The compartmentation of nitrogenous assimilates in suspension cultured cells of carrot.

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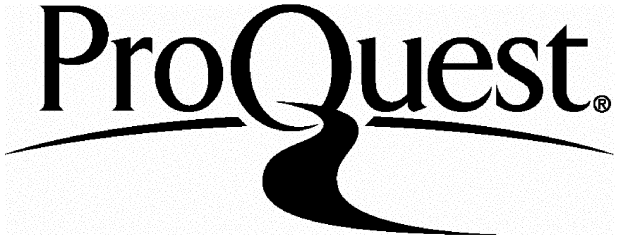

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Dedicated to my parents, brother and sister, nephews and nieces.

Abstract

Little is yet known of the role of compartmentation of assimilate in the regulation of higher plant nitrogen metabolism. In order to investigate the importance of such compartmentation techniques were developed which enabled intact plastids, mitochondria and vacuoles to be prepared from protoplasts derived from carrot (*Daucus carota*) suspension cells. When the amino acid content of each organelle was determined by HPLC (high performance liquid chromatography) the results suggested that certain amino acids were unevenly distributed in the protoplast. Glutamine, valine, phenylalanine and asparagine were primarily vacuolar in distribution while other species, glutamate, Gaba and alanine, were chiefly cytoplasmic. By feeding protoplasts with ammonium chloride containing the stable isotope ^{15}N and preparing organelle fractions at regular intervals thereafter it was possible to monitor the intercompartmental fluxes of freshly synthesised amino acids by HPLC and GC-MS (gas chromatography-mass spectrometry). Feeding with ammonium resulted in a considerable increase in the total amino acid content of protoplasts, chiefly due to increased breakdown of proteins in the vacuole, a response characteristically elicited by the onset of cytosolic acidosis.^{15}N was found to be incorporated chiefly into three different amino acids, Gaba, glutamine and glutamate, which differed in their spatial distribution: freshly synthesised Gaba and glutamate were largely restricted to the cytosol while glutamine was compartmentalised in the vacuole. Of these three Gaba appeared to be the major sink for freshly assimilated nitrogen.

The metabolic responses of protoplasts to feeding with ammonium was studied using *in vivo* ^{31}P-and ^{15}N-NMR (nuclear magnetic resonance). This confirmed that ammonium uptake resulted in a decline in cytosolic pH, which was subsequently restored to its original value. A concomitant increase and decrease were observed in glutamate decarboxylase activity and in the resulting accumulation of Gaba. It is proposed that cytosolic pH is regulated through a biochemical pH stat centred around the decarboxylation of glutamate. ^{15}N-NMR spectra collected from cells made hypoxic prior to the addition of ammonium, to induce cytosolic acidosis, suggested that maintenance of a vacuolar pool of glutamine is an important way in which plant cells conserve nitrogen and presumably also carbon.

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Abbreviations used

Ala	Alanine
ANCA	Automated $^{15}N/^{13}C$ analysis
Asp	Aspartate
ATP	Adenosine triphosphate
Cit	Citrate
ddH_2O	Double distilled H_2O
dH_2O	Distilled H_2O
2,4-D	2,4-Dichlorophenoxyacetic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPPS	(N-[2-Hydroxyethyl]-piperazine-N'-[3-propane-sulfonic acid]
fwt	Fresh weight
G6P	Glucose-6-phosphate
Gaba	γ-aminobutyrate
GAP	Glyceraldehyde-3-phosphate
GC-MS	Gas chromatography/mass spectrometery
Glu	Glutamate
Gly	Glycine
GS/GOGAT	Glutamine synthetase/glutamate synthase
HPLC	High performance liquid chromatography
mal	Malate
MES	(2-[N-Morpholino]ethanesulfonic acid
NAD	β-Nicotine adenine dinucleotide
NADH	β-Nicotine adenine dinucleotide reduced form
NADP	β-Nicotine adenine dinucleotide phosphate
NADPH	β-Nicotine adenine dinucleotide phosphate reduced form
NDP-hexose	Di phosphate nucleotide linked hexose
α-NTP	α-phosphate group of tri-phosphate nucleosides

β-NTP	β-phosphate group of tri-phosphate nucleosides
γ-NTP	γ-phosphate group of tri-phosphate nucleosides
OAA	Oxalacetic acid
2oxg	2-Oxoglutarate
P-choline	Phosphorylcholine
3PG	3-phospho-glycerate
Pyr	Pyruvate
TCA	Tri-carboxylic acid
TES	(N-tris[Hydroxymethyl]methyl-2-aminoethaesulfonic acid
TP	Triose phosphates
Tris	(tris[Hydroxymethyl]aminomethane)
(v/v)	volume per volume
(w/v)	weight per volume

Chapter 1 Introduction

1.1 Compartmentation in plant cells

It would undoubtedly be more agreeable to regard the metabolic processes within a plant cell as arising from chance encounters between haphazardly arranged enzymes and substrate molecules. However there is an irrefutable body of evidence which shows that even amongst the prokaryotes specific metabolic reactions are assigned to discrete regions of cell space (Srere and Mosbach, 1974). Such localisation of metabolites in intracellular compartments is an essential feature of biomolecular organization of higher plant cells, in which metabolic regulation is more dependent on compartmentation than in animal cells (Dennis and Miernyk, 1982).

The study of metabolic compartmentation in plant cells dates from the pioneering work of Gregory and Sen (1937), and Bennet-Clark and Bexon (1943) which implied that the soluble components of plant cells were not evenly distributed within the cell. The advent of ^{14}C-labelled compounds allowed this phenomenon to be studied more closely. Bidwell *et al.* (1964), demonstrated that amino acids derived from protein breakdown were segregated from primary assimilates and destined for deamination as opposed to protein synthesis. Similarly Oaks (1965), and Dougall (1965) described discrete pools of amino acids which differed in their metabolic responsiveness, whilst *en route* to the same metabolic fate. Cowie and McClure (1959) provided probably the first evidence of two distinct amino acid pools in the yeast *Candida utilis*, the first being relatively small and metabolically active providing amino acids for protein synthesis, while the second, larger pool showed all the characteristics of being a reserve pool. Subsequently it was suggested that the rapidly turning-over pool and the reserve pool corresponded to cytoplasm and vacuole, respectively.

Referring to these reports, Lips and Beevers (1965a,b), showed that similar concepts could account for the behaviour of organic acids in corn roots, and Steward and

Bidwell (1966) confirmed that their use of the term "pool" denoted a membrane-delimited cytological feature. The proponents of this theory of compartmentation were not without their critics: Joy and Foulkes (1965) concluded that the patterns of metabolite distribution in barley embryos could best be explained by invoking varying degrees of end product repression, that were responsible for competition between sugars and proteins resulting in an uneven supply of carbon to protein.

From these early works it is possible to describe the theory of compartmentation in terms of three essential features: that the assimilation of exogenously applied solutes results in an uneven distribution within the cell; that any one compound with different routes of synthesis will have different metabolic fates, and finally that alternative routes of synthesis and asymmetrical solute distributions are localised to membrane delimited morphological spaces. The formulation of such a theory led to the concept of compartments at the cellular level becoming firmly established (Oaks and Bidwell, 1970).

1.2 Methods used to study cellular compartments

Following early observations of metabolite compartmentation many methods have been used to investigate the morphological cell space in which the compartmentalised pools might be located. Many of the methods described below were originally developed to investigate vacuolar compartmentation, reflecting the presumed importance of this organelle. The methods employed may be regarded as *direct* techniques, when immediate measurements are made within the compartment, and as *indirect* techniques when compartmentation is inferred from the behaviour of pre-existing and newly synthesised metabolite pools.

1.2.1 *Indirect* evidence for metabolic compartmentation

The (indirect) quantitative analysis of metabolite pools was facilitated by the development of radio-labelled compounds (early studies are reviewed in Oaks and

Bidwell 1970). A general observation from many experiments was that labelled metabolites are not in equilibrium with total cellular pools. Frequently analysis of the kinetics of labelled metabolites suggests that they are contained in one of two distinct pools; a large pool, rapidly turning over, and a smaller, apparently metabolically inactive, storage pool. The latter is repeatedly assumed to be vacuolar.

The indirect analysis of compartments through analysis of the kinetics of exogenously supplied tracers has been approached in two ways. Firstly, labelled substrates are supplied and the fluxes of freshly synthesised metabolites are compared to the unlabelled pre-existing pools. Rhodes *et al.* (1989a) used this method to analyse the labelling kinetics of freshly synthesised amino acid pools in plantlets of *Lemna minor* L. grown on $^{15}NH_4Cl$. These authors concluded that the ratios of unlabelled:labelled amino acids were diagnostic of the size and metabolic activity of the amino acid pools. Secondly, experiments of the "Pulse-chase" type are performed by pre-loading cells with labelled substrates and then providing a source of unlabelled substrate, so that the dissemination of the labelled pools may be followed against an unlabelled background. Cooke *et al.* (1980) used this method to study the compartmentation of amino acids and protein pools in *Lemna*. Plantlets were grown on ^3H_2O, so that transamination reactions resulted in the incorporation of ^3H at position 2 of the amino acid. On transfer to tritium-free medium the fate of the tritiated amino acid was followed. The authors propose that the loss of ^3H from the amino acid through transamination is indicative of the amino acid being localised in a compartment containing transaminases. Consequently amino acids retaining label were regarded as being isolated from transaminase activity i.e. compartmentalised within the vacuole. This method has been recently employed to study the effect of methionine sulphoximine (MSO), an irreversible inhibitor of glutamine synthetase, on the vacuolar storage of amino acids in *Lemna* (Goodchild and Sims, 1990). In similar experiments Lee and Clarkson (1986) and Lee and Drew (1986) were able to study the concurrent short term influx and efflux of nitrate from barley roots by tracer flux analysis of ^{13}N-labelled nitrate. From

analysis of the short term efflux of $^{13}NO_3^-$, the authors claimed that the cytoplasmic NO_3^- pool accounted for some 2% of the total nitrate in the roots.

Studies of the cellular fluxes of labelled metabolites have one major advantage over other methods of compartment analysis in that they allow the study of the dynamics of metabolite compartmentation *in vivo*. However, they do rely upon the assumption that metabolite pools occupying discrete morphological cell space have unique characteristics which allow them to be indentified, i.e. that vacuolar pools are metabolically inactive. This assumption, depending on the time scale of the investigation, is not always correct.

1.2.2 *Direct* evidence for metabolic compartmentation

The recognition that vacuoles are the site of deposition of a number of different substances has been achieved by microscopical methods. Specific staining techiques, with heavy metals providing the contrast for electron microscopical investigations, have shown that a number of inorganic compounds are accumulated in the vacuole in a variety of species (reviewed by Matile and Wiemken, 1985). A more recent development of this approach has been the use of X-ray microanalysis of anion concentrations in the vacuolar and cytoplasmic compartments of *Atriplex spongiosa* (Storey *et al.*, 1983).

Perhaps the most exciting technological development in the analysis of cellular compartmentation has been the direct measurement of metabolite levels *in situ*, using ion sensitive microelectrodes. Such devices have been used to measure a number of different ions in plant cells, including Ca^{2+} (Miller and Saunders, 1987), H^+ (Felle, 1987) and NO_3^- (Miller and Zhen, 1991). This technique allows the direct and highly accurate measurement of ion concentrations *in situ*, its main disadvantage being that intercompartmental measurements are mainly restricted to large unicellular algae such as. *Chara ceratophylla*. A recent adaptation of micro-manipulation technology in the

study of compartmentation has been the development of pressure probes enabling picolitre samples of undiluted vacuolar sap to be taken from cells *in situ*. Malone *et al.* (1991) reported on the use of this technique to measure the concentrations of inorganic ions in the vacuoles of individual cells of intact wheat leaf epidermis.

Several methods exist by which the nature and size of compartmental metabolite pools can be determined, not *in situ*, but after fractionation of cells and protoplasts, following the recognition that standard methods of cell fractionation frequently resulted in the loss of micromolecules from isolated subcellular structures. Wiemken and Nurse (1973) addressed this problem by developing a technique for the fractionation of cells of the food yeast,*Candida utilis*, into vacuolar and cytoplasmic fractions based upon differential permeabilization of the plasmalemma followed by destabilisation of the tonoplast. Gerhadt and Heldt (1984) reported a technique for the fractionation of freeze-stopped spinach into stromal, vacuolar and cytosolic fractions permitting analysis of the subcellular malate and sucrose pools. Fractions were resolved by density gradient centrifugation of freeze-stopped material extracted in organic solvents. The method was unique in so much as it allowed metabolite levels in subcellular compartments other than the vacuole to be determined. It was also rapid and therefore allowed dynamic changes in metabolite levels to be monitored. The isolation of subcellular particles has thus become a principal methodology employed in the study of metabolite pools.

Certain criteria must be met if subcellular particles are to be used to study metabolite compartmentation. The particle should be recovered morphologically intact, it should be free of contaminating material, and the recovery should be quantitative (Price, 1983). Fractionation methods frequently involve two phases; *disruption* of the tissue, and the subsequent *separation* of subcellular particles. With regards to the former the most significant methodological development of recent years has been the use of protoplasts for fractionation into organelles (Thayer, 1985). Innumerable methods of

protoplast disruption have been employed, the exact method being determined by which subcellular fractions one wishes to isolate. In general, fractionation into vacuoles and cytoplasm has been achieved by gentle, controlled lysis of the protoplasts. This may be achieved by reducing the level of osmoticum in their supporting medium (Sasse *et al.*, 1979), by phosphate-dependent osmotic lysis (van der Valk, 1987) or by polybase (DEAE-dextran)-induced lysis (Boudet *et al.*, 1981). All these methods have produced intact vacuoles with varying degrees of contamination from other organelles.

A recent advance in the study of metabolite compartmentation by protoplast fractionation involves the use of evacuolate protoplasts, ordinary protoplasts being evacuolated by ultracentrifugation through a density gradient. By comparison of evacuolate protoplasts with vacuolate protoplasts, pool sizes of cytosolic and vacuolar metabolites can be determined. Using the method initially reported by Griesbach and Sink (1983), Steingraber and Hampp (1986) provided the first report of the use of this technique in the compartmental analysis of a range of metabolites. Their observations were consistent with estimates of pool sizes obtained by other methods. The use of such a system could also facilitate investigation of the part played by the vacuole in those processes where the involvment of the organelle is thought to be minimal. As a further development Wu and Tsai (1992) have recently reported a method for the evacuolation and enucleation of *Brassica* mesophyll protoplasts. The liberation of organelles other than vacuoles does not depend so greatly on controlled protoplast disruption. The method most frequently employed involves forcing the protoplasts through a nylon mesh, either by syringe (Rathnam and Edwards, 1976) or by centrifugation (Hampp, 1980).

Most of the methods given for the subsequent separation of subcellular fractions are based upon density gradient centrifugation. This technique facilitates the resolution of all the major organelles and allows each fraction to be quantitatively related to the protoplast lysate by means of the relative activities of marker enzymes. Recent

developments of this technique have provided a method for the rapid fractionation of protoplasts which allow the dynamics of compartmentation to be studied. Using silicon oil centrifugation Lilley *et al.*, (1982) separated wheat leaf protoplasts into chloroplastic, mitochondrial and cytosolic fractions within seconds of disrupting the plasmalemma. This enabled the authors to study the compartmentation of rapidly turning over metabolites, such as pools of adenine nucleotides.

1.3 Organelles as intracellular compartments

All organelles or cytoplasmic inclusions which play a part in the compartmentation of metabolites must satisfy the following requirements: they should be a discrete, membrane-delimited morphological space, they should show specific permeability, and finally they should possess a well-defined membrane transport system. Traditionally, those organelles visible under the light microscope, namely vacuoles, chloroplasts (and developmental stages thereof), mitochondria and the nucleus, have been regarded as the predominant compartments within the cell. However, the advent of electron microscopy has revealed further organelles, including the endoplasmic reticulum, polyribosomes, golgi apparatus and microbodies, which all share the features necessary to function as compartments for metabolites. Oaks and Bidwell (1970) also acknowledge the existence of hypothetical "microcompartments" comprising groups of chemical derivatives, enzyme aggregates and other non-membrane bound cytoplasmic inclusions, which may also be regarded as compartments. Prior to discussing the nature of the vacuole, chloroplast and mitochondrion and their roles in compartmentation I would like to discuss those organelles whose function as metabolic compartments is less firmly established.

1.3.1.Nuclei

The possession of a well-defined nucleus which contains most of the genetic material and in which mRNA is synthesised is the principal criterion by which eukaryotes are distinguished from prokaryotes. Nuclei possess all the features characteristic of

organelles in which the compartmentation of metabolites is well established: they are membrane bound and possess nuclear pore complexes which allow the selective transport of molecules across the membrane. Despite these features there is no evidence to suggest that the nucleus has a role in the compartmentation of metabolites.

1.3.2 Endoplasmic reticulum

The endoplasmic reticulum (ER) serves as one of the principal biosynthetic sites in plant cells (Jones, 1985). The ER represents a site for glycosylation of many plant proteins (Bollini *et al.*, 1983; Chrispeels *et al.*, 1982), and steps in the metabolism of terpenoids have also been localised to this organelle (Graebe, 1982). Moreover the ER is implicated in the biogenesis of other organelles, due to the concentration of enzymes for phospholipid metabolism it contains (Chrispeels, 1980). However, little evidence exists to suggest that the ER plays a role in the compartmentation of primary metabolites, although some evidence does exist suggesting it may play a role in the sequestration of Ca^{2+}, regulating local concentrations (Hepler, 1983) and influencing the fusion of vesicles on the cell plate and hence cell wall formation. In support of this role, Buckhout (1983) has shown that in the roots of *Lepidium sativum* L. ATP-dependent Ca^{2+} accumulation is localised in the ER.

1.3.3 Golgi bodies

Golgi bodies or dictyosomes are distributed throughout the cytoplasm of the cell, and their apparent function is the synthesis, transport and secretion of non-cellulosic cell wall materials (Chrispeels, 1976). The sulphation of polysaccharides in the brown algae has also been shown to be almost exclusively compartmentalised in the dictyosomes (Evans *et al.*, 1973). It is conceivable that the compartmentalisation of these enzymic systems in the dictyosomes contributes to the overall regulation of phospholipid synthesis, but there is little evidence to support this.

1.3.4 Microbodies

Microbodies, including peroxisomes and glyoxysomes, are bound by a single membrane and represent the loci for many oxidative processes which are not coupled to the phosphorylation of ADP. Peroxisomes are usually found in photosynthetic cells in association with chloroplasts, while glyoxysomes are characteristic of the endosperm of germinating seeds and are found in association with lipid deposits or spherosomes (Beevers, 1979). Thus, both organelles represent loci for the compartmentation of unique enzyme systems. The metabolic role of peroxisomes is confined to the conversion of glycolate to glycine (Kisaki and Tolbert, 1969), whereas the function of the glyoxysomes is one of ß oxidation and glycolysis, effecting the conversion of long chain fatty acids to succinate with catalase destoying the H_2O_2 generated by the fatty acyl CoA dehydrogenase reaction (Cooper and Beevers, 1969). Lips and Assivar (1972) suggested that plant leaf peroxisomes have a role in the reduction of nitrite. This suggestion does not appear to have received much attention, the consensus of opinion being that even in non-green tissue all nitrite reductase activity is plastidial (Emes and Fowler, 1979).

In summary there is a considerable body of evidence implicating the endoplasmic reticulum, golgi bodies, peroxisomes, and glyoxysomes in the compartmentation of a variety of key enzyme systems. Such compartmentation undoubtedly contributes to the overall regulation of metabolic processes. However, there appears to be little evidence that the above organelles are involved in the compartmentation of assimilates.

Before considering those organelles with established functions in compartmentation, it should be noted that although such compartments are dealt with individually here, this does not imply that they always function as separate entities. In fact, in many examples where the compartmentation of processes and metabolites serves a regulatory function, separate compartments are seen to operate in concordance. For example, the germination of fat-containing seeds is marked by a rapid consumption of fatty acids (Wiskich, 1980). Fatty acids are catabolised in the glyoxysomes (Cooper and Beevers,

1969), generating succinate and NADH, both of which are further metabolised in the mitochondria, providing substrates for gluconeogenesis in the cytoplasm. Thus overall there is a flow of reducing equivalents and carbon from fatty acids in the glyoxysome, to the mitochondria, and thence into sucrose in the cytoplasm. Likewise the photorespiratory pathway involves the extensive transport of intermediates between the chloroplasts, peroxisomes and mitochondria.

1.4 Mitochondria as metabolic compartments

Mitochondria possess all the features necessary for a function in metabolic compartmentation; they have a high degree of specific permeability, and well defined transport systems interfacing with the cytosol which regulate the influx and efflux of biomolecules. The compartmental role of the mitochondria in the spatial organisation of oxidative phosphorylation and in the localisation of the TCA and fatty acid cycles is well established.

Early studies implicated the mitochondria in the compartmentation of key organic acids. Lips and Beevers (1965a,b), demonstrated that malate pools in corn roots become differentially labelled when supplied with ^{14}C-labelled bicarbonate and ^{3}H-labelled acetate. A mitochondrial pool of malate was produced from the labelled acetate *via* the TCA cycle whereas a cytoplasmic pool became labelled *via* the dark fixation of acetate. The authors went on to to suggest that the existence of a mitochondrial malate pool permitted the functioning of the TCA cycle even in the presence of inhibitory cytosolic concentrations of malonate (Lips *et al.*, 1966).

Glutamate dehydrogenase (GDH) activity is associated with the mitochondria of higher plants (Stewart *et al.*, 1980), and several authors have presented evidence to suggest that this enzyme, and hence the organelle, could operate primarily in the assimilation or reassimilation of ammonium (Rhodes *et al.*, 1989b, Srirvasta and Singh, 1987 and Yamaya *et al.*, 1986). Meanwhile, a greater body of experimental evidence has been

presented showing that GDH has little or no (re)assimilatory role and that the GS/GOGAT cycle represents the principal pathway for ammonium assimilation in higher plants (Miflin and Lea, 1980; Stewart *et al.*, 1980, Robinson *et al.*, 1991, 1992). Observations that GDH levels are high during senescence of germinating cotyledons (Lea and Joy, 1982) and leaves (Thomas, 1978), have led to the proposal that mitochondria provide a locus for the oxidation of glutamate, providing the cell with reduced nucleotides and carbon skekeltons under circumstances where cytosolic carbon pools are depleted (Cammaerts and Jacobs, 1985). This view was recently strengthened by Robinson *et al.* (1992), who provided evidence that GDH has a catabolic role, remobilising carbon skeletons in sucrose-limited suspension cells of carrot.

Shargool *et al.* (1988), have shown that arginine catabolism, *via* arginase (EC 3.5.3.1), is compartmentalised in the mitochondria whereas the arginine biosynthetic system is extramitochondrial. De Ruiter and Kolloffel (1982) have shown that mitochondrial arginase activity is greatly increased during seed germination, when cytosolic demand for its products, ornithine, and ultimately glutamate,will be high.

In summary, there is considerable evidence to suggest that mitochondria function in the compartmentation of assimilates of both carbon and nitrogen metabolism. The common feature of the examples discussed above is that they all confer a anaplerotic function upon the mitochondria.

1.5 Chloroplasts (and developmental stages thereof) as metabolic compartments

Chloroplasts share many of the characteristics of mitochondria which allow them to function as metabolic compartments. Their role in many aspects of metabolism is well documented. These include the light-driven assimilation of carbon, nitrogen and sulphur (Jensen, 1980), amino acid metabolism (Bryan, 1976), terpene synthesis (Kreuz and Kleining, 1981), fatty acid metabolism (Stumpf, 1980) and complex lipid

anabolism (Mudd and Dezacks, 1981). However I should like to limit my discussion to the role of non-green plastids in metabolic compartmentation of metabolites, as such discussion is pertinent to the data which I will be present in subsequent chapters.

Plastids represent intermediary stages in the development of fully photosynthetic chloroplasts, and are non-photosynthetic. The term plastid encompasses the following structures, as defined by Dennis and Miernyk (1982): proplastids, which are undifferentiated plastids found in meristematic tissues; etioplasts, which are found in the developing leaves of plants grown in the absence of light and can be regarded as structures characteristic of the interrupted progress of chloroplast development; chromoplasts, which are specialized for the accumulation of lipid-soluble pigments, such as carotenoids; amyloplasts, which are given over to the storage of carbohydrate; leucoplasts, which lack pigmentation and are best regarded as mature proplastids, and finally, elaioplasts and proteinoplasts, which are plastids of limited distribution containing large quantities of lipid and protein, respectively.

Despite being of an intermediary nature, plastids represent the site of compartmentation of many metabolic processes and their products. For example, it is now firmly accepted that fatty acid biosynthesis in plants occurs in the plastids (Stumpf, 1980). There is an increasing body of evidence to suggest that the processes providing the substrate for fatty acid biosynthesis, acetyl CoA, are also located in the plastid; acetyl CoA does not readily cross membranes and it was deemed unlikely that plastidial fatty acid synthesis is fed by cytosolic acetyl CoA (Dennis and Miernyk, 1982). Plastids can synthesise fatty acids from acetate, but the necessary enzyme, acetyl CoA synthetase is not present at high concentrations in plant cells. These observaions led Dennis and Miernyk (1982) to suggest that acetate is derived from pyruvate *via* a plastidial pyruvate dehydrogenase complex ($_{p}$PDC) and $_{p}$PDCs have, in fact, been isolated from castor seed endosperm (Thompson *et al.*, 1977), pea leaf (Camp *et al.*, 1988), and etiolated maize and soya bean shoots (Cho *et al.*, 1988). Plastids from endosperm cells also

show glcolytic activity and possess enzymes of the pentose phosphate pathway (Yamada *et al.*, 1974, Simcox *et al.*, 1977). The compartmentation of these enzyme systems in the plastids provides a means of regulating the flow of carbon from photoassimilates to triglycerides. Gluconeogenesis is restricted to the cytoplasm (ap Rees *et al.*, 1975), and compartmentation in the plastids, of the systems described above, thus results in intracellular separation of synthetic and degradative processes sharing the same substrate.

The examples described above illustrate how compartmentation is beneficial to metabolic regulation. Several workers have shown that when glycolytic activity can be detected in two compartments, subcellular site-specific isoenzymes are present (Miernyk and Dennis, 1982, 1984, Pichersky and Gottlieb, 1984). This could provide a means of "coarse" control of the relative activity within the two compartments, as it is probable that the glycolytic isoenzymes are encoded by separate nuclear genes. It can be postulated that compartmentation affords an additional means of "fine" metabolic regulation of glycolysis. Regulation of rate may be brought about by the (in)activation by metabolic intermediates such as, nucleotides, adenylates, P_i etc (Turner and Turner, 1975). Thus, if (iso)enzyme systems are compartmentalised small changes in levels of regulatory molecules will be amplified by virtue of the fact that they are confined within a smaller volume. This will provide a wider gradation of effectors to which the enzymes are sensitive, permitting finer control, especially for isoenzymes systems where the cytosolic and the plastidial components are kinetically, physicochemically and immunologically similar.

Plastids also represent a site of compartmentation for many aspects of nitrogen metabolism in plant cells, including purine and ureide metabolism (Shelp *et al.*, 1983), and the reduction of nitrite and its assimilation into amino acids (Miflin, 1974, Beevers and Hagemann, 1980, Miflin and Lea, 1980). As with the compartmentation of carbon processing, described above, the localisation of some of the events of nitrogen

metabolism in the plastid confers many advantages upon the cell. For example, the branched chain amino acids leucine, valine, and isoleucine are synthesised *via* a common pathway, from pyruvate, located in the plastids (Schulze-Siebert *et al.*, 1984). In this way, branched chain amino acid synthesis is kept distinct from mitochondrial pyruvate metabolism.

Considering the role played by the plastid in the primary assimilation of ammonium, it is firmly established that glutamine synthesis occurs in two compartments in leaf cells higher plants (Mann *et al.*, 1979, Wallsgrove *et al.*, 1979, Hirel and Gadal, 1981). Two major isoforms of glutamine synthetase, GS_1 and GS_2, have been isolated and shown to be localised in the cytosol and the plastid, respectively (Mann *et al.*, 1983, McNally *et al.*, (1983). It has been suggested that GS_1 is responsible for the reassimilation of ammonium released by the photorespiratory conversion of glycine to serine in the mitochondria (Keys *et al.*, 1979, Hirel and Gadal, 1980). Meanwhile GS_2 is thought to function primarily in the primary assimilation of ammonium in the cytosol (Hirel *et al.*, 1982, McNally *et al.*, 1983). Recently mutants lacking GS_2 have been shown to accumulate ammonium at a rate equivalent to that theoretically expected from photorespiration (Lea *et al.*, 1989). Thus it appears that compartmentation can serve to isolate two catabolic processes, as well as separating the catabolic from the anabolic. It is accepted that GS_1 and GS_2 are encoded separately within a small, nuclear multi-gene family (Forde and Cullimore, 1989). This suggests that the relative concentrations of the iso-forms may be regulated independently.

1.6 Vacuoles as metabolic compartments

The interest in vacuoles as potential sites for the localisation of metabolic processes and metabolites reflects the prominent position and central location of the vacuole in the mature plant cell. The term "vacuole" designates an optically empty space delimited by a single membrane, the tonoplast, which interfaces with the cytoplasm on its outer face and the vacuolar solutes (frequently termed the "cell sap"), on its inner face. For a long

time the vacuole was considered an inert compartment, functional only in the maintenence of turgor pressure since the osmotic properties of the cell were attributed to solutes accumulated in the vacuole and to the semipermeability of the tonoplast (Zirkle, 1937). However, evidence has accumulated implicating the vacuole in a variety of functions.

1.6.1 Vacuoles as lytic compartments

The detection of latent, membrane-bound hydrolases in homogenates of rat liver led to the concept of *lysosomes* (De Duve, 1969). Analogous sites of hydrolytic activity were found in yeast and plant cells (reviewed by Matile, 1975) with considerable evidence to indicate that this lytic compartment was in fact the vacuole (Matile, 1976, 1978). Subsequently the vacuole was indeed assigned a detoxification function, providing an area within the cell where potentially cytotoxic compounds were thought to accumulate and be rendered harmless by the action of hydrolases, proteases, esterases, and glycosidases (Boller and Kende, 1979).

There is a considerable body of evidence to suggest that vacuoles not only function in the degradation of unwanted and abnormal metabolites, but that they may also be involved in redistribution of hydrolysates. The vacuole has been shown to be directly involved in the remobilisation of seed protein during germination (Nishimura and Beevers, 1979). Likewise in the senescing corolla of *Ipomoea tricolor* the products of protein and nucleic acid degradation released from the vacuole due to changes in the selective permeability of the tonoplast with ageing appear to be subsequently metabolized in the cytoplasm and exported *via* the sieve tubes (Wiemken, 1975).

There is evidence to suggest that functional proteins may also be imported into the vacuole for reasons other than their degradation. Proteinase inhibitors have been shown to accumulate in the vacuoles of tomato cells in response to wounding (Walker-Simmons and Ryan, 1977; Nelson and Ryan, 1980). In bean leaves ethylene is seen to

induce the accumulation of chitinase in the vacuole, probably representing a defense against fungal attack (Boller and Vögeli, 1984). Recent studies have shown that the targeting of proteins to the vacuole is achieved by virtue of targeting domains within the mature protein (Tague *et al.*, 1990, Saalbach *et al.*, 1991). There is no evidence to suggest that vacuolar proteins can be released into the cytoplasm other than during autolysis (Boller and Wiemken, 1986).

The compartmentation of hydrolytic processes in the vacuole provides a means of removing potentially harmful metabolites from the cytoplasm. The separation of these processes from the cytoplsm by the tonoplast facilitates the organized degradation of biomolecules. Payne and Boulter (1974) compared the *in vivo* degradation of ribosomal RNA in senescent broad bean cotyledons with the degradation in homogenates, observing that the release of RNase from disrupted vacuoles led to an accelerated and disorganized destruction of cytoplasmic ribosomes. Senescence in living tissues is marked by the synthesis of certain proteins in the cytoplasm and the breakdown of others in the vacuole. Thus lytic processes are spatially separated from sites of macromolecular synthesis. Wodzicki and Brown (1973) also noted that during the differentiation of meristem cells of pine into tracheids, the cytoplasm was cleared by autophagic processes of the vacuole. Consequently the compartmentation of lytic processes may be regarded as a fundamental form of regulation of plant development.

1.6.2 Vacuoles as compartments of catabolic processes

The plant cell vacuole has been indentified as the site of a number of catabolic processes. Guy and Kende (1984) have shown that the enzyme catalysing the final stage of ethylene biosynthesis, the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, is localised in the vacuole. The accumulation of alkaloids in the vacuole of plant cells is well documented (Kreis and Reinhard, 1987; Ehmke *et al.*, 1987) and recently Bloom *et al.* (1991) have shown that vacuoles from cells of *Catharanthus roseus* (L.) G.Don have the ability to convert ajmalicine into serpentine,

through the action of basic peroxidases. Finally vacuoles isolated from barley leaf tissue (Wagner *et al.*, 1983) and Jerusalem Artichoke tuber protoplasts (Frehner *et al.*, 1984) have the enzymatic machinery to form fructans. It can be concluded that vacuoles do have a role in catabolic processes, although this is limited, and the older view that they are metabolically inert is being revised.

1.6.3 Vacuoles and the compartmentation of photosynthetic assimilates

The accumulation of secondary photosynthates in the vacuole is one of the best examples their dynamic nature. In leaf cells the primary assimilates of photosynthesis, triose phosphate (TP) and 3-phosphoglycerate (3PG), are exported from the chloroplast stroma to the cytosol where they are generally metabolised onwards to sucrose and malate. These secondary photosynthates are translocated *via* the phloem to regions where they are required for growth and as respiratory substrates. A proportion of these assimilates will also be stored within the cell to be consumed during the dark period. Fixed carbon may be stored as starch in the chloroplast stroma, or as sucrose and malate in the extrachloroplastic space (Martinoia *et al.*, 1984). If newly synthesised assimilates were retained in the cytoplasm, their accumulation would perturb water potential, causing the cytoplasm to swell and disrupting metabolism by dilution of cytosolic metabolites (Kaiser and Heber, 1984). Determination of tracer kinetics with $^{14}CO_2$ have revealed that newly assimilated sucrose exists as two distinct intracellular pools: a cytoplasmic pool which is rapidly exported for translocation, and a vacuolar pool which is slowly exported (Geiger *et al.*, 1983). As would be expected in suspension cultured cells and assimilating protoplasts the need to provide a store for sucrose and malate is greater. It has been shown that the rate of transfer of photosynthetic products from the cytosol across the tonoplast of barley mesophyll protoplasts can approach the rate of carbon fixation (Kaiser *et al.*, 1982). It is interesting to note that newly fixed sucrose is excluded from the vacuole in sweet clover, however (Boller and Alibert, 1983). There is considerable evidence to suggest that the observed vacuolar pools of photoassimilates are not merely surpluses, but

rather that such accumulation represents a dynamic process. Gerhardt and Heldt (1984) observed that vacuolar sucrose and malate pools in spinach leaves (100 and 25 μatom C/mg Chl respectively), developed over 9h of light and were completely depleted after 4h of darkness. The compartmentation of malate in plants showing CAM metabolism provides a striking example of dynamic vacuolar compartmentation. In CAM plants, assimilation of atmospheric CO_2 occurs mainly at night, to minimise water loss by transpiration. Associated with this nocturnal CO_2 fixation is the accumulation of malate in the vacuoles of assimilatory cells. During the subsequent day, malate is exported from the vacuole and decarboxylated, the CO_2 released being reassimilated through the C_3 carbon reduction cycle (Smith, 1987). In addition to providing a temporary storage facility for nocturnally fixed CO_2, the vacuolar accumulation of malate also contributes significantly to the maintenance of tugor pressure. In *Kalanchoë daigremontiana*, despite the decreases in leaf water potentials associated with nocturnal transpiration, leaf turgor pressure always remains constant or increases slightly during the night (Smith and Lüttge, 1985). These major changes in plant water relations are directly dependent upon the processes which bring about the nocturnal accumulation of malate in the vacuole and its subsequent diurnal remobilisation i.e. the malate transport mechanisms at the tonoplast.

1.6.4 Vacuoles and the compartmentation of inorganic compounds

The concentration of ions in the cytoplasm is strictly controlled (Boller and Wiemken, 1986); for example concentrations of Ca^{2+} in the vacuole are frequently reported as being an order of magnitude greater than in the cytoplasm (Macklon, 1984). Ca^{2+} accumulation in the vacuoles of members of the Crassulaceae and Brassicaceae is thought to balance the accumulation of carboxylates in the vacuole (Smith, 1987). Although little is known of the function of vacuolar accumulation of Ca^{2+}, it would be surprising if so conspicuous an organelle as the vacuole played no part in the homeostatis of cytoplasmic Ca^{2+} concentrations in plant cells.

Rebeille *et al.* (1983) have shown that in cells of *Acer pseudoplatanus*, orthophosphate (P_i) is localised within two compartments corresponding to the cytosol and the vacuole. The authors also showed that when cells were transfered to a phosphate-deficient medium, the cytoplasmic [P_i] was maintained at the expense of the vacuolar concentration and they proposed that a tonoplast transport mechanism was responsible for the maintenance of cytoplasmic P_i concentrations. Thus the vacuole appears to play a role in the regulation of those energy transductions requiring or mediated by P_i, in the synthesis of complex molecules for which P_i is an intermediate, and, indirectly, in the regulation of many metabolic sequences that are sensitive to P_i.

The vacuole is also regarded as playing an important role in the regulation of cytoplasmic H^+ concentrations and hence pH (Smith and Raven, 1979). Investigations employing ^{31}P NMR techniques have revealed that when plant cells are exposed to external environments held at different pH values, the pH of the vacuole falls in parallel with decreases in the pH of the external medium, whilst the pH of the cytoplasm remains relatively constant. On the otherhand, anaerobically stressed root tips show a decrease in cytoplasmic pH without a concomitant change in vacuolar pH (Torimitsu *et al.*, 1984, Wray *et al.*, 1985). Thus it appears that at low external pH an influx of protons into the cytoplasm is compensated for by an increase in (H^+) transport into the vacuole.

1.6.5 Vacuoles and the compartmentation of nitrogenous assimilates

Nitrate, the principal nitrogen source of most plants and amino acids, their primary metabolites, can accumulate in large quantities in the vacuoles of higher plant cells. 99% of the nitrate content of barley mesophyll protoplasts was found to be vacuolar (Martinoia *et al.*, 1981), and in the same system Dietz *et al.* (1990) reported that certain amino acids were present at concentrations of 120 mM, with a significant proportion being localised in the vacuole. Many authors have suggested that the value of such

massive vacuolar accumulation lies in the necessity of keeping cytosolic amino acid concentrations as low as possible, whilst maintaining a steady flow of nitrate into the cell in order to keep nitrate reductase stably induced (Shaner and Boyer, 1976; Heimer and Filner, 1971). Despite the recent advances in our understanding of the spatial organization of the enzyme systems of nitrogen metabolism (reveiwed in Hrazdina and Jensen, 1992), our understanding of the spatial separation and dynamics of metabolite pools has been restricted to "static" descriptions of vacuolar amino acid and nitrate pools (Sasse *et al.*, 1979; Boudet *et al.*, 1981; Martinoia *et al.*, 1981; Yamaki, 1984). However, the static image of vacuolar pools has been challenged by the finding that stored amino acids are mobilized from the vacuoles of barley mesophyll protoplasts upon relief from nutrient stress. Such mobilisation appears to be an active process regulated by cytoplasmic ATP levels (Dietz *et al.*, 1989, 1990). This provides an indication that vacuoles can function both as a sink and a source for nitrogenous assimilates, such traffic being regulated by a carrier in the tonoplast. Recently, the dynamic involvement of the vacuole in nitrogen metabolism has been demonstrated by Beck and Renner (1990), who described intracellular fluxes during the growth period of a photoautotrophic suspension culture, and Carroll *et al.* (1992), who followed the dynamics of partitioning of newly assimilated amino acids between vacuolar and cytoplasmic fractions of N-depleted carrot protoplasts when supplied with NH_4^+.

From the numerous examples discussed above, it is evident that compartmentation in the plastids and mitochondria is predominantly of enzyme systems, whilst the vacuole appears to be the principal site for compartmentation of metabolites. That such compartmentation is often of a dynamic nature suggests that the vacuolar pools of certain metabolites should not be regarded as metabolically inert, but as metabolically quiescent. For any morphological cell space to function as a compartment it must constantly oppose the forces of equilibrium and remain capable of accumulating solutes against a concentration gradient. As many metabolic compartments can function in a dual source/sink role, there must be regulation of transport across the delimiting

membrane. Such regulation must underpin the "communication" between compartments, apparent in many aspects of plant metabolism.

1.7 Compartmentation and membrane transport

The dynamic aspect of metabolic compartmentation depends upon the delimiting membrane being energized and actively involved in the transport of ions and metabolites. Here, active transport may be regarded as the movement of a molecule through a membrane against its electrochemical potential gradient, the requisite energy being provided by a coupling to a source of free energy (Reinhold and Kaplan, 1984). At present the most widely favoured hypothesis to account for active transport across plant membranes proposes that the source of free energy necessary for transport is an ion (H^+) gradient (Mitchell, 1979). The electrogenic transport of H^+ without the contransport of cations, results in a proton gradient (δpH) and a membrane potential ($\delta\psi$), both of which additively constitute a *proton-motive force* (*pmf*). The *pmf* is thought to be generated by the action of ATPases at the membrane surface. In mitochondria the translocation of protons is also coupled to electron flow through the cytochrome pathway and in chloroplasts it can originate from light mediated electron flow along the transversely arranged system of electron carriers in the thylakoid. The *pmf* across the tonoplast is generated solely by the action of ATPases and PP_iases however(Poole *et al.*, 1984, Rea and Poole, 1985).

The electrogenic pump generating the *pmf* is regarded as a "primary active" transport system, whereas the flow of metabolites driven by the *pmf* is "secondary active" (Reinhold and Kaplan, 1984). The electrogenically coupled transport of metabolites across plant membranes is mediated by a proteinaceous element of the membrane, termed a carrier. The carrier binds both ion and the substrate: if it carries them both in the same direction it is known as a *symport* process; the transport in opposing directions is known as *antiport*. The nature of membrane energisation and the types of carriers involved in metabolite transport are given in Figure 1.1a. Figures 1.1b-d are

representations of some of the carriers which have been identified as transporting metabolites across the inner mitochondrial membrane, the inner chloroplast membrane and the tonoplast, respectively. The figure is not intended to provide a complete description of our present understanding of plant membrane transport; for the sake of clarity, it has been restricted to proposed transporters of carbon and nitrogenous compounds only.

Figure 1.1

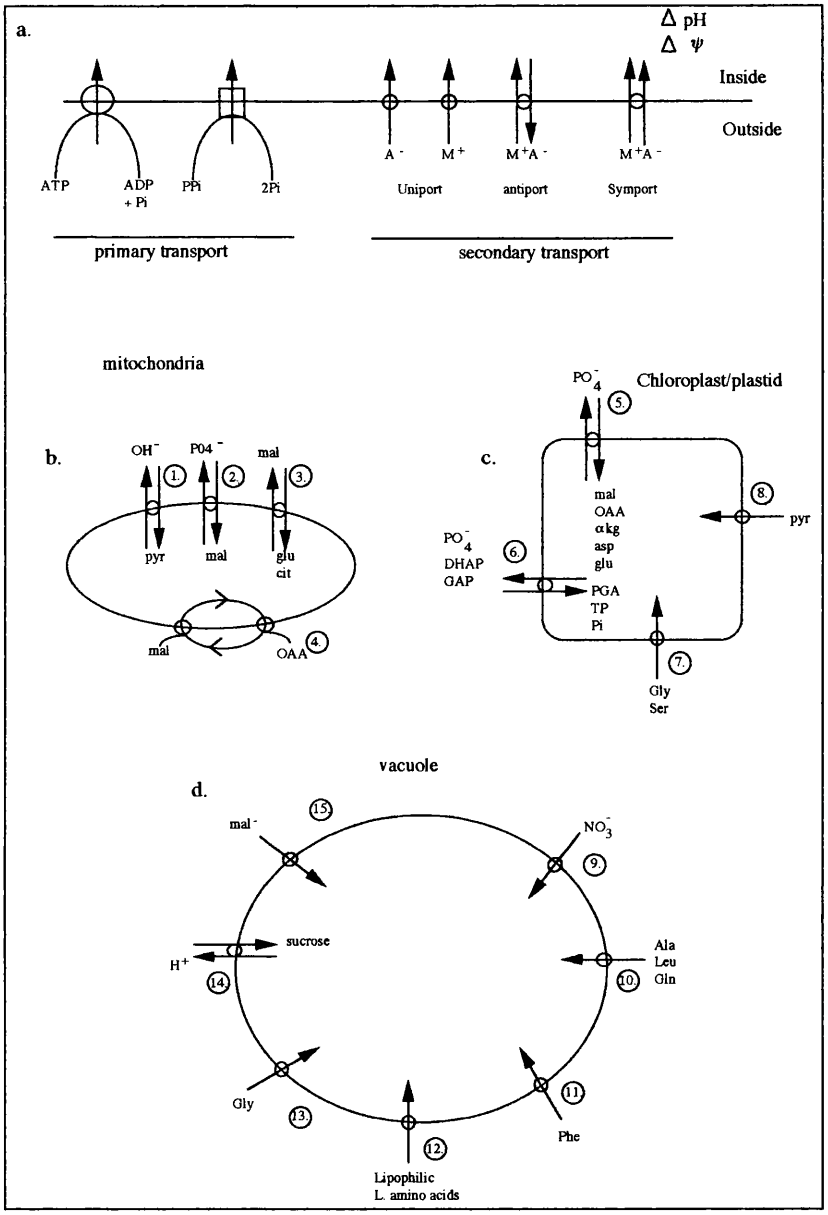

Figure 1.1. The energisation of the plant cell membrane and some of the secondary active carrier mediated transport processes proposed for the inner mitochondrial membrane, the inner chloroplast envelope and the tonoplast. References: [1] Day and Hanson, 1977, [2] Phillips and Williams, 1973, [3] Day and Wiskich, 1977, [4] Zogloweck *et al.*, 1988, [5] Lehner and Heldt, 1978, [6] Fliege *et al.*, 1978, [7] Nobel and Cheung, 1972 [8] Huber and Edwards, 1977, [9] Pope and Leigh, 1987, [10] Dietz *et al.*, 1990, [11] Homeyer and Schultz, 1988 [12] Homeyer *et al.*, 1989, [13] Baker, 1978, [14] Goerlach and Willims-Hoff, 1992, [15] Martinoia *et al.*, 1985.

1.7.1 The influence of metabolite transport on the regulation of metabolism

Figure 1.1b shows some of the carrier mediated active transport processes of the mitochondrial inner membrane. Mitochondria require a constant source of pyruvate to supply the TCA cycle (Hanson and Day, 1980). The existence of a monocarboxylate carrier was first demonstrated in corn mitochondria (Day and Hanson, 1977). It has been proposed that a $PO4^-$/malate antiport would provide malate at a sufficient rate to produce intramitochondrial concentrations of oxaloacetate and pyruvate sufficient to allow rapid TCA cycle activity (Wiskich, 1980). The operation of a malate-oxaloacetate shuttle (analogous to the malate-aspartate shuttle of animal cells) is thought to be responsibe for maintaining a redox gradient between the NADH/NAD systems in the mitochondria and cytosol (Zogloweck *et al.*, 1988). The authors propose that the link of malate and oxaloacetate transport allows the cytosolic ratio of malate/oxaloacetate to remain high, thus keeping the redox potential of cytosolic NADH/NAD high. A significant redox potential gradient across the mitochondrial/cytosol interface is required in order to maintain coupling sites within the respiratory chain.

It can be seen that the carriers described above contribute to the role of the mitochondrion as a metabolic compartment in so much as the malate and pyruvate carriers provide substrate at a sufficient rate to allow rapid TCA cycle activity. The proposed oxaloacetate/malate shuttle also serves to regulate cytosolic malate concentrations whilst maintaining a gradient of reducing equivalents between the cytosol and the mitochondrion.

Figure 1.1c shows some of the proposed carrier-mediated transport processes of the inner chloroplast membrane. The chloroplast is a metabolically flexible compartment; the existence of specific transport processes serves to emphasise this role. The phosphate transporter facilitates the export of fixed carbon in the form of phosphate esters from the stroma to the cytosol, an exchange counterbalanced by the uptake of P_i

from the cytosol (Fliege *et al.*, 1978). Heber and Heldt (1981) have shown that 60% of chloroplastic reactions are directly dependent upon the phosphate carrier. Only one sixth of the triose phosphate assimilated in the chloroplast is available for export to the cytosol however; the remainder is required within the organelle for the regeneration of ribulose bisphosphate (Heber and Heldt, 1981). The phosphate carrier is responsible for fine tuning the export rate of assimilate so as to maintain sufficiently large pools of TP and 3PG to act as carbon cycle intermediates.

It has been observed that during CO_2 assimilation, isolated spinach chloroplasts export mainly TP to the cytosol The efflux of 3PG is restricted, despite its presence in the chloroplasts at a much higher concentration than TP (Lilley *et al.*, 1977). The authors suggest that this is a direct consequence of the pH gradient that exists between the external space and the stroma. In so much as phosphate and phosphate esters are transported as divalent ions, the existence of a pH gradient across the chloroplast envelope will alter the equilibrium between the the divalent and trivalent form of 3PG, resulting in the restriction of its transport *via* the divalent carrier. This provides another illustration of how metabolism and compartment transport processes are integrated, resulting in the regulation of the former.

The co-dependence between P_i and TP transport may also be considered to be contributory to chloroplast function as a storage compartment for starch. In spinach protoplasts, TP exported to the cytosol is transformed primarily to sucrose (Stitt *et al.*, 1980), and the P_i consequently liberated is transported back to the stroma in exchange for more TP. During periods of high carbon fixation, the transformation of TP to sucrose is limited, hence cytosolic P_i falls. The chloroplast ceases to export TP, due to lack of external P_i, and excess fixed carbon is stored as starch (Herold, 1976). Thus it can be seen that the phosphate carrier is at least partly responsible for some of the functions which lead the chloroplast to be regarded as a metabolic compartment.

Figure 1.1d illustrates some of the transport processes that are thought to occur at the tonoplast. The uptake of metabolites across the plasmalemma is energized by the *pmf* across it (Dietz *et al.*, 1990) and much of the evidence suggests that this is also the case for tonoplast transport. Walker and Leigh (1981) indentified tonoplast bound ATPase and PP_iase activities and proposed that their function was to act as proton pumps, energizing the membrane. Convincing evidence is now available showing that both the ATPase and the PP_iase are directly responsible for proton pumping across the tonoplast (Poole *et al.*, 1984, Rea and Poole, 1985). The electrochemical H^+ gradient generated drives the transport of solutes across the tonoplast either by direct coupling to the pH gradient or by secondary transport systems (Sze, 1985, Rea and Sanders, 1987).

Vacuoles have been shown to have the ability to act as both source and sink, that is, to accumulate metabolites and remobilise them on demand. Clearly to function in this way the movememt of molecules must be regulated. Dietz *et al.*, (1990) have described an amino acid carrier from the tonoplast of barley mesophyll cells, which is activated but not energized by ATP. The authors propose that uptake into the vacuole is enhanced in the presence of ATP due to activation of the transporter proteins by ATP binding, as observed in animal systems. Hence vacuolar accumulation will be high when the assimilation of nitrogen into amino acids is maximal. The efflux of amino acids from the vacuole was also shown to be stimulated by ATP (for which no explanation has been offered) but also by decreasing cytosolic levels of amino acids. This latter phenomenon suggests an attractive hypothesis, namely that vacuolar uptake would occur only when cytosolic amino acid levels are high, efflux being catalyzed when cytosolic levels are dangerously low.

Recently tonoplast carriers for glycine (Goerlach and Willims-Hoff, 1992) and arginine and aspartate (Martinoia *et al.*, 1991) have been indentified and it has been suggested that the two systems operate as a "valve" facilitating the accumulation of amino acids at high light intensities (Goerlach and Willims-Hoff, 1992). The precise nature of this

transporter has yet to be discovered although current interest is centred upon voltage-dependent ion channels in the tonoplast (Hedrich *et al.*, 1986, Pantoja *et al.*, 1990).

The most dramatic example of the dynamic nature of the vacuole is given by its role in the accumulation and remobilisation of malate in CAM plants (reviewed by Lüttge, 1987). Buser-Suter *et al.* (1982) provided evidence for a permease in the tonoplast of *Bryophyllum*, and subsequently Martinoia *et al.*. (1985) confirmed that in barley protoplasts the vacuolar accumulation of malate is mediated by a carrier and energized by coupling to the electrogenic transfer of protons, catalysed by an ATPase. Recently the membrane potential has been identified as the predominant component of the *pmf* responsible for driving the uptake of malate into vacuoles isolated from lettuce leaves (Blom-Zandstra *et al.*, 1990).

There is considerable evidence to suggest that the mobilisation of certain metabolites is affected by osmotic factors. When metabolites are accumulated to any great extent in the vacuole they represent a significant proportion of the osmotic potential of the vacuolar sap. Therefore if cytoplasmic volume and turgor are to remain constant, other cytoplasmic solutes must be accumulated to replace them. In red beet disks it has been observed that the mobilisation of vacuolar sucrose is accompanied by the vacuolar accumulation of inorganic salts (Leigh and Wyn Jones, 1986; Perry *et al.*, 1987). Increases in the vacuolar concentrations of sugars and organic acids concomitant with the utilisation of vacuolar NO_3^- have been noted in spinach leaves (Steingröver *et al.*, 1986) can be seen to have important implications for the regulation of the mobilisation of vacuolar solutes, since the rate at which solutes are exported from the vacuole may be limited or controlled by the rate at which replacement solutes are made available in the cytoplasm.

The literature on tonoplast transport is substantial and complex, but certain consistent points do emerge. It is generally accepted that the generation and maintenance of a

steep transtonoplast electrochemical gradient is attributable to the joint action of two electroenzymes, an anion-sensitive ATPase and a cation sensitive PP_iase. The operation of these electroenzymes serves to energise the tonoplast facilitating the transport of NO_3^-, Cl^-, malate and amino acids *via* specific transporters. ATP appears to regulate, but not, energise transtonoplast transport. Finally, the mobilisation of stored solutes appears to be a passive process, regulated by the cytosolic availability of a replacement vacuolar solute or by the cytosolic concentration of the vacuolar solute to be mobilised.

The preceding discussion is intended to illustrate the importance of the intracellular compartmentation of metabolites and enzymes in the regulation of metabolic processes. The principal site of action for the mechanisms which regulate compartmentation appears to be the membranes at which specific, and often carrier-mediated transport of metabolites occurs. This belief is based upon evidence which suggests that plant membrane transport, particularly transtonoplast, is energized by electroenzymes. The mechanisms of transport regulation will presumably involve changes in the mole fraction and capacitance of pairs of adenylates responsible for the transduction of intracellular signals. The relative concentration of the metabolites, and the osmotic potential on either side of the compartment delimiting membrane will also be influential in the regulation of compartmentation. Thus it can be seen that the interfacing of cytosolic processes with compartment membranes which are selectively premeable, affords a means of metabolic self-regulation.

1.8 The aims of the project

Despite considerable advances in our understanding of the major metabolic pathways of nitrogen assimilation in higher plants, there is still uncertainity as to the role played by intracellular partitioning of nitrogenous assimilates, in the organisation and regulation of the events of nitrogen metabolism. The work which will be described in this thesis was carried out with the aim of determining the role of the vacuole, plastids and

mitochondria in the compartmentation of the primary assimilates of nitrogen metabolism. Protoplasts prepared from suspension cells of *Daucus carota* were used as a model system, to facilitate the rapid separation of the constituent organelles from a physiologically viable source. The ultimate goal was to determine the extent to which compartmentation contributes to the overall regulation of nitrogen assimilation, especially under the metabolic perturbations which ensue when nitrogen-depleted plant cells are provided with $NH4^+$ as the sole nitrogen source.

Chapter 2 Materials and Methods.

2.1 Growth conditions of carrot cell cultures

Suspension cultures of *Daucus carota* L.cv Chantenay, originally isolated in 1987, were maintained on Murashige and Skoog medium (Murashige and Skoog, 1962) (Flow Labs., Irvine, Scotland.), modified by the inclusion of 2,4-D (0.2 mg/mL) and kinetin (0.1 mg/mL); this maintenance medium was termed MDK. Cells were subcultured every 14 days by inoculation of 10 volumes of MDK with 1 volume of suspension. Standard cultures of 70 mL were grown in 250 mL flasks, and where larger volumes of cells were required the volumes of media and inocula were increased *pro rata*. "Back up" cultures were maintained as callus on MDK solidified with 1.5% Difco agar. Suspension cultures were maintained in Ehrlenmeyer flasks capped with aluminium foil and kept on an orbital shaker at 25°C, 100 rpm in continuous low density fluorescent light (5 $\mu E/m^2/s$).

For all the methods to follow, benchtop centrifugation (< 9000 *g*) was carried out in a Sigma 2K15 centrifuge with a 6 x 30 mL fixed angle rotor at 4°C. Spectrophotometric determinations, both colorometric and kinetic, were carried out in a Phillips PU 8740 scanning spectrophotometer, blanked against the appropriate buffer.

2.2 Protoplast isolation

Cell suspension cultures in the mid to late exponential phase of growth (11-13d) were aseptically harvested by filtration onto two layers of sterile muslin and transferred to 7 volumes of a solution containing: 1% cellulase R10 (w/v), 0.5% macerozyme R10 (w/v) (Yakult Honsha Co., Tokyo) and 0.01% pectolyase Y23 (w/v) (Seisham Pharmaceutical, Tokyo) in a buffer based upon MDK but modified by the omission of inorganic nitrogen sources (KNO_3 and NH_4NO_3) and containing additionally 0.5 M mannitol as an osmoticum and 50 mM MES at pH 5.7. Enzyme solutions were filter-sterilised using a 0.22 μm membrane. After incubation for 3-4h at 35°C on an orbital

shaker at 100 rpm, liberated protoplasts were collected by centrifugation at 250 *g* for 10 min and washed 3x in the above protoplasting buffer with enzymes omitted.

2.3 Protein determination

Protoplasts, vacuoles or organelle fractions were extracted in three volumes of a buffer containing 25 mM Tris (pH 8.0), 1 mM EDTA, 1 mM mercaptoethanol and 0.01% (v/v) Triton X-100. The soluble protein content were determined by the Bradford method (1976) using a Bio-Rad protein assay kit (Bio-Rad Richmond Va. USA). Standard curves were prepared using 0.1-1.4 mg/mL BSA. For total (TCA insoluble) protein, fractions were extracted 3 times in 10 volumes of 10% (w/v) ice cold TCA, for at least 5 mins in the first two instances and 30 mins in the final extraction. The resulting pellets were solubilised in 0.3 M NaOH at 40°C for 60 min and assayed for total protein using the Folin-phenol reagent after the method of Lowry *et al.* (1951). Standard curves were prepared using 1-10 mg/mL BSA in 0.3 M NaOH.

2.4 Determination of cell and medium ammmonia

Ammonium concentrations in the media were assayed directly by the colorometric method of McCullough (1967), in which 100 μL of appropriately diluted medium were assayed in a total volume of 1.1 mL. Concentrations were determined against a standard curve prepared from 0.1-2 mM NH_4Cl in ddH_2O, and for each set a "spike" sample containing 1 mM NH_4Cl was included to ensure calibration. Intracellular ammonium was assayed by the same method on protoplasts and vacuoles following extraction in 3 volumes of HPLC grade methanol; in this case calibration was achieved against a standard curve of NH_4Cl in methanol.

2.5 Determination of intracellular and medium nitrate

Nitrate was assayed according to the method of Sloan and Sublett (1966). In an initial reduction step 250 μL of the methanol extract described above or appropiately diluted medium was added to 2 mL of 0.4 M NH_3/NH_4 buffer at pH 9.6, to which in turn was

added 1.75 mL 0.1 M $MgCl_2$ and 0.5 g cadmium filings. The reactants were mixed and allowed to stand at room temperature for 30 minutes, after which time aliquots were taken for nitrate analysis, as follows: 1mL 1% (w/v) sulphonic acid and 1 mL 0.02% (w/v) N-(1-napthyl)ethylenediamine (NEDD) were added to 1 mL aliquots of the reduction mixture and incubated at room temperature for 30 mins prior to determining the optical density at 540 nm. Na_2NO_3 (0.1-0.6 μmol) was used to ascertain the efficency of reduction and $NaNO_2$ (10-100 nmol) was used to prepare a standard curve.

2.6 Enzymatic assays

2.6.1 α-mannosidase ((AMS) EC 3.2.1.24) and acid phosphatase ((AP) EC 3.2.3.2)

AMS and AP activities were assayed by determining the release of *p*-nitrophenol (*p*-NP) from their substrates, α-D-mannopyranosidase and *p*-nitrophenyl phosphate respectively, after the method of Wagner (1983). 0.5 mL reaction mixture contained: 8 mM substrate, 10/25 μL protoplast/organelle suspension (20-30 μg soluble protein), 10 mM $CaCl_2$, 10 mM $MgSO_4$ and 100 mM MES/EPPS at pH 5.5. This reaction mixture was incubated at 37°C for 10 min after which the released *p*-NP was visualized by the addition of 0.5 mL 0.1 M NaOH and the optical density at 410 nm determined. The release of *p*-NP was quantified using a standard curve in the range 10-100 μmol *p*-NP. The rate of hydrolysis of the substrate without the enzyme was routinely between 0 and 5% of enzymatic hydrolysis.

2.6.2 Glucose-6-phosphate dehydrogenase ((G6PDH) EC 1.1.1.49)

G6PDH was assayed according to the method of Simcox *et al.* (1977). Reduction of NADP was followed at 340 nm in a 3 mL reaction volume containing: 17 μM NADP, 33 μM glucose-6-phosphate, 0.1% (v/v) Triton X-100, 100 μL protoplast or organelle suspension (100 μg soluble protein approx.) and 20 mM TES at pH 7.5. The reaction was linear with respect to time and enzyme concentration. Control assays, in which the enzyme was replaced by buffer, gave no appreciable change in absorbance. In this,

and all other assays involving the reduction or oxidation of NAD(P)/NAD(P)H, activity is calculated by the use of the millimolar extinction coefficient of NADH, which is 6.22 at 339 nm.

2.6.3 NADH-cytochrome c reductase ((NADH-CR) EC 1.6.2.3) and cytochrome c oxidase ((CYTOX) EC 1.9.3.1)

NADH-CR was assayed by monitoring the oxidation/reduction of cytochrome c at 550 nm, according to the method of Hodges and Leonard (1974). The reduction of cytochrome c was followed in a 3 mL reaction system containing 0.01% (w/v) digitonin, 1.7 μM equine heart cytochrome c, 100 μL protoplast or organelle suspension (100 μg soluble protein approx.) and 50 mM K_2HPO_4/KH_2PO_4 buffer at pH 7.5. The reaction was started by the addition of 100 μM NADH. NADH was omitted from the control.

CYTOX was assayed in the same way except the reaction mixture did not contain NADH and the cytochrome was reduced, prior to initiation of the reaction, by the addition of a minimal number of 4 μL drops of 500 μM sodium dithionate (excess dithionate being removed by bubbling air through the solution for 3 min); in control assays the enzyme was replaced with buffer. Both reactions were linear with respect to time and enzyme concentration. Rates of change in absorbance were determined over 3 min and activities calculated assuming a millimolar extinction coefficient for cytochrome c of 29.5 at 550 nm.

2.6.4 Glutamate dehydrogenase ((GDH) EC 1.4.1.2)

GDH activity was determined by measuring the rate of 2-oxoglutarate dependent NADH oxidation after the method of Robinson *et al.* (1991), in a 1 mL reaction volume containing 150 mM NH_4Cl, 100 mM $CaCl_2$, 300 μM NADH, 50-100 μL protoplast or organelle suspension (50-100 μg soluble protein approx.) and 100 mM Tris at pH 8.2. The reaction was started by the addition of 15 mM 2-oxoglutarate. Control assays, in

which 2-oxoglutarate was replaced with buffer gave rates of change in absorbance of approximately 3% of those achieved in the presence of 2-oxoglutarate.

2.6.5 Triose phosphate isomerase ((TPI) EC 2.6.1.1)

TPI was assayed by coupling the production of glucose-3-phosphate through glucose-3-phosphate dehydrogenase and monitoring the reduction of NAD, after the method of Pichersky and Gottlieb (1984). The assay mixture, in a total volume of 3 mL, contained: 2 mM sodium arsenate, 60 μM NAD, 3 units of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 100 μL protoplast or organelle suspension (100 μg soluble protein approx.) and 20 mM Tris-acetate at pH 7.2. The reaction was started by the addition of 2.5 mM dihydroxyacetone phosphate (DHAP). DHAP was prepared by the hydrolysis of dihydroxyacetone phosphate dimethyl ketal di(monocyclohexylammonium) salt, 25 mg of which was dissolved in 2 mL of dH_2O, to which was added 0.5 g (wet weight) Dowex 50(H^+). This slurry was swirled for 30 s, filtered and the filtrate washed with a minimal amount of ddH_2O, the combined filtrates were kept at 40°C for 4 h to hydrlyse the ketal, and the resulting acidic solution was adjusted to pH 4.5 with K_2CO_3 and stored frozen. TPI activity was linear with respect to time and enzyme concentration, and control assays were run in the absence of G3PDH.

2.6.6 Aspartate amino transferase ((AST) EC 2.6.1.1)

AST was measured by determining the rate of oxidation of NADH when the transfer of the amino group from aspartate to 2-oxaloacetate is linked to the reduction of this product by transamination with malate dehydrogenase, using an optimised diagnostic kit (Sigma chemical Co.). Controls were run with boiled enzyme preparations.

2.7 Extraction of amino acids

Amino acids were extracted from protoplasts and organelles in 100% HPLC grade methanol. For protoplasts approximately 100 mg was extracted in 3 mL of methanol and for organelles 1 mL of methanol per 100 μL suspension was frequently used.

2.8 HPLC determination of amino acid pools

Amino acid pools were characterised from the methanol extracts described above as *o*-phthaldialdehyde (*o*-PA) derivatives by HPLC. The HPLC system (Kontron Instruments, Watford, Herts.) consisted of: a model 420 pump, 460 autosampler, 425 gradient former, 2 cm stainless steel guard column, a C-18 (10 x .45 cm) column and a Milton Roy Fluoro-Monitor 3 fluorescence detector. The whole system was controlled by a 450 multitasking data system.

All reagents, standards, samples and eluents described below were filtered through a 0.45 μm HV filter (Millipore, Bradford, Ma. USA) prior to use. The derivatisation of amino acids was adapted from the method of Joseph and Marsden (1986), in which *o*-PA stock was prepared from 50 mg *o*-PA dissolved in 1 mL methanol and made up to 7.5 mL with 0.4 M borate buffer at pH 9.5. A working *o*-PA reagent was made from 1.5 mL of the above stock to which was added 10 μL of mercaptoethanol, 10 μL of 0.25 mM homoserine (HSER, an internal standard) and 30 μL of sample were derivatised with 40 μL of working reagent. After 2 mins 8 μL of derivatised sample was injected along a gradient generated by two eluents; A, 50 mM Na_2HPO_4/CH_3COONa with 20% (v/v) methanol and 20% (v/v) tetrahydrofuran (pH 8.3), and B, 65% (v/v) methanol. The 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. All eluents were degassed with helium prior to use.

Samples were calibrated against amino acid standards (Sigma, Dorset, UK) supplemented with 0.25 nM glutamine, γ-aminobutyric acid (Gaba), serine and

asparagine. Standards were run every 5 samples and treated in exactly the same way except that only 20 μL were derivatised with 60 μL *o*-PA working reagent.

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

The fate of ^{15}N, fed to protoplasts as an inorganic nitrogen source, was followed by purification of the amino acid extracts described above and the subsequent GC-MS analysis of their tert-butyldimethylsilyl (t.BDMS) derivatives. Methanol extracts were centrifuged for 5 mins at 1300 *g* in a microfuge and the supernatent filtered through a 0.45 μm HV filter as described for HPLC samples. The filtrate was taken to dryness by rotary evaporation and redissolved in 1 mL ddH_2O. The extract was applied to a Bio-Rex sample preparation disc containing AG 50W-X8 cation exchange resin (Biorad, Richmond, Va. USA) and washed with 5 mL ddH_2O to remove organic acids. Sugars and amino acids and amides were eluted with 7 mL 6M NH_4OH. This fraction was lyophilised overnight, resuspended in 1 mL 50% methanol, transferred immediately into silanised glass vials and taken to dryness under N_2(g) with gentle heating to approximately 30^{o}C.

This purified extract was derivatised according to the method of Rhodes *et al.* (1989). A derivatising mixture (40 μL for protoplast samples and 25 μL for organelles) of methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSFA, Pierce Chemical Co.): pyridine: triethylamine (15:15:1) was added to the dry samples which were then heated at 75^{o}C for 30 mins in silanised glass vials capped with teflon septa.

^{15}N incorporation into each amino acid was determined by GC-MS analysis of the t.BDMS derivatives using a VG305 mass spectrometer linked to a Vector 2 data system (Technivent St.Louis, USA). 0.6 μL of sample was run on Varian 3400 gas chromatograph, with an on-column injector cooled with CO_2, and fitted with a 25 M polydimethyl, 0.25 μm film thickness, fused silica capillary column. Helium was used as the carrier gas with a flow rate of 1 mL/min. The column oven was programmed

from 120°C for 1 min, +40°C/min to 280°C. Mass spectra were acquired using an electron energy of 70 eV over a mass range 650 to 100 mass to charge ratio (*m/z*) with a total scan time of 3.2 s.

Incorporation of ^{15}N was calculated after integrating the areas obtained for fragment ions for both labelled and unlabeled amino acids, and expressed as the percentage of ^{15}N above natural abundance, or atom % excess. The use of t. BDMS derivatives allows the determination of the total amount of ^{15}N incorporation into the amino acid amides asparagine and glutamine, and the relative proportions that are singly and doubly labelled.

2.10. ANCA analysis of ^{15}N incorporation into protein

The total ^{15}N incorporation into protein was determined by the technique of automated $^{15}N/^{13}C$ analysis-mass spectrometry (ANCA-MS) using a Europa Scientific Roboprep Tracer Mass system. TCA insoluble extracts of whole protoplasts were prepared as described for the determination of total protein; such pellets were resolubilised in 0.3 M NaOH and 50 μL injected onto Carbosorb and analysed in triplicate with the ANCA-MS in small sample mode, as described by Barrie and Lemley (1989). Samples were calibrated against standards containing $^{15}NH_4Cl$ (99% enriched).

Chapter 3 The isolation and characterisation of vacuoles, plastids and mitochondria from carrot protoplasts.

3.1. Introduction

The vacuole is an organelle characteristic of non-meristematic plant cells whose functions include roles in tissue support, metabolite storage and sequestration, and lytic processes. Much of our recent understanding of such functions has depended on the development of methods for the isolation of intact vacuoles. The first report of large-scale isolation of intact vacuoles from plant tissues appeared some 17 years ago (Wagner and Siegelman, 1975). This technique provided an opportunity to increase our understanding of the physiology and biochemistry of the vacuole in relation to a range of cellular activities. Protoplasting is an essential step in the large scale isolation of vacuoles. Although vacuoles have been liberated from intact tissues (Leigh and Branton, 1976; Boudet *et al.*, 1989, Nishimura and Beevers, 1978), the high shear forces involved disrupt fragile vacuoles making large scale isolation impractical. Protoplasting, however, also has disadvantages, since it involves prolonged exposure of the parent cells to both plasmolysis and wall degrading enzymes, which may alter the biochemical composition and function of the vacuole.

The method for vacuole isolation presented here was adapted from that of Van der Valk (1987), in which gentle phosphate-dependent osmotic lysis, in conjunction with a pH shock, brings about the release of intact vacuoles from previously isolated protoplasts. Further purification of the vacuolar fraction was achieved by density centrifugation in isosmotic medium. These procedures were developed to give the maximum vacuole yield combined with minimum contamination with intact protoplasts, cytoplasmic and other cellular components. The degree of purity of vacuole preparations was assessed both microscopically and by assaying marker enzymes characteristic of vacuoles, mitochondria, plastids and the free cytosolic fraction. The proportion of protoplast

volume occupied by vacuole(s) was determined, again using both microscopic and marker enzyme methods.

The isolation of mitochondrial and plastid fractions was carried out separately from vacuolar fractionation, and involved physical lysis by forcing protoplasts through a mesh. This was necessary since osmotic shock may rupture such organelles. Further purification using isotonic density gradient centrifugation and characterization using marker enzymes was also performed.

3.2 Materials and Methods

3.2.1 Vacuole isolation

Washed protoplasts were harvested by centrifugation (250 *g*, 10 min) and 1 volume of pellet was resuspended in 5 volumes of a lysis buffer containing 100 mM K_2HPO_4, 5 mM DTT, and 6% (v/v) Percoll (Sigma, Dorset, U.K.). After centrifugation for 3 min at 250 *g* vacuoles were collected as a "floating band" in the neck of a babcock bottle.

3.2.2 Determination of relative vacuolar volume

Since protoplasts were multivacuolate, two unrelated methods were employed to permit an accurate estimation of vacuolar volume. Regardless of the method used, it is essential that any measurements are made upon vacuoles experiencing the same osmotic pressure as the protoplasts from which they originated. In the first method 500 μL of purified vacuole suspension was resuspended in 15 mL of protoplasting buffer containing 6% Percoll (v/v) and collected by centrifugation at 250 *g* for 3 min in a 15 mL capacity babcock bottle. This procedure was repeated with 500 μL of a protoplast suspension. 20 μL aliquots of both protoplasts and vacuoles were taken from the top of the babcock bottle and assayed for AMS activity as described previously.

The second method involved direct measurement of the diameters of vacuoles and protoplasts. 100 μL aliquots of both protoplasts and vacuoles were taken after Percoll

flotation in iso-osmotic solutions, mounted under a cover slip, and mean diameters measured at 800x magnification using an eye-piece graticule. Only the largest vacuoles were measured as the method is based upon the assumption that these are derived from univacuolate protoplasts. Mean spherical volumes for protoplasts and vacuole populations were calculated after measurement of 400 diameters.

3.2.3 Assessment of the purity of vacuole preparations

400 μL of a vacuole suspension was prepared from 13-day cultures and made to 2 mL with lysis buffer. 100 μL aliquots were assayed for the following marker enzymes: AMS and AP (vacuole); G6PDH (cytosol); GDH and CYTOX (mitochodrion); NADH -CR (endoplasmic reticulum) and TPI (plastids), as described previously. Separate aliquots were taken for determination of soluble and total protein.

3.3 Mitochondria and plastid isolation

Protoplasts were harvested by centrifugation as above and 1 volume of pellet was resuspended in 6 volumes of ice-cold organelle isolation buffer containing; 0.5 M mannitol, 1% (w/v) BSA and 1 mM EDTA in dH_2O at pH 7.5. The suspension was forced through a 20 μm nylon mesh held over the end of a syringe barrel, into cooled centrifuge tubes using a 40 mm 11/10 disposable hypodermic needle. Unlysed protoplasts and debris were removed from the lysate by centrifugation at 500 *g* for 10 min. The supernatant (hereafter referred to as S_5) was decanted into cooled centrifuge tubes and centrifuged at 15000*g* to pellet motochondria, plastids and other organelles for 10 min in a MSE pegasus 65 ultracentrifuge using a 8 x 50 mL fixed angle aluminium rotor. The supernatant (S_{150}) was decanted off and the resulting pellet (P_{150}) was resuspended gently, using a paint brush, in 1 mL of organelle isolation buffer, and layered onto a discontinuous step gradient of 45% ,22%, 10% (v/v) Percoll in 2 mL steps in a 15 mL Falcon tube (Beckton Dickinson Labware, New Jersey, USA).

Each concentration of Percoll was made by mixing the appropriate volumes of organelle isolation buffer with the same buffer made up in Percoll. Distinct interfaces between the steps were achieved by the "floating cork" method where less dense solutions were pipetted gently onto the surface of a circular piece of cork (with a diameter slightly less than that of the centrifuge tube) floating on the surface of the denser solution. The step gradients were centrifuged at 8000 *g* for 3 min, after which the plastid fraction was recovered from the 10/22% interface and the mitochondrial fraction from the 22/45% interface. Fractions were collected by suction using long bore glass pipettes and either transferred to HPLC grade methanol or assayed for marker enzymes, as appropriate.

3.3.1 Assessment of purity and integrity of the mitochondrial and plastid fractions

The fractions were prepared as described above and the gradient divided into sucessive 500 μL fractions from the top. Each fraction was assayed for the following marker enzymes: AMS, GDH, CYTOX, NADH-CR, TPI, AST, G6PDH, as previously described. Recovery of orgenelles within a specific fraction was determined by comparision of the activiy of the relevant marker enzyme, with the total amount of activity applied to the gradient. Standard marker enzyme assays yield, what will be termed **total** activity, which includes both organelle-bound and soluble activity. In order to calculate the membrane bound component of the total activity the activity of the enzyme in the presence of an osmoticum (0.5 M mannitol) was determined (hereafter refered to as the **soluble** activity), as organelles remain intact the activity can be attributed to soluble enzymes only. The **organelle-bound** activity of a specific marker enzyme is merely the difference between the **total** and the **soluble** activity.

The relative volumes of the plastid and mitochondrial fractions were estimated from a calculation of the non-osmotic volume (NOV) of the protoplasts (Weyers and Fitzsimmons, 1982). Protoplasts were prepared, washed and resuspended in a buffer based upon MDK, containing 50 mM MES at pH 5.7 and either 500, 400, 300, 200 or 150 mM mannitol. The protoplasts were allowed to incubate for 30 mins at 25°C on an

orbital shaker. The diameters of between 30 and 40 protoplasts from each mannitol concentration, were measured using an eye-piece graticule, and volumes calculated assuming sphericity. The calculation of NOV is detailed in the legend to Figure 3.5.

3.4 Results

3.4.1 Vacuole isolation

Protoplasts were routinely obtained at yields of approximately 25% and their appearance reflected the active metabolic state of the cultured cells from which they originated (see Figure 3.1A). The protoplasts were vacuolate in appearance (as they were prepared from stationary phase cells) the majority being multivacuolate.

Wagner (1986) reported that from 80 papers describing the release of vacuoles from protoplasts less than 6% used an unchanged, previously published method. Carrot protoplasts conformed to this precedent and many methods of vacuole release were investigated, including polybase induced lysis (Boudet *et al.*, 1981), reducing the osmoticum of the supporting media (Leigh, 1983) and mechanical disruption. These methods all produced vacuoles but at low efficiency and purity. The method finally developed was based upon phosphate dependent osmotic lysis, as described by Van der Valk (1987), Boller and Kende (1979) and Wagner and Siegleman (1975). This method produced virtually total lysis and the majority of vacuoles were released intact (see Figure 3.1A inset). In any preparation a number of transiently released vacuoles were seen to burst, suggesting that vacuoles from multivacuolate protoplasts are of varying osmotic potential.

Figure 3.1

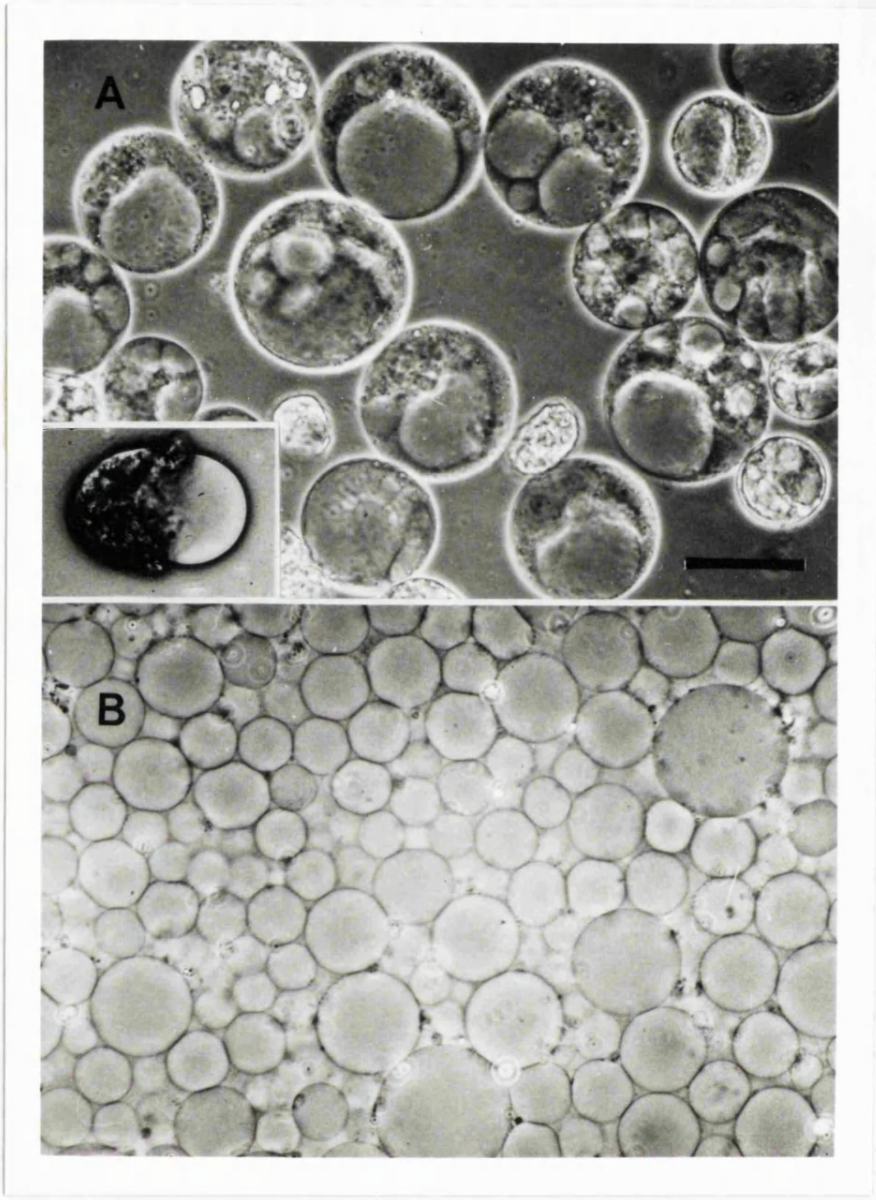

Figure 3.1 Phase contrast micrographs of protoplast (A) and purified vacuole (B) preparations from 12 d old carrot suspension cultures. Inset in A shows vacuole release 3 minutes after the addition of lysis buffer to the protoplast suspension. Scale bar = 15 μm.

Vacuole yields of between 10-15% were obtained using this method. However, problems were encountered in separating released vacuoles from contaminating cytoplasmic debris. This was surmounted by combining the osmotic shock with a pH increase from 5.7 to 8.0, which served to encourage "clumping" of the debris, enabling

vacuoles to be separated from the lysate by differential centrifugation through a 6% Percoll solution. Microscopic investigation revealed that contamination by intact protoplasts was always less than 1% (Figure 3.1B), and little contaminating material was seen adhering to the tonoplast (as confirmed by marker enzyme analysis, see below).

Table 3.1 reports some of the characteristics of the vacuole preparations in relation to the protoplasts from which they came. All calculations are made relative to AMS activities, since AMS was selected as the reference enzyme localised entirely in the vacuole (see discussion). Contamination of vacuole preparations with activities characteristic of cytosol and mitochondria were 2.9% and 2.1% respectively, whilst that associated with plastids (TPI) was 9.6%. Vacuolar content of soluble protein was 16% of that of the protoplast. The specific activity of AMS was 10.8 times higher in vacuoles than in protoplasts, with less than 1% attributable to intact protoplasts. These data are compatible with the assumption that AMS activity is entirely vacuolar and that as a marker it provides a reliable reference for stiochiometric comparisions of solute concentrations and for the calculation of vacuolar volume. A second potential vacuolar marker, AP, was also investigated. While AP activity was largely vacuolar, approximately 20% was found to be cytoplasmic when calculated relative to AMS. Sasse *et al.* (1979), reported that the vacuoles of carrot protoplasts contained only 55% of total AP activity.

Estimation of the vacuole:protoplast volume ratio was made by two methods, as described above. Direct measurement under iso-osmotic conditions of diameters of 400 protoplasts or vacuoles gave a vacuole to protoplast volume ratio of 52.5%, based upon the assumption that larger vacuoles derive from univacuolate protoplasts. Secondly, comparisions were made of AMS activity per unit volume of both vacuole and protoplast preparations under isosmotic conditions. One volume of vacuoles contained on average 1.75 times as much activity as one volume of protoplasts, from

which, calculation yields a vacuole volume equal to 57% of the protoplast volume. The validity of this is based upon the assumption that AMS is 100% vacuolar and that the packing characteristics of each preparation following centrifugation were equivalent.

Table 3.1

Parameter	Protoplasts	Vacuoles	Vacuolar content %
Protein[a]	22.2 +_ 1.9	3.6 +_ 0.4	16.2
nKat AMS/mg[b]	6.1	75.0	1,080
Volume[c]	5.9 +_ 0.8	3.1 +_ 0.4	52.5
nKat AMS/mL[d]	154 +_ 6.5	270 +_ 4.0	175
AMS activity[e]	1.0	1.0	100
AP activity[e]	10.2 +_ 0.7	8.1 +_ 0.9	79.4
GDH activity[e]	0.69 +_ 0.03	0.02 +_ 0.03	2.9
G6PDH activity[e]	0.14 +_ 0.05	0.003 +_ 0.05	2.1
TPI activity[e]	0.52 +_ 0.3	0.05 +_ 0.04	9.6

Table 3.1 Characteristics of protoplast and vacuole preparations.
[a] Total TCA insoluble protein (mg/mL protoplast)
[b] AMS activity per mg soluble protein, mean +_ SD of three representative experiments.
[c] Volumes determined from mean +_ SD diameter determinations from 400 vacuoles ($m^{-3}x10^{-15}$).
[d] AMS activity per mL protoplast or vacuole suspension, which also allows vacuole fresh weight to be calculated as mg protoplast equivalent, mean of three experiments +_ SD .
[e] Enzyme activities as nKat/nKat AMS, mean of three experiments +_SD

3.4.2 Plastid and mitochondrion isolation

Fractions enriched in mitochondria and plastids were prepared by density gradient centrifugation after protoplast disruption. Table 3.2 illustrates the efficiency throughout the differential centrifugation stage in the production of fractions enriched in enzyme activities characteristic of plastids (TPI) and mitochondria (CYTOX and GDH). Successive differential centrifugation steps resulted in at least 60% recovery of mitochondrial activity and 67% recovery of plastidial activity.

Table 3.2

Fraction	Marker enzyme activity			
	AMS	GDH	CYTOX	TPI
			µKat/fraction	
whole extract[a]	27.8	17.8	3.2	9.6
S_5[b]	24.9	15.5	2.7	8.9
S_{150}[c]	27.4	2.8	0.6	3.4
P_{150}[d]	4.03	10.8	2.9	6.4

Table 3.2 The efficiency of recovery of marker enzymes throughout the successive differential centrifugation steps of mitochondrial/plastid isolation procedure.
a One volume of washed protoplasts resuspended in 6 volumes of organelle isolation buffer and lysed by forcing through a 20 μm mesh.
b The supernatant produced by centrifugation of the above brei at 500 *g*.
c The supernatant produced by the centrifugation of S_5 at 15000 *g*.
d The pellet from the above spin,enriched with mitochondrial and plastidial marker enzyme activities.
Differential centrifugation steps result in at least a 60% recovery of mitochondrial activity and a 67% recovery of plastidial activity.

Figure 3.2 shows the distribution of soluble protein following discontinuous density gradient centrifugation of a fraction enriched in mitochondrial and plastidial activity. Two distinct protein bands were observed, the first falling within fractions 6-7

(10/22% Percoll interface, 11.5 mg/fraction) and the second within fractions 10-11 (22/45% Percoll interface, 2.8-3.0 mg/fraction). The soluble protein recovered from fractions 6 and 7 represented 79% of the soluble protein of the P_{150} fraction applied to the gradient.

Figure 3.2

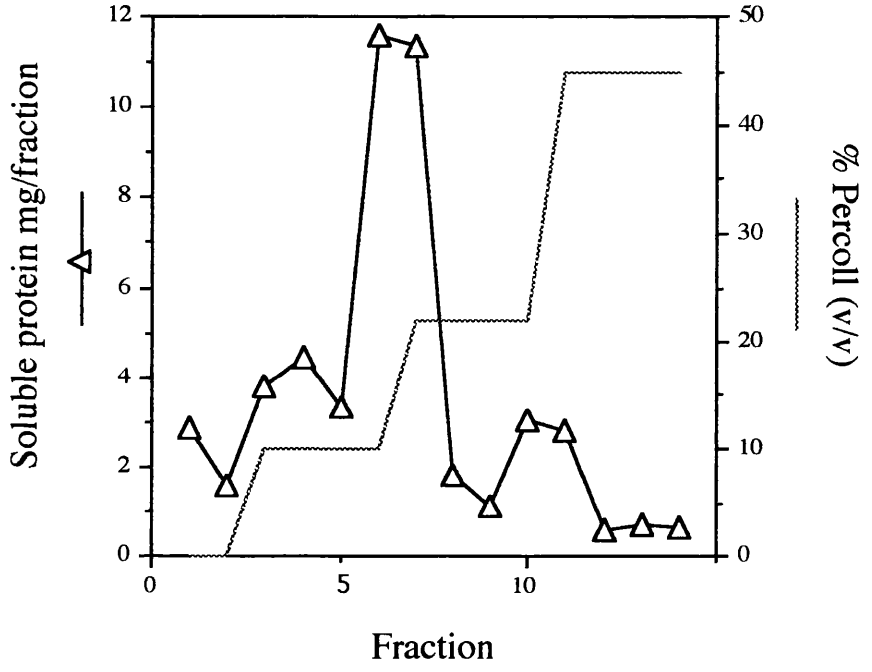

Figure 3.2. The distribution of soluble protein along a discontinuous Percoll gradient following the centrifugation (9000 *g* 3 mins) of an overlayered 1500 *g* fraction (P_{150}).

The distribution of **total** CYTOX and TPI activities following density gradient separation are shown in figure 3.3a. There is a peak of TPI activity coincident with the major protein peak (see Figure 3.2) and a smaller peak across the 22/45% interface. Most of the total CYTOX activity was recovered in fractions 2-3, with a second peak of activity, representing approximately 60% of the first peak, at the 22/45% interface. These data do suggest a degree of separation of the activities, but to determine the extent to which the observed total activities can be attributed to intact organelles, the fractions were assayed for the same markers using the "protection" assay method of Yamada *et al.* (1984). Assays of the **soluble** TPI activity revealed that the majority of

component and presumably plastids, the most enriched fraction occuring at the 10/22% interface. On the other hand majority of the CYTOX activity in this region of the gradient was not **membrane-bound**, having probably been released from intact mitochondria. The majority of the **membrane-bound** CYTOX activity, and thus the most highly enriched mitochondrial fraction was found at the 22/45% interface. A degree of contamination was evident in each fraction, and this is quantified in Table 3.3. Data for GDH is not shown, however approximately 97% of the GDH applied to the gradient co-separated with the peak of CYTOX activity, confirming the mitochondrial activity of these peaks

Figure 3.4 shows the distribution of the marker enzymes AMS and NADH-CR following density gradient centrifugation. AMS activity was uniformly distributed on the gradient (Figure 3.4a) and showed no membrane-bound component, indicative of neglible cross contamination of the fractions with intact organelles. NADH-CR activity was largely membrane bound (presumably as ER) and distributed in two peaks at fractions 7 and 11. (Data on the distribution of the activities of AST is not detailed here, since although most activity was retained in the P_{150} fraction , its distribution on Percoll gradients was uniform , and closely resembled that of AMS. It was clearly not associated with any specific organelle and of no further value as a marker enzyme).

Figure 3.3

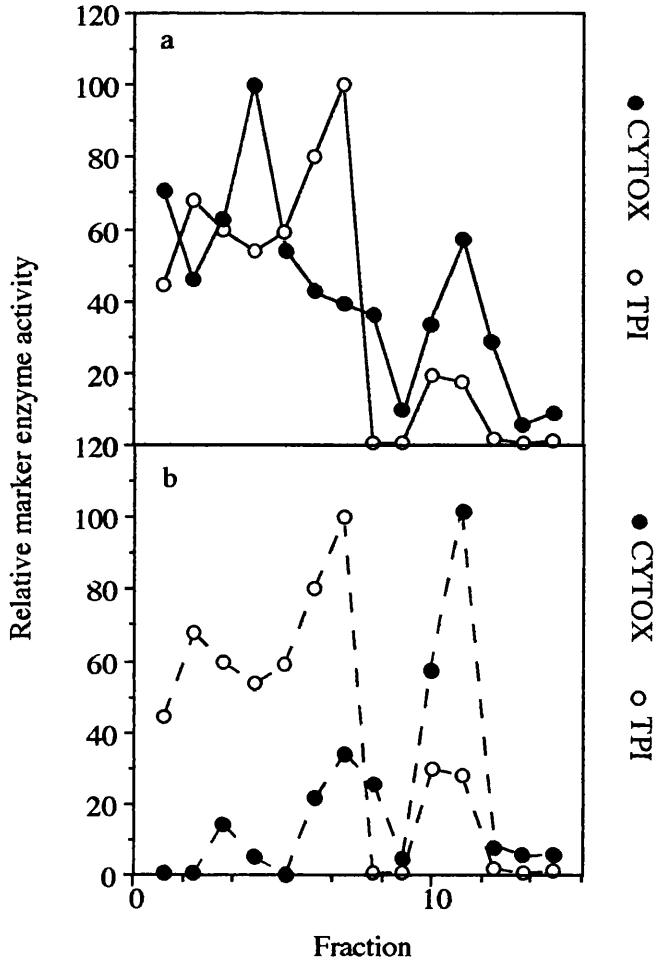

Figure 3.3. The distribution of a plastidial marker (TPI) and a mitochondrial marker (CYTOX) along a discontinous percoll gradient as in Figure 3.4.
Figure 3.3a Shows **total** activity, whereas **Figure 3.3b** gives the **membrane-bound** activity i.e. the difference between **total** and **soluble** activity. Activities are presented as a percentage of the activity of the most enriched fraction. The relative enzyme activities can be converted to specific activities (μkat/fraction) by multiplying the ordinates by 0.0528 in the case of TPI and 0.0289 for CYTOX.

Figure 3.4

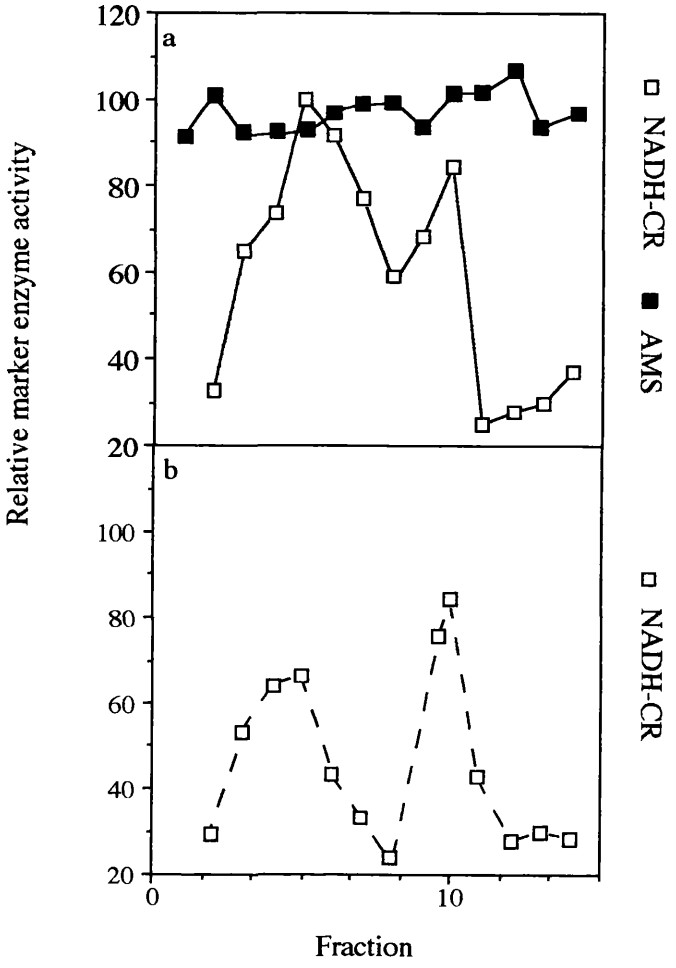

Figure 3.4 The distibution of the ER and vacuolar markers, NADH-CR and AMS respectively, along a discontinuos gradient.
Figure 3.4a shows the distribution of **total** activities
Figure 3.4b shows the **membrane-bound** component of the NADH-CR activity . AMS activities along the gradient had negligible or no **membrane-bound** component. The relative activities can be converted to specific activities (μkat/fraction) by multiplying the ordinates by 0.0374 for NADH-CR and 0.043 for AMS.

Table 3.3

Fraction	Marker Enzyme Activities			
	TPI	CYTOX	NADH-CR	AMS
		µkat/g protopast equivalent[a]		
P_{150}	$1.25^{+}_{-}0.6$	$34.98^{+}_{-}7.3$	$49.35^{+}_{-}14.7$	$45.83^{+}_{-}13.9$
10/22[b]	$0.86^{+}_{-}0.3$	$1.84^{+}_{-}0.7$	$6.88^{+}_{-}2.7$	$6.65^{+}_{-}0.9$
% Rec[c]	77	5	14	15
% intact[d]	64	82	94	nd
22/45[e]	$0.23^{+}_{-}0.1$	$10.08^{+}_{-}3.4$	$36.24^{+}_{-}9.8$	$2.37^{+}_{-}0.3$
% rec[c]	18	31	73	5
% intact[d]	7	87	69	nd

Table 3.3 The activities of specific marker enzymes through successive fractions along a discontinous Percoll gradient

[a] All activities are expressed per g fresh weight protoplasts and are means of at least three separate preparations $^{+}_{-}$ SD

[b] The interface of the 10% and 22% Percoll steps i.e. fraction 7.

[c] That proportion of the total enzyme activity applied to the gradient recovered in the particular fraction

[d] That proportion of the total enzyme activity that can be attributed to intact organelles.

[e] The interface of the 22% and 45% Percoll steps i.e. fraction 11.

Table 3.3 gives the degree of cross contamination of the TPI and CYTOX peaks expressed as specific activities against a fresh weight protoplast equivalent. It illustrates that the majority of the applied TPI activity is recovered at the 10/22% interface; this activity is predominantly attributable to intact organelles and is contaminated with intact mitochondria (≈5%) and membrane bound NADH-CR activity (≈14%). The peak of CYTOX activity at the 22/45% interface represents 31% of that applied, the majority being membrane bound. There is cross contamination with intact plastids (≈7%) and co-separation with a peak of membrane bound NADH-CR activity.

Figure 3.5 shows the stages in the estimation of the the non-osmostic volume (NOV) of carrot protoplasts. The data presented is a representative set, all others giving similar results. "Least squares" linear regression gave a value for NOV of 0.47×10^{-15} m^{-3}, some 8% of the total protoplast volume, where the NOV is the proportion of the protoplasts which is not available for free solute exchange. Intuitively, nuclei, plastids and mitochondria are the principle organelles contributing towards the NOV.

Figure 3.5

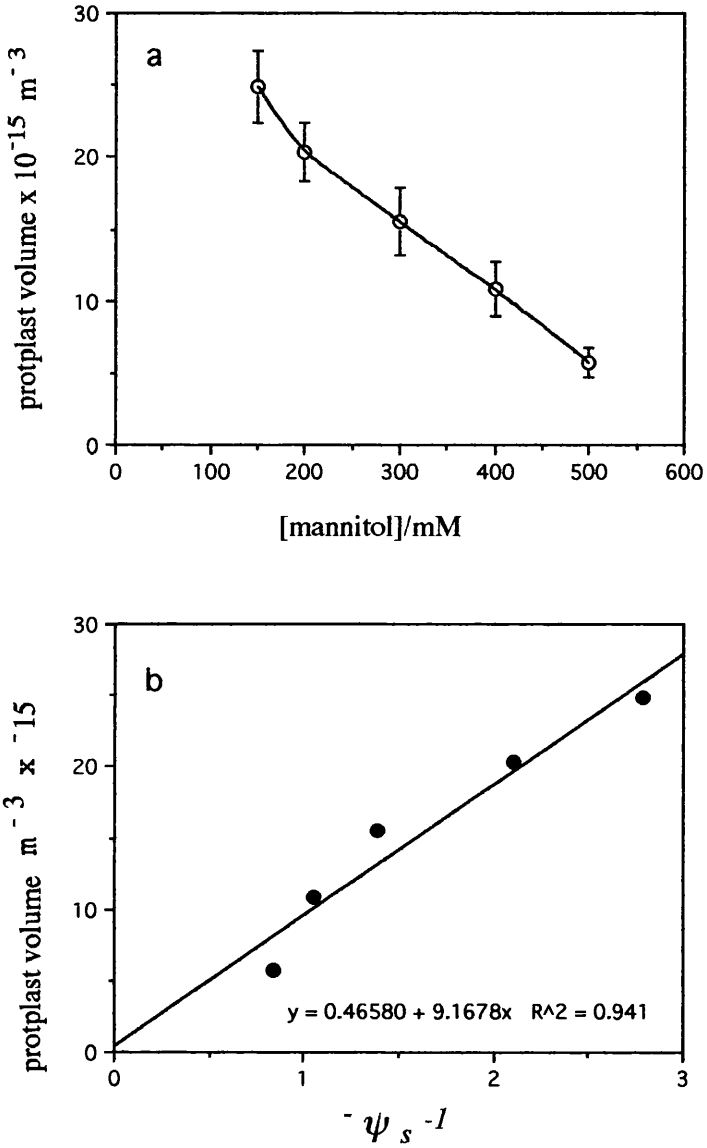

Figure 3.5 The stages in the calculation of the non-osmotic volume (NOV) of carrot protoplasts. Figure 3.5a shows the osmotic effects on carrot protoplast volumes as a function of mannitol concentration in the supporting media and Figure 3.5b shows the relationship as a function of the solute potential (Ψ_S 301 K) of the medium. Ψ_S is calculted from the *Boyle Van't Hoff* equation; $-\Psi_S(V-b)=nRT$, where V and b are the protoplast and non-osmotic volumes respectively, n = the apparent number of osmotically active solute molecules (i.e. the mannitol concentration), R = the gas constant and T = the temperature/K. From this equation a plot of V against Ψ_S^{-1} is a straight line of slope nRT and y intercept b (NOV). The linear regression was fitted by the "least squares" method.

3.5 Discussion.

The results presented here confirm that the method described for the bulk isolation of vacuoles yields suspensions of intact vacuoles, relatively free of contamination from intact protoplasts and enzymes characteristic of other compartments within the protoplast.

Workers have reported a wide range of indices of contamination for vacuole preparations; from 2% (Le-Quoc, 1986), 10% (Boller and Kende, 1979, Kreis and Reinhard, 1985) and 29% (Renaudin *et al.*, 1986). Vacuole preparations obtained by the methods described here were of comparable purity to the best published reports, and it was not necessary to make corrections for contaminating organelles or protoplasts in subsequent calculations of metabolite pool sizes.

The selection of a marker enzyme characteristic of vacuoles was of critical importance for this study, since it constitutes a reference point for comparisons between vacuolar and extravacuolar quantities as well as in calculating vacuolar volume. AMS has been widely used in this role owing to its reported presence exclusively in the vacuole (Boller and Kende, 1979, Kaiser *et al.*, 1986, Martinoia *et al.*, 1981). The close agreement between volume determinations made using AMS data as compared with direct physical methods, and also the much higher specific activity of AMS in the vacuole as compared with the protoplast, confirms that AMS is strictly vacuolar in carrot protoplasts also. This information is applied in future chapters in calculations involving stoichiometric comparisions of vacuolar and cytoplasmic metabolites.

Two methods of estimating vacuolar volume as a proportion of protoplast volume gave very similar results, and a relative vacuolar volume of 55% was adopted for subsequent calculations. Vacuolar volumes of the order of 70-90% have been previously reported for studies of barley mesophyll (Kaiser *et al.*, 1986) and other mature cell types, such as fruit flesh (Yamaki, 1984). The relatively larger proportion occupied by the

cytoplasm in these preparations reflects the higher metabolic activity of proliferating cells as compared with mature cells *in planta.*

Further fractionation of the cytoplasmic fraction was achieved by gentle disruption of the protoplasts, differential centrifugation and iso-osmotic density gradient centrifugation. Recovery and purity were estimated by marker enzyme analysis. The initial stages of disruption and differential centrifugation were effective in producing a fraction containing 60-70% of the marker enzyme activity from an intial lysate with 30x the volume. Separation of this highly enriched fraction on discontinuous Percoll gradient produced visibly distinct, particulate bands at the interfaces, which through protein and marker analysis were shown to be coincident with peaks of activity of enzymes associated with plastids and mitochondria. Several other workers have shown peaks of TPI and CYTOX activity along density gradients which denoted the resolution of plastids and mitochondria respectively (Nishimura *et al.*, 1976, Miflin, 1974, Hampp, 1980)

The cross contamination of the particulate bands was higher than the cross contamination of the vacuole preparations. Purity of the fractions was determined for each preparation, and the preparations were only used for experiments on nitrogen assimilation if the degree of cross-contamination was less than 25%. In this case the degree of contamination was taken into account in the calculation of the relative size of metabolite pools associated with each fraction. However the method presented does resolve plastids and mitochondria simultaneously and quickly, thus sparing the organelles from the prolonged exposure to released vacuolar contents, which could affect membrane integrity and solute concentrations.

An apparent anomaly of the method was observed in which NADH-CR activity (normally associated with endoplasmic reticulum) co-incided with both mitochondrial and plastidially enriched fractions. Studies have established that NADH-CR can be

associated with the endoplasmic reticulum (Colbeau *et al.*, 1971, Moore *et al.*, 1973, Phillip *et al.*, 1976), but it's activity does not appear to be confined to ER (Leigh and Branton, 1976) and has been associated with the outer mitochondrial membrane (Moreau and Lance, 1972).

Repeated estimations of the NOV of carrot protoplasts all gave volumes approximating to 8% of the protoplast. Therefore, assuming the vacuole occupies 55% and the extravacuolar space (the "cytoplasm") 45%, then 37% of the protoplast will be given over to free solute exchange. It was not possible to make microscopical investigations of the isolated plastid and mitochondrial fractions and there was uncertainty in predicting the packing arrangement of the organelles at the Percoll interfaces; consequently it was not possible to estimate the relative volumes of the organelles by the approaches adopted for vacuoles. However, from transmission electron micrographs of sections through carrot suspension cells (Kindly supplied by Dr R Pennel, UCL) it was possible to make rough estimates of the relative contribution each organelle made to the NOV. The nucleus contributed ≈24% to the NOV; plastids in aggregate contributed ≈59%, and mitochondria in aggregate ≈17%. Thus overall relative volumes of 1.9%, 4.7%, 1.4% of the total protoplast volume could be attributed to the nucleus, plastids and mitochondria respectively. Despite uncertainties over the packing arrangement of the organelles, comparisions of marker enzyme activities suggested that the relevant enzymes were contained within volumes accounting for approximately 5% and 2% of the total protoplast volume for the plastid and the mitochondrial marker respectively, estimations which were not to disimilar from those obtained from a calculation of NOV.

Chapter 4. The partitioning of nitrogenous assimilate between cytoplasmic and vacuolar fractions.

4.1 Introduction

The principal advantage of the use of protoplasts in studies of plant metabolism is that it allows gentle cell lysis, enabling the recovery of intact organelles in sufficient quantities to allow the study of localization and subcellular movement of metabolites. Bulk isolation of vacuoles from protoplasts has enabled direct analysis of the role of the vacuole and cytoplasm in the compartmentation of amino acids to be made in a large number of species (Wagner, 1979, Boudet *et al.*, 1981, Thom *et al.*, 1982, Yamaki, 1984, Beck and Renner, 1990).

Mitochondria also function as metabolic compartments by virtue of their specific permeabilty and well defined membrane transport systems, classical examples including the spatial organization of the electron transport chain of oxidative phosphorylation, and the compartmentation of the reactions of the Krebs cycle (Oaks and Bidwell, 1970). Several workers have illustrated the ability of isolated mitochondria to assimilate ammonium or oxidise glutamate (Yamaya *et al.*, 1986, Day *et al.*, 1988) and GDH activity has been asociated with the mitochondria of higher plants (Stewart *et al.*, 1980). These observations suggest that the mitochondrial compartment is involved in higher plant nitrogen metabolism.

Plastids and proplastids from a variety of non green tissues represent sites of subcellular compartmentation. The isoenzymes of glycolysis are divided between the cytoplasmic and plastidial compartments, the enzymatic complement of each compartment differing in its physiochemical, kinetic and immunological properties. Key enzymes of fatty acid synthesis have been shown to be localised within the plastids (Simcox *et al.*, 1977). Furthermore plastids have been implicated in the compartmentation of some of the events of nitrogen assimilation, having been shown to

contain a wide range of transaminases (Ireland and Joy, 1990) and up to 60% of GS in alfalfa and pea (Vézina *et al.*, 1987)

This chapter reports a study of the partitioning of amino acids in plastidial, mitochondrial and vacuolar fractions of suspension cultured carrot cells. The dynamic fluxes of freshly synthesised amino acids across these compartments was investigated by the incubation of protoplasts with both ^{15}N-labelled NH_4Cl and KNO_3 and their subsequent fractionation and amino acid analysis by HPLC and GC-MS.

4.2 Materials and methods

4.2.1 Determination of amino acid pools: sizes and distribution

Cells: The distribution of amino acids in whole cells was determined, for comparison with that within protoplasts. Cell cultures were grown on standard medium for 12 d, then aseptically transfered to MDK medium modified by the omission of inorganic nitrogen sources (NH_4NO_3 and KNO_3) for 6h; a period equivalent to wall-digestion during protoplast preparation to allow the closest comparision with protoplasts. Cells were then collected by centrifugation (250 *g* 10 min) and the pellet extracted in methanol at a ratio of 1g (fwt) to 5 mL methanol for amino acid determinations by HPLC.

Protoplasts and sub-cellular compartments: Protoplasts were prepared from 12-d old cultures. Aliquots containing known fresh weights of pelleted protoplasts were taken for amino acid analysis by HPLC (100 mg protoplasts : 3.0 mL methanol), and for determination of AMS, CYTOX, and TPI activities. The remaining protoplasts were lysed and treated for either vacuole or mitochondria/plastid preparation. Aliquots of purified vacuole preparation were then taken for amino acid analysis by HPLC (100ul vacuoles: 1.0 mLHPLC grade methanol), and for AMS determinations. For determinations on mitochondrial and plastid fractions, 500 μL aliquots were taken from

the 10/22% percoll interface (plastid fraction) and the 22/45% percoll interface (mitochondrial fraction). 100 μL of each fraction was transferred to 1 mL of methanol for amino acid analysis by HPLC, and activities of CYTOX, TPI and were determined on the remainder.

4.2.2 Expression of results

All amino acid quantities in each compartment were calculated as the equivalent content in 1.0 g protoplasts, by reference to the appropriate marker enzyme. Thus, vacuolar quantities per unit of AMS were normalized against the AMS activity of 1.0 g protoplasts in the experiment concerned; plastid and mitochondrial quantities were calculated in the same way using TPI and CYTOX activities respectively. Estimations of concentrations of amino acids in compartments were made assuming relative volumes (see chapter 3) of 55% (vacuole), 4.7% (plastid), 1.4% (mitochondrial) and 37% (cytosolic - by difference).

4.2.3 ^{15}N labelling studies

Washed protoplasts from 2 x 1 L of 12-14d old cultures were resuspended at a density of approximately 10^6/mL in 200 mL of medium similar to N-free MDK (see chapter 2) but modified by the inclusion of an osmoticum (0.5 M mannitol) and 50 mM MES at pH 5.7. The protoplast suspension was incubated at 25°C on an orbital shaker for 1 h. Then, at time zero, filter-sterilised aliquots of a solution of $^{15}NH_4Cl$ (99% ^{15}N, MSD isotopes, Montreal Canada) were added to the culture flask to give a final concentration of 2 mM.

At intervals after time zero, 50 mL aliquots of the suspension were lysed to yield either vacuoles or mitochodria/plastid preparations. Aliquots of each purified organelle fraction were stored in methanol for HPLC and GC-MS determinations, and the appropriate marker enzyme (AMS, TPI or CYTOX) assayed at once. Immediately prior to each lysis time-point, aliquots of the protoplast suspension were taken for immediate

marker enzyme assay, or stored for HPLC and GC-MS determinations, TCA-insoluble protein or ^{15}N-protein determinations. Medium samples were stored for ammonium determinations. In some experiments a similar procedure was followed but with 2 mM $K^{15}NO_3$ as the sole nitrogen source.

4.3 Results

4.3.1 Amino acid pools: sizes and subcellular distribution

Amino acid contents of whole cells, protoplasts, and protoplast sub-compartments from 12 day-old cultures are shown in Table 4.1. The relative proportions of amino acids are broadly similar in both cells and protoplasts derived from them. Concentrations in protoplasts are substantially higher than in cells, reflecting osmotic shrinkage, absence of cell walls and tighter packing of protoplasts as compared with cells. The mean total amino acid contents of vacuole, mitochondria and cytosol broadly correspond to the ratio of the volumes occupied by the respective compartments. Plastids, on the other hand, occupy approximately 4.5% of the protoplast volume but carry only 0.1% of the total amino acid content.

Individual amino acids within protoplasts may be regarded as either primarily cytosolic, i.e., Gaba, alanine, methionine, tyrosine and aspartate; equally distributed between the cytosol and the vacuole, i.e., glutamate, serine, arginine, leucine, histidine, threonine and isoleucine; or primarily vacuolar, i.e., glutamine, valine, phenylalanine and asparagine.

Table 4.1

amino acid	content		relative distribution			
	cells	proto.	vac.	mito.	plas	cyto
	µmol/g		*% total content*			
Gaba	10.7+_ 2.9	38.3	30.3	0.2	+	69.5
Gln	8.2+_ 2.3	26.9	65.4	1.6	0.2	37.7
Ala	3.3+_ 1.1	26.3	31.6	1.9	+	66.5
Glu	8.4+_ 3.2	16.9	43.2	1.1	+	56.2
Ser	7.2+_ 2.6	15.1	55.0	1.3	nd	44.4
Val	4.6+_ 1.7	14.3	79.0	1.5	nd	19.6
Arg	5.2+_ 1.9	13.8	43.5	3.6	0.2	52.9
Leu	2.2+_ 0.7	9.3	55.9	1.2	nd	43.0
His	4.4+_ 0.9	9.2	39.1	1.3	nd	59.6
Thr	3.4+_ 1.1	8.3	57.8	1.9	nd	41.0
Ile	2.9+_ 0.3	5.9	45.8	1.9	nd	52.5
Phe	1.9+_ 0.4	5.5	63.6	2.4	+	36.4
Met	0.7+_ 0.3	4.6	23.9	8.7	+	67.4
Asn	1.2+_ 0.8	4.6	82.6	2.4	nd	15.2
Tyr	0.4+_ 0.3	3.9	28.2	3.3	nd	69.2
Asp	0.7+_ 0.4	2.5	36.0	2.4	nd	68.0

Table 4.1. The amino acid content of N-depleted cells and protoplasts and the relative distribution of the total protoplast content within vacuolar, mitochondrial, plastidial and cytosolic fractions. The results represent the means of at least three experiments +_ SD.
+ < 0.1% nd not detected

No amino acid appeared to be strongly localised within the mitochondria, except for methionine, and possibly arginine and tyrosine. Plastids contained extremely small

amounts of amino acids, with only glutamine and arginine present at above 0.1% of the total protoplast pool.

Each compartment or organelle interfaces directly with the cytosol. Similar amino acid concentrations across the delimiting membrane would suggest diffusion as a principal factor in relative distribution. On the other hand, differences would suggest either that selective accumulation has occured, or that deamination, decarboxylation or transamination reactions are proceeding at different rate on each side of the compartmental membrane. Table 4.2 shows the concentration of individual amino acid within each fraction. The mean total concentrations are broadly similar in three compartments, in the order mitochondria> cytosol>vacuole. The remarkably low concentrations within plastids suggest either selective exclusion, or that the organelle represents a locus for rapid amino acid turnover. Very different observations were made by Hampp *et al.* (1984), who found that in mesophyll oat protoplasts between 25%-35% (according to the light regime) of total aspartate was found in the chloroplast and likewise between 46%-52% of total glutamate, whereas aspartate was not detected in the mitochondria. Presumably these differences can be attributed to the photosynthetic nature of mesophyll protoplasts as compared with the heterotrophic origin of the protoplasts in this study.

While overall concentrations are similar in compartments other than plastids, individual amino acids show large differences in distribution. Of the primarily vacuolar amino acids, valine, phenylalanine and asparagine accumulated in the vacuole at around three times their cytosolic concentrations. The concentration of glutamine, the major vacuolar species, was about 50% higher in the vacuole than in the cytosol.

Table 4.2

amino acid	mean concentration			
	vacuole	mito.	plas.	cytosol
		mM		
Gaba	31.6+- 1.4	6.4+- 1.2	+	71.8+-21.2
Gln	32.6+- 4.5	30.0+- 3.9	1.3+- 0.2	24.2+- 4.1
Ala	15.0+- 1.8	25.7+- 2.4	+	48.5+- 6.5
Glu	13.3+- 1.3	13.6+- 2.0	+	25.6+- 1.3
Ser	15.0+- 2.4	13.6+- 0.9	nd	18.1+- 1.1
Val	20.1+- 4.3	15.7+- 2.8	nd	7.5+- 0.9
Arg	10.9+- 1.7	35.0+- 3.2	0.5+- 0.1	20.1+- 2.4
Leu	9.5+- 0.9	7.9+- 2.2	nd	10.8+- 1.9
His	6.5+- 0.7	8.6+- 2.3	nd	7.3+- 2.0
Thr	8.7+- 0.5	11.4+- 2.0	nd	9.1+- 1.7
Ile	5.0+- 0.9	7.9+- 0.8	nd	8.5+- 1.5
Phe	6.3+- 1.2	9.3+- 0.7	nd	1.9+- 0.4
Met	2.4+- 0.4	28.5+- 0.6	nd	6.5+- 0.2
Asn	6.9+- 0.7	5.3+- 0.2	nd	1.9+- 0.3
Tyr	1.9+- 0.2	9.3+- 0.4	nd	7.5+- 0.9
Asp	1.3+- 0.3	4.3+- 0.2	nd	4.7+- 0.2
Σ	187.0	232.5	1.8	202.2

Table 4.2. The amino acid contents of vacuoles, mitochondria, plastids and the cytosol expressed as concentrations. These data represent means of at least three experiments
+- SD. + <0.5 mM nd not detected.

Within the mitochondria, the major species arginine and glutamate were present at concentrations 66% and 25% respectively higher than in the cytosol. Phenylalanine and methionine were acumulated at more than 4 times the cytosolic concentration, while Gaba was stikingly depleted. The major cytosolic amino acids were Gaba, alanine, glutamate and to a lesser extent serine.

4.3.2 ^{15}N incorporation into amino acid pools: sizes, distribution and kinetics

The distributions described above reflect a static or equilibrium position in which individual pool sizes are derived from a complex series of events involving synthesis, catabolism and interconversions of amino acids. To study the partitioning of newly-synthesized amino acids, kinetic studies in which protoplasts were supplied with 2mM ^{15}N NH_4Cl prior to fractionation were undertaken. Experiments of two kinds were done; firstly, preparations of vacuoles were made at intervals of 2 hours up to 8 hours, to give time-course data on vacuolar and cytoplasmic (extravacuolar) changes. Secondly, full fractionations were made at a single time-point (4 hours) to give data on vacuolar, mitochondrial, plastid and cytosolic pools. The results of each experimental series are described in sequence below.

The first series of experiments produced data on the kinetics of enrichment of amino acid pools in vacuolar and cytoplasmic fractions over an 8 h period. Table 4.3 shows the changes in the amounts within each compartment of total and ^{15}N-labelled amino acids, total and ^{15}N-labelled protein, and the depletion of $^{15}NH_4^+$ from the medium. Total amino acids refers to the sum of the individual amino acids; complete data is not shown for the sake of brevity. During the experiment there was a 40% increase in total cytoplasmic amino acid amount, and a massive three fold increase in total vacuolar amino acids between 0-6h, followed by a sharp decline between 6-8h. The origin of this substantial increase in unlabelled amino acids will be considered later; for the moment it will be suffice to point out that the net protein content of the protoplasts fell by more than 20% over the first 2 h, and increased slowly to approximately the original

by more than 20% over the first 2 h, and increased slowly to approximately the original level after 8 h. Incorporation of ^{15}N into protein was not detected until 6 h, and even after 8 h the amount was less than 1% of that incorporated into amino acids.

Table 4.3

Time	amino acids				protein		
	unlabeled		^{15}N labeled				
	cyto	vac	cyto	vac	Total[a]	^{15}N[b]	media NH_4^+
h			*µmol/g*		*mg/g*	*µmol/g*	*mM*
0	78.3	26.2	0.0	0.0	25.4	0.00	2.00
2	86.4	50.6	5.1	5.3	19.7	0.00	0.71
4	86.6	89.5	17.2	6.4	20.7	0.00	0.45
6	92.6	94.7	15.4	7.4	21.3	0.08	0.38
8	109.2	62.6	17.5	7.8	26.1	0.15	0.42

Table 4.3 Changes in total amino acids, protein, and medium ammonium following addition of 2 mM $^{15}NH_4Cl$ to N depleted protoplasts
[a] TCA extracted protoplasts.
[b] ^{15}N content of total protein was calculated fron the atom % excess values of TCA extracted protoplasts assuming an average protein content of 16% nitrogen.

The first 2 h following the addition of $^{15}NH_4Cl$ were also marked by a significant decrease in the total protein content of protoplasts, amounting to a decrease of 22% of the content at time zero. A loss that was re-established over the remainder of the experimental period. The appearence of ^{15}N in protein between 6-8 h illustrating that

freshly assimilated N is making an, albeit small, contribution to the increases in protein levels after 2 h.

The contributions of individual amino acids from pre-existing pools to this overall increase in amino acids (data not shown) were proportional to their intial concentrations. Thus, Gaba gave the largest increase, followed by glutamine, glutamate and alanine. Vacuolar contents of all amino acids increased more than the corresponding cytoplasmic content relative to their intial levels.

Incorporation of ^{15}N-labelled amino acids in each fraction increased steadily between 0-4h to a maximum where approximately 65% of the total label was cytoplasmic. The quantity of ^{15}N present in amino acids after 4 h was equivalent to that supplied intially as $^{15}NH_4Cl$ (25 μmol/g); consequently assimilation may be regarded as being complete by this time. ^{15}N accumulation above 2 μmol/g was restricted to the four most abundant amino acids; Gaba, glutamine, glutamate and alanine. Eight amino acids (gly, leucine, isoleucine, aspartate. arginine, phenylalanine, serine and asparagine) labelled to less than 1 μmol/g, whilst no accumulation of ^{15}N-labelled valine, histidine, threonine, methionine, lysine or tyrosine was detected by mass-spectrometry. The changes in total and ^{15}N-labelled pool sizes together with atom % excess values are given in Figures 4.1-4.4 for detailed consideration of the accumulation and partition kinetics of, glutamine, glutamate, alanine and Gaba respectively.

Figure 4.1a shows that glutamine increased in both compartments during the course of the experiment, with a substantial efflux of glutamine from the vacuole between 6-8 h. Figure 4.1b and c show that the first enrichment of glutamine appeared after 2 h in the vacuolar pool and marked the vacuolar accretion of freshly synthesised glutamine. By the end of the experimental period 60% of the vacuolar ^{15}N was present as glutamine. In contrast there was little enrichment and accumulation of the glutamine in the

cytoplasm; after 8 h, freshly synthesised glutamine represented only 5% of the total of freshly synthesised amino acids in the cytoplasm.

Figure 4.2a shows a decrease in the cytoplasmic glutamate pool over the first 4h of the experiment suggesting either a high metabolic demand or transfer to the vacuole, since there is a pronounced increase (7 fold) in vacuolar glutamate. The initial response is reversed between 4-8 h when cytoplasmic glutamate increases sharply. Figure 4.2c illustrates a striking difference between the accumulation of freshly synthesised glutamate and glutamine, in that newly synthesised glutamate does not accumulate in the vacuole, constituting less than 6% of the non-glutamine vacuolar label after 2h, and less than 1% after 8 h. Figure 4.2b shows an intial labelling of the vacuolar pool but ^{15}N abundance progressively declines throughout the period of the experiment.

Figure 4.1

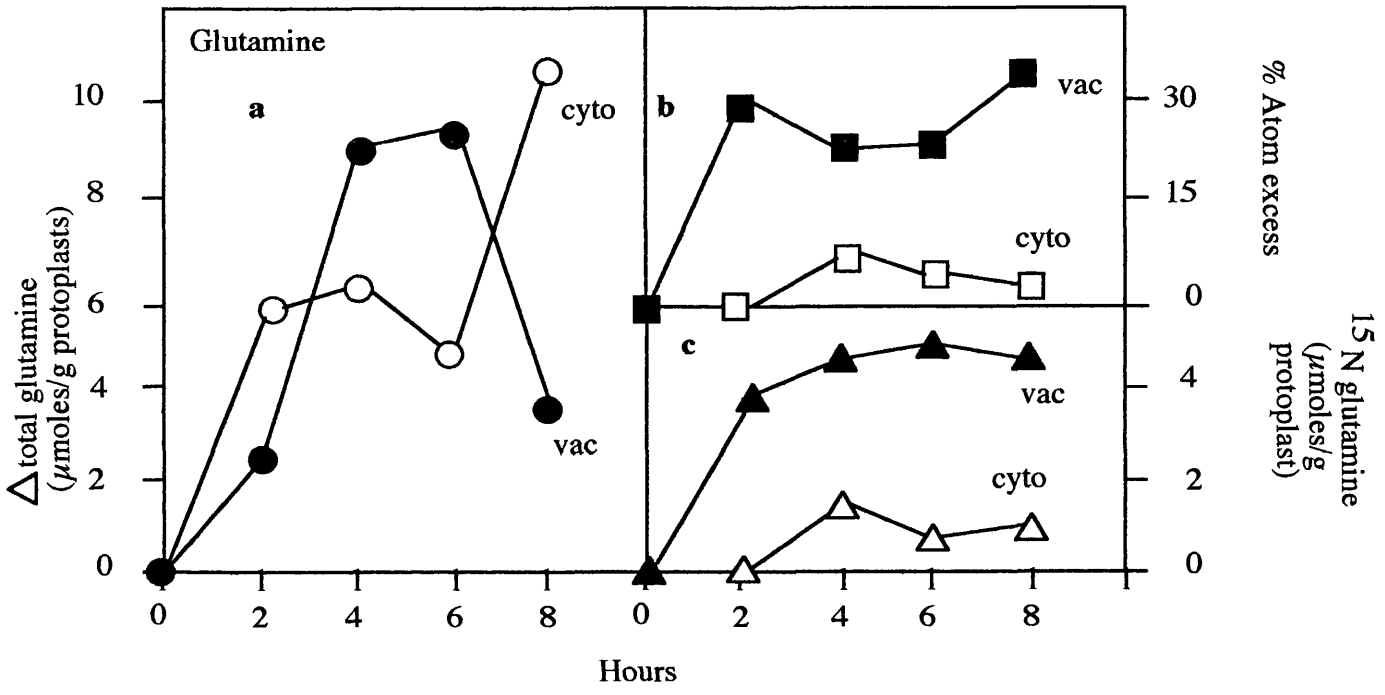

Figure 4.1 The changes in total, ^{15}N-labelled glutamine and its percentage ^{15}N enrichment in both cytoplasmic and vacuolar fractions, at 2 h intervals following the addition of 2 mM $^{15}NH_4Cl$. Figure 4.1a shows the changes in total ($^{15}N + ^{14}N$) glutamine relative to the quantity at time zero.

Figure 4.1b shows the % enrichment of glutamine with ^{15}N and Figure 4.1c the increases in ^{15}N labelled glutamate.

Figure 4.2

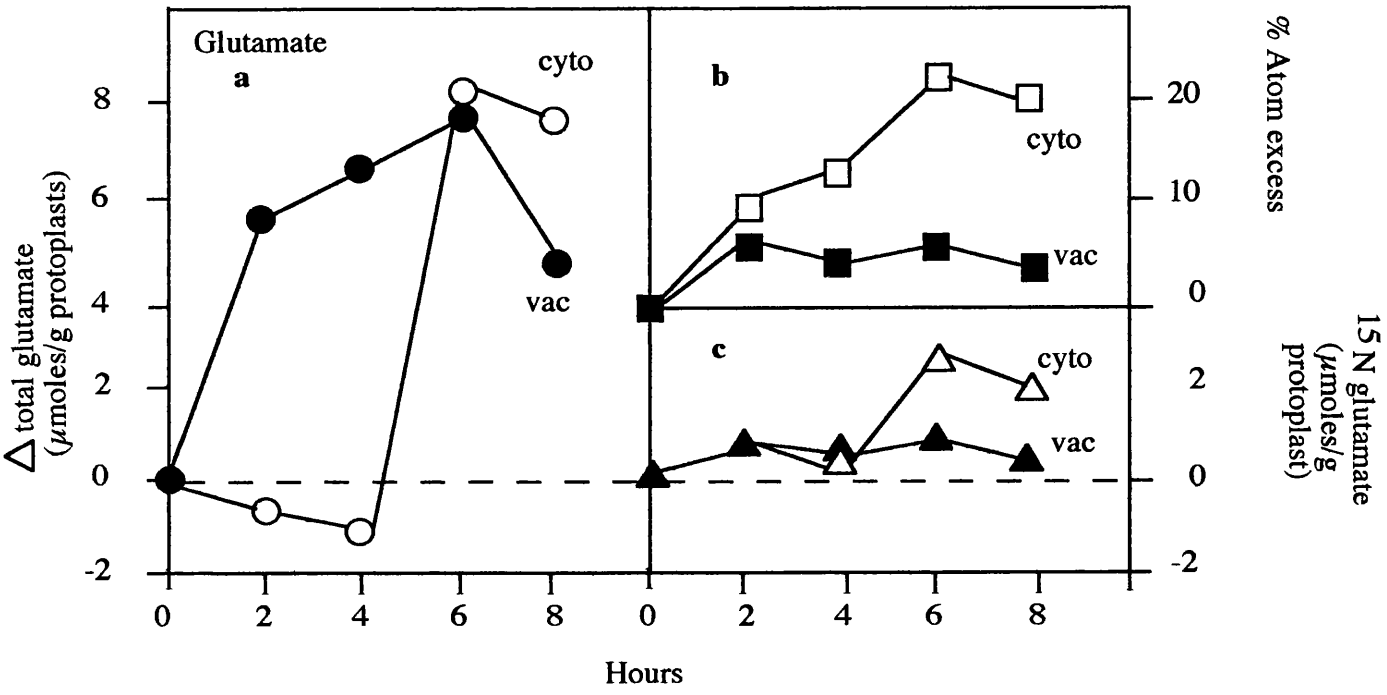

Figure 4.2 The changes in total, ^{15}N labelled glutamate and its percentage ^{15}N enrichment in both cytoplasmic and vacuolar fractions, at 2 h intervals following the addition of 2 mM $^{15}NH_4Cl$. Figure 4.2a shows the changes in total ($^{15}N + ^{14}N$) glutamate relative to the quantitity at time zero. Figure 4.2b shows the % enrichment of glutamate with ^{15}N and Figure 4.2c the increases in ^{15}N labelled glutamate.

Figure 4.3a suggests a substantial movement of alanine from the cytoplasm to the vacuole takes place over the first 6 h, followed by a reversal of this trend to 8 h. The high level of enrichment of vacuolar alanine, rising sharply with time (Figure 4.3b), supports this suggestion, although the accumulation of ^{15}N-labelled alanine in either compartment is small (Figure 4.3c), presumably due to onward metabolism.

Gaba is the most abundant amino acid species in protoplasts (Table 4.1), and shows dramatic changes in level and distribution involving both total and newly-synthesised species. Large increases in total Gaba occurred up to 4 h in both vacuole and cytoplasm (Fig 4.7a). In a striking difference between the compartments, 80% of the increase in total Gaba could be attributed to newly-synthesised molecules, whereas the vacuolar pool showed no enrichment or accumulation at all (Figure 4.4b and c). The enrichment kinetics of this massive cytoplasmic pool (Figure 4.4b) reach a maximum after 4 h; this observation is consistent with the depletion of the $^{15}NH_4^+$ pulse after 4h. ^{15}N-labelling patterns show that there is little interchange between the cytosol and vacuole (Figure 4.4c) and that there is a total exclusion of freshly synthesised Gaba from the vacuole. Gaba clearly represents a major sink for ^{15}N supplied as ammonium. After 4 h, over 50% of the total ^{15}N supplied at time zero was present in Gaba.

Of the remaining amino acids, asparagine, serine, glycine and to a lesser extent arginine, isoleucine, and aspartate all show changes in distribution of total compounds similar to that of alanine, although changes were seldom greater than $\pm$ 1.5 μmol/g. Enrichment with ^{15}N was minimal, at the limit of detection of the mass spectrometer.

Figure 4.3

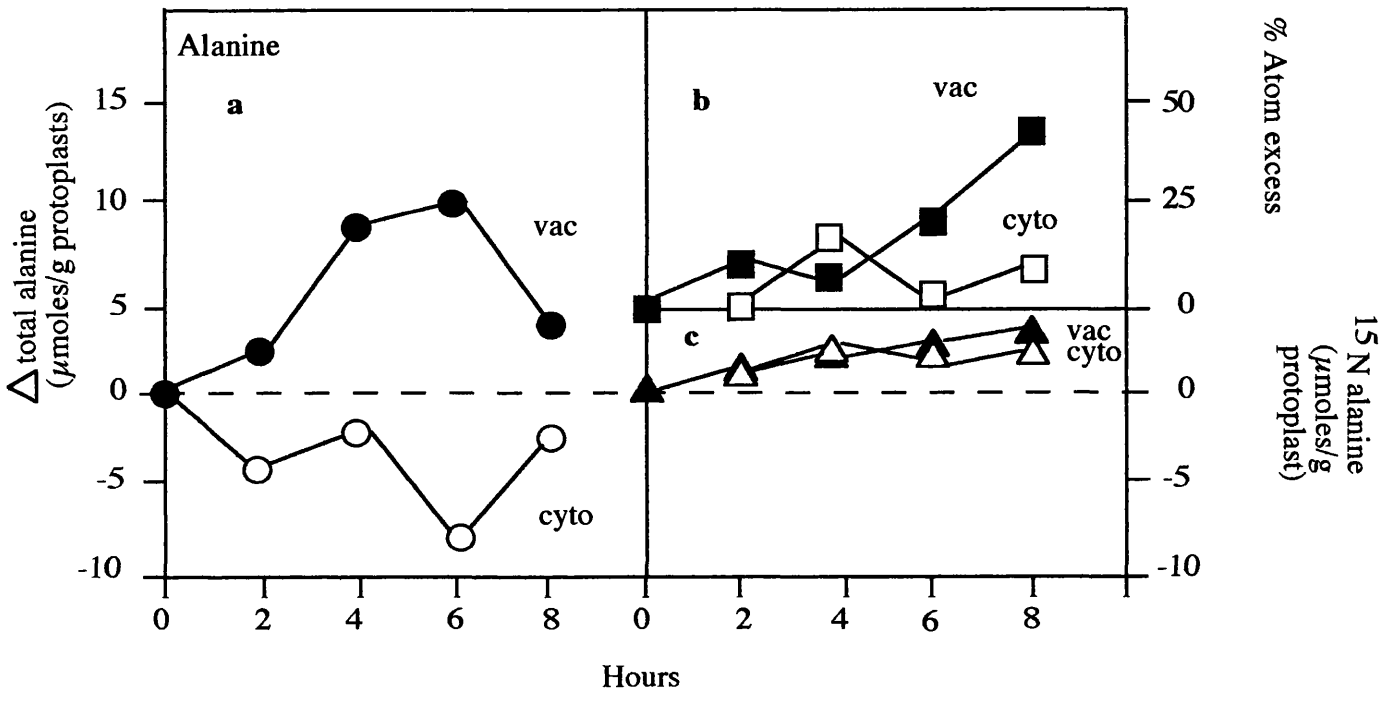

Figure 4.3 The changes in total, ^{15}N labelled alanine and its percentage ^{15}N enrichment in both cytoplasmic and vacuolar fractions, at 2 h intervals following the addition of 2 mM $^{15}NH_4Cl$. Figure 4.3a shows the changes in total ($^{15}N + ^{14}N$) alanine relative to the concentration in each fraction at time zero. Figure 4.3b shows the % enrichment of alanine with ^{15}N and Figure 4.3c the increases in ^{15}N labelled alanine.

Figure 4.4

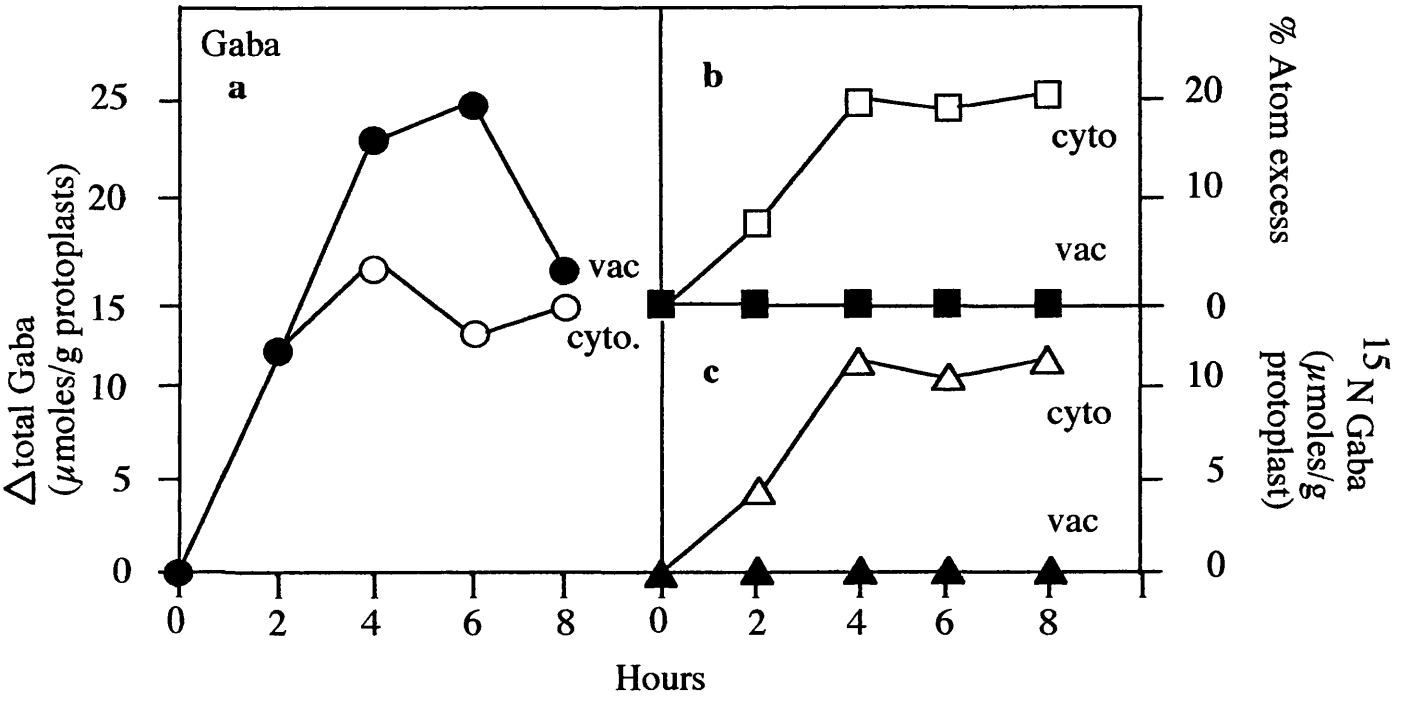

Figure 4.4 Shows the changes in total, ^{15}N labelled Gaba and its percentage ^{15}N enrichment in both cytoplasmic and vacuolar fractions, at 2 h intervals following the addition of 2 mM $^{15}NH_4Cl$. Figure 4.4a shows the changes in total (^{15}N + ^{14}N) glutamate relative to the concentration in each fraction at time zero. Figure 4.4b shows the % enrichment of Gaba with ^{15}N and Figure 4.4c the increases in ^{15}N labelled Gaba.

Attempts were made to determine labelling patterns in similar experiments in which ^{15}N was supplied as $K^{15}NO_3$ rather than NH_4Cl. However, in several experiments of this kind, reliable data on ^{15}N-labelling could not be obtained, owing to very low and non-reproducible ^{15}N-enrichments. All indications were that the assimilation of nitrogen was considerably slower from NO_3^- than from NH_4^+. In the majority of experiments label only began to appear in amino acids some 12 h after the addition of the $K^{15}NO_3$. Under the conditions employed, protoplasts became senescent after this time, and reproducible results could not be obtained. Difficulties in feeding experiments with nitrate as the source of nitrogen were also encountered in studies with *in vivo* NMR (see Chapter 5).

In a second series of experiments vacuole, mitochondria, and plastid fractions were isolated 4 hours after addition of 2mM $^{15}NH_4Cl$ to protoplast suspensions and the enrichment of their amino acid pools determined. Table 4.4 shows the concentrations of total and ^{15}N-labelled amino acids; only amino acid that were consistently enriched in all experiments are included. The patterns of distribution of total amino acids were similar to those described previously (Tables 4.1.-4.2), in that compounds were primarily cytosolic or vacuolar (according to species) with smaller amounts in mitochondria and traces only in plastids. The partitioning of newly-synthesised compounds however shows sharp differences from this pattern. Most remarkably, 80% of the vacuolar glutamine was labelled while other amino acids were either very lightly labelled (alanine) or unlabelled. Again, cytosolic Gaba was the main sink for ^{15}N, newly-synthesised glutamate or alanine were not accumulated to any great extent. The most striking observation is the very high percentage of labelled glutamine, and, to a lesser extent, alanine within plastids. Within mitochondria, high proportions of newly-synthesised methionine and phenylalanine, and to a lesser extent, glutamate were observed,

Table 4.4

amino acid	distribution			
	vacuole	mitochondria	plastid	cytosol
		µmol/g		
Ala				
total	7.4	0.70	0.04	28.4
^{15}N	0.2	0.03	0.02	2.1
Gln				
total	26.4	0.42	0.12	13.5
^{15}N	21.1	0.00	0.10	5.3
Glu				
total	11.0	0.90	0.02	14.2
^{15}N	0.0	0.24	0.00	1.9
Gaba				
total	17.4	0.60	0.01	39.4
^{15}N	0.0	0.01	0.00	27.6
Met				
total	1.7	0.4	0.01	4.8
^{15}N	0.0	0.3	0.00	0.0
Phe				
total	4.1	0.2	0.01	2.9
^{15}N	0.0	0.1	0.0	1.4

Table 4.4. The concentration of total and ^{15}N labelled amino acids in vacuolar, mitochondrial, plastidial and cytosolic fractions from protoplasts incubated in 2 mM $^{15}NH_4Cl$ for 4h. The values given are representative of least five separate data sets.

although glutamine was not enriched at all. The implications of these observations in terms of the sites of ammonium assimilation will be discussed later.

Glutamine synthesised in the presence of $^{15}NH_4^+$ may become labelled with ^{15}N in either the *amino* position, the *amido* position, or both. Since assimilation in carrot cells has been shown to be exclusively through GS (Robinson *et al.* 1991), labelling will occur first in the amido position. Following the formation of labelled glutamate through the GOGAT reaction, a second ^{15}N may be transfered to the amino position, producing a glutamine molecule labelled in both positions; this scheme is illustrated by Figure 4 5. The use of t.BDMS derivatives for GC-MS employed in this study permits the proportion of singly- (amino or amido) and doubly- (amino and amido) labelled glutamine to be determined (Rhodes *et al.*, 1980).

Figure 4.5

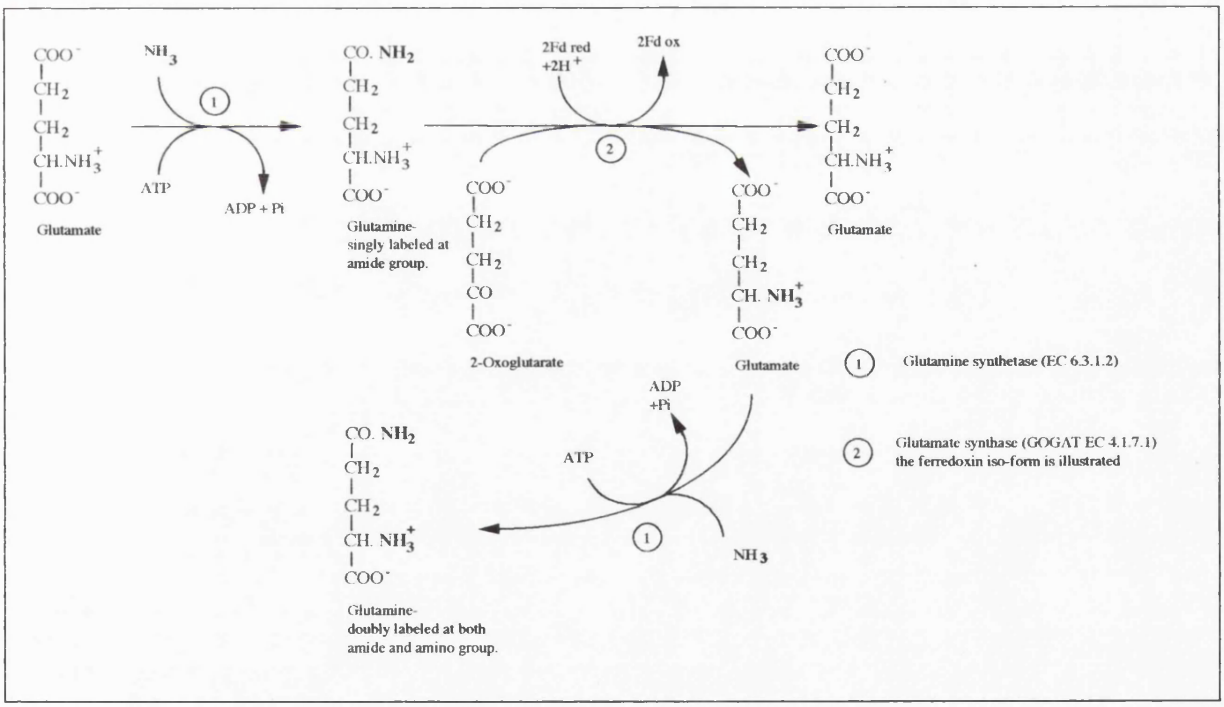

Figure 4.5 The incorporation of ^{15}N label into the *amino* group of glutamate and into the amide group, and the *amide* and *amino* groups of glutamine.

The ratios of doubly:singly labelled glutamine are shown in Table 4.5. In time-course experiments, vacuolar glutamine was predominantly doubly labelled throughout the 8-

hour period. Extravacuolar glutamine, on the other hand, was mainly doubly labelled after 2 hours, but the proportion of singly labelled species increased steadily until after 8 hours only 6% of molecules were doubly labelled. The simplest interpretation is that doubly labelled glutamine was stored in the vacuole in the early part of the experiment as a metabolically isolated pool. Extravacuolar glutamine also became doubly labelled as expected during the early period when $^{15}NH_4^+$ was abundant, but after the depletion of the $^{15}NH_4Cl$ pulse, continued synthesis occurred in the presence of predominantly $^{14}NH_4^+$, leading to the observed increase in single-labelled moiety. Whatever the mechanism, the data clearly shows the strong functional separation between the two compartments in terms of glutamine metabolism. Within the plastid, only 8% of glutamine is double-labelled after 4 hours, despite the very high labelling percentage of 83% (Table 4.4). Plastids thus resemble the extravacuolar situation, but with more rapid loss of double-labelled species.

Table 4.5

compartment	D:S ratio hours after addition of $^{15}NH_4Cl$			
	2	4	6	8
vacuole	14.30	5.04	2.50	11.14
extravacuolar	3.10	0.20	0.10	0.06
plastid		0.08		

Table 4.5 The ratio of doubly (*amino* and *amido*) to singly (*amino* or *amido*) labelled glutamine recovered in vacuolar, extravacuolar and plastidial fractions of carrot protoplasts at 2h intervals after the addition of 2 mM $^{15}NH_4Cl$.

4.4 Discussion

4.4.1 Amino acid distribution in cells and protoplasts

The amino acid content of protoplasts was similar to that of the cells from which they were derived, although concentrations were about 3 times higher in protoplasts due to osmotic shrinkage. Most major amino acids increased in proportion to their cellular concentrations except alanine, which was above, and glutamate, below, the average increase. These differences could be explained by a shift in the equilibrium position of alanine aminotransferase, although other explanations are possible. Gaba, a non-protein amino acid, makes the largest contribution to amino acid pools in cells and protoplasts. Although Gaba accumulation has been reported in response to stress conditions, (Steward *et al.*, 1958, Kishinmani and Windholm, 1987), it is known to be the most abundant amino acid in carrot cell suspensions at all stages of growth (Robinson *et al.*, 1991).

The amino acids involved in nitrogen assimilation differed markedly in their cellular distributions, glutamine being predominantly vacuolar and glutamate cytosolic. These findings are compatible with previous reports on the distribution of glutamate and glutamine. In sweet clover, 22% of the glutamate pool was shown to be vacuolar (Boudet *et al.*, 1981), while in *Lemna* fronds glutamine was inferred to be largely vacuolar and glutamate largely cytosolic (Goodchild and Sims, 1990). In barley mesophyll protoplasts, glutamate was excluded from the vacuoles while most other amino acids were present (Dietz *et al.*, 1990). In the yeast *Saccharomyces cerevisiae* 38% of the total glutamate was vacuolar whilst 85% of the total glutamine and asparagine was restricted to the vacuole (Messenguy *et al.*, 1980).

Other amino acids, particularly valine, and to a lesser extent phenylalanine and asparagine, were mainly present in the vacuole, and at concentrations considerably greater than those observed in the cytosol. Asami *et al.* (1985) noted that up to 95% of free cellular phenylalanine was found in the vacuole of spinach leaf protoplasts. These

findings may reflect the sequestering of aromatic amino acids at a locus removed from their site of synthesis; a process which has been discussed with reference to the chloroplasts by Schulze-Siebert *et al.* (1987).

Asparagine, like glutamine, functions as a nitrogen storage compound (Lea and Miflin, 1980, Sieciechowicz *et al.*, 1988). Although less abundant in carrot protoplasts than glutamine, it is interesting to note that it also is strongly localised in the vacuole, and to a greater extent than any other amino acid. Asparagine synthetase has been detected in the cytosolic fraction of root nodules (Shelp and Atkins, 1984), and Rhodes *et al.* (1987) proposed, from kinetic data, that up to 10% of free asparagine in *Lemna minor* was metabolically inactive and therefore thought to be vacuolar. The much higher proportions of both glutamine and asparagine in carrot protoplasts suggests an important nitrogen storage role for vacuoles in this species at least.

4.4.2 $^{15}NH_4Cl$ feeding studies: effects on overall nitrogen metabolism

Before considering the distributions of individual newly-synthesised amino acids, the effect of supplying ammonium chloride to nitrogen-limited protoplasts will be discussed in order to establish the overall metabolic background to these studies. The dominant metabolic feature during the first 6h at least was a massive increase in total amino acid pools, particularly in the vacuole (where totals increased over 350%), but also in the cytoplasm. Newly-synthesised (^{15}N-labelled) amino acids accounted for only 25% of this increase, even though virtually all of the ^{15}N supplied at time zero, was incorporated into amino acids (Table 4.3). Two possibilities may explain the large increase in unlabelled amino acid pools; either carry-over of nitrate and ammonium, or protein catabolism. Analyses performed at time zero, revealed that the protoplast content of nitrate ion was 7.7 mmol/g and that of ammonium ion 0.2 mmol/g. Consequently, carry-over could account for less than 10% of the observed amino acid increase. On the other hand, total (TCA-insoluble) protein fell by 22% between 0 and 2 h and did not recover to its original level until 8 h (Table 4.3). This decrease in protein

is sufficient to account for the increased amino acids, assuming an average nitrogen content of protein of 16%. No incorporation of ^{15}N into TCA-insoluble protein fractions was detected by ANCA over the first 4 h, only an insignificant amount of protein synthesis occurred even after 8 h. These observations strongly suggest that the addition of labelled ammonium ions coincided with a period of protein catabolism, probably within the vacuole, leading to large accumulations of released amino acids.

It seems likely that these responses are due to cytosolic acidification caused by ammonium ions supplied as the sole form of nitrogen. During preliminary experiments with unbuffered suspensions of either cells or protoplasts, the pH of the culture medium fell by up to 2 units within 4h of the addition of NH_4Cl. The inclusion of MES in the incubation media stabilised the external pH, but may have only served to mask cytosolic acidification.

Kishinmani and Ojima, (1980) noted that responses consistent with the onset of cytosolic a[illegible]osis were induced when suspension cultures of rice cells were fed with ammonium as the only nitrogen source. Increased protease activity has been reported in response to cytosolic acidification (Peoples and Dalling, 1978), and the recognition of the vacuole as a discrete site of hydrolytic activity (see Matile, 1976) suggest that the large increases in vacuolar amino acids arise from proteolysis there. Canut *et al.* (1985) reported that the hydrolysis of intracellular proteins in vacuoles isolated from *Acer pseudoplatanus* cells is induced by a reduction in the pH of the external medium.

The observation that Gaba is the main sink for ^{15}N indicates high activity of the enzyme glutamate decarboxylase. Increases in glutamate decarboxylase activity have been reported in response to cytosolic acidification (Lane and Stiller, 1970; Streeter and Thompson, 1972, Davies, 1980,). The decarboxylation of glutamate to Gaba is a proton consuming reaction, which will tend to act as a metabolic buffer, thereby moderating cytosolic acidification. Reid *et al.* (1985) and Roberts *et al.* (1992) have

both reported that glutamate decarboxylation has a role in the regulation of cytoplasmic pH in maize root tips.

Taken together, the findings discussed above suggest the following metabolic scenario. The assimilation of ammonium ions leads rapidly to proton release and hence cytosolic acidification. Lowered pH stimulates protease activity leading to release of amino acids, primarily within the vacuole but later exported to the cytosol, and simultaneously stimulates glutamate decarboxylase activity. High rates of conversion of glutamate to Gaba tend to oppose acidification by proton consumption, so that Gaba becomes a major product of glutamate both labelled and unlabelled. Further evidence for these ideas is presented in the next chapter.

4.4.3 $^{15}NH_4Cl$ feeding studies: intracellular distribution patterns of individual amino acids

From previous studies it has been shown that in carrot cell suspension cultures, ammonium is assimilated exclusively through glutamine synthetase (GS) (Robinson *et al.*, 1991), consequently glutamine is the primary assimilate of nitrogen metabolism and glutamate its immediate product *via* the GOGAT cycle. Clear evidence was obtained or distinct differences in compartmentation of the pivotal amino acids glutamine and glutamate and the principle products of their continued metabolism. Considering firstly the vacuolar and extracuolar pools, the most striking obervation was the strong accumulation of newly fixed glutamine in the vacuole, compared with the restriction of glutamate and Gaba to the extravacuolar space. Alanine, the other amino acid which is highly enriched, was more evenly distributed. These findings point to characteristic and ordered separation of nitrogenous assimilate between the major cellular volumes. Vacuolar accumulation of glutamine was paralleled by that of asparagine, another reputed storage amino acid. However although over 80% of the cellular asparagine was vacuolar, ^{15}N enrichment was low, presumably due to the massive diversion of

assimilated nitrogen to Gaba, leaving little ^{15}N available for distribution through aminotransferase action.

Two lines of evidence indicate that functional separation as well as spatial separation of the pools exists. Firstly kinetic studies indicate relatively little interchange of freshly synthesised amino acids between pools except in the case of alanine. The observed pattern of enrichment, depletion and re-enrichment of the extra-vacuolar alanine pool suggests rapid turnover, whilst the vacuolar alanine pool shows kinetics characteristic of a slowly accumulating storage pool. The existence similar, metabolically distinct glutamine and alanine pools have been repotrted in *Lemna* Cooke *et al.* (1980), Rhodes *et al.* (1980, 1989a). Secondly the persistence of doubly labelled glutamine in the vacuole while extravacuolar glutamine becomes almost entirely singly labelled makes it very unlikely that these pools are in communication. An obvious interpretation is that the vacuole provides the location for a metabolically quiescent storage pool, while the metabolically labile cytoplasmic pool provides the amino N for glutamate, Gaba and other amino acids. A consequence of this pattern of distribution is that the extravacuolar glutamine is considerably less enriched with ^{15}N than its products glutamate and Gaba. This apparent paradox can be resolved if it is assumed that a sub-compartment of the extravacuolar glutamine pool exists that is relatively small but highly enriched. Such a pool could act as a precursor for glutamate and Gaba.

From the data on cytoplasmic distribution after 4 h of isotopic feeding, one clear candidate emerges, the glutamine content of plastids is small in comparision with other pools, yet is highly enriched to the extent that more than 80% of the toal is ^{15}N labelled (see Table 4.4). In contrast, mitochondrial glutamine was not detectably enriched, and bulk cytoplasmic enrichment was less than half that of the plastid pool. These observations suggest that the plastid may be the site of primary ammonium assimilation *via* the GS pathway. This is consistent with the observations that isoforms of higher plant GS are localised in the chloroplasts or plastids (McNally *et al.*, 1983), as well as

in the cytosol. Other studies indicate that GOGAT enzymes are also located in the plastids. Chen and Cullimore (1989) have shown that two isoforms of NADH glutamate synthase (GOGAT) are located in the plastids of *Phaseolus vulgaris* and the feredoxin dependent form of the enzyme is localised in the chloroplast stroma of tomato xylem parenchyma and epidermal cells (Botella *et al.*, 1988). There is however, no evidence of cytosolic isoforms of GOGAT.

From the above considerations, and the observation that glutamine in the plastids is mostly labelled in the single position, it is possible to propose a scheme for the primary assimilation of ammonium in carrot protoplasts. According to this scheme (represented in Figure 4.6), labelled ammonium is assimilated *via* GS in the plastid, producing amido-labelled glutamine . This is then recycled within the plastid *via* GOGAT to produce two glutamate molecules, one of which will be labelled. A high cytosolic demand for glutamate results in an effux of these products from the plastid, which in conjunction with a depletion of the supplied $^{15}NH_4Cl$ results in the plastidial glutamine pool being predominately singly labelled. The supply of glutamate to the cytosol must be controlled or be met by an influx of glutamate, so as to maintain the plastidial pool necessary for continued GS/GOGAT activity. Once in the cytosol this glutamate pool provides substrate for a number of competing processes; which (in order of decreasing competiveness as suggested by the kinetics of enrichment of their respective products) include decarboxylation to Gaba, amination to glutamine *via* cytosolic GS creating a doubly labelled glutamine pool bound for vacuolar accumulation, and for transamination reactions resulting in the dissemination of nitrogen into other amino acids and ultimately protein.

Figure 4.6

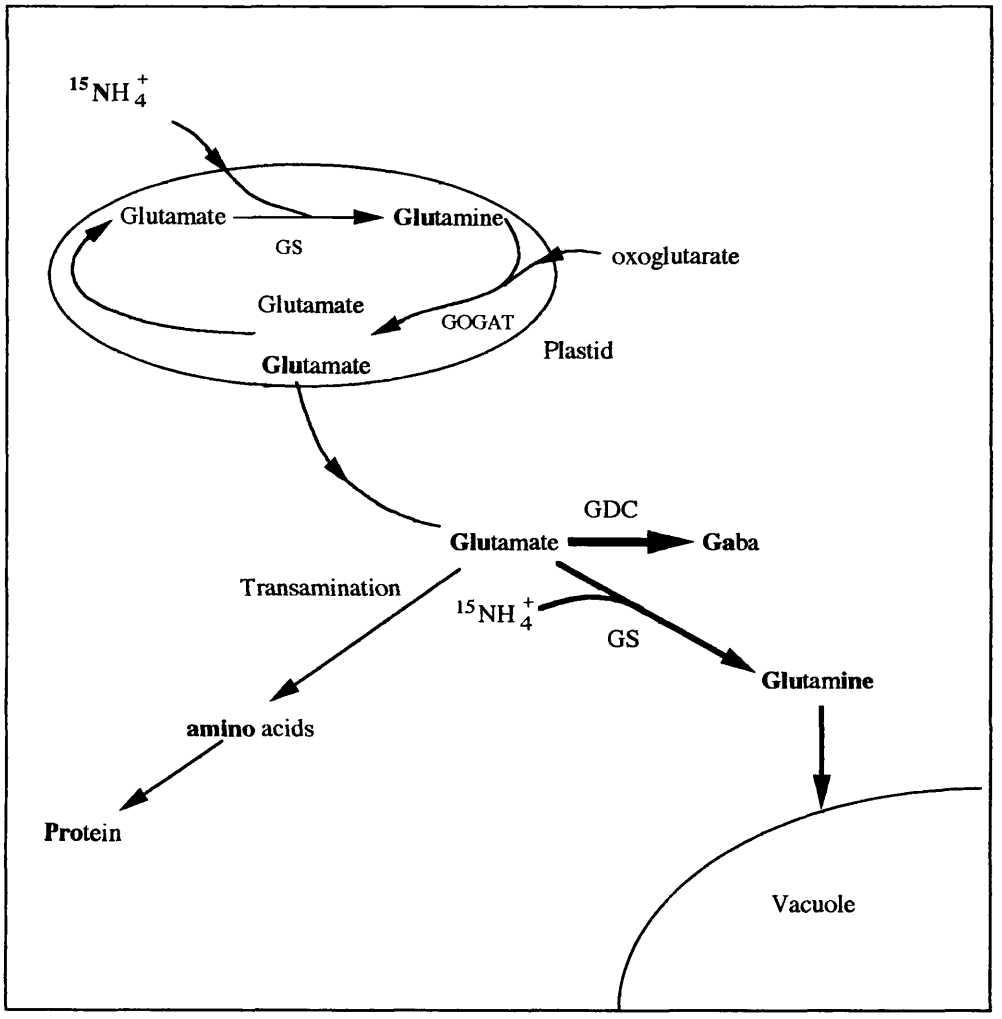

Figure 4.6 A proposed scheme for the compartmentation of the events of primary ammonium assimilation in carrot protoplasts.

Chapter 5 An Investigation into the metabolic consequences of ammonium assimilation in carrot protoplasts and cells using *in vivo* NMR techniques.

5.1 Introduction

The data presented in Chapter 4 show that when ammonium is supplied to N-depleted protoplasts as the sole nitrogen source a range of metabolic changes occur which are characteristic of responses to cytosolic acidification creating a specific metabolic background against which nitrogen is assimilated and partitioned. These changes included a pH drop in unbuffered suspensions, rapid proteolysis, and high rates of Gaba formation. This chapter describes more detailed investigations of these events using *in vivo* NMR techniques and determination of glutamate decarboxylase activity.

An important application of NMR techniques in the study of plant cell metabolism is the direct measurement of cytoplasmic and vacuolar pH (Loughman and Ratcliffe, 1984). Using NMR the resonances of the separate vacuolar and cytoplasmic pools of inorganic phosphate (P_i) in a high resolution ^{31}P NMR spectrum can be related to the pH of the compartments. This technique has been applied to monitor intercellular pH changes in a number of different systems including excised maize root tips (Roberts *et al* ., 1982, 1984, 1992) and in a variety of cell suspension systems including; *Acer pseudoplatanus* (Martin *et al* ., 1982), *Elaeis guineensis* (Hughes *et al* ., 1983), *Rosa damescena* (Murphy *et al* ., 1983) and *Daucus carota* (Fox and Ratcliffe, 1990).

The assimilation of ^{15}N-labelled ammonium by carrot cells and protoplasts was followed by broad band ^{1}H decoupled ^{15}N NMR spectroscopy using the air-lift system described by Fox *et al.* (1989). As the ^{15}N nuclei in amino acids are bonded to ^{1}H nuclei there is an interaction known as coupling which splits the intensity of the resonance producing more than one signal. Since a high level of background noise is unavoidable in *in vivo* systems this makes the ^{15}N signal difficult to detect. Thus to

counter the coupling interaction, ^{1}H decoupling is employed in which the ^{15}N signal is not split and therefore easier to detect. When decoupling is employed there is an enhancement of the signal known as the nuclear Overhauser enhancement (NOE). The NOE factor varies for each individual molecule depending upon the interaction with other molecules. The use of NMR spectroscopy to follow the incorporation of ^{15}N into plant tissues has been reported by Loughman and Ratcliffe (1984), Belton *et al*. (1985), Thorpe *et al.* (1989), Robinson *et al.* (1991) and Fox *et al.* (1992); all these studies emphasise the value of NMR as an analytical tool.

5.2 Materials and methods

5.2.1 Preparation of protoplasts and cells for *in vivo* NMR

Protoplasts were prepared as described in Chapter 2, cells from stationary-phase cultures were harvested by gentle centrifugation (500 *g* 10 min) and both cells and protoplasts were resuspended in fresh medium to give a total volume of 25 mL with a packed cell volume of 50%. The suspensions were transfered to a 20 mm diameter NMR tube and 0.015% (v/v) Antifoam A (Sigma, Dorset UK) added. The suspensions were oxygenated with an airlift system operating with either oxygen or air flow at a rate of 50 mL/min, and allowed to stabilise in the NMR tube for 3h before the acquisition of spectra. ^{15}N-labelled NH_4Cl or KNO_3 were added after the 3h incubation period to an intial external concentration of 20 mM.

5.2.2 ^{31}P and ^{15}N NMR spectroscopy

^{31}P and ^{15}N NMR spectra were recorded at 121.49 MHz and 30.42 MHz respectively on a Brucker CXP 300 spectrometer using a 20 mm diameter selective frequency (^{31}P) or a broad band frequency (^{15}N) probeheads. Tissue suspensions were oxygenated continuously with the air-lift system and the temperature was maintained at 25°C. ^{1}H decoupled ^{15}N NMR spectra were accumulated with a 90° pulse angle, a recycle time of 2 s, a total acquisistion of 30 min or 2 h, and low power decoupling for 1.75 s prior to acquisition to produce the nuclear Overhauser enhancement and high power

decoupling for 0.25 s during the acquisition. ^{31}P spectra were acquired over 30 min with ^{1}H decoupling, a 90° pulse angle, and a 0.8 s recycle time.

5.2.3 *In vitro* glutamate decarboxylase activity

The glutamate decarboxylase (GDC EC 4.1.1.15) activity of N-depleted protoplasts following the addition of 2 mM NH_4Cl was investigated as follows. A protoplast suspension of approx. 10^6/mL in osmotically balanced, buffered, N-free MDK was prepared as detailed in Chapters 2 and 4. NH_4Cl to 2 mM was added to the suspension and aliquots taken initially, and at 2 h intervals thereafter, and assayed for GDC activity. Samples were also taken and fixed in methanol for subsequent HPLC analysis.

GDC was determined *in vitro* by measurement of the release of CO_2 from glutamate. The assay mixture, contained 5 mM glutamate, 10 μM pyridoxal phosphate, 0.01% (v/v) Triton X-100, 0.25% (v/v) Antifoam A, 1 mM aminoxyacetate (AOA, an inhibitor of transamination reactions) and 100 mM K_2HPO_4/KH_2PO_4 at pH 5.8, in 1 mL. This reaction mixture was contained within a gastight side-arm test tube receiving CO_2 free air *via* an Analytical Development Co. (ADC) air supply unit at 250 mL/min. The tube was connected *via* the side arm to an ADC LA2 portable infra red gas analyser (IRGA), and the evolution of CO_2 intiated by the addition of 100 μL of protoplast suspension (20-50 μg soluble protein) *via* a needle and syringe piercing the bung. Steady state CO_2 evolution was measured against a CO_2 free reference. The reaction proved to be linear with respect to time and enzyme concentration.

5.3 Results

5.3.1 ^{15}N-NMR spectroscopy.

^{15}N-NMR spectroscopy was used to follow nitrogen assimilation into amino acids *In vivo* in protoplasts and cultured cells. Two levels of oxygenation were provided, by air-lifting suspensions with pure oxygen (oxygenation) or with air (aeration).

The ^{15}N-NMR spectra obtained from oxygenated protoplasts in the presence of 20 mM $^{15}NH_4Cl$, over a 20 h period are shown in Figure 5.1 (a-e). The label from the exogenous ammonium first appeared in the amide N of glutamine within 30 mins of the addition of $^{15}NH_4Cl$ (Figure 5.1a). This pool steadily increased over an 8 h period, and then declined. Label was observed in the amino-N of glutamate during the second hour of the experiment (Figure 5.1b), and subsequent changes in the size of the pool of labelled glutamate paralleled that of glutamine. The peak corresponding to the resonance of $^{15}NH_4^+$ declined over the first 4 h of isotopic labelling. It should be noted that ^{1}H decoupled ^{15}N-NMR does not allow the extracellular $^{15}NH_4^+$ signal to be distinguished from the intracellular signal. However, since the decline in $^{15}NH_4^+$ is consistent with the dissemination of ^{15}N into amino acids it would be reasonable to assume that the pattern of decline shown by the $^{15}NH_4^+$ signal is consistent with the pool being predominately extracellular. On repeating this experiment , using cells instead of protoplasts, the spectral changes observed over 18 h of isotopic labelling are shown in Figure 5.2a-e. The patterns of incorporation of ^{15}N are very similar to those observed with protoplasts, with the exception that incorporation into Gaba was limited. When these experiments were repeated using 20 mM $^{15}KNO_3$ as with the GC-MS experiments (see Chapter 4) no labelling could be detected at all. Attempts were made to induce nitrate reductase activity by the addition of unlabelled KNO_3 prior to isotopic labelling, but to no avail. As concluded in Chapter 4 this lack of incorporation of ^{15}N can be attributed to a considerably slower rates of nitrate assimilation as opposed to the observed rates of ammonium assimilation.

Figure 5.1

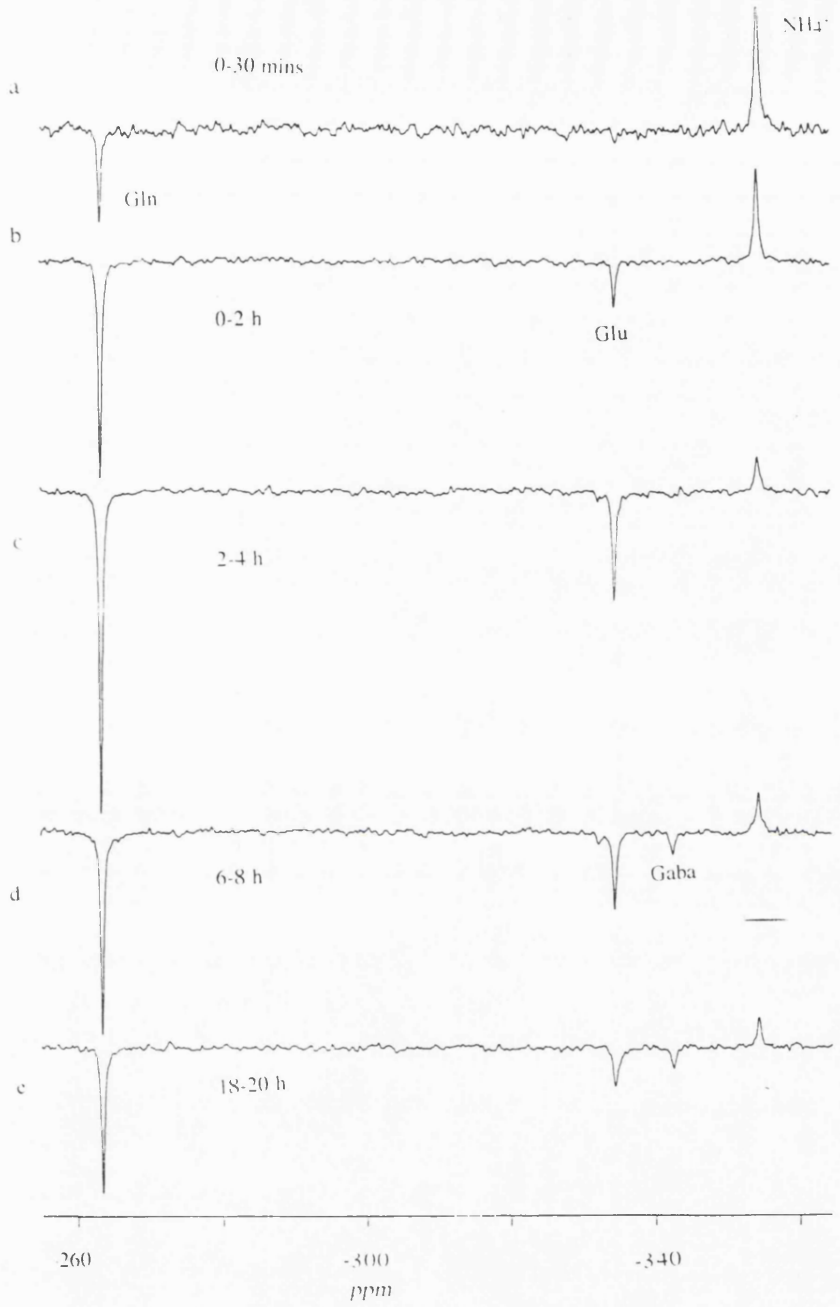

Figure 5.1 Changes in the ^{15}N-NMR spectra of oxygenated, N-depleted carrot protoplasts following the addition of 20 mM $^{15}NH_4Cl$.

Figure 5.2

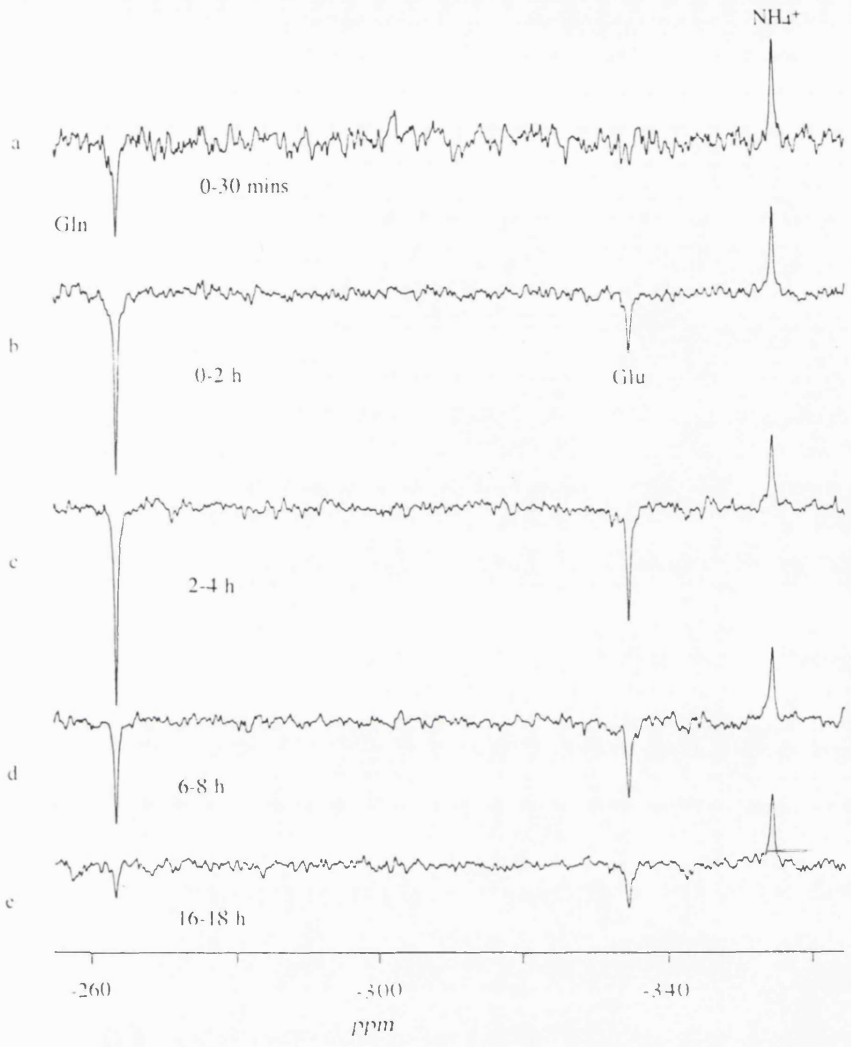

Figure 5.2 Changes in the ^{15}N-NMR spectra of oxygenated, N-depleted carrot cells following the addition of 20 mM $^{15}NH_4Cl$.

It has been observed that reducing the amount of oxygen available to plant tissues can result in cytosolic acidiosis (Lee and Ratcliffe, 1983, Roberts *et al* ., 1982). The effects on pathways of ^{15}N assimilation were studied in aerated protoplasts and cells, in which oxygen availablility to the assimilating tissues was limited by suppling the airlift system with air at 50 mL/min as opposed to oxygen. ^{15}N-NMR spectral changes of aerated carrot protoplasts following the addition of 20 mM $^{15}NH_4Cl$ are shown in Figure 5.3. The changes are broadly similar to those of oxygenated protoplasts (Figure

5.1) except that less accumulation of ^{15}N-glutamine was observed in aerated protoplasts, together with the appearence of ^{15}N-Gaba after 2-4 h.

Figure 5.3

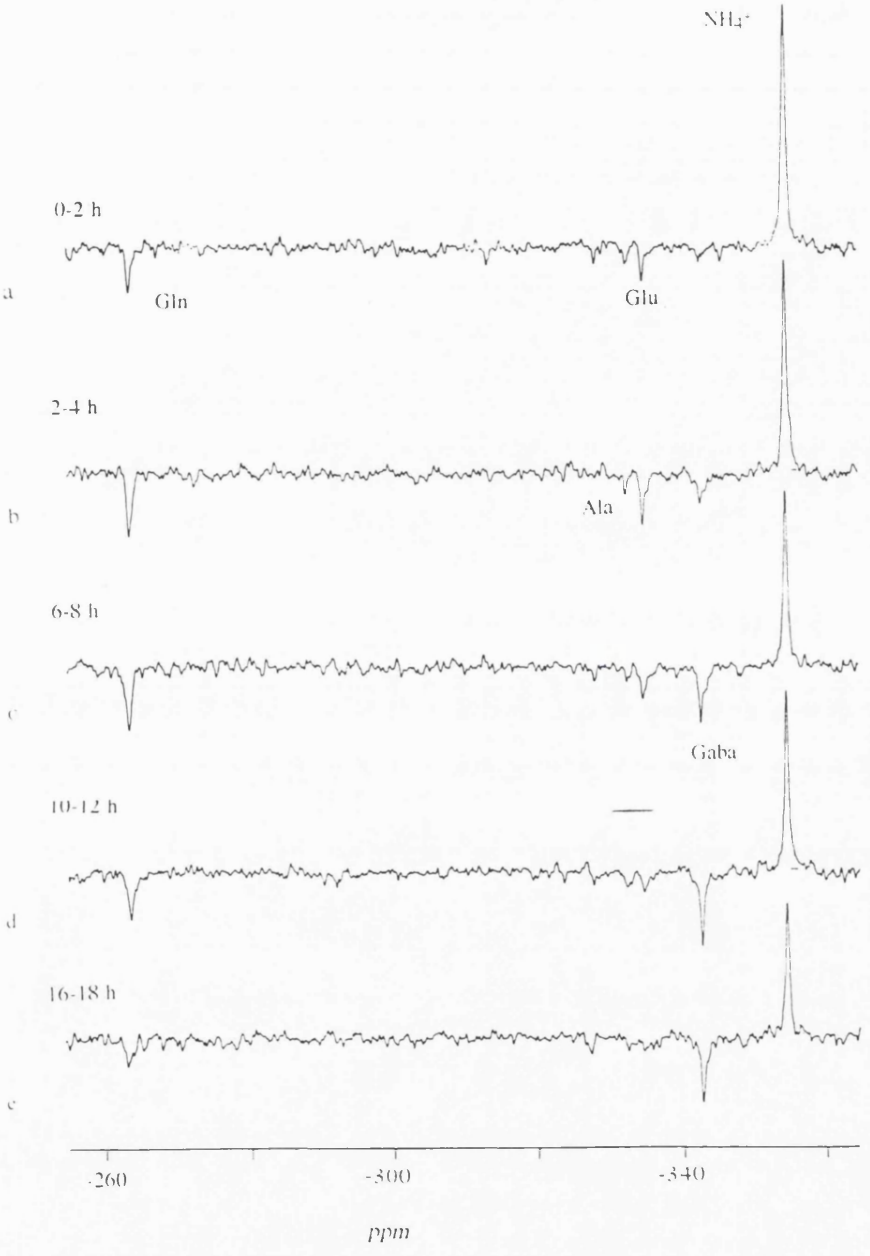

Figure 5.3 Changes in the ^{15}N-NMR spectra of aerated, N-depleted carrot protoplasts following the addition of 20 mM $^{15}NH_4Cl$.

When assimilation patterns in aerated cells (Figure 5.4) are likewise compared with those of oxygenated cells (Figure 5.2) a similar metabolic shift occurs to a more marked

degree. Aerated cells accumulate little ^{15}N-glutamine in comparision with oxygenated cells. Decreasing oxygen availability is associated with a shift from retention of supplied nitrogen as glutamine to its conversion and accumulation as glutamate, alanine and finally Gaba as the major sink. The metabolic patterns established by GC-MS (Chaper 4) are more similar to those obtained by *in vivo* NMR at the lower levels of oxygen availability.

Figure 5.4

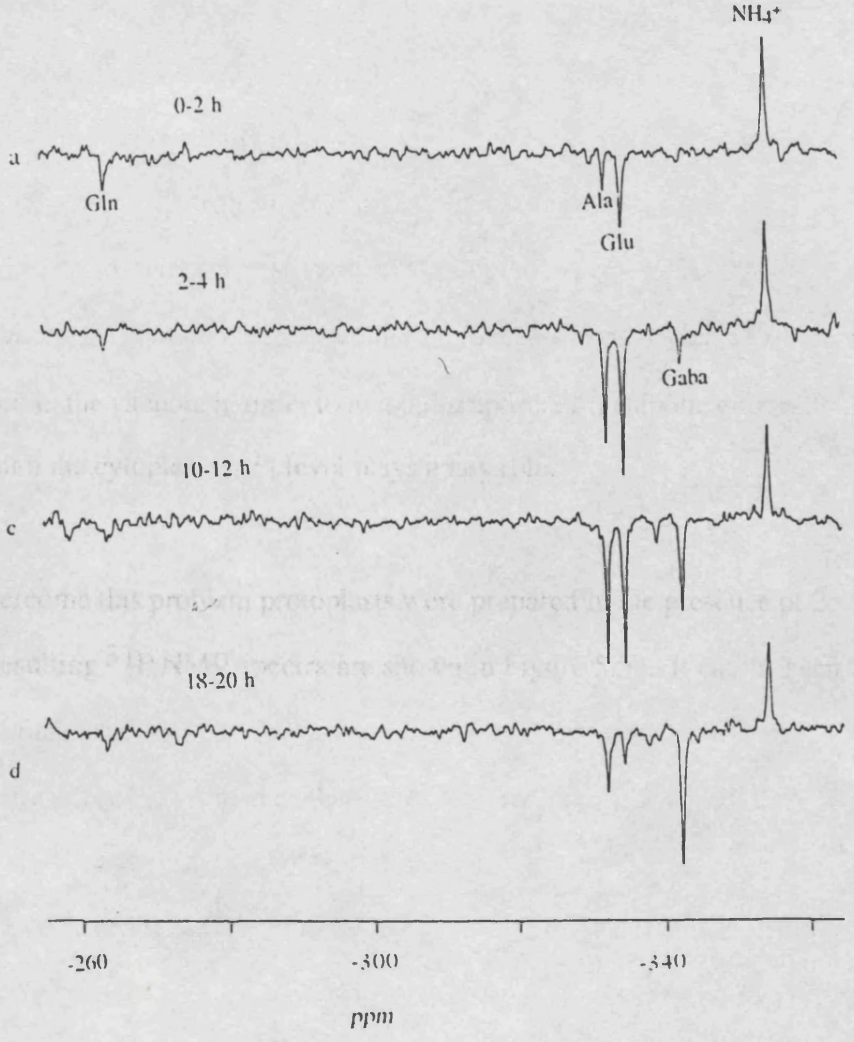

Figure 5.4 Changes in the ^{15}N-NMR spectra of aerated, N-depleted carrot cells following the addition of 20 mM $^{15}NH_4Cl$.

5.3.2 ^{31}P NMR spectroscopy

In vivo ^{31}P NMR was used to investigate changes in cytoplasmic pH of protoplasts and cells. A representative spectrum for oxygenated protoplasts is shown in Figure 5.5a. In all experiments it was observed that the vacuolar P_i resonance was so broad as to swamp species with resonances both upfield and downfield, such that no reliable inferences could be drawn as to the chemical shifts of the cytoplasmic P_i resonance. It was concluded that spectra of sufficient quality could not be obtained from standard protoplast suspensions. This was probably due to the absence of a cell wall, which greatly increases the influence of vacuolar resonance on the overall spectra. In addition it is likely that the protoplasting procedure led to a considerable increase in vacuolar P_i. The vacuole has been shown to be instrumental in the regulation of cytoplasmic P_i levels, by providing a site for temporary storage of P_i (Rebeille *et al.*, 1983). It is probable that protoplasting increases the intracellular P_i concentration to such an extent that it is accumulated in the vacuole in order to avoid disruption of metabolic energy transduction, in which the cytoplasmic P_i level plays a key role.

In an attempt to overcome this problem protoplasts were prepared in the presence of 2 mM EDTA. The resulting ^{31}P NMR spectra are shown in Figure 5.5b. It can be seen that the inclusion of the chelating agent improves the quality of the spectra considerably. Unfortunately EDTA also chelates the Ca^{2+} required to stabilise the plasmalemma,and in its presence the viability of protoplasts was drastically reduced. Consequently it was concluded that interpretable spectra could not be obtained with protoplasts, and the investigation of cytosolic pH was pursued using suspension cultured cells.

Figure 5.5

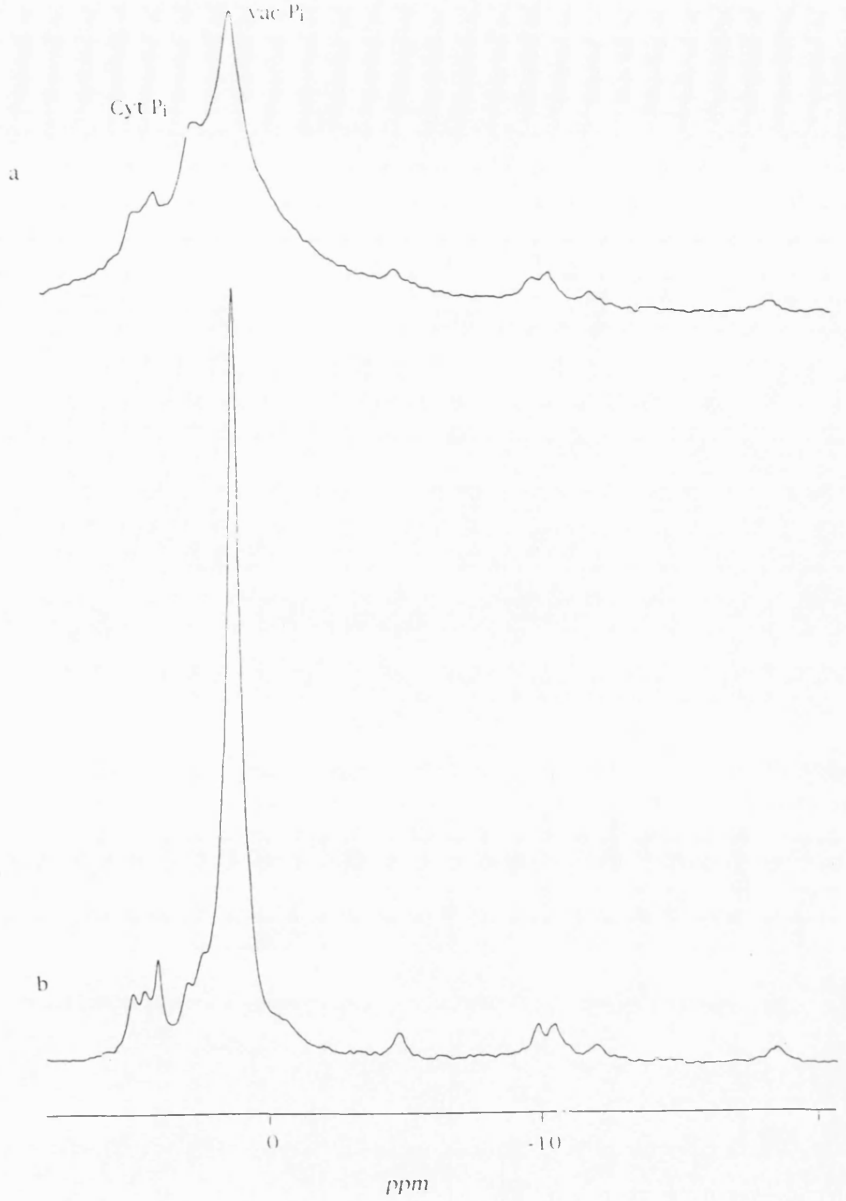

Figure 5.5 ^{31}P-NMR spectra of carrot protoplasts in a) the absence of EDTA, and b) the presence of EDTA

The changes, over 10 h, in the ^{31}P spectra of carrot cells exposed to 20 mM NH_4Cl are shown in Figure 5.6a-c.

Figure 5.6

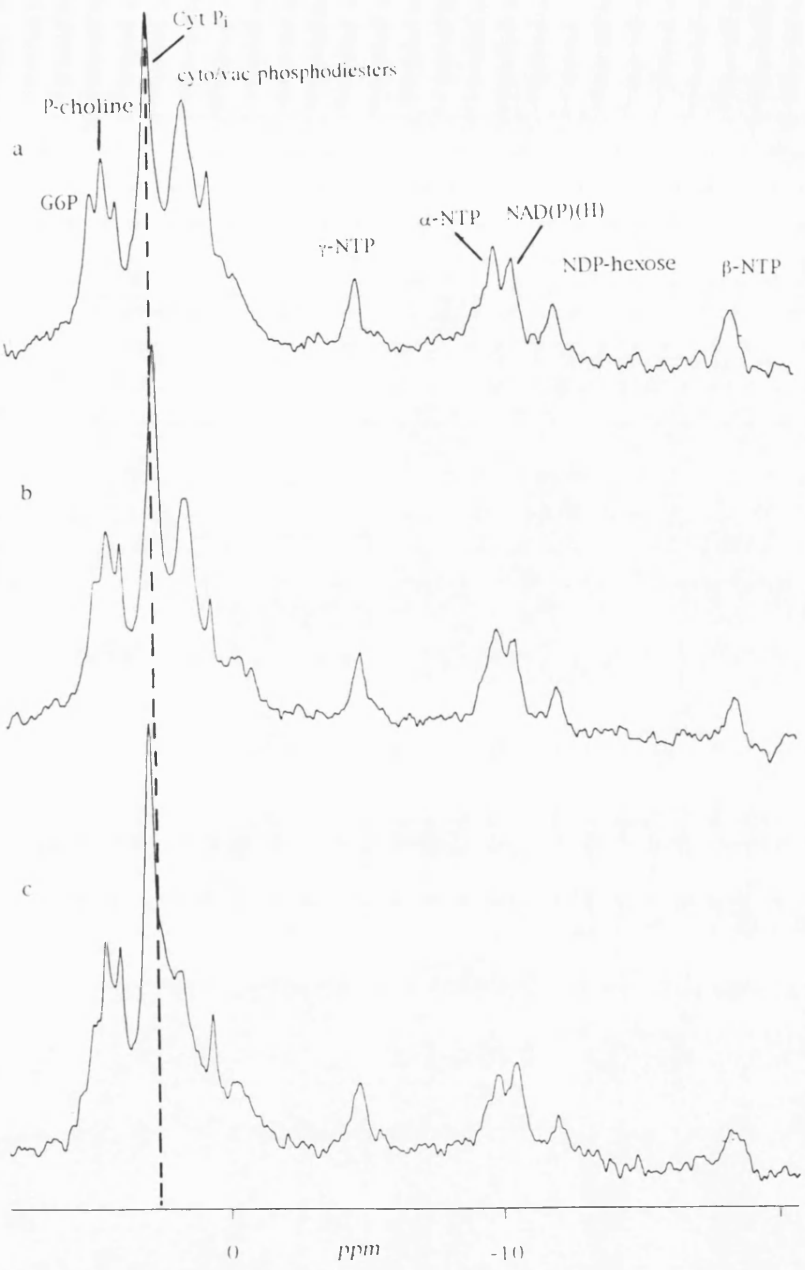

Figure 5.6 ^{31}P-NMR spectra of carrot cells; a) 0-30 min before b) 4-4 h 30 min after, and c) 9 h 30 min-10 h after the addition of 20 mM NH_4Cl . The changes in chemical shift of the cyt. P_i resonance peak relate to the changes in cyt. pH shown in Figure 5.5. The dotted line represents the chemical shift of cyt. P_i at pH 7.5.

Cytoplasmic pH changes were determined from the chemical shifts of cytoplasmic P_i resonances, using calibration curves obtained from P_i solutions that approximated to the ionic conditions in the cytoplasm (Martin *et al.*, 1982). These changes in

cytoplasmic pH as a function of time after the addition of NH_4Cl are given in Figure 5.7. The spectra show that the assimilation of ammonium by cells results in decrease in cytoplasmic pH of approximately 0.1 pH unit after 4 h 30 min.

Figure 5.7

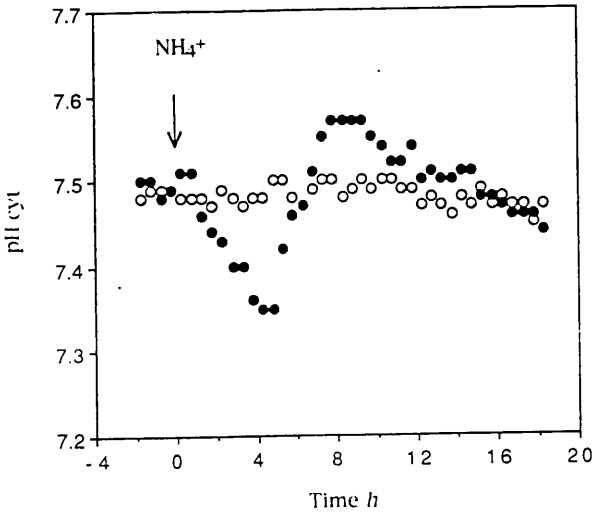

Figure 5.7 Changes in cytosolic pH, determined by ^{31}P-NMR spectroscopy, following the addition of 20 mM NH_4Cl to carrot cells. The open symbols are control measurements of cyt. pH of cells to which no ammonium as added.

This was followed by a gradual restoration of pH to its intial value 10 h after the addition of NH_4Cl. Examination of some of the other resonances suggests that the cells were metabolically viable during the course of the feeding (Figure 5.6a-c). The resonance of the γ-phosphate of NTP (principally ATP) remains constant throughout the experiment, as did the levels of NAD(P)(H) and NDP hexose, although the decreases were observed in the resonances of glucose-6-P, and cytosolic and vacuolar phosphodiesterases.

5.3.3 Glutamate decarboxylase activity

The effects of the addition of 2 mM NH_4Cl to N-depleted protoplasts upon extractable glutamate decarboxylase (GDC) activity and upon the levels of Gaba is shown in Figure 5.8. There is a significant increase in the activity of extractable GDC during the first six hours of feeding, followed by a decrease to approximately 50% of its original activity after 8 h. The timing of these changes correlate closely with the intial rapid increase in Gaba over the first 4 h to a maximum after 6 h. These data suggest that the induction of GDC by addition of ammonium is indeed related to the massive Gaba accumulation observed in the GC-MS experiments.

Figure 5.8

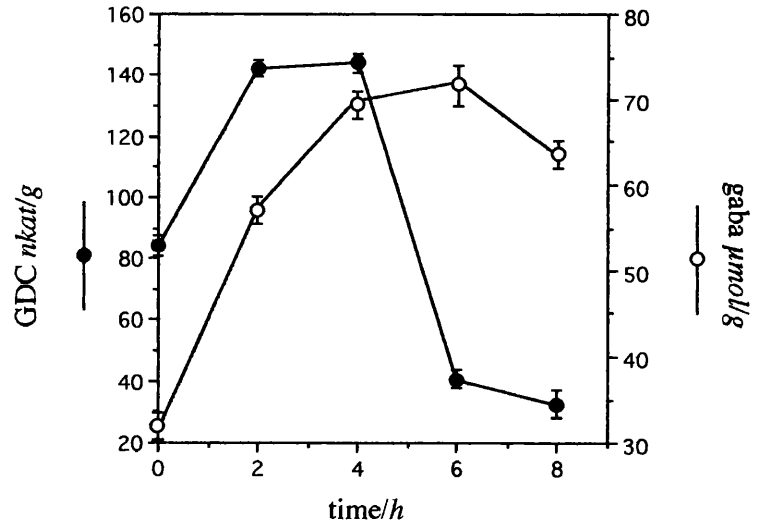

Figure 5.8 *In vitro* GDC activity and Gaba content of carrot protoplasts following the addition of 2 mM NH_4Cl. The specific activities and amino acid contents are means of three experiments +- SD

5.4 Discussion

In vivo ^{15}N NMR spectroscopy has considerable value as an analytical tool allowing the passage of exogenously applied ^{15}N, through amino acids to be followed. However it is not a particulary sensitive technique and detection of labelling is limited to those amino acids which are more abundant Little quantitative information can be obtained from ^{15}N-NMR spectra, and therefore the pathways of assimilation suggested by the spectra will always be viewed in the light of those data obtained from the GC-MS experiments described in Chapter 4.

The results illustrated in Figure 5.1 are consistent with GS/GOGAT being the primary route of ammonium assimilation in protoplasts. Comparison of the spectra obtained from protoplasts (Figure 5.1) and cells (Figure 5.2), illustrates that the principal difference in N assimilation by the two systems, is the incorporation of label into Gaba by protoplasts which is not shown by cells. As mentioned above this cannot be taken to mean that Gaba is not involved in the dissemination of assimilated N in carrot cells. Indeed incorporation of ^{15}N into Gaba by cells has been shown by GC-MS (Robinson *et al* ., 1991). These spectra collected from protoplasts do however confirm the conclusions of chapter 4, that Gaba is a major sink for freshly assimilated N in protoplasts when supplied with NH_4^+.

Streeter and Thompson (1972) provided the first indication that Gaba, arising from the decarboxylation of glutamate, is a characteristic stress metabolite, rather than an intermediate in the synthesis of glutamate from succinate, in many plant cells. The accumulation of Gaba in plant tissues has been noted in response to a variety of environmental perturbations including water stress, mineral deficiency, low temperature and anaerobis (reviewed by Narayan and Nair, 1990). The conclusion drawn from the GC-MS data discussed in Chapter 4 was that carrot protoplasts accumulate Gaba in response to cytosolic acidiosis brought about by the assimilation of NH_4^+.

Attempts were made to investigate cytosolic acidiosis in protoplasts by ^{31}P NMR, but as discussed above protoplasts did not lend themselves to this technique. Alternative approaches to cytosolic pH measurement could include; the use of labelled probes (Walker and Smith, 1975) or turgor resistant microelectrodes (Felle,1987). Given that cells showed very similar patterns of ^{15}N assimilation to protoplasts, the former were used to investigate cytosolic pH changes in response to NH_4^+ assimilation.

The cytoplasmic P_i resonance prior to the addition of NH_4^+ gave a chemical shift correponding to a pH of approximately pH 7.5. A high pH which is characteristic of well oxygenated plant tissues (Pfeffer *et al* ., 1986). The addition of ammonium to cells does result in temporary cytosolic acidiosis, the cells have an inherent capability to restore cytosolic pH, bringing the pH back to the level it was before the addition of NH_4^+.

It has been suggested that the intersection of the activities of two enzymes involved in the addition and removal of a carboxylate group can constitute a *pH stat* (Davies, 1986). The observed accumulation of Gaba and the fluctuations in ctyoplasmic pH upon ammonium assimilation, suggested an investigation of the responses of glutamate decarboxylase.

Changes in GDC activities were consistent with the timing of changes in cytosolic pH. In higher plants Gaba catabolism has been shown to occur via a shunt pathway. A reaction inititiated by the transfer of an amino group from Gaba to 2-oxoglutarate producing succinate semialdehyde (SSA), catalysed by 4-aminobutryate transferase (γ-ABT) and was first noted by Dixon and Fowden (1961). The final stage of the shunt, catalysed by succinate semialdehyde dehydrogenase (SSADH) results in a flux of carbon back into the TCA cycle, N back into glutamate, and the generation of one proton for each Gaba molecule transaminated. It is conceivable that cellular Gaba levels and cytosolic pH are regulated by an interplay between GDC and the γ-AB shunt. Such

a system would represent a *biochemical* pH stat responsible for the *fine* control of cytosolic pH, to contrast with the *biophysical* pH stats thought to be responsible for moderating major fluxes in cytosolic pH, predominately through the active pumping of ions.

Two possible explanations exist to account for the involvement of GDC in pH regulation; that the decline in cytoplasmic pH resulted in a loss of compartmentation of glutamate bringing metabolically inactive pools to GDC. It has been shown that freshly assimilated glutamate is predominately cytosolic in distribution. GDC is regarded as a soluble cytosolic enzyme (Streeter and Thompson, 1972, Narayan and Nair, 1990), and so this explanation seems less likely. Secondly, the decrease in cytosolic pH could be responsible for the activation of GDC, leading to proton consumption and Gaba accumulation. Certainly GDC has been shown to have an acidic pH optimum (Narayan and Nair, 1986) and this seems the most likely explanation. A drop in cytosolic pH has been shown to stimulate the activity of $NADP^+$ malic enzyme responsible for the decarboxylation of malate to pyruvate (Davies 1986) and was also thought to be involved in the regulation of cytosolic pH.

A comparison of the metabolic profiles of protoplasts suggested by the GC-MS experiments and the NMR spectra imply that the protoplasts used in the former were approaching a state of hypoxia. The protoplast suspensions were not oxygenated, but presented a much larger surface area for gaseous exhange than available in the NMR tube. The oxygen availibility to the protoplasts used in the GC-MS experiments could have been improved by decreasing the density of the incubating suspension. This was not carried out in practice due to the economic constraints imposed by the availability of ^{15}N-labelled nitrogen sources.

In protoplasts, to which oxygen availability is reduced (Figure 5.4), the continued assimilation of N from $NH4^+$ is pushed towards its *pH stat* function (the accumulation

of Gaba) rather than providing the precursors for transamination and protein synthesis. Reducing the oxygen tension within the system results in an initial transfer of N, when added as NH_4^+, *via* the glutamine amide to alanine and glutamate. Cytosolic acidification promotes the synthesis of glutamate and alanine (at the expense of glutamine) intially, but further metabolism of N is restricted by its diversion into Gaba. In similar experiments Roberts *et al.* (1992), have shown (by *in vivo* ^{13}C NMR) that pretreatment of maize root tips with ammonium prior to initiating hypoxia results in a transfer of carbon from glutamine to Gaba, an over-accumulation of which, was regarded as being indicative of cell death. Leading to the conclusion that the excessive diversion of nitrogen (and presumably carbon) into Gaba, can be avoided by establishing and maintaining a metabolically quiescent pool of these elements. The vacuolar accumulation of freshly synthesised glutamine (as observed by GC-MS) provides a likely candidate for this role. ^{15}N NMR spectra revealed that against a metabolic background of cytosolic acidiosis and recovery, a residual pool of glutamine was established. The spectra collected do not allow a subcellular location to be assigned to this pool (although techniques have been developed whereby the differential chemical shifts of ^{15}N-containing species in different pH environments, allow resonances to be designated as vacuolar or extravacuolar in origin). However it is possible to conclude from those data presented in Chapter 4, that this resonance is predominantly of a vacuolar origin. Hypoxia prevents such a glutamine pool becoming established or maintained.

Chapter 6 A General Discussion

The major events of higher plant metabolism have been elucidated, their intermediate stages determined, and relationships to one another proposed. Thus we have succeded in providing a qualitative description of ***what is***. However such descriptions cannot provide information on the metabolic responsiveness of such systems to perturbations in the plant cells external and internal environment. Without such quantitative information, questions such as: ***how much*** of a particular metabolite is produced under specific circumstances; or ***why*** a reaction proceeds at a different rate or produces a different end product remain largely unanswered.

Answers to some of the above questions can be provided by quantitative investigations into metabolic regulation. Metabolic pathways may be defined in terms of the kinetic and themodynamic properties of their constitutive enzymes. Therefore insights into their regulation can be obtained through studies of the phenomology of enzyme:substrate interactions. This is not the only regulatory mechanism worthy of investigation, as plant cells can exhibit a second level of control, afforded by the prevention of certain interactions and the encouragment of others through compartmentation of metabolites and enzymes.

The work presented in this thesis, provides an illustration of the role of compartmentation in the regulation of a variety of metabolic processes. It also provides and validates methods to facilitate the study of the size and distribution of amino acid pools between subcellular compartments, and to investigate the fluxes of freshly synthesised amino acids to and from these compartments. Finally it provides a description of one particular metabolic environment against which nitrogen is assimilated and partitioned and suggests reasons why this partitioning contributes to the overall regulation of the assimilation of nitrogen when supplied as ammonium. The purpose of this final chapter being to bring together the salient features of the work

described, to illustrate the questions arising, and to discuss possible solutions suggested by the data.

The principal novel feature of the work described is the use of protoplasts as starting material for cellular fractionation studies involving ^{15}N assimilation. The major advantage of this approach is that one may isolate vacuoles, mitochondria and plastids from the same starting material; indeed, this is the only approach possible at present for the isolation of vacuoles in bulk. Disadvantages of the use of protoplasts concern the extensive manipulations involved in thier preparation and the possibility that their responses may not be typical of "normal' cells *in planta*. In order that meaningful insights may be gained from experiments with protoplasts several requisites should be fulfilled; that efficient fractionation methods may be developed; that protoplasts remain capable of reasonable rates of nitrogen assimilation; and that the metabolic background accompanying such assimilation be described as far as possible. If these requirements are met, the results may be considered, at least , as valid possibilities for wider application under the circumstances described.

The assessment of fractionation methods rested upon marker enzyme analysis, both for an indication of purity of the organelle preparations and so that the amounts recovered in each fraction can be quantitatively related to the intact protoplast. Vacuolar preparations of high quality and purity were obtained after an extensive development of procedures; plastid and mitochondrial fractions although substanstially enriched were less pure. Fortunately differences between these fractions were generally large and clear, and so cross-contamination seems unlikely to distort the broader picture. Aerated protoplasts proved capable of rapid nitrogen assimilation from ammonium ions, although the rate of assimilation from nitrate was too low for useful results to be obtained; consequently all results refer to ammonium assimilation, which inevitably introduces a metabolic bias. Consequently application of the methodology presented in the opening chapters provides assimilatory protoplasts from which subcellular fractions

can be obtained efficiently, quantitatively and in sufficient quantities to allow the static and dynamic nature of their associated amino acid pools to be determined.

The equilibrium distribution of amino acid pools gave clear evidence that amino acids are compartmentalised into morphologically discrete cell space. The most striking features being; the restriction of relatively large amounts of Gaba to the cytosol, and the clear separation of glutamate and glutamine, the latter being predominantly vacuolar and the former largely cytosolic. It is interesting to note that the two amino acids which are localized strongly towards the vacuole, namely glutamine and asparagine, carry uncharged side chains, whereas the predominantly cytosolic protein amino acids (glutamate and aspartate) carry polar, negatively charged side chains. The plastids and mitochondria have relatively minor roles in the compartmentation of large pools of amino acids, reflecting their function as catabolic and anabolic compartments, where amino acids are formed and nitrogen disseminated, rather than stored.

The amino acid pools described above undergo considerable changes when protoplasts are supplied with NH_4Cl as the sole N source. Principally, there is a considerable increase in the total amino acid content , notably in the vacuolar fraction , which can only be accounted for by the catabolism of existing protein reserves. The results of an investigation into the metabolic fate of freshly assimilated nitrogen revealed that the major sink for freshly assimilated ammonium is a cytosolic Gaba pool, which amounts to a considerable diversion of nitrogen away from protein and nucleic acid precursors. It is proposed that such a shortfall in cytosolic amino N is met by protein catabolism, principally in the vacuole. Which raises the question ; why is the majority of assimilated N incorporated into, what amounts to, a metabolic "dead end"?

The responses observed are characteristic metabolic responses to cytosolic acidification. The decarboxylation of glutamate to Gaba is a proton consuming reaction, catalysed by glutamate decarboxylase. The activity of which was shown to increase and decrease in

accordance with the decline and subsequent restoration of cytosolic pH, following the addition of NH_4Cl. Thus it is proposed that the diversion of amino-nitrogen from glutamate into Gaba constitutes a biochemical pH stat.

Gaba is not the only sink for ^{15}N in the data described, a significant quantitiy of ^{15}N is incorporated into other amino acid pools, compartmentalised into discrete regions of cell space. Why is this happening when considerable amounts of cellular energy are being expended to restore cyosolic amino acid levels through vacuolar protein catabolism?

It is proposed that the compartmentation of freshly assimilated nitrogen affords a means of spatially amd functionally separating assimilates providing a degree of metabolic plasticity. The diversion of nitrogen into Gaba in order to fullfil a pH stat function creates a considerable drain upon cytosolic glutamate resources, the replenishment of which will presumably be dependent upon the presence of a pool of its immediate precursor, glutamine. Therefore in answer to the above question, it is suggested that the compartmentation of a glutamine pool represents a nitrogen conservation strategy, allowing carrot cells to operate a Gaba based biochemical pH stat effectively, and to propitate the continued metabolism of nitrogen following stabilisation of cytosolic pH. The assimilation of nitrogen in higher plants is inextricably linked to that of carbon (Oaks, 1992). In so much as carbohydrates are required, not only to provide carbon skeletons, but also to provide the reducing power required for nitrate and nitrite reductase and the formation of glutamate. In this respect the observed compartmentation of amino acids may also contribute to the regulation of carbon metabolism. The maintenance of a vacuolar glutamine pool provides a source of nitrogen which may be mobilised without removing carbon, as 2-oxoglutarate, from the TCA cycle in the generation of glutamate. Hypoxia prevents cells establishing or maintaining such a glutamine pool, presumably through either, an effect upon the

transport of glutamine into the vacuole, or through an inhibitory effect upon cytosolic GS.

Protoplasts prepared from suspension-cultured cells of *Daucus carota* are capable of rapidly assimilating nitrogen when supplied as ammonium. Such assimilation results in a temporary perturbations in cytosolic pH status and evokes metabolic responses compatable with such responses. The most characteristic of which being a diversion of assimilated nitrogen into the non-protein amino acid Gaba, which is proposed represents the proton consuming stage of a biochemical pH stat. Against this metabolic background of cytosolic acidosis and moderation, freshly assimilated nitrogen is directed towards discrete compartments. Of particular note are the striking patterns of compartmentalisation shown by the pivotal amino acids, glutamine and glutamate; with the primary synthesis of the two species restricted to the plastid, and the subsequent restriction of the freshly synthesised amide to the vacuole. It is proposed that such compartmentation allows cellular metabolism to be resumed following the relaxation of cytosolic pH stress.

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