000rpm and the cells resuspended in MDK medium containing either 0, 1% or 2% w/v sucrose (duplicate flasks). A sample was removed from alternate duplicates at daily intervals. The cells were analysed for GDH activity and the medium was analysed for ammonium content. After 4 days sterile sucrose solution was added to one of the sucrose-free treatments to give a final concentration of 2% w/v sucrose. The remaining sucrose-free treatment was left without sucrose as a control.

Figure 5.1

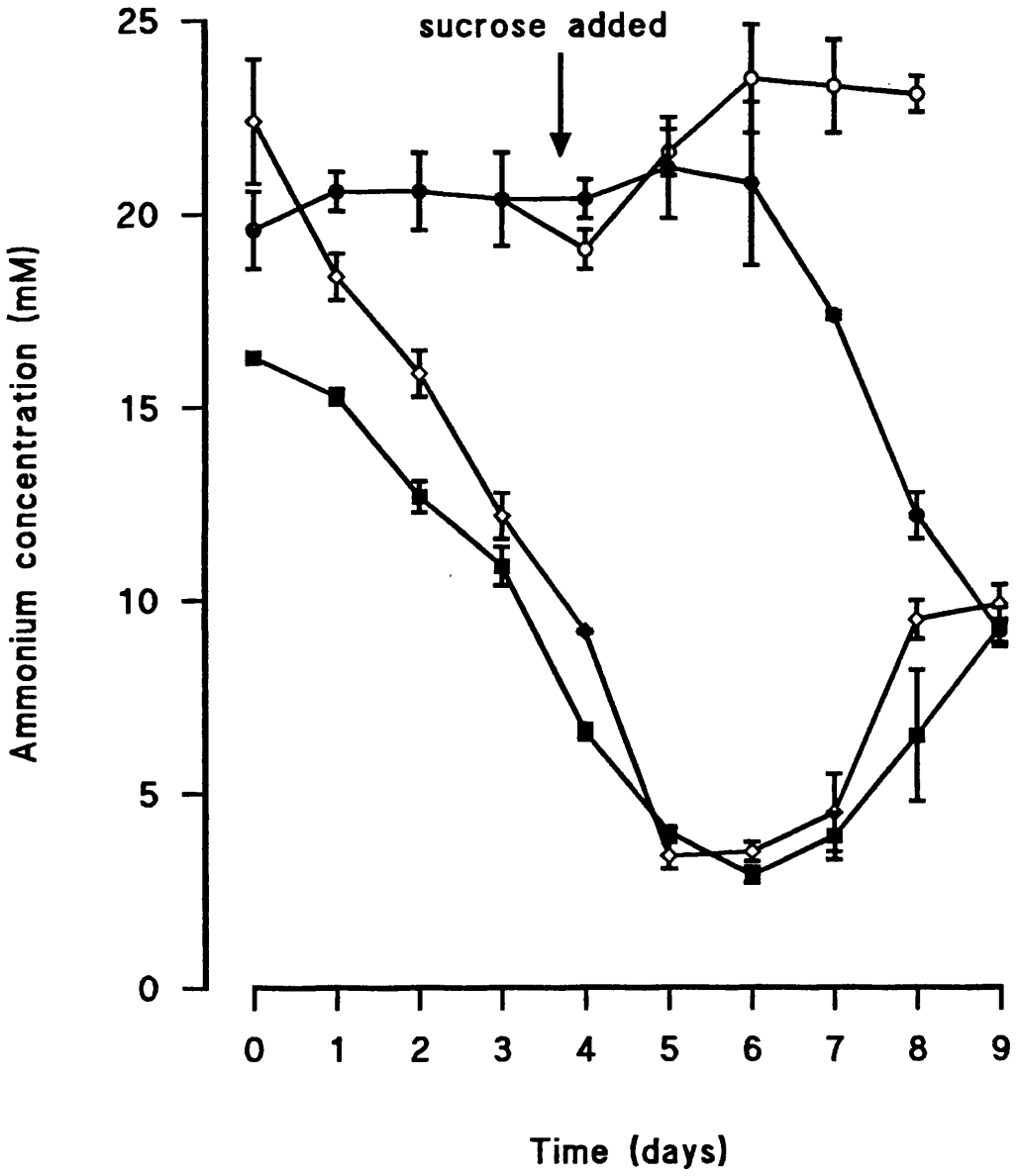

28
24
20
16
12
8
4
0
Enzyme activity (nkats/mgprotein)
sucrose added
0
1
2
3
4
5
6
7
8
9
Time (days)

with double the standard inoculum load and consequently entered stationary phase around d 7). The sucrose-free cultures showed an immediate increase in GDH activity and levels continued to rise until d 3 when they reached 18.33 nkats/mgprotein. On d 4 one subset of the sucrose-free culture flasks was injected with sucrose solution to produce a 2% sucrose concentration. In these flasks the level of GDH activity fell dramatically following the addition of sucrose but continued to rise in the sucrose-free control treatment. By d 6 the level of activity was 8.34 nkat/mgprotein in the treatment with added sucrose compared with 22.67 nkat/mgprotein in the sucrose-free treatment (Fig 5.1). GDH activity continued to fall in the cultures with added sucrose until d 9 when the experiment was concluded. The sucrose starved cells began to show severe necrosis from day 6 and by d 8 all the cells were dead.

The media ammonium concentrations were determined for all treatments and the results are shown in Figure 5.2. In the treatments containing sucrose the ammonium levels declined at a steady rate until d 6, after which there was a release of ammonium into the medium. This is the same pattern of ammonium utilisation/production observed in normal MDK cultures. In the sucrose-free treatments there was a very slight increase in the ammonium concentration over the first 4 days. Following the addition of sucrose to one of the sucrose-free treatments there was a lag of 2 days before ammonium was assimilated and between d 6-9 the medium ammonium concentration fell rapidly. In the sucrose-free treatment the medium ammonium continued to increase and the level remained high until the cells died.

The findings suggest a close link between sucrose concentration, GDH activity and ammonium release which correlates well with the data for normal MDK cultures where GDH levels rise when sucrose becomes limiting (Chapter 3). These results show that GDH is derepressed under conditions of sucrose starvation and this effect on GDH coincides with the release of ammonium from the cells into the medium.

Figure 5.2 Changes in medium ammonium concentration (mM) during growth in medium containing 0%, 1% and 2% sucrose (w/v).



(symbols as for figure 5.1)

Effect of sucrose limitation on nitrogen metabolism of cells grown on various nitrogen sources.

In the previous experiment, the effect of sucrose starvation was investigated in cultures containing the standard nitrogen input, namely 39.4 mM NO_3^- (as potassium nitrate) and 20.6 mM NH_4^+ (as ammonium chloride). In order to investigate the influence of different nitrogen sources within the culture, the experiment was repeated with the standard nitrogen input replaced by different sources; full experimental details are given in Table 5.1. Samples were taken initially and at daily intervals for 3 days and determinations made of the specific activity of GDH; medium ammonium concentration; medium pH; cell fresh weight and soluble amino acid content of the cells.

The data in Figures 5.3 to 5.5 shows the GDH activity, medium ammonium concentration and medium pH for the ten treatments, the data for the sucrose-free treatments is shown in part (a) of the figure and the data for the 2% sucrose treatments is in part (b). In the sucrose free treatments there was little change in cell fresh weight over the 3 days (Table 5.4), but the GDH activity, medium ammonium and medium pH all increased over the same period (Figs 5.3A, 5.4A and 5.5A). The addition of 10mM 2-oxoglutarate to the ammonium and glutamine treatments did not alter these trends. The most dramatic increase in GDH specific activity occurred in the nitrate treatment and the two treatments with 2-oxoglutarate (Fig 5.4A); GDH activity increased by over 300% in these treatments. The largest increases in medium ammonium were shown by the ammonium, glutamine and nitrate treatments (Fig 5.3A). In the ammonium treatment (Fig 5.3) the media ammonium concentration increased from 10mM to 15mM over 3 days. The pH values for the sucrose-free treatments were in the range 7.05 - 7.74 by the third day (Fig 5.5A) compared with 3.9 - 5.4 for the sucrose treatments (Fig 5.5B).

In the presence of sucrose the cells grew well and there was a decline in GDH activity, medium ammonium and medium pH.

Table 5.1

Effect of sucrose limitation on nitrogen metabolism in cells grown on various nitrogen sources, Treatments investigated.

Nitrogen source (10mM)	Sucrose Concentration (w/v)	Additions
ammonium chloride	0%	
ammonium chloride	2%	
potassium nitrate	0%	
potassium nitrate	2%	
L-glutamine	0%	
L-glutamine	2%	
L-glutamate	0%	
L-glutamate	2%	
ammonium chloride	0%	10mM 2-oxoglutarate
L-glutamine	0%	10mM 2-oxoglutarate

Experimental details.

10 flasks of MDK medium were preinnoculated, grown for 4 days, collected and washed as above (Fig 5.1). The cells were then transferred to 10 flasks containing 50 ml of N-free medium, 6 flasks without sucrose and 4 flasks containing 2% w/v sucrose. The flasks were returned to the shaker to equilibriate. After 1 hour sterile media containing the various nitrogen sources and 2-oxoglutarate were added to the flasks to give the final concentrations shown above, (total volume 65 ml).

At time 0 and at daily intervals samples were taken aseptically to determine cell fresh weight, specific activity of GDH, soluble amino acid pools, medium ammonium concentration and medium pH.

Figures 5.3 - 5.5
Effect of sucrose concentration on cultures grown in media containing various nitrogen sources in the presence and absence of 2% (w/v) sucrose over a 3 day period.

Figure 5.3
Changes in medium ammonium concentration (mM)

Figure 5.4
Changes in the specific activity of GDH (nkats/mg protein)

Figure 5.5
Changes in medium pH.

a) Treatments with 0% sucrose

- ● 10 mM ammonium chloride
- □ 10 mM potassium nitrate
- ▲ 10 mM L-glutamine
- ◇ 10 mM L-glutamate
- ○ 10 mM ammonium chloride + 10 mM 2-oxoglutarate
- ▼ 10 mM L-glutamine + 10 mM 2-oxoglutarate

b) Treatments with 2% (w/v) sucrose

- ● 10 mM ammonium chloride + 2% sucrose
- □ 10 mM potassium nitrate + 2% sucrose
- ▲ 10 mM L-glutamine + 2% sucrose
- ◇ 10 mM L-glutamate + 2% sucrose

Figures 5.3 - 5.5
Effect of sucrose concentration on cultures grown in media containing various nitrogen sources in the presence and absence of 2% (w/v) sucrose over a 3 day period.

Figure 5.5
Changes in medium pH.

a) Treatments with 0% sucrose

- ● 10 mM ammonium chloride
- □ 10 mM potassium nitrate
- ▲ 10 mM L-glutamine
- ◇ 10 mM L-glutamate
- ○ 10 mM ammonium chloride + 10 mM 2-oxoglutarate
- ▼ 10 mM L-glutamine + 10 mM 2-oxoglutarate

b) Treatments with 2% (w/v) sucrose

- ● 10 mM ammonium chloride + 2% sucrose
- □ 10 mM potassium nitrate + 2% sucrose
- ▲ 10 mM L-glutamine + 2% sucrose
- ◇ 10 mM L-glutamate + 2% sucrose

Figure 5.3a

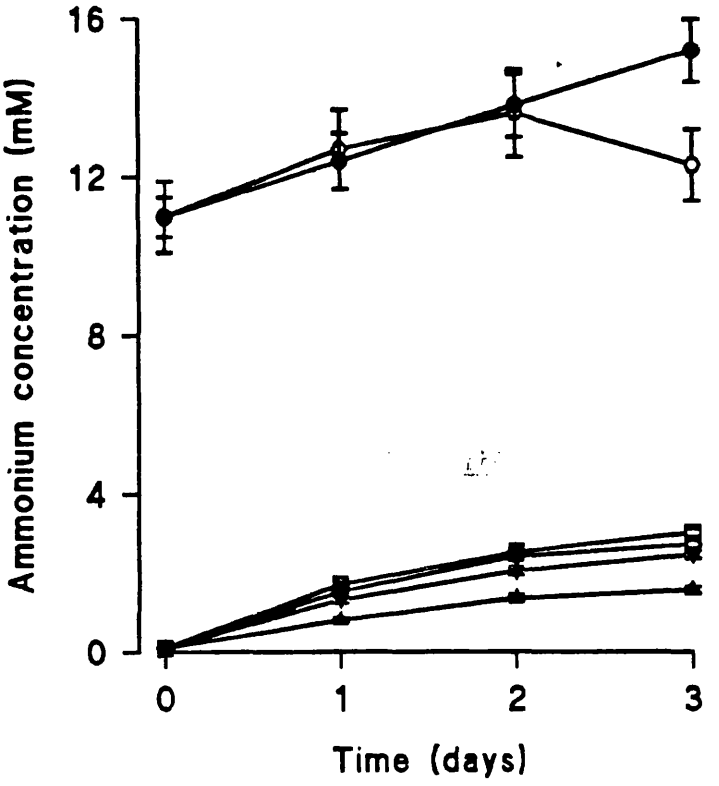
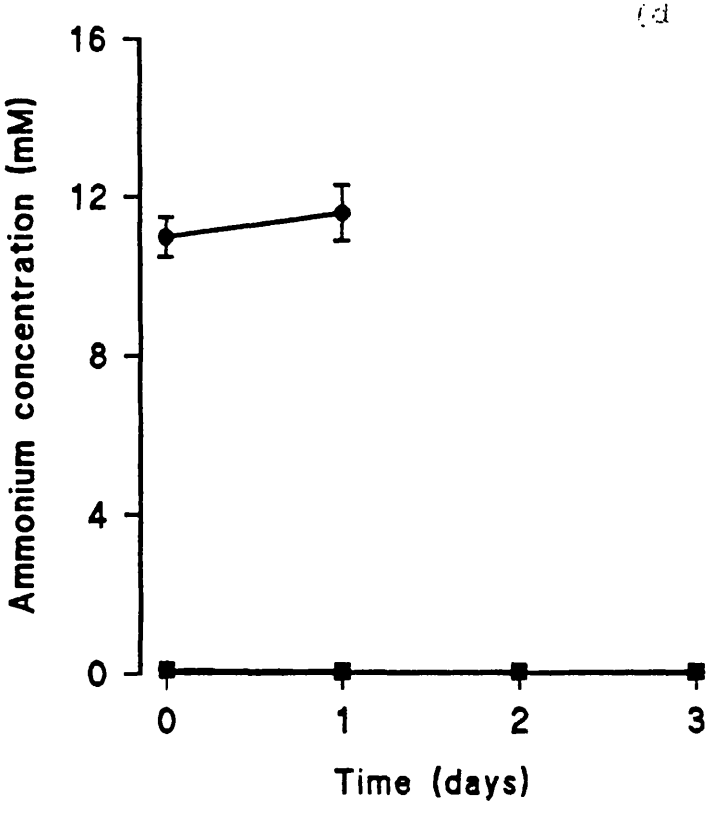


Figure 5.3b



Figures 5.3 - 5.5

Effect of sucrose concentration on cultures grown in media containing various nitrogen sources in the presence and absence of 2% (w/v) sucrose over a 3 day period.

Figure 5.4

Changes in the specific activity of GDH (nkats/mg protein)

a) Treatments with 0% sucrose

- ● 10 mM ammonium chloride
- □ 10 mM potassium nitrate
- ▲ 10 mM L-glutamine
- ◇ 10 mM L-glutamate
- ○ 10 mM ammonium chloride + 10 mM 2-oxoglutarate
- ▼ 10 mM L-glutamine + 10 mM 2-oxoglutarate

b) Treatments with 2% (w/v) sucrose

- ● 10 mM ammonium chloride + 2% sucrose
- □ 10 mM potassium nitrate + 2% sucrose
- ▲ 10 mM L-glutamine + 2% sucrose
- ◇ 10 mM L-glutamate + 2% sucrose

Figure 5.4a

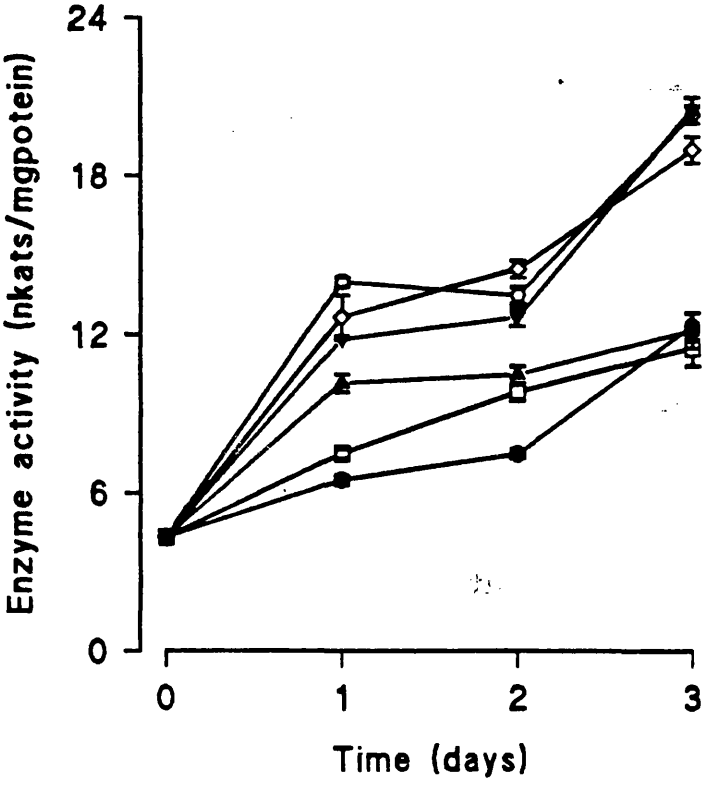
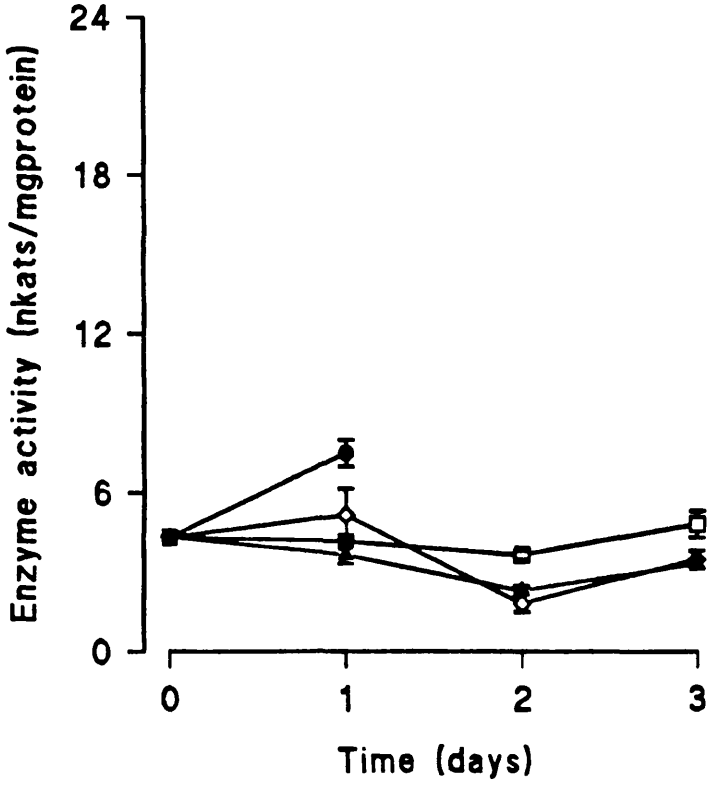


Figure 5.4b



Figures 5.3 - 5.5
Effect of sucrose concentration on cultures grown in media containing various nitrogen sources in the presence and absence of 2% (w/v) sucrose over a 3 day period.

Figure 5.3
Changes in medium ammonium concentration (mM)

a) Treatments with 0% sucrose

- ● 10 mM ammonium chloride
- □ 10 mM potassium nitrate
- ▲ 10 mM L-glutamine
- ◇ 10 mM L-glutamate
- ○ 10 mM ammonium chloride + 10 mM 2-oxoglutarate
- ▼ 10 mM L-glutamine + 10 mM 2-oxoglutarate

b) Treatments with 2% (w/v) sucrose

- ● 10 mM ammonium chloride + 2% sucrose
- □ 10 mM potassium nitrate + 2% sucrose
- ▲ 10 mM L-glutamine + 2% sucrose
- ◇ 10 mM L-glutamate + 2% sucrose

Figure 5.5a

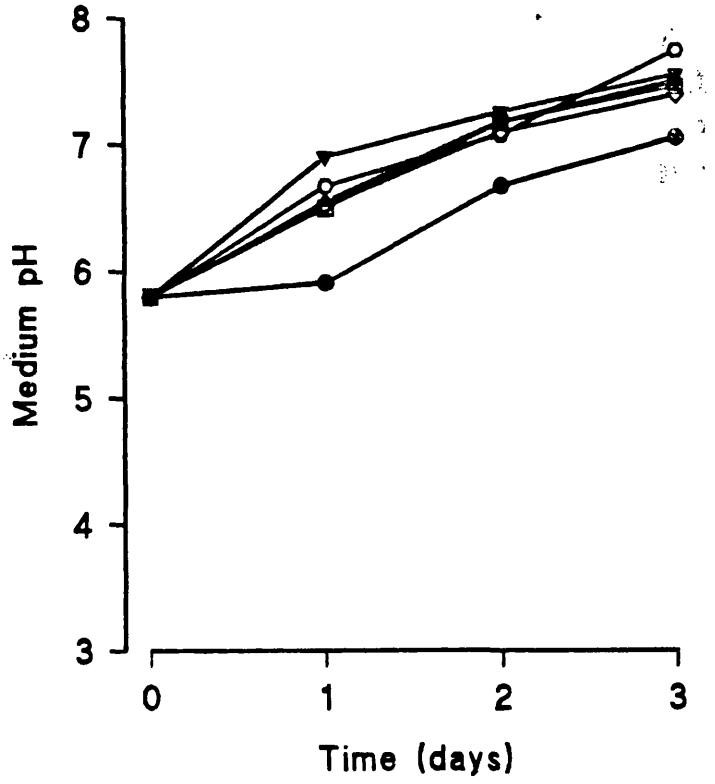
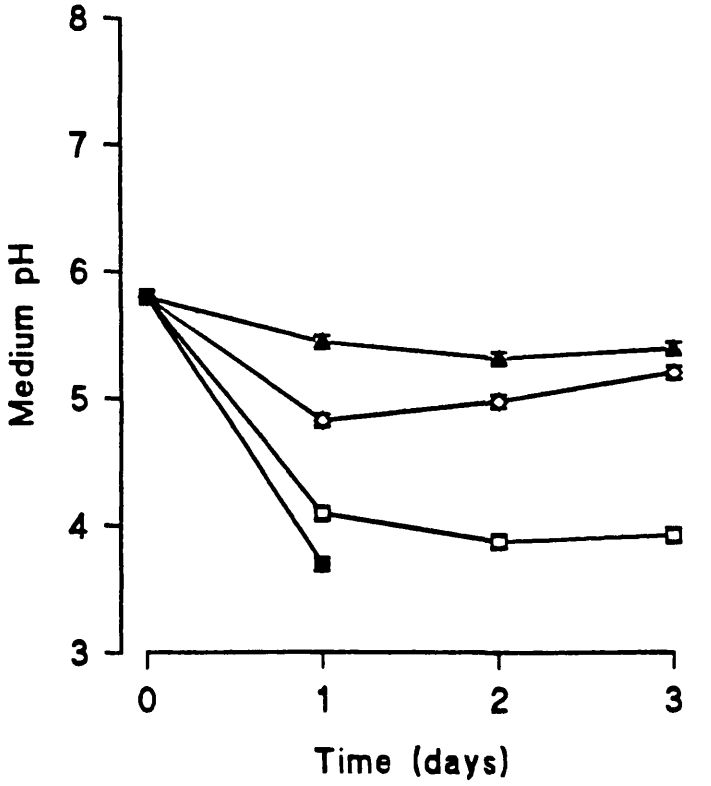


Figure 5.5b



The nitrate and glutamine treatments showed the largest increase in fresh weight over the 3 days (Table 5.4). In the ammonium treatment with sucrose the cells died within 24 h. Cell cultures are known to be unable to grow in media containing ammonium as a sole nitrogen source (see comments above, Chapter 3). It was therefore only possible to obtain data for d 1 and it was difficult to quantitate some of this data due to cell collapse making measurements of fresh weight and therefore GDH and amino acid content inaccurate. The medium pH fell dramatically from 5.8 to 3.7 (Fig 5.5B) and the medium ammonium increased slightly over the 24 hours (Fig 5.3B).

The results from this experiment confirm that sucrose has a marked effect on GDH activity. GDH is derepressed under conditions of carbon limitation and this effect is seen in cells grown on a variety of nitrogen sources.

The effect of sucrose concentration on the soluble amino acid pool was also examined in this experiment. Table 5.2 shows the amino acid concentrations (umols/gfw) for the 10 treatments over the first 24 hours. In all cases the sucrose-free treatments show a higher concentration of soluble amino acids than the 2% sucrose treatments. In the treatments without sucrose the total amino acid pools increased by between 107 and 195% over 24 hours. Table 5.3 shows the percentage increase over the 24 hour period for each amino acid, negative values indicate a decrease. The data shows that although there was an overall increase in total amino acid concentration in the sucrose free treatments it was not uniform. The asparagine, serine, histidine, threonine, arginine, tyrosine, valine, isoleucine and lysine pools increased by over 100% in all the sucrose free treatments. However, glutamate, glutamine, aspartate, alanine and GABA showed much smaller increases. This pattern of accumulation mirrors that seen during the stationary phase of culture when only certain amino acids accumulate probably as a result of protein catabolism. Glutamate, glutamine, alanine and GABA do not accumulate under these conditions.

Table 5.2

Changes in the soluble amino acid concentration (umols/gfw) during 24 hours growth in medium containing various nitrogen sources in the presence and absence of 2% sucrose.

(Treatments 0 time 0, A ammonium, AS ammonium + sucrose, N nitrate, NS nitrate +sucrose, Gln glutamine, GlnS glutamine + sucrose, Glu glutamate, GluS glutamate + sucrose, AO ammonium + 2-oxoglutarate, GlnO glutamine + 2-oxoglutarate)

	Concentration (umols/gfw)				
Treatment	0	A	AS	N	NS
Amino acid					
Glutamate	1.73	2.79	1.09	2.04	0.92
Glutamine	1.51	1.94	1.23	1.08	0.48
GABA	0.63	1.29	0.66	1.03	1.20
Alanine	1.00	1.88	0.97	1.27	0.17
Arginine	0.33	1.18	0.64	1.11	0.64
Serine	0.81	2.79	0.99	2.89	0.80
Methionine	0.36	0.85	0.66	0.67	0.31
Glycine	0.44	0.29	0.64	0.91	0.35
Valine	1.01	3.91	1.09	3.85	0.57
Aspartate	0.75	1.44	0.76	1.18	0.47
Leucine	0.49	1.97	0.85	1.61	0.42
Isoleucine	0.54	2.38	0.80	2.43	0.47
Threonine	0.98	3.18	0.92	3.46	0.75
Phenylalanine	0.43	1.41	0.76	0.79	0.46
Asparagine	0.68	2.79	0.85	2.45	0.52
Tyrosine	0.43	1.32	0.78	0.99	0.29
Histidine	1.96	5.38	0.92	4.64	2.25
Lysine	0.61	1.44	0	1.42	0.82
Total	14.69	38.23	14.61	33.82	11.89

Table 5.2 continued

	Concentration (umols/gfw)					
Treatments	Gln	GlnS	Glu	GluS	AO	GlnO
Amino acid						
Glutamate	4.02	0.97	3.22	1.29	1.98	4.29
Glutamine	2.15	1.20	1.71	0.52	1.33	2.35
GABA	1.59	0.89	2.88	1.16	0.82	1.44
Alanine	2.39	1.27	1.83	1.03	0.90	1.79
Arginine	1.07	0.33	0.95	0.31	0.75	1.32
Serine	2.21	0.82	3.10	0.87	2.61	3.23
Methionine	0.56	0.30	0.62	0.30	0.43	0.76
Glycine	0.89	0.56	0.20	0.44	0.73	1.29
Valine	3.12	1.01	4.45	0.71	3.15	4.53
Aspartate	1.24	0.42	1.51	0.43	1.30	2.29
Leucine	1.42	0.50	1.85	0.41	1.48	2.09
Isoleucine	1.90	0.58	2.66	0.48	2.11	2.91
Threonine	2.91	1.19	3.45	0.95	3.55	4.23
Phenylalanine	0.80	0.40	0.95	0.34	0.73	1.29
Asparagine	2.17	0.60	2.68	0.46	2.35	2.94
Tyrosine	0.89	0.46	1.01	0.34	0.78	1.03
Histidine	4.13	2.46	5.04	1.79	4.28	5.47
Lysine	0	3.59	3.57	3.46	1.13	0
Total	33.46	17.55	41.68	15.29	30.41	43.25

The addition of 2-oxoglutarate to the ammonium-sucrose free treatment resulted in a smaller increase in the total pool compared with the ammonium alone. In the glutamine-sucrose free treatment 2-oxoglutarate produced an increase in the amino acid pools, particularly aspartate, asparagine, serine, glycine, threonine, arginine, valine, phenylalanine, isoleucine and leucine. This treatment showed the highest overall increase in amino acid concentration.

There was very little change in total amino acid pool size in the plus sucrose treatments, the overall net change being less than 20% in all cases (Table 5.3). In all the sucrose treatments the glutamate and glutamine pools declined slightly. In the ammonium treatment (Table 5.2) arginine, tyrosine, methionine, phenylalanine and leucine accumulated but the glutamate, glutamine, histidine and lysine pools decreased. In the glutamine treatment there was an accumulation of lysine, and the glutamate and nitrate treatments showed increased GABA. Arginine also accumulated in the nitrate treatment. Over the next 48 hours the accumulation of amino acids continued at a slower rate in the sucrose free treatments and there was little change in the sucrose treated cells.

From this data it is clear that high levels of GDH and accumulation of certain amino acids are all responses of the cells to low sucrose levels. The fact that similar results are seen in stationary phase cells suggests that this too is a response to carbon starvation.

The results from these experiments show that GDH is derepressed under conditions of sucrose limitation. The active catabolism of protein and production of ammonia which accompany this derepression suggested the possibility that GDH was active in the catabolism of glutamate. This was investigated by studying the metabolism of [^{15}N]glutamate using <u>in vivo</u> NMR, ANCA-MS and conventional GC-MS.

Table 5.3

Changes in the soluble amino acid pools (expressed as percentage increase) over 24 hours, during growth in medium containing various nitrogen sources in the presence and absence of 2% sucrose.

(Treatments 0 time 0, A ammonium, AS ammonium + sucrose, N nitrate, NS nitrate +sucrose, Gln glutamine, GlnS glutamine + sucrose, Glu glutamate, GluS glutamate + sucrose, AO ammonium + 2-oxoglutarate, GlnO glutamine + 2-oxoglutarate. Negative numbers indicate a decrease from time 0)

	Increase (%)			
Treatments	A	AS	N	NS
Amino acid				
Glutamate	62	-37	19	-47
Glutamine	28	-19	-28	-68
GABA	107	6	65	92
Alanine	88	-3	27	83
Arginine	262	96	240	97
Serine	244	22	255	-2
Methionine	135	82	86	-13
Glycine	-33	46	109	-20
Valine	286	7	280	-44
Aspartate	92	1	57	-37
Leucine	304	74	231	-13
Isoleucine	343	49	352	-12
Threonine	226	-6	255	-23
Phenylalanine	232	78	87	8
Asparagine	314	26	263	-23
Tyrosine	211	83	132	-32
Histidine	174	-53	137	15
Lysine	135	-100	132	34
Total pool	161	-1	131	-19

Table 5.3 continued

	Increase (%)					
Treatments	Gln	GlnS	Glu	GluS	AO	GlnO
Amino acid						
Glutamate	133	-44	86	-25	15	149
Glutamine	42	-20	13	-65	-12	56
GABA	154	42	361	85	31	131
Alanine	139	27	83	3	-10	79
Arginine	230	0	193	-5	131	307
Serine	172	1	281	7	222	298
Methionine	54	-16	70	-17	19	111
Glycine	103	29	-55	0	67	196
Valine	208	0	339	-30	211	347
Aspartate	65	-44	101	-43	73	206
Leucine	192	2	279	-17	204	328
Isoleucine	253	7	395	-10	293	442
Threonine	198	22	254	-2	264	334
Phenylalanine	89	-6	124	-19	72	204
Asparagine	221	-12	297	-32	248	336
Tyrosine	109	7	138	-19	84	142
Histidine	110	26	157	-9	118	179
Lysine	-100	486	483	464	85	-100
Total pool	128	20	184	4	107	195

Table 5.4.

Changes in cell fresh weight (g/flask) during 3 days growth in medium containing various nitrogen sources in the presence and absence of 2% sucrose.

	Cell fresh weight (g/flask)			
Time (days)	0	1	2	3
Treatment				
ammonium	1.3	1.41	1.59	1.71
ammonium + sucrose	1.3	0.86	-	-
nitrate	1.3	1.67	1.51	1.82
nitrate + sucrose	1.3	2.08	3.38	5.25
L-glutamine	1.3	1.57	1.64	1.77
L-glutamine + sucrose	1.3	2.24	4.19	6.68
L-glutamate	1.3	1.51	1.52	1.42
L-glutamate + sucrose	1.3	2.03	3.78	6.29
ammonium + 2-oxoglutarate	1.3	1.83	1.54	1.51
L-glutamine + 2-oxoglutarate	1.3	1.41	1.69	1.41

CHAPTER 6: GLUTAMATE METABOLISM IN CARROT CELL CULTURES.

The results from the investigations into ammonium assimilation and the effect of sucrose starvation in these cells suggested that GDH might be active in the catabolism of glutamate. This mode of action for the enzyme has been suggested by many workers mainly in response to the weight of evidence which shows that GDH does not have a role in ammonium assimilation in higher plants. Previously it had not been possible to demonstrate conclusively that glutamate is catabolised to ammonium *in vivo* and this role for GDH had remained speculative. Using suspension cell cultures it was possible to investigate the metabolism of ^{15}N glutamate and therefore determine any catabolic action of GDH by assessing ^{15}N enrichment of ammonium produced by the cells. In order to maximise the accumulation of ammonium the experiment was carried out in the presence of two inhibitors, MSO and aminooxyacetate (AOA), an inhibitor of transamination reactions. AOA was used to lessen the transfer of the labelled amino group to other amino acids. In addition MSO was added so that any ammonium that was produced from the labelled glutamate would remain in the cells or medium and not be assimilated into glutamine via GS. It was not possible to detect [^{15}N]ammonium at low concentrations using conventional GC-MS so *in vivo* NMR and ANCA-MS were used to determine the ^{15}N enrichment of ammonium.

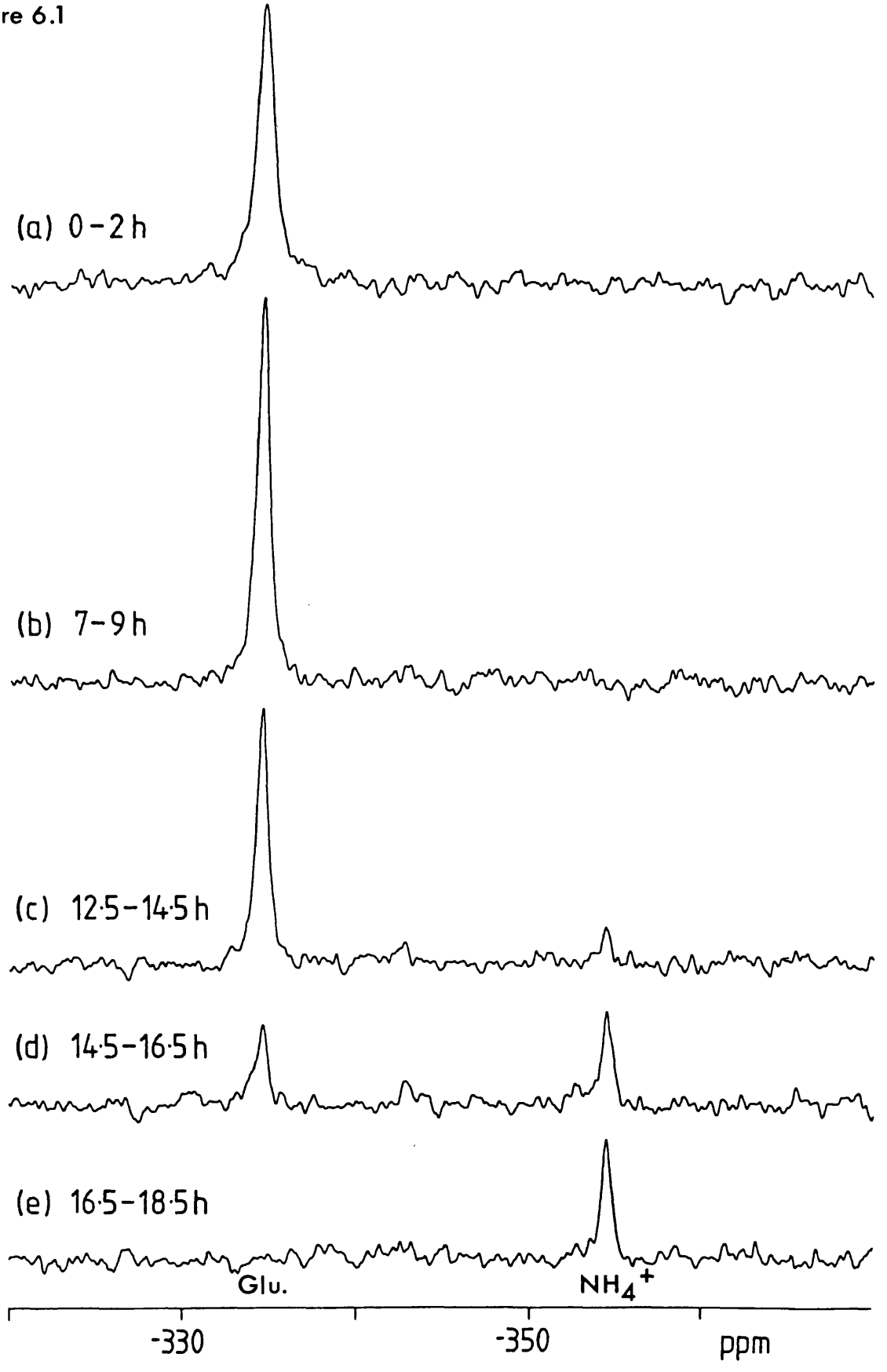
In vivo NMR of ^{15}N glutamate.

The metabolism of ^{15}N-labelled glutamate by cells pretreated with 2 mM MSO and AOA was followed over 24 h using *in vivo* ^{15}N NMR spectroscopy (Figure 6.1). The increase in peak height and reduction in linewidth of the glutamate resonance between 2 and 9 hours occur as glutamate is taken up from the medium into the cells. [^{15}N] ammonium was detected at 13.5 hours and its concentration increased over the next 4 hours, while the glutamate resonance decreased. These results show that glutamate can be catabolised to ammonium in the presence of

Figure 6.1

^{1}H-coupled ^{15}N NMR spectra of 11 d carrot cells following the addition of 20 mM [^{15}N]glutamate. The spectra were obtained in two hours over the time intervals indicated. The increase in peak height and reduction in linewidth of the glutamate resonance (-334.8 ppm) between (a) and (b) was due to the uptake of glutamate from the external medium at pH 5.6 to the cytoplasm at pH 7.6. The properties of the ammonium resonance (-354.6 ppm) are discussed in the text.

Figure 6.1



GDH.

The long interval between the addition of glutamate and the appearance of label in ammonium can be explained by the slow uptake of glutamate into the cells. The effect of pH on the linewidth of the glutamate and ammonium resonances in ^{1}H-coupled spectra is shown in figure 6.2. At pH 7.5 (approximately the pH of the cytoplasm) both the glutamate and ammonium resonances have narrow linewidths, whereas at pH 5.5 (approximately the pH of the vacuole and the external medium) the resonances are very broad, making detection difficult with the short acquisition times used. Over the first 8 h following the addition of glutamate , the resonance became narrower reflecting the uptake of glutamate from the external medium at pH 5.6 to the cytoplasm at pH 7.6.

Experiments using longer recycle times were carried out in an attempt to quantify the amount of ammonium produced (data not shown). At best, the ammonium resonance contained 30% of the label. Quantification, however proved difficult if not impossible, much of the signal may have gone undetected due to the effect of pH on the linewidth of the ammonium resonance (Fig 6.2). Ammonium in the vacuole or in the external medium would not have been readily detected because of the effect of low pH on the linewidth. Removing the cells from the external medium and raising the pH to 7.5 revealed the presence of a small amount of ammonium (approximately 10% of the label). ^{14}N NMR studies have shown that at least 97% of the ammonium is located in the vacuole in maize root segments (Lee and Ratcliffe, submitted). It is therefore likely that much of the ammonium, which would have been present in the vacuole would have gone undetected.

In a series of similar experiments, using two different cell lines, the intensity of the ammonium signal represented 15-30% of the original glutamate signal. The results for the *in vivo* NMR suggest that up to 10% of the label is present in the medium and 15-30% is present as cellular ammonium; however for the reasons described above, an exact measurement of the

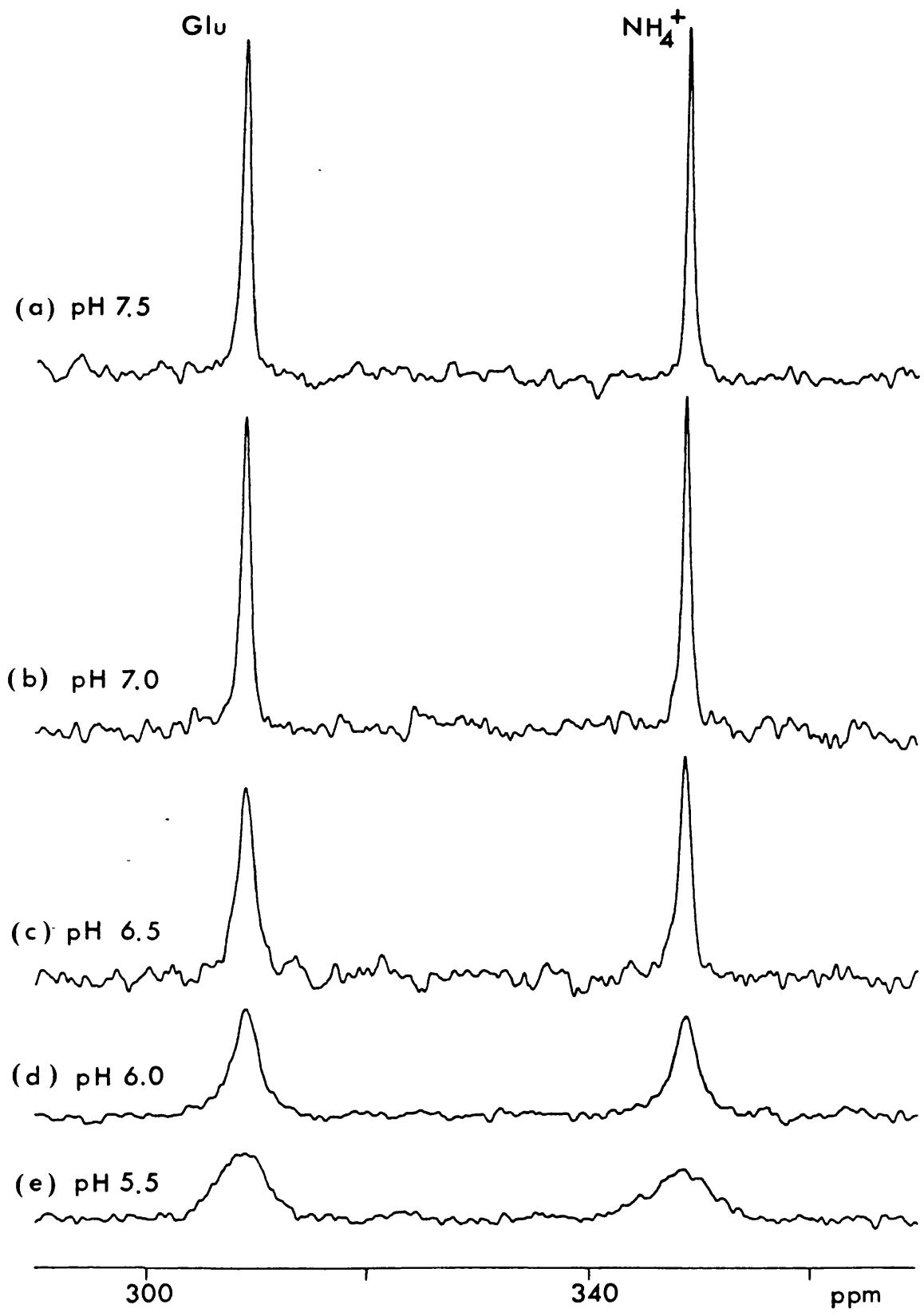
Figure 6.2

The effect of pH on the ^{1}H coupled ^{15}N NMR signal of 20mM [^{15}N]glutamate and 20mM [^{15}N]ammonium.

The spectra were accumulated with a 30° pulse angle and a 20s recycle time for 2h 40min for (a),(b) and (c) and for 8h for (d) and (e)

Each spectrum was obtained in 2.5 h in five 30 min blocks. The 30 min blocks were interleaved with one another over a 7.5 h time interval 2 h after the addition of ammonium chloride.

Figure 6.2



fraction of the label that accumulated in ammonium was not possible by this method. The technique is also limited to detection of the more abundant amino acids. ^{1}H-decoupled spectra showed that GABA also became labelled (Fig 6.3). The absence of the remaining amino acids from these spectra is due to their concentrations being below the detection limits of *in vivo* NMR. In order to determine the ammonium and amino acid enrichment more precisely the experiment was repeated and the results analysed by conventional GC-MS and ANCA-MS.

Metabolism of [^{15}N]glutamate determined by conventional GC-MS and ANCA-MS.

The metabolism of [^{15}N]glutamate was investigated over a 24 hour period, following transfer of stationary phase cells to medium containing 5mM [^{15}N]glutamate in the presence or absence of either 1 mM MSO, 1 mM AOA or both inhibitors. Samples were taken at 12,18 and 24 hours and incorporation of ^{15}N into ammonium and amino acids was determined using conventional GC-MS and ANCA-MS analysis (full experimental details are given in Table 6.1). The specific activities of GS and GDH at 12 and 24 hours for each treatment are shown in Table 6.1. GS was completely inhibited after 12 hours in both MSO treatments but the activity increased in the control and AOA treatments. GDH activity decreased by nearly 50% in all the treatments over 24 hours.

The cell and medium ammonium concentrations are shown in Table 6.2. In the MSO cultures ammonium is produced at a rate of 1.66 umols/h/gfw (MSO) and 1.39 umols/h/gfw (AOA/MSO). The aminooxyacetate treatment showed an increase in cellular ammonium at 12 hours but this was utilised by 18 hours.

Table 6.3 shows the distribution of ^{15}N in the cells after 12 hours. Uptake of[^{15}N]glutamate was reduced by 25% in the MSO treatment and 19% by the AOA/MSO treatment. In the AOA treatment the glutamate uptake increased slightly. In the MSO and AOA/MSO treatments between 9 and 10% of the ^{15}N label was recovered as [^{15}N]ammonium in the medium. This corresponds to

Figure 6.3

^{1}H-decoupled ^{15}N NMR spectra of 11 d carrot cells following the addition of 20 mM [^{15}N]glutamate. The spectra were obtained in two hours over the time intervals indicated. The spectra show that ^{15}N is incorporated into GABA as well as ammonium (see Figure 6.1).

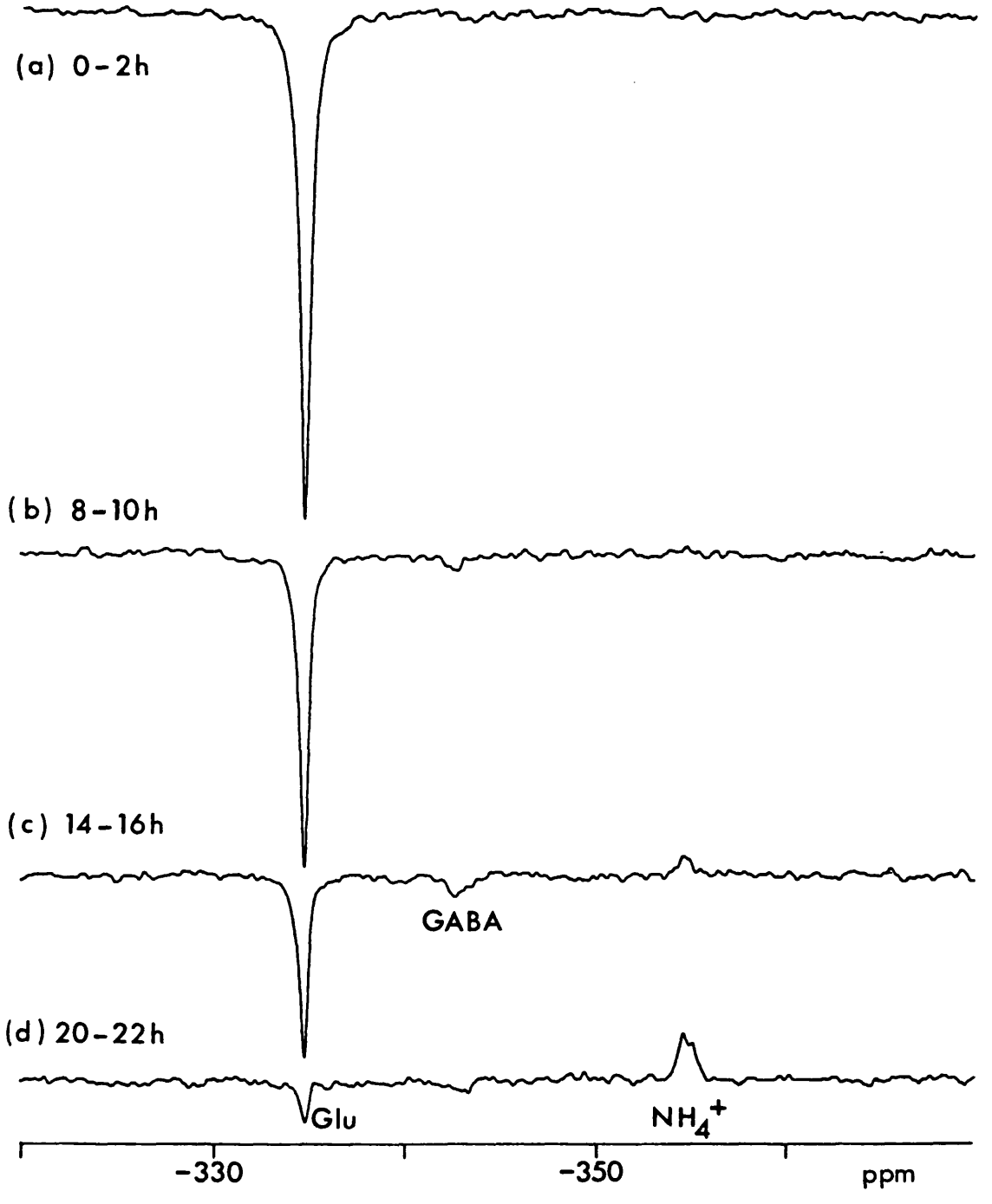


Table 6.1

Changes in the specific activities of GS and GDH during 24 hour treatment with 5mM [^{15}N]glutamate in the presence and absence of 1mM MSO, 1mM AOA and both inhibitors.

	Enzyme activity (nkat/mgprotein)							
Treatment	Control		AOA		MSO		AOA/MSO	
Hours	GS	GDH	GS	GDH	GS	GDH	GS	GDH
0	1.2 ±0.03	16.8 ±0.3	1.2 ±0.03	16.8 ±0.3	1.2 ±0.03	16.8 ±0.3	1.2 ±0.03	16.8 ±0.3
12	1.00 ±0.01	10.2 ±0.2	1.2 ±0.01	10.3 ±0.2	0	10.7 ±0.2	0	10.5 ±0.2
24	2.17 ±0.03	9.3 ±0.2	2.33 ±0.03	9.7 ±0.2	0	8.9 ±0.2	0	9.1 ±0.2

Methods.

Stationary phase cells (d 10) were collected, washed and resuspended in nitrogen free medium as described for ^{15}N ammonium assimilation (Chapter 4). After centrifugation the cells were resuspended in medium containing 5mM ^{15}N glutamate with no inhibitors (control), 1mM MSO (M), 1mM AOA (A) and 1mM AOA and MSO (AM). Samples were taken at 0,12,18 and 24 hours. Cells were extracted and the activities of GS and GDH determined. Amino acid concentration and incorporation of label were determined by HPLC and GC-MS. The total ^{15}N incorporation into the cells was determined using the ANCA system. Total media ammonium was measured colourimetrically and after concentration the ^{15}N enrichment was determined by ANCA-MS. The media glutamate concentrations were determined by HPLC.

Table 6.2

Changes in cell and medium ammonium concentration during 24 hour treatment with 5mM [^{15}N]glutamate in the presence and absence of 1mM MSO, 1mM AOA and both inhibitors.

	Treatment			
	Control	AOA	MSO	AOA/MSO
Cell ammonium (umols/gfw)				
0 hours	2.5±0.1	2.5±0.1	2.5±0.1	2.5±0.1
12 hours	1.2±0.05	5.2±0.2	8.5±0.25	6.0±0.15
18 hours	1.9±0.08	1.5±0.05	8.1±0.2	7.9±0.2
24 hours	1.1±0.05	0.6±0.005	10±0.3	8.8±0.2
Medium ammonium (mM)				
0 hours	0.25±0.04	0.25±0.04	0.25±0.04	0.25±0.04
12 hours	0.2±0.01	0.19±0.01	1.57±0.07	1.75±0.06
18 hours	0.02	0.03	1.81±0.08	1.84±0.07
24 hours	0.02	0.02	2.56±0.1	2.68±0.11

12% of the total ammonium produced by the MSO cells and 9% of that from the AOA/MSO cells. No ammonium was detected in the medium in either the control or the AOA treatment. The ^{15}N label in ammonium only includes that secreted into the medium and not the ammonium present in the cells. It was not possible to measure the labelling of the cell ammonium because the concentration was below the detection limit of the ANCA-MS.

Incorporation of label into soluble amino acids was determined by GC-MS. The total incorporation of label is reduced in all the inhibited treatments, especially the MSO treatment which gave values half that of the control (Table 6.3). However, as a proportion of uptake, soluble amino acids accounted for: control 29%, AOA treatment 23%, MSO treatment 19% and AOA/MSO treatment 21%. The largest proportion of the label was detected as insoluble nitrogen, which represented 63% to 65% of the total uptake for all the treatments except the AOA/MSO. The AOA/MSO value of 46% is particularly low and is due to an unusually low total nitrogen value. this explains the poor recovery rate for this treatment.

Table 6.4 shows the distribution of the ^{15}N label in the amino acid fraction after 12 hours (the values are given in umols/gfw and percentage of the labelled pool, incorporation in atom % excess is given in Table 6.5). In the AOA treatment ^{15}N labelling was significantly reduced in glutamate, methionine, aspartate, serine and isoleucine. The percentage labelling into GABA, histidine and tyrosine was increased by this treatment. The proportion of total glutamine label increased slightly in the AOA treatment, from 19% to 23%. Analysis of the position of the label showed that in the control the majority of label was present in the amino-N but treatment with AOA produced a more equal distribution: 55% amino-N, 45% amide-N. The label in the amide group suggested that [^{15}N]ammonium was produced from the [^{15}N]glutamate and reassimilated via GS. In the MSO treatment, glutamate represented a larger percentage of the amino acid pool compared to the control and AOA treatment and there was no

Table 6.3.

Distribution of ^{15}N in cells after 12 hour incubation with [^{15}N]glutamate.

Cells were cultured in the presence of 5 mM [^{15}N]glutamate ± 1 mM MSO or 1 mM AOA.

	Concentration (umol^{15}N/gfw)			
	Control	AOA	MSO	AOA/MSO
Glutamate uptake	19.8	21.0	15.0	16.0
Ammonium in medium	0	0	1.5	1.4
Soluble amino acids	5.8	4.8	2.9	3.3
Insoluble nitrogen	12.7	13.2	9.9	7.3
Total recovered	18.5	18.0	14.3	12.0
Total recovered (%)	93	86	95	75

Table 6.4.

Incorporation of ^{15}N label into amino acids after 12 hour treatment with [^{15}N]glutamate in the presence and absence of inhibitors.

Cells were cultured in the presence of 5 mM [^{15}N]glutamate ± 1 mM MSO or 1 mM AOA. Percentages represent the proportion of the total ^{15}N labelled pool present as each amino acid.

	^{15}N incorporation (umols/gfw)			
Amino acid	Control	AOA	MSO	AOA/MSO
Glutamate	0.57	0.17	0.48	0.26
Glutamine				
-amino	0.99	0.61	0	0
-amide	0.11	0.5	0	0
Alanine	0.67	0.57	0.19	0.29
GABA	1.93	2.05	0.71	1.84
Valine	0.14	0.13	0.1	0.08
Leucine	0.1	0.08	0.09	0.07
Isoleucine	0.12	0.06	0.13	0.09
Methionine	0.1	0.04	0.03	0
Serine	0.32	0.15	0.2	0.16
Threonine	0.26	0.18	0.18	0.18
Phenylalanine	0.04	0.04	0.04	0.04
Aspartate	0.41	0.07	0.45	0.19
Asparagine				
-amino	0.02	0.04	0.04	0.04
-amide	0	0	0	0
Tyrosine	0.01	0.03	0.01	0
Histidine	0	0.06	0.03	0
Arginine	0	0	0.22	0
Proline	0	0	0	0
Total	5.79	4.78	2.9	3.36

Table 6.4 cont.

Incorporation of ^{15}N label into amino acids after 12 hour treatment with [^{15}N]glutamate in the presence and absence of inhibitors.

Cells were cultured in the presence of 5 mM [^{15}N]glutamate ± 1 mM MSO or 1 mM AOA. Percentages represent the proportion of the total ^{15}N labelled pool present as each amino acid.

	^{15}N incorporation (% of total pool)			
Amino acid	Control	AOA	MSO	AOA/MSO
Glutamate	9.8	3.6	16.8	8.0
Glutamine				
-amino	17.1	12.8	0	0
-amide	1.9	10.5	0	0
Alanine	11.6	11.9	6.6	8.9
GABA	33.3	42.9	24.5	56.8
Valine	2.4	2.7	3.4	2.5
Leucine	1.7	1.6	3.1	2.2
Isoleucine	2.1	1.3	4.5	2.8
Methionine	1.7	0.8	1.0	0
Serine	5.5	3.1	6.9	4.9
Threonine	4.5	3.8	6.2	5.6
Phenylalanine	0.7	0.8	1.4	1.2
Aspartate	7.1	1.5	15.5	5.9
Asparagine				
-amino	0.5	0.8	1.4	1.2
-amide	0	0	0	0
Tyrosine	0.1	0.6	0.3	0
Histidine	0	1.3	1	0
Arginine	0	0	7.6	0
Proline	0	0	0	0
Total	100	100	100	100

Table 6.5.

Incorporation of ^{15}N label into amino acids (atom % excess) during 24 hour treatment with [^{15}N] glutamate in the presence and absence of AOA and MSO.

(Treatments Control no inhibitors, AOA 1mM AOA, MSO 1mM MSO, AOA/MSO imM AOA + 1mM MSO)

12 hours

	Incorporation of ^{15}N (atom % excess)			
Treatment	Control	AOA	MSO	AOA/MSO
Amino acid				
Glutamate	25	16	25	21
Glutamine -amino	9	6	0	0
-amide	1	5	0	0
GABA	41	31	35	32
Alanine	37	30	26	26
Arginine	0	0	7	2
Serine	19	12	15	12
Methionine	18	10	7	0
Glycine	0	0	0	0
Valine	8	8	5	4
Aspartate	24	9	25	17
Leucine	15	13	15	12
Isoleucine	16	11	17	14
Threonine	13	11	10	10
Phenylalanine	9	10	8	10
Asparagine -amino	1	2	2	2
-amide	0	0	0	0
Tyrosine	2	5	2	0
Proline	0	0	0	0
Histidine	0	2	1	0
Lysine	0	0	0	0

Table 6.5 cont.

Incorporation of ^{15}N label into amino acids (atom % excess) during 24 hour treatment with [^{15}N] glutamate in the presence and absence of AOA and MSO.

18 hours

	Incorporation of ^{15}N (atom % excess)			
Treatment	Control	AOA	MSO	AOA/MSO
Amino acid				
Glutamate	38	20	33	31
Glutamine -amino	8	7	0	0
-amide	1	3	0	0
GABA	39	32	31	32
Alanine	43	31	31	33
Arginine	0	2	0	0
Serine	23	12	16	10
Methionine	21	12	2	10
Glycine	0	0	0	0
Valine	12	10	6	5
Aspartate	23	9	20	15
Leucine	22	17	15	13
Isoleucine	23	16	19	15
Threonine	16	15	11	10
Phenylalanine	16	14	8	11
Asparagine -amino	3	2	2	1
-amide	0	0	0	0
Tyrosine	7	7	1	2
Proline	0	0	0	0
Histidine	3	3	1	0
Lysine	0	0	0	0

Table 6.5. cont.

Incorporation of ^{15}N label into amino acids (atom % excess) during 24 hour treatment with [^{15}N] glutamate in the presence and absence of AOA and MSO.

24 hours

	Incorporation of ^{15}N (atom % excess)			
Treatment	Control	AOA	MSO	AOA/MSO
Amino acid				
Glutamate	32	10	26	25
Glutamine -amino	8	9	0	0
-amide	2	2	0	0
GABA	38	33	25	31
Alanine	41	21	23	27
Arginine	0	3	0	0
Serine	26	7	8	11
Methionine	20	0	2	2
Glycine	0	0	7	0
Valine	15	9	7	6
Aspartate	24	8	11	4
Leucine	24	12	7	14
Isoleucine	20	15	16	17
Threonine	17	9	6	10
Phenylalanine	16	4	6	11
Asparagine -amino	2	2	0	2
-amide	0	0	0	0
Tyrosine	8	7	0	4
Proline	0	0	0	0
Histidine	3	6	0	1
Lysine	0	0	0	0

incorporation of ^{15}N into glutamine. There was a decrease in the proportion of label in GABA and alanine and an increase in the remaining amino acids, especially leucine, isoleucine, phenylalanine, aspartate and arginine. In the AOA/MSO treatment over 50% of the label is present as GABA, which reflects the fact that the glutamate decarboxylase enzyme is unaffected by MSO or AOA.

The ^{15}N incorporation into amino acids at 18 and 24 hours is given in Tables 6.5 and 6.6. In most cases the incorporation of ^{15}N into amino acids is lower in the inhibitor treatments, the exceptions being GABA, arginine and histidine in the AOA treatment and GABA in the AOA/MSO treatment.

Table 6.7 shows the changes in amino acid concentration over the 24 hours for all four treatments, expressed as (umols/gfw). The total pool size declined in all the treatments over the 24 hours. In the control the decline was 33%, while in the presence of inhibitors larger declines were observed, namely: AOA 44%, MSO 47% and AOA/MSO 44%.

When glutamate is provided as the sole nitrogen source the role of glutamine as a key amino acid in nitrogen metabolism is lessened. In all treatments there was a decline in the glutamine pool size over a 24 hour period, whereas in cells grown on nitrate or ammonium there would be an increase in this pool. The glutamine pool declined fastest in the MSO treatments in which a drop of 81% was observed as compared with falls of 54% in the control and 62% in the AOA treatment. The labelling data for glutamine shows that no glutamine is synthesised in the presence of MSO. In the AOA treatment glutamine production is comparable with that in the control.

The other amino acids which decrease in all the treatments are arginine, histidine, asparagine, tyrosine, valine, phenylalanine, isoleucine and leucine. The labelling data shows that there is very little label incorporated into these amino acids suggesting that there is very little synthesis of these amino acids over the 24 hours.

Table 6.6.

Incorporation of ^{15}N label into amino acids (umols/gfw) after 18 hour treatment with [^{15}N]glutamate in the presence and absence of AOA and MSO.

	Incorporation of ^{15}N (umols/gfw)			
Treatment	Control	AOA	MSO	AOA/MSO
Amino acid				
Glutamate	1.94	0.38	1.16	0.68
Glutamine -amino	0.64	0.57	0	0
-amide	0.09	0.25	0	0
GABA	1.33	1.60	0.58	1.53
Alanine	1.22	0.62	0.34	0.52
Arginine	0	0.06	0	0
Serine	0.38	0.13	0.22	0.11
Methionine	0.07	0.03	0	0
Glycine	0	0	0	0
Valine	0.19	0.14	0.09	0.07
Aspartate	0.26	0.06	0.20	0.11
Leucine	0.14	0.08	0.09	0.08
Isoleucine	0.18	0.07	0.15	0.09
Threonine	0.31	0.26	0.21	0.18
Phenylalanine	0.06	0.04	0.04	0.04
Asparagine -amino	0.07	0.04	0.05	0.02
-amide	0	0	0	0
Tyrosine	0.04	0.04	0.01	0.02
Proline	0	0	0	0
Histidine	0.1	0.09	0.03	0
Lysine	0	0	0	0
Total	7.02	4.46	3.17	3.45

Table 6.6. cont.

Incorporation of ^{15}N label into amino acids (umols/gfw) after 24 hour treatment with [^{15}N] glutamate in the presence and absence of AOA and MSO.

	Incorporation of ^{15}N (umols/gfw)			
Treatment	Control	AOA	MSO	AOA/MSO
Amino acid				
Glutamate	0.96	0.11	0.65	0.4
Glutamine -amino	0.46	0.42	0	0
-amide	0.11	0.09	0	0
GABA	0.91	1.41	0.29	1.24
Alanine	0.66	0.20	0.17	0.30
Arginine	0	0.08	0	0
Serine	0.32	0.05	0.08	0.09
Methionine	0.05	0	0	0
Glycine	0	0.03	0.03	0
Valine	0.16	0.08	0.09	0.07
Aspartate	0.25	0.04	0.09	0.02
Leucine	0.09	0.03	0.04	0.08
Isoleucine	0.11	0.05	0.11	0.10
Threonine	0.29	0.12	0.10	0.15
Phenylalanine	0.04	0.02	0.02	0.04
Asparagine -amino	0.03	0.03	0	0.03
-amide	0	0	0	0
Tyrosine	0.03	0.02	0	0.02
Proline	0	0	0	0
Histidine	0.08	0.14	0	0.02
Lysine	0	0	0	0
Total	4.55	2.92	1.67	2.56

Table 6.7

Changes in the soluble amino acid concentration (umols/gfw) during 24 hour treatment with 5mM [^{15}N] glutamate in the presence and absence of AOA and MSO.

(Treatments C = control, A = AOA, M =MSO, AM =AOA/MSO)

	Concentration (umols/gfw)						
Treatments	0	12C	18C	24C	12A	18A	24A
Amino acid							
Glutamate	2.96	2.26	5.11	3.00	1.04	1.88	1.09
Glutamine	12.38	11.03	9.08	5.70	10.10	8.20	4.67
GABA	2.14	4.71	3.41	2.41	6.62	5.00	4.26
Alanine	1.98	1.82	2.83	1.62	1.90	2.00	0.94
Arginine	3.94	3.16	3.13	2.64	3.21	2.94	2.75
Serine	1.30	1.71	1.65	1.22	1.27	1.09	0.70
Methionine	0.24	0.57	0.36	0.26	0.41	0.26	0.10
Glycine	0.64	0.97	0.75	0.48	0.84	0.71	0.47
Valine	2.74	1.80	1.57	1.08	1.57	1.35	0.83
Aspartate	0.72	1.71	1.14	1.05	0.80	0.69	0.53
Leucine	1.24	0.67	0.62	0.39	0.61	0.45	0.26
Isoleucine	1.06	0.74	0.77	0.55	0.57	0.46	0.31
Threonine	1.06	1.98	1.95	1.71	1.66	1.75	1.38
Phenylalanine	0.72	0.47	0.36	0.26	0.30	0.28	0.23
Asparagine	2.70	2.45	2.23	1.63	2.22	2.03	1.54
Tyrosine	0.84	0.62	0.56	0.37	0.59	0.51	0.33
Histidine	3.86	3.16	3.22	2.62	2.92	2.89	2.35
Lysine	0	0	0	0	0	0	0
Total	40.52	39.82	38.73	26.99	36.62	32.49	22.73

Table 6.7 cont.

Changes in the soluble amino acid concentration (umols/gfw) during 24 hour treatment with 5mM [^{15}N] glutamate in the presence and absence of AOA and MSO.

	Concentration (umols/gfw)					
Treatments	12M	18M	24M	12AM	18AM	24AM
Amino acid						
Glutamate	1.93	3.53	2.48	1.26	2.19	1.59
Glutamine	6.50	5.43	2.37	6.84	4.97	2.42
GABA	2.03	1.86	1.15	5.75	4.79	4.02
Alanine	0.73	1.11	0.76	1.11	1.59	1.10
Arginine	3.15	3.27	2.89	3.34	3.14	3.07
Serine	1.33	1.37	0.99	1.32	1.08	0.80
Methionine	0.43	0.21	0.15	0.39	0.19	0.14
Glycine	0.83	0.77	0.44	0.80	0.77	0.44
Valine	2.06	1.45	1.31	1.94	1.36	1.21
Aspartate	1.80	1.01	0.81	1.11	0.73	0.56
Leucine	0.60	0.62	0.64	0.58	0.58	0.57
Isoleucine	0.75	0.77	0.69	0.66	0.62	0.56
Threonine	1.75	1.95	1.63	1.75	1.76	1.53
Phenylalanine	0.48	0.45	0.39	0.41	0.39	0.35
Asparagine	2.23	2.31	1.69	2.25	2.13	1.66
Tyrosine	0.35	0.53	0.40	0.56	0.50	0.39
Histidine	2.98	3.40	2.57	2.95	2.92	2.33
Lysine	0	0	0	0	0	0
Total	29.89	30.05	21.36	33.00	29.70	22.73

In the control the general decline in amino acid pools is offset by increases in some of the key amino acids. The pools of glutamate, aspartate, alanine and GABA increase over the first 18 hours, although in general the three inhibitor treatments show much smaller increases in these amino acids. However, GABA is an exception since this pool increases in both the AOA treatments when compared to the control. Synthesis of GABA does not involve transamination and in both AOA samples this amino acid accumulates. The presence of AOA effects the synthesis of aspartate, alanine, serine, methionine and isoleucine and produces a channelling into GABA. This is clear from both the labelling data and the amino acid pool sizes.

The reduced uptake of glutamate from the medium by the MSO treated cells is reflected in the fact that only the threonine, aspartate and GABA (AOA/MSO) pools increase. The remaining amino acids decline less than those in the control.

These experiments offer strong evidence to support the view that GDH is involved in the catabolism of glutamate. The production of [^{15}N]ammonium from [^{15}N]glutamate has been demonstrated both <u>in vivo</u> by NMR and <u>in vitro</u> using the ANCA system. Both methods enabled detection of [^{15}N]ammonium and using ANCA it has also been possible to quantify the release of ammonium into the medium. The labelled ammonium recovered from the medium accounted for 10% of the label taken up by the cells. In the presence of GS inhibitors this ammonium can be detected in the media, whereas if GS is active, ammonium produced by this reaction is reassimilated into the amide of glutamine.

From the <u>in vivo</u> NMR experiments it seems probable that up to 30% of the cellular ammonium is labelled in these cells. If this is the case then up to 27% of the label taken up by the cells would be recovered as ammonium. Unfortunately it is not possible to accurately determine the enrichment of the cell

ammonium using ANCA-MS.

The results demonstrate clearly that GDH is active in the catabolism of glutamate to ammonium in these cells and it seems that this reaction occurs in response to carbon starvation of the cells. The catabolic action of GDH would provide carbon skeletons to ensure continued metabolic activity in these cells under conditions of carbon stress. When sucrose levels are replenished the activity of GDH declines.

CHAPTER 7: DISCUSSION.

In this general discussion, the metabolism of glutamate in carrot cell cultures will be discussed and a model for the role of GDH in higher plants proposed. The implications of this model in relation to previously published work will then be considered.

One of the findings in this thesis demonstrates that although GDH was present at high levels in these carrot cells no role in the assimilation of ammonium could be assigned to the enzyme. Results obtained using the GS inhibitor MSO in combination with ^{15}N labelling studies confirmed that ammonium was assimilated via the GS pathway and provided no evidence to support a role for GDH in the assimilation of ammonium. These results are consistent with the majority of the published work which shows that primary ammonium assimilation occurs solely through the consecutive action of the enzymes glutamine synthetase and glutamate synthase (Rhodes et al 1980; Woo et al 1982; Fentem et al 1983; Berger and Fock 1983; Ta et al 1984; Martin et al 1986; Rhodes et al 1986; Sieciechowicz et al 1989).

Rhodes et al (1989) had suggested that the inhibitor MSO might have an effect on the supply of 2-oxoglutarate to the mitochondria and therefore reduce the activity of GDH by a substrate limitation. This possibility seemed unlikely since MSO does not appear to have any secondary inhibitory effect on GDH in those plants which have been shown to assimilate ammonium via the GDH pathway, for example in the ectomycorhizal fungi of the genus Cenococcum (Genetet et al, 1984; Martin et al, 1988) and in Peltigera aphthosa (Rai et al, 1981). Nevertheless, if MSO does have a substrate limitation effect on GDH this would obviously have profound implications for the acceptance of the role of GS in ammonium assimilation and was therefore investigated. The assimilation of [^{15}N]ammonium was followed in the presence and absence of 2-oxoglutarate, and although 2-oxoglutarate stimulated the

incorporation of ammonium in the control, assimilation of labelled ammonium was not observed in the presence of MSO (Chapter 4). These results show that there is no assimilation of ammonium under conditions where GS is inhibited even when the cells are supplied with excess 2-oxoglutarate and ammonium. This would appear to rule out even a minor role for GDH in ammonium assimilation.

Having shown that GDH was not active in the assimilation of ammonium, a possible catabolic role for the enzyme was investigated. The enzyme was found to be derepressed by conditions of carbon limitation, an effect that was demonstrated in cells grown on a wide range of nitrogen sources (Chapter 5). These experiments have shown that under conditions of carbon limitation GDH activity increases by a factor of ten. The derepression of GDH under conditions of carbon limitation has been widely reported (Oaks et al, 1980; Nauen and Hartmann, 1980; Sahulka and Lisa, 1980; Ratajczak et al, 1981; Tassi et al, 1984).

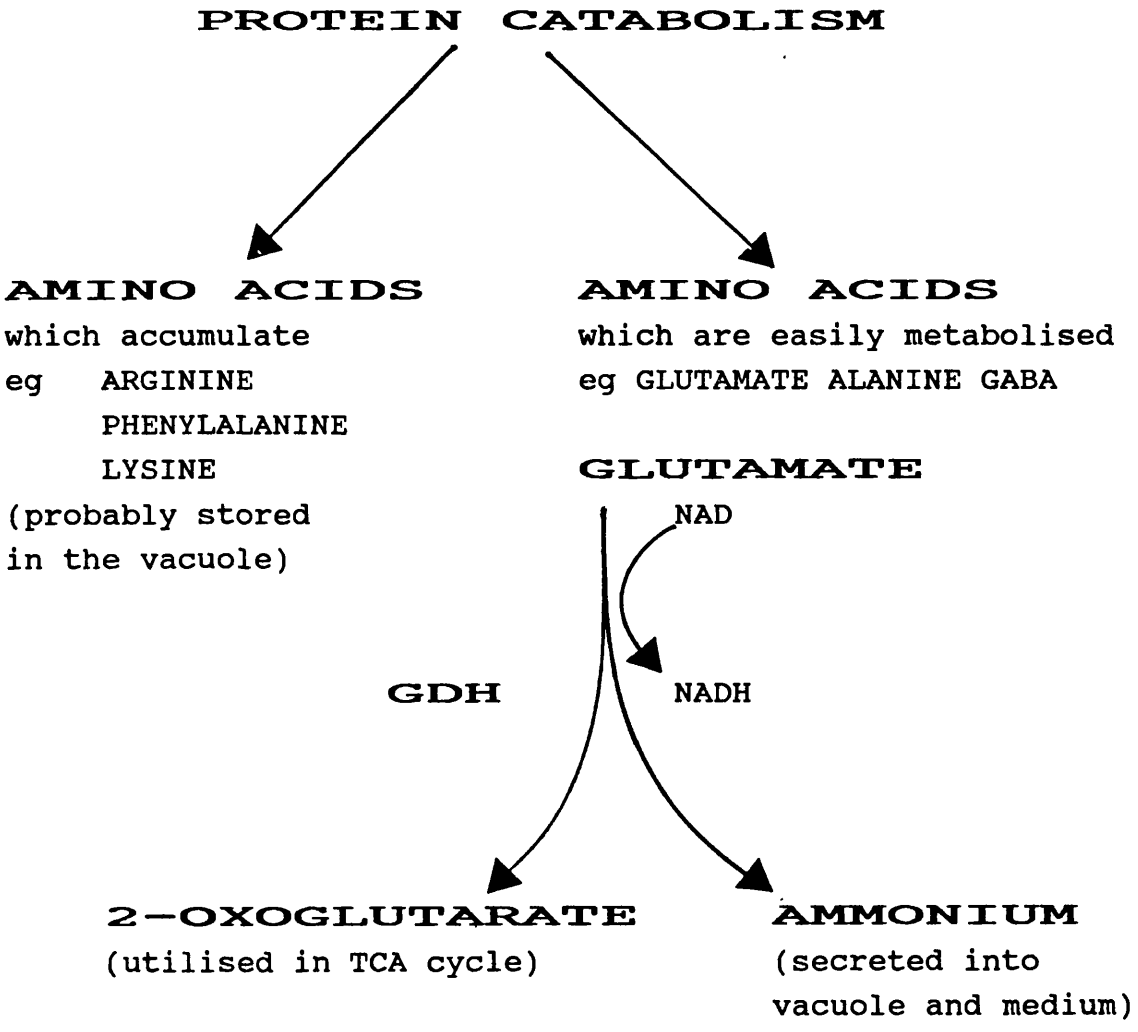
Although GDH has been widely assumed to be active in the catabolism of glutamate, this has not been demonstrated in higher plants. In this investigation <u>in vivo</u> NMR was used in conjunction with ANCA-MS and conventional GC-MS techniques to demonstrate unequivocally that ^{15}N glutamate is catabolised to [^{15}N]ammonium in carrot cells. If GS is active the [^{15}N] ammonium is detected in the amide-N of glutamine and if GS is inhibited ammonium is released into the medium. The combination of <u>in vivo</u> NMR and ANCA-MS techniques enabled the detection of ^{15}N enrichment of ammonium at relatively low concentrations. The use of NMR demonstrated that [^{15}N]glutamate catabolism was occurring <u>in vivo</u> but did not allow accurate measurement of the ammonium produced. Using ANCA-MS it was possible to determine the quantity of [^{15}N]ammonium that was secreted into the medium by the cells. Conventional GC-MS techniques were useful for determining the incorporation of ^{15}N into amino acids but did not allow measurement of the enrichment of $^{15}NH_4^+$ since this ion has the same mass as water and is therefore difficult to detect at low

concentrations. The combination of the three techniques provided a clear demonstration that GDH is active in the catabolism of glutamate in these cells. These techniques could also be applied to a range of other experimental tissues and could be used to investigate the role of GDH in, for example, root tissues or senescing organs.

The conclusion from these experiments is that GDH is active in the catabolism of glutamate and that this reaction is amplified under conditions of carbon limitation. During the stationary phase of culture the cells have exhausted the medium supply of sucrose concentration, and growth ceases. As a result of carbon limitation within the cell the enzyme GDH becomes derepressed and there is also a net catabolism of cell protein. The amino acids which are released as a result of protein catabolism fall into two groups, those which accumulate within the cell, for example arginine, phenylalanine, isoleucine and lysine, and those which are do not accumulate such as glutamate, glutamine, alanine and GABA. These latter amino acids are easily metabolised within the cell to provide carbon skeletons for respiration. The release of the amino group as ammonium can occur through the action of amino acid oxidases or dehydrogenases. The enzyme GDH occupies a central role in this process catalysing the deamination of glutamate to 2-oxoglutarate and ammonium. The ammonium produced by this process accumulates both within the cell and in the external medium. When the cells are placed in fresh medium containing sucrose the activity of GDH declines, net protein catabolism ceases and the ammonium level within the cell is reduced. At the same time the enzymes of ammonium assimilation, GS and GOGAT, increase in activity and the active assimilation of ammonium and nitrate is initiated. This model for the role of GDH (Figure 7.1) implies that the enzyme is primarily an enzyme of carbon metabolism and its role in higher plants is to ensure that there are sufficient carbon skeletons for effective functioning of the TCA cycle, particularly under conditions where carbon is limited.This catabolic role for GDH implies an important regulatory function in carbon and nitrogen metabolism.

Figure 7.1

MODEL FOR THE ROLE OF GLUTAMATE DEHYDROGENASE IN CARROT CELL CULTURE CELLS.



Since GDH catalyses a reversible reaction but no assimilatory activity has been demonstrated it must be assumed that regulation *in vivo* prevents the production of glutamate. The experiments described in this thesis suggest that this regulation does not involve the concentration of ammonium or 2-oxoglutarate, since the addition of these two substrates to cells did not promote the assimilation of ammonium by GDH. It therefore seems likely that the enzyme is regulated by the availability of reduced pyridine nucleotides. Yamaya et al (1984) reported concentrations of 0.21 mM for NADH and 6.37 mM for NAD in mitochondria isolated from corn shoots. It seems possible that the ratio of NAD/NADH could prevent GDH from assimilating ammonium and regulate the deamination of glutamate. Davies and Teixera (1975) showed that the deamination of glutamate in isolated pea seedling mitochondria was inhibited by low concentrations of NADH. As a result of these experiments they suggested that GDH was controlled by the ratio of NAD/NADH in the mitochondria. If the concentration of TCA cycle acids in the mitochondria is high then the ratio of NAD/NADH is low and deamination would be inhibited. However, when the concentration of TCA cycle acids falls (ie when cells are carbon limited) the ratio of NAD/NADH would rise and the deamination of glutamate would occur allowing replenishment of the TCA acids at the expense of glutamate (Davies and Teixera, 1975). Joy (1988) argues that although the properties of the enzyme *in vitro* do not support a catabolic role this would be possible in a situation where the products (particularly ammonium and NADH) could be continuously removed.

Effective regulation of the catabolic action of GDH would be required in order to ensure that excessive depletion of the glutamate pool does not occur in cells which are not sucrose starved. Ferguson and Sims (1971) showed that when *Candida utilis* was grown on glutamate media there was extensive derepression of NAD-GDH in order to provide the yeast with ammonium for amide synthesis. When these cultures were transferred to glutamine or ammonium media there was rapid

inactivation of GDH in order to maintain the concentration of the glutamate pool (Ferguson and Sims, 1971). The fall in GDH activity when carbon starved carrot cells are provided with sucrose (Chapters 3 and 5) suggests that a comparable regulation may be occurring in these carrot cells.

This model of GDH activity has implications for much of the work previously reported concerning the role of GDH in higher plants. It may also explains why the enzyme is found in high levels in senescing organs and root tissues, since carbon limitation and protein catabolism may be occurring in both these tissues. Several studies have shown that treatment with high concentrations of ammonium produces elevated GDH levels in higher plants (Gamborg and Shyluk, 1970; Jain and Shargool, 1987; Barash et al, 1973, 1975; Cammaerts and Jacobs, 1985; Rhodes et al, 1976; Kanamori et al, 1972;). The general conclusion from these studies has been that the high level of ammonium present in these tissues enables GDH to operate in the assimilation of ammonium. This suggests that the role of GDH in higher plants is to prevent ammonium toxicity, although this view is not supported by inhibitor studies. For example, in 1976. studies with Lemna minor (Rhodes et al, 1976) showed that GDH levels increased by 60% when cells were grown on medium containing 10 mM ammonium compared with 0.1 mM. At the time it was suggested that GDH might be active in preventing ammonium toxicity by assimilating some of this ammonium. However, in a later paper, Rhodes et al (1980) ruled out this possibility after they had shown that ^{15}N incorporation into amino acids was prevented in the presence of MSO.

An alternative hypothesis is that the high concentration of ammonium present in these tissues is producing carbon limitation by diverting carbon away from carbohydrate reserves and into amino acids and protein. The diversion of ^{14}C carbon from carbohydrate to amino acids in response to elevated ammonium levels has been shown in Chlorella vulgaris (Miyachi and Miyachi, 1985). It is possible that the high levels of GDH observed after treatment with ammonium are actually a

response to the carbon limitation produced by the increased ammonium levels rather than as a direct result of treatment with ammonium. This hypothesis is supported by results from Cammaerts and Jacobs (1985) who found that the de novo GDH synthesis was induced in Arabidopsis thaliana plantlets by elevated ammonium levels. However, the addition of sucrose to the medium reduced the ammonium dependent induction of GDH. In soybean tissue culture cells, Jain and Shargool (1987), reported that cells grown on high ammonium as a sole source of nitrogen showed a rapid utilisation of ammonium accompanied by an equally fast depletion of the carbon supply. Once the ammonium and carbon supply was exhausted GDH levels increased. The addition of either 2-oxoglutarate or glutamate to these cells inhibited the apparent induction of GDH and also enabled the cells to continue to grow on high ammonium as a sole nitrogen source (Jain and Shargool 1987). These results suggest that the GDH derepression is occurring as a result of carbon limitation caused by excess ammonium levels.

The observation that GDH activity increases during senescence also fits the model proposed above for GDH. The situation in the senescing leaf can be compared in certain respects to that observed in the stationary phase carrot cells. As photosynthesis declines the senescing leaf becomes limited with respect to carbon supply. The catabolism of leaf protein produces amino acids, some of which accumulate, whereas others such as glutamate are rapidly catabolised. In 1978, Thomas put forward a scheme detailing the reactions which could convert the nitrogen from protein catabolism into amide nitrogen for transport from the senescing leaf to other parts of the plant. The fact that glutamate and aspartate do not accumulate as a result of protein catabolism suggests that they are readily metabolised as they are released from protein and their nitrogen is transferred to the amide pool. Thomas suggested that glutamate was catabolised to ammonium via GDH and that this ammonium was then reassimilated via GS into glutamine and transported from the leaf. Towards the end of senescence there is a large increase in tissue ammonium accompanied by a continuing increase in GDH activity. This

was attributed (Thomas 1978) to ammonium induction of GDH as reported by Barash et al (1973), however from the model proposed in this thesis the continued increase in GDH activity would be due to derepression of GDH as a result of carbon limitation.

Support for a catabolic role for GDH during senescence is widespread (Cammaerts and Jacobs, 1985; Berger et al, 1985; Kar and Feierabend, 1984). Kar and Feierabend reported that GDH represented the major path for the liberation of ammonium from amino acids in senescing wheat leaves since no L-amino oxidase activity was detected. Cammaerts and Jacob (1985) noted a pronounced activation of NAD-GDH during senescence in Arabidopsis thaliana leaves and suggested that the oxidation of glutamate could provide the cells with reduced nucleotides and carbon for carbohydrate metabolism in circumstances where the chloroplasts are inactivated. During senescence there is a decline in GS and particularly GOGAT activities over the same period that GDH activity increases (Simpson and Dalling, 1981). This has led to the suggestion that GDH might be important in the reassimilation of ammonium in late senescence (Simpson and Dalling, 1981; Groat and Vance, 1981; Kang and Titus 1980). However, Berger et al (1985) showed that the GS activity was sufficient to account for reassimilation of all photorespiratory ammonium produced during senescence, and demonstrated that when ^{15}N ammonium was fed to senescing wheat leaves it was incorporated first into the amide of glutamine. In the same study, the senescing wheat leaves were also incubated with ^{15}N glutamate. This produced a slight enrichment of the ammonium pool suggesting that mitochondrial deamination of ^{15}N glutamate via GDH had occurred (Berger et al, 1985)

It seems that the model proposed for a catabolic role for GDH fits much of the published data for GDH activity in senescing tissues. GDH is active in senescing tissues to allow mobilisation of carbon and nitrogen from protein into forms which can be utilised or transported from the leaf. If this is the case then the high levels of ammonium accumulation observed in the senescing leaf are produced by the catabolic

action of GDH, rather than the activity of the enzyme being induced by the ammonium levels. The fate of the products of glutamate oxidation depend on the status of GS in these tissues; if GS is active then ammonium is reassimilated into glutamine and transported from the leaf. However, if GS is inactive the ammonium released from glutamate accumulates in the leaf, this seems to occur both during the latter stages of senescence (Thomas, 1978; Kang and Titus, 1980) and if senescing leaves are kept in the dark (Kar and Feierabend, 1984) . The 2-oxoglutarate and reduced nucleotides would be utilised within the leaf for carbohydrate metabolism.

High GDH activity is also associated with root tissues in higher plants, especially the mature regions of the root. Oaks et al (1980) found that high levels of GDH activity in mature roots were associated with levels of high ammonium concentration and suggested that in these circumstances GDH was active in the assimilation of ammonium. The addition of sucrose to these roots brought about a reduction in GDH activity (Oaks et al, 1980), suggesting that the original derepression of GDH was caused by carbon limitation. It seems possible that these results could equally be interpreted by the catabolic model for GDH activity. In this case GDH is derepressed in mature roots as a result of carbon limitation. The oxidation of glutamate to provide carbon skeletons also results in the production of ammonium, which may explain why the levels of cell ammonium are high.

A catabolic role for GDH could also explain the results obtained during investigation of a GDH deficient mutant of Zea mays by Rhodes (1989). This mutant shows marked changes in shoot/root ratio and a reduced level of ^{15}N assimilation under normal growth conditions, but no ammonium assimilation is observed in the presence of MSO. This implies that the although assimilation occurs via the glutamate synthase pathway the absence of GDH has a profound effect on growth. If the role of GDH in higher plants is the oxidation of glutamate then a deficiency in the catabolism of glutamate, might impair growth by reducing the potential of the cells to

recycle the products of protein catabolism. This GDH mutant could be useful in establishing a catabolic role for GDH in higher plants and deserves further investigation.

The published work described both in this conclusion and in the introduction shows little evidence for a role of GDH in ammonium assimilation in higher plants. The highest levels reported via an MSO insensitive pathway amount to less than 1% of the assimilation observed via the glutamate synthase pathway. The evidence presented here shows conclusively that GDH is not active in the assimilation of ammonium but can operate in the catabolism of glutamate in higher plant cells. GDH is derepressed under conditions of carbon limitation and responsible for ensuring an adequate supply of carbon skeletons and reduced nucleotides under conditions of carbon stress. The model proposed here suggests that GDH may be an important enzyme in the regulation of nitrogen and carbon metabolism in higher plants.

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