THE ENZYMATIC AND IMMUNOLOGICAL DETECTION OF THE PLANT 2-OXOACID DEHYDROGENASE COMPLEXES

by

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SUMMARY

Specific properties, including subcellular localisation, kinetic parameters and subunit composition, of the plant 2-oxoacid dehydrogenase complexes (pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxoacid dehydrogenase complexes) were investigated by applying enzymatic and immunological techniques. The intracellular distribution of this family of multienzyme complexes within pea root and leaf tissue confirmed the presence of a mitochondrial and plastid pyruvate dehydrogenase complex (PDC). The first extensive screening of plant organelles for the presence of PDCs sister complexes, 2-oxoglutarate dehydrogenase (OGDC) and branched-chain 2-oxoacid dehydrogenase (BCDC) complexes, was performed and indicated that these multienzyme complexes are confined to mitochondria. This distribution was based on enzymatic detection and addressed numerous potential limiting factors such as low *in vivo* concentrations of an active complex and the presence of NADH oxidases masking activity. The activities recorded for mitochondrial and plastid PDCs were consistent with previously reported values, OGDC activity was approx. 20% of mitochondrial PDC activity and BCDC-catalysed reaction displayed a very low reaction rate resulting from low concentrations of an active complex in the mitochondrial extracts.

Initial velocity data for potato mitochondrial PDC and OGDC and pea chloroplast PDC-catalysed reactions were collected and the apparent Michaelis constants agreed with previously reported values. This family of multienzyme complexes were shown to be inhibited by the products of the reaction sequence, NADH and acyl CoA, displaying greatest sensitivity to the NAD^+:NADH ratio. The 2-oxoacid dehydrogenase-catalysed reactions have previously been shown to function by a ping-pong mechanism and the inhibition patterns observed in this investigation were consistent with such a mechanism; acyl CoA and and NADH were competitive inhibitors versus their steady state precursors whilst product inhibition versus the 2-oxoacid substrate was uncompetitive.

The availability of high-titre polyclonal antisera to the 2-oxoacid dehydrogenase complexes and to specific subunits isolated from bovine heart facilitated the immunological probing of plant mitochondrial and plastid extracts. Immuno-reactive plant polypeptides, which were shown to be associated with a high M_r aggregate i.e. a multienzyme complex, were detected and their M_r values compared to the analogous mammalian enzymes. Immunological investigations suggests that the E2 subunit of pea and potato mitochondria and pea chloroplast PDC carry one lipoyl domain paralleling the situation in the analogous yeast enzyme and contrasting the two lipoyl bearing domains which are carried by the mammalian subunit. M_r comparisons also indicate that the E2 subunit of plant OGDC and BCDC, like their mammalian and prokaryotic counterparts contain one lipoyl domain. No equivalent antigenic species were identified in chloroplast extracts supporting an exclusive mitochondrial location of OGDC and BCDC as indicated by enzymatic investigations.

Immunological detection of the E3 subunit, a common enzyme to each 2-oxoacid dehydrogenase complex and the glycine decarboxylase complex (GDC), identified distinct mitochondrial and plastidic forms of the plant enzyme indicating that organelle-specific forms are expressed in the plant system.

Separation of solubilised, heat-treated potato tuber mitochondrial protein by anion exchange chromatography resolved three distinct E3 activities. These activities appear to represent distinct forms of E3 arranged as α_2 and β_2 homodimers and an $\alpha\beta$ heterodimer (M_r α, 59,000 and M_r β, 56000) . Comparison of mitochondrial E3 profiles from photosynthetic and non-photosynthetic tissue suggest that the potential isoforms are differentially expressed in different tissues. Such observations indicated the three forms of the E3 display complex selectivity. Consequently, potato mitochondrial E3 isoforms may exhibit specificity at tissue, organelle and complex levels.

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ABBREVIATIONS

APAD	3-acetylpyridine adenine dinucleotide
ADPG	adenosine 5'-diphosphate glucose
approx.	approximately
BCDC	branched-chain 2-oxoacid dehydrogenase complex
Bicine	N,N,-bis[2-hydroxyethyl]-glycine
BSA	bovine serum albumin
cm	centimetres
cv.	cultivar
DCPIP	2,6 dichlorophenol -indo-phenol
DTT	dithiothreitol
Da	Daltons
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
FITC	fluorescein isothiocyanate
g	gravity
h	hour
MES	2-(N-morpholino)ethane-sulphonic acid
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
MOPS	3-(N-morpholino)propane-sulphonic acid
NAD^+	nicotinamide adenosine dinucleotide
NADH	nicotinamide adenosine dinucleotide hydride
$NADP^+$	nicotinamide adenosine dinucleotide phosphate
nkat	nanokatal

nm	nanometre
nmol	nanomoles
Nycodenz	5-(N-2,3-dihydroxypropylacetamido)2,4,6 triiodo-N,N'-bis(2,3-dihydroxypropyl)-isophthalamide
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PGA	3-phosphoglycerate
PLP	pyridoxal phosphate
PMSF	phenylmethylsulphonyl fluoride
PVP	polyvinyl pyrolidone
s	second
SDS	sodium dodecyl sulphate
TES	[hydroxy-1,1-bis(hydroxymethyl)ethyl] amino ethane sulphonic acid
THF	tetrahydrofolate
TPP	thiamine pyrophosphate
Tricine	N-[2-hydroxy-1,1-bis(hydroxy)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
Tween 20	polyoxyethylenesorbitan monolaurate
µm	micrometre
v/v	volume to volume
w/v	weight to volume

DECLARATION

I hereby declare that the work presented in this thesis is my own, except where otherwise cited or acknowledged. No part of this thesis has been previously presented for any other degree.

Ailsa Carmichael

May, 1994

CHAPTER ONE

INTRODUCTION

1.1 THE 2-OXOACID DEHYDROGENASE COMPLEXES

The pyruvate dehydrogenase (PDC), 2-oxoglutarate dehydrogenase (OGDC) and branched-chain 2-oxoacid dehydrogenase (BCDC) complexes, members of the 2-oxoacid dehydrogenase family, are multienzyme assemblies occupying key regulatory positions in central metabolism (Fig. 1.1). They are composed of multiple copies of at least three different enzymes which co-ordinate the catalysis of the respective 2-oxoacid substrates to the appropriate CoA derivative (Reed, 1974; Yeaman, 1989; Perham, 1991).

PDC catalyses the irreversible oxidative decarboxylation of pyruvate to acetyl CoA. As the bridge between glycolysis and the tricarboxylic acid (TCA) cycle, PDC is of prime physiological importance. It commits the carbon atoms originating from glucose to one of two fates: either entry into the TCA cycle and consequent oxidation by TCA enzymes with the concomitant generation of ATP or incorporation into a range of cellular components via biosynthetic pathways which utilise acetyl CoA as the substrate. OGDC is a component enzyme of the TCA cycle converting 2-oxoglutarate to succinyl CoA. The irreversibility of this reaction targets OGDC for a regulatory role, and indeed it is responsible for controlling the flux of carbon around the TCA cycle in its latter stages. In addition the activity of this complex supplies succinyl CoA for the biosynthesis of porphyrins (in mammalian cells only), lysine and methionine. BCDC catalyses the committed step in the degradation of branched-chain amino acids leucine, isoleucine and valine. It may also be involved in the catabolism of methionine and threonine as 2-oxobutyrate and 4-methylthio-2-oxobutyrate (Jones & Yeaman, 1986) are oxidatively decarboxylated by BCDC. In mammals this multienzyme activity is of nutritional significance in controlling the levels of branched-chain amino acids, converting excesses into acyl CoA derivatives and thereby recycling carbon atoms back into central metabolism.

As the 2-oxoacid dehydrogenase complexes occupy key positions in regulatory metabolism abnormalities in their activities have serious clinical repercussions. Maple syrup urine disease results from deficiency in BCDC activity (Dancis *et al.*, 1963). Patients with this genetic disorder have increased levels of branched-chain 2-oxoacids

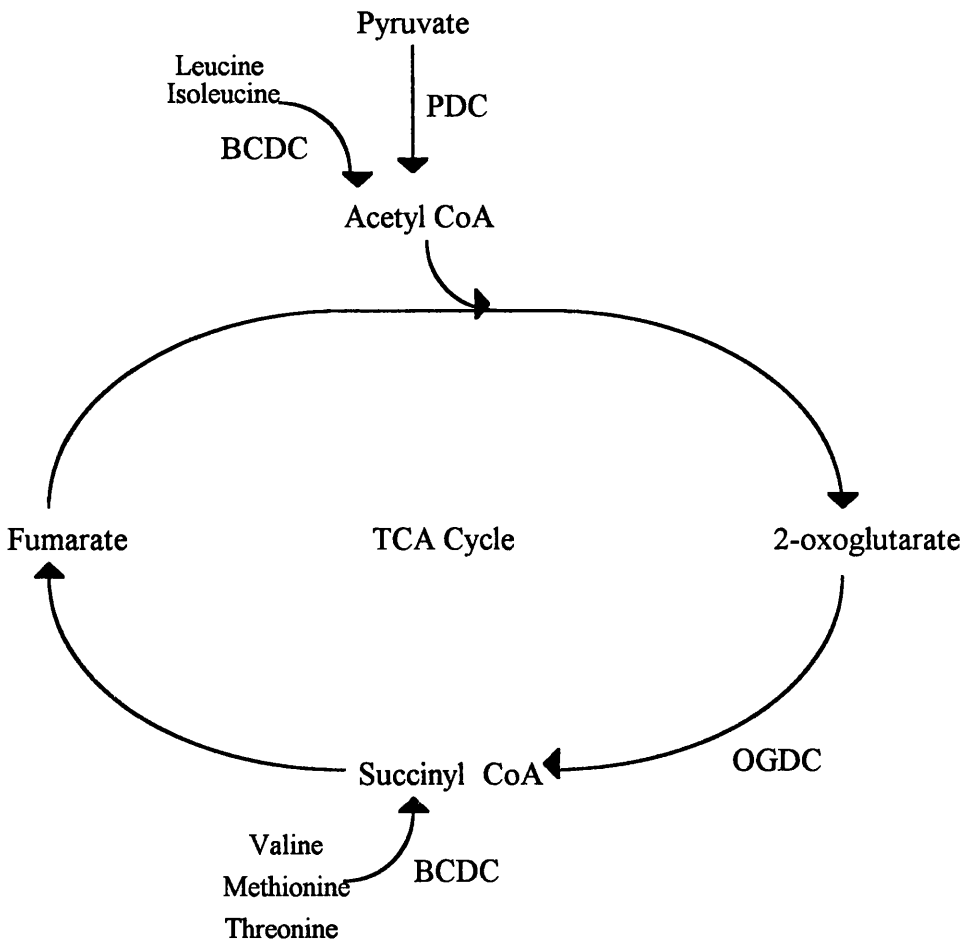


Figure 1.1: Partial representation of the tricarboxylic acid cycle illustrating the positions of the 2-oxoacid dehydrogenase complexes in central metabolism.

and amino acids and these elevated levels results in ketoacidosis and, in severe cases, mental retardation. In addition, deficiency in PDC activity is characterised by bouts of lactic acidosis (Butterworth, 1985; Stansbie *et al.*, 1986). The autoimmune disease, primary biliary cirrhosis, is a chronic and often fatal condition which results in cholestasis and liver cirrhosis (Gershwin *et al.*, 1988). Component enzymes of all three 2-oxoacid dehydrogenase complexes, the E2 enzymes, in particular the E2 component of PDC, have been identified as autoantigens (Fussey *et al.*, 1988).

The decarboxylation and dehydrogenation of 2-oxoacids is catalysed by the 2-oxoacid dehydrogenase complexes in a multistep process involving the sequential and co-ordinated actions of three separate enzymes (Reed, 1974; Yeaman, 1989): a substrate specific dehydrogenase (E1) employing thiamine pyrophosphate (TPP) as a catalytic cofactor, a dihydrolipoamide acyltransferase (E2) whose cofactor, lipoamide, is covalently attached, and a FAD^+ requiring dihydrolipoamide dehydrogenase (E3). The E1 component catalyses the first reaction, decarboxylating the 2-oxoacid and transferring the resulting acyl group onto the covalently bound lipoyl moieties of E2. E2 possesses transacylase activity, depositing the acyl group onto the CoA acceptor leaving the lipoyl domain in a reduced state. In order to re-enter the catalytic cycle the oxidised form is regenerated by the action of the E3 subunit which transfers the reducing equivalents onto NAD^+ via its FAD^+ cofactor (Fig. 1.2).

In PDC the E1 component is a pyruvate dehydrogenase (EC 1.2.4.1), in OGDC the dehydrogenase component is specific to 2-oxoglutarate (EC 1.2.4.2) and the analogous enzyme in BCDC (EC 1.2.4.4) is capable of interacting with at least five different 2-oxoacids, all of which are breakdown products of the branched-chain amino acids, methionine and threonine (Table 1.1). Similarly the E2 component is complex specific. E2 from PDC is an acetyltransferase (EC 2.3.1.12), a succinyltransferase (EC 2.3.1.61) appears in OGDC and the E2 from BCDC (no EC number) has a wider substrate specificity, capable of transferring a range of acyl groups. In mammals, the E3 (EC 1.8.1.4) component is common to all complexes, having the same role in each, that of reoxidising the lipoamide prosthetic group of E2 (Yeaman, 1989).

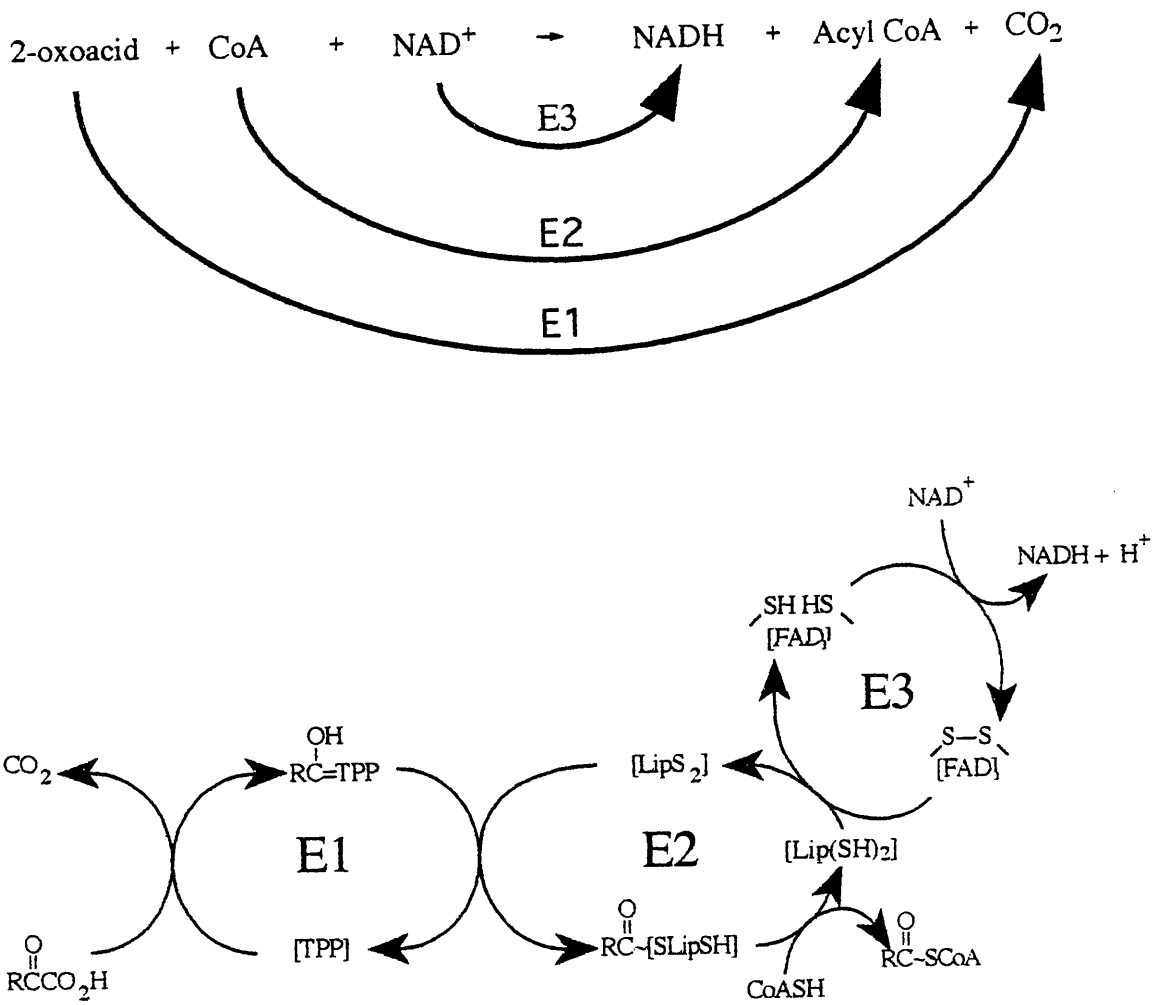


Figure 1.2: Schematic representation of the reaction mechanism of the 2-oxoacid dehydrogenase complexes highlighting the specific reactions catalysed by the component enzymes.

2-OXOACID DEHYDROGENASE	2-OXOACID SUBSTRATE	PRODUCT: ACYL CoA DERIVATIVE
PDC	Pyruvate	Acetyl CoA
	2-oxobutyrate	Propionyl CoA
OGDC	2-oxoglutarate	Succinyl CoA
BCDC	4-methyl 2-oxopentanoate	3-methyl butyryl CoA
	3-methyl 2-oxopentanoate	2-methyl butyryl CoA
	3-methyl 2-oxobutyrate	2-methyl propionyl CoA
	2-oxobutyrate	Propionyl CoA
	4-methylthio-2-oxobutyrate	3-methylthiopropionyl CoA
	Pyruvate	Acetyl CoA

Table 1.1: 2-oxoacid substrate and acyl CoA product of the 2-oxoacid dehydrogenase complexes.

A number of E2 polypeptides form the structural core of all three complexes (Fig. 1.3) and the peripheral subunits E1 and E3 are attached to this central core by non-covalent interactions (Oliver & Reed, 1982). The multimeric core of PDC from eukaryotes and Gram-positive bacteria is composed of sixty E2 polypeptides and has icosohedral symmetry forming a pentagonal dodecahedron. In contrast twenty four interacting E2 polypeptides form the octahedral core of all known OGDCs, BCDCs and also PDCs from Gram-negative bacteria (Perham *et al.*, 1987). The number of peripheral subunits is variable, however a ratio of 2:2:1 for E1:E2:E3 has been reported by a number of groups (Reed, 1974; Yang *et al.*, 1985; Perham, 1991). The resulting assemblages have large M_r values ranging from 5 to 10 million, in a similar league to ribosomes, with dimensions of 200-400Å. Consequently, they have undergone close scrutiny by electron microscopy where the highly symmetrical core and ordered distribution of the peripheral subunits have been observed (Junger & Reinauer, 1972).

An additional component of mammalian PDC, protein X, has been identified (De Marcucci & Lindsay, 1985; Jilka *et al.*, 1986). During resolution this polypeptide co-fractionates with E2 and was originally thought to be a proteolytic fragment of the E2 subunit. Extensive immunological and structural studies have now identified component X as a distinct polypeptide. It contains at least one lipoyl moiety which can be reductively acetylated *in vitro*; however it is unclear if it participates in the catalytic activity of the native complex (Hodgson *et al.*, 1986; Neagle *et al.*, 1989; Rahmatullah *et al.*, 1989). Although the precise role of component X has yet to be determined the current consensus is that it plays a structural role, that of aiding the binding of E3 subunits to the E2/X core (Powers-Greenwood *et al.*, 1989). Preferential digestion of E2s lipoyl domains has shown, however, that component X can support intact PDC activity at 20% the activity of the native complex (Lawson *et al.*, 1991a). Consequently, the function of Xs lipoyl domains in the native complex has yet to be firmly established. The component enzymes of the 2-oxoacid dehydrogenase complexes can be easily separated by SDS polyacrylamide gel electrophoresis (Fig. 1.4). If milder conditions are employed, e.g. gel filtration at high ionic strength, the complexes can be resolved into catalytically active individual enzyme components. These can then be re-constituted into

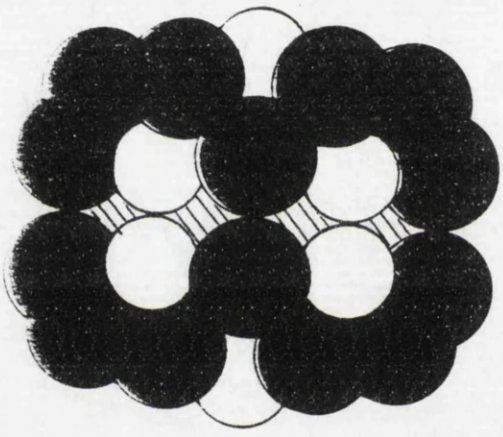


Figure 1.3: Model of the native *Escherichia coli* PDC. Figure is reproduced from Stryer (3rd Edition, p381). The peripheral subunits E1 (●) and E3 (O) are non-covalently bound to the central multimeric E2 (⊗) core.

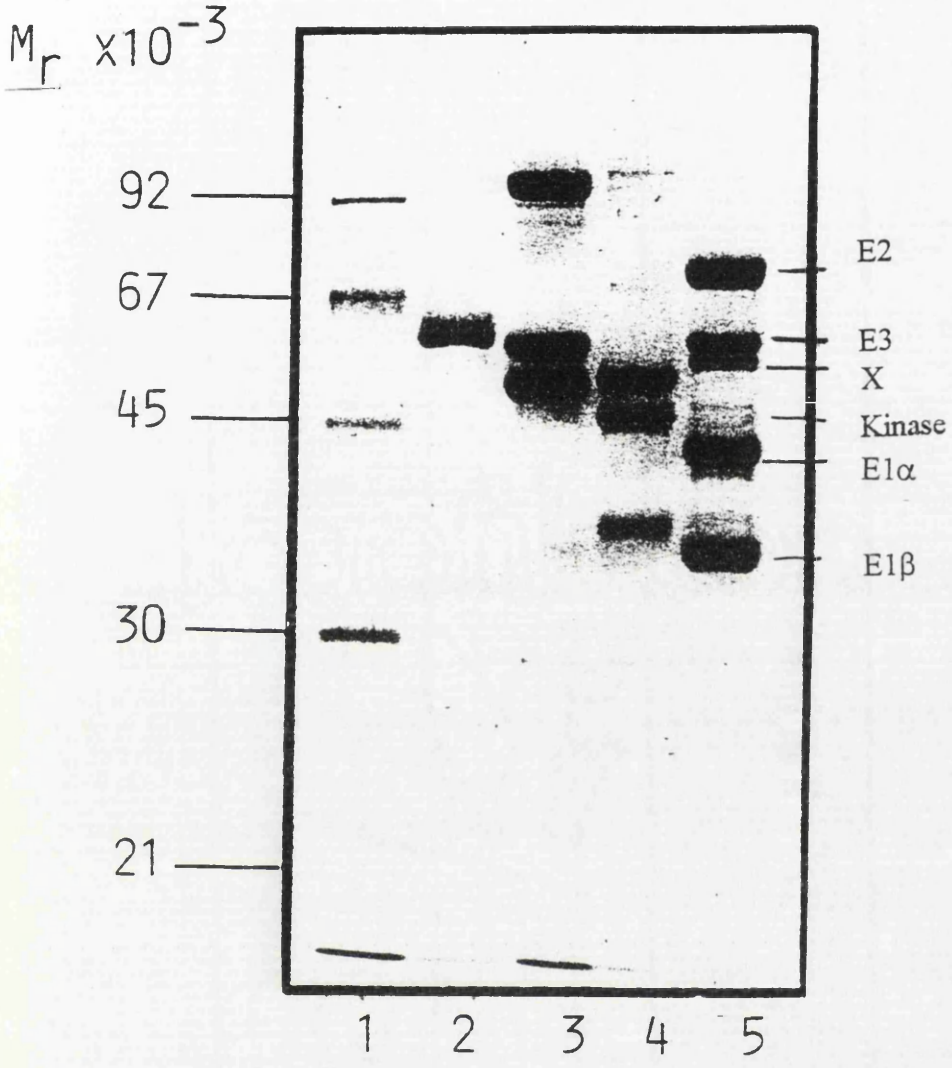


Fig. 1.4: Separation by SDS polyacrylamide gel electrophoresis of the component enzymes of the 2-oxoacid dehydrogenase complexes isolated from bovine heart (Clarkson & Lindsay, 1991). Lane1, M_r markers; Lane 2, dihydrolipoamide dehydrogenase (E3); Lane 3, bovine heart OGDC; Lane 4, bovine heart BCDC; Lane 5, bovine heart PDC.

a fully competent intact complex indicating that these multienzymes are truly self-assembling.

The 2-oxoacid dehydrogenase complexes exemplifies a very sophisticated multienzyme system. Three enzymes, self assembling in a highly organised manner and thus co-ordinating a complex reaction sequence with maximum efficiency, is highly interesting from a structural viewpoint. In addition they generate great interest biochemically owing to their significant regulatory role in central metabolism. A closer examination of the individual subunits and their interactions is therefore merited.

1.2 COMPONENT ENZYMES OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

2-oxoacid Dehydrogenase Component (E1):

The E1 component of the 2-oxoacid dehydrogenase complexes catalyses the initial decarboxylation of the 2-oxoacid and is the rate determining step of the overall reaction (Walsh *et al.*, 1976). It is also the only irreversible reaction in the multistep activity and, although the precise mechanistics remain elusive, it is thought to occur via a covalent adduct of TPP, whereby a molecule of CO_2 is released and 2-(1-hydroxyethylidene)-TPP is formed (Reed, 1974). The E1 enzyme also catalyses the reductive transacylation of the lipoic acid cofactor of the E2 enzyme.

The E1 component of OGDCs and of PDCs from Gram-negative bacteria exist as homodimers of M_r 2 x approx.100,000 (Koike & Koike, 1976). There is no evident sequence similarity between the E1 components of PDC and OGDC from prokaryotes even when considering both enzymes from the same source (Darlison *et al.*, 1984). A common sequence motif in TPP binding has been identified, however, and is present in all known E1 sequences and in other TPP binding proteins (Hawkins *et al.*, 1989).

The E1 component from BCDCs and eukaryotic and Gram-positive PDCs is composed of two non-identical subunits termed α and β. The α and β polypeptides from PDC display M_r values of 41,000 and 35,000 (Matuda *et al.*, 1983) and the analogous enzymes which oxidatively decarboxylate the branched-chain 2-oxoacids have M_r values

46,000 and 38,000 respectively (Pettit *et al.*, 1978). These subunits aggregate to form tetramers, $\alpha_2\beta_2$, with a total M_r value of approx. 150,000 (Reed *et al.*, 1985). There is some evidence to suggest that the α subunit catalyses the decarboxylation reaction and the β subunit catalyses the reductive acylation (Roche & Reed, 1972) although this has yet to be firmly established. It has recently been postulated that the α and β chains are derived from the splitting of a common ancestral gene from which all E1 decarboxylases have ultimately originated (Matuda *et al.*, 1991). Sequence data across a broad phylogenetic spectrum, stretching into the plant kingdom, will have to be considered before the evolutionary pathway can be uncovered.

Dihydrolipoamide Dehydrogenase (E3):

The E3 enzyme catalyses the reversible oxidation of lipoamide coupled to the reduction of one molecule of NAD^+ allowing E2 to re-enter the catalytic cycle. During the redox reaction catalysed by E3 electrons are shuttled from lipoamide via the flavin cofactor and ultimately discharged onto NAD^+ forming reducing power in the form of NADH (Ghisla & Massey, 1989; Williams, 1992).

The E3 component is usually common in all members of the 2-oxoacid dehydrogenase complexes. Exceptions to the rule are two species of *Pseudomonas* which express genetically distinct forms of dihydrolipoamide dehydrogenase designated LPD-Glc and LPD-Val (Sokatch *et al.*, 1981; Sokatch & Burns,1984). This was first demonstrated in *P. putida* where two E3 enzymes were isolated which displayed complex specificity (Burns *et al.*, 1989a). LPD-Val (M_r 49,000) is the E3 component of BCDC and LPD-Glc (M_r 56,000) is specific for OGDC, GDC and possibly PDC. The two forms of dihydrolipoamide dehydrogenase produced different peptide patterns on proteolysis (Sokatch *et al.*, 1983). In addition, a *P. putida* mutant lacking LPD-Val retained normal PDC and OGDC activities but was deficient in BCDC activity demonstrating that the two forms are not interchangeable. *P. aeruginosa* also exhibits complex specific isoforms of dihydrolipoamide dehydrogenase with M_r values 50,000 and 54,000 analogous to LPD-Val and LPD-Glc respectively (McCullet *et al.*, 1986).

Burns *et. al.* (1989b) isolated a third dihydrolipoamide dehydrogenase from *P. putida* which they have provisionally termed LPD-3. LPD-3 exhibits a distinct M_r value, amino acid composition and N-terminal sequence from LPD-Val and LPD-Glc. Although its function remains unclear it has been demonstrated that LPD-3 restored PDC and OGDC activities in mutants defective in LPD-Glc. Complete PDC activity was recovered whereas LPD-3 restoration of OGDC activity was 60% as effective as LPD-Glc. Although LPD-3 can replace LPD-Glc as the E3 component of PDC and OGDC, the natural role of this dihydrolipoamide dehydrogenase in *P. putida* remains an enigma. It has been postulated that it is affiliated with either an unknown multienzyme complex or one that has escaped characterisation. One candidate is 2-ketoadipate dehydrogenase which, in Pseudomonads, is involved in lysine and pipicolic acid oxidation (Fothergill & Guest, 1977).

A form of E3, distinct from the *lpd* gene product which provides E3 for PDC and OGDC has been purified from *E. coli* (Richarme, 1989). It has an M_r value of 46,000, very similar to the LPD-Val from *P. putida*, and may be involved in galactose transport. This calls to question the possibility of an additional role for E3 in other sytems distinct from its association with multienzyme complexes.

The E3 subunit exists as homodimers (M_r 2x55,000) and each dimer carries one non-covalently bound molecule of FAD^+ (Williams, 1992). Unlike the E1 and E2 enzymes, which have only been identified as components of the 2-oxoacid dehydrogenase complexes, the E3 subunit is also found in association with the glycine cleavage system, designated the L-protein therein (Motokawa & Kikuchi, 1974). This flavoprotein has also been found in the bloodstream form of *Trypanosoma cruzi* (Lohrer & Krauth-Siegel, 1990), *T. brucei* (Danson *et al.*, 1987) and in archaebacteria (Danson *et al.*, 1984) organisms which are known to lack the 2-oxoacid dehydrogenase complexes. Presumably in these organisms E3 is not affiliated with a multienzyme system and carries out a yet unknown function as an independent, solitary enzyme. E3 is located exclusively in the plasma membrane of *T. cruzi* and *T. brucei*. This led to the postulate that E3 may be involved in glucose transport (Danson *et al.*, 1987), thus paralleling the situation in *E. coli* from where a form of dihydrolipoamide dehydrogenase has been

implicated in galactose and maltose transport across the membrane (Richarme & Heine, 1985; Richarme, 1985; Richarme, 1989).

E3 has been extensively studied from a wide range of sources. Sequence data is available at both the amino acid and the nucleotide level and the three dimensional structures of E3 from *A. vinelandii* (Schierbeck *et al.*, 1989), *P. putida* (Mattevi *et al.*, 1992a), *P. fluorescens* (Mattevi *et al.*, 1992b) and yeast (Takenata *et al.*, 1988) have been determined. Such a wealth of information has led to the complete characterisation of the domain and subunit organisation and of the catalytic site.

Dihydrolipoamide Acyltransferase (E2):

Although the E1 and E3 subunits can be considered as 'stand alone' enzymes E2 activity is inextricably linked to the activities of the other component enzymes. The E2 enzyme of the 2-oxoacid dehydrogenase complexes fulfils a number of essential roles: it forms the highly symmetrical core of the complexes, it carries the covalently bound lipoyl cofactor, it catalyses the acyltransferase reaction, it binds the peripheral subunits and it couples their activities by providing a flexible lipoyl group and domain which can visit various active sites. Limited proteolysis of the E2 component has revealed that it consists of several functional domains (Bliele *et al.*, 1979; Reed & Hackert, 1990; Perham, 1991) which are illustrated in Fig. 1.5. These include a compact inner catalytic domain which houses the active site of the enzyme and also provides anchorage for the E1 subunits. The catalytic domain, which is located at the C-terminus, also interacts with additional catalytic domains of other E2 polypeptides thus maintaining the central multimeric core. There follows a distinct region of polypeptide, thought to exist as a folded domain, which is responsible for the binding of E3 subunits. A variable number of lipoyl domains are located in tandem repeat at the N-terminus. This extended outer domain contains the lysine groups to which the lipoic acid cofactors are covalently attached via a thioester linkage.

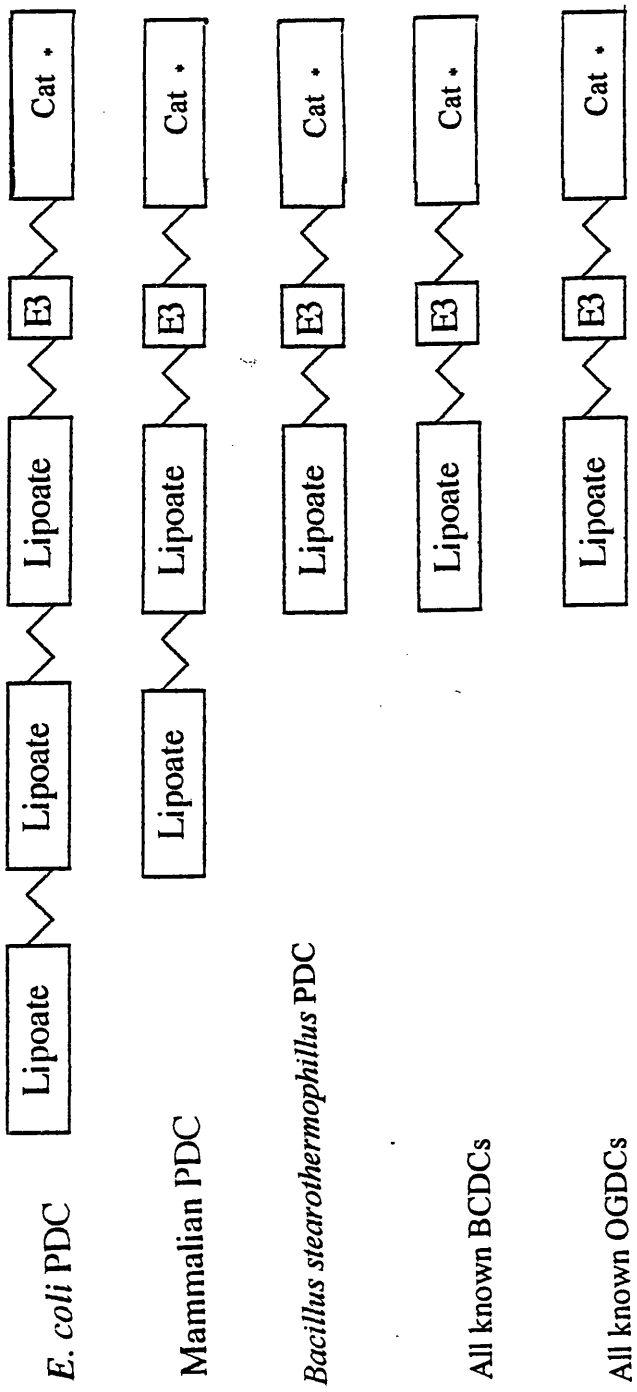


Figure 1.5: Schematic representation of the E2 component of *Escherichia coli* mammalian and *Bacilus stearothremophillus* PDC and all known OGDCs and BCDCs. The individual domains; inner catalytic domain (Cat), the E3 binding domain (E3) and the lipoic acid containing domain (Lipoate) are connected via linker regions (∿). The putative active site is denoted with a *.

The modular arrangement of the E2 subunit has hindered the growth of well ordered crystals (Fuller *et al.*, 1979). Recently, however, three dimensional structure determination has been focused on individual domains and NMR techniques were employed to solve the structures of the E2 lipoyl domain of *Bacillus stearothermophilus* PDC (Dardel *et al.*, 1991) and the E3 binding domain on the E2 component of *E. coli* OGDC (Robin *et al.*, 1992). The structure of the catalytic domain of E2 from *Azotobacter vinelandii* PDC has also been determined by X-ray crystallography (Shulze *et al.*, 1991; Mattevi *et al.*, 1992c) and structure analyses has demonstrated a similarity to chloramphenicol acetyltransferase (CAT).

The number of lipoyl moieties on the E2 enzyme varies with the complex and the organism. E2 from all OGDCs and BCDCs studied to date contain one lipoyl domain. This was initially determined by cross-linking studies using phenylene-o-bismaleimide in the presence of the 2-oxoacid substrate (Hodgson *et al.*, 1988). The 2-oxoacid substrate induces acylation of lipoyl domains on the E2 enzyme leading to the generation of thiol groups. Subsequent reaction with phenylene-o-bismaleimide results in the crosslinking of E2 subunits via the covalently attached lipoic acid groups. The E2 component from OGDC and BCDC form only dimers which is consistent with the presence of one lipoyl domain. In contrast the E2 subunit of mammalian PDC is capable of forming dimers, trimers and higher aggregates, suggesting that the component enzyme carries at least two lipoyl bearing domains. The gene encoding the E2 subunit of human PDC has since been cloned and sequenced and contains a coding region for two lipoyl domains (Thekkumkara *et al.*, 1988)

The E2 subunit of *E. coli* PDC is unique in carrying three highly conserved lipoate containing domains (Stephens *et al.*, 1983). These additional lipoyl moieties appear to be superfluous as deletion of two of the three domains by site-directed mutagenesis does not affect the assembly of a functional PDC which exhibits full catalytic activity (Guest *et al.*, 1985). The reason as to why *E. coli*, and several other organisms, contain these apparently redundant lipoyl domains has yet to be firmly resolved, they may come into play when substrate or cofactor concentrations are extremely limiting. The effects of multiple lipoyl domains on the activity of PDC was

investigated by constructing E2 chains with one to nine lipoyl domains (Machado *et al.*, 1992). Plasmids containing these constructs, and coding regions for the other component enzymes of PDC, were subsequently introduced into *E. coli* strains lacking a PDC complex. In each case an active complex was expressed and normal subunit stoichiometries were recorded. The complexes containing 1-3 lipoyl domains exhibited optimal PDC activity whereas lower activity was monitored in the complexes displaying more than three lipoyl containing domains. Machado *et al.* (1992) suggested that this deleterious effect resulted from under-lipoylation and the presence of unlipoylated domains interferes with component enzyme interaction. NMR studies of these genetically-reconstructed PDC complexes show that the E2 chain containing three lipoyl domains displays greater mobility than the other complexes (Machado *et al.*, 1993) . This indicates that active-site coupling will be maximal with the presence of three domains as normally carried by the E2 subunit of *E. coli* PDC.

The lipoamide cofactor, covalently bound to a lysine residue, is very flexible, forming 14Å 'swinging arms' which facilitate interaction between the three active sites. Extra mobility is provided by the linker regions which connect the three domains. These regions of the polypeptide are characterised by being rich in proline, alanine and charged amino acid residues (Reed & Hackert, 1990; Perham, 1991).The extreme flexibility of the E2 core structure aids active site coupling, a general feature of the 2-oxoacid dehydrogenase complexes (Hackert *et al.*, 1983; Perham *et al.*, 1987). After acylation of the lipoamide the newly generated acyl group can take a variety of routes through the multimeric core before it is discharged onto the CoA acceptor. Activated intermediates are therefore efficiently transferred, optimising catalytic activity and minimising the occurrence of side reactions. This highlights the benefits of coupling sequential reactions by providing the appropriate microenvironment of the multienzyme complex.

1.3 REGULATION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

As the 2-oxoacid dehydrogenase complexes catalyse key metabolic reactions, their activities are subject to strict control. In bacteria, complex activity is thought to be regulated primarily by end product inhibition (Bremer, 1969). The mammalian complexes are also prone to end product inhibition (Parker & Weitzman, 1973; Yeaman, 1986). Specifically the appropriate CoA derivative inhibits the E2 polypeptide and NADH inhibits the E3 component. The effects are negated by CoA and NAD^+ respectively.

With regard to PDC and BCDC, complex control is exerted mainly via reversible phosphorylation of the E1α subunit (Yeaman, 1986; 1989). OGDC is not susceptible to covalent modification and activity is governed by a range of allosteric effectors. OGDC is predominantly sensitive to the concentration of free Ca^{2+} which markedly decreases the K_m for 2-oxoglutarate (McCormack & Denton, 1979). In addition, an increase in the ADP:ATP ratio stimulates the activity of this TCA cycle multienzyme complex (Rutter & Denton, 1988). Dictation of OGDC activity by the energy status of the cell is of physiological significance. Energy metabolism can be effectively regulated by increasing the flux of carbon atoms around the TCA cycle and away from other biosynthetic pathways, when the energy demands of the cell are high.

Mammalian PDC and BCDC are controlled in part by reversible phosphorylation mediated by complex specific kinases and phosphatases (Yeaman, 1986; Reed & Yeaman 1987). In each case multiple sites on the E1α subunit are phosphorylated inactivating the complex. Three serine residues on the E1α subunit of PDC are subject to phosphorylation (Yeaman *et al.*, 1978; Koike *et al.*, 1988) whereas two serine residues, corresponding to sites one and three on PDC, on the BCDC enzyme are phosphorylatable (Cook *et al.*, 1983a; 1983b; 1984). Phosphorylation of one primary site is responsible for inactivation and it has been suggested that multisite phosphorylation provides a mechanism for controlling the reactivation of the inactive complex (Sugden *et al.*, 1978). The principal phosphorylation site on both PDC and BCDC has been identified as site 1 (Fig. 1.6).

BCDC Ile-Gly-His-His-Ser(P)-Thr-Ser-Asp-Asp-

PDC Tyr-His-Gly-His-Ser(P)-Met-Ser-Asp-Pro-

Fig. 1.6: Primary structure of phosphorylation site one (P) on the E1α polypeptide of PDC and BCDC.

Site one (Ser(P)) is located at the carboxyl-terminal third of the E1α subunit from PDC and BCDC and the sites are very similar in primary structure (Paxton *et al.*, 1986). Despite this homology their respective kinases display complex specificity.

Characterisation of the BCDC-kinase was initially hampered owing to purification difficulties (Cook *et al.*, 1985). Recently, however, BCDC-kinase has been purified from rat heart and liver and is a monomeric enzyme of M_r value 44,000. (Shimomura *et al.*, 1990; Popov *et al.*, 1991). BCDC-kinase is insensitive to NAD^+:NADH and CoA:Acyl CoA ratios and the major regulatory mechanism influencing activity is inhibition by the 2-oxocaid substrates (Paxton & Harris, 1984). BCDC-kinase has been cloned and sequenced from a rat heart cDNA library (Popov *et al.*, 1992). This was the first mitochondrial protein kinase to be cloned and, surprisingly, it displayed little homology to other eukaryotic serine/threonine protein kinases. A high degree of similarity was detected, however, between rat BCDC-kinase and prokaryotic histidine protein kinases.

PDC-kinase has been purified to homogeneity and consists of two subunits α and β with M_r values 48,000 and 45,000 respectively. The α subunit is directly involved in catalysis and the β subunit is believed to regulate activity (Stepp *et al.*, 1983). Unlike the analogous BCDC regulatory enzyme, PDC-kinase activity is stimulated by the products of the complex reaction sequence, acetyl CoA and NADH and this stimulation is antagonised by CoA and NAD^+ (Pettit *et al.*, 1975). Both pyruvate and TPP, presumably by blocking the active site of the E1 substrate, inhibit the kinase (Pratt & Roche, 1979). The successful cloning and sequencing of rat PDC-kinase has recently been achieved (Popov *et al.*, 1993) and, as with BCDC-kinase, shows greatest homology

with prokaryotic histidine protein kinases which may reflect the evolutionary origin of mitochondria.

Dephosphorylation and concomitant reactivation is mediated by complex specific phosphatases. The regulatory enzymes affiliated with both PDC and BCDC have been purified and the native enzymes have M_r values of 150,000 and 460,000 respectively (Teague *et al.*, 1982). PDC-phosphatase has an absolute requirement for Mg^{2+} and is stimulated by Ca^{2+}. It consists of a catalytic subunit of M_r value 50,000 and a 90,000 subunit which tightly binds one molecule of FAD^+ the function of which is unclear (Teague *et al.*, 1982).

In comparison with PDC-phosphatase, information concerning the BCDC-phosphatase is limited. The catalytic subunit of M_r value 33,000 has been purified; however the function and properties of the remaining subunits have yet to be elucidated (Damuni *et al.*, 1984; Damuni & Reed, 1987). Control of mammalian PDC and BCDC by covalent modification is therefore governed by a complex regime. The complexity of the mechanism is highlighted in Fig. 1.7 which illustrates the interaction of the positive and negative effectors of PDC-kinase and phosphatase activities.

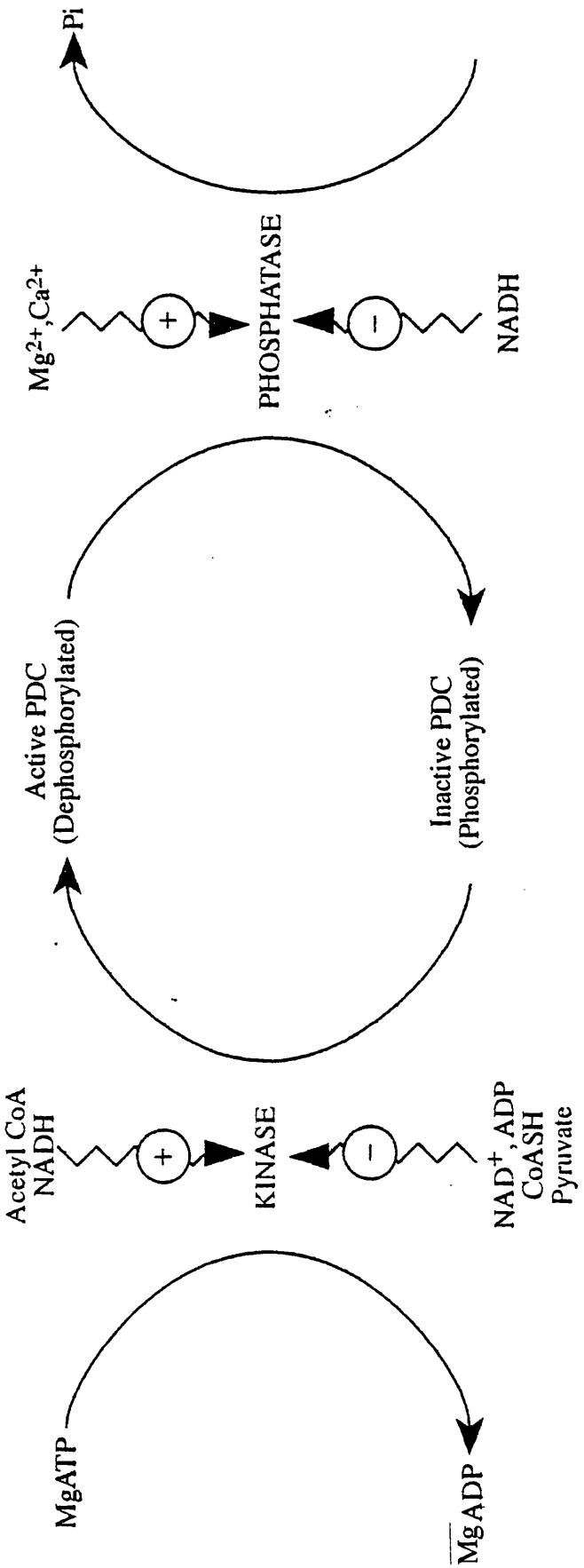


Figure 1.7: Diagramatic representation of the complex regulatory regime controlling the activity of mammalian PDC kinase and phosphatase.

1.4 2-OXOACID DEHYDROGENASE COMPLEXES IN PLANTS

Characterisation of plant 2-oxoacid dehydrogenase complexes has lagged behind that of their mammalian and bacterial counterparts and has to date focused on PDC. This situation has developed owing to the formidable task of purifying the plant multienzyme complexes and stems from the low number of mitochondria per fresh weight of tissue as compared with mammalian cells. An intact plant complex, PDC, has been purified to homogeneity only once and was described by the workers as a 'Herculean effort' (Rubin & Randall, 1977a). Half a tonne of broccoli was harvested to yield a mere 1mg of mitochondrial PDC. Although these authors raised antisera to broccoli mitochondrial PDC and subsequently probed other plant extracts, a gel of the purified complex, showing the constituent proteins of PDC as resolved by gel electrophoresis, was not presented in the publication. Consequently, the paucity of information concerning the plant complexes is understandable. The limited information available on the plant complexes is reviewed in this section and this is mainly dedicated to PDC, to which the majority of research has so far been focused. It is necessary to examine this, however, as it forms a basis for the investigations into the other complexes described in this thesis.

1.5 PLANT PYRUVATE DEHYDROGENASE COMPLEX

Plants are unique in possessing two distinct spacially separate forms of PDC, one in the mitochondrial matrix and the other located within the plastid stroma (Camp & Randall, 1985; Camp *et al.*, 1988). The occurrence of spatially separate forms of PDC in plants is far from extraordinary when considering the greater complexity of subcellular compartmentation compared to mammalian and microbial cells. These two forms of the multienzyme assembly have been shown to exhibit differences in substrate specificity, pH optima and Mg^{2+} ion requirements (Miernyk *et al.*, 1985). In addition, a recent report has demonstrated distinct differences in subunit composition between pea mitochondrial and plastid PDC (Taylor *et al.*, 1992). As in other eukaryotic systems, plant mitochondrial PDC (mtPDC) is involved in respiratory processes, channelling carbon

atoms into the TCA cycle and generating specific carbon skeletons. In plant cells, unlike mammalian cells and microbes, *de novo* fatty acid synthesis occurs within the the plastid compartment (Ohlrogge *et al.*, 1979), with plastid PDC (pPDC) providing acetyl CoA for this biosynthetic pathway (Camp & Randall, 1985).

1.6 PLANT MITOCHONDRIAL PYRUVATE DEHYDROGENASE COMPLEX

Plant mtPDC has been detected in a wide variety of plant tissues including pea (Randall *et al.*, 1981), potato (Crompton & Laties, 1971), spinach (Rao & Randall, 1980), cauliflower and broccoli (Randall, 1982; Rubin & Randall, 1977a; Rubin *et al.*, 1978) and displays similar properties to PDC from a non-plant source. Rubin and Randall (1977a) successfully purified mitochondrial PDC from broccoli by treatment with protamine sulphate, ultracentrifugation, and polyethylene glycol fractionation. After ultracentrifugation, which concentrates the multienzyme complex, PDC activity was confined to the pellet indicating that broccoli mtPDC has an M_r value of several million, as found in other organisms. The purified complex was found to have an absolute requirement for pyruvate (K_m 0.25mM), TPP, NAD^+ (K_m 0.11mM), CoA (K_m 54μM) and Mg^{2+}, with an optimal activity at pH 7.8. $NADP^+$ was declared a poor substitute for NAD^+; however, Mg^{2+} could be replaced with other divalent cations, Ca^{2+} or Mn^{2+}. 2-oxoglutarate was shown to be an ineffective substrate; however, broccoli mtPDC, like its mammalian counterpart, was capable of interacting with 2-oxobutyrate (K_m 1.66mM) at approximately one third the rate obtained with pyruvate.

1.7 REGULATION OF PLANT MITOCHONDRIAL PYRUVATE DEHYDROGENASE COMPLEX

Plant mtPDC occupies a key regulatory position with regard to both energy metabolism and the generation of carbon skeletons. Consequently, its activity is governed by a complex regulatory regime. Fine control of plant mtPDC, like all PDCs studied to date is achieved by end product inhibition (Mierynk & Randall, 1987b). The end products of the complex reaction, NADH and acetyl CoA, inhibit competitively with respect to NAD^+ and CoA displaying typical K_i values of 19μM and 54μM respectively (Miernyk & Randall, 1987b). The K_i values for acetyl CoA are generally greater than the K_m values for CoA and a linear increase in inhibition is observed with increasing ratios of acetyl CoA:CoA. In contrast K_i values for NADH are consistently smaller than K_m values for NAD^+ and with steadily increasing ratios of NADH:NAD^+ a logarithmic increase in inhibition has been reported (Randall *et al.*, 1977; Thompson *et al.*, 1977a). Such a behavioural pattern implies that plant mtPDC, like mammalian and microbial complexes, is very sensitive to the NAD^+:NADH ratio.

Plant mtPDC is also subject to control by covalent modification by reversible phosphorylation (Randall *et al.*, 1981; 1989; 1990). This exciting regulatory feature has been extensively studied and was the first example of a phosphorylation-dephosphorylation mechanism controlling the activity of a plant enzyme. Hence the control of biological catalysts by reversible phosphorylation was extended into the plant kingdom. This regulatory phenomenon has been shown to be operative across a broad range of species. Mitochondrial PDC from the green leaves of spinach (Rao & Randall, 1980), pea (Randall *et al.*, 1981), rye and fescue, the florets of broccoli and cauliflower and from the developing endosperm of *Ricinus*, (Rapp & Randall, 1980) have all been reported to display regulation by reversible phosphorylation. The existence of such a mechanism was verified by demonstrating a Mg-ATP dependent inactivation of PDC (Rubin & Randall, 1977b). When incubating with Mg-[γ-^{32}P]-ATP loss of activity is coupled with the incorporation of label into a 43,300Da subunit which cross-reacts with anti-broccoli PDC. This cross-reacting polypeptide corresponds to the phospho-E1α

subunit of mammalian PDC which has a slightly lower M_r of 41,000 (Matuda *et al.*, 1983).

Although it has been firmly established that mtPDCs from the plant kingdom are subject to regulation by reversible phosphorylation, structural information is limited. Antibodies were prepared to a synthetic peptide corresponding to a 14 amino acid tryptic fragment containing phosphorylation sites 1 and 2 of bovine PDC (Miernyk & Randall, 1989). Bovine, porcine and yeast PDC cross-react with these polyclonal antibodies; however no cross-reaction is observed with pea mitochondrial and plastid PDC. The synthetic peptide acts as a substrate for the mammalian PDC kinase, whereas the plant PDC-kinase will not catalyse the transfer of a phosphoryl group onto the same tryptic fragment. This observation suggests that the phosphorylation sites on mammalian and plant mtPDC are distinctly different.

The precise location of the phosphorylation site on the E1α subunit of plant mtPDC has yet to be firmly established. Miernyk and Randall (1987a) implicated a threonine as the phosphorylated residue, unlike the mammalian enzyme where multiple serine residues are phosphorylated (Yeaman *et al.*, 1978). They constructed an 8 amino acid polypeptide corresponding to phosphorylation site 1 of the bovine complex. This construct did not act as a substrate for plant PDC-kinase, however substitution of a serine for a threonine results in an effective phosphoryl acceptor. Investigations suggest that, as with the E1α component of mammalian PDC, multiple sites are phosphorylated on the plant enzyme. Preliminary sequence data on the E1α subunit of plant mtPDC is required before the precise nature of the phosphorylation mechanism can be unravelled. Randall and co-workers are presently occupied in the cloning and sequencing of the E1 subunits of pea mtPDC (Gemel *et al.*, 1993; Luethy *et al.*, 1993). It is therefore envisaged that the specific phosphorylated residues on the plant mitochondrial E1α enzyme will be identified in the near future.

Mammalian PDC-kinase exists as an αβ heterodimer. It has been proposed that the α subunit (M_r 48,000) is directly involved in catalysis and the β subunit (M_r 45,000) regulates activity (Stepp *et al.*, 1983). With respect to the plant PDC-kinase a single subunit, thought to represent the catalytic subunit, of M_r value 53,000, has been

identified by photoaffinity labelling with 8-azido-[α-^{32}P]ATP (Miernyk & Randall, 1988). The regulatory enzyme from pea mitochondria exhibits a K_m for Mg-ATP of 2.5μM (Randall *et al.*, 1989), which is substantially lower than the value for the mammalian enzyme. When considering typical ATP concentrations in the mitochondrial matrix it is clear that the kinase has to be subject to strict control or PDC would always be inactivated (phosphorylated). A complex regulatory scheme is in operation and several mitochondrial metabolites have been identified as potential negative modulators of plant PDC-kinase activity, including acetyl CoA, NADH, citrate, Na^+ and pyruvate (Miernyk & Randall, 1987a; Schuller & Randall, 1989). Inhibition of the kinase by acetyl CoA and NADH is extraordinary as it favours an active complex and works against control by feedback inhibition. In contrast mammalian PDC-kinase activity is, as expected, stimulated by acetyl CoA and NADH. The basis for the difference between the mammalian and plant regulatory enzymes has yet to be established.

Pyruvate has been identified as a potent inhibitor of plant mtPDC-kinase, displaying a typical K_i of 60μM (Budde & Randall, 1988; Schuller & Randall, 1990). Inhibition by pyruvate is enhanced by TPP. Kinase activity is stimulated by mM and μM levels of [K^+] and [NH_4^+] respectively (Schuller & Randall, 1989). Schuller *et al.* (1993) reported the stimulatory effect of [K^+] and [NH_4^+] on PDC-kinase activity in dialyzed extracts of pea leaf mitochondria. Kinetic analyses indicated that the monovalent cation stimulation resulted from a lowering of the K_m for ATP without affecting the V_{max}.

The stimulatory effect of NH_4^+ ions is an exciting phenomenon as it introduces a potential photorespiratory control of the TCA cycle during photosynthesis. Glycine, a product of photorespiration, is transported to mitochondria from chloroplasts where it is oxidised to serine with the concomitant production of NH_4^+ ions (Lorimer & Andrews, 1981). Consequently, mitochondria from illuminated tissue exhibit increased levels of ATP, glycine oxidation and NH_4^+ ions. These conditions stimulate kinase activity and inactivate the complex. This provides a mechanism for conserving carbohydrate when the cell is photosynthetically active by minimising the flux of carbon around the TCA cycle. The potential communication between organelles is certainly an attractive hypothesis as it provides a control mechanism which does not interfere with glycine oxidation and

oxidative phosphorylation which sustain mitochondrial ATP levels. Preferential inactivation of plant mtPDC in the presence of NH_4^+ appears to be restricted to species which display C_3 metabolism. Species which metabolise carbon through the C_4 pathway exhibit very low levels of photorespiration by shuttling oxaloacetate or malate into the chloroplasts of bundle-sheath cells. Consequently, increased levels of NH_4^+ ions in the mitochondrial matrix is not an effective indicator of a photosynthetically active cell and, accordingly, NH_4^+ ions appear to have no effect on the activity of mtPDC-kinase from C_3 plant species (J.G. Lindsay & H.G. Nimmo, Glasgow University, personal communication).

The reactivation of phosphorylated plant mtPDC is catalysed by PDC-phosphatase. Unlike the specific kinase which is tightly associated with plant mtPDC, the PDC-phosphatase exhibits a weak association and is consequently lost during partial purification. This, coupled with the apparent lability of the regulatory enzyme, has hampered its thorough characterisation. Rapp *et.al.* (1987) adopted a gentler isolation procedure and successfully demonstrated that PDC-phosphatase is an intrinsic component of plant mtPDC. Phosphatase activity has an absolute rquirement for divalent cations, with activation by $Mg^{2+} > Mn^{2+} > Co^{2+}$. Micromolar concentrations of Ca^{2+} inhibit the phosphatase (Randall *et al.*, 1990) in contrast to the mammalian counterpart where Ca^{2+} exerts a stimulatory effect (Pettit *et al.*, 1972). It is becoming obvious that divalent cations play an important role in metabolic regulation. Such control is often modulated via carrier proteins, calmodulin in the case of Ca^{2+}. Calmodulin, however, does not enhance the Ca^{2+} stimulated effect and the application of EGTA does not totally negate the inhibition (Budde *et al.*, 1987; Miernyk & Randall, 1987c). Other inhibitors of the plant PDC-phosphatase are F^- and orthophosphate (Randall *et al.*, 1990). Extensive investigations are necessary before the complete regulatory mechanism can be appreciated.

These collective studies provide firm evidence that plant mtPDC is receptive to control by reversible phosphorylation and product inhibition and *in vitro* experimentation has uncovered a highly complex regulatory regime governing PDC-kinase and phosphatase activities (Fig. 1.8).

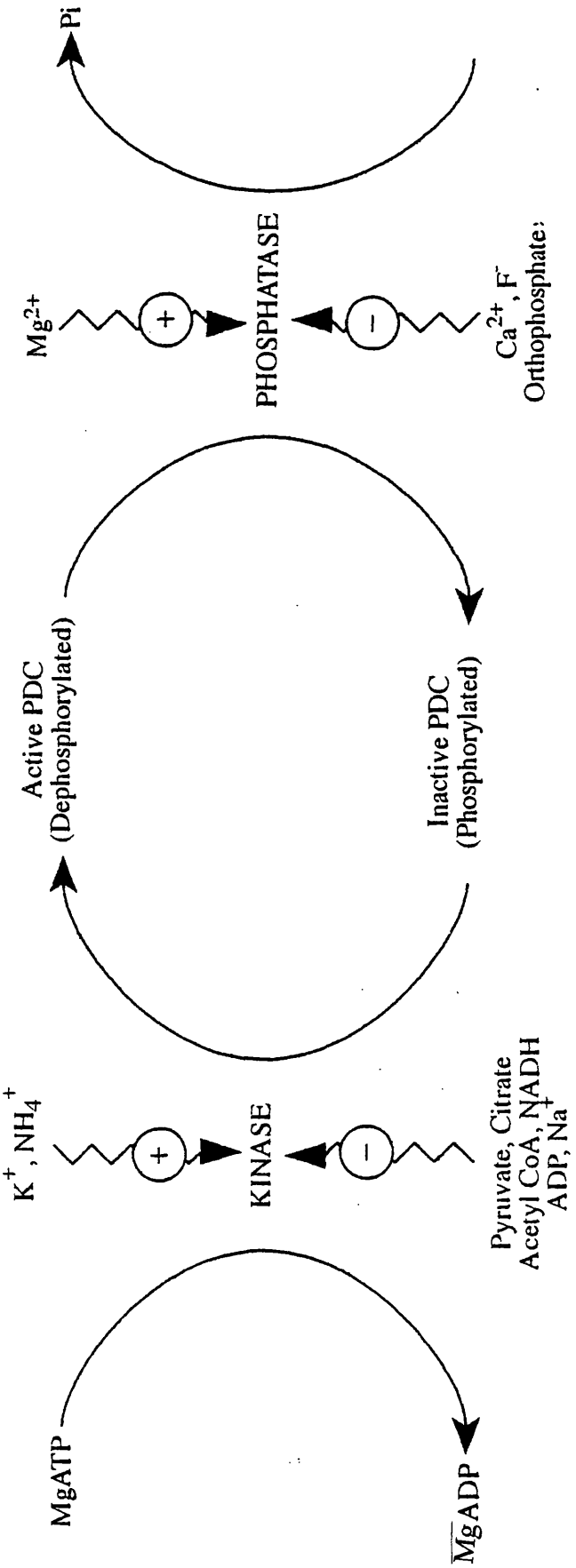


Figure 1.8: Diagramatic representation of the complex regulatory regime controlling the activity of plant mitochondrial PDC kinase and phosphatase.

1.8 SUBUNIT COMPOSITION OF PLANT MITOCHONDRIAL PYRUVATE DEHYDROGENASE COMPLEX

M_r value determination of the component enzymes of plant mtPDC was first undertaken by Randall and co-workers (Camp & Randall, 1985). This was achieved using antibodies raised against mtPDC from the floral buds of broccoli which they had purified during the late seventies (Rubin & Randall, 1977a). Analysis of pea mtPDC by SDS-PAGE and subsequent Western blotting revealed subunits of M_r 97,000, 67,400, 58,100, 43,300 and 37,000. It was assumed that the 58,100Da subunit represented the E3 component as it also cross-reacted with antiporcine E3, and the 43,000 and 37,000Da cross-reacting species corresponded to the E1α and β subunits respectively. The 67,400 immuno-reactive polypeptide exhibits a similar M_r value to the mammalian E2 subunit. The 97,000Da band may be the E1 subunit of OGDC which is often a contaminant of PDC preparations. This can be considered as a tentative identification of certain component enzymes as subunit specific antisera were unavailable to verify the nature of each immunoreactive polypeptide.

Immunological analysis of pea mtPDC using antibodies to the native bovine heart PDC identified four prominant cross-reacting proteins (Taylor *et al.*, 1992). Further analysis employing subunit specific antisera allowed the nature of the bands to be determined (Table 1.2). Antibovine E2 from PDC cross-reacted with a 54,000Da polypeptide, the mammalian subunit has an M_r of 74,000. As the size difference is consistent with the M_r of a single lipoyl domain, it is possible that E2 from plant PDC possesses only one lipoyl moiety, as in E2s from yeast PDC and mammalian OGDC and BCDC, as opposed to the two domains covalently attached to the E2 of mammalian PDC. Pea mitochondrial protein displayed no appreciable cross-reactivity with antisera to the E1 component of bovine heart PDC (Taylor *et al.*, 1992). A strong immune response was observed with exposure to antibodies to yeast E1 from PDC. The immunoreactive polypeptide (M_r 41,000) correlates with a 43,300Da pea mitochondrial protein which was identified as E1α by phosphorylation studies (Miernyk *et al.*, 1985).

PDC Subunit	M_r Values (kDa)			
	Mammalian	Pea chloroplast	Pea Mito-chondria	Yeast
E2	74	50	50	58
E3	55	52	67	56
X	50	48	67	50
E1α	41	N.D.	41	45
E1β	36	N.D.	N.D.	35

Table 1.2: M_r estimation of the component enzymes of pea mitochondrial and plastid PDC with comparison to mammalian and yeast PDC. N.D.= non-detectable. Plant subunits were identified by immunological methods by Taylor *et al.* (1992).

By probing pea mitochondrial protein with anti-E3 serum Taylor *et al.* (1992) identified an immunoreactive poylpeptide displaying an M_r value of 67,000, greater than the analogous mammalian enzyme, M_r value of 55,000. In addition antisera raised against component X from bovine heart, cross-reacted with a pea mitochondrial protein of the same M_r (67,000). Although these could represent distinct polypeptides, it is possible that component X sequences have become integrated into the E3 gene. A similar situation has recently been reported in mammalian OGDC where a component X-like domain is located at the N-terminal region of the E1 subunit (Rice & Lindsay, 1992).

1.9 PLASTID PYRUVATE DEHYDROGENASE COMPLEX

A wide variety of chloroplast products, including fatty acids and isoprenoids are ultimately derived from acetyl CoA (Camp & Randall, 1985). As the chloroplast envelope is impermeable to CoA derivatives an intracytoplasmic source of acetyl CoA was initially considered.

Acetyl CoA sythetase activity supplies plastids with acetyl CoA, however an additional potential source stems from the activity of a plastid PDC. During the early years of this research contradictory results were published. Roughman (1979) reported the absence, or trace amounts at best, of PDC in spinach chloroplasts. In contrast other groups recorded significant levels of PDC in chloroplasts isolated from peas, butter lettuce and non-green plastids of castor oil seeds (Reid *et al.*, 1975, 1977). The existence of a plastid located PDC remains a point of contention as acetate was found to be the preferred precursor of chloroplast acetyl CoA (Roughman, 1979). Nevertheless the presence of a plastid PDC has been extensively supported over the last decade.

Reid *et al.* (1975) separated the organelles from developing *Ricinus* endosperm by rate-zonal sedimentation on discontinuous sucrose gradients. Two distinct peaks of PDC activity were detected, one associated with mitochondrial marker enzymes and the other appearing in the fraction containing plastid marker enzyme activity. This dual location of PDC was also observed in peas. Although the specific activity of plastid PDC

is consistently lower than plant mtPDC, it is sufficient to fulfil the demands of fatty acid synthesis.

Camp and Randall (1985) achieved a 75-fold purification of pea chloroplast PDC by a series of PEG fractionations. Since the validity of a PDC localised in chloroplasts was debated at this time, it was essential to authenticate the identity of this multienzyme complex. They unequivocally established that the observed oxidative decarboxylation of pyruvate was a function of PDC activity by showing an absolute requirement for NAD^+ and CoA. These authors also reported that antibodies to broccoli mtPDC only partially inactivated the pea pPDC. This indicated that the observed activity was a function of a plastid located PDC and did not arise from mitochondrial contamination. The partially purified multienzyme complex was unable to interact with 2-oxoisovalerate or 2-oxoisocaproate, ruling out the possibility of a BCDC-mediated reaction utilising pyruvate as substrate. The complex was capable of utilising 2-oxobutyrate at approximately 10% the rate of pyruvate, paralleling the situation in plant mitochondrial, mammalian and microbial PDC. $NADP^+$, which is the predominant electron acceptor within chloroplasts, was a poor substitute for NAD^+, displaying a rate of only 4% of that with NAD^+. The activity of PDC can be measured in such a way as to quantify each of the products: by feeding with 1-[^{14}C]-pyruvate incorporation of radiolabel into CO_2 can be assessed, and formation of acetyl CoA and NADH can be determined by HPLC analysis. Camp and Randall (1985) quantified the three products and, in accordance with a PDC reaction mechanism, observed a 1:1:1 stoichiometry of CO_2:acetyl CoA:NADH.

1.10 REGULATION OF PLASTID PYRUVATE DEHYDROGENASE COMPLEX

Product inhibition:

Pea chloroplast PDC was found to be subject to product inhibition, a property it has in common with all other PDCs (Thompson *et al.*, 1977a; 1977b). The final products of the overall reaction, acetyl CoA and NADH, are linear competitive inhibitors with respect to CoA and NAD^+, displaying K_i values of 20μM (Camp & Randall, 1988).

Camp and Randall (1988) also observed a linear increase in inhibition of pea chloroplast PDC with escalating concentrations of acetyl CoA, whereas a logarithmic increase in inhibition was discerned with increasing NADH concentrations. This implies that the system is more sensitive to inhibition by NADH.

Regulatory enzymes are often prone to inhibition by the end products of the metabolic pathway. As plastid PDC activity supplies a primary precursor for the biosynthesis of fatty acids and isoprenoids, it is conceivable that the end products of these pathways exert feedback inhibition. The end products of lipid metabolism, free fatty acids, acyl CoA, acyl-ACPs, complex lipids, complex isoprenoids e.g. carotenoids, abscisic acid, and gibberellins, have yet to be assessed in detail for their potential control of plastid PDC.

Metabolite regulation:

Unlike plant mtPDC, which is subject to fine control by adenylates, there is no evidence that plastid PDC is prone to metabolite influence. Plastid metabolites tested for their effect on PDC activity included intermediates of glycolysis, the TCA cycle and the photorespiratory carbon cycle (Camp & Randall, 1988). With the exception of phosphoglycolate, all failed to modulate PDC activity *in vitro.* Similarly pea chloroplast PDC was declared insensitive to a wide range of chloroplast metabolites including intermediates of the reductive pentose-phosphate pathway and amino acid biosynthesis. Exceptions to the rule are oleic acid, which inhibits PDC activity, and palmitic acid which exerts a stimulatory effect (Camp & Randall, 1988).

Protein turnover:

Preliminary investigations are underway to ascertain whether protein turnover plays a part in the control of plastid PDC. Camp and Randall (1988) uncovered a potential developmental programme which governed plastid PDC activity in *Ricinus* endosperm and green tissue. In maturing endosperm tissue, peak PDC activity is observed during a period of maximum storage lipid accumulation, whilst optimal chloroplast PDC activity is correlated with leaf expansion, a stage in development

associated with extensive lipid synthesis. These authors also reported the reduction of plastid PDC activity to undetectable levels when the tissue is fully mature or senescent.

Light/dark transitions:

When considering the role of PDC in the plastid compartment, that of supplying acetyl CoA for fatty acid biosynthesis, a regulatory regime coincident with optimal conditions for this biosynthetic pathway is predicted. Within the plant cell *de novo* fatty acid synthesis is restricted to the plastid stromal phase and, in photosynthetic tissue, is light stimulated (Sauer & Heise, 1987; Treede *et al.*, 1986). In the dark the chloroplast stroma has a neutral pH, concentrations of divalent cations are low, and 'reductant' charge is a scarce commodity. When the chloroplast is photosynthetically active, i.e. upon illumination, there is an alkanisation of the stromal phase to a pH of approx. 8, an increase in concentration of Mg^{2+} and Ca^{2+} from 3 to 5mM, and production of reducing equivalents (Camp & Randall, 1985). In accordance with these conditions, plastid PDC has been shown to have an alkaline pH optimum and a higher divalent cation requirement than its mitochondrial counterpart (Randall *et al.*, 1989).

Treede and Heise (1985) reported that in spinach chloroplast extracts concentrations exceeding 5mM of free Mg^{2+} increases the apparent V_{max} of the oxidative decarboxylation of pyruvate to acetyl CoA without appearing to influence the K_m for pyruvate. This high divalent cation requirement distinguishes chloroplast PDC from the plant mitochondrial complex where concentrations of Mg^{2+} greater than 5mM are ineffective, or even inhibitory, with respect to PDC activity.

Hence there is increasing evidence supporting the activation of plastid PDC by light, induced by indirect effectors such as pH and a high divalent cation requirement. This regulatory regime is not confined to chloroplasts, but is shared with plastid PDC from non-green tissue. The control of plastid PDC by light/dark transitions is a unique regulatory mechanism amongst all PDCs studied to date.

Covalent Modification:

Plastid PDC, unlike its mitochondrial counterpart, does not appear to be regulated by covalent modification (Williams & Randall, 1979; Camp & Randall, 1985). As all eukaryotic PDCs studied to date are subject to control by this mechanism, the potential phosphorylation of plastid PDC by an intrinsic PDC-kinase was extensively investigated. Camp & Randall (1985) did not record an ATP-dependent phosphorylation with the concomitant inactivation of pea chloroplast PDC. In addition, incubation with pea mtPDC-kinase, beef heart protein kinase or phosphorylase kinase in the presence of ATP did not inactivate the complex. These authors did report that a cAMP-dependent protein kinase was capable of catalysing the transfer of ^{32}P from [γ-^{32}P]-ATP to pea chloroplast PDC. This incorporation, however, was not accompanied by inactivation. Miernyk *et al.* (1988) incubated pea chloroplasts and leucoplasts from *Ricinus* endosperm with a variety of phosphoryl donors, including ATP. Nucleotide dependent inactivation of plastid PDC was not observed and, in addition, immunoprecipitated plastid PDC failed to incorporate ^{32}P from [γ-^{32}P]-ATP. In agreement with other reports (Miernyk *et al.*, 1988), the application of exogenous heterologous protein kinases failed to suppress plastid PDC activity.

The obvious conclusion is that chloroplast PDC, in conjunction with all prokaryotic PDCs, is not susceptible to control by reversible phosphorylation. When considering the role of PDC within the chloroplast compartment, modulating multienzyme activity by covalent modification would be counterproductive. An ATP-dependent inactivation of PDC would preferentially occur during periods of illumination owing to increased levels of ATP when the chloroplast is photosynthetically active. This is inconsistent with PDC proposed function, that of supplying acetyl CoA for fatty acid synthesis, and also works against other regulatory factors in operation, e.g. pH and Mg^{2+} levels.

1.11 SUBUNIT COMPOSITION OF PLANT CHLOROPLAST PYRUVATE DEHYDROGENASE COMPLEX

Pea mitochondrial and chloroplast PDC, although differing in pH optima and Mg^{2+} requirements, share common substrates, cofactors and products. Immunologically, however, they are distinct entities. Based on precipitation of chloroplast PDC with antibodies raised against the mitochondrial multienzyme complex from broccoli the two forms of mulienzyme complex appear to be only partially homologous (Camp & Randall, 1985). Only a proportion of pea chloroplast PDC, 40%, was precipitated. Plastid PDC was more rigorously characterised by these authors by employing the Western blotting procedure using anti-broccoli PDC serum (Camp & Randall, 1985). Differences between partial purifications of pea mitochondria and chloroplast PDC were detectable, namely a diffuse band appearing in chloroplast protein at 66,000Da and the absence of the 43,000 and 37,000Da cross-reacting polypeptides in the chloroplast preparation. Common immunoreactive proteins of M_r 98,000 and 58,000 were observed. The 58,000Da band is thought to represent the E3 subunit, however the identity of the 98,000Da protein remains elusive. It could be the E1 subunit of OGDC which is a common contaminant of PDC preparations; hence this enzyme would be responsive to a cross-reaction with antisera raised to broccoli mtPDC contaminated with E1 subunit of OGDC. When antibodies to the *E. coli* multienzyme complex were assessed for their immune-response with pea chloroplast PDC, no cross-reaction was observed.

Although immunological analysis of pea chloroplast PDC was undertaken by Camp and Randall (1985) exploiting broccoli mtPDC antisera not all the component enzymes were identified and the presence of component X was not investigated. Taylor *et al.* (1992) carried out extensive immunological analyses of pea chloroplast PDC using antibodies to the native bovine heart PDC. Resulting Western blots revealed four prominent bands with a strong immune-response in the M_r range 45,000-50,000.

To characterise these immunoreactive polypeptides more stringently antibodies to specific subunits of mammalian PDC were employed. Chloroplast protein showed no cross-reaction with antisera to mammalian or yeast E1. In contrast a 41,000Da band was

detected in pea mitochondrial protein with antiserum to the E1 subunit of yeast PDC. This polypeptide has been identified as the E1α subunit, as Camp & Randall (1985) classed a mitochondrial protein of similar M_r value as the E1α component of PDC by phosphorylation studies. In view of the different methods of regulation between plant mitochondrial and plastid forms of PDC, it is far from surprising that the respective E1 components from these organelles are antigenically distinct polypeptides.

The E2 component of pea chloroplast PDC was identified by Taylor *et al.* (1992) at M_r 50,000, quite distinct from the mammalian enzyme (M_r 67,000). Again the M_r value is similar to the analogous yeast enzyme and is consistent with the presence of only one lipoyl domain. It is possible that the plant enzyme contains one lipoyl moiety, paralleling the situation in mammalian OGDC and BCDC, as opposed to two which are present on the E2 from mammalian PDC.

A pea chloroplast protein gave a strong immune response with antibodies to component X from bovine heart PDC (Taylor *et al.*, 1992). Further characterisation of this chloroplast protein is required before the function of X in plastid PDC can be resolved. Unlike the E3 from pea mitochondria, which is detected at an M_r value of 67,000, a pea chloroplast protein of M_r 52,000 cross-reacted with mammalian anti-E3 in accordance with the M_r value of mammalian enzyme. This observation, coupled with the revelation that pea chloroplast and mitochondrial PDC express differing salt sensitivities (A. Taylor, personal communication), suggests the existence of organelle-specific forms of this enzyme.

1.12 PLANT 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

In comparison with PDC, research into plant OGDC and BCDC has barely entered its infancy, with only a few tentative reports concerning these plant complexes emerging in past years. As a consequence characterisation of plant PDC's sister multienzyme complexes has been far from systematic.

Prior to 1970 attempts to monitor a plant OGDC-catalysed reaction was hampered by the presence of high levels of NADH oxidase activity (Poulsen & Wedding,

1970). The assessment of purification techniques by enzymatically detecting increases in specific activity was therefore impractical as reaction rates were masked by NADH oxidation. Coupled with this, efforts to purify plant OGDC applying techniques which had proved successful with the mammalian multienzyme complex, e.g. pH and protamine precipitation or gel filtration resulted in the partial or complete dissociation of the E3 component from the remainder of the complex. This may have been a consequence of the purification procedure imposed on a complex to which the peripheral enzymes are only loosely associated. It could however reflect the *in vivo* situation: there is a possibility that only a proportion of the E3s are associated with the 2-oxoacid dehydrogenase complexes, leaving a percentage of unbound subunit free in the matrix phase. To date the reality remains unclear, nevertheless, in order to reach a level of intact complex activity where the potential rates of E1 and E2 are not limited by E3, the introduction of mammalian E3 is essential. This is indicative of E3 dissociation from the core structure.

This phenomenon lead Poulson and Wedding (1970) to concentrate their efforts on the purification of a partial OGDC complex, the E1/E2 core, from cauliflower mitochondria. By a process of ammonium sulphate fractionation they purified the 'subcomplex'. Consequently, intact OGDC activity was only discernable with the addition of exogenous E3 purified from pig heart. Therefore mammalian E3, which is specific for lipoate, must associate with the cauliflower core assemblage. This partial complex displayed very similar properties to the mammalian and bacterial counterparts previously isolated. It had an absolute requirement for 2-oxoglutarate, CoA, TPP, exogenous E3 and consequently NAD^+. As bound TPP was found to be absent from the cauliflower OGDC E1/E2 subcomplex it was possible to ascertain that the preferred cofactor of E1 was Mg^{2+} conjugated to TPP. Mg^{2+}-TPP was found to dissociate readily from the complex and may reflect one of the control mechanisms which is operative in plants.

Cauliflower OGDC has been reported to be activated by AMP (Wedding & Black, 1971; Wedding & Black, 1977; Craig & Wedding, 1980). This nucleotide stimulation increases maximum velocity by as much as 3-fold and decreases the K_m for 2-oxoglutarate by 10-fold. The multienzyme complex from cauliflower, in common with all other 2-oxoacid dehydrogenase complexes characterised to date, has an absolute

divalent cation requirement. Ca^{2+}, Mg^{2+} and Sr^{2+} can all be utilised by cauliflower mitochondrial OGDC; however a strong preference for Mg^{2+} is displayed. In the presence of AMP, OGDC activity is stimulated and equal rates are attained regardless of which divalent cation is introduced. It has been postulated that AMP results in the tighter binding of Mg^{2+}-TPP to the E1 component of cauliflower OGDC. This observation was also documented by Craig and Wedding (1980).

Pyruvate has also been reported to exert an inhibitory effect on the activity of plant OGDC (Dry & Wiskich, 1985; 1987). CoA is utilised by both PDC and OGDC resulting in competition between the two multienzyme complexes for available substrate. It has been proposed that the observed pyruvate inhibition of OGDC is a consequence of PDC and OGDC interaction via a limited CoA pool size in the mitochondrial matrix.

In the plant cell OGDC activity has only been detected in the mitochondrial compartment. To our knowledge it is located exclusively in mitochondria; however the only other organelle to be screened for the presence of OGDC is the chloroplast. To date enzymatic detection of OGDC in chloroplasts has never been reported. It has not been part of a long-term research programme and therefore the absence of this multienzyme from the chloroplast compartment cannot be completely ruled out. Optimal assay conditions may not have been reached, or concentrations of the complex may be so low that enzymic detection is impractical, or the multienzyme may be controlled by a subtle regulatory regime which has yet to be appreciated.

1.13 PLANT BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEX

BCDC activity has particular relevance with respect to protein turnover as it catalyses a committed step in the breakdown of branched-chain amino acids, formed by transamination of leucine, isoleucine and valine, and is also thought to be involved in the catabolism of methionine and threonine. Consequently, BCDC plays a vital role in the course of steady state protein turnover. Plant BCDC has never been the subject of extensive investigation. Information with regard to structure, subunit composition,

regulation and intracellular location is extremely limited, and even lags behind characterisation of plant OGDC.

BCDC activity in plant cells has only been detected in the peroxisomes of mung bean hypocotyls (Gerbling & Gerhardt, 1988; 1989). These authors demonstrated a CoA- and NAD^+-dependent reaction with the concomitant formation of NADH and acyl CoA. The cofactor requirements, product analyses, and the observed stoichiometries are consistent with a BCDC-catalysed reaction. By detecting intermediate acyl CoAs by HPLC, Gerbling and Gerhardt (1989) proposed pathways for the peroxisomal degradation of 2-oxoisocaproate, 2-oxoisovalerate and 2-oxo-3-methylvalerate. Their results infer that branched-chain amino acids are oxidatively decarboxylated by BCDC activity. The degradation of fatty acids, once activated by long-chain acyl CoA synthetase or, by the evidence of this report, BCDC, by β-oxidation is a basic metabolic function of higher plant peroxisomes. After formation of the appropriate acyl CoA from branched-chain 2-oxoacid, a BCDC-catalysed reaction, intermediates are further metabolised by β-oxidation to acetyl CoA. This is the final product of branched-chain 2-oxoacid degradation in higher plant peroxisomes.

Gerbling and Gerhardt (1988) reported the absence of BCDC activity in mitochondria and plastids from mung bean hypocotyls and suggested that plant BCDC is located exclusively in peroxisomes. By assessing NADH formation they have unambiguously recorded peroxisomal BCDC activity. Specific activities were in the pmol NADH/sec/mg range, as opposed to activities of nmol NADH/sec/mg consistently recorded for mitochondrial PDC. It should be noted, however, that their search for plant BCDC is confined to the organelles of mung bean hypocotyls. In addition it is feasible that a plant mitochondrial located BCDC is predominately inactivated by a phosphorylation mechanism. This would parallel the situation with mammalian BCDC where, in certain tissue, a large proportion of the multienzyme complex is in the inactive phosphorylated state. To our knowledge reactivation of a prospective plant mitochondrial or plastid BCDC by stimulating the phosphatase has never been documented. Consequently, the presence of a latent mitochondrial or plastid BCDC cannot be discounted at present.

1.14 GLYCINE DECARBOXYLASE

A fourth multienzyme complex worthy of note is glycine decarboxylase (GDC) or the glycine cleavage system. This complex has been purified from plants (Walker & Oliver, 1986; Bourguignon *et al.*, 1988), animals (Kikuchi & Hiraga, 1982) and bacteria (Klien & Sagers,1966; 1967) and catalyses the reversible cleavage of glycine forming CO_2, 5,10-methylene tetrahydrofolate, NH_4^+ and NADH. As with the 2-oxoacid dehydrogenase complexes, glycine is oxidatively decarboxylated in a multi-step process involving the sequential actions of a number of distinct enzymes. GDC consists of four proteins designated P, H, T and L (bacterial counterpart nomenclature P1 to P4 respectively). In eukaryotes GDC is restricted to mitochondria where it is believed to exist as a labile multicomponent complex maintaining a loose association with the inner membrane.

Glycine metabolism plays an important role in plants with respect to photorespiration with GDC occupying a key position in this pathway (Fig. 1.9). The photorespiratory pathway involves the co-operation of three organelles; glycolate, formed in chloroplasts, is shuttled to leaf peroxisomes where it is converted by peroxisomal enzymes to glycine. Glycine then migrates to leaf mitochondria and is subsequently decarboxylated by GDC (Fig. 1.9). Although photorespiration results in the net consumption of energy, GDC does salvage reducing power which is fed into the mitochondrial electron transport chain.

In common with other enzymes involved in photosynthetic and photorespiratory processes, GDC synthesis is light dependent. Low amounts of its constituent enzymes are present in etiolated leaves. Upon illumination however, there is a rapid synthesis of GDC (Day *et al.*, 1985; Walker & Oliver, 1986). In addition Gardestrom *et al.* (1980) observed that glycine cleavage in spinach is confined to leaf mitochondria indicating that GDC is also subject to spatial regulation. This spatial control was also recorded in pea by Walton and Woolhouse (1986) who reported that GDC activity in non-photosynthetic tissue was 2-5% of that in the leaves. Finally Macherel *et al.* (1990) and Kim and Oliver (1990) recorded negligible levels of GDC activity in non-photosynthetic pea tissue. In

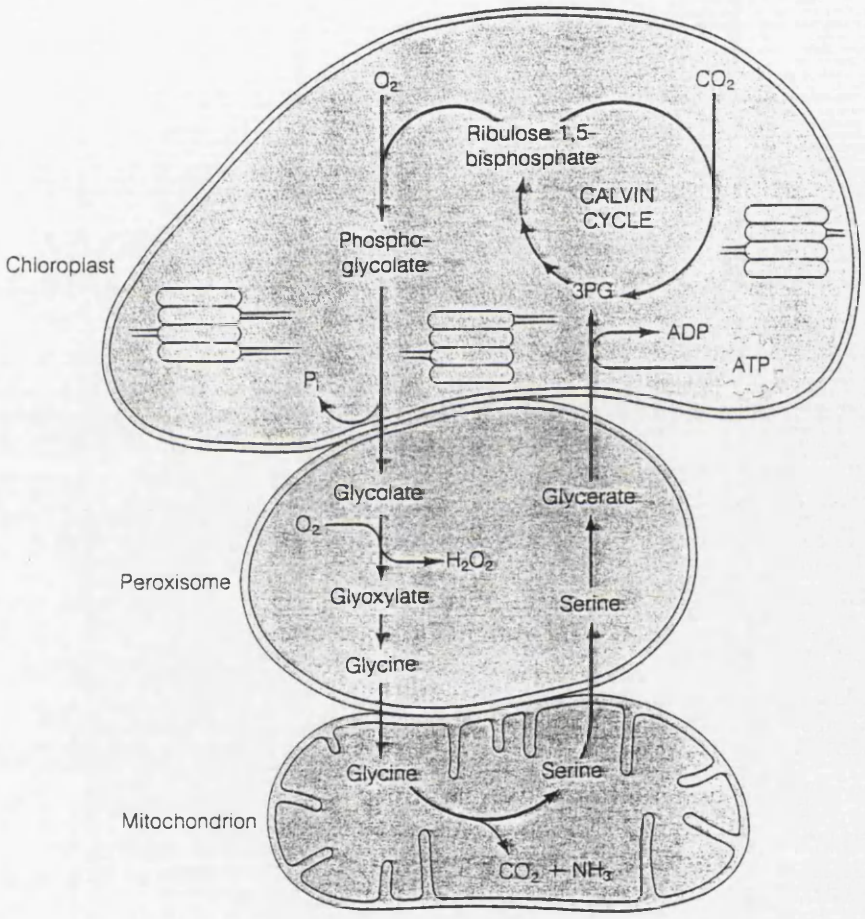


Figure 1.9: Photorespiratory pathway illustrating the co-operation of chloroplast, peroxisomes and mitochondria. Reproduced from Biochemistry (Mathews & van Holde, p666)

contrast GDC constitutes one third of the soluble matrix protein of pea leaf mitochondria (Oliver *et al.*, 1990).

Many parallels can be drawn between GDC and the 2-oxoacid dehydrogenase complexes. Analogies become apparent when examining the mechanistics of the reaction sequence (Fig. 1.10). Glycine binds to the P-protein via the pyridoxal phosphate (PLP) cofactor and is decarboxylated. The resulting aminomethyl moiety is transferred onto the lipoic cofactor of the small heat-stable H-protein. Although the P-protein catalyses the true decarboxylation reaction, the reaction rate is slow when it acts independently. The H-protein binds prior to CO_2 release increasing the rate of decarboxylation by several orders of magnitude. The next enzyme in the sequence, the T-protein, facilitates the release of ammonia from the methylamine intermediate bound to the H-protein. Tetrahydrofolate serves as an acceptor for the remaining 1-carbon unit, forming 5,10-methylenetetrahydrofolate. The mitochondrial isoenzyme of serine hydroxymethyl-transferase (SHTM; EC 2.1.2.1) then transfers the C_1 group of 5,10-methylene tetrahydrofolate to a second glycine molecule to yield serine. The lipoic acid moiety on the H-protein is left in a reduced state and the oxidised form is regenerated by the action of the L-protein, a flavin-requiring dihydrolipoamide dehydrogenase, resulting in formation of the final product of the complex reaction sequence, NADH. In common with the reaction mechanism of the 2-oxoacid dehydrogenases, GDC employs a lipoic acid containing component enzyme. The gene encoding the H-protein of pea mitochondrial GDC has been cloned and sequenced and was found to be strongly light-induced, a factor generally displayed by photorespiratory enzyme systems (Kim & Oliver, 1990; Macherel *et al.*, 1992). The amino acid sequence was determined and displayed strong homology to the analogous enzyme from chicken liver especially surrounding the lysine residue which binds the lipoyl moiety. The sequences surrounding the lipoate attachment site of H-proteins and other lipoate containing proteins, e.g. the E2 enzymes of the 2-oxoacid dehydrogenase complexes, are highly conserved (Yeaman, 1989). The primary structure of the H-protein has been characterised by combined liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry (Merland *et al.*, 1993) and displays an M_r value of 14,000. The lipoic acid

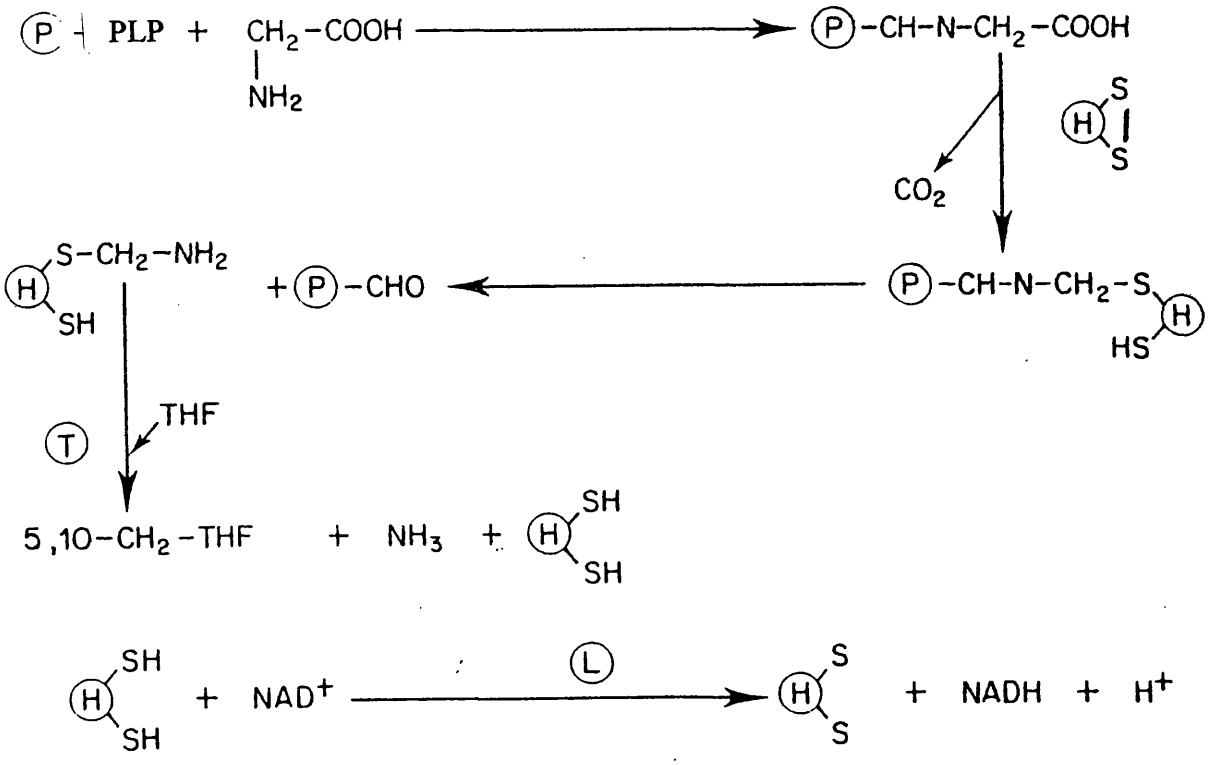


Figure 1.10: Reaction mechanism of glycine decarboxylase complex. P, H, T and L in the circles represent the component enzymes in volved in the catalysis of glycine. THF = tetrahydrofolate.

cofactor was located, attached to a lysine residue, as in the E2 enzymes of the 2-oxoacid dehydrogenase complexes.

Both the glycine cleavage system and the 2-oxoacid dehydrogenase complexes utilise a dihydrolipoamide dehydrogenase, designated the L-protein and E3 respectively. It is GDCs' employment of dihydrolipoamide dehydrogenase that is of particular relevance to this investigation. Dihydrolipoamide dehydrogenase, as a component enzyme associated with PDC, OGDC, BCDC and GDC, has been the focus of extensive research. An important question when considering this conservative enzyme is whether an ubiquitous form of dihydrolipoamide dehydrogenase exists, i.e. the same gene product, or are complex and organelle specific forms of the enzyme expressed?

Turner *et al.* (1992) succesfully purified the L-protein (dihydrolipoamide dehydrogenase) of GDC from pea leaf mitochondria and obtained a single band of M_r 60,000 on SDS-PAGE. Using antibodies to the purified dihydrolipoamide dehydrogenase they identified possitive cDNA clones from a pea leaf and embryo λgt11 expression library. The deduced amino acid sequence exhibited high homology to dihydrolipoamide dehydrogenase from *E. coli*, yeast and humans. No spatial regulation of dihydrolipoamide dehydrogenase was observed as comparable levels of dihydrolipoamide dehydrogenase were recorded in the leaves, stem and embryo indicating that the enzyme is required by other systems in these tissues. Consequently, their research points to a single plant dihydrolipoamide dehydrogenase which associates with the 2-oxoacid dehydrogenase complexes and GDC.

Concurrent with the above study Bourguignon *et al.* (1992) also isolated and characterised a cDNA clone encoding the L-protein of pea leaf mitochondrial GDC. In agreement with Turner *et al.* (1992) the evidence indicates that in higher plants a single form of dihydrolipoamide dehydrogenase is expressed.

Recent unpublished work on dihydrolipoamide dehydrogenase at Glasgow University suggests that the situation in higher plants, such as potatoes, may be more complex. Bourguignon *et al.* (1988), whilst characterising the component enzymes of pea leaf mitochondrial GDC recorded a single dihydrolipoamide dehydrogenase activity released from a Mono Q HR 5/5 column. Contrary to this, Millar (Glasgow University, personal communication), by expanding the phosphate gradient, resolved three distinct peaks of potato mitochondrial dihydrolipoamide dehydrogenase activity by anion exchange chromatography. These observations suggest that a number of isoenzymes may be involved in the reoxidation of lipoyl moieties in some plants. This oberservation is currently the focus of intensive investigations in order to fully characterise these differing forms of dihydrolipoamide dehydrogenase in potato mitochondria.

1.15 AIMS OF THIS THESIS

With the wealth of information available on the mammalian and microbial 2-oxoacid dehydrogenase complexes, it is clear that, with the exception of PDC, the plant counterparts have been somewhat neglected. This project attempts to redress the balance and tackles such areas as intracellular distribution, basic kinetic characterisation and subunit composition of the multienzyme complexes from pea and potato.

Enzymatic analyses of the plant complexes is performed, unambiguously locating the 2-oxoacid dehydrogenase complexes in specific organelles. In addition the availability of antiserum to the bovine heart 2-oxoacid dehydrogenase complexes and to specific subunits facilitates the immunological detection of this multienzyme family from a plant source. This avenue of investigation, which relies on sufficient homology between the two kingdoms, allows M_r estimation of the of the respective plant component enzymes.

The latter section of the research programme concentrates on the E3 subunit from potato mitochondria. Ongoing research at Glasgow University has uncovered a unique situation regarding this enzyme. As E3 is involved in the catalysis of both 2-oxoacids and glycine the possibility of complex specific isoforms of potato mitochondrial E3 is addressed.

It is clear that 2-oxoacid dehydrogenase complexes occupy strategic positions in central metabolism. Owing to the irreversibility of their reaction sequences these multienzyme complexes also exert regulatory powers over many biosynthetic pathways. Consequently, charaterisation of the plant 2-oxoacid dehydrogenase complexes will aid our basic understanding of plant cellular metabolism.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS

All chemicals were obtained from Sigma (Poole, Dorset) unless otherwise stated in the text. Thirum treated pea seeds (*Pisium sativum* L., cv. Little Marvel) were purchased from Sharpes International Seeds Ltd. (Sleaford, Linconshire) and and other plant material from a local market (Rosemans, Glasgow Fruit Market)

METHODS

2.1 GROWTH OF PLANTS

Pea seeds (*Pisium sativum* L., cv. Little Marvel) were grown in moist vermiculite at 200mEm^{-2}s^{-1} in a growth chamber with a 12 h photoperiod (22^{0}C light/18^{0}C dark). Root material was harvested from 4 day old seedlings in order to avoid the development of an extensive lateral root system. Leaf tissue was collected 11 days after planting allowing full expansion of primary leaves.

Potato tubers (*Solium tuberosum*, cv. Maris Piper) were placed in the dark to initiate the growth of sprouts. Sprouts were removed with approx. 3-4cm of tuber tissue, planted in pots of compost and grown at 200μmolm^{-2}s^{-1} of photosynthetically active light in a growth chamber with a 12 h photoperiod (22^{0}C light/18^{0}C dark). After the primary leaves had emerged (approx. 1 week) the plants were re-potted and leaf tissue was collected for harvesting approx. 3 weeks later.

2.2 ISOLATION OF ORGANELLES

2.2.1 MITOCHONDRIAL ISOLATION

Mitochondria from both root/tuber and leaf tissue were isolated by a process of differential centrifugation employing a modified version of mitochondrial preparation described by Day *et al.* (1988). All procedures were carried out at 4°C. Tissue was ground in a polytron homogeniser (System Technik, Ruschlikon, Switzerland) in 3-4 volumes of grinding buffer containing 0.3M sucrose, 10mM KH_2PO_4, 2mM EDTA, 2mM $MgCl_2$, 1%(w/v) polyvinyl pyrrolidone 40 (PVP-40), 1%(w/v) bovine serum albumin (BSA) and 25mM sodium phosphate buffer. 30mM iso-ascorbic acid was added fresh on the day of preparation and the pH adjusted to 7.6. The homogenisation process was restricted to four short bursts at speed five on the polytron to limit organelle damage.

The homogenate was filtered through six layers of pre-wetted muslin into cold centrifuge tubes containing protease inhibitors (final concentrations: 1mM phenylmethylsulphonyl fluoride (PMSF), 1mM benzamidine-HCl and 10μM leupeptin) and centrifuged at 1000.*g* for 5 min in a Sigma 3K-20 centrifuge. The supernatant was retained and centrifuged for 20 min at 12000.*g*. The crude mitochondrial pellet was resuspended using a hand homogeniser in 30ml of wash buffer (0.3M sucrose, 1mM glycine, 0.1%(w/v) BSA and 10mM [2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino ethane-sulphonic acid (TES) pH 7.2). The 1000.*g* and 12000.*g* centrifugations were repeated and the mitochondrial pellet resuspended in approximately 2ml of wash buffer. The mitochondrial extract was then layered onto a 30%(v/v) solution of Percoll, buffered with 10mM potassium phosphate pH 7.2 containing 1mM EDTA and 0.1%(w/v) BSA, and centrifuged at 40000.*g* for 30 min in an ultracentrifuge (OTD-65B; Sorvall Instruments, Stevenage Herts., U.K.). The mitochondria formed a distinct broad band near the bottom of the self generated gradient.

The mitochondrial fraction was retrieved with a Pasteur pipette, care was taken when removing the leaf mitochondrial fraction as the upper region of the gradient was heavily laden with membrane fragments, presumably of chloroplast origin. The

mitochondrial fraction was diluted in 5 volumes of wash buffer minus glycine and centrifuged at 12000.*g* for 15 min to remove the Percoll. The upper section of the supernatant was removed and the pellet further diluted with wash buffer minus glycine and BSA. This series of dilution and centrifugation steps (12000.*g*/15 min) was continued until a firm mitochondrial pellet was formed. The final pellet was resupended in a minimal volume of wash buffer minus glycine and BSA.

2.2.2 CHLOROPLAST ISOLATION

Chloroplasts were harvested from leaf tissue according to a slightly modified method described by Murphy & Leech (1977; 1978). All procedures were carried out at 4°C. Using 3-4, four sec bursts on a polytron homogeniser (speed 3) approx. 100g of leaf tissue was ground in 3-4 volumes of grinding buffer (described in 2.2.1). The homogenate was filtered through six layers of pre-wetted muslin, a protease inhibitor mixture added (1mM PMSF, 1mM benzamidine-HCl and 10μM leupeptin), then the samples were centrifuged at 200.*g* for 3 min in a Sigma 3K-20 centrifuge. The supernatant was subsequently centrifuged at 3020.*g* for 3 min. The mitochondria were confined to the supernatant while the chloroplast fraction was pelleted.

The crude chloroplast pellets were gently resuspended with a Pasteur pipette in approximately 5ml of buffer A (0.3M sorbitol, 4.4mM sodium pyrophosphate, 1mM EDTA, 3.5mM $MgCl_2$ and 50mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine) pH 8.0; 2-mercaptoethanol (13.5mM) was introduced immediately prior to use). Chloroplast pellets were washed twice by resuspending in buffer A and repelleting, the final pellet was resuspended in 10ml of Buffer A and layered onto a 10ml volume of buffer B (as buffer A, except 0.6M sorbitol) and centrifuged at 420.*g* for 5 min. The resulting pellet was resuspended in a minimal volume of buffer A with the addition of 1mg/ml dithiothreitol (DTT).

Intact chloroplasts were further purified by isopycnic centrifugation in Percoll gradients following a slightly modified procedure of Mourioux & Douce (1981). A continuous gradient of Percoll medium (50%(v/v) Percoll, 330mM sorbitol, 2mM EDTA

and 0.15%(w/v) BSA and 50mM 3-(N-morpholino) propane-sulphonic acid (MOPS) pH 7.8) was pre-formed by centrifuging at 10000.*g* in a Sigma 3K-20 centrifuge for 100 min. The crude chloroplast suspension was subsequently loaded onto the gradients and centrifuged at 5000.*g* for 5 min. Intact chloroplasts formed a broad band near the bottom of the gradient whereas stripped chloroplasts and extrachloroplastic membranes failed to enter the gradient and were confined to the sample-gradient interface. Intact chloroplasts were diluted in 5 volumes of buffer A and recovered by centrifugation (3020.*g*/3 min). The final pellet was resuspended in a minimal volume of buffer A.

2.2.3 PEROXISOME ISOLATION

Peroxisomes from pea root and leaf tissue were prepared following a procedure described by Behari & Baker (1993) with slight modifications. All procedures were carried out at 4°C. Tissue was gently homogenised in grinding buffer (2.2.1) for 4 x 5 sec using a polytron at speed 2 and filtered through six layers of pre-wetted muslin. A protease inhibitor mixture was introduced (1mM PMSF, 1mM benzamidine-HCl and 10μM leupeptin) and the homogenate was then subjected to a series of 1000.*g* and 12000.*g* centrifugations, as in mitochondrial preparation (2.2.1). The resulting pellet was resuspended in 2-4ml of buffer A (0.5M sucrose, 10mM KCl and 1mM EDTA and 25mM 2-(N-morpholino)ethanesulphonic acid (MES) pH 6.0) and loaded onto a step gradient of 10ml of 35%(w/v) 5-(N-2,3-Dihydroxypropylacetamido)-2,4,6-Triiodo-N,N'-bis(2,3-Dihydroxypropyl)-isophthalamide (Nycodenz) dissolved in buffer A overlaid with 8ml 25%(w/v) Nycodenz in buffer A.

The gradient was spun at 32000.*g* in a swing-out rotor in an Sorvall ultracentrifuge for 2.5 h , fractionated from the bottom and the 1ml fractions assayed for hydroxypyruvate reductase activity. The fractions exhibiting maximal activity were pooled, diluted in 5 volumes of buffer B (0.5M sucrose, 10mM KCl and 1mM $MgCl_2$, 52mM MES-KOH pH 6.0,) and centrifuged for 30 min at 12000.*g*. The resulting pellet was gently resuspended with a Pasteur pipette in a minimal volume of buffer B.

2.2.4 ROOT PLASTID ISOLATION

Root plastids were prepared as described by Emes & England (1986). All procedures were carried out at 4°C. Root tissue was homogenised in an equal volume of buffer A (0.33M sorbitol, 1mM EDTA, 1mM $MgCl_2$ and 0.1% BSA(w/v) and 50mM Tricine-NaOH pH 7.9) for 20 sec using a polytron at speed 3. The brie was filtered through six layers of pre-wetted muslin, protease inhibitor mixture introduced (1mM PMSF, 1mM benzamidine-HCl and 10μM leupeptin) and the sample centrifuged at 200.*g* for 1 min in a Sigma K12 centrifuge. The supernatant was decanted and centrifuged for 3 min at 4000.*g* in a fixed angle rotor. Each crude plastid pellet was subsequently gently resuspended in 10ml buffer A minus BSA. Each 10ml aliquot was underlayed with 10ml of 10%(v/v) Percoll in buffer B (0.33M sorbitol and 50mM Tricine-NaOH pH 7.9) and centrifuged in a swing out rotor for 5 min at 4440.*g*. The supernatant was discarded and the resultant plastid pellet resuspended in a minimal volume of buffer B.

2.3 ELECTRON MICROSCOPY

Isolated organelle preparations were spun at 12000.*g* for 5 min in a bench top microfuge and resuspended in fibrinogen. Thrombin was added which initiates the formation of a fibrin clot. This was then cut into smaller pieces and fixed in 3%(w/v) glutaraldehyde in 0.2M phosphate buffer for 3 h. The specimens were then rinsed (3x10 min) in 0.2M phosphate buffer and postfixed for a 1 h period in 2%(w/v) osmium tetroxide. The organelles were then dehydrated through a graded series of ethanols to 100% and subsequently embedded in spurr resin and polymerised at 60°C for 20 h. Sections were cut with a diamond knife on an L.K.B.111 microtome and picked up on 300 mesh copper grids. The individual sections were stained with methanolic uranyl acetate and lead citrate and viewed on a Zeiss 902 electron microscope.

2.4 PROTEIN DETERMINATION

Protein concentrations were estimated by a modification (Markwell *et al.*, 1978) of a method originally described by Lowry *et al.* (1951) using BSA fraction V as a standard.

2.5 ASSAY PROCEDURES

2.5.1 SUCCINATE DEHYDROGENASE ASSAY: (EC 1.3.99.1)

Mitochondrial marker enzyme.

Succinate dehydrogenase activity was determined spectrophotometrically adopting a modified method of Veeger *et al.* (1969). The reaction mixture comprised 0.12M sodium phosphate buffer (pH 7.5) containing 1.7mM KCN, 0.05%(w/v) phenazine methosulphate (freshly made due to light sensitivity) and protein extract; 50µl of 0.005%(w/v) 2,6 dichlorophenol-indo-phenol (DCPIP) was introduced to the reaction mixture which was then placed in a 1cm light path in a Phillips Pye Unicam SP8-500 UV/VIS spectrophotometer and the absorbance at 600nm recorded for 2 min. The reaction was initiated with 60mM sodium succinate and the decrease in absorbance at 600nm determined (extinction coefficient for DCPIP: $21 mM^{-1}cm^{-1}$).

2.5.2 HYDROXYPYRUVATE REDUCTASE ASSAY: (EC 1.1.1.81)

Peroxisomal marker enzyme.

Hydroxypyruvate reductase was assayed spectrophotometrically by monitoring the consumption of NADH following the protocol described by Tolbert (1970). The final 1ml assay mixture contained 0.2mM NADH, 5mM potassium phosphate buffer pH 6.2 and variable amounts of organelle protein. Reactions were initiated by the addition of 1mM hydroxypyruvate and the decrease in absorbance at 340nm monitered on a Phillips

Pye Unicam SP8-500 UV/VIS spectrophotometer (extinction coefficient for NADH: $6.22mM^{-1}cm^{-1}$)

2.5.3 ADP-GLUCOSE PYROPHOSPHORYLASE ASSAY: (EC 2.7.7.27)

Plastid marker enzyme.

ADP-glucose pyrophosphorylase activity was determined spectrophotometrically employing the method described by Jouret & Douce (1985). The 1ml assay mixture contained 1mM pyrophosphate, 5mM NaF, 1mM adenosine 5'-diphosphoglucose disodium salt (ADPG), 5mM 3-phosphoglycerate acid (PGA), 5mM $MgCl_2$, 2mM DTT, 0.5mM $NADP^+$, 0.8units phosphoglucomutase and 0.7units glucose-6-phosphate dehydrogenase and 45mM Tricine pH 7.5. Reactions were initiated by addition of varying amounts of organelle protein and the production of NADPH determined by monitoring the increase in absorbance at 340nm recorded on a Phillips Pye Unicam SP8-500 UV/VIS spectrophotometer (extinction coefficient for NADH: $6.22mM^{-1}cm^{-1}$)

2.5.4 2-OXOACID DEHYDROGENASE COMPLEX ASSAY:

2-oxoacid dehydrogenase activity was determined spectrophotometrically following a modified method of Camp and Randall (1985). The final 1ml assay mixture contained 0.2mM thiamine pyrophosphate (TPP), 5mM $MgCl_2$, 1.4mM β-NAD^+, 2.6mM cysteine-HCl, 0.12mM lithium CoA (freshly prepared), 50mM Tricine pH 8.0 and varying amounts of solubilised organelle protein. The specific 2-oxoacid dehydrogenase complex reactions were initiated by the addition 0.15mM of the appropriate 2-oxoacid substrate; pyruvate (PDC), 2-oxoglutarate (OGDC) or 2-oxobutyrate, 2-oxoisocaproate, 2-oxovalerate, 4-methylthio-2-oxobutyrate and 2-oxo-β-methyl-n-valerate (BCDC). The 2-oxoacids were prepared fresh to avoid the formation of dimers. The production of NADH was monitored at 340nm using a Phillips Pye Unicam SP8-500 UV/VIS spectrophotometer (extinction coefficient for NADH: $6.22mM^{-1}cm^{-1}$)

2.5.5 DIHYDROLIPOAMIDE DEHYDROGENASE (E3) ASSAY: (EC 1.8.1.4)

Dihydrolipoamide dehydrogenase activity was determined spectrophotometrically following a method of Reed *et al.* (1958). The final 1ml assay mixture contained 100mM phosphate bufer (pH 7.8), 0.8mM β-NAD^+ and organelle extract. Reactions were initiated by introduction of 0.2mg of dihydrolipoamide (for preparation see 2.6) and the production of NADH monitored by recording the increase in absorbance at 340nm using a Phillips Pye Unicam SP8-500 UV/VIS spectrophotometer (extinction coefficient for NADH: 6.22$mM^{-1}cm^{-1}$).

2.6 PREPARATION OF DIHYDROLIPOAMIDE

Dihydrolipoamide was prepared following a method of Reed *et al.* (1958). 400mg of lipoamide (thiocotic acid) was dissolved in 80%(v/v) methanol at 4°C. 400mg of sodium borohydride was dissolved, again at 4°C, in 2ml of distilled water and added to the lipoamide/methanol. The solution was stirred at room temperature until it went clear (approx. 45 min).

The solution was subsequently acidified with 0.25M HCl to pH 2.0 and extracted with 40ml chloroform. Dihydrolipoamide, restricted to the bottom layer, was dried down under N_2. The material was redissolved by stirring on a hot plate in 40-60ml of toluene/heptane (2.5:1.0). The solution was again dried down under N_2 until a minimal volume of liquid remained which was allowed to crystallise naturally and stored at -20°C. For use approx. 7mg of dihydrolipoamide was dissolved in 1ml of ethanol.

2.7 SODIUM DODECYL SULPHATE (SDS) POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Prior to electrophoresis, crude organelle proteins were precipitated by the addition of 80%(v/v) acetone and storage at -20°C for 1 h. Samples were centrifuged at 12000.*g* for 20 min in a Sigma 3K-20 centrifuge to pellet precipitated protein. After aspiration of residual acetone with N_2, the pellets were resuspended in sample buffer (2.5%(w/v) SDS, 10%(w/v) sucrose, 10mM DTT, 0.01%(w/v) pyronin Y and 62.5mM Tris-HCl pH 6.8) and dissociated by boiling for 3 min. Proteins were subsequently resolved on 11.5%(w/v) polyacrylamide gel slabs (16x16x0.5cm), with 5%(w/v) stacking gel, in the discontinuous buffer system of Laemmli (1970). Prestained SDS-PAGE molecular weight markers were introduced, which are electrophoretically transferred onto nitrocellulose filters, to estimate M_r values; β-Galactosidase, fructose-6-phosphate kinase, pyruvate kinase, ovalbumin, lactic dehydrogenase, triosephosphate isomerase displaying apparent M_r values of 116,000, 96,400, 80,400, 55,700, 43,700 and 38,800 respectively.

2.8 WESTERN BLOTTING

After plant proteins were separated by gel electrophoresis the proteins were electrophoretically transferred, in the presence of buffer containing 50mM glycine, 0.02% SDS, 25mM Tris-base and 20%(v/v) methanol buffer system, onto a nitrocellulose filter as previously described by De Marcucci *et al.* (1985). The filters were subsequently incubated with the appropriate primary antiserum diluted 50-fold with wash buffer (155mM NaCl, 0.5%(v/v) Tween 20 and 20mM Tris-HCl pH 7.2) and immunoblotted with ^{125}I-labelled protein A. Immunoreactive polypeptides were visualised by exposure to Fuji Medical X-ray film (Fuji Photo Film Co., Ltd.) at -80°C.

2.9 SILVER STAINING

After SDS-PAGE on 11.5%(w/v) gel slabs (16x16x0.1cm), plant proteins were visualised by silver staining following a method described by Heukeshoven & Dernick (1985). Protein was fixed by immersing the gel in fixing solution (40%(v/v) ethanol and 10%(v/v) acetic acid). The fixing process, which precipitates the protein allowing SDS to diffuse out, takes at least 30 min. The gel was then transferred to an incubation medium (30%(v/v) ethanol, 500mM sodium acetate trihydrate, 12mM sodium thiosulphate and 0.5%(w/v) glutaraldehyde). After washing with distilled water the silver solution was introduced (5mM silver nitrate with 0.01%(w/v) formaldehyde) for 40 min. After a distilled water wash, the proteins were visualised by placing in developing solution (90mM sodium carbonate with 0.005%(w/v) formaldehyde) for approximately 40 min. The reaction was terminated by the addition of 40mM EDTA for 10 min and finally washed in distilled water. For long term storage the gels were placed in 10%(v/v) glycerol.

2.10 *IN SITU* IMMUNOFLUORESCENT LOCALISATION OF ANTIGENS

In situ immunofluorescent localisation of antigens was carried out as described by Marrison & Leech (1992).

2.10.1 FIXING AND EMBEDDING

2-3mm root and leaf sections were cut from 5-day old pea seedlings and immediately placed in fixative (3%(w/v) paraformaldehyde, 50%(v/v) ethanol and 5%(v/v) acetic acid) at room temperature for approximately 14 h. The fixative was then removed and the tissue dehydrated by a series of 15 min ethanol incubations: 75%(v/v), 85%(v/v), 4 x 95%(v/v) and 4 x 100%. The final ethanol mix was made 10%(v/v) with respect to Polyethylene glycol (PEG) 1500 (Fisons, Loughborough) and rotated in a water bath at

56°C for 30 min. The tissue was subsequently infiltrated with PEG by placing in increasing PEG/ethanol solutions at 56°C: 30 min in 20%(v/v), 30 min in 50% (v/v), 30 min in 70%(v/v), 1 h in 100% and overnight in 100% PEG. The samples were then placed in fresh PEG (100%) at 56°C for at least 14 h. The sections were then positioned in peel-away moulds (Polysciences Inc., Moulton Rd., Hampshire), embedded with molten PEG and allowed to set overnight at room temperature. For long term storage the blocks were placed in a dessicated box at 4°C.

2.10.2 PREPARATION OF POLY-L-LYSINE COATED SLIDES

To ensure that the tissue sections remained fixed to the slide during the immunolabelling procedure it was essential to use slides coated with poly-L-lysine. Glass slides were thoroughly cleaned prior to treatment by washing in acid and alcohol (1%(w/v) HCl and 70%(v/v) ethanol) for 2 h. The slides were subsequently soaked in poly-L-lysine hydrobromide (50µg/ml) at room temperature for 5 min. After wrapping in tin foil they were then heated at 80°C for 2 h.

2.10.3 SECTIONING

The samples, now embedded in PEG, were removed from the moulds and mounted onto wooden blocks using molten PEG and allowed to set well at 4°C. Using a stainless steel blade on a microtome, 7µm sections of pea root and leaf tissue were cut. These were carefully layered onto poly-L-lysine slides, sprayed lightly with distilled water to aid adhesion, and dried down at 40°C overnight.

2.10.4 IMMUNOFLUORESCENT LABELLING

The slides were soaked in distilled water to remove the PEG surrounding the tissue sections. After the slides were allowed to air dry the sections were rehydrated by 1 min incubations through a series of ethanol dilutions (100%, 90%(v/v), 70%(v/v) and 50%(v/v)) to distilled water and finally phosphate buffered saline (PBS: 0.16M NaCl, 8mM Na_2HPO_4, 2.7mM KCl and 1.5mM KH_2PO_4). The tissue were subsequently incubated with the particular antibody (diluted 1:50 and 1:100 with PBS with 0.5%(w/v) BSA) in a moist environment at 4°C overnight.

After the incubation period, the antibody was drained from the slide and sections washed for 15 min intervals in PBS with 0.5%(w/v) BSA, PBS with 0.01%(v/v) Tween 20 and finally PBS. Sections were then incubated with the secondary antibody (1:40 dilution of FITC-congugated goat anti-rabbit antiserum) in a moist environment at 4°C for 1 h. The slides were then washed, as previously described, and mounted with Cutifluor (glycerol/PBS, Agar Scientific, Stansted, Essex). After sealing with nail varnish the slides were viewed through a Nikon Microphot FXA microscope with a fluorescence attachment and a high pressure merury lamp. FITC was excited at 495nm with a 460nm auxiliary filter to reduce autofluorescence and photomicrographs were taken using Kodak Ektachrome 400 colour slide film. For long term storage slides were kept in a dessicated container at 4°C.

2.11 PREPARATION OF ANTISERUM

Antisera to the native 2-oxoacid dehydrogenase complexes isolated from bovine heart were prepared as described by De Marcucci *et al.* (1985). Subunit specific antisera were prepared following the protocol described by De Marcucci & Lindsay (1985). The high specificity of antisera has previously been verified by immunoblotting purified mammalian 2-oxoacid dehydrogenase complexes with the respective antisera (De Marcucci *et al.*, 1985).

For the immunofluorescent studies purified IgG antisera to the bovine heart 2-oxoacid dehydrogenase complexes were utilised. A 10ml column of QAE-Sephadex was

equilibrated with 0.1M Tris-HCl pH 6.5. Approximately 1ml of antiserum was applied to the gel matrix and eluted with the same buffer. 1ml fractions were collected and the absorbance at 280nm recorded. The fractions exhibiting absorbance values above 0.8 were pooled and concentrated in dialysis tubing against PEG 6000. The IgG antisera was stored at -70^{0}C until use.

CHAPTER THREE

INTRACELLULAR DISTRIBUTION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

3.1 INTRODUCTION

The plant 2-oxoacid dehydrogenase complexes have not been extensively characterised and the limited infomation available has focussed primarily on PDC. As this family of multienzyme complexes occupy key positions in cellular metabolism characterisation of their structural, physical and regulatory properties are crucial to the understanding of energy metabolism, amino acid turnover and fatty acid and isoprenoid biosynthesis.

Research so far has been hindered by the difficulty in purifying these multienzyme complexes from plant tissue. Conventional purification schemes have proven unsuccessful apparently resulting from low levels of organelle protein and the lability of the complexes when the organelles are disrupted (Poulsen & Wedding, 1970; Randall *et al.*, 1990). Coupled to this is the complexity and ill-defined nature of certain plant organelles, e.g. plastids. In addition the release of phenolic compounds, which are abundant in plant tissue, and endogenous proteases interfere with the purification of intact and viable plant enzymes.

With respect to the preliminary characterisation of the plant 2-oxoacid dehydrogenase complexes, knowledge of their intracellular distribution is of fundamental importance. Within the plant cell PDC is reported to have a dual location being present in mitochondria and plastids (Camp & Randall, 1985; Mierynk *et al.*, 1985). Plant OGDC, as a component multienzyme of the TCA cycle, has only been identified in mitochondria (Wedding & Black, 1971; Dry & Wiskich, 1980; Neuburger *et al.*, 1984; Dry & Wiskich, 1987) and limited investigations indicate that mung bean BCDC is restricted to peroxisomes (Gerbling & Gerhardt, 1988; 1989).

The mitochondrial and plastidic forms of PDC have been the subject of extensive investigations as documented in chapter one. Although PDC has been characterised from a wide range of plants, focusing predominately on pea, information regarding this multienzyme complex from species exhibiting C_4 metabolism is limited. Mitochondrial PDC (mtPDC) plays a pivotal role in the control of intermediary metabolism. It serves as the primary entry point into the TCA cycle and is therefore central to energy metabolism

and the production of carbon skeletons. Consequently its activity has to be governed by a strict regulatory regime, the full complexity of which has yet to appreciated. Control of plant mtPDC is achieved, in part, by reversible phosphorylation of the E1α subunit (Randall *et al.*, 1981; Miernyk & Randall, 1987a; 1987c). Phosphorylation with concomitant inactivation is facilitated by a specific kinase which is itself influenced by numerous metabolites. It was recently reported that the PDC-kinase activity is stimulated by the monovalent cation, NH_4^+ (Schuller & Randall, 1989; Schuller *et al.*, 1993). This significant observation suggests that mtPDC will be preferentially inactivated in the presence of a photorespiratory metabolite. Carbon flow through the TCA cycle is therefore restricted when the tissue is photosynthetically active thus conserving cellular energy.

Plant species exhibiting a C_4 pathway display very low rates of photorespiration as CO_2 is shuttled to the chloroplasts of bundle-sheath cells in the form of oxoaloacetate or malate. CO_2 then enters the Calvin cycle with high concentrations of CO_2 accelerating the carboxylase action of Ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO) and limiting the oxygenase activity. Consequently, levels of glycine metabolism and NH_4^+ ions in the mitochondrial matrix are ineffective indicators of a photosynthetically active cell and preliminary unpublished observations (J.G. Lindsay & H. G. Nimmo, Glasgow University, personal communication) suggest that PDC kinase from a C_4 source is not stimulated by NH_4^+ ions. PDC from C_3 species have therefore evolved a unique regulatory system amongst all PDCs studied to date to complement their carbon metabolism.

PDC is also located in the plastid compartment of plant cells supplying acetyl CoA for fatty acid biosynthesis. Acetyl CoA synthetase also provides an intracytoplasmic source of acetyl CoA (Leidrogel & Bauerle, 1986). The acetyl CoA sythetase and PDC supply routes were assessed for their efficiency in supplying acetyl CoA for fatty acid biosynthesis. Acetate, the primary substrate for acetyl CoA synthetase was found to be the preferred substrate for long-chain fatty acid synthesis, consequently, the existence of plastid PDC remained a point of contention for many years. Localisation of PDC in the plastid compartment has now been firmly established, however, by Randall and co-

workers (Camp & Randall, 1985, Miernyk *et al.*, 1985) who have documented its structural, catalytic and regulatory properties which are distinct from mtPDC.

In plants OGDC, as a component multienzyme of the TCA cycle, is believed to be confined to the mitochondrial matrix displaying approx. 20% of the activity of mtPDC (Cho *et al.*, 1988). The apparent dissociation of the component enzymes of plant OGDC has hampered its purification; however, a 2-oxoglutarate-lipoyl succinyltransferase 'subcomplex' (E1-E2) has been isolated from cauliflower mitochondria (Poulsen & Wedding, 1970). In common with other TCA cycle enzymes, cauliflower mtOGDC has been reported to be controlled in part by energy charge. Specifically, AMP stimulates the complex, linking the activity of this respiratory multienzyme complex with the energy status of the cell. In addition, pyruvate appears to exert an inhibitory effect on the activity of plant OGDC (Dry & Wiskich, 1985; 1987). Research has indicated that pyruvate does not directly interfere with OGDC activity. The inhibition is thought to arise through competition for endogenous CoA, since PDC and OGDC utilise CoA from a common matrix pool and turnover of one acylCoA product (acetyl CoA from PDC activity) appears to dramatically affect the activity of the other (OGDC activity). This observed interaction between mitochondrial multienzyme complexes adds to the subtlety of the overall control mechanism.

The only organelles reported to contain BCDC are peroxisomes. However, as previously stressed, only one group has searched for a plant BCDC and this was restricted to the organelles of mung bean hypocotyls (Gerbling & Gerhardt, 1988). This limited study indicates that the branched-chain amino acids, resulting from the transamination of leucine, isoleucine and valine, are oxidatively decarboxylated by BCDC in peroxisomes. The resulting activated intermediates are subsequently primed to enter the β-oxidation pathway which ultimately produces acetyl CoA. The obvious assumption from this study (Gerbling & Gerhardt, 1988; 1989) is that, in the plant cell, the route of amino acid breakdown is restricted to peroxisomes suggesting that other cellular sites direct their branched-chain amino acids to a second compartment for degradation.

It is therefore clear that the 2-oxoacid dehydrogenase complexes play a central role in the co-ordination of energy metabolism and amino acid turnover. A closer

examination of these complexes is required to appreciate fully the intricacies of plant cellular metabolism. The first aspect to be investigated in this study was the subcellular location of this family of multienzyme complexes.

This chapter concerns itself with the intracellular distribution of the 2-oxoacid dehydrogenase complexes within pea tissue and was assessed adopting two different modes of investigation. Firstly, enzymatic detection which provides unequivocable evidence of an active multienzyme complex, and secondly immunological detection. This latter method exploits antibodies raised against the 2-oxoacid dehydrogenase complexes isolated from bovine heart, allowing the routine identification of immuno-reactive plant polypeptides in specific organelles by immunofluorescent techniques. An immunological cross-reaction of the antisera with plant polypeptides does not confirm unambiguously the existence of the appropriate plant multienzyme complex. However, the detection of immuno-reactive species does signify the potential presence of plant 2-oxoacid dehydrogenase complexes and therefore may serve as a useful tool in the assessment of intracellular distribution.

3.2 ISOLATION OF ORGANELLES

A range of organelles comprising root and leaf mitochondria, peroxisomes and plastids were isolated from pea tissue (*Pisum sativum* L. cv. Little Marvel). Isolation procedures, as described in Materials and Methods (2.2), were based on differential centrifugation. Mitochondria were further purified by isopycnic centrifugation on self-generating Percoll gradients. Although functional plant organelles are routinely isolated by centrifugation on sucrose density gradients, yields are low due to extensive osmotic damage by high sucrose concentrations. In contrast the use of a non-toxic silica sol gradient (Percoll) provides an isoosmotic environment throughout the isolation procedure resulting in high yields of intact functional mitochondria (Neuburger *et al.*, 1982).

Percoll gradients were also employed to purify further the plastid fraction. Chloroplast extracts were centrifuged on pre-formed gradients (0-50%(v/v)) and this proved essential as regards the separation of intact organelles from ruptured material. Intact chloroplasts formed a dense band near the bottom of the gradient whereas stripped thylakoids and extrachloroplastic membranes failed to enter the gradient and were restricted to the sample/gradient interface.

Isolation of a pure preparation of peroxisomes is notoriously difficult owing to heavy contamination from mitochondria. Consequently, an additional purification step after differential centrifugation was necessary. The use of Percoll gradients results in low yields of contaminated peroxisomes (A. Baker, Cambridge University, personal communication). However, centrifugation on pre-formed Nycodenz gradients has been reported as an excellent route for the isolation of rat liver peroxisomes (Imanaka *et al.*, 1987) glyoxisomes from sunflower cotyledons (Behari & Baker, 1993) and tobacco leaf peroxisomes (Onyeocha *et al.*, 1993). Therefore, peroxisomes from pea root and leaf tissue were further purified on Nycodenz gradients following the procedure outlined in Materials and Methods (2.2.3).

3.3 ESTIMATION OF PURITY OF PLANT ORGANELLES

Before each of the organelle preparations could be subjected to enzymatic and immunological studies, it was essential to assess the level of their integrity and determine the extent of contamination from other organelles.

Mammalian dihydrolipoamide dehydrogenase (E3) is not a cytoplasmic enzyme and is restricted to organelles. Consequently endogenous activity will be stimulated by the application of detergent. Assuming that plant E3 is also confined to specific compartments and the organelle membrane is impermeable to exogenous substrates and cofactors, latent enzyme activity will indicate the level of organelle intactness. With the exception of peroxisomes, each organelle preparation was assayed for E3 activity, as described in Materials and Methods (2.5.6), in buffer containing 0.5M sucrose in the presence and absence of 0.25%(w/v) Triton X-100. As peroxisomes exhibited very low E3 activity, thought to arise from contamination from other organelles, hydroxypyruvate reductase activity, which is specifically located in peroxisomes, in the presence and absence of detergent was recorded for this fraction. The degree of organelle intactness was estimated by calculating latent E3 and hydroxypyruvate reductase activity as follows:

$$\frac{\text{(Activity with Triton X-100) - (Activity without Triton X-100)}}{\text{(Activity with Triton X-100)}} \times 100\%$$

The results, shown in Table 3.1, demonstrate that a high level of organelle intactness was maintained by following the isolation protocols described in Materials and Methods (2.2). E3 activity was dependent on the addition of detergent confirming that this enzyme is compartmentalised within pea mitochondria and plastids.

Each organelle preparation was assessed for cross-contamination with the following marker enzymes; succinate dehydrogenase (mitochondrial marker), hydroxypyruvate reductase (peroxisomal marker) and ADP-glucose pyrophosphorylase (plastidic marker), the specific activities of which were monitored during the isolation procedure. Assay procedures are documented in Materials and Methods (2.5) and the

Organelle Preparation	% Organelle intactness
Root Mitochondria	95
Leaf mitochondria	100
Root Peroxisomes*	92*
Leaf Peroxisomes*	85*
Root Plastids	96
Chloroplasts	70

Table 3.1: Percent intactness, as indicated by latent E3 and hydroxypyruvate reductase (*) activity, of organelles isolated from pea (Var. Little Marvel). Isolation procedures, based on differential centrifugation, and determination of E3 and hydroxypruvate reductase activity, in the presence and absence of 0.25%(w/v) Triton X-100, were determined as described in Materials and Methods (2.2 and 2.5 respectively). Percent organelle intactness was calculated by:

$$\frac{(\text{Activity+Triton})-(\text{Activity-Triton})}{\text{Activity+Triton}} \times 100\%.$$

The data presented were recorded during a single preparation. Activities and degree of intactness were monitored, however, during at least three independent preparations and did not vary more than ±5%.

Preparation	Succinate Dehydrogenase Units of Activity	Hydroxypyruvate Reductase Units of Activity	ADP-glucose Pyrophosphorylase Units of Activity
Root homogenate	44	88	140
Root mitochondria	16.89	0.28	3.25
% Recovery	38.37	0.32	2.32
Root peroxisomes	N.D.	3.34	N.D.
% Recovery	—	3.80	—
Root Plastids	1.2	0.2	107.06
% Recovery	0.27	0.22	76.38

Preparation	Succinate Dehydrogenase Units of Activity	Hydroxypyruvate Reductase Units of Activity	ADP-glucose Pyrophosphorylase Units of Activity
Leaf homogenate	27	43	68
Leaf mitochondria	6.01	N.D.	N.D.
% Recovery	22.48	—	—
Leaf peroxisomes	N.D.	3.29	N.D.
% Recovery	—	7.65	—
Chloroplasts	0.25	N.D.	40.22
% Recovery	0.93	—	58.74

Table 3.2: Distribution and % recovery of marker enzyme activities in organelles isolated from pea (Var. Little Marvel) by differential centrifugation. Succinate dehydrogenase (mitochondrial marker), hydroxypyruvate reductase (peroxisomal marker) and ADP-glucose pyrophosphorylase (plastid marker) activities were determined as documented in Materials and Methods (2.5). Total activities are expressed in units where 1unit represents 1nmol NADH/sec. N.D.= not detectable. The data presented was recorded during a single preparation. Activities and % recoveries were monitered, however, during at least three independent preparations and did not vary more than ±5%.

distribution and % recoveries of these marker enzyme activities are displayed in Table 3.2.

It is clear that the individual preparations resulted in the preferential isolation of the respective organelles. Approx. 40% and 20% of succinate dehydrogenase activity was recovered in the root and leaf mitochondrial fractions respectively illustrating the high performance of Percoll in the isolation of mitochondria. Negligible mitochondrial marker enzyme activity was recorded in peroxisomal and plastid extracts.

Following the plastid isolation procedure outlined in Materials and Methods (2.2), 60-70% of the marker enzyme activity (ADP-glucose pyrophosphorylase) was retrieved in plastid fractions. ADP-glucose pyrophosphorylase activity was undetectable in peroxisomes originating from both root and leaf tissue. Root mitochondria, however, appeared to be slightly contaminated with plastids with approx. 2.5% of plastid marker enzyme activity recovered in the isolate.

Hydroxypyruvate reductase activity was undetected in both mitochondrial and plastid fractions whilst peroxisomes exhibited a mere 3-8% recovery of marker enzyme activity. It has previously been reported that purification of peroxisomes on Nycodenz gradients results in low yields of intact organelles (A. Baker, Cambridge University, personal communication). It is clear that peroxisome isolates were devoid of mitochondrial and plastid contamination as illustrated by marker enzyme distribution. It appears that yield has to be sacrificed for an uncontaminated preparation of peroxisomes exhibiting a high level of integrity.

In addition the purity of individual fractions was independently assessed by electron microscopy, each isolate being prepared as described in Materials and Methods (2.3). Micrographs, typical of each organelle preparation, are presented in Figs. 3.1A to 3.1F.

Electron micrographs of the mitochondrial preparations (Figs. 3.1A and 3.1B) indicated that there appeared to be slight contamination from peroxisomes; however, the most abundant species were mitochondria. The purity of the isolate was also reflected by undetectable levels of hydroxypyruvate reductase activity (peroxisome marker) in these fractions.

Root plastid fractions (Fig. 3.1C) exhibited no mitochondrial contamination, bu peroxisomes again appeared to be the major contaminant with a few large catala crystals visible. In addition there was considerable contamination from membranou material, the origin of which was unclear.

Isolated pea chloroplasts displayed a high level of membrane intactness (Fig 3.1D). Low levels of broken thylakoids were present and appeared to be trapped between intact chloroplasts. Contamination from other organelles in this isolate wa minimal confirming the marker enzyme distribution data.

Finally, it is clear that root and leaf peroxisomes exhibited minimal contaminatio from other organelles (Figs. 3.1E and 3.1F respectively). These preparations were however, heavily contaminated by other membranous material.

Examination of marker enzyme distribution, electron micrographs and the latency of E3 and hydroxypyruvate reductase activity indicated that, by following the purification protocols described in Materials and Methods (2.2), the individual organelle preparations were relatively free from contaminating species and a high level of organelle intactness was maintained. The main cause for concern was contamination from broken membranes. This membranous material may have originated from ruptured tonoplast or plasmalemma. They may also have originated from other ruptured organelles thus presenting a major problem which has to be considered.

As the 2-oxoacid dehydrogenase complexes are loosely associated with the inner membranes such contamination may interfere with immunological and enzymatic studies of these multienzyme complexes. The plastid and peroxisomal marker enzymes are present in the soluble phase of their respective compartments. Consequently, it should be borne in mind that assessment of activity in individual extracts does not allow estimation of broken plastid and peroxisomal membrane contamination. As succinate dehydrogenase (mitochondrial marker) is membrane associated, contamination from broken mitochondrial membranes would be detected when examining the distribution of marker enzyme activity. Negligible succinate dehydrogenase activity was recorded in the other organelle isolates indicating that the membranous contamination was not of mitochondrial origin.

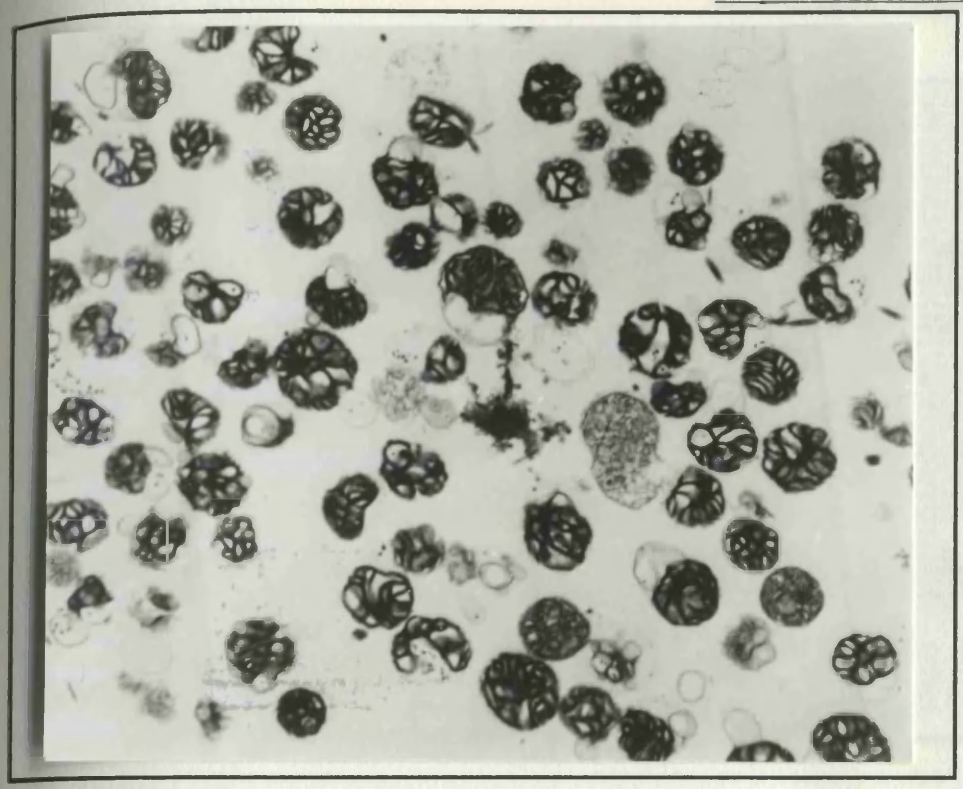


Figure. 3.1A: Electron micrograph of pea root mitochondrial extracts (x11000). Isolation and electron microscopy procedures are described in Materials and Methods (2.2 and 2.3 respectively) and apply to Figs. 3.1A-3.1F.

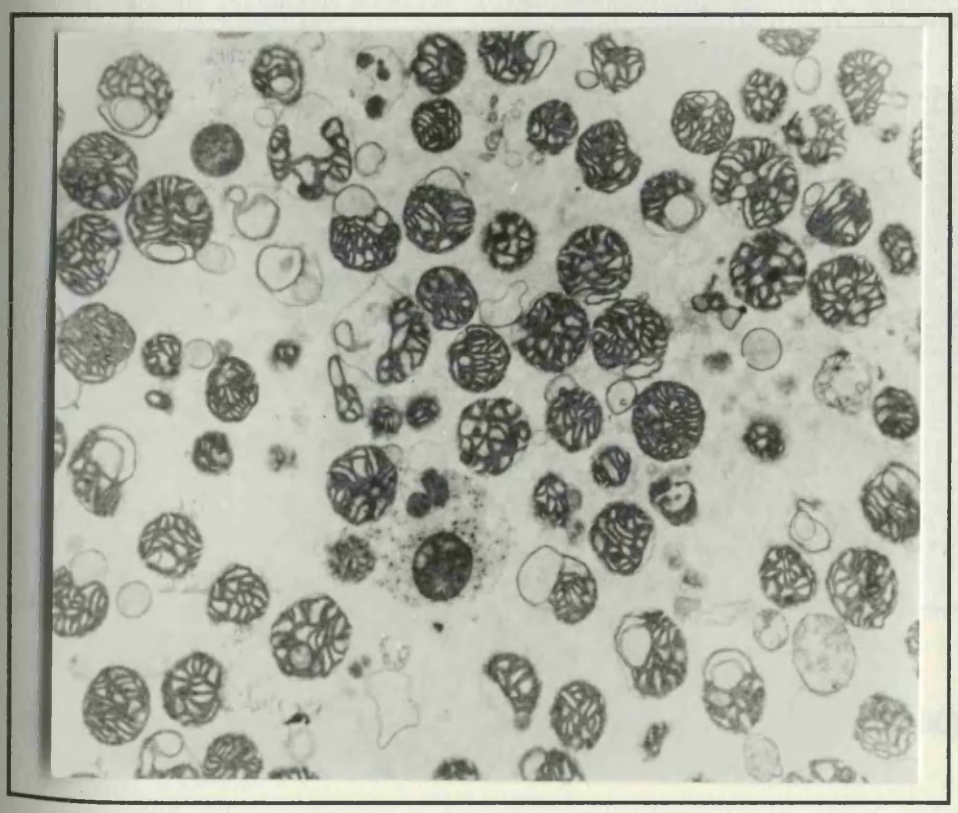


Figure 3.1B: Electron micrograph of pea leaf mitochondrial extracts (x11000).

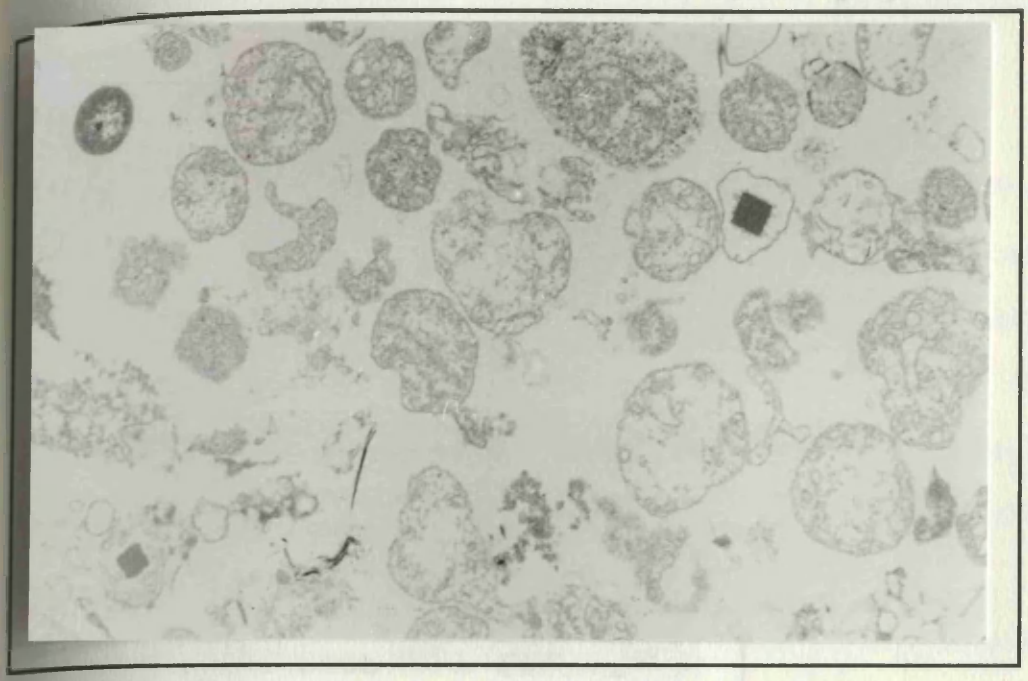


Figure 3.1C: Electron micrograph of pea root plastid extracts (x11000).

Figure 3.1D: Electron micrograph of pea chloroplasts extracts (x11000).

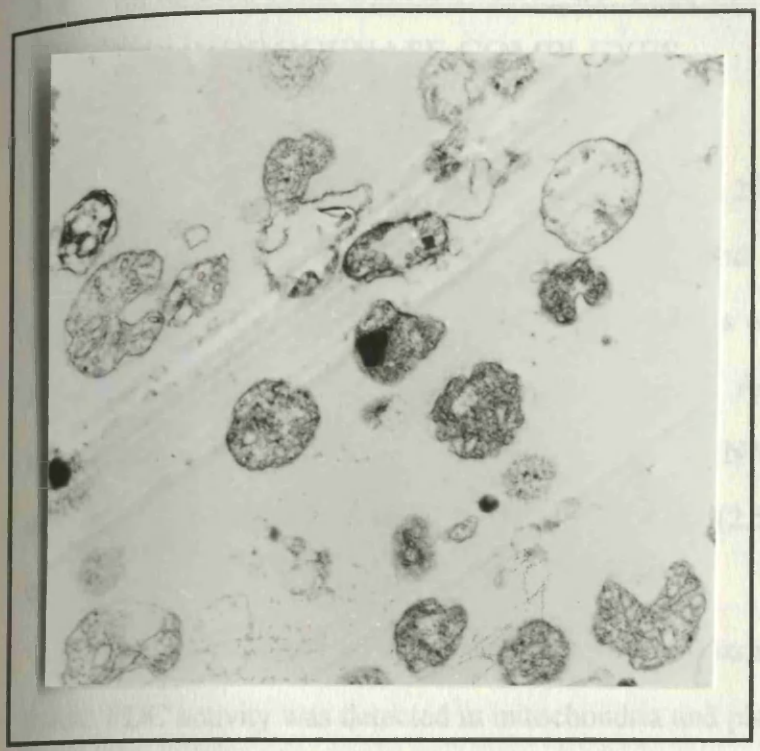


Figure 3.1E: Electron micrograph of pea root peroxisome extracts (x11000).

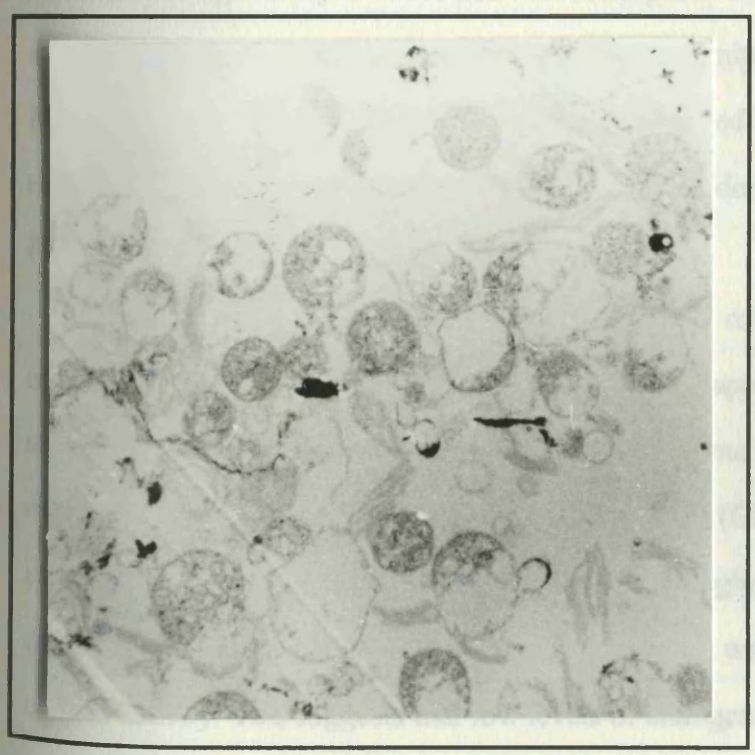


Figure 3.1F: Electron micrograph of pea leaf peroxisome extracts (x11000).

3.4 ENZYMATIC DETECTION OF THE PLANT 2-OXOACID DEHYDROGENASE COMPLEXES

The organelle extracts were solubilised with 0.2%(w/v) Triton X-100 (0.5%(w/v) Triton X-100 for the chloroplast preparation) and the membranes removed by centrifugation (12000.*g*/20 min). These soluble extracts were subsequently assayed for 2-oxoacid dehydrogenase complex activity. Activities were determined spectrophotometrically monitoring the production of NADH at 340nm. Standard assay conditions are documented in Materials and Methods (2.5.4) and were adhered to unless otherwise stated.

Table 3.3 illustrates the distribution of the 2-oxoacid dehydrogenases within pea tissue. PDC activity was detected in mitochondria and plastids originating from both leaf and root tissue. Mitochondrial PDC activities ranging from 4.5 to 5.0 nmol NADH/sec/mg protein were consistently recorded. Root plastid and chloroplast PDC activities were generally 10-15% and 5-10% respectively of the equivalent mitochondrial multienzyme activities. The activities recorded for mitochondrial and plastid forms of PDC were in accordance with these previously reported by Camp & Randall (1985). The reactions, found to be NAD^+-, CoA- and pyruvate-dependent, were consistent with a PDC-catalysed reaction (Table 3.4).

OGDC activity was detected in both leaf and root mitochondria displaying rates of 0.7 and 1.12 nmol NADH/sec/mg respectively, approx. 20% of that recorded for mtPDC. Similar relative activities for these multienzyme complexes have previously been reported for maize and soybean PDC and OGDC (Cho *et al.*, 1988) The observed reaction maintained a complete dependence for 2-oxoglutarate and NAD^+ (Table 3.5). In the absence of CoA the OGDC-catalysed reaction was reduced to one third of the control activity. This suggests that low levels of endogenous CoA may have been present in the organelle preparation.

Typical traces of pea root mitochondrial PDC and OGDC activities are illustrated in Fig. 3.2.

ORGANELLE PREPARATION	PDC ACTIVITY	OGDC ACTIVITY	BCDC ACTIVITY
Root mitochondria	5.00	1.12	N.D
Leaf mitochondria	4.89	0.71	N.D
Root peroxisomes	N.D	N.D	N.D
Leaf peroxisomes	N.D	N.D	N.D
Root plastids	1.63	N.D	N.D
Chloroplasts	0.45	N.D	N.D

TABLE 3.3: Distribution of 2-oxoacid dehydrogenase activities in organelles isolated from pea tissue (Var. Little Marvel). Isolation procedures and assay conditions are documented in Material and Methods (2.2 and 2.5.4 respectively). Specific multienzyme complex activities are expressed in nmol NADH/sec/mg of organelle protein. N.D.=not detectable. The data reported were recorded during a single preparation, however, activities were monitored during at least three independent preparations and did not vary more than ±10%.

Assay conditions	Root mitochondria Relative PDC Activity (%)	Leaf mitochondria Relative PDC Activity(%)	Root plastids Relative PDC Activity (%)	Chloroplasts Relative PDC Activity (%)
Control (complete assay mixture)	100	100	100	100
-organelle protein	0	0	0	0
- pyruvate	0	0	0	0
- NAD^+	0	0	0	0
- CoA	2.2	8.7	7.5	10.7

TABLE 3.4: Substrate requirements of pea mitochondria and plastid PDC. Organelle preparations were obtained by differential centrifugation and PDC activities determined as described in Materials and Methods (2.2 and 2.5.4 respectively). Activities, recorded in triplicate, are expressed as relative values (% of control activity).

Assay conditions	Root mitochondria Relative OGDC Activity (%)	Leaf mitochondria Relative OGDC Activity (%)
Control (complete assay mixture)	100	100
-organellular protein	0	0
-2-oxoglutarate	0	0
-NAD^+	0	0
-CoA	18.7	33

TABLE 3.5: Substrate requirements of pea mitochondria OGDC. Organelle preparations were obtained by differential centrifugation and OGDC activities determined as described in Materials and Methods (2.2 and 2.5.4 respectively). Activities, recorded in triplicate, are expressed as relative values (% of control activity).

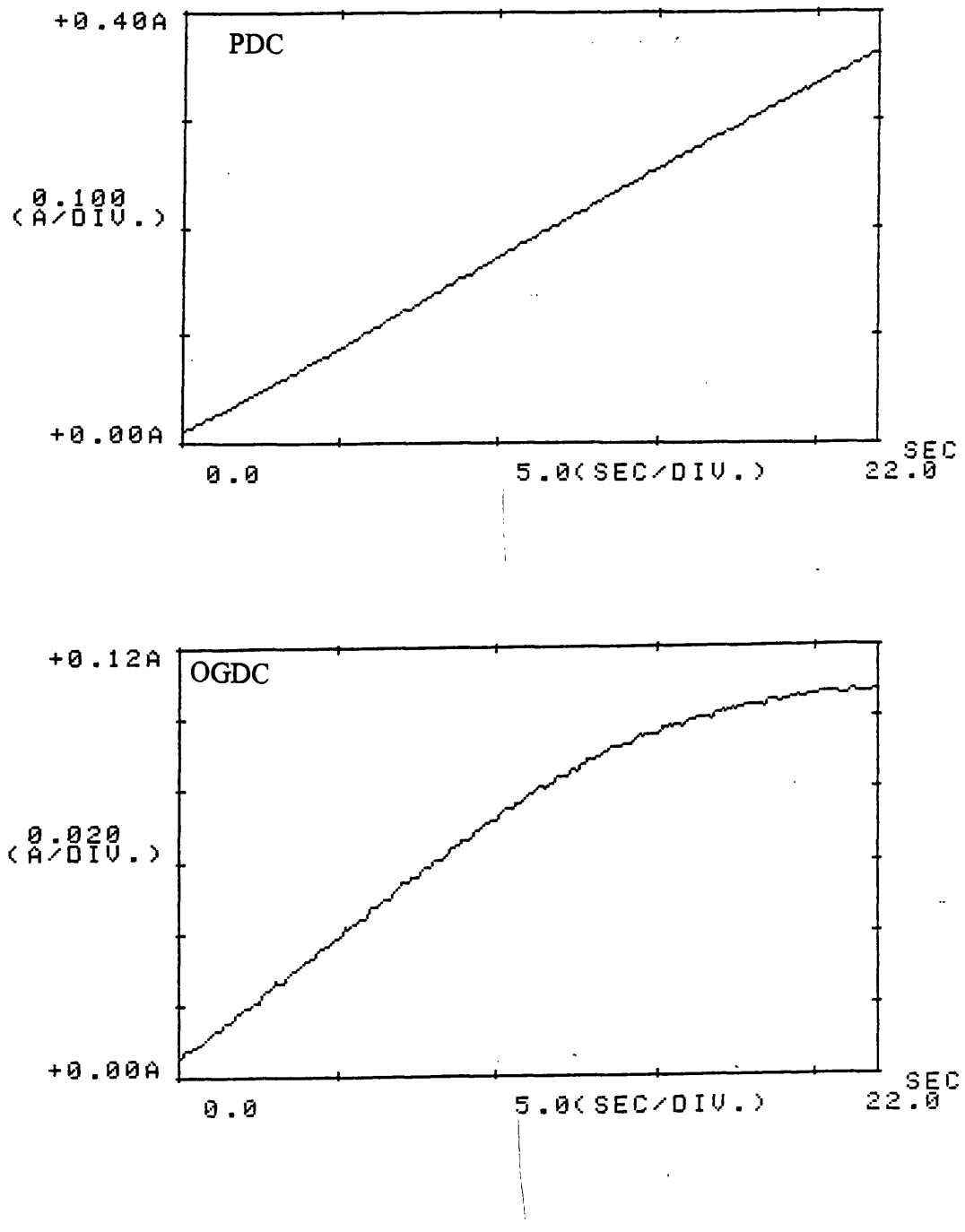


Figure 3.2: Typical traces of pea root mitochondrial PDC and OGDC-catalysed reactions. The production of NADH was monitored on a Shimadzu UV-160A spectrophotometer under standard assay conditions as described in Materials and Methods (2.5.4).

This range of organelles was also evaluated for BCDC activity. A BCDC-catalysed reaction was undetectable in the organelles screened. Throughout the investigation very low levels of BCDC activity were occasionally discernible in mitochondria isolated from both pea leaf and root tissue. This enzymatic detection, however, was inconsistent and the reason for this is unclear. Considering the metabolic role of BCDC, its involvement in branched-chain amino acid breakdown, it is feasible that this multienzyme system was developmentally suppressed in the tissue screened. It is possible that BCDC is synthesised or activated in senescent or developing endosperm tissue which exhibits extensive protein breakdown.

In summary, PDC activity was detected in leaf and root mitochondria, root plastids and chloroplasts. OGDC activity was confined to mitochondria and results also tentatively suggest that BCDC activity was restricted to the mitochondrial compartment.

3.5 EXAMINATION OF THE FACTORS INFLUENCING ENZYMATIC DETECTION

The reason for the lack of 2-oxoacid dehydrogenase complex activity in the organelles screened may be manifold:

1. Detection of 2-oxoacid dehydrogenase complex activity is masked by NADH oxidases.

2. Solubilisation of the organelles has disrupted the multienzyme complexes.

3. The multienzyme complexes are present at low concentrations limiting enzymatic detection.

4. Optimal assay conditions have not been attained.

5. 2-oxoacid dehydrogenase complexes are inactivated

6. 2-oxoacid dehydrogenase complexes are not located within these compartments, having no role in the specific metabolic function of these organelles.

3.5.1 NADH OXIDATION

The level of NADH oxidation in each organelle preparation was measured under standard 2-oxoacid dehydrogenase assay conditions with the introduction of 1.4mM NADH (Table 3.6). Levels of NADH oxidation in mitochondrial, peroxisomal and root plastid extracts were similar, oxidising between 0.1 and 0.3 nmol NADH/sec/mg of protein. Oxidation of NADH by chloroplast extracts was approx. 4-fold lower. The levels of NADH oxidation, although appearing low, may significantly interfere with the spectrophotometric analysis of 2-oxoacid dehydrogenase complex activity especially when monitoring very low reaction rates e.g. OGDC and BCDC activities.

In order to eliminate this potential problem NAD^+ was replaced with 3-acetylpyridine adenine dinucleotide ($APAD^+$). $APAD^+$ is utilised by mammalian and microbial 2-oxoacid dehydrogenase complexes whilst most NADH oxidases are unable to interact with the reduced form of this analogue of NAD^+. Assuming that plant complexes behave in a similar manner then the addition of $APAD^+$ to the reaction mixture should allow the detection of an active complex without the interference of NADH oxidases. $APAD^+$-dependent 2-oxoacid dehydrogenase complex activities in the screened organelles and purified bovine heart PDC and OGDC are presented in Table 3.7.

It is clear that purified bovine heart PDC and OGDC utilised $APAD^+$. Equivalent rates were approx. 75% and 60% respectively of the NAD^+-dependent reaction rates. Pea mitochondrial OGDC and PDC and plastid PDC utilised $APAD^+$ at approx. 25% of the NAD^+-catalysed reaction, indicating that the plant complexes can interact with this analogue of NAD^+ although at lower rates than the mammalian complexes. The application of $APAD^+$ did not lead to the detection of BCDC activity in the isolated organelles. In addition, an $APAD^+$-dependent OGDC-catalysed reaction in the peroxisomal and plastid compartments was not observed.

ORGANELLE PREPARATION	NADH OXIDATION (nmol NADH consumed/sec/mg protein)
Root mitochondria	0.30
Leaf mitochondria	0.25
Root peroxisomes	0.20
Leaf peroxisomes	0.22
Root plastids	0.18
Chloroplasts	0.06

TABLE 3.6: NADH oxidation in organelles isolated from pea (Var. Little Marvel) indicated in Materials and Methods (2.2). The extent of NADH oxidation was determined in the standard 2-oxoacid dehydrogenase assay mixture with the addition of 1.4mM NADH. Activity is expressed in nmol of NADH consumed/sec/mg of organelle protein. The data presented are representative of single organelle preparations, however, activities were monitored during at least three independent isolations and did not vary more than ±5%.

Organelle Preparation	Multienzyme Complex	NAD^+Dependent 2-Oxoacid Dehydrogenase Activity	$APAD^+$Dependent 2-Oxoacid Dehydrogenase Activity
Root mitochondria	PDC	4.75	1.27
	OGDC	1.08	0.34
	BCDC	N.D.	N.D.
Leaf mitochondria	PDC	3.89	1.05
	OGDC	1.12	0.28
	BCDC	N.D.	N.D.
Root plastids	PDC	1.38	0.32
	OGDC/BCDC	N.D.	N.D.
Chloroplasts	PDC	0.47	0.08
	OGDC/BCDC	N.D.	N.D.
Root/leaf peroxisomes	PDC/OGDC/ BCDC	N.D.	N.D.
Bovine heart	PDC	93	72
	OGDC	68	40

TABLE 3.7: NAD^+- and $APADH$-dependent 2-oxoacid dehydrogenase activity in organelles isolated from pea (Var. Little Marvel). Isolation and assay procedures are as described in Materials and Methods (2.2). Activities are expressed in nmol NADH or $APADH_2$/sec/mg protein. N.D.= not detectable. The data presented are representative of single organelle preparations, however, activities were monitored during at least three independent isolations and did not vary more than ±1%.

It is clear that NADH oxidation presents a potential problem when determining 2-oxoacid dehydrogenase complex activity. This interference was especially prevalent when attempting to record very low 2-oxoacid dehydrogenase complex activity e.g. BCDC-catalysed reaction. Although the use of $APAD^+$ does alleviate the problem of endogenous NADH oxidases, the resulting assays lost a degree of sensitivity. $APAD^+$-dependent rates were approx. 25% that of NAD^+-dependent activity indicating that it is a less effective substrate for plant PDC and OGDC than for the mammalian complexes. In addition the plant organelle extracts were capable of oxidising APADH. Rates, however, were lower than the oxidation of NADH. Consequently, the use of $APAD^+$ to overcome NADH oxidation was not considered an effective course of action. The masking of activity by NADH oxidation has therefore to be borne in mind when assessing crude plant organelle extracts for 2-oxoacid dehydrogenase complex activity.

3.5.2 DISRUPTION OF THE MULTIENZYME COMPLEXES

It is possible that solubilisation of the organelles by inclusion of the detergent Triton X-100 in the extraction buffer has disrupted the 2-oxoacid dehydrogenase complexes. In such a case, although the entire complement of enzymes may be present, they no longer have the ability to catalyse the complete reaction sequence. Consequently, the organelles were ruptured by three freeze-thaw cycles. Samples were frozen by placing in liquid N_2 for 2 min and were allowed to thaw at room temperature. Membranes were removed by centrifugation (12000.*g*/10 min) and the activities of PDC, OGDC and BCDC were measured under standard assay conditions (Table 3.8).

As with Triton X-100 solubilised extracts, mitochondrial and plastid PDC and mitochondrial OGDC activities were recorded when organelles were ruptured by freeze-thaw cycles. In each case multienzyme complex activity was generally around 50% of activities attained with Triton X-100-solubilised extracts and may reflect the efficiency of detergent solubilisation in dissociating the complexes from mitochondrial and plastid membranes. When adopting the freeze/thaw method of organelle disruption,

Preparation	Multienzyme Complex	Complex Activity (Triton X-100 Disruption)	Complex Activity (Freeze-Thaw Disruption)
Root mitochondria	PDC	17.0	6.3
	OGDC	4.8	2.8
	BCDC	N.D.	N.D.
Leaf mitochondria	PDC	13.1	7.0
	OGDC	5.0	1.8
	BCDC	N.D.	N.D.
Root plastids	PDC	4.8	2.6
	OGDC/BCDC	N.D.	N.D.
Chloroplasts	PDC	2.8	1.6
	OGDC/BCDC	N.D.	N.D.
Root/Leaf peroxisomes	PDC/OGDC/BCDC	N.D.	N.D.

Table 3.8: 2-oxoacid dehydrogenase complex activity in organelles isolated from pea. Extracts were either disrupted with 0.25%(w/v) Triton X-100 or by a series of freeze and thaw cycles (frozen three times in liquid N_2 and thawed at room temperature). In each case membranes were removed by centrifugation at 10000.*g*/10 min. In each case 50µl of extract was assayed as described in Materials and Methods (2.5.4) and activities are expressed in nmol NADH/sec/ml. N.D.= not detectable. The data presented are representative of single organelle preparations, however, activities were monitored during at least three independent isolations and did not vary more than ±5%.

2-oxoacid dehydrogenase complex activity remained undetected in the peroxisomal compartment as did plastidic OGDC and BCDC and mitochondrial BCDC.

It may be that both these techniques (solubilisation of the organelles with Triton X-100 or repeated freeze-thawing) disrupts the multienzyme complexes and hence intact organelles were assessed for 2-oxoacid dehydrogenase complex activity. Increasing concentrations of Triton X-100 were introduced to the assay media to disrupt the organelles allowing exogenous substrates and cofactors entry. Fig. 3.3 displays root mitochondrial and plastid PDC and root mitochondrial OGDC activities as a function of Triton X-100 concentration in the assay mixture. Optimal activities are observed in the 0.05 to 0.2%(w/v) range. Concentrations exceeding 0.5%(w/v) appear to exert a deleterious effect on PDC and OGDC activities. BCDC and plastid and peroxisomal OGDC activities were undetectable in the intact organelles with Triton X-100 concentrations in the assay media ranging from 0 to 1%(w/v). This indicates that the lack of 2-oxoacid dehydogenase activity is not a function of multienzyme disruption.

With reference to the mammalian, microbial and plant 2-oxoacid dehydrogenase complexes, the most common cause of complex disruption is the dissociation of the E3 subunit. Considering the plant complexes the application of purified mammalian E3, which may associate to the E2 core, would allow maximum intact activity to be attained. Consequently 25, 50 and 100units/ml of purified pig heart E3 was introduced into the reaction mixture and 2-oxoacid dehydrogenase complex activity again determined. The application of exogenous mammalian E3, however, failed to stimulate 2-oxoacid dehydrogenase complex activity in these organelle extracts.

3.5.3 PARTIAL PURIFICATION OF PLANT 2-OXOACID DEHYDROGENASE COMPLEXES

Low concentrations of the 2-oxoacid dehydrogenase complexes within the solubilised extracts would limit enzymatic detection, e.g. a BCDC-catalysed reaction. Consequently, a basic partial purification scheme was employed in an attempt to obtain a 'concentrated' fraction of multienzyme activity. The mammalian 2-oxoacid

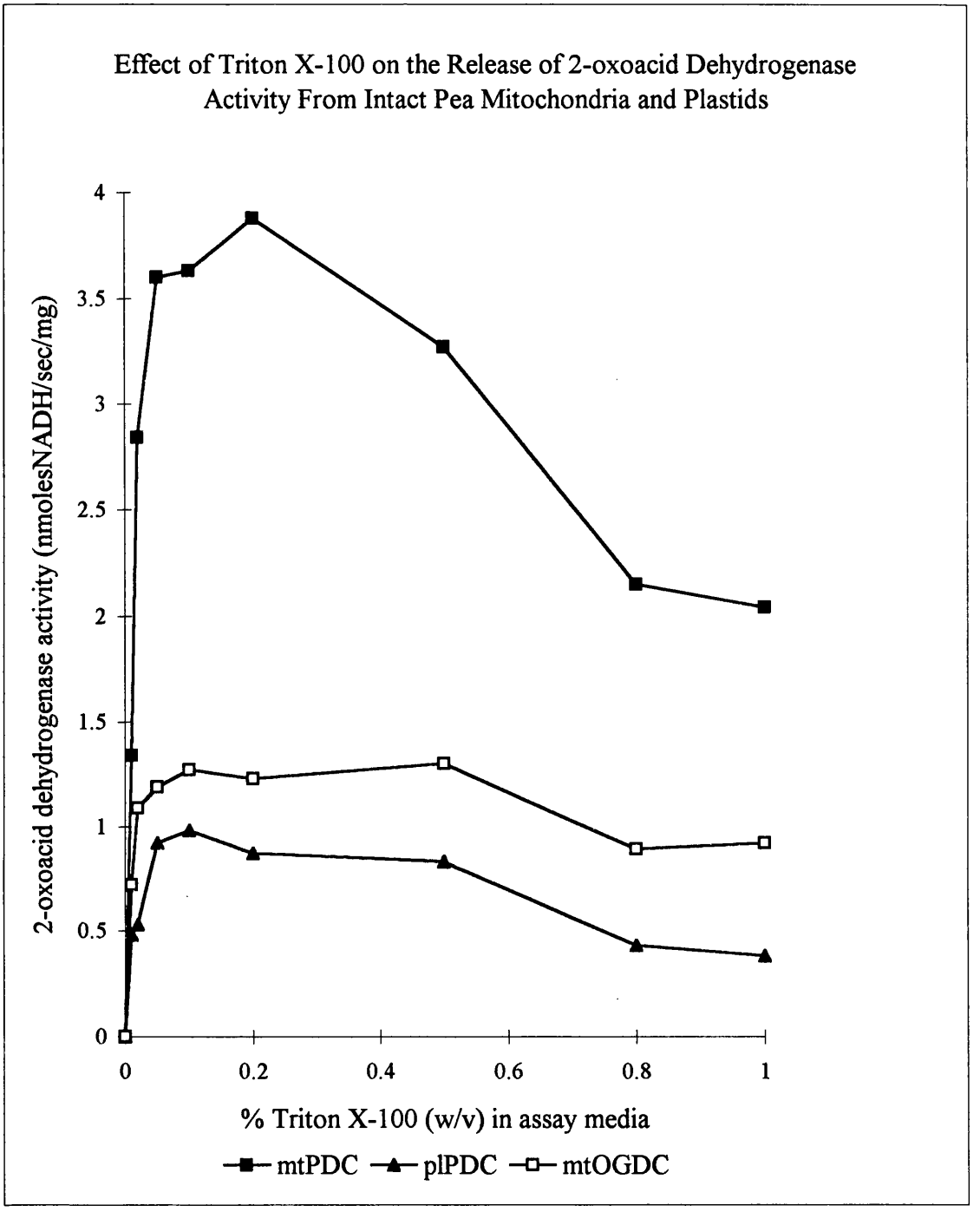


Figure 3.3: Effect of Triton X-100 on pea root mitochondrial PDC and OGDC and root plastid PDC activities. PDC and OGDC activity was determined under standard assay conditions with the introduction of Triton X-100 (0-1%(w/v)) to the reaction mixture. Activities are expressed in nmol NADH/sec/mg protein.

dehydrogenase complexes have M_r values of several million and are routinely isolated by high speed centrifugation. The plant 2-oxoacid dehydrogenases are reported to have M_r values in a similar range to their mammalian counterparts and consequently will be pelleted under similar conditions. Organelle preparations were therefore solubilised with 0.5%(w/v) Triton X-100 and centrifuged at 10000.*g* for 10 min to remove insoluble material. The solubilised extracts were then subjected to a high centrifugal force (100000.*g*/5hr) and the resulting pellets resuspended in a minimal volume of resuspension media in order to retain maximum concentration of the multienzyme complexes. This should alleviate potential problems of low, *in vivo*, concentrations of the complexes. Each partially purified organelle preparation was assayed for 2-oxoacid dehydrogenase activity (Table 3.9).

By following this basic purification scheme a 2-fold increase in the specific activity of mitochondrial and plastid PDC was observed. Over 60% of plastid and root mitochondrial PDC activities were recovered during the partial purification scheme. Recovery of leaf mtPDC was slightly lower, approx. 55%. In contrast mitochondrial OGDC exhibits an approx. 1.5-fold purification and approx. 45% of intact activity was recovered after high speed centrifugation. Plastid and peroxisomal OGDC and BCDC activities remained undetected in the 'concentrated' fraction, however a BCDC catalysed reaction was discernable in the final root mitochondrial pellet with DL-2-oxomethylvalerate as substrate. This suggests that the enzymatic detection of a mitochondrial located BCDC may be hampered by low *in vivo* concentrations of an active complex.

One disadvantage of this partial purification scheme is that the complexes are prone to disruption due to the relatively high concentration of Triton X-100. With the mammalian multienzyme complexes there is a tendency for a proportion of the peripheral subunits, E1 and E3, to dissociate from the core structure. These dissociated component enzymes fail to pellet and remain free in the supernatant. The distribution of E3 activity throughout the partial purification procedure for each of the organelles screened is also included in Table 3.9.

Preparation	Multienzyme Activity	Step1: Units of Activity S.A.	T.A.	Step2: Units of Activity S.A.	T.A.	Step3: Units of Activity S.A.	T.A.	% Recovery
Root Mitochondria	PDC	3.7	21.0	N.D.		6.5	14.3	68.2
	OGDC	0.7	4.1	N.D.		0.8	1.8	45.1
	BCDC	N.D.		N.D.		0.05	0.09	—
	E3	54.2	309.0	36.9	137.8	72.6	159.7	96.3
Leaf Mitochondria	PDC	3.6	27.8	N.D.		6.0	15.6	55.8
	OGDC	0.8	6.6	N.D.		1.2	3.2	48.8
	BCDC	N.D.		N.D.		N.D.		—
	E3	44.7	348.6	38.2	153.8	68.4	177.8	95.1
Root Plastids	PDC	1.2	12.02	N.D.		2.3	8.2	68.5
	OGDC/BCDC	N.D.		N.D.		N.D.		—
	E3	12.9	123.0	7.9	26.5	19.2	65.3	74.6
Chloroplasts	PDC	0.3	3.4	N.D.		0.8	2.6	78.3
	OGDC/BCDC	N.D		N.D.		N.D		—
	E3	3.8	48.4	2.5	10.5	7.5	23.0	69.2
Root & Leaf peroxisomes	PDC/OGDC BCDC/E3	N.D.		N.D.		N.D.		—

Table 3.9: Partial purification of the 2-oxoacid dehydrogenase complexes from organelles isolated from pea (Var. Little Marvel). Purification steps: step 1, Triton X-100 solubilised protein; step 2, supernatant after 100000.*g*/5hr centrifugation; step 3, resuspended pellet. Total activities (T.A.) and specific activities (S.A.) were determined as described in Materials and Methods (2.5.4) and are expressed in nmol NADH/sec/total volume and nmol NADH/sec/mg respectively. The percent recoveries of the 2-oxoacid dehydrogenase complexes and E3 were recorded. N.D.= not detected. The data presented is representative of single organelle preparations, however, activities were monitored during at least three independent isolations and did not vary more than ±7%.

Approximately 50% of E3 activity was confined to the final pellets and the remaining activity was detected in the supernatant fraction. It should be noted that the origin of the E3 in the supernatant is unknown, as PDC, OGDC, BCDC and GDC all utilise E3 in their catalytic cycle. In addition 'free' E3, not affiliated with a multienzyme system, may be present in the soluble phase of the organelles. It cannot be discounted, however, that E3 has dissociated from the 2-oxoacid dehydrogenase complexes thus restricting enzymatic detection. To overcome this potential problem, again pig heart E3 was introduced into the reaction mixture to satisfy maximum activity. This course of action was ineffectual in the stimulation of the 2-oxoacid dehydrogenase complex activity in the 'concentrated' fractions. At present, however, it is unclear if mammalian E3 can associate with the plant complexes and reoxidise lipoyl moieties on the E2 enzymes. Poulsen & Wedding (1970) have reported that an E1-E2 'subcomplex' of cauliflower mtOGDC is capable of utilising pig heart E3. Unpublished observations (Millar & Khan, Glasgow University, personal communication) indicate, however, that E3 isolated from potato and pea cannot reoxidise the lipoyl domains of mammalian PDC, suggesting E3 specificity between the two kingdoms.

3.5.4 OPTIMAL ASSAY CONDITIONS

2-oxoacid dehydrogenase complex activity would remain undetected if the imposed assay conditions had not been optimised. Activity was assessed with increasing concentrations of the three substrates, NAD^+, CoA and the appropriate 2-oxoacid by 2- and 5-fold to determine if substrate concentrations were limiting. In addition the concentration and the nature of the divalent cation was varied. $MgCl_2$ was replaced with $CaCl_2$ and $MnCl_2$ at 1mM and 5mM. Furthermore, the pH of the assay media was fixed at 6.5, 7, 7.5, 8 and 8.5 using the appropriate buffers (MES, TES and Tricine). In each case 2-oxoacid dehydrogenase complex activity remained undetected suggesting that the lack of 2-oxoacid dehydrogenase complex activity in specific organelles was not a result of non-optimal assay conditions.

The activities of OGDC and BCDC were also determined replacing NAD^+ with $NADP^+$. This is of particular relevance to any chloroplast multienyzme complex as $NADP^+$ is the predominant electron acceptor within this organelle. In addition $NADP^+$-specific forms of PDC and OGDC have been reported in *Euglena gracilis* and in certain denitrifying *Pseudomonas* strains (Inui *et al.*, 1984; Lochmeyer & Fuchs, 1990). The substitution of NAD^+ with $NADP^+$ did not stimulate BCDC activity or peroxisomal and plastid OGDC activity. Furthermore negligible mitochondrial PDC and OGDC and plastid PDC activities were recorded when $NADP^+$ was introduced as the electron acceptor.

3.5.5 INACTIVATION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

Enzymatic detection of the 2-oxoacid dehydrogenase complexes would be unattainable if the complexes were inactivated. This is of particular relevance to BCDC, whose mammalian counterpart in certain tissue, e.g. bovine heart, is predominately in an inactive phosphorylated state. In mammals PDC and BCDC are in part controlled by covalent modification where a Mg-ATP dependent phosphorylation mechanism is operable, with phosphorylation of the E1α subunit resulting in inactivation of the complex (Yeaman, 1986; 1989). There is extensive evidence supporting an ATP-dependent phosphorylation scheme controlling the activity of plant mitochondrial PDC (Randall *et al.*, 1981; 1989). With this link into the plant kingdom it is feasible that plant BCDC activity, in accordance with its mammalian counterpart, is regulated by reversible phosphorylation.

In an attempt to reactivate a potentially phosphorylated plant BCDC solubilized pea organelle protein was incubated for 30 min with 5, 10, 50 and 100 units of alkaline phosphatase (1 unit will hydrolyse 1μmol of p-nitrophenyl phosphate/min). This has proven successful with mammalian BCDC; hence it is feasible that this unspecific phosphatase would remove any phosphate groups on the E1α subunit of plant BCDC

resulting in the reactivation of the multienzyme complex. This course of action, however, failed to stimulate BCDC activity in the range of organelles screened.

Unlike mammalian PDC and BCDC, OGDC is not regulated by covalent modification. It was therefore considered unlikely that the plant counterpart would be inactivated by a phosphorylation mechanism. Previous reports have detected an AMP stimulation of cauliflower mtOGDC (Craig & Wedding, 1980). Consequently, extracts of the organelles screened were incubated for 10 min with 1mM AMP; however, OGDC-catalysed reactions in the plastid and peroxisomal compartments were not recorded.

3.5.6 ABSENCE OF THE 2-OXOACID DEHYDROGENASE COMPLEXES IN SPECIFIC ORGANELLES

It has to be considered that the lack of specific 2-oxoacid dehydrogenase complex activity in the range of organelles screened is that the particular multienzyme complex is absent in that compartment of the plant cell. In such a case the specific multienzyme system has no role in the metabolic processes of the organelle.

Finally, a developmental programme may govern the regulation of 2-oxoacid dehydrogenase expression. Consequently, if organelles were isolated from peas when activity was suppressed or the proteins were yet to be synthesised, they would escape enzymatic detection. The existence of developmental programmes in the control of plant enzymes, including PDC, has been well documented (Miernyk *et al.*, 1988). One recent example is GDC, where Northern blot analysis indicates that initiation of pea GDC synthesis occurs approximately 9-10 days after germination (R. Douce, Grenoble, personal communication).

Organelles were isolated from 4 and 9 day old root and leaf tissue respectively. PDC and OGDC are key respiratory multienzyme complexes and consequently, it was considered unlikely that they would be developmentally suppressed in such active tissue. As BCDC is involved in the breakdown of branched-chain amino acids control of activity by a developmental programme is feasible. During the course of the plant's life BCDC activity plays a role in maintaining steady state protein turnover. It is foreseeable that

maximum activity would be expressed by tissue which exhibited extensive protein breakdown e.g. during scenesence or in developing endosperm tissue.

3.6 IMMUNOLOGICAL DETECTION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

The availability of polyclonal antisera to the native PDC, OGDC and BCDC and to their individual subunits isolated from bovine heart, provided a useful tool in the investigation of the intracellular distribution of the plant 2-oxoacid dehydrogenase complexes. Since the antibody-antigen interactions, in this case, span a broad phylogenetic spectrum the occurrence of non-specific interactions has to be borne in mind. In addition, it is possible that the constituents of the plant 2-oxoacid dehydrogenase complexes are antigenically distinct from their mammalian counterparts. Nevertheless, this course of investigation was considered viable as Taylor *et al.* (1992) reported specific cross-reactions of pea mitochondrial and chloroplast protein with high-titre antiserum to bovine heart PDC.

The intracellular distribution of the plant 2-oxoacid dehydrogenase complexes within pea was assessed adopting immunofluoresence techniques. By applying this technique to transverse sections of pea root and leaf tissue, species which cross-react with antibodies to the mammalian 2-oxoacid dehydrogenase complexes can be analysed *in situ*. This approach was adopted in collaboration with J. Marrison and R. Leach (York University) who have recently developed techniques for the subcellular localisation of antigens by immunofluoresence (Marrison & Leach, 1992); tissue sections are challenged with antisera and antigens subsequently identified after visualisation with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies.

Five day old root and leaf tissue was fixed and embedded in polyethyleneglycol as documented in Materials and Methods (2.10). In collaboration with J. Marrison and R. Leach (York University) the samples were sectioned and probed with IgG antisera to the 2-oxoacid dehydrogenase complexes isolated from bovine heart (prepared at Glasgow University as described in Materials and Methods section 2.11). Immuno-

reactive regions were subsequently visualised with FITC-conjugated goat anti rabbit antiserum.

Root sections were incubated with antisera to mammalian PDC and OGDC followed by FITC-conjugated secondary antibodies (Figures 3.4A and 3.4B respectively). In each case a fluorescent signal was concentrated in small pinpoints generally situated at the periphery of the longitudinal root cells. This pattern of cross-reaction indicates a specific mitochondrial localisation (J. Marrison & R. Leach, York University, personal communication). Root sections were also exposed to PBS/BSA, as opposed to the primary antibody, prior to incubation with FITC-conjugated goat anti-rabbit serum. No mitochondrial signal was observed in the control section (Fig. 3.4C) authenticating the specific antibody binding to mitochondrial located antigens

No immunological cross-reactions were observed when challenging root and leaf sections with anti-BCDC serum. Immunofluorescent probing of leaf sections, however, identified a mitochondrial localisation of OGDC (Figure 3.4D). The mitochondrial signal (again identified as fluorescent pinpoints) can clearly be seen against a background of dull yellow chloroplast autofluorescence.

Surprisingly, leaf mitochondria and plastids did not respond to anti-PDC serum. Extensive evidence supports a PDC located in both these compartments (Miernyk *et al.*, 1988), including enzymatic detection reported in this investigation. Antibodies to the native PDC display reactivity primarily to the E2 subunit. Consequently, low concentrations or unaccessibility of the antigen may have interfered with immunological detection of PDC in leaf mitochondria and plastids. The absence of immunofluorescent labelling may also have been a function of non-optimal antibody dilutions. This phenomenon was supported by challenging leaf sections with E3 antibodies which specifically localised in chloroplasts (Fig. 3.4E) which were situated in the peripheral layer of cytoplasm of these highly vacuolated cells. Again the absence of non-specific binding of the primary antibodies was demonstrated by the control section (Fig. 3.4F) where the autofluorescing chloroplasts are clearly distinct from the FITC signal.

Fig. 3.4: Localisation of PDC (3.4A) and OGDC (3.4B) in pea root tissue and OGDC (3.4D) and E3 (3.4E) in pea leaf tissue. 7µm sections of five day old root and leaf tissue were labelled with appropriate anti bovine heart 2-oxoacid dehydrogenase complex serum and visualised with FITC-conjugated secondary antiserum. Control pea root and leaf sections (3.4C and 3.4F respectively) were exposed to PBS/BSA prior to incubation with FITC-conjugated secondary antiserum. Sections were viewed (x100) using a Nikon Microphot FXA ultra violet microscope. Photomicrographs were taken using Kodak Ektachrome 400colour slide film with automatic exposure setting.

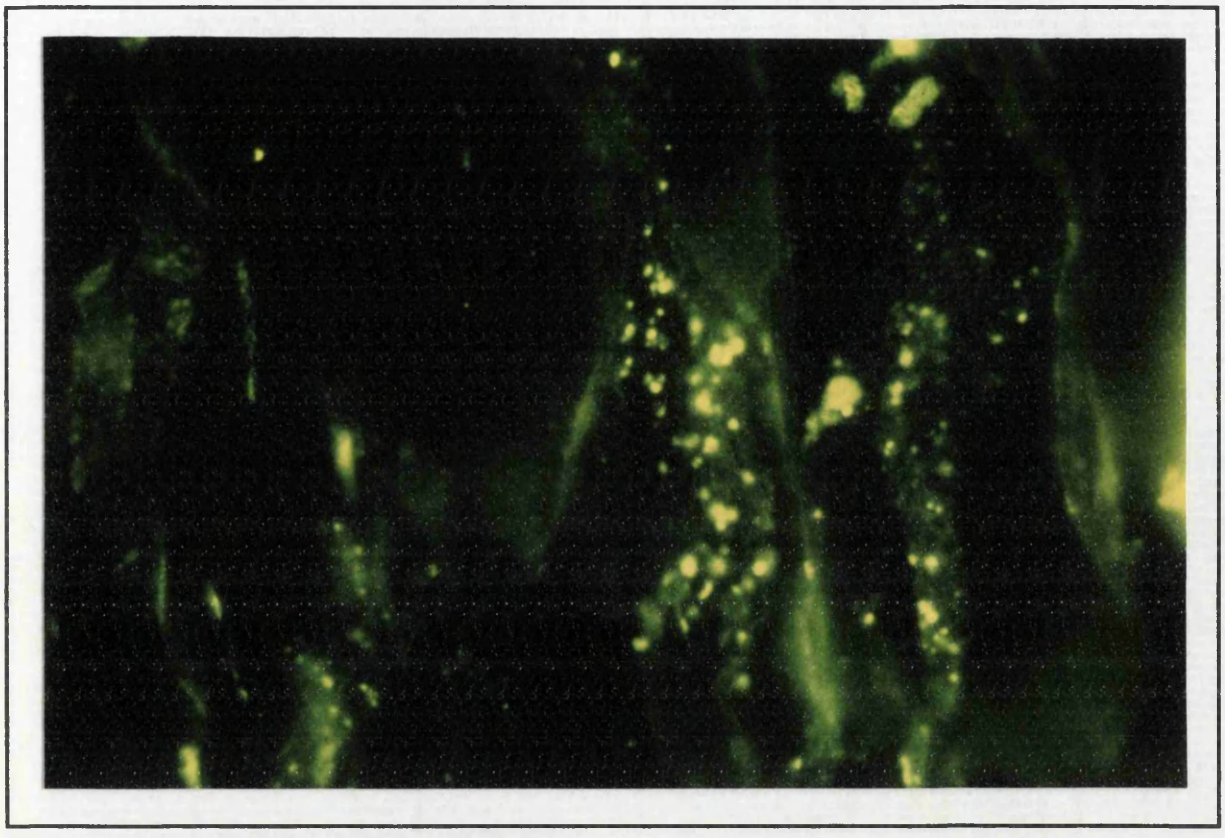


Figure 3.4A: Root tissue probed with anti-PDC.

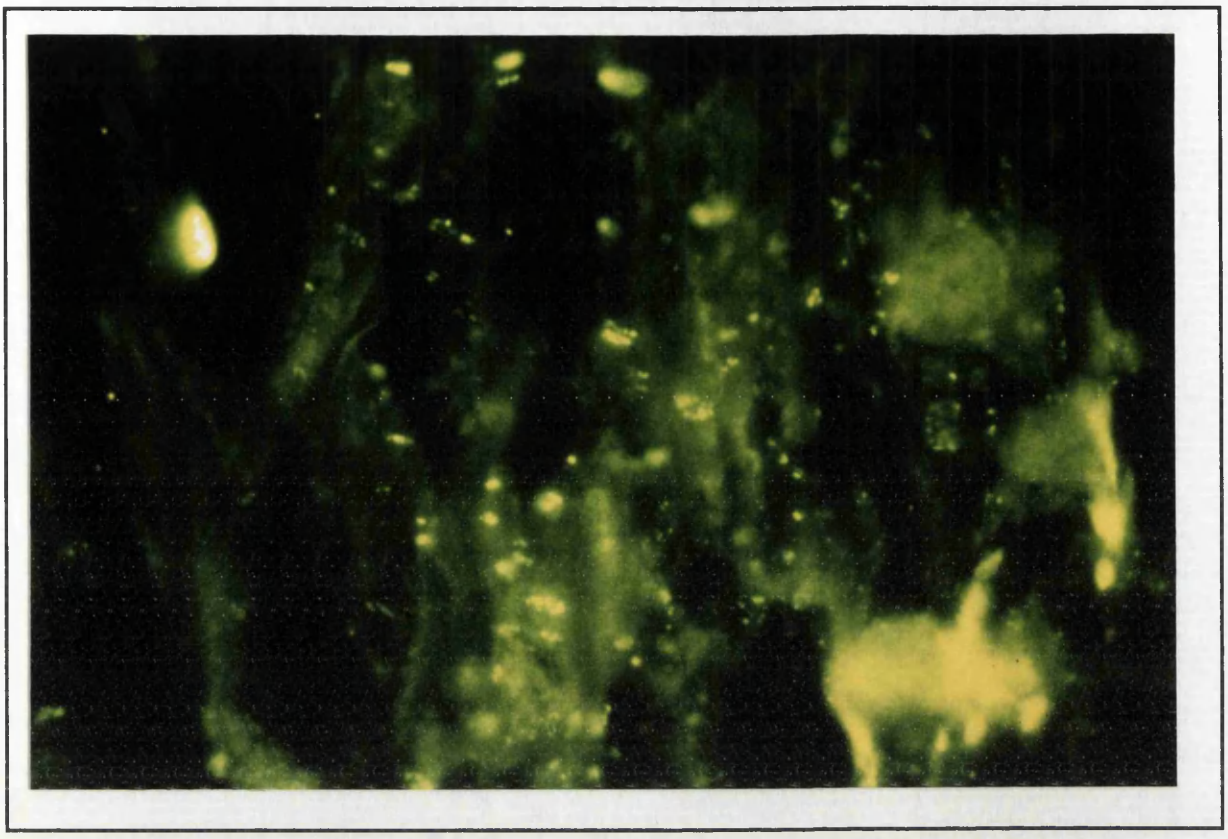


Figure 3.4B: Root tissue probed with anti-OGDC.

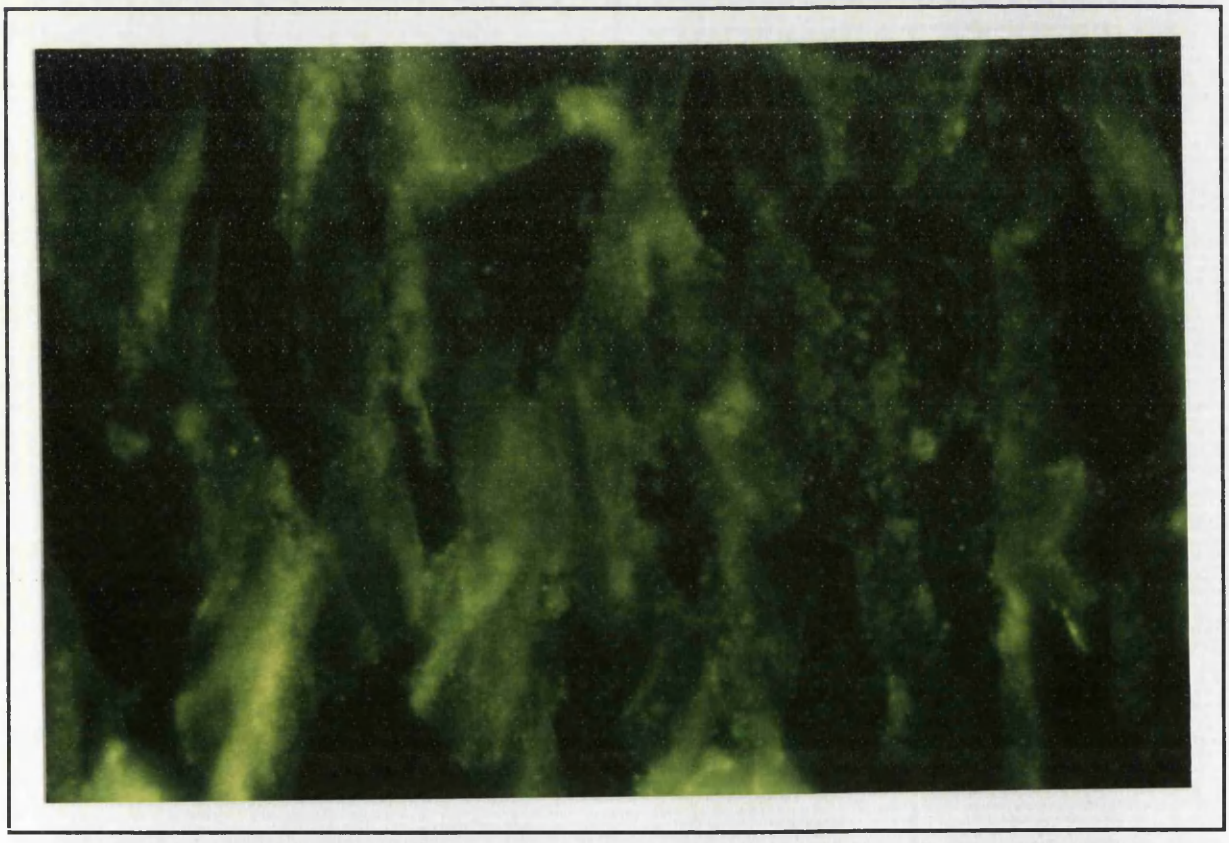


Figure 3.4C: Control root section.

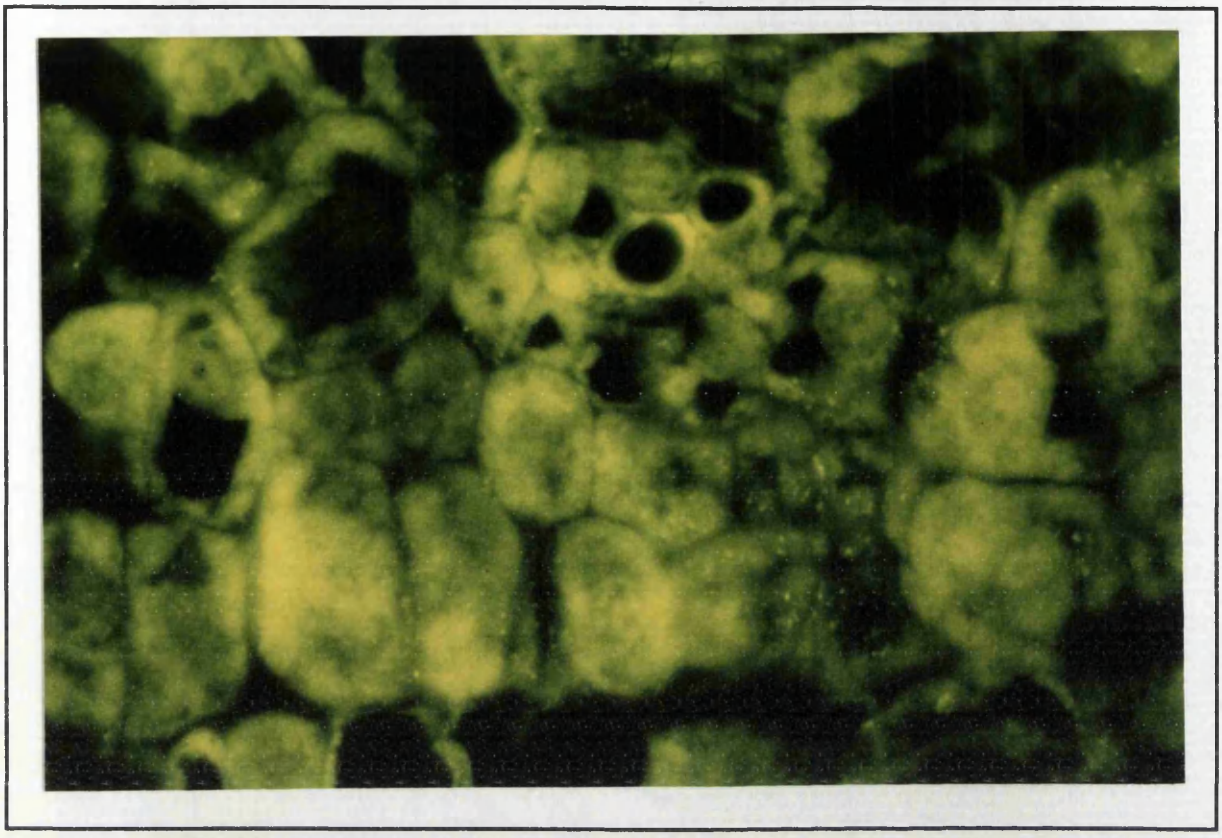


Figure 3.4D: Leaf tissue probed with anti-OGDC.

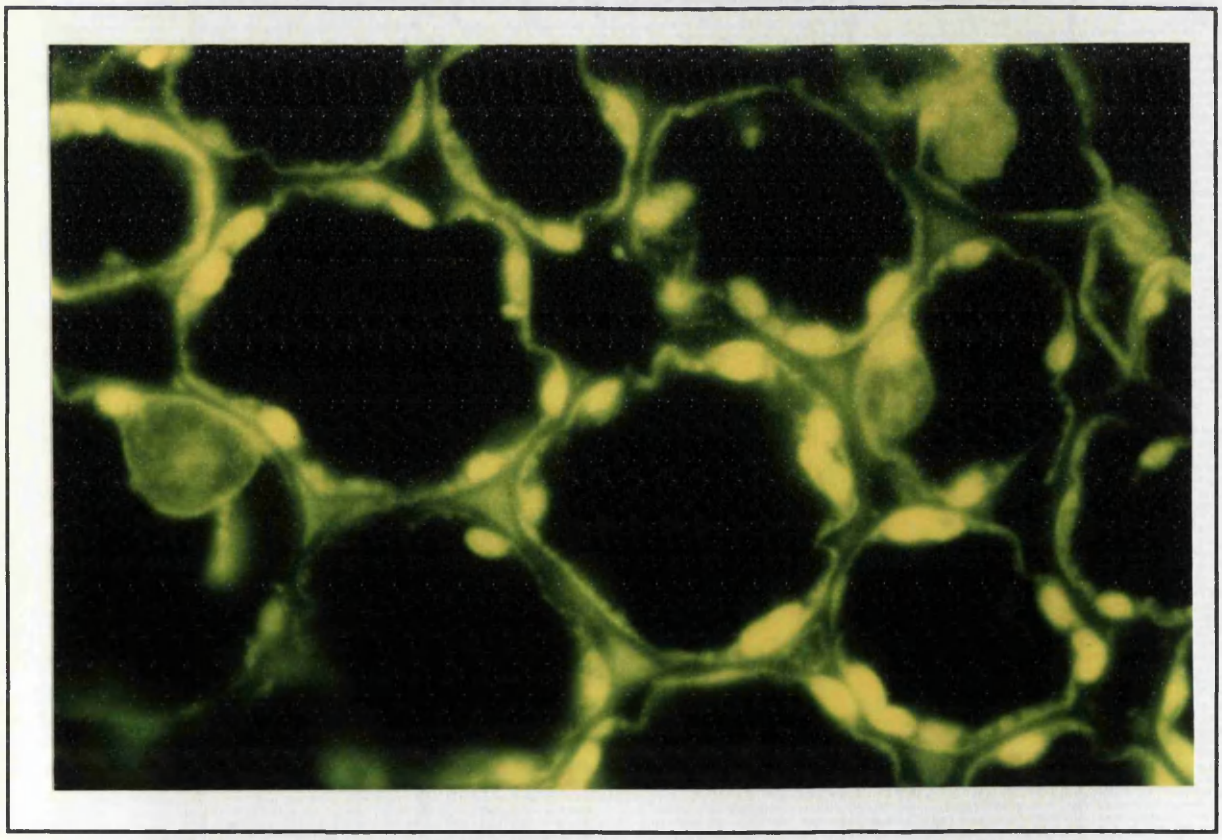


Figure 3.4E: Leaf tissue probed with anti-E3.

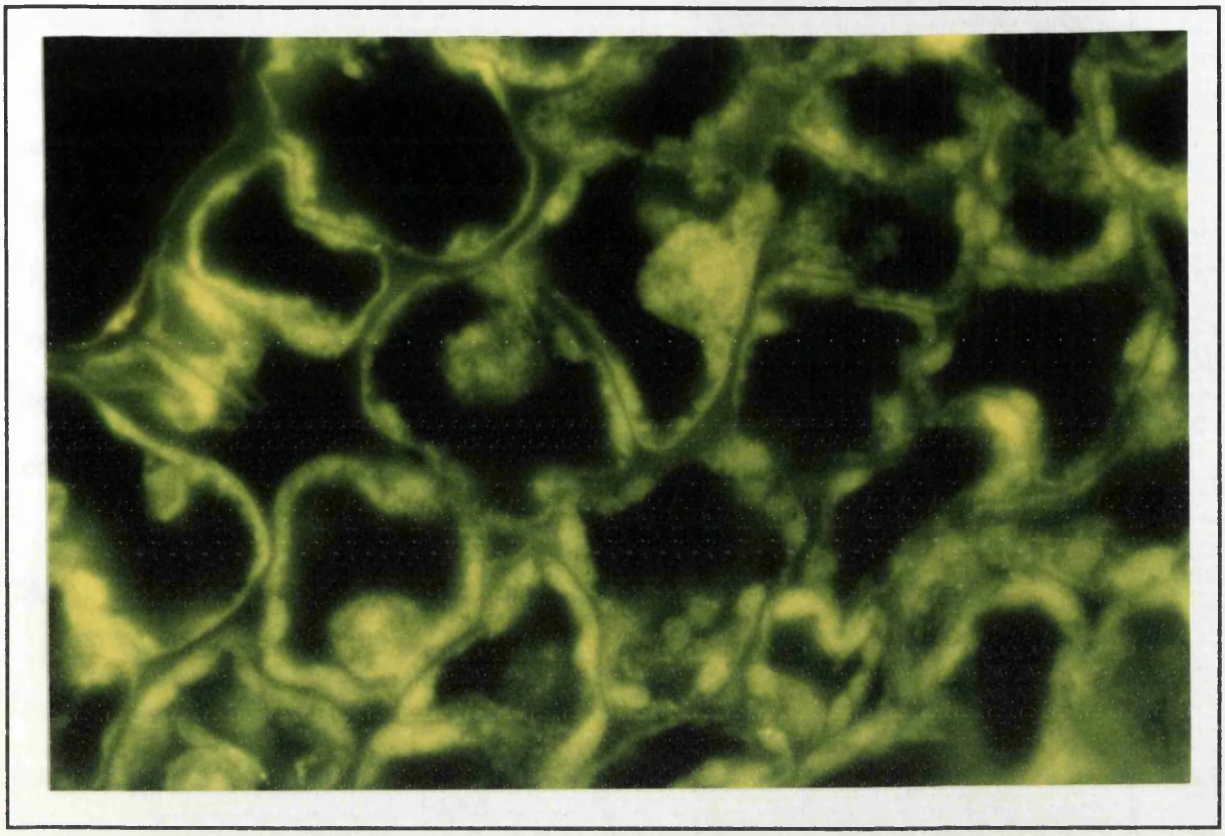


Figure 3.4F: Control leaf section.

Examination of the intracellular distribution of the 2-oxoacid dehydrogenase complexes in pea by adopting an immnuological strategy detected a mitochondrial located PDC and OGDC and a chloroplast E3. These observations are supported by enzymatic investigations reported in this chapter. Pea root and leaf tissue did not respond to BCDC antibodies, however, the plant multienzyme complex may be antigenically distinct from the anaogous mammalian complex. In this case, therefore, the utility of immunofluorescent labelling as a tool for the investigation of subcellular localisation is limited by the absence of antisera to the purified plant 2-oxoacid dehydrogenase complexes.

3.7 INTRACELLULAR DISTRIBUTION OF PEA 2-OXOACID DEHYDROGENASE COMPLEXES

The subcellular localisation of the 2-oxoacid dehydrogenase complexes within the organelles of pea was investigated by enzymatic and immunological techniques (Table 3.10). From the collated data it is clear that PDC is located in mitochondria and plastids from both root and leaf tissue. In addition, OGDC is confined to root and leaf mitochondria displaying approx. 20% the activity of mtPDC. Although no cross-reactivity was detected when challenging root and leaf sections to antiserum to the mammalian BCDC, enzymatic analyses tentatively suggests that BCDC is specifically located in mitochondria, not only in peroxisomes as previously reported (Gerbling & Gerhardt, 1988: 1989). Gerbling & Gerhardt (1988) recorded BCDC rates in the pmoles/sec/mg and it should be noted that the changes in absorbance procured by such low reaction rates would not register on the spectrophotometer employed in this investigation. Consequently, it is plausible enzymatic detection of pea peroxisomal BCDC was limited by low *in vivo* concentrations of an active complex.

Organelle Preparation	Multienzyme Complex	Enzymatic Detection	Immunological Detection
Mitochondria	PDC	+	+
	OGDC	+	+
	BCDC	?	-
Plastids	PDC	+	-
	OGDC	-	-
	BCDC	-	-
	E3	+	+
Peroxisomes	PDC/OGDC/ BCDC	-	-

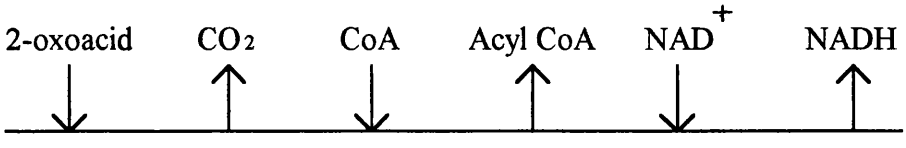
Table 3.10: Enzymatic and immunological detection of the 2-oxoacid dehydrogenase complexes within organelles isolated from pea (*Pisium sativum* L., cv. Little Marvel) by differential centrifugation (Materials and Methods, 2.2). Multienzyme complex activities were monitored spectrophotometrically, measuring the production of NADH (Materials and Methods, 2.5.4). Pea 2-oxoacid dehydrogenase complexes were also detected immunologically by applying immunofluorescence techniques as described in Materials and Methods (2.10), challenging tissue sections to antiserum raised against the complexes isolated from bovine heart.

4.1 INTRODUCTION

The 2-oxoacid dehydrogenase complexes consist of three enzymes: a 2-oxoacid dehydrogenase, a dihydrolipoamide acyltransferase and a dihydrolipoamide dehydrogenase designated E1, E2 and E3 respectively (Reed, 1974; Perham, 1991). The oxidative decarboxylation of specific 2-oxoacids demands the co-ordinated actions of each enzyme and the catalytic cycle involves three active sites, on three separate proteins, resulting in a complex mechanism. Basic kinetic analyses, initial velocity and product inhibition experiments, were performed on the plant complexes in order to characterise the reaction mechanism.

Kinetic characterisation of the plant 2-oxoacid dehydrogenase complexes has to date been focused on PDC. The kinetic mechanism of plant mitochondrial and plastidic PDC has been documented by a number of groups and substrate interaction studies are consistent with a three site ping-pong mechanism (Randall *et al.*, 1977; Miernyk & Randall, 1987b; Thompson *et al.*, 1977a). Data collected from initial velocity and product inhibition studies on mammalian PDC (Tsai *et al.*, 1973), OGDC (Hamada *et al.*, 1975) and BCDC (Boyer & Odessey, 1991) are also consistent with this mechanism.

During a reaction functioning by a ping-pong mechanism, first described by Cleland (1973), a modified form of the enzyme is formed together with the first product before the second substrate can be catabolised. The 2-oxoacid dehydrogenase complex reaction sequence involves a number of reactions catalysed at distinct active sites and the ping-pong nature can be illustrated diagramatically:



Ping-pong mechanisms can be confirmed by analysing initial velocity patterns of one substrate at varying concentrations of a second substrate. Double reciprocal plots will result in a series of parallel lines if the enzyme catalysed reaction is functioning by a ping-pong mechanism (Fig. 4.1).

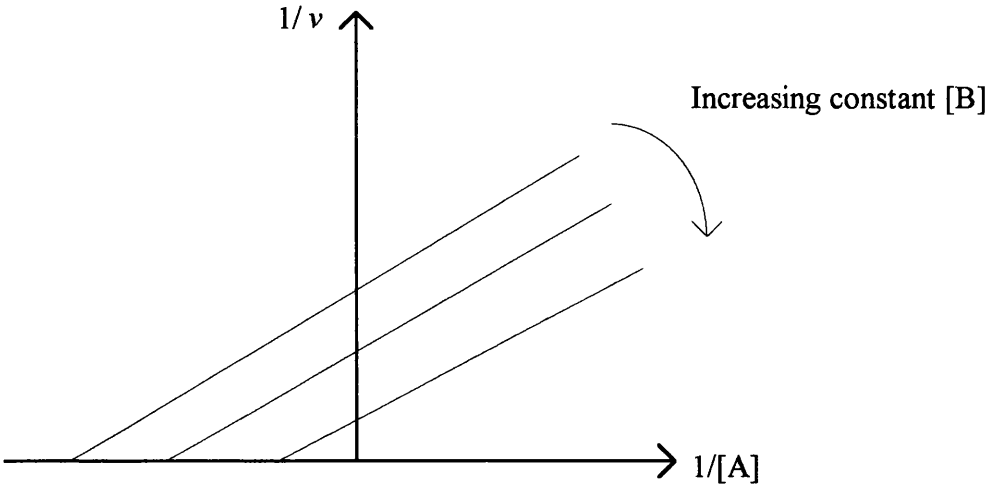


Figure 4.1: Double reciprocal plot obtained for an enzyme catalysed reaction functioning by a ping pong mechanism. Plot of $1/v$ versus 1/[A] at several concentrations of B, where A and B are substrates of a multiple substrate reaction.

Such initial velocity patterns have been reported for a number of plant mitochondrial PDCs, including cauliflower, broccoli and pea, and plastid PDC from soybean, maize and pea (Randall *et al.*, 1977; Thompson *et al.*, 1977b; Miernyk & Randall, 1987b). Typical K_m values are presented in Table 4.1.

Both plant mitochondrial and plastid PDCs, in common with their mammalian and microbial counterparts, are subject to inhibition by the products of the overall reaction. Inhibition by NADH versus NAD^+ and acetyl CoA versus CoA is competitive for all plant PDCs studied to date, while inhibition by NADH or acetyl CoA versus pyruvate is uncompetitive. This inhibitory behaviour reflects the distinct active sites of the multienzyme complex and correlates with the inhibition patterns predicted by a ping-pong mechanism. Typical K_i values for the product inhibitors are also included in Table 4.1. As with prokaryotic and other eukaryotic PDCs, the plant mitochondrial and plastid multienzyme complex is very sensitive to the NAD^+:NADH ratio as demonstrated by the low K_i value (NADH) compared with the K_m value (NAD^+).

Plant Source	K_m(Pyr) μM	K_m(NAD^+) μM	K_m(CoA) μM	K_i(NADH) μM	K_i(Acetyl CoA) μM
Pea leaf mtPDC	57	122	4	18	10
Broccoli floret mtPDC	250	110	6	13	19
Cauliflower floret mtPDC	207	125	7	34	13
Pea leaf pPDC	120	36	10	9	16
Castor seed endosperm pPDC	62	130	6	27	23
Maize shoot pPDC	120	16	4	12	18

Table 4.1: Michaelis constants for a range of mitochondrial (mt) and plastid (p) PDCs from a variety of plant tissue: pea leaf mitochondria (Miernyk & Randall, 1987b), broccoli floret mitochondria (Rubin & Randall, 1977a), cauliflower floret mitochondria (Randall *et al.*, 1977), pea leaf plastids (Camp *et al.*, 1988), castor seed endosperm plastids (Thompson *et al.*, 1977a) and maize shoot plastids (Cho *et al.*, 1988).

Kinetic characterisation of the plant 2-oxoacid dehydrogenase complexes has been previously reported (Thompson et al., 1977b; Miernyk & Randall, 1987b). Initial velocity data were collected, however, for the 2-oxoacid dehydrogenase complex catalysed reactions investigated in this study and the kinetic constants compared to previously documented values. Inhibition of the complexes by the products of the reaction sequence, acyl CoA and NADH, was also assessed and the inhibition patterns compared with the mechanism's predicted behaviour.

4.2 PLANT MITOCHONDRIAL 2-OXOACID DEHYDROGENASE COMPLEXES

The kinetic analyses of the plant 2-oxoacid dehydrogenase complexes demands an extensive supply of protein. Consequently, a range of plant material was screened for both ease of organelle isolation and product yield. Following the procedure outlined in Materials and Methods (2.2), mitochondria were isolated from pea roots, cauliflower florets and the tubers of potato, turnip and swede (purchased from a local market). Specific PDC, OGDC and BCDC activities were recorded as reported in Materials and Methods (2.5.4) for each mitochondrial preparation (Table 4.2). Although pea root mitochondria exhibited the highest PDC and OGDC specific activities (approx. 5.0 and 2.5 nmol NADH/sec/mg respectively) the routine harvesting of 200-400g of root tissue was extremely labour intensive. Consequently, potato tuber mitochondria, which displayed comparable specific activities, were selected for kinetic analyses. The variety Maris Piper was chosen as an all year round supply was guaranteed. Potato mitochondrial extracts were prepared for electron microscopy as described in Materials and Methods (2.3). Typical micrographs are displayed in Fig. 4.2A and demonstrate the absence of contaminating organelles. The isolates, however, appear heavily contaminated with membranous material.

An additional advantage in opting for potato tuber mitochondria is the detection of a BCDC-catalysed reaction. The specific activity of potato mitochondrial BCDC was determined for each of the five primary BCDC substrates; 2-oxobutyrate, 2-

Material	Pea	Potato	Cauliflower	Turnip	Swede
Weight (kg)	0.2	1	1	1.5	0.5
Volume of extract (ml)	0.75	3	3	2	2
[Protein] (mg/ml)	4.5	9.8	9.6	8.2	5.6
PDC Activity	5.67	4.89	0.12	1.29	1.96
OGDC Activity	1.24	1.05	0.03	0.3	0.42
BCDC Activity	N.D.	0.08	N.D	N.D.	0.04

TABLE 4.2: Isolation of mitochondria from pea roots, cauliflower florets and potato, turnip and swede tubers. Isolation procedures were based on differential centrifugation with additional purification on Percoll gradients as described in Materials and Methods (2.2). Protein concentrations were determined following a modified method of Lowry *et al.* (1951) using BSA fraction V as a standard. Activities of the 2-oxoacid dehydrogenase complexes, expressed in nmol NADH/sec/mg, were determined spectrophotometrically monitoring the production of NADH under standard assay conditions as documented in Materials and Methods (2.5.4). Specific activities are representative of a single preparation, however rates were monitored during at least three independent preparations and did not differ by more than ±5%. N.D.= not detectable.

BCDC Substrate	NAD^+-dependent Activity	$APAD^+$-dependent Activity
2-oxobutyrate	0.54	0.22
2-oxoisocaproate	N.D.	0.04
2-oxovalerate	N.D.	0.02
4-methylthio-2-oxobutyrate	N.D.	0.04
DL-2-oxomethylvalerate	0.21	0.17

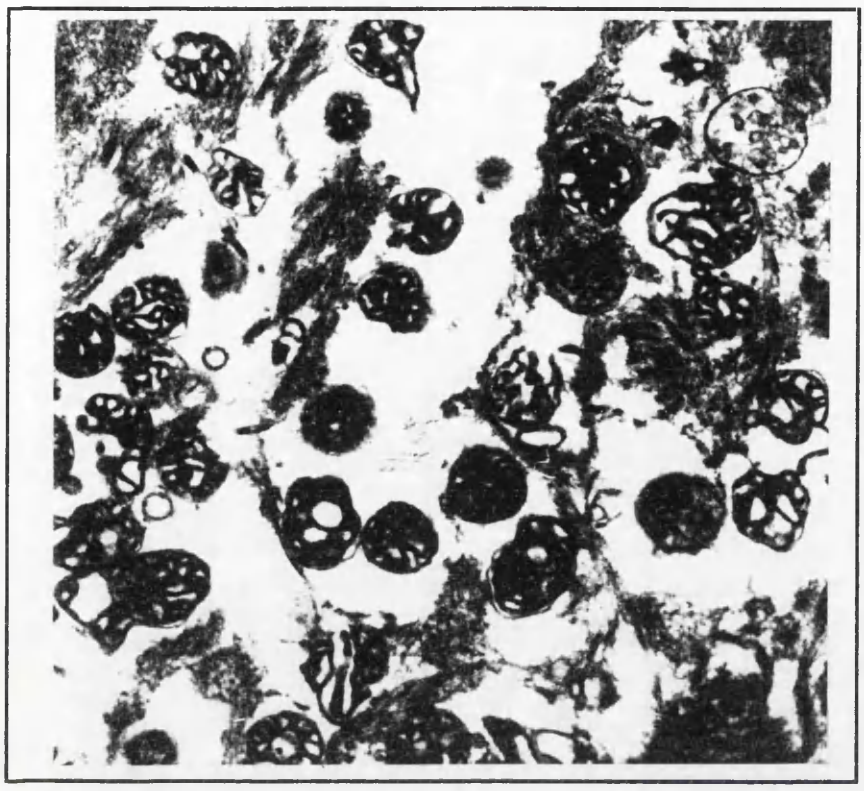
TABLE 4.3: NAD^+- and $APAD^+$-dependent BCDC activity in potato mitochondria. Standard assay conditions were adopted as described in Materials and Methods (2.5.4) and reactions were initiated by the addition of one of the five branched-chain 2-oxo acids. Activities are expressed nmol . Specific activities are representative of a single preparation, however rates were monitored during at least three independent preparations and did not differ by more than ±5%. N.D.= not detectable.

NADH or APADH/sec/mg.

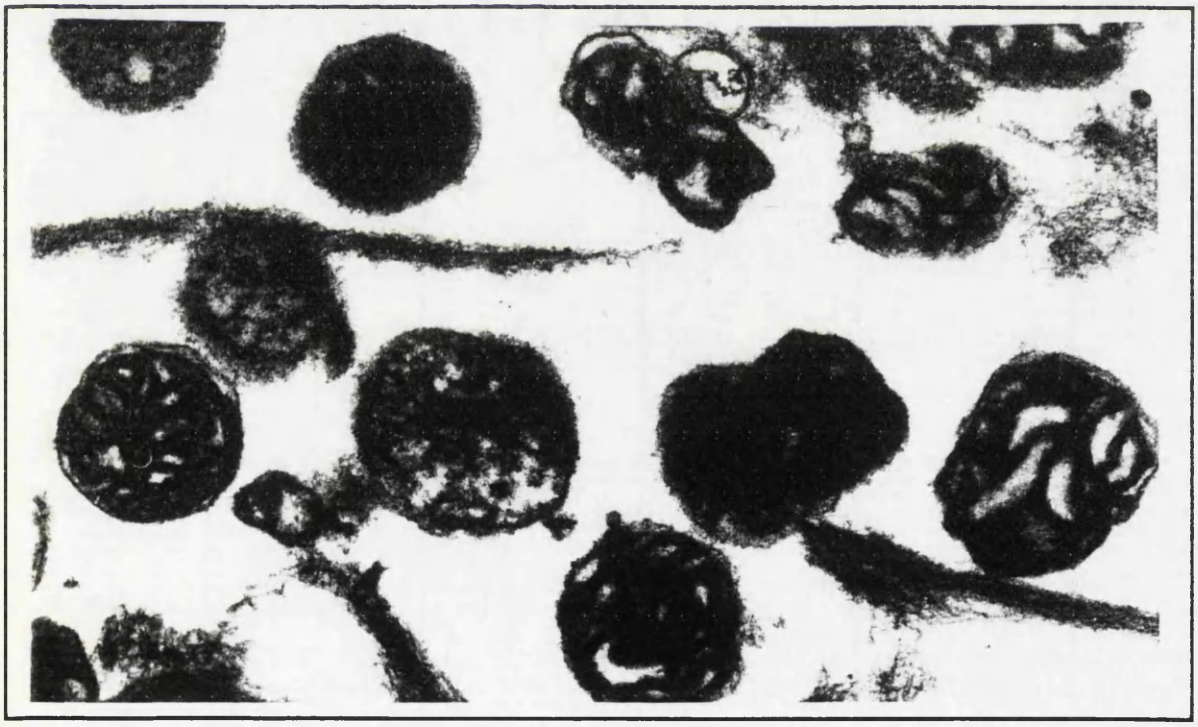
oxoisocaproate, 2-oxovalerate, 4-methylthio-2-oxobutyrate and DL-2-oxo-methylvalerate (Table 4.3). 2-oxobutyrate gives rise to the highest specific activity, 0.22 nmol NADH/sec/mg, however, plant PDC can also oxidatively decarboxylate 2-oxobutyrate at approx. 50% the rate of pyruvate (Miernyk & Randall, 1987b). Consequently, the oxidative decarboxylation of 2-oxobutyrate cannot be attributed solely to a BCDC-catalysed reaction. When NAD^+ was substituted with $APAD^+$, 2-oxoisocaproate, 2-oxovalerate and 4-methylthio-2-oxobutyrate dependent BCDC-catalysed reactions were detectable. These activities were both extremely low and inconsistent with rates ranging from 0.02 to 0.06 nmol NADH/sec/mg. DL-2-oxo-methylvalerate dependent BCDC activity, however, was approx. 5-fold greater, NAD^+-dependent rates of 0.2 nmol NADH/sec/mg were consistently recorded. This parallels the situation in mammalian BCDC where DL-2-oxo-methylvalerate exhibits the lowest K_m (Boyer & Odessey, 1991).

4.3 PLANT PLASTID PYRUVATE DEHYDROGENASE COMPLEX

Chloroplasts were isolated from nine day old pea leaves and E3 and PDC and specific activities of approx. 5 and 0.25 nmol NADH/sec/mg respectively were consistently recorded. Chloroplasts were also prepared from three week old potato leaves. Electron micrographs indicated that few intact potato chloroplasts survived the isolation procedure and stripped thylakoid membranes were predominant in the isolate. As potato contains a high starch content it was suspected that the disruption of large starch grains during the initial tissue homogenisation ruptured organelles. This problem was greatly ameliorated by placing the plants in the dark for 48 h, thus lowering the starch content, prior to harvesting. A vital stage in preparation was the separation of the intact species from thylakoid fragments by centrifugation on Percoll gradients as described in Materials and Methods (2.2.2). Samples from the intact chloroplast band and the stripped thylakoid debris were prepared for electron microscopy as described in Materials and Methods (2.3) and typical micrographs are presented in Fig. 4.2B and 4.2C respectively.



(x11000)



(x36000)

Fig. 4.2A: Electron micrographs of potato tuber mitochondrial isolates. Isolation and electron microscopy procedures are described in Materials and Methods (2.2 and 2.3 respectively).

Fig. 4.2B: Electron micrograph (x11000) of intact potato chloroplasts separated by centrifugation through Percoll gradients as described in Materials and Methods (2.2.2). Isolates were fixed and prepared for microscopy as described in Materials and Methods (2.3).

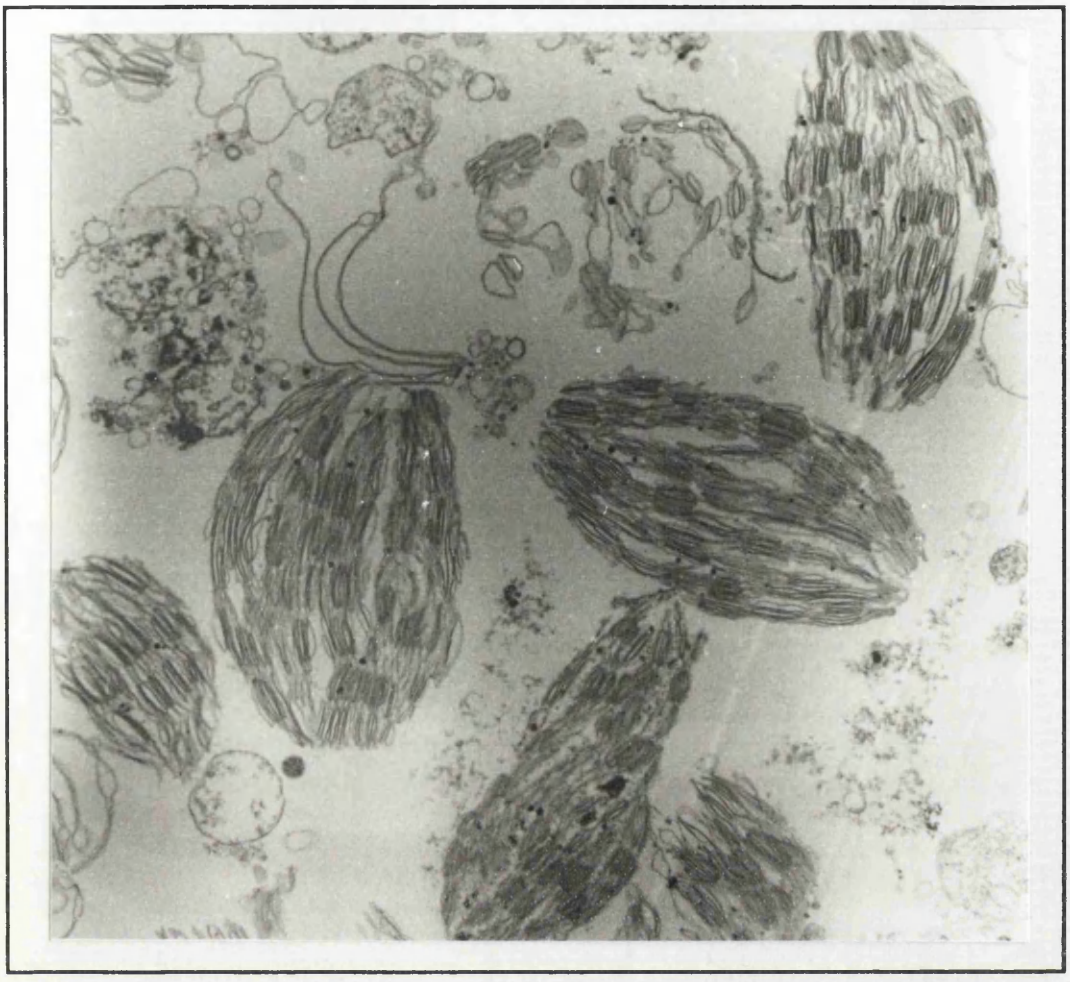


Fig. 4.2C: Electron micrograph (x11000) of stripped potato thylakoid membranes separated by centrifugation through Percoll gradients as described in Materials and Methods (2.2.2). Isolates were fixed and prepared for microscopy as described in Materials and Methods (2.3).

Although E3 activity was consistently recorded in potato chloroplasts (0.5-1.5 nmol NADH/sec/mg), PDC activity was undetected. It was presumed that the spectrophotometric monitoring of potato chloroplast PDC activity was hampered by low concentrations of the multienzyme complex in the extract. Scaling up the preparation was not considered feasible as growth room facilities restricted a large constant supply of potato leaves. In addition the concentration of extract used to monitor PDC activity spectrophotometrically was limited due to the high background absorbance of the chloroplast suspension.

Pea tissue was therefore selected to investigate the kinetic properties of chloroplast PDC. Typical pea chloroplast PDC activities ranged from 0.2-0.5 nmol NADH/sec/mg. This low production of NADH limited kinetic analyses as spectrophotometric detection of a PDC-catalysed reaction with limiting concentrations of substrates was impractical. Consequently, chloroplast extracts were solubilised with 0.5%(w/v) Triton X-100, the insoluble material removed by centrifugation (12000.*g*/20 min), and the soluble fraction subjected to a high centrifugal force (100000.*g*/5 h). The pellet was resuspended in a minimal volume of resuspension buffer, as described in Materials and Methods (2.2.2). Kinetic data for plastid PDC were subsequently collected from this 'concentrated' fraction, which displayed PDC activities of approx. 1-1.5 nmol NADH/sec/mg.

4.4 EFFECT OF pH ON THE ACTIVITY OF PLANT 2-OXOACID DEHYDROGENASE COMPLEXES

The pH optima for potato mitochondrial PDC (mtPDC) and OGDC and pea chloroplast PDC (pPDC) were determined by assessing the specific activities of PDC and OGDC-catalysed reactions at pHs ranging from 5.0 to 9.0 (Fig. 4.3). As numerous authors had reported that glycylglycine buffers exert a deleterious effect on the activity of the 2-oxoacid dehydrogenase complexes (Miernyk & Randall, 1987b), MES, TES, tricine, bicine and CHES were used to hold the reaction mixture at the appropriate pH. The relationship between pH and the specific activity of potato mitochondrial PDC and

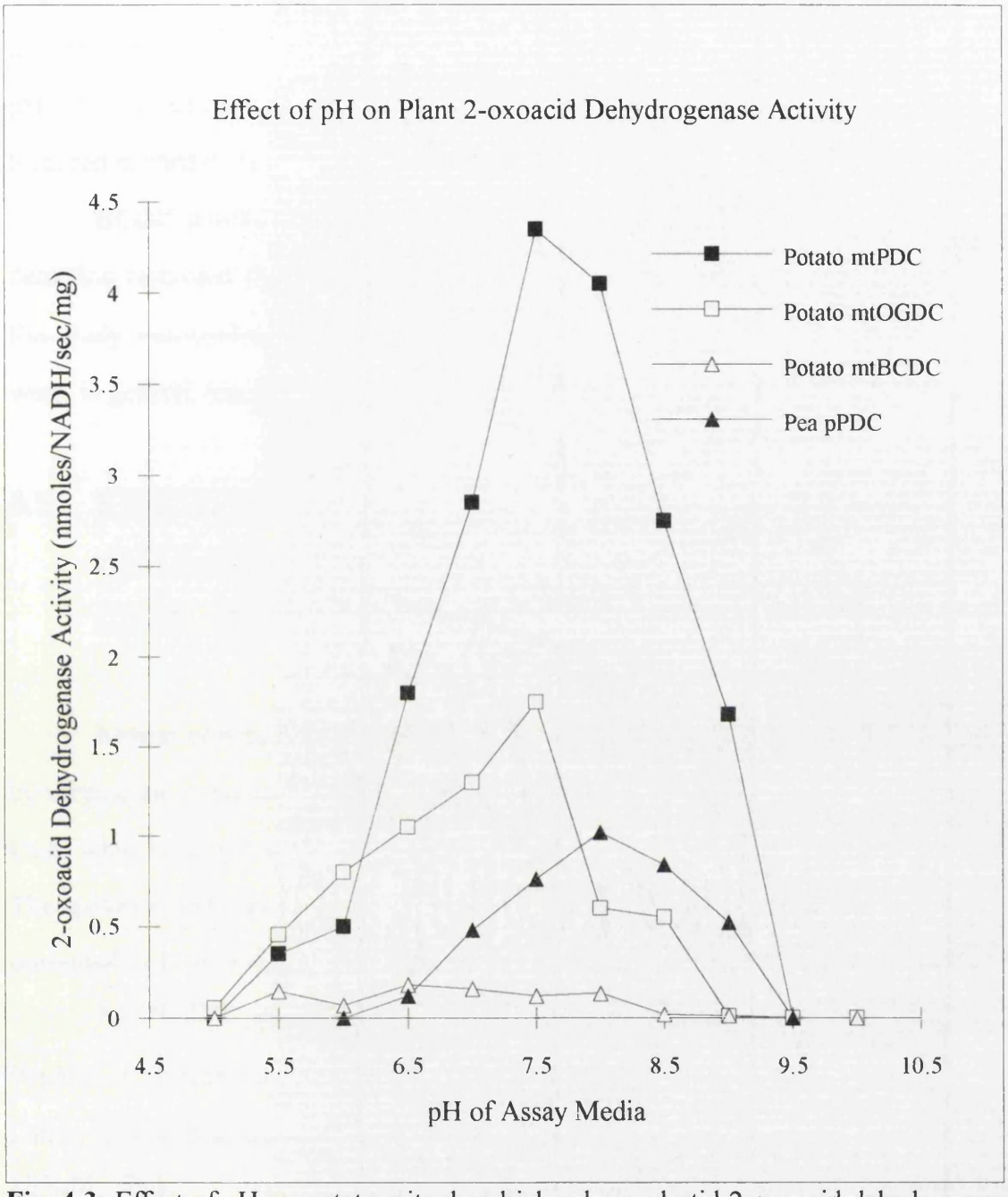


Fig. 4.3: Effect of pH on potato mitochondrial and pea plastid 2-oxoacid dehydrogenase activity. PDC, OGDC and BCDC activities were determined as described in Materials and Methods (2.5.4) with the following buffers used to hold the assay medium at the appropriate pH: pH 5.0-6.5, MES, pH 6.5-8.0, TES, pH 8.0-9.0, Bicine and pH 9.0-10.0, CHES. Specific activities are representative of a single preparation, however rates were monitored during at least three independent preparations and did not differ by more than ±5%.

OGDC is typical of a biological catalyst, forming a bell shaped curve. Potato mtPDC displayed optimal activity at pH 7.5. pH has a similar effect on potato mtOGDC, maximum activity being observed again at pH 7.5. Pea pPDC activity was optimised at pH 8.0. This alkaline pH optimum coincides with previously reported values for PDCs localised in plastid compartments (Camp & Randall, 1985).

BCDC activity was too low to estimate the pH optimum. This limited enzymatic detection restricted the kinetic analyses of potato mitochondrial BCDC. Consequently this study was confined to potato mitochondrial PDC and OGDC as BCDC activities were, in general, insufficient to generate accurate kinetic data.

4.5 KINETIC CONSTANTS FOR PYRUVATE DEHYDROGENASE AND 2-OXOGLUTARATE DEHYDROGENASE COMPLEX-CATALYSED REACTIONS

Kinetic constants were estimated for potato mtPDC and OGDC and pea pPDC by varying the concentration of one of the substrates, pyruvate/2-oxoglutarate, NAD^+ or CoA, while holding the concentrations of the other substrates at fixed, saturating levels. The apparent Michaelis constants were derived from the Lineweaver-Burk plots and are presented in Table 4.4.

$K_m(NAD^+)$ and K_m(pyruvate) values for potato mtPDC were estimated at 90μM and 130μM respectively, coinciding with previous reported values (Crompton & Laties, 1971). Michaelis constants determined for pea chloroplast PDC were similar, 185μM (NAD^+) and 180μM (pyruvate). K_m(CoA) values determined in this investigation were 32μM and 25μM for potato mtPDC and pPDC respectively, higher than the corresponding documented values. Previously reported K_m(CoA) constants were determined for partially purified preparations of mitochondrial and plastid PDC. In contrast, kinetic analyses carried out in this investigation were performed on mitochondrial and plastid extracts which may have contributed to the higher K_m(CoA).

Initial velocity experiments conducted for the potato mtOGDC-catalysed reaction estimated Michaelis constants of 445μM, 100μM and 20μM for 2-oxoglutarate, NAD^+

Michaelis constant	Potato tuber mitochondrial PDC μM	Potato mitochondrial OGDC μM	Pea chloroplast PDC μM
K_m(Pyruvate)	90	-	185
K_m(2oxoglutarate)	-	445	-
K_m(NAD^+)	130	100	180
K_m(CoA)	32	20	25
K_i(NADH)	20	18	15
K_i(Acetyl CoA)	20	-	20
K_i(Succinyl CoA)	-	40	-

Table 4.4: Initial velocity and inhibition constants for potato tuber mitochondrial PDC- and OGDC and pea chloroplast PDC-catalysed reactions. K_m values, calculated from Lineweaver-Burk plots. Inhibition constants were calculated as described in the text from Lineweaver-Burk plots displayed in Figs. 4.4 to 4.9. K_m and K_i values are representative of a single preparation; however kinetic constants were determined for at least three independent preparations and did not differ by more than ±10%.

and CoA. The recorded value for 2-oxoglutarate was relatively high for a primary substrate of an enzyme catalysed reaction. Mammalian OGDC is stimulated by Ca^{2+} which markedly decreases its K_m for 2-oxoglutarate. Potato mtOGDC may display similar properties to its mammalian counterpart. Consequently, omission of Ca^{2+} from the assay media may have contributed to the high K_m(2-oxoglutarate) value recorded in this investigation.

As the concentrations of secondary or tertiary substrates were not varied when determining K_m values, this initial velocity data does not provide evidence supporting a ping-pong mechanism. As initial velocity patterns supporting this mechanism have been extensively reported for the plant 2-oxoacid dehydrogenase complexes (Thompson *et al.*, 1977a; Miernyk & Randall, 1987b) it was decided to restrict ping-pong confirmation to product inhibition analyses.

4.6 PRODUCT INHIBITION OF PYRUVATE DEHYDROGENASE AND 2-OXOGLUTARATE DEHYDROGENASE COMPLEX-CATALYSED REACTIONS

All PDCs and OGDCs characterised to date from mammalian, microbial and plant sources are inhibited by the products of the overall reaction. As the initial velocity data obtained for all 2-oxoacid dehydrogenase complexes is consistent with a three-site ping-pong mechanism (Randall *et al.*, 1977; Miernyk & Randall, 1987b), product inhibition of potato mitochondrial PDC and OGDC and pea chloroplast PDC in accordance with this reaction mechanism was predicted. In such a case products binding at the same active site as the substrate molecule (e.g. acyl CoA and CoA and NADH and NAD^+) are expected to exhibit competitive inhibition patterns. In a similar vein, uncompetitive inhibitory behaviour is predicted if the product and substrate binding sites are distinct (acyl CoA and pyruvate and NADH and pyruvate).

A series of experiments were designed to investigate inhibition of the potato mtPDC, mtOGDC and pea chloroplast PDC-catalysed reactions by NADH and the appropriate acyl CoA. The initial velocity of reaction was measured under standard assay

conditions as described in Materials and Methods (2.5.4), varying the concentration of one of the substrates. The inhibitor, NADH or acyl CoA was introduced at two different concentrations immediately prior to the initiation of the reaction.

NADH and acetyl CoA competitively inhibited potato mtPDC with respect to their steady state precursors (Fig. 4.4). In contrast, when pyruvate concentration was varied in the presence of each of the products, plots of 1/Activity versus 1/[Pyruvate] yielded a series of parallel lines indicative of uncompetitive inhibition of mtPDC (Fig. 4.5). The kinetic analyses were performed on crude extracts and the reaction rates, in particularly in the presence of inhibitor, were relatively low. The resulting experimental error has led to slight anomalies in the inhibition patterns, e.g. the divergence of the parallel lines. The observed competitive and uncompetitive inhibition patterns (Fig. 4.4 and 4.5 respectively) were accepted, however, as numerous authors had reported similar inhibitory behaviour of the plant 2-oxoacid dehydrogenase complexes (Randall *et al.*,1977; Miernyk & Randall, 1987b). This holds true for product inhibition of potato mtOGDC and pea chloroplast PDC.

Potato mtOGDC was inhibited by NADH and succinyl CoA and all substrate-product combinations produced identical modes of inhibition to potato mtPDC. Figs. 4.6 and 4.7 depict the competitive and uncompetitive inhibition patterns of NADH and succinyl CoA versus their steady state precursors and 2-oxoglutarate respectively. As with potato mtPDC the pea chloroplast multienzyme complex was inhibited by the products of the overall reaction, NADH and acetyl CoA. Inhibition by NADH was competitive with respect to NAD^+ (Fig. 4.8A) and uncompetitive with respect to pyruvate (Fig. 4.9A). In addition, in conjunction with potato mtPDC, competitive and uncompetitive inhibitory patterns were observed when acetyl CoA inhibited versus CoA and pyruvate respectively (Figures 4.8B and 4.9B).

The inhibitory behaviour observed for PDC and OGDC is consistent with a ping-pong mechanism. Inhibition of potato mitochondrial PDC and OGDC and pea chloroplast PDC by NADH and the appropriate acyl CoA versus CoA and NAD^+ respectively was not uncompetitive as predicted by a ping-pong mechanism. Such anomalous behaviour has previously been reported for both plant and mammalian 2-oxoacid dehydrogenase

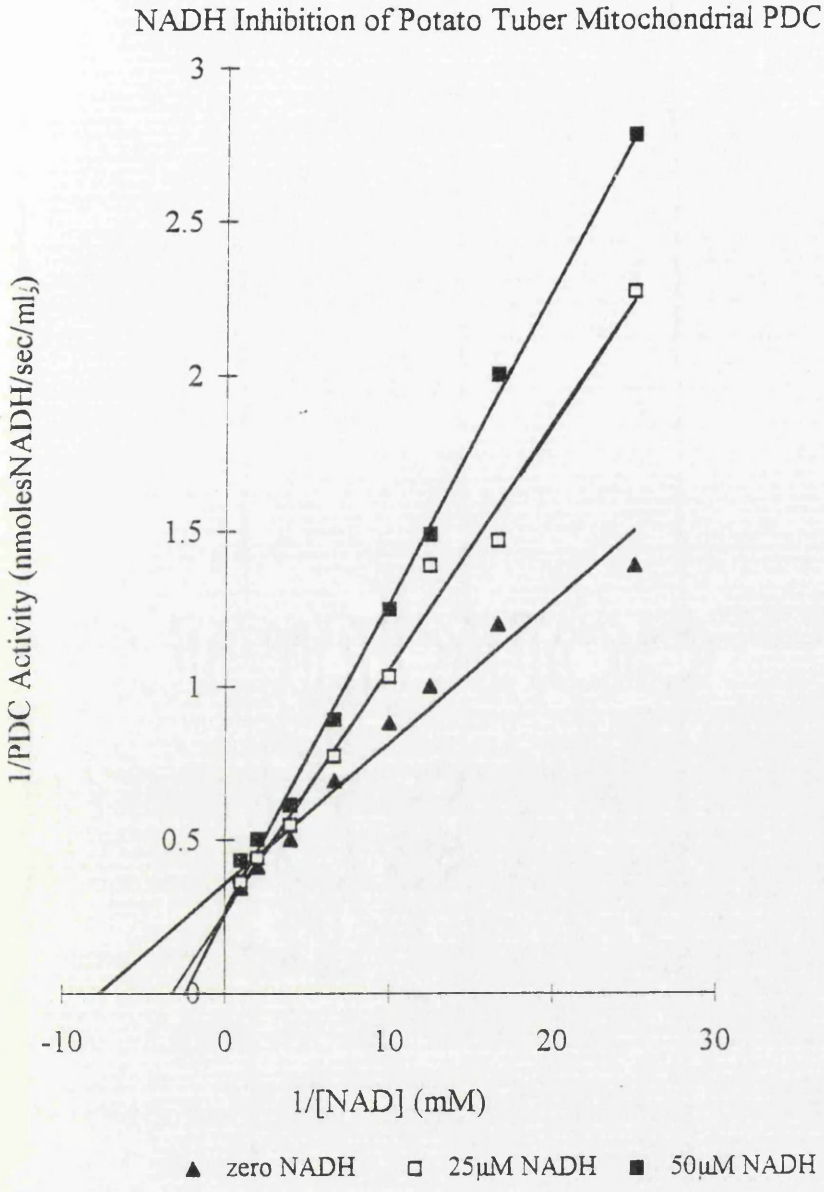


Figure 4.4A: Double reciprocal plot of velocity of a mtPDC-catalysed reaction versus [NAD^+] at zero, 20μM and 40μM NADH. PDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

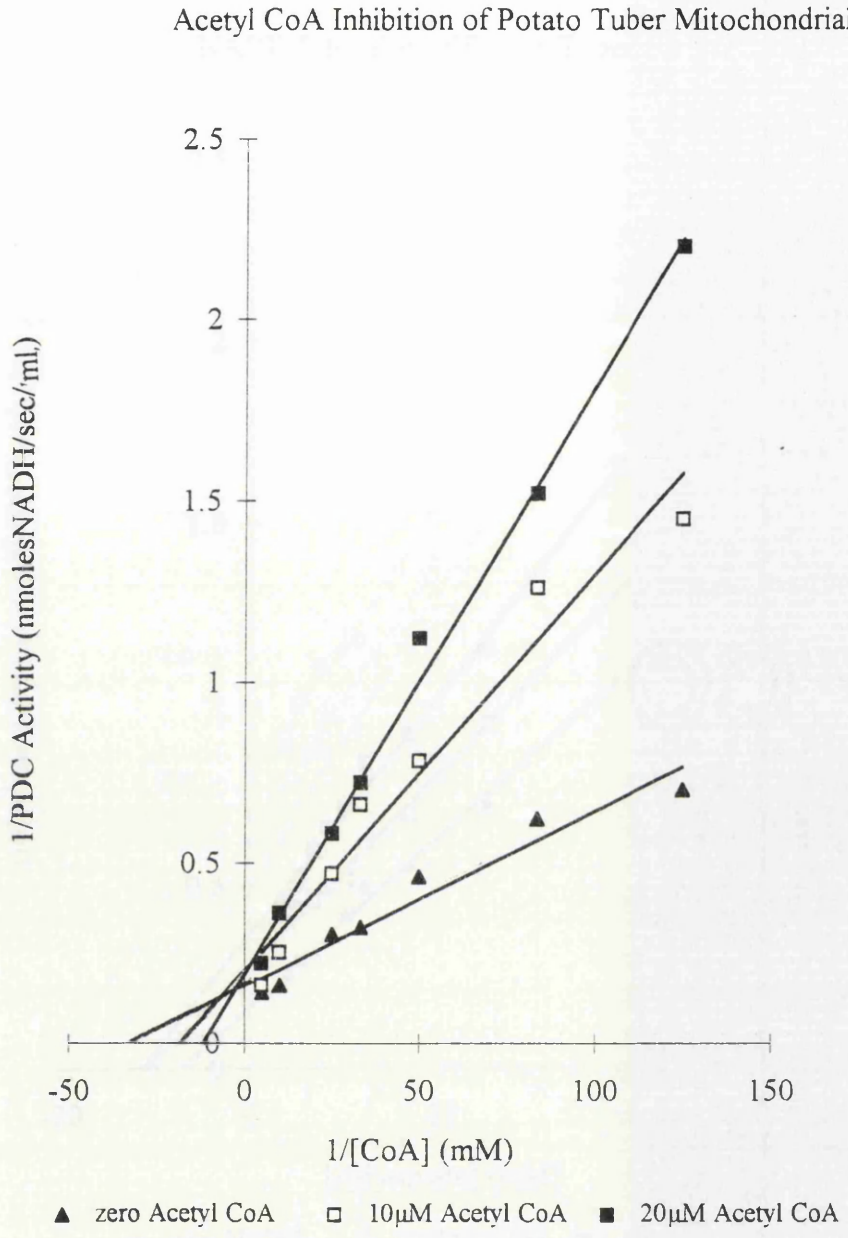


Figure 4.4B: Double reciprocal plot of velocity of a mtPDC-catalysed reaction versus [CoA] at zero, 10μM and 20μM acetyl CoA. PDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of CoA. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

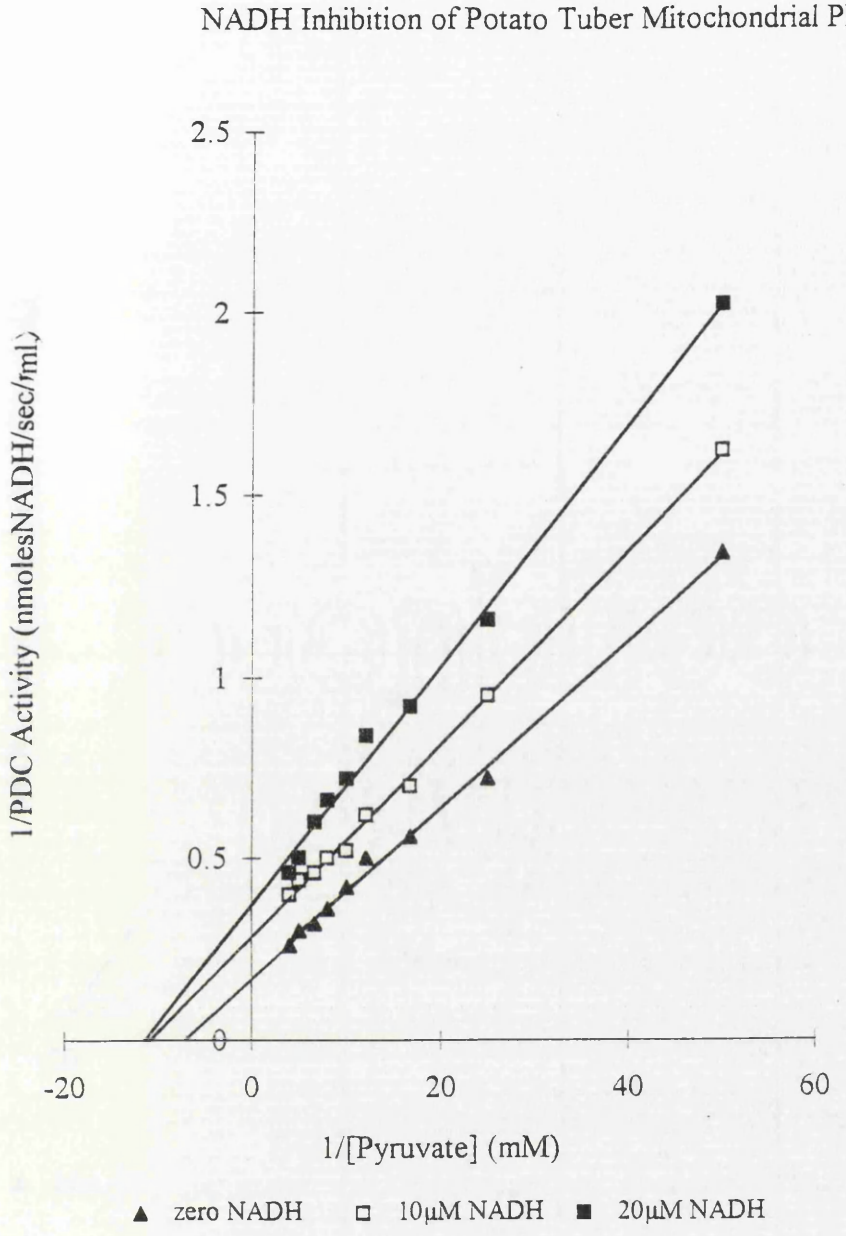


Figure 4.5A: Double reciprocal plot of velocity of a mtPDC-catalysed reaction versus [pyruvate] at zero, 20μM and 40μM NADH. PDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of pyruvate. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

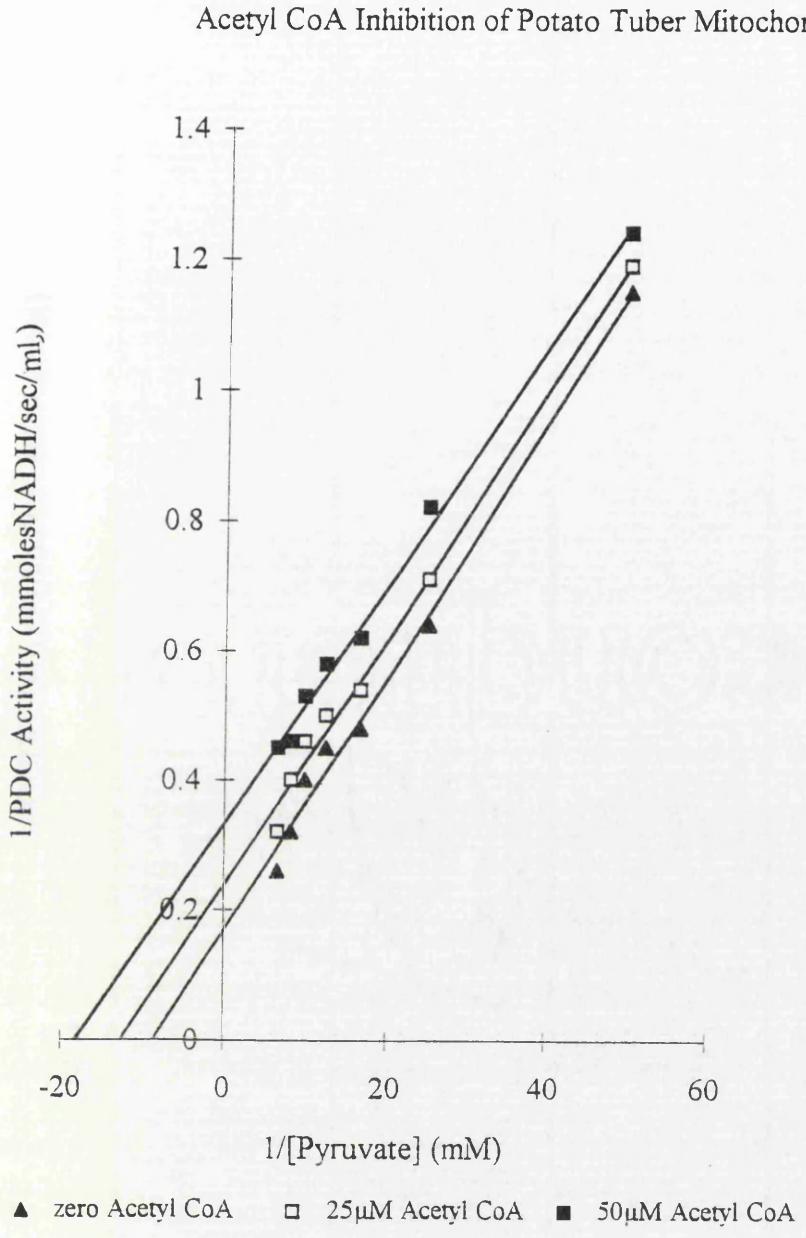


Figure 4.5B: Double reciprocal plot of velocity of a mtPDC-catalysed reaction versus [pyruvate] at zero, 20μM and 40μM acetyl CoA. PDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of pyruvate. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

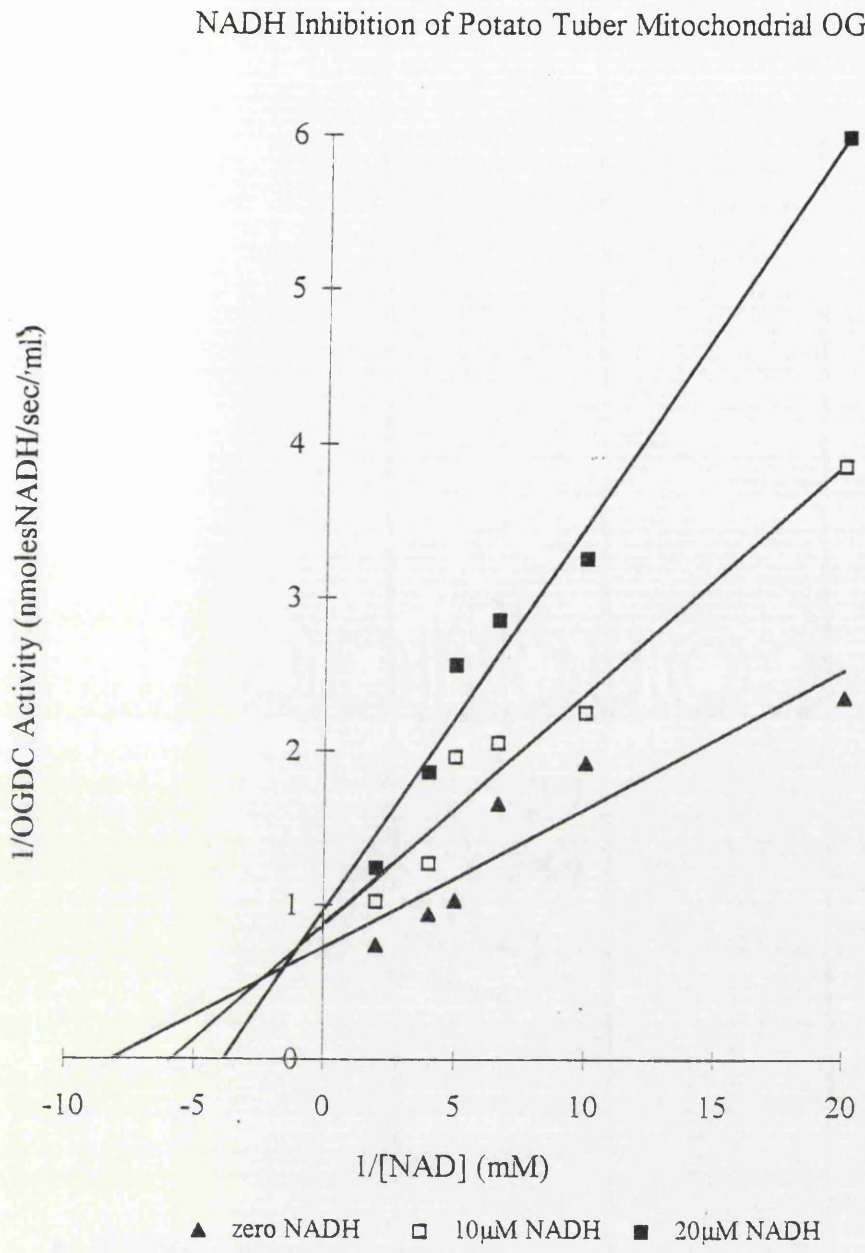


Figure 4.6A: Double reciprocal plot of velocity a mtOGDC-catalysed of reaction versus [NAD^+] at zero, 10μM and 20μM NADH. OGDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

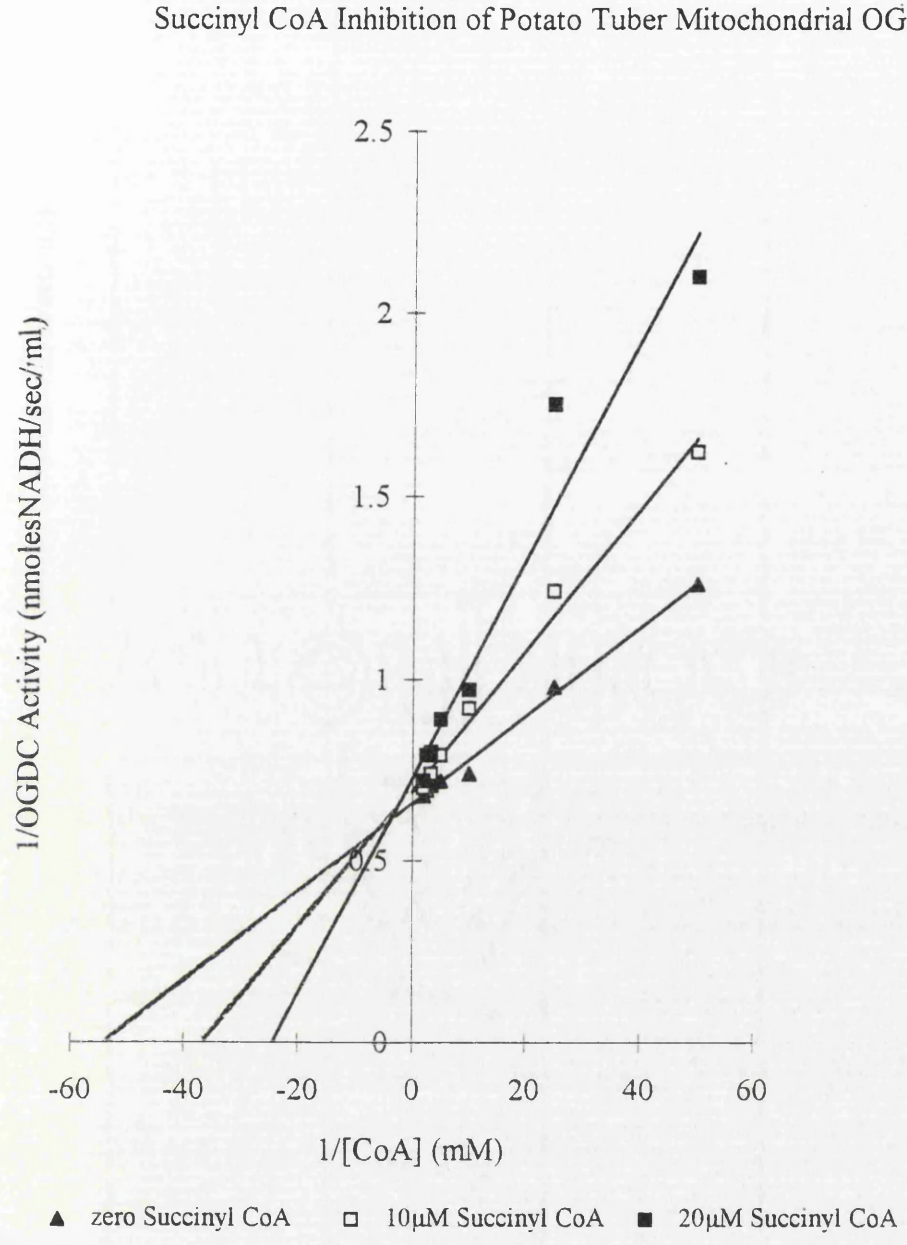


Figure 4.6B: Double reciprocal plot of velocity of a mtOGDC-catalysed reaction versus [CoA] at zero, 10μM and 20μM succinyl CoA. OGDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of CoA. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

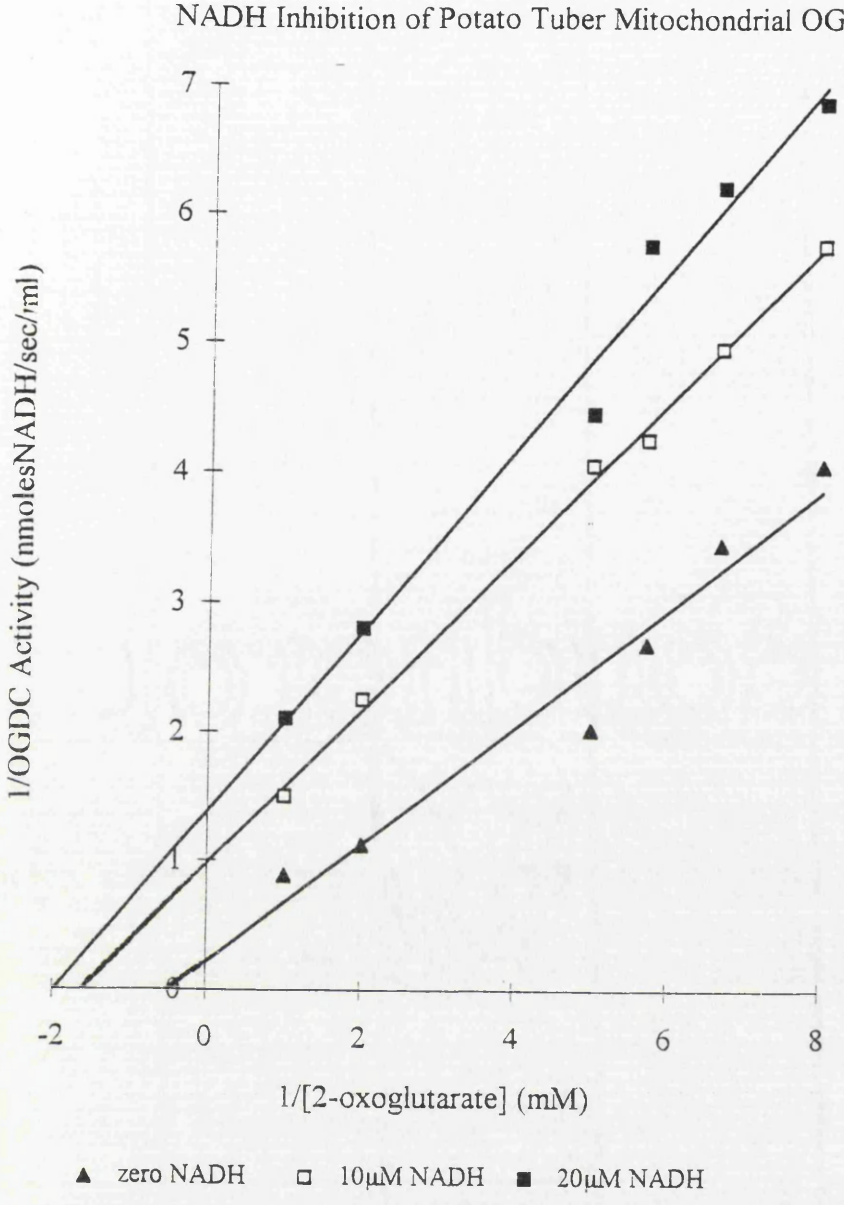


Figure 4.7A: Double reciprocal plot of velocity of a mtOGDC-catalysed reaction versus [2-oxoglutarate] at zero, 10μM and 20μM NADH. OGDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of 2-oxoglutarate. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

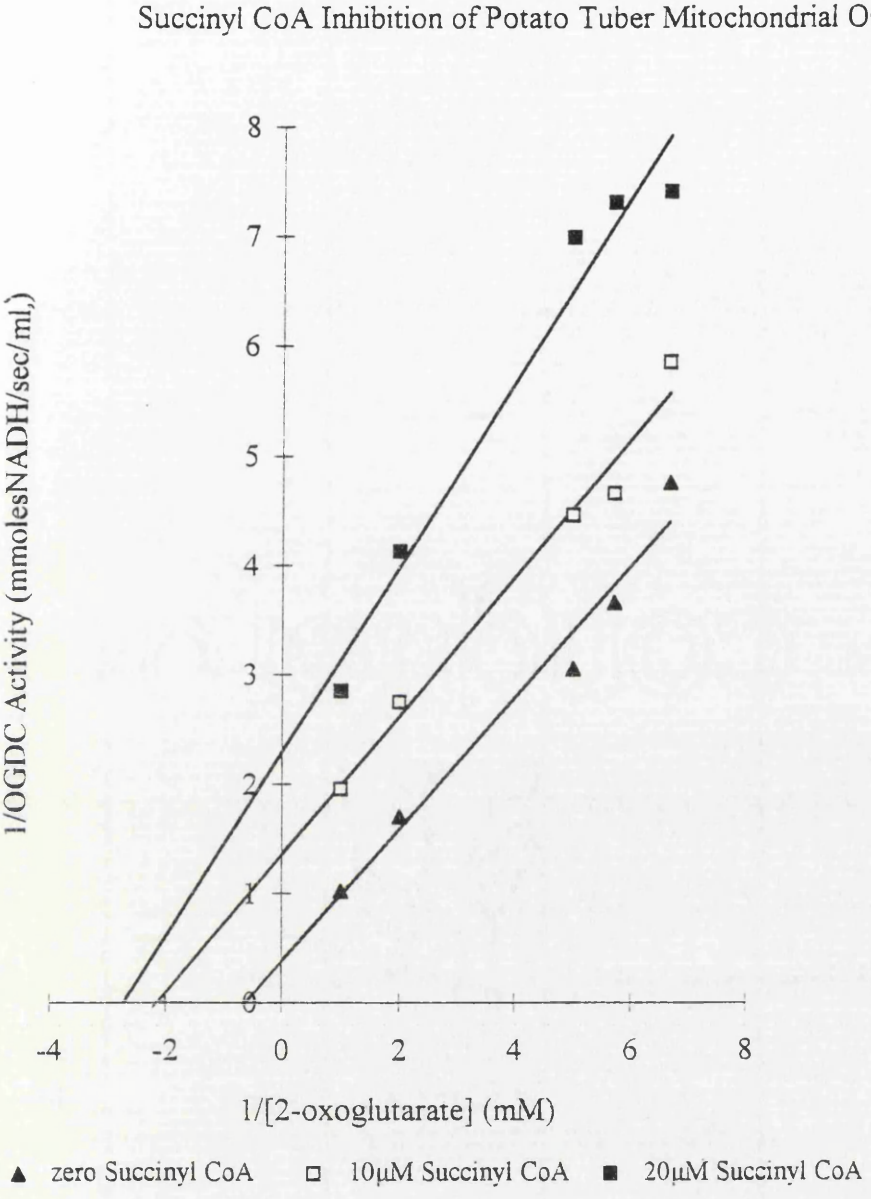


Figure 4.7B: Double reciprocal plot of velocity of a mtOGDC-catalysed reaction versus [2-oxoglutarate] at zero, 10μM and 20μM succinyl CoA. OGDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of 2-oxoglutarate. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

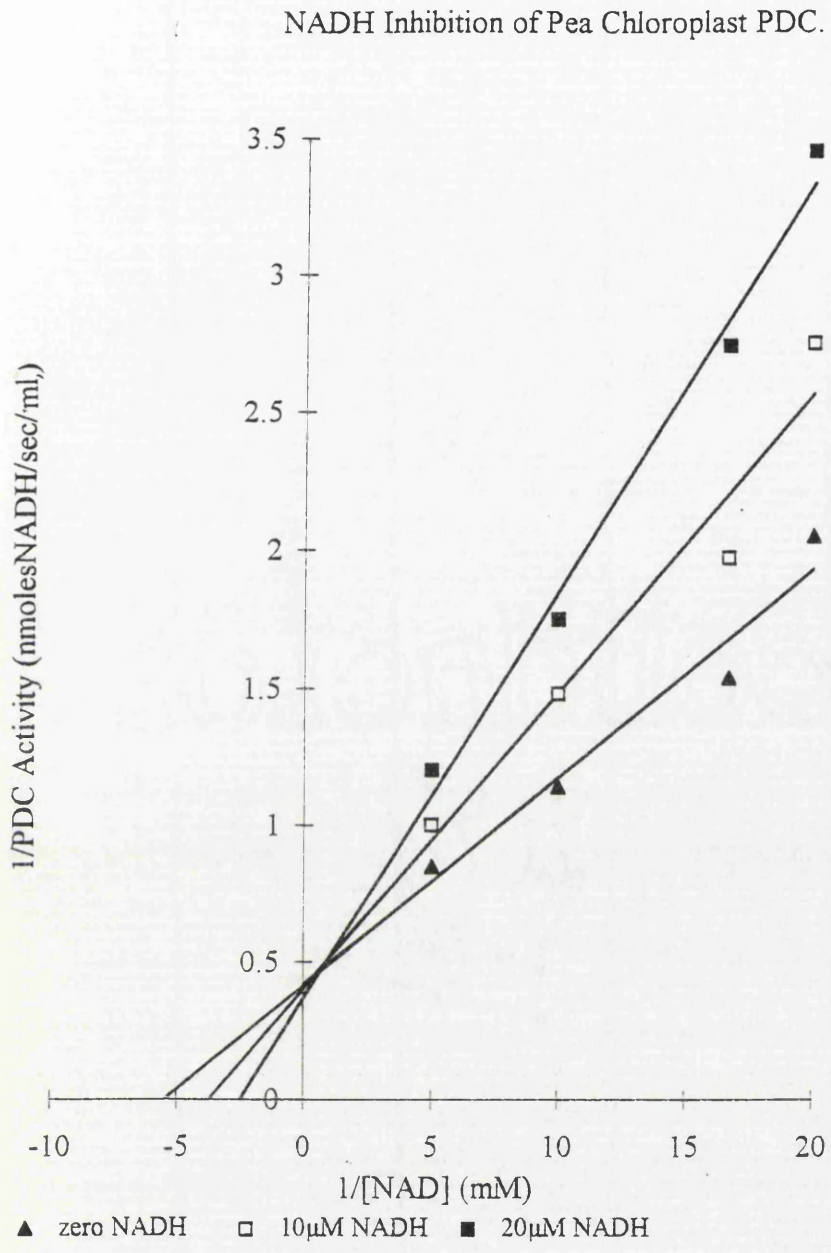


Figure 4.8A: Double reciprocal plot of velocity of a pPDC-catalysed reaction versus [NAD^+] at zero, 10μM and 20μM NADH. PDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of NAD^+. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

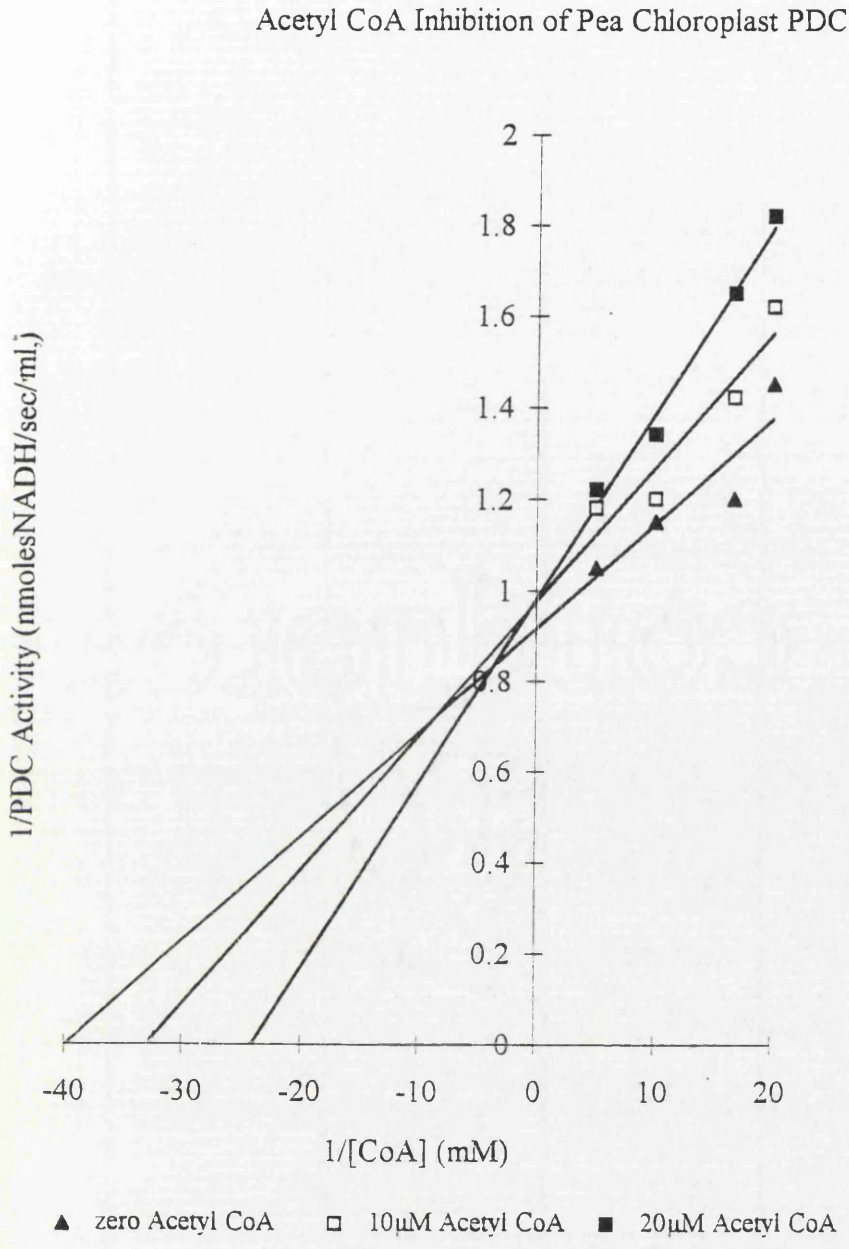


Figure 4.8B: Double reciprocal plot of velocity of a pPDC-catalysed reaction versus [CoA] at zero, 10μM and 20μM acetyl CoA. PDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of CoA. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

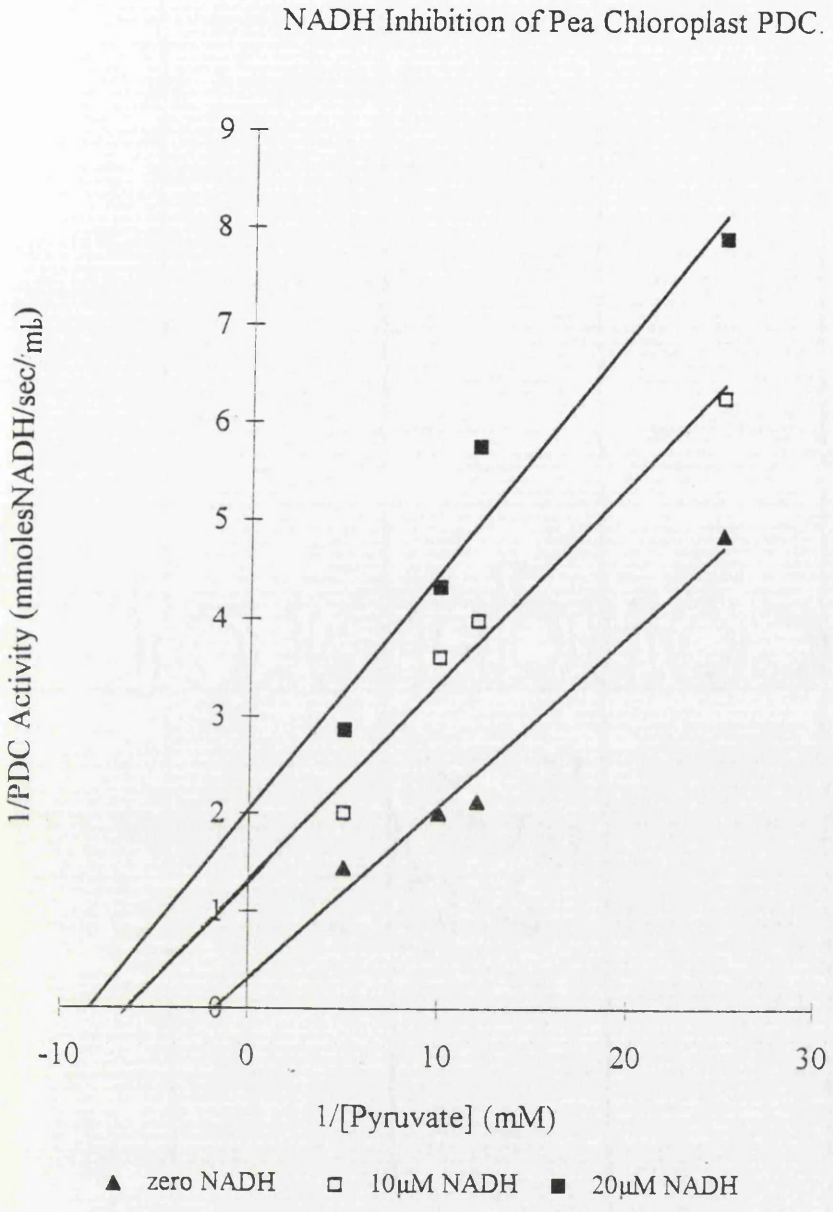


Figure 4.9A: Double reciprocal plot of velocity of a pPDC-catalysed reaction versus [pyruvate] at zero, 10μM and 20μM NADH. PDC activity, expressed in nmol NADH/sec/ml, was determined spectrophotometrically under standard assay conditions except with varying concentrations of pyruvate. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

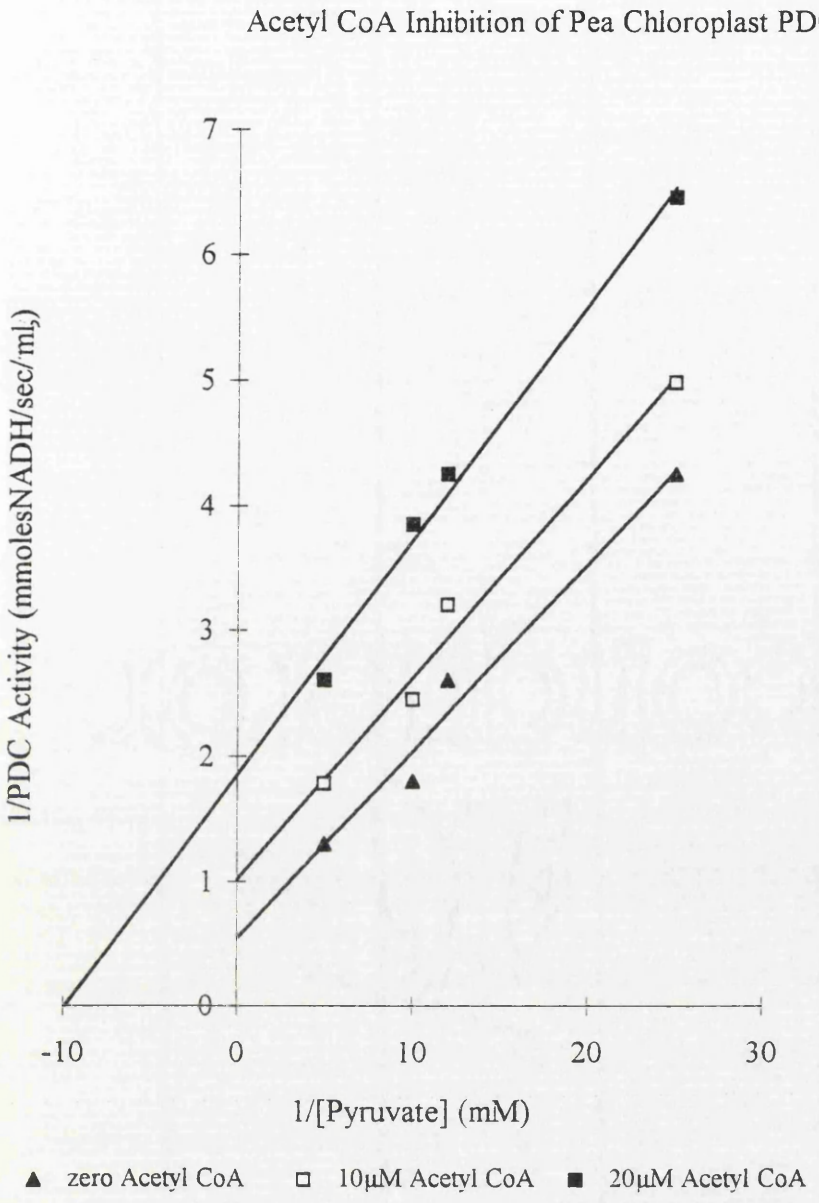
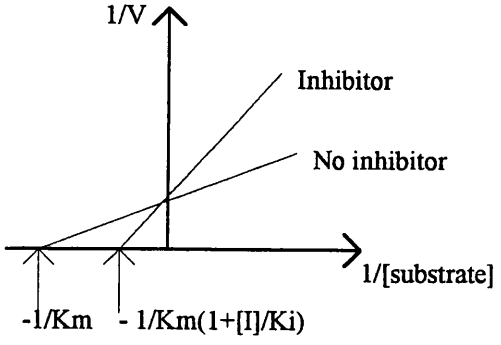


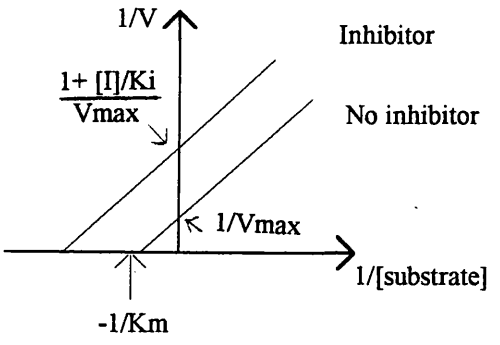
Figure 4.9B: Double reciprocal plot of velocity of a pPDC-catalysed reaction versus [pyruvate] at zero, 10μM and 20μM acetyl CoA. PDC activity, expressed in nmol NADH/sec/mg, was determined spectrophotometrically under standard assay conditions except with varying concentrations of pyruvate. Best fit lines were calculated by regression analyses using a least squares method (LINEST function, EXCEL).

complexes. It is thought to result from either steric hindrance due to the close proximity of the E2 and E3 active sites or conformational changes in E2, resulting from the binding of CoA or acyl CoA, interfering with NAD^+ or NADH binding to E3 (Tsai *et al.*, 1973).

As the substrate-product interactions have been analysed within the framework of Michaelis-Menten kinetics the K_i values can be easily calculated by estimating the K_m and Vmax in the presence of inhibitor for competitive and uncompetitive inhibition respectively:



COMPETITIVE INHIBITION



UN-COMPETITIVE INHIBITION

K_i values for the product inhibition of potato mitochondrial PDC and OGDC and pea chloroplast PDC are presented in Table 4.4. In accordance with previously documented values for plant mtPDCs (Crompton & Laties, 1971; Miernyk & Randall, 1987b), inhibitor constants for NADH and acetyl CoA of 20μM were determined for potato mtPDC-catalysed reaction. Product inhibition of pea pPDC estimated K_i values of 15μM and 20μM for NADH and acetyl CoA respectively, again agreeing with Randall's calculated constants (Camp *et al.*, 1988). Potato mtOGDC was also subject to product inhibition displaying values of 18μM (NADH) and 40μM (Succinyl CoA).

For each 2-oxoacid dehydrogenase complex-catalysed reaction analysed in this investigation, K_i(acyl CoA) and K_m(CoA) were very similar. In contrast, K_i(NADH) values were lower than the calculated $K_m(NAD^+)$. This indicates that each system, in common with all other 2-oxoacid dehydrogenase complexes, is primarily sensitive to the NAD^+:NADH ratio.

4.7 KINETIC ANALYSES OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

Examination of potato mtPDC and OGDC-cayalysed reactions as a function of pH over a range of 5.0 to 10.0 revealed a peak at pH 7.5 with 50% maximal activity at 6.8 and 8.5 and 6.0 and 7.8 respectively. Pea chloroplast PDC displayed maximal activity at pH 8.0, agreeing with previously reported pH optima for plastid located PDCs. The observed alkaline pH optima supports the proposed role of PDC within the plastid compartment; supplying acetyl CoA for fatty acid biosynthesis. This pathway is light driven and hence will be operative during periods of illumination i.e. when the cell is photosynthetically active. Photosynthesis increases the alkalinity of the stromal phase and increases the concentration of divalent cations and these conditions have been shown to stimulate plastid PDC activity.

Under optimal conditions of pH, divalent cations and cofactors, the kinetic constants of mitochondrial and chloroplast PDC agreed favourably with previous reports. The kinetic properties of potato mitochondrial OGDC had not been elucidated prior to this investigation. K_m values estimated for CoA and NAD^+ were very siminlar to the PDC reaction sequence whilst the K_m(2-oxoglutarate) value was high, 445μM, compared to a K_m(pyruvate) of 90μM and 185μM for mitochondrial and plastid PDC respectively. Mammalian OGDC displays a high K_m(2-oxoglutarate) which is dramatically reduced in the presence of Ca^{2+}. It is possible that the plant multienzyme complex is stimulated in a similar manner and consequently, the absence of Ca^{2+} may have contributed to the high K_m (2-oxoglutarate) value.

The 2-oxoacid dehydrogenase activities investigated were inhibited by the products of the overall reaction, NADH and acyl CoA, and the inhibitory beheviour was consistent with a ping pong mechanism. Competitive inhibition patterns were observed when varying the concentration of CoA and NAD^+ in the presence of fixed concentrations of acyl CoA and NADH respectively. Inhibition patterns were uncompetitive when acyl CoA and NADH were introduced with varying concentrations of the respective 2-oxoacid substrate.

In common with all other 2-oxoacid dehydrogenase complexes studied to date potato mtPDC and OGDC and pea pPDC activities are prone to regulation by end product inhibition displaying greatest sensitivity to the NADH:NAD^+ regime. Mitochondrial PDC, unlike its chloroplast counterpart, is also subject to reversible inactivation by phosphorylation (Randall *et al.*,1977; 1981). A potential phosphorylation scheme controlling the activity of plant OGDC has never been investigated, however, the mammalian complex is not prone to control by covalent modification. With the exception of mtPDC, the extensive screening of metabolites for their regulatory powers has yet to be performed on the plant complexes. As these multienzyme systems occupy prime sites in central metabolism it is envisaged that different layers of regulatory mechanisms, including end product inhibition, are operative allowing fine *in vivo* control of the 2-oxoacid dehydrogenase complexes.

CHAPTER FIVE

SUBUNIT COMPOSITION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

5.1 INTRODUCTION

The structural organisation of mammalian 2-oxoacid dehydrogenase complexes has been extensively documented (Yeaman, 1986; 1989). By comparison information on the size, organisation and subunit structure of the plant complexes is limited and has focused mainly on PDC (Camp & Randall, 1985; Taylor *et al.*, 1992). Subunit composition can be investigated immunologically employing antisera to the 2-oxoacid dehydrogenase complexes and Western blotting techniques allows estimation of the M_r values of immuno-reactive polypeptides. Antisera to the mammalian 2-oxoacid dehydrogenase complexes and to specific subunits were available in Glasgow allowing the immunological investigation of the subunit composition of this multienzyme family from pea and potato reported in this chapter.

This line of investigation depends upon sufficient homology between the 2-oxoacid dehydrogenase complexes from the mammalian and plant kingdoms; the plant multienzyme complexes may be so distantly related to their mammalian counterparts that little or no cross-reactivity will be detected. From the outset therefore, the immunological probing of the plant complexes with antisera to the mammalian counterparts was limited by the potential lack of homology and the occurrence of non-specific interactions. In addition low concentrations and poor reactivity of antibody to antigen may interfere with immunological detection of the plant 2-oxoacid dehydrogenase complexes. This research programme was considered viable, however, as Taylor *et al.* (1992) reported specific cross-reactions of pea mitochondrial and chloroplast protein with antiserum to specific subunits of PDC isolated from bovine heart.

Several groups have previously identified subunits of plant mitochondrial and plastid PDC by immunological techniques. Randall and co-workers raised antisera to purified broccoli mtPDC (Rubin & Randall, 1977a). Partially purified pea mtPDC was challenged with this antisera and five immuno-reactive polypeptides were identified with M_r values 97,700, 67,400, 58,100, 43,000 and 37,300 (Camp & Randall, 1985). Additional experiments established the nature of two polypeptides: the 58,100Da protein

cross-reacted with anti-porcine E3 antiserum and was subsequently identified as the dihydrolipoamide dehydrogenase component while the 43,000Da protein was recognised as the E1α subunit by phosphorylation studies (Miernyk *et al.*, 1985). As the E1α subunit of plant mtPDC is susceptible to phosphorylation with concomitant inactivation, incubation of pea mtPDC with Mg-[γ-^{32}P]ATP was performed to trace the phosphorylated polypeptide. Loss of catalytic activity was paralleled by the accumulation of radio-label in a 43,000Da species, indicating that this polypeptide was probably the E1α subunit. The 97,700Da cross-reacting species may represent the E1 subunit of OGDC. OGDC is a major contaminant of purified preparations of PDC, consequently, antiserum raised to broccoli mtPDC may also display reactivity to component enzymes of OGDC. The identity of the 67,400 immuno-reactive polypeptide remains unresolved; its M_r is very similar to the E2 subunit of mammalian PDC, however, species detected in the M_r range of 65,000-70,000 are generally considered to be artefacts (see section 5.4).

In addition, immunological analyses of pea chloroplast PDC with antibodies to broccoli mtPDC was carried out by Camp and Randall (1985). In common with the pea mitochondrial complex, this antiserum cross-reacted with a 98,000 and 58,000Da pea chloroplast protein. The 58,000Da subunit was identified as E3 as it displayed additional cross-reactivity with anti-porcine E3 antibodies. In contrast to the pea mitochondrial immuno-reactive profile reported by these authors, cross-reacting chloroplast proteins of M_r values 43,000 and 37,000 were absent.

Taylor *et al.* (1992) exploited antisera to specific subunits of mammalian PDC to provide a more detailed analysis of the subunit composition of pea mitochondrial and plastid PDC. This study identified differences in subunit composition between the mammalian, pea mitochondrial and pea chloroplast multienzyme complex. Antisera to the E2 subunit of mammalian PDC cross-reacted with 50,000Da pea mitochondrial and chloroplast protein. Mammalian E2 has an M_r value of 74,000 and carries two lipoyl domains. The lower M_r of the plant enzyme may reflect the presence of only one covalently attached lipoyl domain as in yeast PDC and mammalian OGDC and BCDC.

The use of anti mammalian E3 serum resulted in the immunological detection of a chloroplast protein with a M_r value of 52,000, very similar to the mammalian subunit

(55,000), however, the equivalent immuno-reactive mitochondrial polypeptide displayed a M_r of 67,000. In addition, a species of identical M_r value cross-reacted with antibodies to component X. Component X was identified as an immunologically distinct subunit of mammalian PDC (M_r, 51,000) and is believed to aid E3 binding to the E2 core assemblage (Powers-Greenwood *et al.*, 1989; Lawson *et al.*, 1991b; Neagle & Lindsay, 1991). A 48,000Da pea chloroplast protein cross-reacted with anti X-serum and this was the first report of the potential presence of component X in plant PDC.

The dual binding of antisera to mammalian E3 and X to a 67,000Da pea mitochondrial protein may reflect potential X sequences integrated in pea mitochondrial E3 resulting in the high M_r form of the two subunits. Such a phenomenon has been demonstrated by Rice and Lindsay (1992) who identified a component X-like domain integrated into the N-terminal region of the E1 subunit of mammalian OGDC.

Taylor *et al.* (1992) reported negligible cross-reactivity of pea mitochondrial and chloroplast protein with antibodies to mammalian E1 from PDC. Incubation with antiserum to the analogous subunit from yeast identified a strong immuno-reactive pea mitochondrial polypeptide of M_r value 41,000. Taking into account the phosphorylation experiments conducted by Miernyk *et al.* (1985), this protein was recognised as the E1α subunit. The mammalian, pea mitochondrial and chloroplast E1 components of PDC appear to be antigenically distinct, whereas a close relationship between yeast and pea mitochondrial E1 was documented. Such observations may provide information concerning the evolutionary origin of plant mitochondria and chloroplasts.

A detailed analyses of plant OGDC and BCDC was undertaken paralleling the immunological investigations of plant PDC undertaken by Taylor *et al.* (1992). Antibodies to specific subunits of mammalian OGDC and BCDC were utilised to identify immuno-responsive plant mitochondrial proteins. The use of subunit specific antisera allows the estimation of M_r values of the prospective subunits of the plant 2-oxoacid dehydrogenase complexes.

5.2 IMMUNOLOGICAL DETECTION OF PLANT PYRUVATE DEHYDROGENASE COMPLEX BY WESTERN BLOTTING

5.2.1 INTACT PYRUVATE DEHYDROGENASE COMPLEX

Potato tuber and pea root mitochondrial and pea chloroplast proteins were precipitated with 80%(v/v) acetone and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). After transferring onto a nitrocellulose filter, proteins were incubated with antibodies to intact bovine heart PDC. Antibody-antigen associations were subsequently visualised by incubation with ^{125}I-labelled protein A as described in Materials and Methods (2.8). Fig 5.1 illustrates the banding patterns of immuno-reactive polypeptides after blotting against antiserum to the intact complex.

The component enzymes of mammalian PDC (lanes 1,2) were clearly resolved by SDS-PAGE and immunoreactive polypeptides were identified with M_r values of 69,000, 56,000, 50,000, 41,000, and 36,000, corresponding to the E2, X, E3, E1α and E1β subunits respectively. An intense cross-reaction was observed with the E2 and E1 component enzymes, the E1 component enzymes appear as one intense cross-reaction owing to overloading of bovine heart PDC, whereas E3 and X displayed weaker cross-reactivity. An intense cross-reaction was detected with an M_r value 53,000 when potato mitochondrial (lanes 3,4), pea mitochondrial (lanes 5,6) and pea chloroplast (lanes 7,8) proteins were exposed to intact PDC antiserum. Mitochondrial extracts derived from both potato and pea displayed additional species with M_r values 73,000, 70,000, 42,000 and 36,000 which displayed minor reactivity.

Antisera to the intact bovine heart PDC initially displays reactivity predominantly to the E2 and E1 subunits only (De Marcucci *et al.*, 1985). The E3 and X components cross-react, however, when exposed to later bleeds of antiserum. This phenomenon is illustrated in the tracks of of bovine heart PDC (Fig 5.1, lanes 1,2) where an intense cross-reaction is detected with E2 and E1 whilst the remaining subunits display weaker cross-reactivity. As the E1 component of plant PDC is antigenically distinct from the

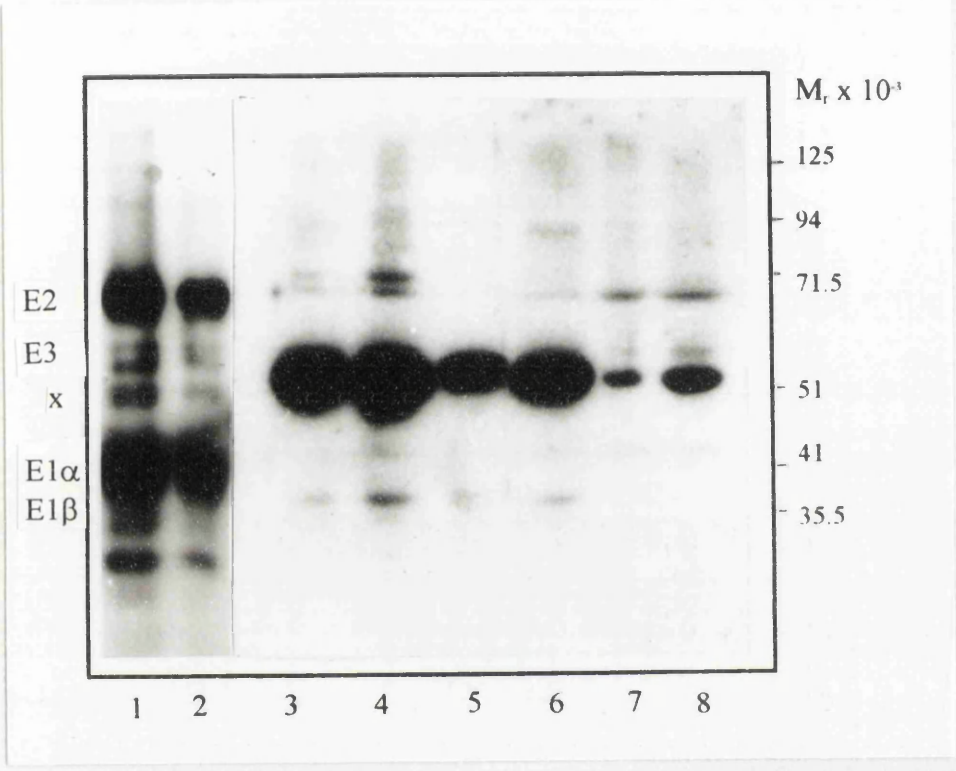


Figure 5.1: Immunological detection of PDC in extracts of potato and pea mitochondria and pea chloroplasts.
Organelle proteins were electrophoresed in 10%(w/v) SDS polyacrylamide gel slabs and electrophoretically transferred onto a nitrocellulose membrane. Membranes were incubated with anti-PDC serum and antibody-antigen associations were detected by autoradiography following incubation with ^{125}I-labelled protein A as described in Materials and Methods (2.8). Lanes 1,2, purified mammalian PDC (0.5μg and 0.2μg respectively); Lanes 3,4, potato mitochondria (15μg and 30μg respectively); Lanes 5,6, pea mitochondria (15μg and 30μg respectively); Lanes 7,8, pea chloroplasts (15μg and 30μg respectively).

analogous mammalian subunit (Taylor *et al.*, 1992) it is possible that the immunological probing of pea and potato mitochondrial and pea chloroplast extracts with intact PDC antiserum only detects the E2 subunit of plant PDC (53,000). Plant protein was therefore challenged with subunit specific antiserum in order to identify individual components of the plant multienzyme complex.

5.2.2 THE E2 SUBUNIT OF PLANT PYRUVATE DEHYDROGENASE COMPLEX

Pea and potato mitochondrial and pea chloroplast proteins were incubated with antiserum to the E2 subunit of mammalian PDC (Fig 5.2). The high specificity of the antiserum is demonstrated in the track of purified bovine heart PDC (lane 1) where a single band was detected; the E2 component enzyme of M_r 68,000. In each case, a single immuno-reactive polypeptide of M_r value 53,000 was identified, in agreement with the report by Taylor *et al.* (1992) and complementing the immuno-reactive profile resulting from incubation with antibodies to intact PDC. The chloroplast antigenic species exhibited a weaker cross-reactivity than the equivalent mitochondrial species. This suggests that either the chloroplast cross-reacting polypeptide is antigenically distinct from its mitochondrial counterpart displaying weaker reactivity to the mammalian antiserum or that lower levels of antigen are expressed in the chloroplast compartment. Lower levels of chloroplast PDC is supported by enzymatic investigations; pea chloroplast PDC displayed approx. 10% the activity of pea mtPDC (section 3.4). The lower M_r of the plant component enzyme suggests that the plant mitochondrial and plastidic subunit may carry only one lipoyl containing domain paralleling the situation in yeast PDC and mammalian OGDC and BCDC.

The use of bovine heart subunit specific antisera allows only the tentative identification of certain plant proteins as the E2 component enzyme of PDC. In order to class the immuno-reactive species unambiguously as a subunit of this multienzyme complex a more rigorous characterisation is required. It has been extensively documented that plant PDC, in common with their mammalian and microbial

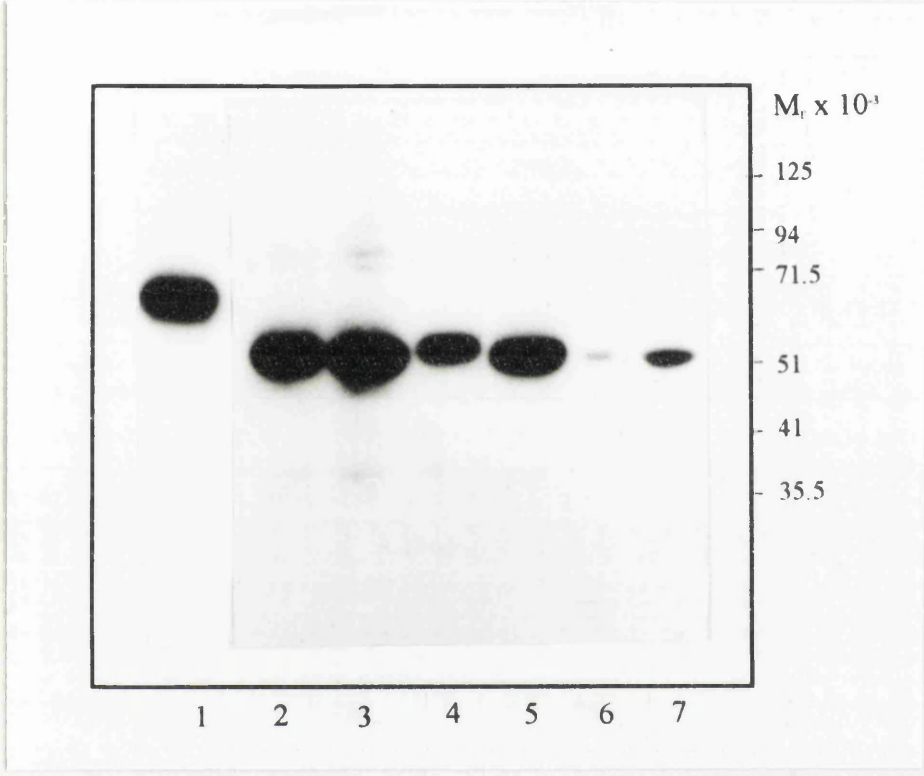


Figure 5.2: Immunological detection of the E2 subunit of PDC in extracts of potato and pea mitochondria and pea chloroplasts.
The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to the E2 component of mammalian PDC. Lane 1, purified mammalian PDC (0.5µg); Lanes 2,3, potato mitochondria (15µg and 30µg respectively); Lanes 4,5, pea mitochondria (15µg and 30µg respectively); Lanes 6,7, pea chloroplasts (15µg and 30µg respectively).

counterparts, have M_r values of several million (Rubin & Randall, 1977a; Camp & Randall, 1985). Consequently, in order to reinforce the information gained from the Western blot analyses, investigations were designed to demonstrate that the cross-reacting species were associated with a high M_r aggregate.

Approximately 10mg of solubilised pea root mitochondrial and pea chloroplast proteins were applied in turn to a Superose 12 column equilibrated with 20mM potassium phosphate (pH 7.2) with 1mM DTT and 5mM NaCl. Proteins were subsequently eluted from the gel permeation column which was connected to a Pharmacia FPLC system and the elution of PDC, OGDC and E3 and activities were tracked enzymatically. The distribution of these activities is illustrated in Fig 5.3.

Pea mitochondrial PDC and OGDC activities were eluted in the void volume (6-7ml) in accordance with a M_r value of several million, whereas E3 activity formed two elution peaks: one at 6-7ml and another at 10-11ml. The void volume E3 activity is that which is associated with intact PDC and OGDC and the second peak reflects 'free' E3 (eluted in accordance with M_r 110,000 as indicated by M_r markers). The M_r of E3 as determined by gel filtration compared to SDS-PAGE (M_r, 58,000, section 5.3) is consistent with its presence as homodimers. Fractionation of pea stromal protein by gel filtration resulted in an identical E3 profile; however, PDC activity was not detected in the gel filtration fractions. It was assumed that intact chloroplast PDC was eluted in the void volume fraction which contained E3 activity with low multienzyme concentrations limiting enzymatic detection.

Specific fractions from the gel permeation analysis were concentrated by centrifugation through Centricon tubes with a 30,000Da retentive membrane and prepared for SDS-PAGE. After gel electrophoresis and transfer to nitrocellulose the samples were challenged with antisera to the E2 subunit of mammalian PDC (Fig 5.4). It is clear that the cross reacting species (M_r 53,000) was confined to the void volume fractions. This indicates that the cross-reacting polypeptides are associated with a high M_r complex, e.g. PDC.

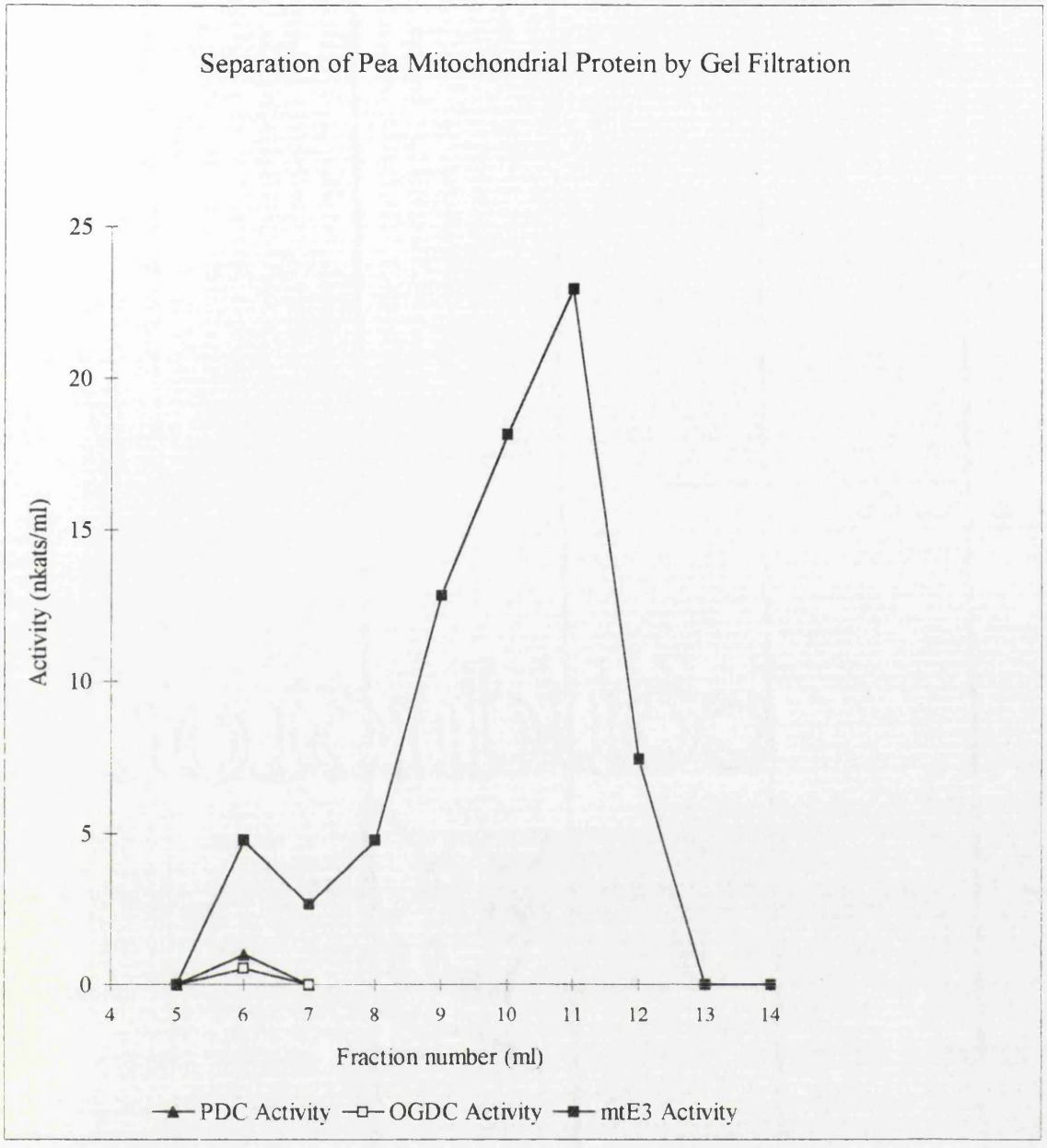


Figure 5.3: Separation of pea mitochondrial PDC, OGDC and E3 activities by gel filtration.
A Triton X-100 solubilised mitochondrial extract was applied to a Superose 12 column connected to a Pharmacia FPLC system. Protein was eluted with 20mM phosphate buffer pH7.2 with 1mM DTT and 1mM NaCl at a flow rate of 0.5ml/min. Fractions were assayed for PDC, OGDC and E3 activity as described in Materials and Methods (2.5). Activities are expressed in nkats/ml.

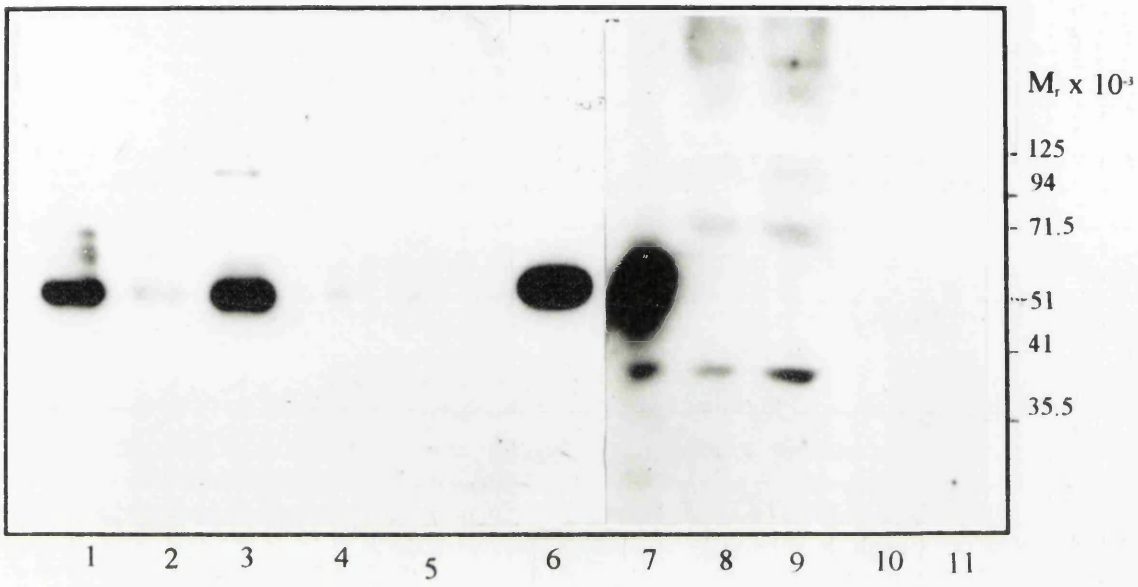


Figure 5.4: Immunological detection of the E2 subunit of PDC in pea mitochondrial and chloroplasts extracts separated by gel filtration.
Triton X-100 solubilised protein was applied to a Superose 12 column connected to a FPLC system and eluted with potassium phosphate buffer (pH 7.2) containing 1mM DTT and 5mM NaCl. Fractions (1ml) were collected and specific fractions were electrophoresed and immunoblotted as described in Fig. 5.1 except that the primary antibody was to the E2 component of mammalian PDC. Lane 1, pea mitochondria (20μg); Lane 2, fraction 5, gel filtered pea mitochondria (15μg); Lane 3, fractions 6-7 gel filtered pea mitochondria (15μg); Lane 4, fractions 8-10 gel filtered pea mitochondria (15μg); Lane 5, fractions 11-13 gel filtered pea mitochondria (15μg); Lane 6, pea chloroplasts (20μg); Lane 7, fractions 6-7 gel filtered pea chloroplasts (15μg); Lane 8, fraction 8-9 gel filtered pea chloroplasts (15μg); Lane 9, fractions 10-11 gel filtered pea chloroplasts (15μg); Lane 10, fractions 12-13 gel filtered pea chloroplasts (15μg); Lane 11, fractions 14-15 gel filtered pea chloroplasts (15μg).

5.2.3 THE E1 SUBUNIT OF PLANT PYRUVATE DEHYDROGENASE COMPLEX

No immunological cross-reactions were observed when exposing plant mitochondrial and plastid protein to antiserum to the E1 subunit of mammalian PDC. The component enzyme from the plant multienzyme complex is therefore immunologically distinct from the analogous mammalian subunit. Taylor *et al.* (1992) also reported negligible cross-reactivity of pea mitochondrial and chloroplast protein with anti E1-serum while an immunological reaction was detected when probing mitochondrial extracts with antiserum to the E1 subunit of yeast PDC. This observation suggests that the plant mitochondrial component enzyme displays greater homology to the yeast subunit.

5.3 IMMUNOLOGICAL DETECTION OF DIHYDROLIPOAMIDE DEHYDROGENASE (E3)

Immunological detection of the E3 subunit, a component enzyme associated with each 2-oxoacid dehydrogenase complex, was also carried out (Fig 5.5). The E3 subunit of mammalian OGDC and PDC (lanes1 and 8, 9 respectively) was identified with an M_r value of 55,000. In agreement with Taylor *et al.* (1992) a single pea chloroplast cross-reacted with anti-E3 serum displaying a very similar M_r value to the mammalian subunit (52,000). The immuno-reactive mitochondrial and chloroplast species were distinct suggesting that the chloroplast signal results from a plastid polypeptide and not from mitochondrial contamination.

Taylor *et al.* (1992) reported a single cross-reaction (M_r 67,000) of pea mitochondrial protein with anti-E3 serum. In contrast two immuno-reactive potato and pea mitochondrial proteins were detected in this investigation displaying M_r values of 67,000 and 58,000 (lanes 2, 3 and 6, 7 respectively). This cross-reacting doublet was

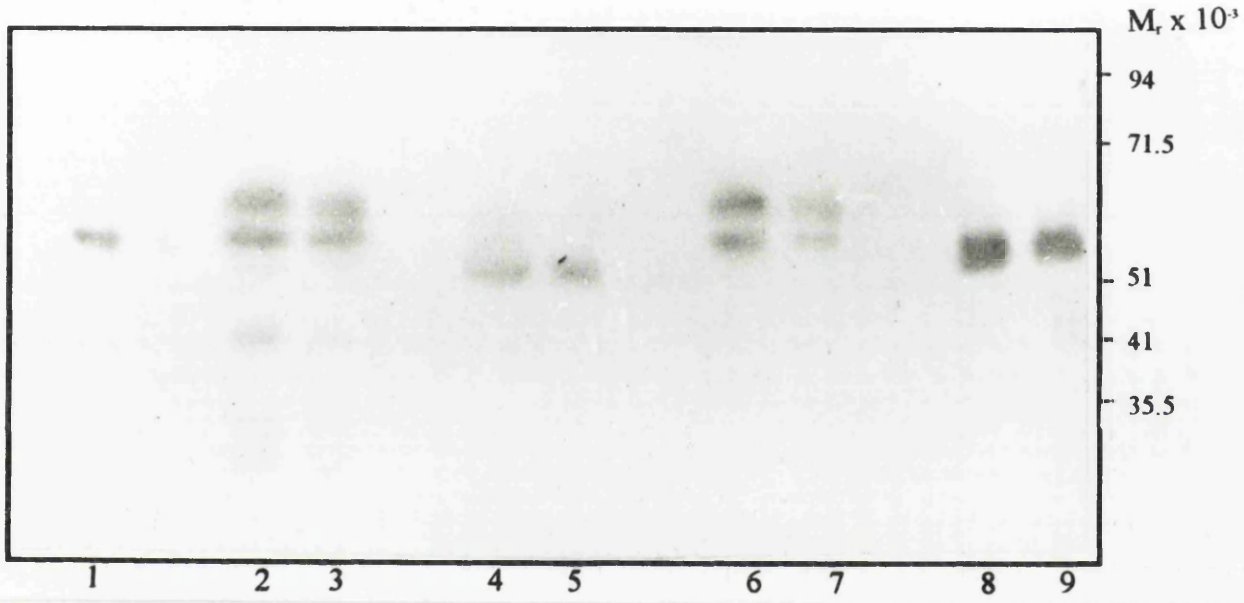


Figure 5.5: Immunological detection of E3 in extracts of potato and pea mitochondria and pea chloroplasts.
The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to mammalian E3. Lane 1, purified mammalian OGDC (0.5µg); Lanes 2,3, potato mitochondria (15µg and 30µg respectively); Lanes 4,5, pea chloroplasts (15µg and 30µg respectively); Lanes 6,7, pea mitochondria (15µg and 30µg respectively); Lanes 8, 9, purified mammalian PDC.

also identified in mitochondria isolated from turnip and cauliflower (results not shown). It was assumed that the 67,000Da species was an artefact as this immuno-reactive species was consistently identified when challenging with a battery of subunit specific antisera (section 5.4). The 58,000Da polypeptide was considered the true antigenic species; Camp & Randall (1985) identified a pea mitochondrial polypeptide of identical M_r value which cross-reacted with antibodies raised to broccoli mtPDC and porcine E3.

In addition, potato mitochondrial E3 has been purified to homogeniety by anion exchange chromatography (R. Millar, Glasgow University, personal communication). Potato mitochondrial E3 comprises two non-identical subunits α and β, displaying M_r values 58,000 and 56,000 respectively, and are arranged as α_2 and β_2 homodimers and an $\alpha\beta$ heterodimer. As a consequence of the small difference in M_r, these subunits were identified on Western blots as a single cross-reacting band (Fig. 5.5). Each polypeptide can be visualised, however, by silver staining (Fig. 6.3; R. Millar, personal communication).

Pea and potato mitochondrial E3 displays a higher M_r value than the analogous chloroplast antigenic species. This observation suggests that organelle-specific form of E3 are expressed in the plants.

5.4 NON-SPECIFIC ANTIBODY BINDING

Throughout this investigation species were consistently identified in the M_r range of 65,000-70,000. These species were often detected in silver stained polyacrylamide gels (e.g. Fig. 6.3, lanes 3, 4, 5) and frequently cross-reacted with a range of antiserum (e.g. Fig. 5.8, 5.9). Such anomalous bands have been detected by a number of groups (H.G. Nimmo; J.G. Coggins, Glasgow University, personal communication). Although these non-specific interactions in this M_r range are especially prevalent in plant extracts, they are generally assumed to represent a common laboratory contaminant e.g keratin. For this reason immuno-reactive polypeptides in the M_r range 65,000 to 70,000 which responded to antibodies raised to the mammalian 2-oxoacid dehydrogenase were

considered to be spurious and futher investigations (examination of gel filtered plant extracts) were required to determine the nature of these cross-reacting species.

5.5 IMMUNOLOGICAL DETECTION OF PLANT 2-OXO-GLUTARATE AND BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEXES BY WESTERN BLOTTING

5.5.1 INTACT 2-OXOGLUTARATE AND BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEXES

Pea and potato mitochondrial and pea chloroplast proteins were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and exposed to antiserum to intact bovine heart OGDC (Fig 5.6). The E1, E3 and E2 component enzymes of bovine heart OGDC (lane1) were detected with M_r values 100,000, 55,000 and 48,000 respectively. Chloroplast protein (lanes 6, 7) showed no appreciable cross-reactivity with antiserum to mammalian OGDC. Potato mitochondrial protein (lanes 2,3) cross-reacted strongly with antisera to the intact OGDC; four prominent bands were detected displaying M_r values 128,000, 55,000, 47,000 and 36,000. The pea mitochondrial immuno-reactive profile was surprisingly distinct from potato; a single cross-reacting species (M_r 36,000) was detected in mitochondria isolated from pea (lanes 4,5).

Distinct differences in pea and potato mitochondrial cross-reacting species were also observed when blotting with antiserum to the mammalian BCDC (Fig. 5.7); two intense cross-reacting species with M_r values 100,000 and 58,000 were identified in tracks of of potato mitochondria (lanes 1, 2, 3) whereas immuno-reactive pea mitochondrial proteins displayed M_r values of 70,000, 67,000, 58,000 and 50,000 (lanes 6, 7). Two chloroplast polypeptides of M_r values 70,000 and 38,000 were detected (lanes 4, 5)

The large differences in immuno-reactive profile observed between pea and potato mitochondrial extracts when incubating with antibodies to the native OGDC and BCDC are extremely surprising and raised the possibility that the cross-reactions were

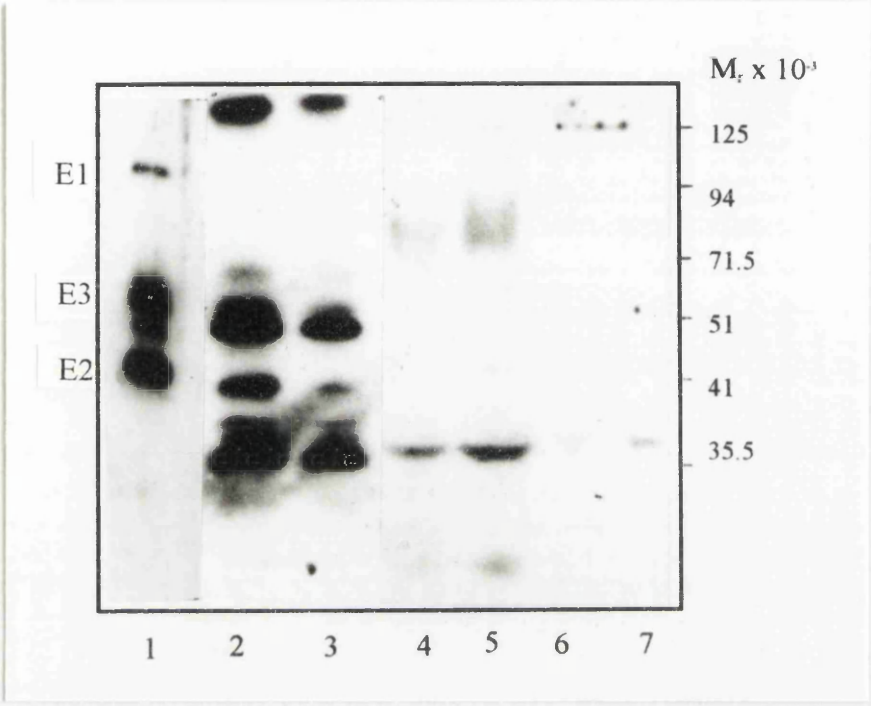


Figure 5.6: Immunological detection of OGDC in extracts of potato and pea mitochondria and pea chloroplasts.
The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to mammalian OGDC. Lane 1, purified mammalian OGDC (0.5µg); Lanes 2,3, potato mitochondria (15µg and 30µg respectively); Lanes 4,5, pea mitochondria (15µg and 30µg respectively); Lanes 6,7, pea chloroplasts (15µg and 30µg respectively).

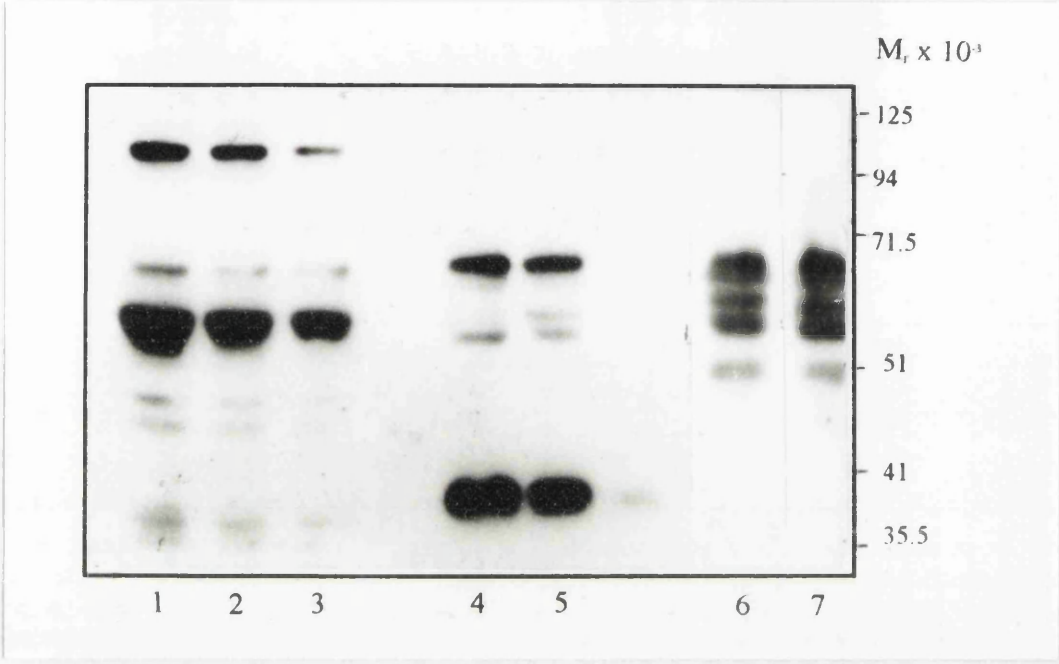


Figure 5.7: Immunological detection of BCDC in extracts of potato and pea mitochondria and pea chloroplasts.
The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to mammalian BCDC. Lanes 1,2,3, potato mitochondria (30µg, 20µg and 10µg; respectively); Lanes 4,5, pea chloroplasts (30µg and 15µg respectively); Lanes 6,7, pea mitochondria (30µg and 15µg respectively).

non-specific artefacts. Enzymatic data reported in this investigation (section 3.4) indicate that pea mitochondrial levels of OGDC and BCDC are very low compared with PDC. Accordingly, the immunological detection of plant OGDC and BCDC demanded a longer exposure period, which accentuates non-specific signals, than the immunological detection of PDC. Consequently, when probing organelle extracts, the sensitivity of this technique limits the detection of antigens which are expressed at low levels. Taking into consideration the strong possibility of non-specific interactions, antisera to specific subunits mammalian OGDC and BCDC were utilised to determine if any of the cross-reactions represented component enzymes of plant multienzyme complexes.

5.5.2 THE E2 SUBUNIT OF PLANT 2-OXOGLUTARATE AND THE BRANCHED-CHAIN 2-0X0ACID DEHYDROGENASE COMPLEXES

Fig 5.8 shows the banding profile of blotting pea and potato mitochondrial and pea chloroplast protein with antisera to the E2 subunit of mammalian OGDC. The high specificity of the antisera is demonstrated in lane 1 where purified mammalian OGDC displays only one visible band: the E2 subunit with M_r value 48,000. Three pea and potato mitochondrial polypeptides (lanes 2, 3 and 6, 7 respectively) with M_r values 67,000, 55,000 and 47,000 cross-reacted with these antibodies. In contrast, pea chloroplast protein (lanes 4,5) cross-reacted with anti-E2 serum and a single intense band, M_r 67,000, was detected. The 67,000Da species is possibly a function of non-specific binding (section 5.4). This would suggest the absence of antigen in the chloroplast compartment and is supported by enzymatic investigations (section 3.4). Mitochondrial polypeptides of M_r values 55,000 and 47,000 are therefore candidates for the E2 component enzyme of plant OGDC. Gel filtered material was incubated with antiserum to the E2 subunit of OGDC in order to determine if either species was associated with a high M_r multienzyme complex (Fig. 5.9).

Immuno-reactive polypeptides of M_r 70,000 and 66,000 were detected in each fraction, thus calling into question the nature of these cross-reacting species. The results suggest that the immuno-reactive doublet is not specifically associated with a high M_r

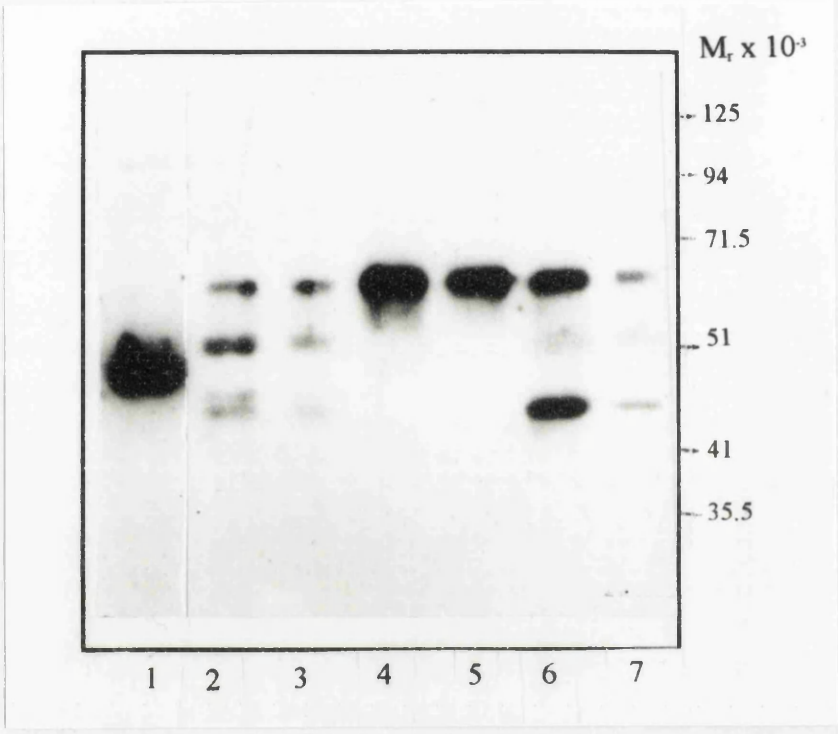


Figure 5.8: Immunological detection of the E2 subunit of OGDC in extracts of potato and pea mitochondria and pea chloroplasts.
The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to the E2 component of mammalian OGDC. Lane 1, purified mammalian OGDC (0.5μg); Lanes 2,3, pea mitochondria (30μg and 20μg respectively); Lanes 4,5, pea chloroplasts (30μg and 20μg respectively); Lanes 6,7, potato mitochondria (30μg and 20μg respectively)

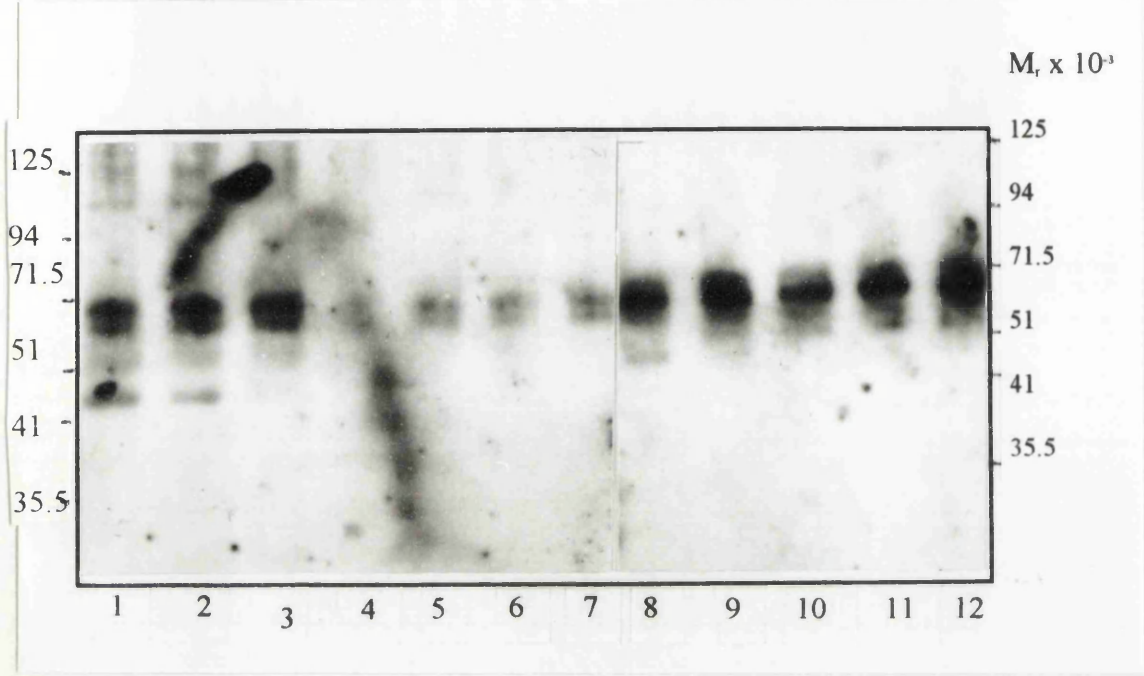


Figure 5.9: Immunological detection of the E2 subunit of OGDC in pea mitochondria and chloroplasts extracts separated by gel filtration.

Pea mitochondrial and chloroplast extracts were separated by gel filtration as described in Fig. 5.4. The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to the E2 component of mammalian OGDC. Lane 1, pea mitochondria (20μg); Lane 2, fraction 6, gel filtered pea mitochondria (15μg); Lane 3, fraction 7 gel filtered pea mitochondria (15μg); Lane 4, fractions 8-9 gel filtered pea mitochnodria (15μg); Lane 5, fractions 10-11 gel filtered pea mitochondria (15μg); Lane 6, fractions fractions 12-13 gel filtered pea mitochondria (15μg); Lane 7, fractions 14-15 gel filtered pea mitochondria (15μg); Lane 8 pea chloroplasts (20μg); Lane 9, fractions 6-7 gel filtered pea chloroplasts (15μg); Lane 10, fraction 8-9 gel filtered pea chloroplasts (15μg); Lane 11, fractions 10-11 gel filtered pea chloroplasts (15μg); Lane 12, fractions 12-13 gel filtered pea chloroplasts (15μg).

aggregrate. Detection of an additional band (M_r 47,000), was restricted to the void volume pea mitochondrial fractions and therefore may represent the genuine component enzyme of plant OGDC. This M_r value is very similar to the mammalian counterpart which carries one lipoyl bearing domain. This cross-reacting species was not detected in gel filtered pea chloroplast fractions nor was an OGDC-catalysed reaction recorded in plastid isolates (section 3.4) suggesting the absence of OGDC in plastid compartment.

The results of immunoblotting with antisera to the E2 subunit of mammalian BCDC are presented in Fig 5.10. This antisera cross-reacted with a 70,000Da chloroplast species and two potato and pea mitochondrial species of M_r values 70,000 and 58,000. The nature of the 58,000Da species remains unresolved, however it displays an identical M_r to a mitochondrial polypeptide which cross-reacted with anti-E3 serum. The M_r value of the 70,000Da cross-reacting species suggest a spurious nature which was confirmed by probing gel filtered pea mitochondrial and chloroplast protein with antiserum to the E2 subunit of BCDC (Fig. 5.11); cross-reacting species of M_r values 70,000 and 67,000 were identified in fractions 5-15ml indicating that the immunoreactive polypeptides are not specifically associated with a high M_r aggregate. A cross-reacting band of M_r 50,000, similar to the M_r of the mammalian subunit, was confined to the void volume fractions of gel filtered pea mitochondrial extracts and may represent the E2 component of BCDC. No such cross-reaction was detected in the equivalent chloroplast fractions suggesting the absence of BCDC in the plastid compartment.

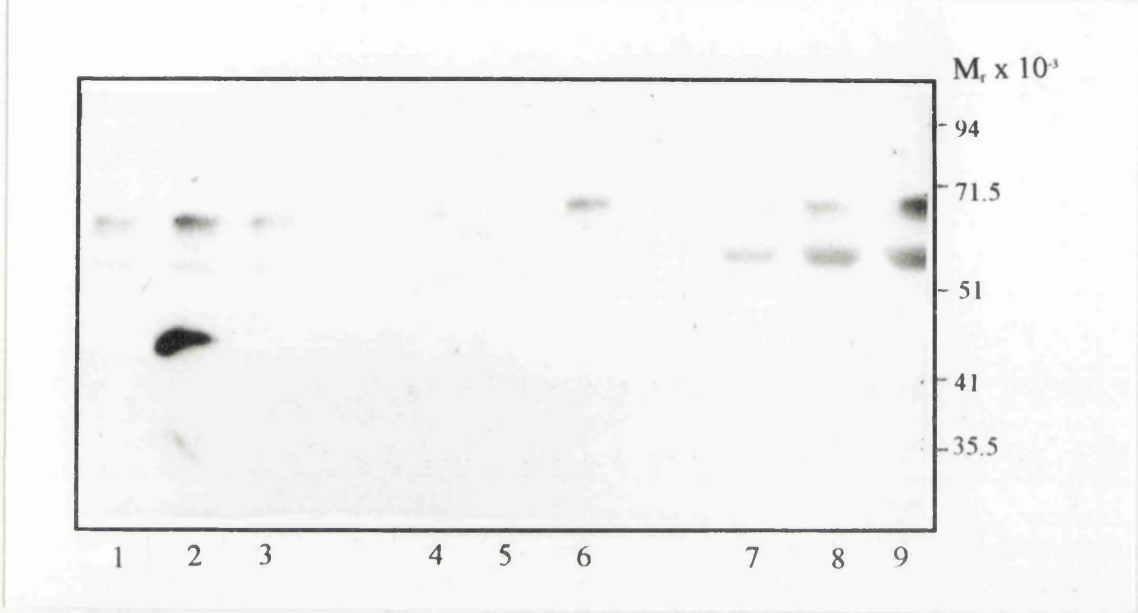


Figure 5.10: Immunological detection of the E2 subunit of BCDC in extracts of potato and pea mitochondria and pea chloroplasts.
The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to the E2 component of mammalian BCDC. Lanes 1,2,3, pea mitochondria (10μg, 20μg and 30μg respectively); Lanes 4,5,6, pea chloroplasts (10μg, 20μg and 30μg respectively); Lanes 7, 8,9, potato mitochondria (10μg, 20μg and 30μg respectively).

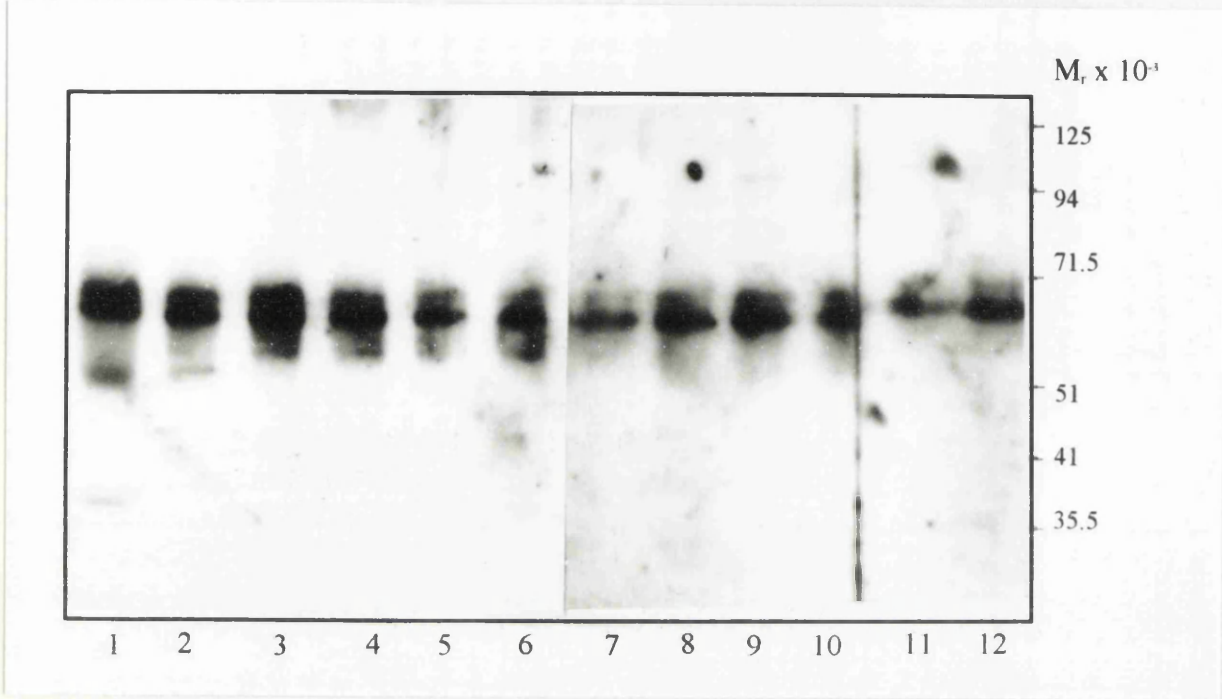


Figure 5.11: Immunological detection of the E2 subunit of BCDC in pea mitochondria and chloroplasts extracts separated by gel filtration.
Pea mitochondrial and chloroplast extracts were separated by gel filtration as described in Fig. 5.4. The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to the E2 component of mammalian BCDC. Lane 1, pea mitochondria (20µg); Lane 2, fraction 7, gel filtered pea mitochondria (15µg); Lane 3, fraction 8 gel filtered pea mitochondria (15µg); Lane 4, fraction 9 gel filtered pea mitochnodria (15µg); Lane 5, fractions 10-11 gel filtered pea mitochondria (15µg); Lane 6, fraction 12-13 pea mitochondria (20µg); Lane 7, pea chloroplasts (20µg); Lane 8, fractions 6-7 gel filtered pea chloroplasts (15µg); Lane 9, fractions 8-9 gel filtered pea chloroplasts (15µg); Lane 10, fractions 10-11 gel filtered pea chloroplasts (15µg); Lane 11, fractions 12-13 gel filtered pea chloroplasts (15µg); Lane 12, fractions 14-15 gel filtered pea chloroplast (15µg).

5.5.3 THE E1 COMPONENT OF PLANT 2-OXOGLUTARATE AND BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEXES

Fig 5.12 illustrates the results of blotting pea and potato mitochondrial and pea chloroplast protein (lanes 2,3,4, 5,6,7 and 8,9,10 respectively) with antisera to the E1 subunit of OGDC. In sharp contrast to the mammalian enzyme (lane , M_r value 100,000) plant protein from both organelles contain two cross-reacting species with M_r values 70,000 and 67,000. These cross-reacting bands do not complement the immuno-reactive profile obtained when blotting with intact antiserum and were considered to be artefacts resulting from non-specific interactions (section 5.4). This indicates that the E1 component of plant OGDC is immunologically distinct from the mammalian component enzyme. A weak cross-reaction was detected, however, in a track of potato mitochondria (lane 2) displaying an M_r value of 100,000, identical to the mammalian counterpart, and may represent the genuine E1 component of potato mtOGDC. A cross-reacting band of similar M_r was not identified in pea mitochondria, however, hence no definitive conclusions could be drawn about the nature and identity of the plant enzyme at this stage.

As with the E1 component enzyme of PDC, no immunological cross-reactions were observed when plant mitochondrial and plastid protein were challenged with antiserum to the E1 subunit of mammalian BCDC. This suggests that the equivalent plant enzyme is antigenically distinct from its mammalian counterpart or that low levels of BCDC in the organelle extracts have limited immunological detection.

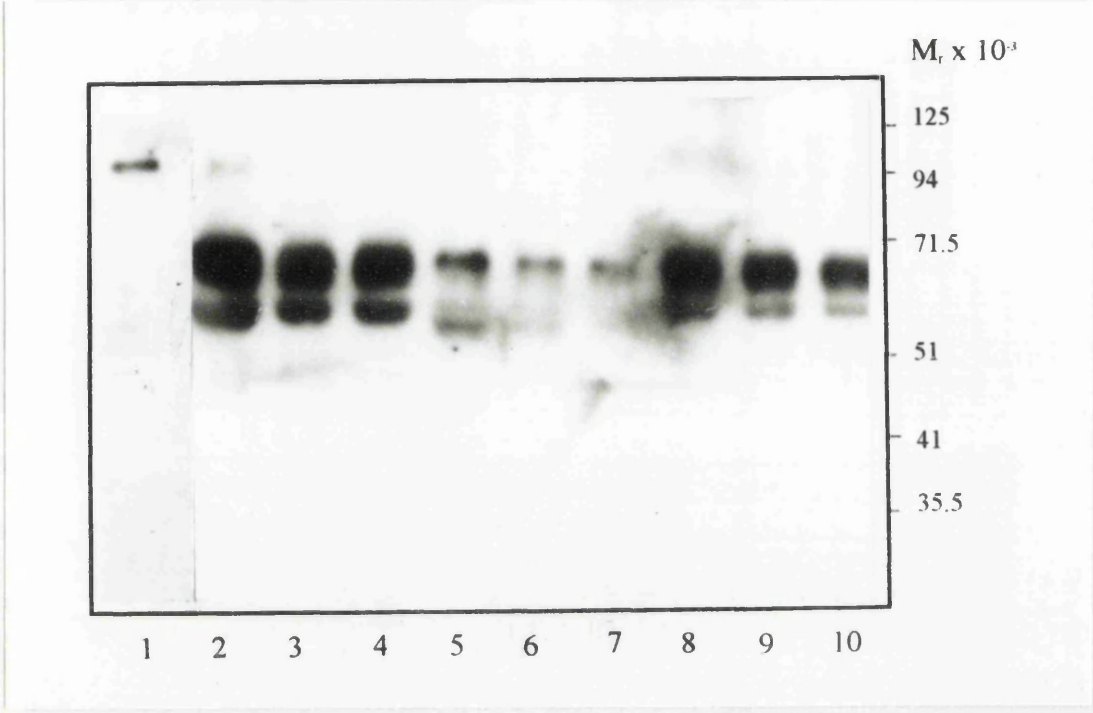


Figure 5.12: Immunological detection of the E1 subunit of OGDC in extracts of potato and pea mitochondria and pea chloroplasts.
The immunoblotting procedure is described in Fig. 5.1 except that the primary antibody was to the E1 component of mammalian OGDC. Lane 1, purified mammalian OGDC (0.5µg); Lanes 2,3,4, potato mitochondria (30µg, 25µg and 15µg respectively); Lanes 5,6,7, pea mitochondria (30µg, 25µg and 15µg respectively); Lanes 8,9,10, pea chloroplasts (30µg, 25µg and 15µg repectively).

5.6 IMMUNOLOGICAL ANALYSES OF THE PLANT 2-OXOACID DEHYDROGENASE COMPLEXES

Probing plant mitochondrial and plastid extracts with antiserum to specific subunits of the 2-oxoacid dehydrogenase complexes isolated from bovine heart has provided interesting insights into this family of plant multienzyme complexes. Challenging pea and potato mitochondrial and pea chloroplast protein with antiserum to the E3 subunit identified immuno-reactive polypeptides of M_r value 52,000 and 58,000 in plastid and mitochondrial fractions respectively. These distinct cross-reacting species demonstrate the absence of cross-contamination between the organelle isolates and suggests that organelle specific forms of E3 are expressed in the plant system. The prospective plant mitochondrial E3 displays a higher M_r than its mammalian and chloroplast counterparts and the nature of this enzyme is further investigated in the next chapter.

Exposure of plant mitochondrial and plastid extracts to antiserum raised against the E2 subunit of PDC identified a single cross-reacting species of M_r value 53,000. This immuno-reactive polypeptide was shown to be associated with a high M_r aggregate indicating that this species represents the E2 component of PDC. It was felt that the immunological cross-reaction of chloroplast protein signified a genuine immuno-reactive plastid species and was not a consequence of mitochondrial contamination as a distinct difference in M_r value between mitochondrial and plastid species reactive towards anti-E3 serum was recorded.

The M_r value of the immuno-reactive polypeptides detected when probing with antiserum to the E2 subunit of PDC suggests that the enzyme from plant mitochondria and plastids may carry a single lipoyl domain paralleling the situation in the analogous yeast enzyme. In addition, although the E1 subunit of plant PDC was antigenically distinct from its mammalian counterpart, Taylor *et al.* (1992) recorded a specific cross-reaction of pea mitochondrial protein with antibodies to the E1 component of yeast PDC. These combined observations support a greater homology to E1 and E2 from yeast

PDC. Such immunological comparisons provide an approach for studying the evolutionary origin of mitochondria and plastids.

As with PDC, the E1 components of plant OGDC and BCDC were immunologically distinct from the mammalian subunits. Immunological reactivity was observed when challenging plant mitochondrial and plastid extracts with antiserum to the E2 subunit of these multienzyme complexes. Analyses of gel filtered pea mitochondrial and chloroplast protein suggested that cross-reacting species in the M_r range of 65,000-70,000 were artefacts. Pea mitochondrial species, which was shown to be associated with a high M_r aggregate, of M_r values 48,000 and 50,000, responded to antiserum to the E2 subunits of OGDC and BCDC respectively. These observations suggest that these immuno-reactive polypeptides, which are absent in the chloroplast fractions, represent the E2 component of plant mitochondrial OGDC and BCDC. The M_r values are very similar to the mammalian enzymes and suggests that the E2 subunit of plant mitoochondrial OGDC and BCDC contains one lipoyl domain.

The immunological detection of the plant 2-oxoacid dehydrogenase complexes complements the enzymatic data documented in chapter 3. Each avenue of investigation supports a dual location of PDC, in mitochondria and plastids, and indicates that OGDC and BCDC are confined to mitochondria.

CHAPTER SIX

PLANT DIHYDROLIPOAMIDE DEHYDROGENASE

6.1 INTRODUCTION

Dihydrolipoamide dehydrogenase (E3) catalyses the reoxidation of lipoic acid residues via a FAD^+ cofactor coupled to the reduction of NAD^+. E3, active as a dimer, contains a redox-active disulphide bridge which is directly involved in catalysis. Dihydrolipoamide dehydrogenase is a member of the family of disulphide oxidoreductases which comprises glutathione, trypanothione and mercuric reductases (Karplus & Shulz, 1987; Schiering *et al.*, 1991; Kuriyan *et al.*, 1991a, 1991b), and is involved in the catalytic cycle of the 2-oxoacid dehydrogenase complexes and the glycine cleavage system designated E3 and L-protein respectively. In higher plants it has yet to be determined if a single E3 is associated with PDC, OGDC, BCDC and GDC, or, if isoenzymes exist which are selective to a particular multienzyme complex. This section of research is concerned with the investigation of whether E3 from the plant 2-oxoacid dehydrogenase complexes and the L-protein associated with GDC share a common identity, or, are there a number of E3 polypeptides, which may represent complex-specific isoenzymes, present in the plant system.

E3 has been isolated and characterised from a wide range of prokaryotic and eukaryotic sources. The three dimensional structure of a number of prokaryotic E3s have been determined by X-ray diffraction. Crystallographic analyses of *Azotobacter vinelandii* (Mattevi *et al.*, 1991) and *Pseudomonas putida* (Mattevi *et al.*1992b) have identified a dimeric organisation of identical subunits (M_r 50,000). The catalytic centre has been positioned at the interface between the two subunits and the NAD^+ and FAD^+ binding sites form two channels separated by the flavin ring.

The most extensively studied is E3 from *E. coli* where the single structural gene (*lpd*) has been sequenced (Stephens *et al.*, 1983) and associates with PDC and OGDC. In general a common E3 is utilised by PDC, OGDC, BCDC and GDC. Two species of *Pseudomonas*, however, express two distinct E3s (LPD-val and LPD-glc) from separate genes (*lpd*-val and *lpd*-glc). These two forms display complex selectivity; LPD-glc is the E3 associated with PDC and OGDC and the L-protein for GDC while LPD-val is the specific E3 for BCDC (Sokatch & Burns, 1984).

Dihydrolipoamide dehydrogenase has been identified in the bloodstream form of *Trypanosoma cruzi* and *T. brucei* (Danson *et al.*, 1987 & Lohrer *et al.*, 1990). These eukaryotic parasites generate ATP from glycolysis and do not possess 2-oxoacid dehydrogenase complexes. In these organisms, dihydrolipoamide dehydrogenase is located specifically in the plasma membrane and may be involved, as in a form of the enzyme in *E. coli* which aids galactose and maltose transport (Richarme, 1989), in the shuttling of sugars across the membrane. This report calls into question the possibility of a role for eukaryotic dihydrolipoamide dehydrogenase distinct from the reoxidation of lipoyl residues on the 2-oxoacid dehydrogenase and glycine decarboxylase complexes.

With respect to mammalian E3, reconstitution experiments, amino acid analyses and immunological cross reactivity, indicate that the 2-oxoacid dehydrogenase multienzyme family utilise a single E3 enzyme (Matuda *et al.*, 1983). It is thought that GDC also employs the same gene product. However, the L-protein readily dissociates from this multienzyme system, therefore, dihydrolipoamide dehydrogenase specifically associated with mammalian GDC has never been isolated. Consequently, definitive evidence in the form of reconstitution experiments, is not available to confirm that mammalian GDC and the 2-oxoacid dehydrogenase complexes utilise the same dihydrolipoamide dehydrogenase.

The possibility of E3 isoenzymes in mammalian tissue remains a point of contention. Immunological observations indicate that mammalian cells may express multiple forms of E3. Carothers *et al.* (1987) identified two immunologically distinct E3s in rat liver mitochondria. They raised antisera against E3 from highly purified PDC and from commercially available porcine heart E3. Since the commercial purification scheme does not involve isolation of a specific 2-oxoacid dehydrogenase complex or GDC, it is possible that there is a co-purification of potential isoenzymes. The resulting antisera precipitated rat liver mitochondrial E3 whereas anti PDC-E3 serum failed to completely inactivate rat liver and pig heart E3. In addition anti PDC-E3 had no effect on GDC activity whereas anti pig heart-E3 inhibited glycine oxidation. Immunological investigations, therefore, suggest that rat liver mitochondria contain two forms of E3, one of which may be the L-protein involved in the decarboxylation of glycine.

The presence of eukaryotic E3 isoenzymes is further suggested by the study of human genetic disorders which affect the 2-oxoacid dehydrogenase complexes and GDC. If a unique E3 is utilised by all four complexes then abnormalities in the enzyme would lead to elevated levels of pyruvate, 2-oxoglutarate, branched chain 2-oxoacids and glycine. Patients with E3 deficiency, however, although having excesses of the 2-oxoacids, display normal glycine metabolism (Koga *et al.*, 1986).

Recent investigations concerning plant E3 indicates that the 2-oxoacid dehydrogenase complexes and GDC share a common enzyme. Walker and Oliver (1986) raised monoclonal antibodies to pea leaf mitochondrial E3 and reaction of this antisera with PDC and GDC produced identical inhibition profiles. Recently Bourguignon *et al.* (1992) cloned and sequenced the L-protein of pea mitochondrial GDC and collected data supporting a single E3 enzyme. Northern blot analyses demonstrated that the transcript encoding the H-protein of GDC was strongly light induced, conforming to a component enzyme of a photorespiratory multienzyme system, whilst induction of L-protein mRNA was minimal. In addition, comparable levels of mRNA encoding the L-protein were detected in leaves, stem and embryo.

Turner *et al.* (1992) independently cloned and sequenced dihydrolipoamide dehydrogenase associated with pea leaf mitochondrial GDC. In contrast with the report by Bourguignon *et al.* (1992), two copies of the same gene were identified. These authors also noted negligible light stimulation of L-protein mRNAs and spacial regulation of E3 was not manifest. These observations suggest that the enzyme is required by other complexes, e.g. the 2-oxoacid dehydrogenase complexes, in non-photosynthetic tissue thus supporting an ubiquitous E3 enzyme shared amongst the 2-oxoacid dehydrogenase complexes and GDC.

The existence of a single plant E3 has yet to be firmly established. Although analyses of pea leaf mitochondrial E3 indicates a single plant enzyme, recent research in Glasgow University has provided increasing evidence supporting the existence of a number of distinct forms of E3 polypeptides in the plant system thus paralleling the situation in *P. putida*. Separation of solubilised potato tuber mitochondrial extracts by anion exchange chromatography resolved three distinct peaks of E3 activity (R. Millar,

personal communication). This chapter investigates the possibility of multiple forms of plant E3 and considers any physiological basis of potential isoforms of dihydrolipoamide dehydrogenase.

6.2 SEPARATION OF PEA AND POTATO MITOCHONDRIAL E3 BY ANION EXCHANGE CHROMATOGRAPHY

Pea root and potato tuber mitochondrial protein were separated by anion exchange chromatography in order to illustrate the separation of the distinct E3 activities (R. Millar, personal communication). Triton X-100-solubilised mitochondrial extracts were heat treated (65°C/10 min) and the denatured material removed by centrifugation (11000.*g*/20 min) prior to loading onto a Mono Q HR 5/5 column. Since E3 is thermotolerant, enzyme activity is unaffected whilst specific activity increases 5-10 fold. Protein was eluted from the anion exchange column connected to a Pharmacia FPLC system employing a 10-400mM phosphate gradient (pH6.8) containing 1mM EGTA and 1mM mercaptoethanol and E3 activity determined as described in Materials and Methods (2.5.5).

Three distinct peaks of E3 activity were resolved at 150mM, 185mM and 220mM phosphate (Fig. 6.1). Typical E3 activity ratios of peaks one, two and three of approximately 1:3:3 were consistently observed. Although three E3 activities were also separated from pea mitochondrial extracts (Fig. 6.2), at 95mM, 130mM and 180mM phosphate, the peak ratios were distinctly different from potato mitochondrial E3. 95% of E3 activity was released in the second peak, however, peaks one and three, although minor, contained genuine E3 activity.

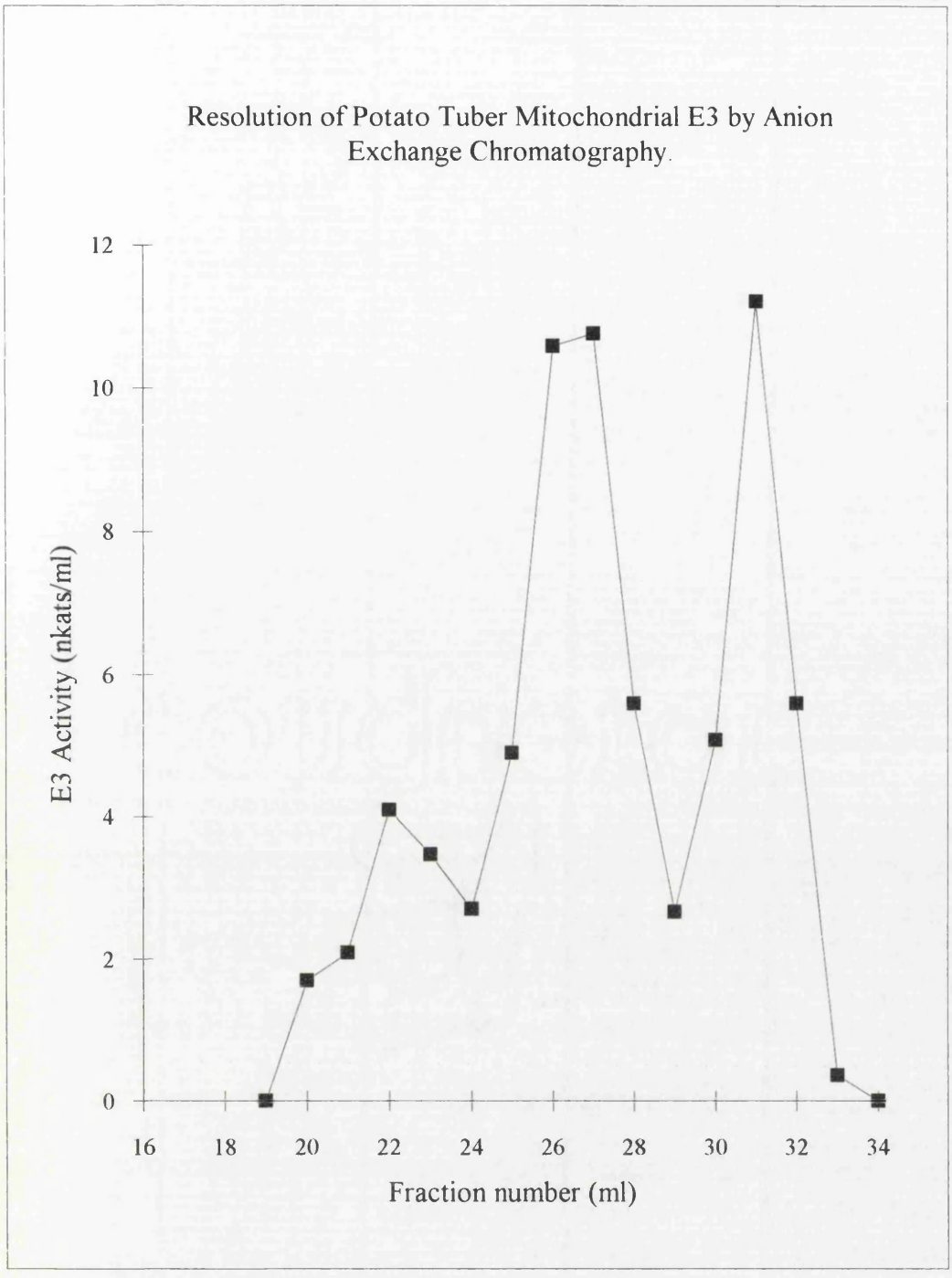


Figure 6.1: Resolution of potato tuber mitochondrial E3 by anion exchange chromatography.
Extracts were solubilized with 0.25%(w/v) Triton X-100 and heat treated prior to loading onto a MonoQ HR 5/5 column connected to a FPLC system. Protein was eluted at a flow rate of 0.5ml/min along a phosphate gradient (10-400mM, pH 6.8) and 60 1ml fractions were collected. E3 activities, expressed in nkats/ml, were determined as described in Materials and Methods (2.5.5).

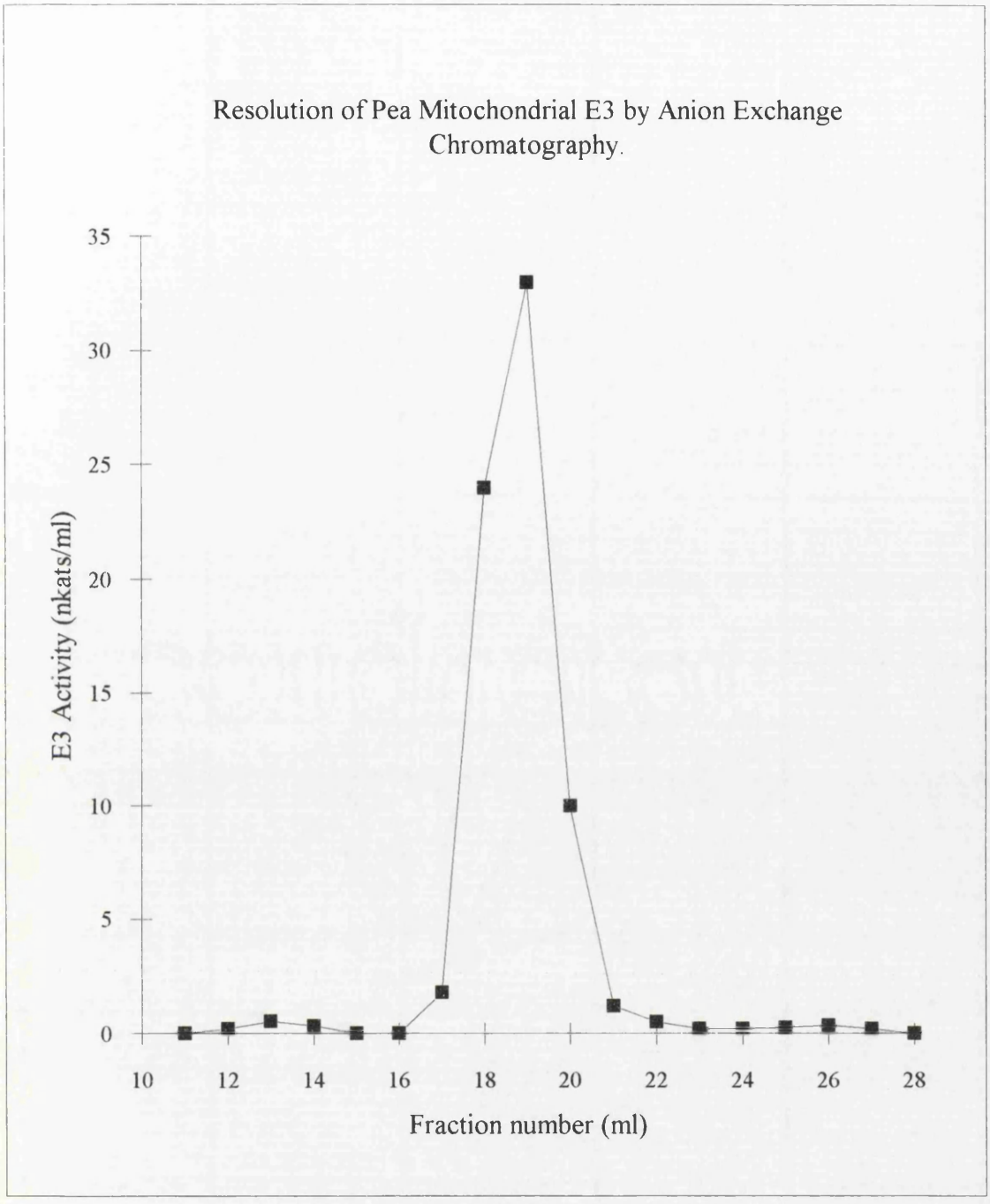


Figure 6.2: Resolution of pea root mitochondrial E3 by anion exchange chromatography.
Extracts were solubilized with 0.25%(w/v) Triton X-100 and heat treated prior to loading onto a MonoQ HR 5/5 column connected to a FPLC system. Protein was eluted at a flow rate of 0.5ml/min along a phosphate gradient (10-400mM, pH 6.8) and 60 1ml fractions were collected. E3 activities, expressed in nkats/ml, were determined as described in Materials and Methods (2.5.5).

The peak fractions containing potato tuber mitochondrial E3 activity were concentrated, the protein precipitated with 80% acetone (-20°C/1 h) and prepared for SDS-PAGE. After electrophoresis proteins were visualised by silver staining (Fig 6.3) as described in Materials and Methods (2.9). Multiple bands were detected in the track of solubilised potato mitochondria (lane 1) whereas thermotolerant polypeptides were visualised in heat-treated mitochondrial extracts (lane 2). A 22,000Da species was observed in each sample and the identity of this polypeptide is unknown. A sharp band of M_r value 59,000 was also detected in fractions containing E3 activity confined to peak one (lanes 3, 4, 5) and a 67,000Da polypeptide which may be an artefact as species in the M_r range 65,000-70,000 were consistently detected throughout the investigation (section 5.4). Two bands of M_r values 59,000 and 56,000 were identified after electrophoresis of fractions containing E3 activity confined to peak two (lanes 7, 8). Polypeptides of M_r values 22,000Da and 30,000Da were also detected. These species were also observed in fractions containing peak three E3 activity along with a 56,000Da polypeptide.

The 59,000Da and 56,000 polypeptides have been recognised as E3 subunits as Millar (Glasgow University, unpublished observations) purified potato mitochondrial E3 to homogeneity and identified polypeptides of similar M_r values. These cross-reacted with antiserum to mammalian E3 indicating that they represent potato mitochondrial dihydrolipoamide dehydrogenase; possibly an α and β subunit. As 'free' E3 activity was eluted from a molecular sieve in accordance with an M_r value of approx. 110,000Da (Section 6.4), polypeptides resolved from the distinct E3 activities may be arranged *in vivo* as α_2 and β_2 homodimers and an $\alpha\beta$ heterodimer.

The resolution of the three distinct E3 activities is consistent with previous finding suggesting that a number of E3 isoenzymes exist in potato mitochondria. It is unclear if this phenomenon holds any physiological relevance in pea mitochondria as 95% of E3 activity was resolved, on anion exchange chromatography, in one peak. This may be a result of non-optimal elution conditions; however, it is also possible that the existence of three independent E3 activities is restricted to tetraploid species made manifest by cross-breeding. The three distinct forms of potato E3 may, as in *P. putida*,

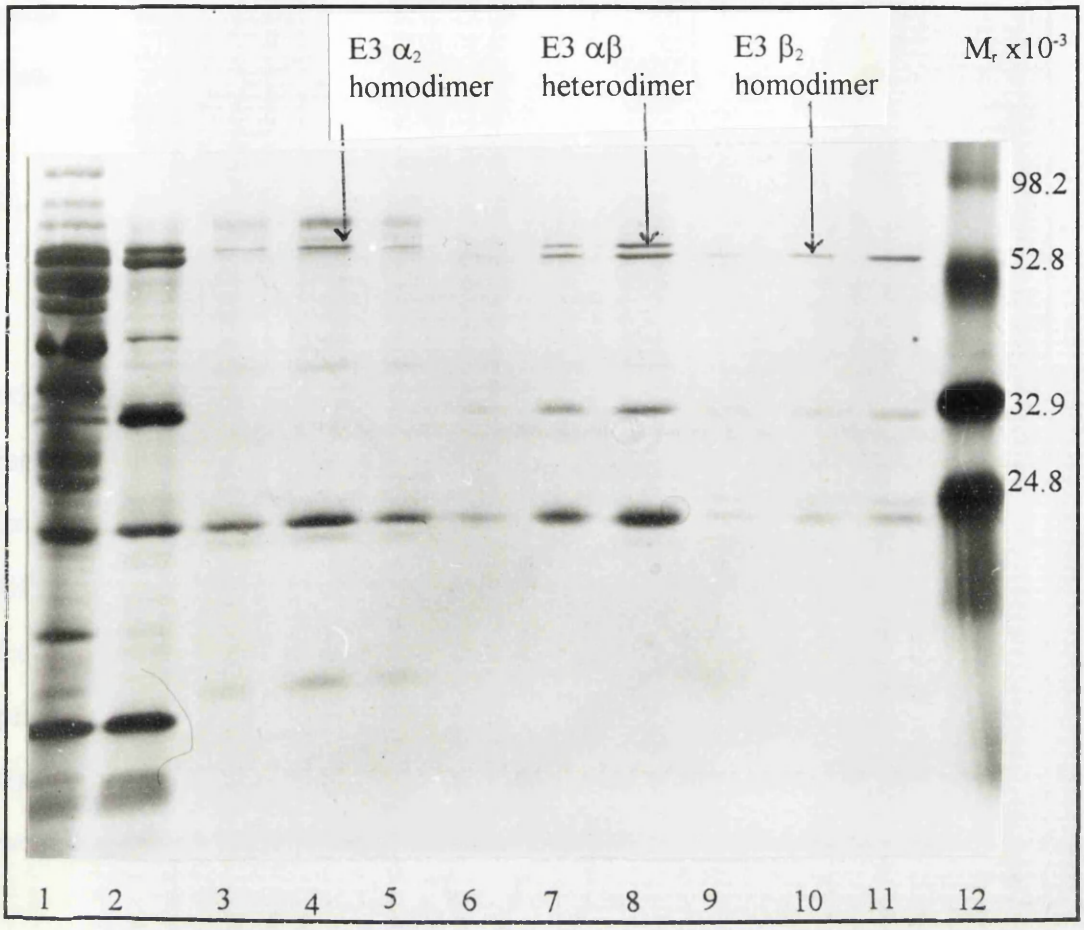


Figure 6.3: Silver stain of peak potato mitochondrial E3 activities, separated by anion exchange chromatography, as resolved by SDS-PAGE.
Triton X-100-solubilised potato mitochondrial extracts were applied to a Mono Q HR 5/5 column connected to a FPLC system and eluted with a phosphate gradient as described in in Fig. 6.1. Peak E3 fractions were concentrated and prepared for SDS-PAGE as described in Materials and Methods (2.7). After electrophoresis proteins were visualised by silver staining as described in Materials and Methods (2.9). Lane 1, Triton X-100-solubilised potato mitochondria (5µg); Lane 2, heat treated potato mitochondria (5µg); Lanes 3,4,5, peak 1 E3 activity; Lanes 6,7,8, peak 2 E3 activity; Lanes 9,10,11, peak 3 E3 activity; Lane 12, molecular weight markers (B.D.H), serum albumin (M_r, 98,200), ovalbumin (M_r,52,800), carbonic anhydrase (M_r,32,900), trypsin inhibitor (M_r,24,800).

pertain to complex specific E3 enyzmes. This last experimental section explores the feasibility of this concept.

6.3 ANALYSES OF POTATO AND PEA CHLOROPLAST E3

To date the only 2-oxoacid dehydrogenase complex reported in the chloroplast compartment is PDC (Camp & Randall, 1985). The absence of other members of this family of multienzyme complexes in plastid fractions is also confirmed by enzymatic investigations reported in chapter 3. In contrast plant mitochondria houses PDC, OGDC, GDC and potentially BCDC. Consequently heat treated solubilised chloroplast protein from potato and pea leaves were applied in turn to a Mono Q HR 5/5 column and protein eluted along a 10-400mM gradient of phosphate (pH 6.8). E3 activity in each fraction was determined as described in Materials and Methods (2.5.5) and comparisons were made with mitochondrial profiles.

A single peak of E3 activity (eluted at 180mM and 185mM) was resolved from pea and potato chloroplast extracts respectively (Fig. 6.4) consistent with the presence of a single E3-requiring system, PDC, in the chloroplast compartment. It is possible that the chloroplast E3 is distinct from the mitochondrial enzymes as immunological detection of pea mitochondrial and plastid E3 suggests that organelle-specific forms are expressed (section 5.3).

6.4 ANALYSES OF E3 RESOLVED BY GEL FILTRATION

It was noted that when solubilised pea and potato mitochondrial protein were separated by gel filtration, two active E3 fractions were eluted (section 5.2.2). Potato tuber mitochondrial protein was solubilised with 0.25%(w/v) Triton X-100 and applied to a Superdex 200 gel filtration column. Protein was eluted with 50mM imidazole pH 6.8 with 1mM DTT and PDC, OGDC and E3 activities determined as described in Materials and Methods (2.5). PDC and OGDC activities were recorded in a single fraction whereas E3 activity was eluted in two separate peaks, at 37-39ml and 56-60ml (Fig. 6.5). From

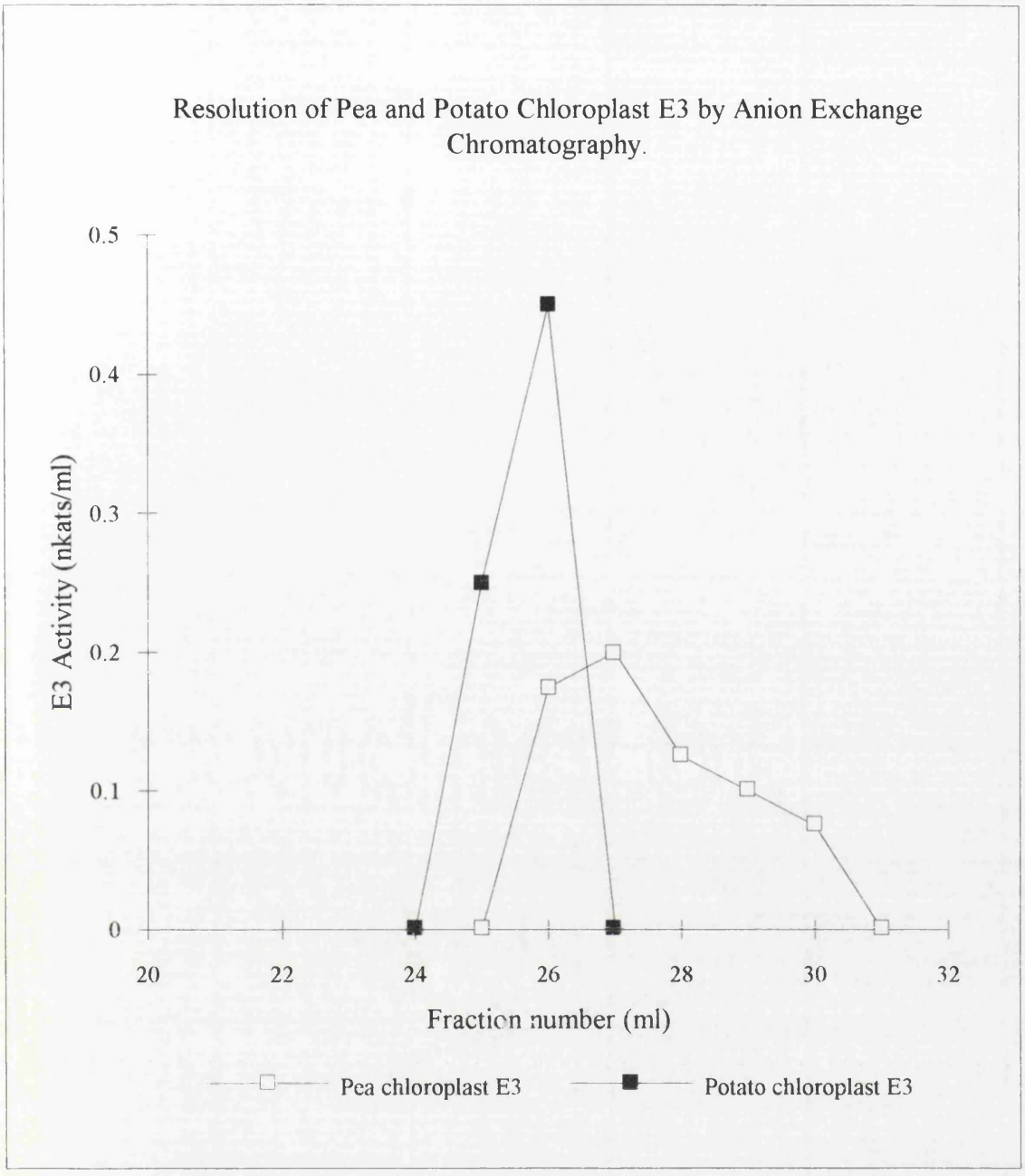


Figure 6.4: Resolution of pea and potato chloroplast E3 by anion exchange chromatography.
Extracts were solubilized with 0.5%(w/v) Triton X-100 and heat treated prior to loading onto a MonoQ HR 5/5 column connected to a FPLC system. Protein was eluted at a flow rate of 0.5ml/min with a gradient of phosphate gradient (10-400mM, pH 6.8) as described in Fig. 6.1 and 60 1ml fractions were collected. E3 activities, expressed in nkats/ml, were determined as described in Materials and Methods (2.5.5). No pea and potato chloroplast E3 activity was detectable in fractions 1-23 and 32-60.

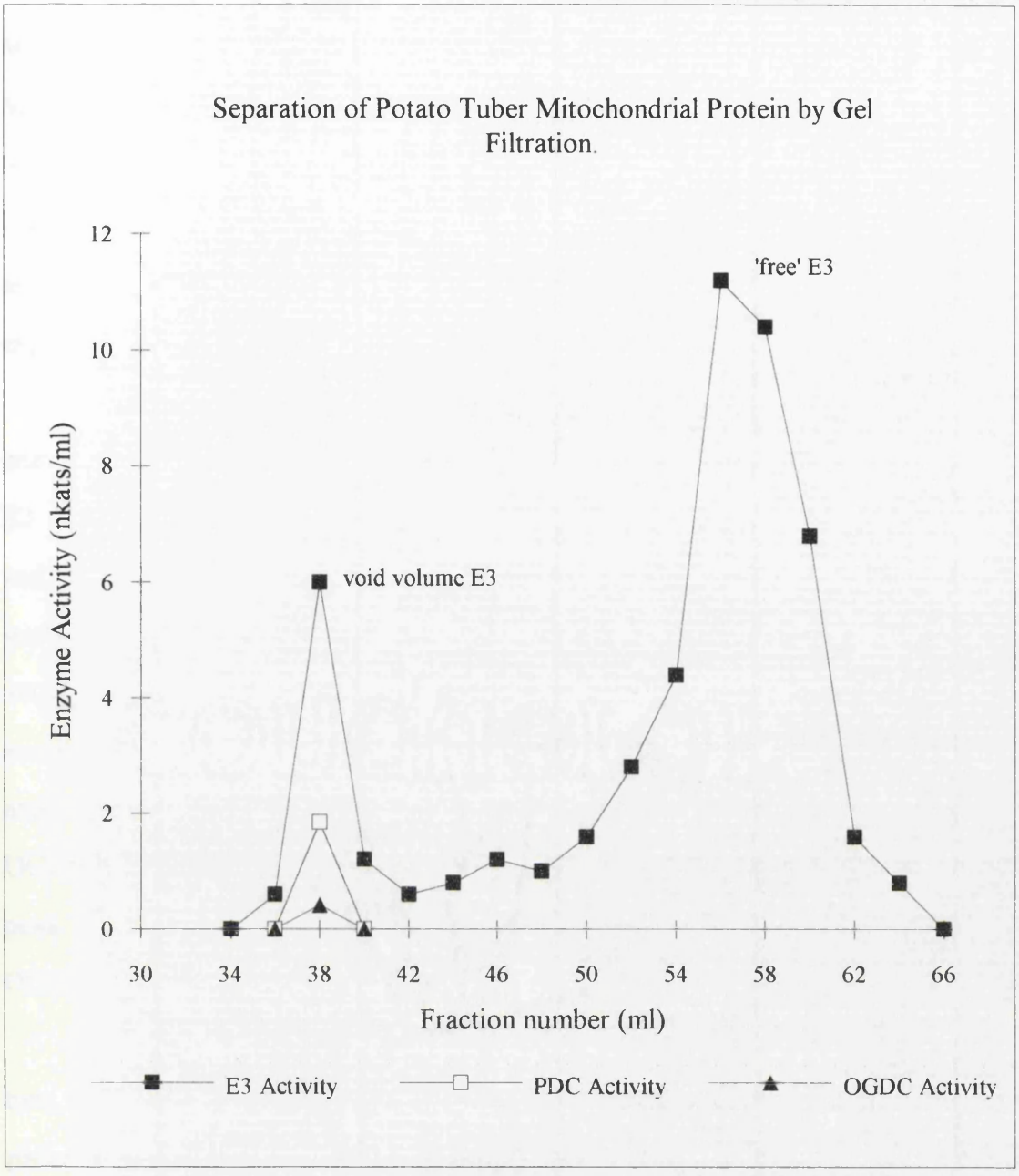


Figure 6.5 Separation of potato tuber mitochondrial E3 by gel filtration.
Potato mitochondrial extracts were solubilised with 0.25%(w/v) Triton-X100 prior to loading onto a 100ml Superdex 200 gel filtration column. Protein was eluted with 100ml of elution buffer (1mM EGTA, 1mM DTT and 50mM imidazole, pH 6.8) with at a flow rate of 1ml/min and E3 activity determined as described in Materials and Methods (2.5.5).

M_r calibration it is clear that the void volume E3 is that which is associated with a high M_r aggregate, i.e. PDC and OGDC, and the second peak represents dissociated, 'free', E3. This distribution of activity provides a base for investigation of complex specificity. Unlike the 2-oxoacid dehydrogenases which exhibit a highly organised architecture, associations between GDCs component enzymes are weak. This information was exploited when examining the gel filtration data.

Bourguignon *et al.* (1988) reported that, on gel filtration, the component enzymes of GDC are eluted separately in relation to their molecular mass. Consequently, E3 associated with GDC (L-protein) would be exclusively located in the 'free' E3 peak and void volume E3 activity would contain the potential isoforms associated exclusively with the 2-oxoacid dehydrogenase complexes. It should be noted that although there was 100% recovery of E3 activity on gel filtration, recovery of PDC and OGDC activity stood at 60% and 45% respectively. As all E3 activity was accounted for it can be assumed that, during fractionation, a proportion of E3 enzymes associated with PDC and OGDC were stripped from their core structures and subsequently eluted in the 'free' E3 peak. Therefore enzyme activity confined to this second peak had a mixed origin: PDC, OGDC and GDC.

The void volume E3 and the 'free' E3 fractions were heat treated and applied in turn to the Mono Q column. Protein was eluted with a 10-400mM linear gradient of phosphate (pH 6.8), as previously described, and E3 activity determined (Fig. 6.6). As with total mitochondrial protein, three active peaks were resolved from the 'free' E3 fractions, containing mixed origin E3. In contrast when void volume E3 was separated by anion exchange only two peaks were resolved. With the premise that GDCs L-protein was absent from this fraction, the data indicates that GDC exclusively or selectively employs the third E3 peak activity in its catalytic cycle.

It is clear that this tentative selectivity requires further confirmation, e.g. sequence and reconstitution data, which is at present being investigated by other members of the research group. Some preliminary data were collected, however, to investigate the possibility of complex specific E3 polypeptides.

Resolution of Potato Tuber Mitochondrial E3, Separated by Gel Filtration, by Anion Exchange Chromatography.

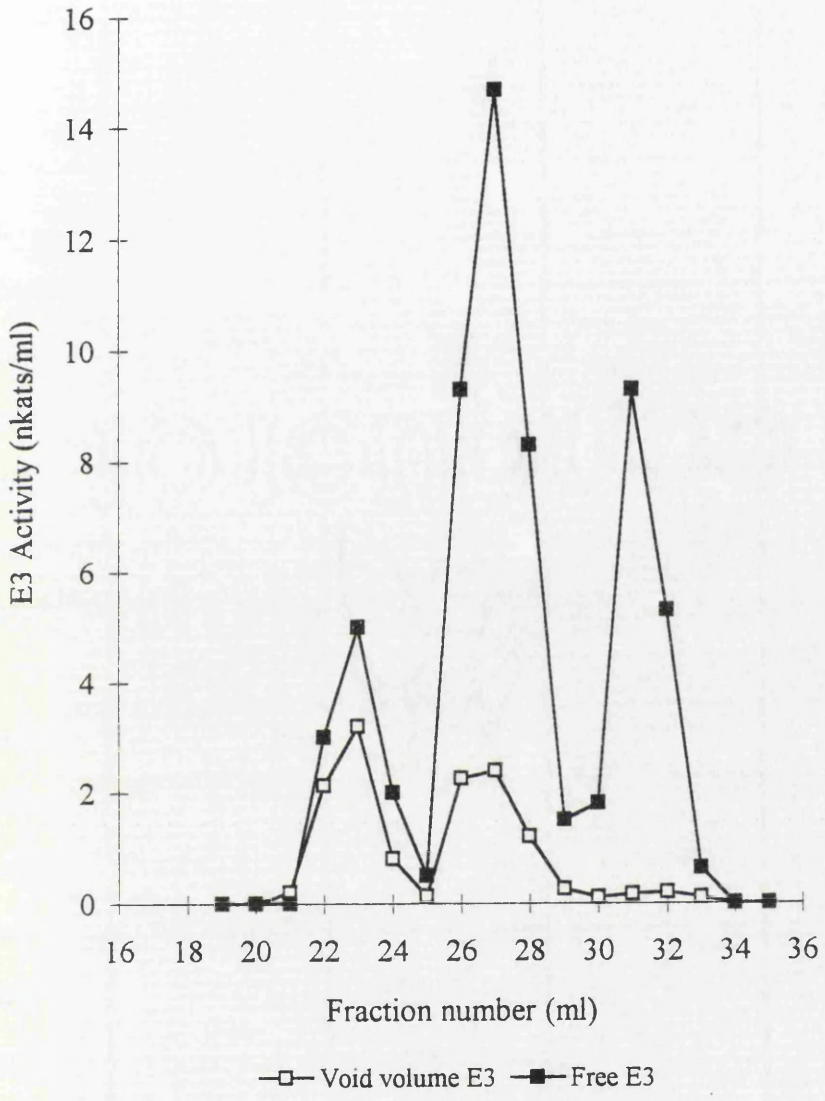


Figure 6.6: Resolution of gel filtered potato tuber mitochondrial E3 by anion exchange chromatography.
Void volume and 'free' E3 gel filtered fractions were applied to a Mono Q HR 5/5 column and protein eluted with a phosphate gradient as described in Fig. 6.1. E3 activity, expressed in nkats/ml, was determined as described in Materials and Methods (2.5.5).

6.5 ANALYSES OF E3 FROM PHOTOSYNTHETIC AND NON-PHOTOSYNTHETIC TISSUE

It has been previously reported that GDC constitutes up to 30% of leaf mitochondrial protein. In contrast, levels in non-photosynthetic tissue are approximately 2-5% of that in the leaves (Walker & Oliver, 1986). Initially it was predicted that comparison of the E3 profiles, resolved by anion exchange chromatography, from photosynthetic and non-photosynthetic tissue would allow the identification of an E3 activity displaying potential GDC selectivity. From the literature, however, it is clear that although expression of the H-protein of the glycine cleavage system is strongly light induced, the L-protein (E3) transcript is not upregulated (Bourguignon *et al.*, 1992; Turner *et al.*, 1992). Comparison of the E3 profile resulting from Mono Q resolution of mitochondria from photosynthetic and non-photosynthetic tissue was performed, however, in order to determine any differences in the relative concentrations of the isoforms.

Mitochondria were isolated from tubers and leaves of potatoes and, as before, the solubilised heat-treated protein applied in turn to a Mono Q column. Protein was eluted with a 10-400mM linear gradient of phosphate (pH6.8) as previously described and the distribution of E3 determined (Fig. 6.7). Three E3 activities were resolved from potato leaf mitochondrial protein and, compared to the tuber mitochondrial profile, a substantial increase in the magnitude of peak one was observed (Table 6.1) indicating tissue-specific expression of the E3 isoforms.

It was essential to determine the integrity of potato leaf mitochondria as disrupted organelles, which would allow leakage from the matrix phase, may limit direct comparisons between the E3 profiles from photosynthetic and non-photosynthetic tissue. As the 2-oxoacid dedydrogenase complexes are believed to be associated with the inner membrane it is possible that their component enzymes would be retained in mitochondrial fractions of even damaged organelles. GDC, however, is notoriously labile and associations are weak. It is feasible, therefore, that the component enzymes of GDC

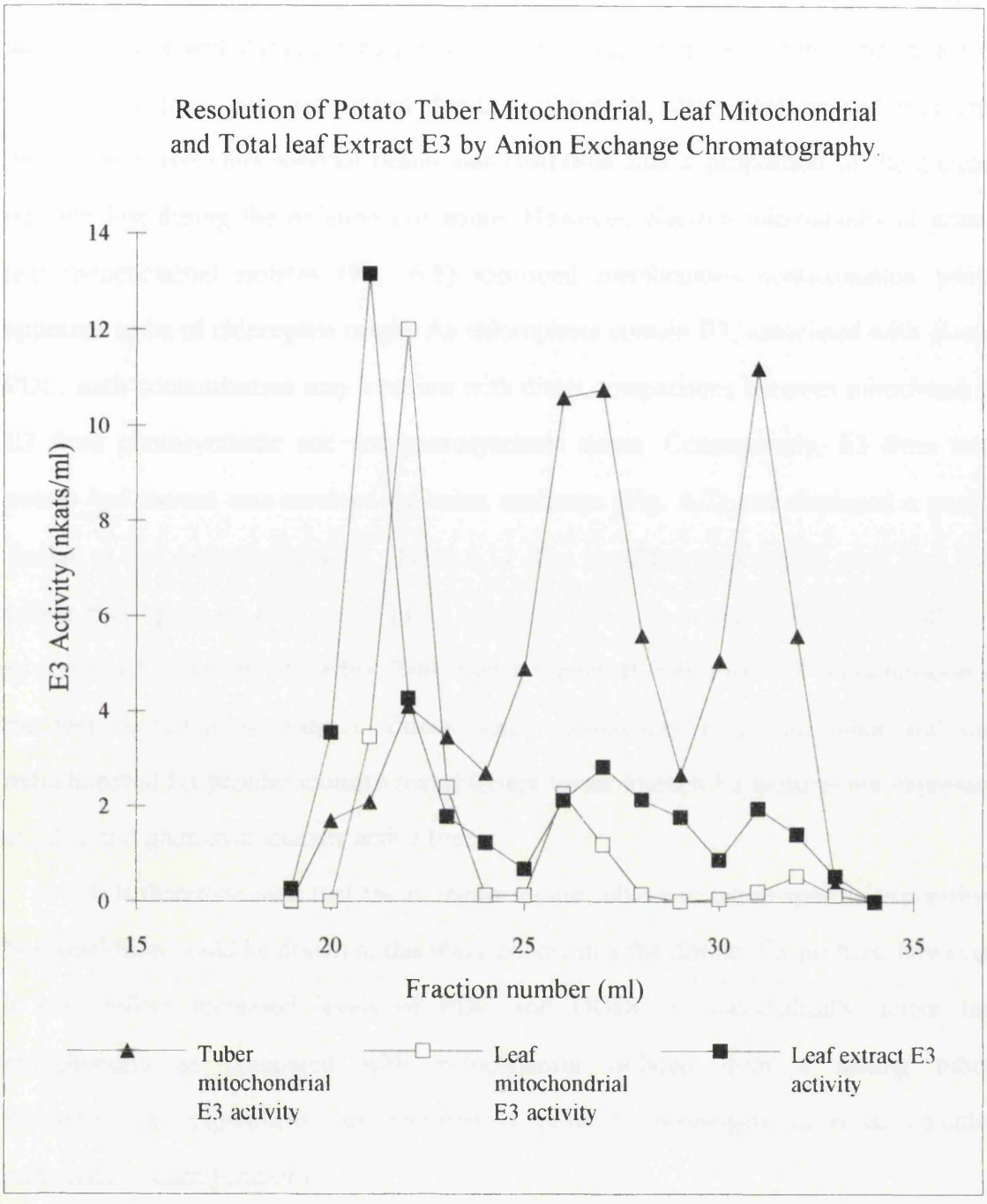


Figure 6.7 Resolution of potato tuber mitochondrial, leaf mitochondrial and total leaf E3 by anion exchange chromatography. Extracts were solubilized with 0.25%(v/v) Triton X-100 and heat treated prior to loading onto a MonoQ HR 5/5 column connected to a F.P.L.C system. Protein was eluted at a flow rate of 0.5ml/min along a phosphate gradient and E3 activities determined as described in Materials and Methods (2.5.5).

would be preferentially lost from 'leaky' mitochondria. Possible loss of a proportion of E3 would interfere with direct comparisons between potato tuber and leaf mitochondrial E3.

E3 activity was determined during leaf mitochondrial isolation and was only detectable in the mitochondrial pellets demonstrating that a proportion of the enzyme was not lost during the isolation procedure. However, electron micrographs of potato leaf mitochondrial isolates (Fig. 6.8) identified membranous contamination which appeared to be of chloroplast origin. As chloroplasts contain E3, associated with plastid PDC, such contamination may interfere with direct comparisons between mitochondrial E3 from photosynthetic and non-photosynthetic tissue. Consequently, E3 from total potato leaf extract was resolved by anion exchange (Fig. 6.7) and displayed a profile similar to leaf mitochondrial E3 (Table 6.1). The shoulder recorded in peak two may reflect chloroplast E3 (section 6.3) thus contributing to the increased levels of peak two as compared to leaf mitochondria. This suggests minimal chloroplast E3 contamination in the leaf mitochondrial extract. Consequently, differences in potato tuber and leaf mitochondrial E3 profiles indicate that different levels of each E3 isoform are expressed in tuber and photosynthetically active tissue.

It is therefore clear that the E3 isoforms are subject to tissue-specific expression. No conclusion could be drawn at this stage concerning the distinct E3 profiles, however, it may reflect increased levels of PDC and OGDC in metabolically active leaf mitochondria as compared with mitochondria isolated from a resting tuber. Reconstitution experiments are required in order to investigate potential complex selectivity of each form of E3.

Protein Source	E3 Peak Ratios (1^{st}:2^{nd}:3^{rd})
Potato tuber mitochondria	1:2.6:2.7
Potato leaf mitochondria	1:0.2:0.05
Total potato leaf extract	1:0.2:0.15

Table 6.1 Potato tuber mitochondria, leaf mitochondria and total leaf extract were solubilised with 0.25%(w/v) Triton X-100 heat-treated (65°C/10 min) and applied to a MonoQ HR 5/5 column. Protein was eluted at a flow rate of 0.5ml/min with a phosphate gradient (10-400mM) and E3 activity determined as described in Materials and Methods (2.5.5). In each case three peaks of E3 activity were resolved and activity ratios (1^{st}:2^{nd}:3^{rd}) determined.

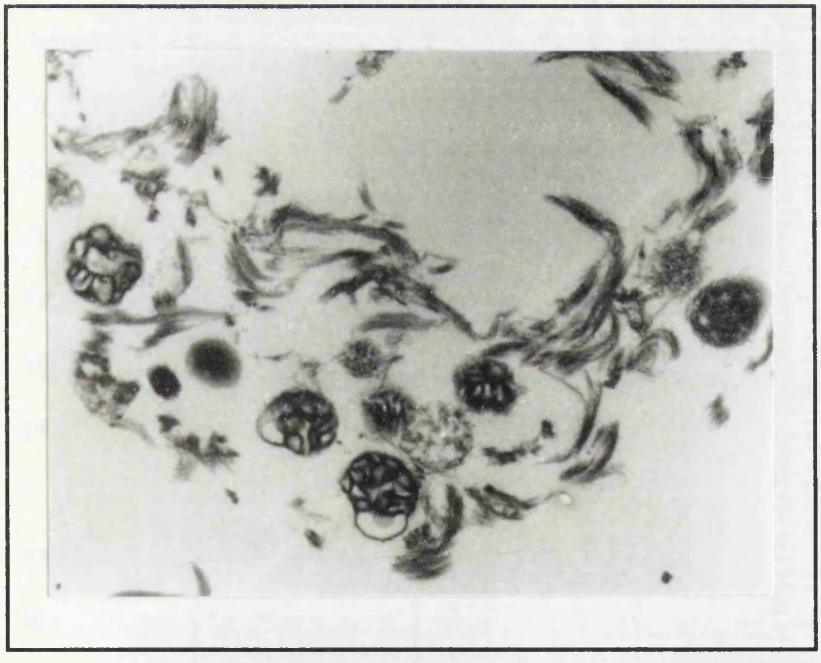


Figure 6.9: Electron micrograph of potato leaf mitochondria (x11000). Isolation and electron microscopy techniques are described in Materials and Methods (2.2 and 2.3 respectively)

6.6 PLANT DIHYDROLIPOAMIDE DEHYDROGENASE

Three distinct potato mitochondrial E3 activities were clearly resolved by anion exchange chromatography supporting work by R. Millar (Glasgow University) who first recorded this phenomenon. Further analyses characterised the independent E3s as α_2 (M_r α, 59,000), β_2 (M_r β, 56,000) homodimers and an $\alpha\beta$ heterodimer. Investigations were performed to ascertain the phyiological significance of distinct E3s. A single E3 activity was detected when solubilised potato chloroplasts, organelles which are reported to contain one E3 requiring system (PDC), were resolved by anion exchange chromatography. This observation instigated a research programme investigating the possibility of complex specificity focussing on a potential E3 species (L-protein) associated with GDC.

Potato mitochondrial extracts were separated by gel filtration in order to obtain fractions (void volume fractions) containing E3 associated with the 2-oxoacid dehydrogenase complexes only. The L-protein of GDC was considered to be absent from these fractions as Bourguignon *et al.* (1988) reported that, on gel filtration, the component enzymes of GDC are eluted separately in relation to their molecular mass. Analyses of the gel filtration data suggests that peak three preferentially associates with GDC. The next obvious route of research would be to determine, by reconstitution studies, whether any of the three E3 activities preferentially stimulate the activity of a particular multienzyme system.

Immunological detection (section 5.4) and analysis of E3 as resolved by anion exchange chromatography indicate that distinct mitochondrial and plastidic forms of E3 are expressed. In addition comparison of mitochondrial E3 from photosynthetic and non-photosynthetic sources suggest that tissue-specific expression of E3 isoforms is manifest. Extensive investigaions at Glasgow University are currently underway in order to establish the physiological role of these E3 isoforms.

CHAPTER SEVEN

DISCUSSION

7.1 INTRODUCTION

The 2-oxoacid dehydrogenase complexes occupy prime sites in central metabolism and are amongst the most sophisticated multienzyme systems known. Acetyl CoA, a product of PDC activity, serves as the primary entry point into the TCA cycle while OGDC is itself an intrinsic component of this respiratory cycle. Consequently, these multienzyme complexes play crucial roles in regulating energy metabolism. In addition the products of these complex reaction sequences, the CoA derivatives, act as primary substrates for other metabolic routes e.g. lipid and isoprenoid biosynthesis. The remaining member of this multienzyme family, BCDC, which catalyses a committed step in the degradation of branched-chain amino acids, methionine and threonine, plays a fundamental role in co-ordinating protein turnover.

GDC, an important multienzyme system in plant metabolism, is involved in the photorespiratory pathway cleaving glycine in the mitochondrial matrix and producing reducing power, in the form of NADH, and ultimately serine, via serine hydroxymethyltransferase (SHMT) activity. GDC shares common properties with the 2-oxoacid dehydrogenase complexes; this multienzyme system employs a dihydrolipoamide dehydrogenase and a lipoic acid containing component enzyme (H-protein). The H-protein is a small heat stable protein and essentially acts as a substrate in the cleavage of glycine rather than a component enzyme which is specifically bound to the complex. Such a loose association results in an inefficient system in which excessive H-protein, up to 30-fold more than the other component enzymes, is expressed (R. Douce, Grenoble, personal communication) to facilitate the glycine catabolism.

As these multienzyme complexes generate such biochemical and structural interest, the regulatory properties, structure and assembly of the mammalian and microbial 2-oxoacid dehydrogenase complexes have been extensively documented. With respect to the plant complexes limited information is available. This research programme has investigated the general properties of this multienzyme family from plant sources focusing on aspects of sub cellular localisation, kinetic characterisation of the reaction mechanism and subunit composition. Enzymatic and immunological studies performed on

the 2-oxoacid dehydrogenase complexes from pea and potato have uncovered a number of interesting features regarding the plant multienzyme family.

7.2 INTRACELLULAR DISTRIBUTION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

A range of organelles, isolated from pea, were screened for the presence of the 2-oxoacid dehydrogenase complexes. Enzymatic and immunological techniques were employed to detect specific members of this multienzyme family in mitochondria, plastids and peroxisomes from root and leaf tissue. Unequivocal evidence of a mitochondrial and plastid PDC was derived through enzymatic studies. By assessing substrate and cofactor requirements it was clear that the production of NADH in these extracts was a function of a PDC-catalysed reaction and the specific activities recorded agreed with previously reported values (Camp & Randall, 1985; Miernyk & Randall, 1987b).

The existence of a mitochondrial and plastid located PDC in a wide range of tissue, including pea, has been previously documented (Camp & Randall, 1985). Mitochondrial PDC (mtPDC) is primarily involved in respiratory processes supplying TCA cycle oxidation with acetyl CoA, whilst pPDC supplies acetyl CoA for the generation of fatty acids. Plants are unique amongst other eukaryotes by compartmentalising fatty acid biosynthesis in plastids. The fatty acid pathway utilises acetyl CoA as a building block for the assembly of long chain fatty acids. Although fatty acids can incorporate radio-label from acetate there is extensive evidence supporting a chloroplast located PDC which supplies an internal source of acetyl CoA. It is unclear if PDCs primary substrate, pyruvate, is transported into the chloroplasts or whether it is derived from 3-phosphoglycerate produced by the Calvin cycle.

Although chloroplast fatty acid synthesis is light stimulated by approx. 30-fold, fatty acids are also synthesised in plastids from non-photosynthetic tissue. Accordingly, PDC activity has been identified in plastids derived from developing *Ricinus* endosperm. PDC activity has been recorded in plastid fractions from leaves and storage tissue, however, to our knowledge, this is the first report of a PDC located in pea root plastid

isolates. In this instance pyruvate, essential for PDC synthesis of acetyl CoA, can either be imported or generated from the glycolytic pathway.

As a TCA multienzyme complex, OGDC activity in pea was exclusive to mitochondria displaying approx. 20% the activity of mtPDC with substrate and cofactor requirements verifying that the observed production of NADH was a consequence of OGDC activity. Such relative PDC and OGDC activities had previously been reported (Cho *et al.*, 1988). PDC activity is greater than her sister complex as not all acetyl CoA is fed into the TCA cycle. A proportion, dependent on cellular requirements, is utilised by other biosynthetic pathways, e.g. lipid synthesis. When CoA was omitted from the assay mixture pea mtOGDC remained active, displaying one third the activity of that with saturating substrate concentrations, suggesting that residual endogenous CoA in the solubilised mitochondria was sufficient to generate activity. As this phenomenon was not observed when analysing pea mitochondrial and plastidic PDC these observations indicate that mtOGDC exhibits a higher affinity for the CoA substrate than PDC. This was supported by kinetic data which estimated a lower K_m(CoA) value for potato mtOGDC than the corresponding values for potato mtPDC and pea pPDC.

This investigation included the first extensive screening of organelles for the presence of OGDC and activity was not detected in the peroxisomal or plastid compartments. Numerous interfering factors which could limit enzymatic detection, in particular the presence of NADH oxidases, complex disruption and low *in vivo* concentrations of an active complex, were thoroughly investigated and suggested that OGDC is indeed absent from these organelles having no role in the specific metabolism of the organelle.

To our knowledge plant BCDC activity has only been reported by one group (Gerbling & Gerhardt, 1988; 1989) who recorded activity in the peroxisomes of mung bean hypocotyls. Within the context of our own study pea peroxisomal located BCDC activity was not identified even when examining 'concentrated' organelle fractions. BCDC was recorded, however, in solubilised pea mitochondrial fractions which had been concentrated by high speed centrifugation. This indicated that BCDC in pea is located in mitochondria and low *in vivo* concentrations of an active complex restricted enzymatic

detection. This was supported by the detection of a BCDC-catalysed reaction in potato mitochondria. Although enzymatic analyses detected a mitochondrial BCDC in pea and potato, Gerbling & Gerhardt (1988) noted the absence of a BCDC-catalysed reaction in mitochondria from mung bean hypocotyls and thereby proposed that branched-chain amino acid breakdown is restricted to peroxisomes. This would ultimately suggest that during the course of amino acid breakdown other organelles export specific material to peroxisomes for degradation. Although co-operation between organelles is a common occurrence, e.g. the photorespiratory pathway, BCDC activity produces CoA derivatives which could ultimately be fed into the TCA cycle, thus rationalising the existence of a mitochondrial BCDC reported in this investigation.

BCDC activity or synthesis may be governed by a developmental programme. In view of its involvement in the catabolism of branched-chain amino acid, methionine and threonine, this multienzyme complex may have been developmentally suppressed in the tissue screened. Maximal activity is predicted during periods of extensive protein breakdown e.g. in senescent or developing endosperm tissue. If this is the case assessment of such tissue would alleviate problems arising from limited enzymatic detection allowing the characterisation of plant BCDC.

7.3 KINETIC ANALYSES OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

Kinetic investigations performed on potato mitochondrial and pea chloroplast PDC-catalysed reactions provided initial velocity data which agreed favourably with previously documented values. Potato mitochondrial OGDC, which had not been kinetically analysed prior to this investigation, displayed similar properties to mtPDC. In addition the observed reactions were inhibited by the products of the overall reaction sequence, NADH and acyl CoA, exhibiting greatest sensitivity to the NAD^+:NADH ratio, and the inhibitory behaviour was consistent with a three site ping-pong mechanism.

7.4 IMMUNOLOGICAL DETECTION OF THE 2-OXOACID DEHYDROGNEASE COMPLEXES

The availability of polyclonal antisera to specific subunits of the 2-oxoacid dehydrogenase complexes isolated from bovine heart provided a useful tool for the investigation of subunit composition of the plant 2-oxoacid dehydrogenase complexes. Immunological detection of this family of multienzyme complexes supported the enzymatic data collected in this report; PDC has a dual location in the plant cell, being present in both mitochondria and plastids whilst OGDC and BCDC are confined exclusively to the mitochondrial compartment. M_r comparisons between the mammalian component enzymes and their respective immuno-reactive plant proteins were performed employing the Western blot procedure. Numerous differences, described below, were detected providing interesting insights into the subunit composition of the plant multienzyme complexes.

Antisera to the E2 subunit of mammalian PDC cross-reacted with a 53,000Da pea and potato mitochondrial and pea chloroplast protein, which is lower than the M_r value of the mammalian subunit (74,000). It was essential to verify that this immuno-reactive species represents the E2 subunit of plant PDC and was not a function of non-specific interactions. It was subsequently shown, by probing pea mitochondrial and plastid protein separated by gel filtration, that the polypeptides responding to antiserum to the E2 subunit of PDC were indeed associated with a high M_r aggregate. This indicated that these polypeptides did represent component enzymes of the plant multienzyme complex and the M_r value suggests that the subunit from pea and potato mitochondria and pea chloroplast may carry a single lipoyl domain paralleling the situation in the analogous yeast enzyme. The E2 subunit from plant mitochondrial and plastid PDC, therefore, potentially shows greater similarity to the yeast enzyme than to the mammalian. This has particular relevance to the mitochondrial enzyme as it supports the proposed evolutionary origin of plant mitochondria; the endosymbiont capture theory of Gram-positive bacteria. Further evidence supporting a greater homology to the yeast multienzyme complex is supplied by immunological detection of the E1 component of

PDC. Whilst the E1 component of potato and pea mitochondrial and pea chloroplast PDC was antigenically distinct from the mammalian counterpart, Taylor *et al.* (1992) reported an immunological cross-reaction of pea mitochondrial protein with antiserum to the E1 subunit of yeast PDC.

Although investigation of the subunit composition of plant PDC by immunological methods was successful, similar characterisation of OGDC and BCDC, particularly when employing antisera to the native complexes, was restricted by the interference of non-specific interactions. The Western blotting technique was limited by sensitivity and the low concentrations of OGDC and BCDC in comparison to PDC, as indicated by enzymatic data, led to the detection of numerous spurious cross-reacting species. Bands were detected in the M_r range 65,000-70,000 which were not specifically associated with a high M_r aggregate and were therefore considered to be artefacts. This highlights the limitations of probing plant extracts with mammalian antibodies and stresses the necessity for the preparation of antisera to the plant 2-oxoacid dehydrogenase complexes which would overcome non-specific interactions.

The use of antisera to specific subunits of mammalian OGDC and BCDC did permit preliminary characterisation of the plant multienzyme complexes; immuno-reactive polypeptides responding to antiserum to the E2 subunit of OGDC and BCDC (M_r values 47,000 and 50,000 respectively) were detected in the void volume fractions of gel filtered pea mitochondria and may represent the E2 component enzymes of plant complexes. The estimated M_r values are consistent with the mammalian counterparts which carry a single lipoyl domain. These immuno-reactive polypeptides were absent in the chloroplast fraction, supporting enzymatic data which indicated that pea OGDC and BCDC are confined exclusively to mitochondria.

Plant mitochondrial and plastid extracts displayed no appreciable cross-reactivity with anti-E1(BCDC) whilst a potato mitochondrial immuno-reactive species of M_r value 100,000, identical to the mammalian subunit, was identified when probing with antiserum to the E1 subunit of OGDC.

Immunological analyses of the E3 subunit identified a cross-reacting pea chloroplast polypeptide of M_r value 52,000, comparable to the mammalian enzyme (M_r

55000) whilst the immuno-reactive pea and potato mitochondrial polypeptide had an M_r value of 58,000. The M_r values of the mitochondrial and chloroplast immuno-reactive polypeptides are distinct and their respective E3s display differing salt sensitivities (Taylor, Glasgow University, personal communication). These observations support the existence of organelle specific forms of plant E3. It is therefore possible that the other component enzymes also display organelle specificity and are encoded by separate genes expressing mitochondrial and plastid targeting sequences.

7.5 DIHYDROLIPOAMIDE DEHYDROGENASE

The separation of three distinct E3 activities from potato mitochondria by anion exchange chromatography is one of the most significant findings reported in this investigation. It is postulated that these independent potato mitochondrial E3s take the form of an α_2 and β_2 homodimers and an $\alpha\beta$ heterodimer and may represent complex specific isoforms. Although this phenomenon has been observed in pea mitochondria separated by anion exchange, 95% of E3 activity was eluted in one fraction. Consequently, it is uncertain whether this holds any physiological significance in this species. The fact that multiple E3 activities are restricted, at present, to potato mitochondria may be accounted for by the tetraploid nature of the cross-breed variety of potato investigated. To examine this hypothesis the screening of other tetraploid species is currently underway. Only one form of E3 was identified in potato chloroplasts, however, suggesting that the mtE3 isoforms are not a function of cross-breeding.

With respect to potato, observations within the context of this project suggest that the distinct forms of mitochondrial E3 do hold physiologically relevance and may be complex specific isoforms:

1. One peak of E3 activity was observed when potato chloroplast protein was separated by anion exchange chromatography. Enzymatic studies conducted in this thesis indicated that the only 2-oxoacid dehydrogenase complex located within the chloroplast compartment is PDC. This coupled with the certitude that GDC is a mitochondrial

multienzyme system suggests that plastids have a requirement of a single prospective E3 isoform.

2. Solubilised potato mitochondrial protein was applied to a gel filtration column to separate E3 associated with intact PDC and OGDC activity and 'free' dissociated E3 which was postulated to contain predominately the L-protein of GDC. These two fractions were subsequently resolved by anion exchange chromatography and produced distinct E3 activity profiles; the third E3 activity was not recorded in the void volume E3 sample.

3. Potato leaf and tuber mitochondrial E3s were resolved by anion exchange chromatography and produced distinctly different E3 activity profiles. This indicates that the occurrence of tissue-specific expression of the E3 isoforms, suggesting that different tissues have different isoform requirements.

These observations suggested that the distinct E3 activities preferentially associate with particular multienzyme systems. The identification of potential potato mitochondrial E3 isoforms provides the ground-work for further investigations concerning possible complex specificity. In the near future it is predicted that sequence data and reconstitution experiments will establish the physiological role of each E3 isoform.

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