

Transport and metabolism of symplastic and apoplastic  
ascorbate during oxidative stress

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A thesis prepared in requirements for the degree of Doctor of Philosophy  
(Ph.D.), University of Edinburgh 2008

## Declaration

This thesis has been composed by myself and the work, of which if is a record, has been carried out by myself. All sources of information have been specifically acknowledged by means of a reference.

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## Acknowledgements

The research for this thesis was funded by the British Biotechnology and Biological Sciences Research Council (BBSRC), UK.

There are many others that must be thanked. In no particular order of appearance...

Prof. S.C. Fry has provided much appreciated advice, ideas and support over the years. I also thank him for maintaining an admirable level of tact and diplomacy when faced with the unenviable task of bringing out the perfectionist in me.

Nele Horemans, Jeremy Barnes, Karen Jackson (see also section on more social IMPS members) and David D'Haese have all given up their time to discuss ideas with me and provide me with advice.

I would particularly like to thank Mikael Attal, without whom there can be no doubt that this would have been a lesser thesis. This man has cooked dinner, shopped, washed-up and cleaned the flat for me for the last two months, took the desiccating chamber and pump back down to Newcastle for me and then (other boyfriends take note here) he offered to Edit My References For Me. He simply (ahem) rocks. Like a pegmatite, apparently.

Sandra Sharples and Shona Lindsay provided invaluable advice on things scientific but, most importantly, entertaining company during and after working hours. Indeed, the walls of the tea room may reverberate with the echoes of exchanged pleasantries for years to come. My memories as a co-demonstrator on the Plant Science 3 field trips still make me break into laughter at apparently random moments of the day. My memories of Friday nights after Assembly in my second year are still mostly missing. Without you, other more social IMPS members and my flat mate, Lorna Street, it would have been a much less amusing thesis.

I also thank Jo Critchley without whose extreme body-locating skills there might not have been any thesis at all, as well as all the other Mountain Rescue and RAF folk involved in scraping my carcass off the mountainside and getting it to hospital.

My family fed me, left me alone when required and kept me topped up with gin and wine when required whilst I was writing up. It cannot have been an easy task. They all deserve many thanks.

To my sister, Zoë.



### **List of abbreviations**

EDTA	Ethylenediaminetetraacetic acid
DHA	Dehydro L-ascorbate
PPO	2,5-diphenyloxazole
Gal-L	Galactono-1.4-lactone
TFA	Trifluoroacetic acid
DMF	Dimethylformate
DKG	2,3-diketo-L-gulonate
HPLC	High-pressure liquid chromatography
DCPIP	Dichlorophenolindophenol

## Abstract

Reactive oxygen species (ROS) are produced continuously in plants and act as important signalling molecules in many cellular processes including stress and defence responses. ROS can arise from external sources as well as being generated by the plant. Pollutants, such as ozone, enter the leaf via stomata and dissolve in the apoplast. ROS can arise both intracellularly and apoplastically: superoxide is produced during photosynthesis as well as by the plasma membrane bound NADPH oxidase during the oxidative burst. Under abiotic stresses such as drought or high light-intensity, superoxide production from photosynthetic electron flow is increased. Ascorbate plays a crucial role in symplastic and apoplastic ROS metabolism. Intracellular ascorbate metabolism is highly regulated; it is coupled to glutathione oxidation and reduction and is under tight enzymic control. Export of ascorbate into the apoplast increases during ozone-induced stress. The apoplast redox state is considered to be more variable than the symplasm. Ascorbate is thought to be taken up from the apoplast in its oxidised form, DHA, via specific carriers, implying tight regulation of apoplastic/symplastic ascorbate transport.

An apoplastic ascorbate breakdown pathway has recently been described by Green and Fry (2005). Ascorbate is oxidised and hydrolysed to yield oxalate via two novel intermediates, cyclic oxalyl L-threonate (cyc.ox.thr.) and 4-*O*-oxalyl-L-threonate (ox.thr.) A novel esterase is thought to catalyse the hydrolysis of ox.thr. to oxalate. Dehydro-L-ascorbate DHA was also hydrolysed to L-2,3-diketogulonate (DKG) which broke down to two unidentified compounds, C and E. It was not known whether this pathway operated intracellularly and how increased ROS production might affect flux through this pathway. The pathway, described, in the culture medium of 5-day-old rose cell suspension cultures but had not been investigated *in planta*.

Intracellular and extracellular metabolism of [<sup>14</sup>C]ascorbate in [<sup>14</sup>C]ascorbate-loaded cells was investigated in response to oxidative stress induced by 0.1 and 1 mM H<sub>2</sub>O<sub>2</sub> and 1 and 10 μM methyl viologen (MV<sup>2+</sup>). The symplasm became more oxidised in response to 0.1 mM H<sub>2</sub>O<sub>2</sub>; DHA levels increased and ascorbate decreased, but ox.thr. and oxalate, products of irreversible ascorbate breakdown, did not accumulate. Symplastic ox.thr. and oxalate accumulated in response to MV<sup>2+</sup> and 1 mM H<sub>2</sub>O<sub>2</sub>. Ox.thr. and oxalate were observed *in-planta*. Flux through the pathway was increased in transgenic tobacco plants which overexpressed the cell wall-located enzyme ascorbate oxidase, suggesting that the redox state of the apoplast could increase apoplastic ascorbate breakdown via ox.thr. The rate of production of oxalate *in vivo* compared to *in vitro* studies suggested that the esterase was located to the symplasm as well as the apoplast. Oxalate did not appear to be metabolised further. Compounds C and E were neither observed *in planta* nor in 10-day old rose cell cultures. DKG and cyc.ox.thr. were present only in low levels.

Export of <sup>14</sup>C in [<sup>14</sup>C]ascorbate loaded cells increased in response to 1 and 5 mM H<sub>2</sub>O<sub>2</sub>. Increased export was characterised by a rapid response during the first 2 min of H<sub>2</sub>O<sub>2</sub> exposure. In *Arabidopsis* and rose cell suspension cultures, export was often observed to occur in series of pulses. The amplitude of pulses increased within the first 2 min of H<sub>2</sub>O<sub>2</sub> exposure. This was not thought to be a result of membrane disruption. <sup>14</sup>C appeared to be exported as [<sup>14</sup>C]ascorbate and taken up as [<sup>14</sup>C]DHA, with minimal oxidation in the culture medium.

These results provide more insight into intracellular ascorbate breakdown via ox.thr. and suggest that oxalate could accumulate in response to oxidative stress in plants. The export of ascorbate/DHA in pulses in response to H<sub>2</sub>O<sub>2</sub> hints at novel mechanisms of regulation of ascorbate/DHA transport across the plasma membrane.

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## **1. Introduction**

### **1.1 The history and importance of ascorbate**

The importance of fresh fruit, particularly oranges and lemons, had been recognised for centuries as a preventative of scurvy, although vitamin C was not isolated until the 1920's. Many dietary studies at the turn of the last century were aimed at discovering the antiscorbutic factor which could aid in the treatment of scurvy. Vitamin C was considered to be an essential factor or 'vitamine' because of its antiscorbutic properties. It acquired its name from being the third essential factor following the discovery of 'A' and 'B' which were shown to be essential components in the diet of rats. Vitamin C refers to two compounds, ascorbate and dehydroascorbate (DHA), whose chemical properties are introduced below. Later studies on guinea pigs, which unlike rats cannot synthesise ascorbate, saw real progress being made towards its isolation as a dietary factor (Davies *et al.* 1991). Albert Szent-Györgyi reported the extraction of a reducing sugar from the adrenal cortex, orange juice and cabbage water (Szent-Györgyi 1928) and the structure was published in 1933 (Haworth and Hirst 1933). Its importance as a dietary factor is accepted as fact, yet a century later its relative merits are still the subject of debate (Podmore *et al.* 1998).

Vitamin C is chemically the most simple of vitamins yet has an extraordinarily diverse array of biological functions. It is ubiquitous throughout the plant and animal kingdoms, with ascorbate analogues being synthesised by fungi. In addition to primates (humans included), some fish, insects, fruit bats and guinea pigs are incapable of synthesising vitamin C and must obtain it through the diet (Davies *et al.* 1991). This is due to genetic divergence of functional biosynthetic pathway enzymes, indicating ascorbic acid may have an interesting evolutionary history.

## 1.2 Chemistry of vitamin C

### 1.2.1 Chemistry of ascorbate

L-Ascorbate, or *L-threo*-hex-2-enono-1,4-lactone, is a very effective biological reducing agent. It is a diacid, consisting of a 5-membered lactone ring with an ene-diol group at C-2 and C-3. The antiscorbutic properties of ascorbate are a function of the ene-diol group. Unusually, the pK values of the hydroxy groups are far removed from each other: the hydroxy group at C-3 has a pK of 4.7 whilst the hydroxy group at C-2 has a pK of 11.8. Ascorbate is therefore usually present as a monoanion at most physiological pH values. Ascorbate acts as an electron donor by providing 1 or 2 electrons to reactive oxygen species (ROS), thereby arresting oxidative chain reactions. Donation of one electron produces the ascorbate radical monodehydroascorbate (MDHA). MDHA is less damaging than other free radicals as it has a redox potential of only +282 mV as opposed to the highly oxidising species  $\cdot\text{OH}$ , which has a redox potential of +2310 mV. MDHA is relatively stable as the single electron is highly delocalised. This stability of MDHA is an essential characteristic of ascorbate as it allows it to be reduced back to ascorbate by accepting an electron (Fig. 1.1) and via the action of MDHA reductase before it disproportionates to DHA plus ascorbate (Beuttner and Schafer 2004). Loss of the second electron produces DHA (Fig. 1.1) which *in-vivo* can be reduced back to ascorbate *in vivo* via the ascorbate–glutathione (GSH) cycle (Halliwell and Foyer 1976; see section 1.6.1).

The complex redox chemistry of ascorbate means that it can also act as a pro-oxidant as well as an antioxidant (Fry 1998). The reduction potential of ascorbate is sufficient to reduce catalytic transition metals such as  $\text{Fe}^{3+}$  or  $\text{Cu}^{2+}$ . The reduced metals can, in the presence of  $\text{O}_2$ , initiate oxidation reactions (Beuttner and Schafer 2004; see 1.3.2).

Ascorbate breakdown occurs most readily under oxidising conditions at alkali pHs (Davies *et al.* 1991). Under such conditions, the *in-vitro* oxidation of ascorbate occurs in two

stages: firstly formation of DHA, then the formation of an un-named product which can be hydrolysed between C-2 and C-3 to yield threonate and oxalate (Isbell and Frush 1979).

#### 1.2.2 Chemistry of dehydroascorbate

Around the time of the discovery of ascorbate it was noted that ascorbate could be oxidised to a product (DHA) with two fewer hydrogen atoms, a product which could be reduced back to ascorbate (Davies *et al.* 1991). This is a critical characteristic of DHA chemistry which allows ascorbate to be recycled in the plant cell via the ascorbate–glutathione cycle (see 1.6.1). Unlike ascorbate, which primarily undergoes oxidation–reduction reactions, DHA can be hydrolysed, reduced or oxidised. Oxidation of DHA *in vitro* is known to result in the formation of threonate (Deutsch 1998). During hydrolysis the lactone bond at C-1 is broken, the ring is opened and the carboxylic acid L-2,3-diketogulonate (DKG) is formed (Fig. 1.1), a process which occurs slowly between pH 2 and pH 4 but much faster at higher, physiological pH values (Penney and Zilva 1943). This makes DHA unstable in aqueous solutions. Hydrolysis to DKG is thought to be irreversible *in vivo* (Deutsch 2000).

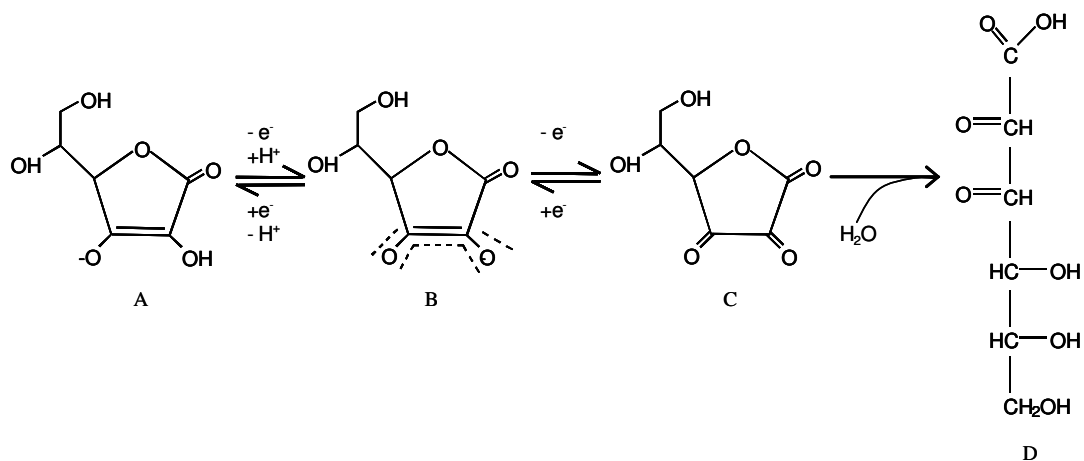
The potential for signalling properties of DHA has been suggested; Fotopoulos *et al.* (2008) show evidence for the involvement of DHA signalling in stomatal closure (see 1.4.2), while Tyburski *et al.* (2008) show that DHA may well be involved in the manifestation of the effects of auxin (see 1.6.7). As a result of the ability of DHA to act as an electron acceptor, DHA is thought to be integral in the formation of disulphide bonds between cysteine residues and therefore in protein folding (Bánhegyi *et al.* 2003).

#### 1.2.3 Chemistry of L-2,3-diketogulonate

DKG has also been reported to be unstable in aqueous solutions (Kagawa 1962, Deutsch 2000). In the presence of oxygen, under alkali conditions, threonate and oxalate are reported to be the breakdown products of DKG, whilst under slightly acid conditions, the formation of

xylonate, lyxonate and CO<sub>2</sub> was observed (Kanfer *et al.* 1960, Kagawa 1962). In the presence of H<sub>2</sub>O<sub>2</sub>, threonate, oxalate and CO<sub>2</sub> were reported (Simpson and Ortwerth 2000). Li *et al.* (2001) describe the non-enzymic conversion of DKG into two δ-lactones; 2,3-diketo-gulonolactone and 3,4-diketo-gulonolactone, the latter being thought to possess antioxidative properties (see 1.6.7).

Deutsch (1998, 2000) pointed out that oxidative decarboxylation might be associated with the consumption of oxygen and therefore confer some antioxidant properties on DKG. The addition of rat liver decarboxylase impacted little on the *in-vitro* rate of formation of oxalate and threonate but increased the production of lyxonate and xylonate (Kagawa 1960, Kagawa 1962) suggesting at least two distinct breakdown pathways of DKG.



**Figure 1.1** The reversible oxidation reactions of ascorbate and the hydrolysis of DHA  
A, ascorbate monoanion; B, MDHA; C, DHA; D, DKG

### 1.3 Ascorbate function in plants

#### 1.3.1 Reduction of ROS produced during photosynthesis

Ascorbate fulfils a critical role as an antioxidant in plants. Although the antioxidant nature of ascorbate is essential to most areas of plant metabolism, it is particularly important in photosynthesis. The reduction of O<sub>2</sub> by ferredoxin at photosystem I (PSI) produces O<sub>2</sub><sup>-</sup>, whilst

the chloroplast electron transport chain generates singlet oxygen (Foyer 1983, Asada 1999).  $O_2^-$  dismutates into  $H_2O_2$ , a reaction which is catalysed by superoxide dismutase (SOD) (Lamb and Dixon 1997). Enzymes essential to carbon fixation such as fructose -1,6-bisphosphatase are particularly sensitive to attack by  $H_2O_2$  (Foyer and Noctor 2005). In the presence of transition metals,  $H_2O_2$  produces hydroxyl radicals ( $\cdot OH$ ).  $\cdot OH$  are highly reactive and, left unchecked, are capable of causing localised oxidative damage (Lamb and Dixon 1992). Ascorbate accumulates to high concentration (10 – 50 mM) in chloroplasts (Foyer *et al.* 1983, Asada 1999) depending on growth conditions. The formation of  $\cdot OH$  is prevented by the reduction of  $H_2O_2$  to  $H_2O$  and by the action of catalase in peroxisomes. This reaction is catalysed in chloroplasts by ascorbate peroxidase (APX) and is coupled to the oxidation of ascorbate to form MDHA. MDHA accepts electrons from the photosynthetic electron transport chain thereby being reduced back to ascorbate but reduction of MDHA is also catalysed by MDHA reductase as part of the ascorbate-glutathione cycle (see 1.6.1). The combination of reactions catalysed by SOD and APX are known as the Mehler-peroxidase cycle.

Ascorbate can protect photosynthetic machinery from oxidation by ROS through its role as an enzyme co-factor as well as that of antioxidant. Ascorbate acts as a co-factor for the enzyme violaxanthin de-epoxidase. Violaxanthin is a carotenoid pigment in the thylakoid membrane. De-epoxidation of violaxanthin produces zeaxanthin (Young 1991). This reaction allows excess energy from high light intensities to be quenched (Ruban *et al.* 1993). During high light intensities, photosynthesis can become saturated. If excess light energy is not quenched, the rate of ROS production increases. Increased electron flow from PSII to PSI decreases the pH of the thylakoid lumen, a process which stimulates activity of violaxanthin de-epoxidase (Pfundel *et al.* 1992). As excess light energy is quenched, reduction of  $O_2$  is decreased and so too is the generation of  $O_2^-$ ,  $H_2O_2$  and singlet oxygen.

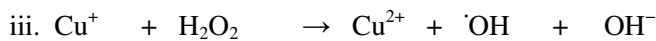
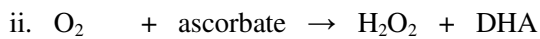
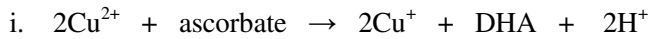
### 1.3.2 Apoplastic ascorbate and growth

Ascorbate may be involved in modulating growth. Ascorbate is a co-factor for prolyl hydroxylase, the enzyme involved in synthesis of hydroxyproline rich proteins, a group of cell wall structural proteins (Myllyla *et al.* 1978, Smirnoff 1996).

Secondly, as an antioxidant, ascorbate is thought to play an important role in determining the extent of oxidative cross linking between cell wall structural components. Following a pathogen elicitor-induced oxidative burst, H<sub>2</sub>O<sub>2</sub> mediated cross linking of cell wall proteins (Lamb and Dixon 1997, Brisson *et al.* 1994) and polysaccharides (Fry *et al.* 2000). Monolignol radicals are generated in the apoplast from presence of H<sub>2</sub>O<sub>2</sub> and cell wall peroxidases. Ascorbate can scavenge these radical reducing them back to monlignols (Fry *et al.* 2000). Kärkönen and Fry (2006) showed that H<sub>2</sub>O<sub>2</sub> could be generated from ascorbate breakdown products DHA and DKG in *Picea abies* cell suspension cultures. H<sub>2</sub>O<sub>2</sub> produced from ascorbate breakdown could potentially stimulate lignification. Ascorbate oxidase (AO) catalyses the production of MDHA from ascorbate (Smirnoff 2000). AO activity is associated with rapidly growing plant tissue (Lin and Varner 1991, Smirnoff 1996). Non-enzymic ascorbate breakdown produces H<sub>2</sub>O<sub>2</sub> (Fry 1998). Despite the potential for H<sub>2</sub>O<sub>2</sub> production from increased ascorbate breakdown in plants with a more oxidised apoplast, these plants showed evidence of cell elongation and had more biomass than wild type (WT) plants (Pignocchi *et al.* 2003). A theoretical wall loosening role for DHA has been proposed. DHA could undergo decarboxylation reactions with side chains of cell wall proteins or the amino termini of polypeptide chains thereby reducing cross linking involving proteins in the cell wall (Lin and Varner 1991). Apoplastic MDHA can accept electrons exported via a cytochrome b (Asard *et al.* 1994). Addition of MDHA to onion root cells has been observed to stimulate H<sup>+</sup>ATPase (Gonzalez-Reyes *et al.* 1994). This could contribute to growth by acidification of the apoplast or,

as Kato and Esaka (2000) have proposed, by stimulation of ion and nutrient uptake, which would raise the intracellular osmotic pressure and lead to cell expansion.

In some circumstances, ascorbate can act as a pro-oxidant, generating  $\cdot\text{OH}$  in the reaction sequence shown below (Equation (i) to (iii) from Dumville and Fry 2003).



In the apoplast  $\cdot\text{OH}$  may participate in polysaccharide scission and so are thought to aid cell wall loosening. Fry (1998) used a mixture of  $\text{H}_2\text{O}_2$ ,  $\text{Cu}^{2+}$  and ascorbate to yield  $\cdot\text{OH}$  and so demonstrated polysaccharide scission *in vitro*. Schopfer (2001) showed that application of such a mixture *in-vivo* resulted in increased wall extensibility. The amount of  $\text{Cu}^{2+}$ , as opposed to other transition metals, was found to increase during ripening in tomato tissue, along with an increased rate of ascorbate export to the apoplast (Dumville and Fry 2003).  $\cdot\text{OH}$  are good candidates for targeted polysaccharide scission as their production can be very localised, possibly depending on  $\text{Cu}^{2+}$  distribution (Fry *et al.* 2002). When the prerequisites of  $\text{H}_2\text{O}_2$ , ascorbate and  $\text{Cu}^{2+}$  are met then  $\cdot\text{OH}$  could be an important mechanism of fruit softening and cell wall extension (Fry *et al.* 2002). It is conceivable that the presence of AO could help control ascorbate levels so that, once formed,  $\cdot\text{OH}$  were not reduced by ascorbate. An increase in the irreversible oxidation of ascorbate in the presence of AO might potentially increase apoplastic oxalate. A cell wall loosening role has been proposed for oxalate whereby oxalate binds to  $\text{Ca}^{2+}$ , disrupting bridges between polygalacturonate chains in the cell wall (see section 1.6.5).



### 1.3.3 Ascorbate and development

Ascorbate appears to be an essential factor in the developmental cycle of plants. During embryogenesis higher levels of ascorbate (as well as an increased ascorbate:DHA ratio) correlate with higher mitotic activity; dormant seeds contain only DHA whilst quiescent centres, where cell division is very low, also have higher levels of DHA. As the redox status of symplastic ascorbate is thought to be much more stable than that of the apoplast (Noctor and Foyer 2005), apoplastic ascorbate is unlikely to directly control developmental processes, although quiescent centres have high levels of activity of the cell wall enzyme AO (Pignocchi *et al.* 2003). AO in the quiescent centre is thought to be involved in keeping ascorbate in its oxidised state, DHA, indicating the importance of the ascorbate redox state in cell division. Exogenous ascorbate has been shown to stimulate division in the quiescent centre of onion root tips (Kerk and Feldman 1995). Adjacent meristematic areas have higher rates of cell division and a more reduced ascorbate pool (Kerk and Feldman 1995).

The effect of variation in ascorbate: DHA ratios in the cell cycle seems to be focussed on the transition from G1 to S phase (Potters *et al.* 2002), the point at which a cell either enters into a new cycle or not (Francis 1992). Studies in a variety of plant systems point to the role of DHA as the active component in a delayed transition from G1 to S phase (Potters *et al.* 2002). Tobacco BY-2 cells treated with DHA showed a delay in cell cycle progression whilst fewer cells divided when ascorbate was added. The intracellular redox state was unaffected as DHA seemed to be re-reduced to ascorbate in the symplasm (Potters *et al.* 2000). It was speculated that if progress through the cell cycle were delayed by higher concentrations of intracellular DHA, DHA might be acting as a signal transmitting information about the extracellular redox state and therefore the extracellular environment (Potters *et al.* 2000). Redox signalling between the apoplast and symplasm is likely to be central to many environmental stimuli and is discussed in more detail in section 1.4.

Ascorbate is closely linked to the biosynthesis of hormones, as it is known to be a co-factor for two enzymes in gibberellic acid (GA) synthesis and one in abscisic acid (ABA) synthesis. GA induces flowering in short day conditions whilst ABA is thought to work antagonistically to GA. The *vtc-1* mutant, which has around 30% of the ascorbate content of wild-type (WT) plants, has lower levels of GA and an elevated ABA content (Barth *et al.* 2006). L-Galactono-1,4-lactone (GalL) is the precursor to ascorbate in the ascorbate biosynthetic pathway described by Wheeler *et al.* (1998). Under a 16-h photoperiod, flowering is earlier in these mutants but feeding GalL can delay flowering. Ascorbate is hence likely to be an important factor in several developmental pathways although the biological mechanisms of the interactions are not yet known.

#### **1.4 Ascorbate and ROS metabolism**

##### 1.4.1 ROS production during biotic and abiotic stress

ROS are small molecules, usually with an unpaired electron and are highly reactive and short-lived. They include the hydroxyl radical ( $\cdot\text{OH}^-$ ), superoxide ( $\text{O}_2^-$ ) and hydrogen peroxide,  $\text{H}_2\text{O}_2$ . ROS are produced in response to biotic stresses such as wounding or pathogen attack and abiotic stresses such as drought or exposure to pollutants in a phenomenon known as the oxidative burst. During the oxidative burst, the plasma membrane-bound enzyme NADPH oxidase forms an active complex with other plasma membrane-associated proteins and reduces  $\text{O}_2$  to  $\text{O}_2^-$ . NADPH oxidase activity is also induced by  $\text{Ca}^{2+}$  binding. This is followed by rapid dismutation of  $\text{O}_2^-$  to yield  $\text{H}_2\text{O}_2$  (Lamb and Dixon 1997, Orozco-Cardenas *et al.* 2001). A direct negative effect of ROS on bacterial viability and germination of fungal spores has been noted (Lamb and Dixon 1997). Oxalate oxidase enzymes are induced by pathogen elicitors (Langebartels *et al.* 2002). Metabolism of oxalate, a breakdown product of ascorbate, to  $\text{CO}_2$  can also produce  $\text{H}_2\text{O}_2$  (see 1.5.6).

Human activities have increased the ambient concentrations of gases such as SO<sub>2</sub> and O<sub>3</sub> in the troposphere. Air-borne pollutants enter plants via the stomata and become dissolved in the apoplast. In the case of SO<sub>2</sub>, this forms sulphite and bisulphite. These are reactive compounds that would oxidise lipids and proteins. This is avoided by reduction to sulphide (Takahama *et al.* 1992). Exposure to increased O<sub>3</sub> concentrations triggers an oxidative burst in plants. Lesions that resemble programmed cell death (PCD) are thought to arise from the induction of pathogen defence responses by O<sub>3</sub> (Langebartels *et al.* 2002).

As much of the oxidative burst is thought to be generated in the apoplast, then apoplastic ascorbate levels will profoundly affect the intensity and duration of an oxidative burst. Apoplastic ROS are known to induce an influx of Ca<sup>2+</sup> to the cytosol. This can be prevented by the addition of exogenous ascorbate in cell suspension cultures (Kawano *et al.* 2000), presumably through the ROS scavenging activity of ascorbate. Reduction of ROS by ascorbate will directly affect oxidative damage but as both ROS and Ca<sup>2+</sup> are associated with signalling pathways, the indirect effects will be manifold.

#### 1.4.2 ROS signalling

Over recent years there has been a growing appreciation of the effect of changes in the redox state of cellular compartments and of the targeted oxidation of cellular machinery on signalling and metabolic co-ordination between organelles (Foyer and Noctor 2005). Exogenous H<sub>2</sub>O<sub>2</sub> in *Arabidopsis* cell suspension cultures stimulated mitochondrial production of H<sub>2</sub>O<sub>2</sub>. Oxidative damage associated with this H<sub>2</sub>O<sub>2</sub> production seemed to be concentrated in proteins associated with the mitochondrial electron transport chain, TCA cycle and ATP synthesis (Sweetlove *et al.* 2002). Metabolic changes in all three of these processes are considered likely to have repercussions on ascorbate metabolism and cellular homeostasis. The last step in ascorbate biosynthesis is catalysed by galactono-1,4-lactone dehydrogenase and is coupled to the

mitochondrial electron transport chain (Nunes-Nesi *et al.* 2007). Mutants deficient in enzymes of the TCA cycle show elevated rates of photosynthesis and ascorbate accumulation (Nunes-Nesi *et al.* 2007). Sulphur containing amino acids are particularly sensitive to oxidation by ROS. Some are re-reduced by specific enzymes (Foyer and Noctor 2005). Such oxidation and reduction of proteins could potentially constitute a reversible redox state-controlled signal.

H<sub>2</sub>O<sub>2</sub> had been proposed to be an important ROS messenger as it is one of the most abundant ROS in plants, a less reactive oxidant than O<sub>2</sub><sup>-</sup> and longer lived than O<sub>2</sub><sup>-</sup> (Bienert *et al.* 2006). Its duration can also be controlled directly by SOD, APX and ascorbate. Production of O<sub>2</sub><sup>-</sup> and therefore H<sub>2</sub>O<sub>2</sub>, is controlled by NADPH oxidase, an enzyme whose activity can be modulated by Ca<sup>2+</sup> (Neill *et al.* 2002b). The release of Ca<sup>2+</sup> into the cytosol from intracellular stores in response to diverse forms of stress that elicit an oxidative burst is closely linked to the production of H<sub>2</sub>O<sub>2</sub>, with H<sub>2</sub>O<sub>2</sub> appearing to be generated downstream of Ca<sup>2+</sup> maxima (Cessna and Low 2001). It has been proposed that cytosolic Ca<sup>2+</sup> could be involved in cross tolerance and stress acclimation whereby exposure to one stress can lead to increased tolerance of subsequent stresses (Cessna *et al.* 2001) though how this is linked to changes in ROS and ascorbate metabolism is not yet clear. H<sub>2</sub>O<sub>2</sub> is thought to act downstream of abscisic acid (ABA) (Fotopoulos *et al.* 2008) in ABA-induced stomatal closure, a process which has also been linked to changes in cytosolic Ca<sup>2+</sup> concentration. Tobacco plants overexpressing DHA reductase had lower levels of H<sub>2</sub>O<sub>2</sub> in guard cells and increased stomatal conductance. This was attributed to an increased rate of regeneration of ascorbate from DHA, meaning more ascorbate was available to reduce H<sub>2</sub>O<sub>2</sub> (Chen and Gallie 2004). However, recent results by Fotopoulos *et al.* (2008) suggest a role for DHA signalling downstream of H<sub>2</sub>O<sub>2</sub>.

H<sub>2</sub>O<sub>2</sub> is known to have signalling properties (discussed in 1.4.2) and, acting downstream of ABA (Zhang *et al.* 2006), leads to a reduction in stomatal conductance (Chen and Gallie 2004). Addition of 100 µM H<sub>2</sub>O<sub>2</sub> to tobacco epidermal strips prompted stomatal closure but so

too did 1 mM DHA. Tobacco plants overexpressing AO had higher background levels of H<sub>2</sub>O<sub>2</sub> and a more oxidised apoplastic ascorbate. Epidermal strips of these leaves were less sensitive with respect to stomatal closure not only to H<sub>2</sub>O<sub>2</sub> but also to DHA. Plants over-expressing AO had higher levels of abscisic acid (ABA) than WT plants, a trait shared with the *vtc-1* mutant (Fotopoulos *et al.* 2008). Likewise, the apoplastic ascorbate: DHA ratio in the *vtc-1* mutant was more reduced (Veljovic-Jovanovic *et al.* 2001). However, whether signalling resulting in stomatal closure occurs as a direct response to DHA, increased DHA content from oxidation of ascorbate by H<sub>2</sub>O<sub>2</sub> or an effect of DHA on ABA signalling is not yet clear.

The size of the intracellular and apoplastic ascorbate pools will have a profound impact on the duration, intensity and location of any ROS derived signal. The amount of reduced ascorbate in the apoplast will affect the amount of H<sub>2</sub>O<sub>2</sub> that enters the cell and therefore the extent to which it oxidises proteins that could be involved in redox signalling. Control of SOD, APX and ascorbate biosynthesis enzymes will be essential to modulating ROS signals as well. Signals derived from H<sub>2</sub>O<sub>2</sub> production are likely to depend on Ca<sup>2+</sup> levels as well as cytosolic ascorbate levels and rates of ascorbate transport into the apoplast.

#### 1.4.3 ROS metabolism in cell cultures

The rate of ROS metabolism in experimental systems varies considerably. *Arabidopsis* cell suspension cultures quickly metabolised 20 mM H<sub>2</sub>O<sub>2</sub>; the half-life was only 2 – 5 min (Neill *et al.* 2002a). Sweetlove *et al.* (2002) reported oxidation of membranes and proteins in *Arabidopsis* cell suspension cultures exposed to H<sub>2</sub>O<sub>2</sub> but found concentrations of 88 mM to be sub-lethal with no evidence of oxidative damage to mitochondrial proteins at lower concentrations. However, the half life of H<sub>2</sub>O<sub>2</sub> in isolated *Arabidopsis* protoplasts was extended to 1 h (Neill *et al.* 2002a). This shows how the cell wall is likely to be an important site of ROS

metabolism, in keeping with the idea that the apoplast is the first point of contact with pollutants such as SO<sub>2</sub> and O<sub>3</sub>.

An important factor in H<sub>2</sub>O<sub>2</sub> metabolism is the rate at which H<sub>2</sub>O<sub>2</sub> enters the cell. Studies on H<sub>2</sub>O<sub>2</sub> entry to mammalian cells have shown that H<sub>2</sub>O<sub>2</sub> enters the cell via diffusion but that this diffusion is limited by the degree of permeability of a membrane and the gradient of H<sub>2</sub>O<sub>2</sub> concentration across it (Antunes and Cadenas 2000). More rapid, controlled movement of H<sub>2</sub>O<sub>2</sub> across membranes through aquaporins has been suggested (Neill *et al.* 2002b). Evidence from Bienert *et al.* (2006) implies variation in the specificity of aquaporins for either H<sub>2</sub>O<sub>2</sub> or H<sub>2</sub>O.

### **1.5 Distribution of ascorbate between the symplast and apoplast**

Some of the consequences of ascorbate and DHA on growth and cell division have been discussed in section 1.3.4, whilst redox signalling has been introduced in section 1.4.2. The intracellular redox state is considered to be much more constant than in the apoplast (Pignocchi and Foyer 2003). As NADPH is not present in the apoplast (Noctor and Foyer 1998), ascorbate is regenerated intracellularly via the ascorbate–glutathione cycle. Therefore ascorbate and DHA transport systems between the apoplast and symplast will exert a strong influence over the redox state of the apoplast and so are likely to be under tight control.

When plants were fumigated with ozone, the apoplastic ascorbate content of leaves increased (Burkey *et al.* 2005, Luwe and Heber 1995). A negative correlation was found between the degree of export of ascorbate to the apoplast and ozone-induced damage between species and between individuals in a single population (Burkey *et al.* 2005), indicating the importance of ascorbate as an apoplastic antioxidant and the importance of ascorbate plasma membrane transport systems. The *vtc-1* mutant has around 23% of the apoplastic ascorbate content of WT plants and is more sensitive to ozone (Veljovic-Jovanovic *et al.* 2001). Apoplastic ascorbate + DHA is typically 10% of the total ascorbate + DHA pool size (Pignocchi and Foyer

2003). Ascorbate concentrations have been found to be highest in the cytosol, around 40 mM depending on growth conditions (Rautenkranz *et al.* 1994). Steep gradients in ascorbate concentration across the plasma membrane indicate a requirement for controlled ascorbate and DHA transport especially as DHA, being uncharged, might diffuse more readily through membranes (Rautenkranz *et al.* 1994).

Uptake of ascorbate and DHA into isolated barley protoplasts (Rautenkranz *et al.* 1994) and plasma membrane vesicles (Horemans *et al.* 1998a) showed saturation kinetics, indicating carrier-mediated uptake of ascorbate and DHA. Ionophores were found to inhibit uptake of both ascorbate and DHA (Rautenkranz *et al.* 1994, Horemans *et al.* 1998b) suggesting proton gradient-driven transport. Inhibition of plasma membrane H<sup>+</sup>ATPase resulted in almost complete inhibition of ascorbate transport (Kollist *et al.* 2001). However, other data from Bichele *et al.* (2000) suggest that diffusion of DHA across the plasma membrane could occur rapidly enough to supply apoplastic demand for ascorbate need to reduce ROS, even during ozone exposure.

Rautenkranz *et al.* (1994) found that DHA was taken up more rapidly in cell suspension cultures than ascorbate. That DHA is the preferred form in which ascorbate is taken up was subsequently found to be the case in plasma membrane vesicles (Horemans *et al.* 1998a, c), isolated protoplasts (Horemans *et al.* 1998b) and leaves (Kollist *et al.* 2001). A high degree of structural specificity by carriers for ascorbate and/or DHA was shown; uptake of ascorbate by cells was inhibited by the addition of iso-ascorbate and DHA but not ascorbate -2-sulphate or 5,6-*O*-cyclohexylidene-ascorbate (Horemans *et al.* 1998b). The addition of DHA to the bathing medium of plasma membrane vesicles stimulated export of ascorbate, leading to the proposal of a counterflow mechanism (Horemans *et al.* 1998c).

Little is known about how and under what circumstances ascorbate or DHA carriers are controlled. DHA uptake is stimulated following an oxidative burst in mammalian cells (Laggner *et al.* 2000). Cd<sup>2+</sup> is taken up via Ca<sup>2+</sup> channels (Horemans *et al.* 2007). Sub-lethal

concentrations of  $\text{Cd}^{2+}$  could induce an oxidative burst from stimulation of NADPH oxidase activity in *Arabidopsis* cell suspension cultures. Uptake of DHA was also inhibited by addition of  $\text{Cd}^{2+}$ . When NADPH oxidase activity and the resulting oxidative burst was blocked with dephenyl iodonium (DPI), DHA uptake continued to be inhibited by  $\text{Cd}^{2+}$  but when  $\text{Ca}^{2+}$  channels were blocked by the addition of  $\text{La}^{3+}$ ,  $\text{Cd}^{2+}$  no longer affected DHA uptake (Horemans *et al.* 2007). This seems to suggest that changes in cytosolic  $\text{Ca}^{2+}$  concentration from extracellular influxes of  $\text{Ca}^{2+}$  could, in part, regulate transmembrane DHA movements. That  $\text{Ca}^{2+}$  regulates such a diversity of plant responses has been attributed to specific responses to amplitude, frequency and location of  $\text{Ca}^{2+}$  signals (Cessna and Low 2001). Therefore, although increases in cytosolic  $\text{Ca}^{2+}$  concentrations from intracellular  $\text{Ca}^{2+}$  stores appeared to be followed by  $\text{H}_2\text{O}_2$  production (Cessna and Low 2001), this does not necessarily implicate  $\text{H}_2\text{O}_2$  production in the regulation of DHA plasma membrane transport.

DHA and glucose appear to share a transporter in the mitochondrial membrane (Szarka *et al.* 2004). Similarities were seen in the uptake of glucose in response to DPI and  $\text{La}^{3+}$  in tobacco cell cultures (Bourque *et al.* 2002) as were seen with DHA in *Arabidopsis* cells (Horemans *et al.* 2007). Plasma membrane DHA transport is known to occur via glucose transporters in mammalian cells (Vera *et al.* 1993). However recent data (Horemans *et al.* 2008) has shown conclusively that DHA and glucose do not share a transporter in the plant plasma membrane. The evidence summarised in this section suggests that ascorbate and DHA transport between the cytosol and the apoplast will be a critical part in communication between these two compartments and an essential part of plant responses to environmental cues.

### **1.6 Regulation of the ascorbate pool**

Ascorbate can represent up to 10% of the soluble carbohydrate content of leaves (Noctor and Foyer 1998) and so can be considered as an important part of carbohydrate metabolism in



plants. Regulation of the ascorbate pool size can therefore be expected to be under tight control. Broadly speaking there are three ways in which ascorbate levels can be regulated: biosynthesis, regeneration and breakdown. These three processes and some of the evidence surrounding their regulation are discussed below.

#### 1.6.1 Regeneration and regulation of ascorbate in the ascorbate–glutathione cycle

Once ascorbate has been oxidised to MDHA or DHA, regeneration to ascorbate can occur (Noctor and Foyer 1998). Regeneration of MDHA can occur in the presence of an electron donor such as NADPH or ferredoxin (Noctor and Foyer 1998). Regeneration of ascorbate from MDHA in the apoplast is possible via a cytochrome b electron transporter (Asard *et al.* 1994) but is also catalysed by the enzyme MDHA reductase (Asada 1999).

As neither glutathione nor NADPH is present in the apoplast, DHA must be transported across the plasma membrane and reduced back to ascorbate in the symplasm. This process is coupled to the oxidation of glutathione. Oxidised glutathione (GSSH) can be reduced back to GSH by GSSH reductase, for which NADPH is the electron donor (Noctor and Foyer 1998). The reduction of H<sub>2</sub>O<sub>2</sub> and oxidation of ascorbate to DHA, catalysed by APX, and oxidation of glutathione forms the basis of the ascorbate–glutathione cycle, the essential part of which is that neither ascorbate nor glutathione is consumed, only H<sub>2</sub>O<sub>2</sub> and NADPH. The reduction of DHA is catalysed by DHA reductase, though it is interesting that other proteins such as thioredoxin (Morell *et al.* 1997) can also reduce DHA. Disulphide bonds can be reversibly oxidised and reduced (Buchanan and Balmer 2005) and so are likely to donate electrons for DHA reduction. Without the ability to regenerate ascorbate the reduction of ROS would lead to the accumulation and breakdown of DHA. Ascorbate can represent up to 10% of the soluble carbohydrates in chloroplasts (Smirnoff 1996) so as the breakdown of DHA is an irreversible process this could

lead to an important loss of carbon from the ascorbate pool. If unregulated this would almost certainly disrupt carbon metabolism and growth.

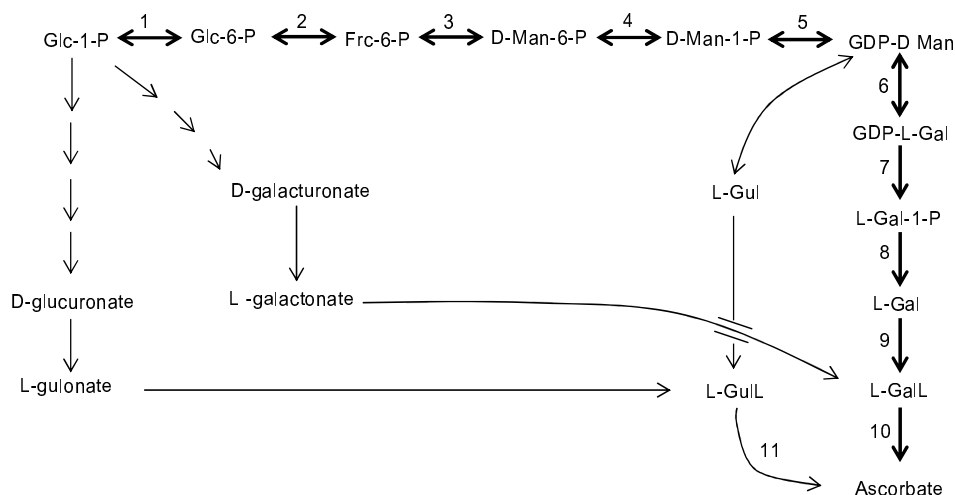
Regulation of the ascorbate–glutathione cycle has been studied mostly with respect to APX. Increased APX activity has been reported in response to abiotic stress (reviewed by Shigeoka *et al.* 2002). Stress results in ROS accumulation. As APX catalyses the reduction of H<sub>2</sub>O<sub>2</sub> by ascorbate, APX regulation could be an important determinant of the longevity of an H<sub>2</sub>O<sub>2</sub> signal. Several forms of APX exist: cytosolic APX (cAPX), thylakoid APX (tylAPX), stromal APX (stAPX) and mitochondrial APX (mtAPX) (Mittler *et al.* 1999, Asada 1999). These isoforms appear to be independently regulated in response to environmental stimuli; drought and high light stress increased cAPX but not either stAPX or tylAPX (Mittler *et al.* 1999). *capx* and *tylapx* mutants were more sensitive to oxidative stress than the double *capx/tylapx* mutant, as measured by root growth. Mutant phenotypes in terms of abiotic stress response, growth and development showed little overlap (Miller *et al.* 2007). Regulation of ascorbate regeneration is likely to be complex and depend on sub-cellular localisation. The enzyme AO also catalyses the oxidation of ascorbate to MDHA (Foyer 1983). It is unclear how this enzyme is involved in the ascorbate–glutathione cycle, although its cell wall location (Pignocchi *et al.* 2003) may prevent any direct involvement as the ascorbate–glutathione cycle is not thought to operate in the apoplast.

#### 1.6.2 Biosynthesis of ascorbate in higher plants

Isherwood *et al.* (1953) first demonstrated the conversion of GalL to ascorbate and proposed that this step was specific to the mitochondria. It was not until much later that the ascorbate biosynthetic pathway which progressed via galactonate came to be defined in its entirety by Wheeler *et al.* (1998) and the last step, the oxidation of galactono-1,4-lactone, was found to be catalysed by an enzyme, L-galactono-1,4-lactone dehydrogenase (GLDH), located to

the inner mitochondrial membrane. There is evidence for some variations on this pathway. D-Glucos-2-ulose has been found to act as a direct precursor to ascorbate (Davey *et al.* 1999). L-Gulonolactone and D-galacturonolactone were also shown to be viable precursors to either GalL or L-ascorbate in *Arabidopsis* cell suspension cultures (Davey *et al.* 1999). Although L-gulonolactone did not act as a substrate for GLDH purified from mitochondria (Oba *et al.* 1995), Bartoli *et al.* (2000) suggested that there may exist an isoform of GLDH in microsomes for which L-gulonolactone was the preferred substrate.

Whilst other routes for ascorbate biosynthesis may well exist in higher plants, recent evidence leaves little doubt that the pathway described by Wheeler *et al.* (1998) is an essential part of plant primary metabolism. Mutants of the recently characterised GDP- L-galactose phosphorylase, which catalyses the step between GDP- L-galactose and L-galactose-1-phosphate, became stunted and bleached shortly after germination but could be rescued by the addition of exogenous ascorbate (Dowdle *et al.* 2007). Evidence suggests that L-gulose, L-gulonolactone and D-galacturonic acid are precursors to ascorbate, though the majority of ascorbate does appear to be synthesised via L-galactonolactone (Wheeler *et al.* 1998, Dowdle *et al.* 2007).



**Figure 1.2** Summary of the proposed ascorbate biosynthetic pathways (adapted from Dowdle *et al.* 2007)

The GDP-mannose pathway, first proposed by Wheeler *et al.* (1998), is thought to be the main route of ascorbate biosynthesis and is highlighted in bold. Some steps from the uronic acid pathways and the L-gulose branch have been omitted. Enzymes of the uronic acid pathways whose activity has not been dwelt on in the text have also been omitted. Enzymes: 1, phosphoglucomutase; 2, phosphoglucose isomerase; 3, phosphomannose isomerase; 4, phosphomannomutase; 5, GDP-mannose pyrophosphorylase; 6, GDP-mannose-3', 5'-epimerase; 7, GDP-L-Gal pyrophosphorylase; 8, L-Gal 1-phosphate phosphatase; 9, L-Gal dehydrogenase; 10, L-GalL dehydrogenase; 11, L-GulL oxidase or dehydrogenase. Abbreviations: Glc, glucose; Frc, fructose; Man, mannose; Gal, galactose; Gul, gulose; GulL, gulonolactone; GalL, galactono-1,4-lactone.

### 1.6.3 Regulation of ascorbate biosynthesis

Since the elucidation of the main ascorbate biosynthetic pathway (Wheeler *et al.* 1998), evidence has been accumulating that suggests that flux is likely to be controlled at several points of the pathway. Whilst increased concentrations of L-galactose and L-GalL resulted in an increase of the ascorbate pool size in pea seedlings, increased concentrations of mannose did not (Wheeler *et al.* 1998). Incorporation of D-[U-<sup>14</sup>C]glucose into the ascorbate pool was decreased when in pea seedlings which were also fed cold ascorbate (Pallanca and Smirnoff 2000). Exogenous ascorbate inhibited the incorporation of D-[U-<sup>14</sup>C]mannose into the ascorbate pool of Arabidopsis cell suspension cultures (Wolucka and Van Montagu 2003). The activity of GLDH that had been purified from spinach leaves was found to be inhibited by the addition of ascorbate (Mieda *et al.* 2004). These observations indicate several possible points of feedback inhibition of

ascorbate biosynthesis and hint at steps between mannose and galactose being important control points.

The involvement of mitochondrial electron transfer in the last step of ascorbate biosynthesis has been touched upon briefly (see 1.4.2). Electron transfer between L-galactonolactone and its electron acceptor, cytochrome *c*, may represent another source of regulation of ascorbate biosynthesis. KCN was found to block ascorbate biosynthesis but not to affect GLDH activity (Bartoli *et al.* 2000). The rate-limiting step in this case would be the degree of reduction of cytochrome *c* and therefore its ability to accept electrons from L-galactonolactone. This in turn raised the possibility of interaction between ascorbate biosynthesis and the TCA cycle as both are known to donate electrons to the mitochondrial electron transfer chain (Bartoli *et al.* 2000). The alternative oxidase (AOx) acts as an electron acceptor in the mitochondrial electron transfer chain but by-passes cytochrome *c* (Moller 2001). Overexpression of AOx decreased mitochondrial H<sub>2</sub>O<sub>2</sub> production whereas H<sub>2</sub>O<sub>2</sub> increased in plants underexpressing AOx (Maxwell *et al.* 1999). H<sub>2</sub>O<sub>2</sub> is intimately involved in signalling and ascorbate metabolism (see 1.4.2, 1.3.1) and so it is perhaps possible that further regulation of ascorbate synthesis could come from H<sub>2</sub>O<sub>2</sub> generation and AOx activity.

Ascorbate biosynthesis is under either light or circadian clock control. Leaf ascorbate levels have a diurnal rhythm, accumulating to a maximum in tobacco leaves in the latter half of the 16-h photoperiod and then decreasing in the dark (Dutilleul *et al.* 2003). GLDH transcript levels in tobacco did not show a diurnal pattern (Pignocchi *et al.* 2003) though this was in contrast to a later study in *Arabidopsis* where transcript levels of several enzymes of the ascorbate biosynthetic pathway (GMP, GPP, VTC-1 AND GLDH) changed in parallel to leaf ascorbate content throughout the photoperiod (Yabuta *et al.* 2007). Yabuta *et al.* (2007) examined links between photosynthesis, carbon metabolism and the ascorbate pool size. Inhibition of photosynthesis led to a down-regulation of the gene for the above enzymes as well

as a decrease in the ascorbate pool size. Sucrose, L-galactose and GalL were fed to leaves kept in the dark so that decreased photosynthesis in the dark was separated from the decreased availability of carbon. The separate effects of carbon availability and photosynthesis were examined in the light in sucrose-insensitive mutants in which supplementary sucrose does not stimulate photosynthesis. In both cases, exogenous carbon sources failed to raise leaf ascorbate content. This led the authors to suggest that photosynthetic electron transfer could be an essential factor in regulating the ascorbate pool size.

Jasmonate is important in wounding responses of plants, upregulating genes associated with signalling. Jasmonate is thought to be involved in the regulation of H<sub>2</sub>O<sub>2</sub> accumulation (Orozco-Cardenas *et al.* 2001). Wolucka *et al.* (2005) found an almost two-fold increase in the intracellular ascorbate content of *Arabidopsis* cells 21 hours after the addition of MeJA. More D-[U-<sup>14</sup>C]mannose was incorporated into ascorbate in MeJA treated cells than in control cells, suggesting that MeJA might stimulate ascorbate biosynthesis. Results in other cell culture systems in the study by Wolucka *et al.* (2005) were inconclusive as to the effect of MeJA on ascorbate biosynthesis. However, the potential for the involvement of MeJA in ascorbate metabolism seen in this paper lead to the inclusion of MeJA as a candidate elicitor compound in latter parts of the present study.

#### 1.6.4 Ascorbate breakdown pathways in higher plants

The environment of the apoplast is more oxidising than that of the cytosol (Foyer and Noctor 2005). Once ascorbate has been oxidised to DHA in the apoplast, DHA can either be transported across the plasma membrane back into the cytosol (Horemans *et al.* 1998b) or broken down in the apoplast. Further breakdown of DHA is irreversible and so constitutes permanent loss of material from the ascorbate pool. There appear to be two catabolic pathways operating in plants, yielding either threonate or tartrate. In the culture medium of rose cell

suspension cultures DHA was observed to be either hydrolysed to DKG or oxidised to a novel compound cyclic oxalyl threonate (cyc.ox.thr.) (Green and Fry 2005).

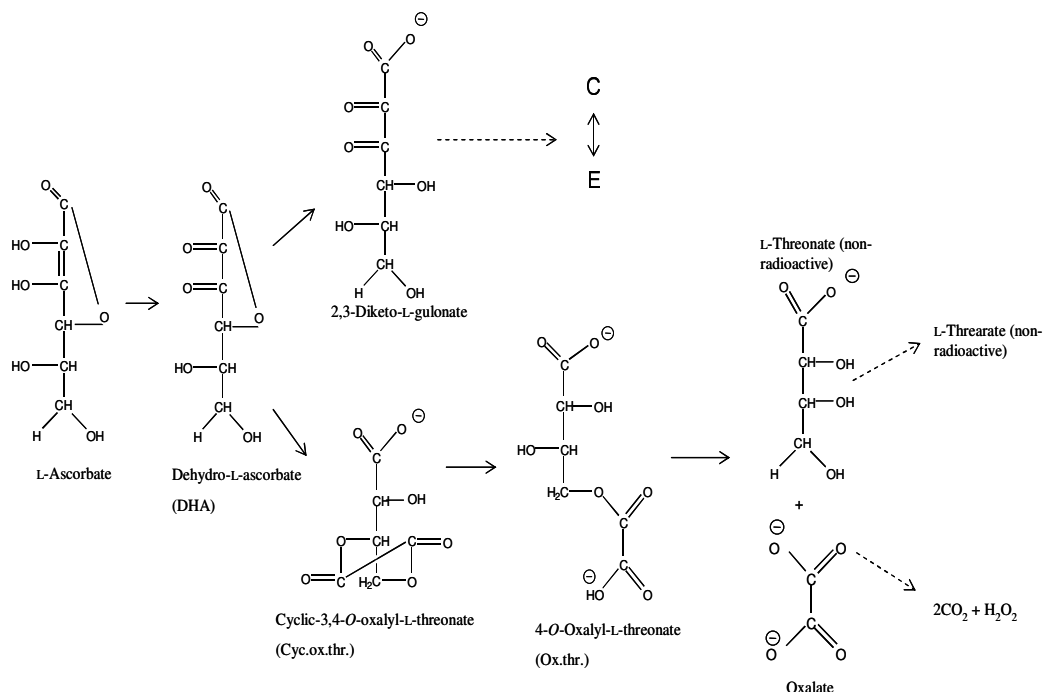
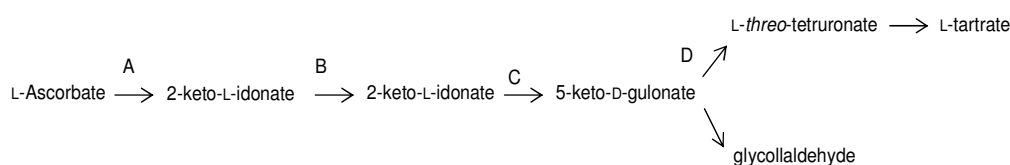


Figure 1.3 The apoplastic ascorbate breakdown pathway proposed by Green and Fry (2005). Dashed arrows represent reactions that were not observed directly in *Rosa* cell suspension cultures. Cyclic-2,3-*O*-oxalyl-L-threonate (not shown) was proposed as a precursor to cyclic-3,4-*O*-oxalyl-L-threonate with migration of the acyl group resulting in the formation of cyclic-3,4-*O*-oxalyl-L-threonate (see text). An enzyme was proposed to catalyse hydrolysis of the ester bond of 4-*O*-oxalyl-L-threonate (see text).

In the proposed pathway, oxidation of the keto group at C-3 of DHA to a carboxyl group results in breakage of the C-2–C-3 bond. The ester bond between C1 and C4 remains intact and another ester bond forms between C-2 and C-5 to form cyclic-2,3-*O*-oxalyl-L-threonate. Finally, the acyl group containing the former C-1 of DHA is transferred from C-4 to C-6 to form cyclic-3,4-*O*-oxalyl-L-threonate (cyc.ox.thr.). The ester bond between C-2 and C-5 is then broken to yield oxalyl-L-threonate (ox.thr.). Hydrolysis of the ester bond in oxalyl threonate leads to the

formation of oxalate and threonate. As [1-<sup>14</sup>C]ascorbate was fed to cells, threonate and any subsequent oxidation products were not visualised.

By feeding [4-<sup>14</sup>C]ascorbate or [6-<sup>14</sup>C]ascorbate to *Pelargonium* apicies, Williams and Loewus (1978) demonstrated that ascorbate was a precursor to tartrate. When 2-keto- L- [1-<sup>14</sup>C]idonic acid, 5-keto- L- [1-<sup>14</sup>C]idonic acid, L- [1-<sup>14</sup>C]idonic acid, [1-<sup>14</sup>C]DHA and [1-<sup>14</sup>C]ascorbate were fed to grape berries, a large proportion of the label was seen to accumulate as L- [<sup>14</sup>C]tartrate (Saito and Kasai 1984). This led to a pathway for the synthesis of tartrate from ascorbate (shown below) being proposed by Loewus (1999). An enzyme thought to catalyse the step from L-idonic acid to 5-keto- D-gluconic acid was reported by DeBolt *et al.* (2007).



**Figure 1.4 The proposed pathway of ascorbate breakdown in *Vitis vinifera* (adapted from DeBolt *et al.* (2007)**  
 A, lactone hydrolysis; B, reduction of keto group at C-2; C, oxidation of alcohol group at C-5; D, cleavage of C-4–C-5. Oxidation of the aldehyde group yields tartrate.

Both pathways appeared to be operating in grapes, as radiolabelled oxalate and L-tartrate were found after [1-<sup>14</sup>C]ascorbate had been fed to grape berries (DeBolt *et al.* 2004). Just over half the radiolabel was recovered as [<sup>14</sup>C]tartrate but berries also contained crystals of calcium oxalate.

Little is known about either how irreversible ascorbate breakdown is regulated or how this relates to ascorbate biosynthesis and regeneration. Enhanced flux through the breakdown pathway would presumably involve balance between regeneration of ascorbate from DHA and either oxidation or hydrolysis of DHA. The existence of a DHA hydrolase was proposed when



more rapid hydrolysis of DHA to DKG was observed in freshly prepared cauliflower homogenates than in boiled ones (Tewari and Krishnan 1960) though this received little subsequent attention.

The pathway discovered by Green and Fry (2005) was found to operate non-enzymically but overall flux through the pathway and at particular points, such as the hydrolysis of oxalyl threonate, was considerably faster in spent cell culture medium than in freshly autoclaved medium. Although there are at least two apparently independent ascorbate breakdown pathways in plants, as this pathway followed the non-enzymic set of reactions it was therefore proposed likely to be a universal plant ascorbate breakdown pathway and not specific to particular taxa (Green and Fry 2005).

#### 1.6.5 The functions of ascorbate breakdown products in plants

Understanding the function of the breakdown products of ascorbate can give some insight into how, and under what conditions, ascorbate breakdown might be regulated. Relative to tartrate and oxalate levels, little ascorbate was present in WT grape berries, suggesting that one of the principal roles of ascorbate in grapes is as a precursor to these compounds (DeBolt *et al.* 2004). The biological function of tartrate is less clear than those of oxalate, discussed below, although its abundance in an edible fruit would suggest it is somehow associated with frugivory.

Oxalate forms calcium oxalate crystals in many plant species (Franceschi and Nakata 2005). Defence against herbivory seems to be an important function of calcium oxalate crystals; calcium oxalate-deficient mutants of *Medicago truncata* were grazed preferentially by moth larvae and injury to mouthparts was observed in larvae grazing on WT plants (Korth *et al.* 2006). Correlations had been observed between selective grazing by gazelles and the distribution of calcium oxalate in leaves of desert lilies (Ruiz *et al.* 2002). Growth in desert environments could be expected to put high demands on antioxidants such as ascorbate. Results obtained by Ruiz *et*

*al.* (2002) suggested a trade-off between growth and number of crystals formed in leaves. Calcium in soils was not a limiting factor to crystal production, implying that oxalate supply was. If ascorbate breakdown is taken to be the primary pathway for oxalate synthesis, then this implies regulation of the amount of ascorbate diverted into calcium oxalate crystal formation.

Other roles have been proposed for calcium oxalate crystals in plants such as calcium sinks. The size and number of crystals were found to respond to changes in calcium concentration; in some plants it may be a dynamic and reversible response to environmental changes (Franceschi and Nakata 2005, Nakata 2003). A cell wall loosening role has been proposed for oxalate; apoplastic oxalate could form calcium oxalate crystals thus decreasing the level of cross linking via calcium bridges between polygalacturonate chains in the cell wall (Lin and Varner 1991, Kato and Esaka 2000). If the hydrolysis of oxalyl threonate to oxalate is indeed under enzymatic control then this raises possibilities of developmental regulation of this enzyme such as in rapidly expanding tissue or in fruits accumulating crystalline calcium oxalate deposits.

There are few reports of biological roles of either DKG or threonate. DKG can convert into two lactones, DKG-3,4-lactone and DKG-2,3-lactone. Both of these lactone possess ene-diol groups and DKG at high concentrations was found to protect yolk lipoprotein against oxidation, although a direct interaction between the lactones and the lipoprotein was not observed (Li *et al.* 2000) so the antioxidant properties of these lactones could not be established directly. Calcium threonate has been reported to stimulate the uptake of ascorbate by human lymphoma cells (Fay and Verlangieri 1991).

#### 1.6.6 Regulation of ascorbate breakdown

Oxidation of DHA to cyclic-2,3-*O*-oxalyl- L-threonate (Green and Fry 2005) or hydrolysis of DHA to DKG (Penney and Zilva 1945) represent the first committed steps in

ascorbate breakdown. Therefore the proportion of the total ascorbate + DHA pool that is present as DHA will affect the degree of ascorbate breakdown that is possible. Factors influencing and regulating the redox state of the ascorbate pool have been discussed in sections 1.4.3, 1.5 and 1.6.1.

An interesting question is the influence of AO on the rate of ascorbate breakdown. AO is localised to the cell wall but it is not clear whether the oxidation of ascorbate to MDHA, which is catalysed by AO, and the subsequent non-enzymic disproportionation of MDHA to DHA and ascorbate could influence the rate of ascorbate breakdown in the apoplast.

There are several lines of evidence to suggest that AO is auxin-regulated. Expression of AO was increased when 2,4-D was added to *Cucurbita* fruit tissue (Esaka *et al.* 1992). Kisu *et al.* (1997) found a region in the AO promoter of cloned from *Cucurbita*. that showed characteristics of being regulated by auxin. AO is known to be particularly abundant in certain species with rapidly expanding fruits such as cucumber, pumpkin and courgette (Esaka *et al.* 1992). AO also showed developmental regulation, being most highly expressed in growing tissue such as young tobacco leaves and growing seedlings (Esaka *et al.* 1992, Kato and Esaka 1996). Whether stimulation of AO activity by auxin has an effect on levels of ascorbate oxidation products other than DHA, either in the symplasm or apoplast, is not known.

More recently, in the roots of tomato seedlings grown on auxin impregnated media, auxin, in growth-stimulating doses, was found to decrease the activity of dehydroascorbate reductase, whilst stimulating the activity of ascorbate oxidase. DHA formed a greater proportion of the total ascorbate pool than in control roots (Tyburski *et al.* 2008). The biological process by which the effect of AO becomes manifest is something which is yet to be fully resolved.

### 1.7 Objectives and outlines of the present study

The objective of this study was to examine the ascorbate breakdown pathway described by Green and Fry (2005) and export of ascorbate and/or ascorbate breakdown products in response to an oxidative stress. Flux through the pathway under conditions of oxidative stress was compared to flux through the pathway in control cell cultures. The rate of turnover of individual metabolites was measured. Increased flux through the irreversible ascorbate breakdown pathway that had been proposed by Green and Fry (2005) in response to stress would be taken as an indication that the rate of irreversible ascorbate breakdown was an important part of ascorbate metabolism. If the rate of metabolism of any one particular compound in the breakdown pathway differed, in response to some stimuli, from control cultures, then active regulation of the pathway at certain stages could be considered likely. Previously (Green 2003, Green and Fry 2005), ascorbate breakdown had been studied in the apoplast. In the present study, the effect of external stimuli on ascorbate metabolism in both the apoplast and symplast was examined. As intra and extracellular communication was known to be important, especially in plants responses to oxidative stress (Noctor and Foyer 2005), transport of ascorbate metabolites across the plasma membrane in response to stimuli was also followed.

The stimuli chosen were those either known to induce an oxidative stress, those thought to be involved in signalling in other areas of ascorbate metabolism or those which might potentially be involved in feedback signalling in the breakdown pathway.  $\text{H}_2\text{O}_2$  and methyl viologen ( $\text{MV}^{2+}$ ) were used to induce an oxidative stress.  $\text{H}_2\text{O}_2$  acts as a direct oxidant but also as a signalling compound (Neill *et al.* 2002b).  $\text{MV}^{2+}$  is a herbicide which accepts electrons from ferredoxin in PSI, thus blocking electron flow between PSII and PSI and generating superoxide anions (Asada 1992). Methyl jasmonate and salicylic acid, both in defence responses and regulation of the oxidative burst, were chosen as signalling compounds that could potentially affect ascorbate metabolism and metabolites of the ascorbate breakdown pathway. These results

form the basis of sections 3.5 – 3.7, with preliminary studies to these experiments detailed in sections 3.3 and 3.4.

Section 3.8 concerns the transport of  $^{14}\text{C}$ -labelled metabolites across the plasma membrane in response to  $\text{H}_2\text{O}_2$  and in response to the addition of metabolites of the ascorbate breakdown pathway.

The effect of the ascorbate redox state on flux through the breakdown pathway was investigated in tobacco plants overexpressing AO. These plants have been characterised (Pignocchi *et al.* 2003, Sanmartin *et al.* 2003, Fotopolous *et al.* 2008) and are discussed in sections 1.3.2 and 1.4.2. The results are presented in section 3.9.

Firstly though, the oxidative and non-oxidative breakdown of ascorbate was studied *in vitro*. Although this pathway has been partially characterised previously (Green and Fry 2005) some questions surrounding it remained unanswered. This forms the basis of sections 3.1 and 3.2.

## 2 Materials and methods

### 2.1 Materials

#### 2.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, United Kingdom), Fischer Chemicals (Loughborough, United Kingdom) or BDH AnalaR Chemical Ltd. (Poole, United Kingdom). L-[1-<sup>14</sup>C]ascorbate and [<sup>14</sup>C]oxalate were purchased from Amersham Pharmacia Biotech U.K. Ltd. Xylulose-5-phosphate was kindly provided by Roland Wohlgemuth. All water used was de-ionised.

### 2.2 Tissue culture

#### 2.2.1 Cell suspension culture media

##### 2.2.1.1 *Rosa sp.* culture medium

Rose cell suspension culture medium was prepared in batches containing CaCl<sub>2</sub> (74 mg/l), KH<sub>2</sub>PO<sub>4</sub> (140 mg/l), KCl (750 mg/l), NaNO<sub>3</sub> (850 mg/l), MnSO<sub>4</sub>·7H<sub>2</sub>O (250 mg/l), MnSO<sub>4</sub>·4H<sub>2</sub>O (1 mg/l), H<sub>3</sub>BO<sub>3</sub> (0.2 mg/l), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.5 mg/l), KI (0.1 mg/l), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.02 mg/l), CoCl<sub>2</sub>·6H<sub>2</sub>O (0.01 mg/l), NaMoO<sub>4</sub>·2H<sub>2</sub>O (0.02 mg/l), FeCl<sub>3</sub>·6H<sub>2</sub>O (5.4 mg/l), NaEDTA·2H<sub>2</sub>O (7.4 mg/l), 2,4-D (1.0 mg/l), kinetin (0.5 mg/l), and either glucose (20 g/l) or glycerol (20 g/l). The pH was adjusted to 6.0 using 1 M NaOH and divided into 50 ml portions in 250 ml conical flasks.

##### 2.2.1.2. *Arabidopsis thaliana* culture medium

Murashige (1962) basal salt with minimal organics (4.4 g/l Sigma number M-6899) was combined with  $\alpha$ -naphthalenacetic acid (0.05 mg/l), kinetin (0.05 mg/l) and a glucose carbon source (2 % w/v). The solution was adjusted to pH 5.8 with 1 M KOH.

##### 2.2.1.3 Tomato culture medium

Murashige et al. (1962) basal salt with minimal organics (4.4 g/l Sigma number M-6899) was combined with IAA (10 mg/l), kinetin (0.05 mg/l), nicotinic acid (0.5 mg/l),

pyridoxine (0.5 mg/l), thiamine (0.1 mg/l), glycine (2.0 mg/l) and a glucose carbon source (2% w/v). The solution was adjusted to pH 5.8 with 1 M NaOH. All culture medium was dispensed into 250-ml conical flasks.

#### 2.2.1.4 Maize culture medium

A sucrose carbon source (2 %) was added to Murashige et al. (1962) basal salt with minimal organics (4.4 g/l Sigma number M-6899). The pH was brought to pH 5.8 with 1M NaOH. All flasks were loosely capped at the neck with a foam bung, covered with two layers of aluminium foil and autoclaved (121°C, 15 min).

### **2.2.2 Maintenance of Cell Suspension Cultures**

Cells were shaken at 25 °C under a continuous source of moderate artificial light. Cell suspension cultures were subcultured fortnightly under sterile conditions. The whole contents of one 14-day-old cell culture was divided equally between four flasks of fresh medium. Arabidopsis cell suspension cultures were subcultured every 5 days into fresh medium (1:10 v/v old culture: fresh medium). Unless specifically stated otherwise, 10-day-old rose cells were used in experiments. At this age cells were expected to have reached the end of the exponential growth phase. The Arabidopsis cell culture was found to be slightly faster growing than the rose cell line, reaching the end of the exponential growth phase at approximately 7 days, so when experiments were repeated in Arabidopsis, 7-day-old cell cultures were used.

## **2.3 Paper electrophoresis**

### 2.3.1 One dimensional paper electrophoresis

Samples were loaded on to Whatman 3MM paper or Whatman no.1 paper. Orange G was loaded as an external marker. The paper was wetted with a buffer that was either pH 2.0 (FAW 1:35:355 v/v/v), pH 3.5 (PyAW, 1:10:189 v/v/v) or pH 6.5 (PyAW 33:1:300 v/v/v). Chelating agents were added to running buffers for the electrophoresis of <sup>14</sup>C-labelled material. The

formation of insoluble oxalate salts during electrophoresis result in the formation of a radioactive streak of [<sup>14</sup>C]oxalate. Formation of this streak was minimised by the addition of chelating agents to running buffers: EDTA (final concentration 5 mM) was added to pH 6.5 and pH 3.5 buffers, thiosulphate (final concentration 5 mM) was added to pH 2.0 running buffer. The paper was placed in a glass tank and hung from a trough at the top that contained the appropriate buffer. The bottom of the paper was immersed in the same buffer. The tank was filled with an immiscible coolant (for electrophoresis at pH 6.5, toluene was used; for electrophoresis at pH 2.0 and pH 3.5, white spirit was used). A voltage of 3.0 or 3.5 kV was applied through the buffer for 30 – 40 minutes in order to separate compounds based on their charge:mass ratios.

The mobility of compounds separated by paper electrophoresis was calculated relative to the mobility of orange G. Neutral compounds move a short distance from the origin as a result of electro-endo-osmosis. The position of neutral compounds was taken as a reference point for calculation of the mobility of compounds with a net charge. Relative mobility (*m*OG) was calculated as the distance migrated from a neutral marker such as glucose or DHA, divided by the distance migrated by orange G from the neutral marker.

### 2.3.2 Two dimensional paper electrophoresis

Paper electrophoresis was carried out as described above but with only one sample loaded on to the origin. The electrophoretogram was allowed to dry overnight, and then carefully sewn to a second paper so that the line of segregated compounds was aligned with the new origin. Electrophoresis was then carried out for a second time as described above.

## **2.4 Descending paper chromatography**

Samples were loaded on to Whatman 3MM paper or Whatman no. 1 paper. The paper was placed in a glass tank and hung from a trough situated at the top of the tank that contained



butanol:acetic acid:water (12:3:5 v/v/v) solvent. The tank was then sealed with a glass lid and the chromatogram removed after 18 h.

## **2.5 Detection of radioactive standards and metabolites**

### 2.5.1 Detection of $^{14}\text{C}$ -labelled compounds by autoradiography

$^{14}\text{C}$ -Labelled samples that had been subjected to paper electrophoresis were detected by autoradiography on Kodak BioMax MR-1 film. The film was exposed for 1 – 6 weeks.

### 2.5.2 Detection of $^3\text{H}$ -labelled compounds by flouorography

Paper electrophoretograms were dipped through a solution of PPO (7 %) in ether. Kodak BioMax MR-1 film was positioned with the matt side down and fogged by a single flash from an orange hand-held flash gun (Amersham, UK) at a distance of 1 – 2 m from the film. An exposure time of 4 – 10 weeks was necessary so that  $^3\text{H}$ -labelled metabolites of [ $^3\text{H}$ ]Gal-L could be visualised with pre-fogged films.

### 2.5.3 Quantification of radioactivity by scintillation counting

Radioactivity was assayed in a Beckman LS 6500 or a Beckman LS 5000 CE multi-purpose scintillation counter (beckmancoulter.com). Aqueous samples were assayed using a 10:1 (v/v) ratio of ‘Optiphase Hisafe’ scintillation fluid (Wallac scintillation products, perkinelmer.com) to aqueous sample. ‘OptiScint Hisafe 3’ scintillation fluid (2 ml) was added to dry paper samples.

## **2.6 Detection of non-radioactive standards and metabolites**

### 2.6.1 Detection of compounds with silver nitrate staining

When completely dry, paper electrophoretograms and paper chromatograms were dipped through three solutions (a–c). The paper was dried for 15 min each time before it was dipped through the next solution. Papers were dipped twice through solution b.

- (a)  $\text{AgNO}_3$  (5 mM in acetone; a small volume of  $\text{H}_2\text{O}$  was used to redissolve any precipitate).
- (b)  $\text{NaOH}$  (0.125 mM in 96 % ethanol).
- (c)  $\text{Na}_2\text{S}_2\text{O}_3$  (10 % w/v in water).

The paper was washed for at least 1 h and then dried overnight.

#### 2.6.2 Detection of compounds with molybdate staining

Concentrated  $\text{HCl}$  (3 ml) was added dropwise with rapid stirring to 17 ml of an 11.8 % (w/v) ammonium molybdate solution. Perchloric acid (6 ml, 60 % v/v) was added dropwise to the solution and the precipitate stirred until it dissolved. Acetone (180 ml) was added. The paper was dipped through this solution, dried and the spots that appeared were marked. The paper was exposed to direct sunlight 10 min and any newly formed spots were marked.

### **2.7 Lactonisation and acidification of [ $^3\text{H}$ ]GalL and [ $^3\text{H}$ ]galactonate**

A solution mostly comprising of [ $^3\text{H}$ ]galactonate with some [ $^3\text{H}$ ]GalL was treated with 4M TFA for up to 1 h. TFA was removed by drying under reduced pressure. Solid [ $^3\text{H}$ ]GalL was redissolved in water immediately prior to addition to cell cultures. [ $^3\text{H}$ ]GalL in the original solution was converted to [ $^3\text{H}$ ]galactonate by the addition of 10  $\mu\text{l}$  0.1 M  $\text{NaOH}$  for 1 h. Solutions were then neutralised with equal volumes of 0.1 M acetic acid.

### **2.8 Analysis of oxidation pathways *in vitro***

#### 2.8.1 Preparation of catalase solution

Catalase was dissolved in water to give a concentration of 0.1 % (w/v). This was divided into aliquots, frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

#### 2.8.2 Oxidation of non-radioactive ascorbate

Ascorbate was oxidised *in vitro* using  $\text{H}_2\text{O}_2$  and buffered using a lutidine (pH 7.0) in an ascorbate: $\text{H}_2\text{O}_2$ :buffer 1:2:4 molar ratio. Lutidine was chosen as it would be volatilised as

samples were dried into paper for electrophoresis. Samples were removed at time points and oxidation was stopped by the addition of excess catalase and then frozen in liquid nitrogen. Samples were stored at  $-80^{\circ}\text{C}$  and analysed within a 2 week period of sampling, thus minimising degradation ascorbate and DHA.

### 2.8.3 Oxidation of [ $^{14}\text{C}$ ]ascorbate

[ $^{14}\text{C}$ ]Ascorbate, mixed with non-radioactive ascorbate was oxidised under strong oxidising conditions by treatment with  $\text{H}_2\text{O}_2$  in either an [ $^{14}\text{C}$ ]ascorbate: $\text{H}_2\text{O}_2$  1:2 molar ratio or a 1:10 molar ratio. Samples were removed at time points and oxidation was stopped by the addition of excess catalase then frozen in liquid nitrogen. [ $^{14}\text{C}$ ]Ascorbate, mixed with non-radioactive, was oxidised under mild oxidising conditions by treatment with water and exposure to air. Samples were frozen in liquid nitrogen, stored at  $-80^{\circ}\text{C}$  for less than two weeks prior to analysis.

### 2.8.4 Preparation and oxidation of DHA

DHA was dissolved in 100 % DMF (a range of non-ionic solvents were tested. After 1 hr at room temperature DHA was found to be most stable in DMF, data not shown) and stored at  $-80^{\circ}\text{C}$ . A 0.5 ml bed volume Dowex-1 x 4 100-200 mesh (acetate $^{-}$  form) column was prepared. Aliquots of 2 M DHA in DMF were diluted in 0.5 ml water and passed down the column followed by 2 ml of 2 mM pyridine acetate buffer (pH 4.0) as the eluent. The eluate (0.5 – 1.0 ml) was collected and immediately mixed with 0.02 moles of  $\text{H}_2\text{O}_2$ . Samples were removed at intervals and added to excess catalase, then frozen in liquid nitrogen.

### 2.8.5 Preparation of DKG –method 1

DHA (40  $\mu\text{l}$ , 3 M) dissolved in 100 % DMF was hydrolysed with 0.2 ml 0.75 M NaOH for 30 s. Hydrolysis was stopped by the addition of 0.1 ml 1.5 M acetic acid. A Dowex-1 x 4 column was prepared as above. The solution was passed down the column followed by 3 ml of 2

mM pyridine acetate buffer (pH 4.0). The eluate was rejected. DKG was eluted from the column with 2 ml of 1.0 M pyridine acetate buffer (pH 4.0).

#### 2.8.6 Preparation of DKG –method 2

DHA was hydrolysed to DKG as described. Solid  $\text{CaCl}_2$  was added in a DHA: $\text{CaCl}_2$  1:2 molar ratio. Ethanol (5 °C) was added until it accounted for 80 % of the reaction mixture (Penney and Zilva 1945). The resulting  $\text{CaDKG}_2$  precipitate was filtered off, washed in ether and dried onto paper. Once removed from the paper the salt was sufficiently stable to dry to a solid under reduced pressure.

#### 2.8.7 Preparation of DKG –method 3

Ascorbic acid was added to a solution of  $\text{KIO}_3$  in a 3:1 ascorbate: $\text{KIO}_3$  molar ratio (Kagawa 1962). 1 M KOH was added over a period of 30 minutes until the yellow-brown solution was colourless. The solution was precipitated in ethanol chilled to  $-80^\circ\text{C}$ . The white precipitate of KDKG was filtered through Whatman no. 1 paper under vacuum in a Buchner funnel at 4 °C and stored at  $-80^\circ\text{C}$ .

#### 2.8.8 Oxidation of DKG

DKG (2 ml, approximate concentration 500 mM) prepared using method 1 was oxidised with 0.02 mol  $\text{H}_2\text{O}_2$  immediately after elution off the Dowex-1 column. Samples were removed over time, added to excess catalase and frozen in liquid nitrogen. DKG prepared using method 2 was used to prepare compound 2; the oxidation was not followed over time. When the solution was buffered at pH 6.0 with a pyridine acetate buffer, DKG prepared using method 3 was oxidised in a DKG: $\text{H}_2\text{O}_2$ :buffer 1:2:4 molar ratio. Otherwise 0.3 M solutions of DKG were oxidised with 1 M  $\text{H}_2\text{O}_2$ .

### 2.8.9 Removal of catalase from solutions

Catalase was removed from solutions with either an Amicon ultra 10k centrifugal device (Millipore corporation) or a PD10 desalting column (GE Healthcare). Centrifugal devices were pre-rinsed once with 0.1 M NaOH and once with water to remove traces of triethylene glycol. Solutions were passed through centrifugal devices by spinning at 4000 g for 10 minutes. PD10 columns were rinsed with 25 ml water. The catalase containing solution was loaded on to the column in 2.5 ml, followed by 3.5 ml water. The first 2.5 ml of eluate was rejected and the next 3.0 ml of eluate was collected.

### **2.9 Collection analysis of gases evolved during the oxidation of DKG**

DKG prepared using method 3 (150 mM final concentration) was oxidised with H<sub>2</sub>O<sub>2</sub> (final concentration 300 mM) in a vial with a crimp cap and airtight rubber seal. Solutions were added through a syringe and hypodermic needle. As solutions were added, the equivalent volume of air was removed through a second syringe and hypodermic needle in an effort to maintain ambient pressure. During gas collection the rubber seal was pierced with a hypodermic needle attached to a syringe in which gas accumulated. Gas was collected for 1 h. The rubber seal was pierced with a second needle and 1ml of gas was injected manually into a 8310 gas chromatograph (Perkin-Elmer) using a helium carrier gas. Gases were separated on a Poropak™ column and detected with a thermal conductivity detector at 250 °C. Peak maxima were collected using a Peaksimple data acquisition system.

### **2.10 Isolation and analysis of compounds**

#### 2.10.1 Elution of compounds from paper electrophoretograms

The section of paper that contained the sample of interest was cut out, rolled up and placed in the barrel of a 5-ml syringe. The barrel was placed into a 15-ml centrifuge tube and was wetted with water. The tube was then centrifuged (3000 rpm, 10 min). The elution process was repeated four times (Eshdat and Mirelman 1972).

### 2.10.2 Analysis and Isolation of compounds with HPLC

Organic acids were separated on a Dionex high-pressure liquid chromatograph (HPLC) machine (Dionex, Sunnyvale, CA, USA) with a Rezex ROA Organic Acid anion exclusion column (Phenomenex Torrance, CA, USA). Samples were eluted in an isocratic 47 mM H<sub>2</sub>SO<sub>4</sub> eluent with a 30 min run time. The eluent suppressed negative charges on organic acids so that compounds were separated by ion exclusion and hydrophobic interactions with the column. The negative charge was less easily suppressed on compounds with a low pK<sub>a</sub>, meaning that these compounds tended to elute faster than those with a higher pK<sub>a</sub>. Compounds were detected by UV-absorbance at 210 nm and 250 nm. For the identification of compounds, a 40 µl injection volume was used. For purification of identified compounds, 80 µl was injected. Peaks were collected with an AS50 autosampler (Dionex, Sunnyvale, CA, USA). Injection and collection of peaks of interest was repeated until sufficient quantities of a compound had been collected. The column was washed multiple times with the 47 mM H<sub>2</sub>SO<sub>4</sub> eluent when compounds eluted off paper had been passed through to column so that contamination of the next sample with residual compounds on the column was avoided.

### 2.10.3 Hydrolysis of oxalyl threonate

Oxalyl threonate (concentration unknown) was hydrolysed with 1.0 M NaOH for up to 320 min. Samples were removed at time points and neutralised with 1.0 M acetic acid.

### 2.10.4 Periodate oxidation of cyclic oxalyl threonate

[<sup>14</sup>C]Cyclic oxalyl threonate (unknown concentration) was treated with NaIO<sub>4</sub> (final concentration 10 mM) in the presence of a 250 mM NH<sub>4</sub><sup>+</sup> formate buffer at pH 3.7. Samples were removed over time and added to 1 % ethane-1, 2-diol then frozen in liquid nitrogen to stop the reaction.

#### 2.10.5 Synthesis of 5-keto-xylonate

2-Keto-xylulose was synthesised from xylulose-5-phosphate for use as an external marker during paper electrophoresis. Xylulose-5-phosphate (final concentration 0.2 mM) was mixed with TEMPO (final concentration 1.7 % w/v) before oxidation with NaOCl (final concentration 0.1 M) for 20 min. Reaction products were separated by paper electrophoresis at pH 6.5. A small portion of the reaction products was stained with a molybdate stain. The migration distances of reaction products were compared to the positions of external markers. When the electrophoretic mobility of the xylulose-5-phosphate had been ascertained, the unstained remainder was eluted from the paper electrophoretogram, dried under reduced pressure and redissolved in water. The phosphate group was then removed by incubation for 24 hours with acid phosphatase (final concentration 1 % w/v) dissolved in an acetic acid:pyridine:water solution (1:1:98 v/v/v). Products were separated by paper electrophoresis at pH 6.5. A small portion of reaction products were stained. Compounds were eluted from the area corresponding to the position of the product identified as 2-keto-xylulose and dried under reduced pressure.

#### **2.11 Detection of ascorbate in cell extracts**

Cell culture samples (2 ml) were removed from cultures at time intervals after incubation with GalL. The culture medium was filtered off and the cells were washed in water and weighed. The cells were then resuspended in a 50 % solution of 10 % (w/v) metaphosphoric acid. After 10 min incubation the suspension was centrifuged at 3000 rpm for 2 min. The supernatant was diluted to 10% (v/v) with 5 % (w/v) metaphosphoric acid. The ascorbate content of samples was assayed by the reduction of DCPIP by ascorbate; 0.5 ml of the diluted supernatant was mixed with 0.5 ml of 0.01 % DCPIP. The absorbance was measured at 519 nm 20 s later.

## **2.12 Measurement of the longevity of H<sub>2</sub>O<sub>2</sub> in cell cultures**

H<sub>2</sub>O<sub>2</sub> was measured using the xylenol orange assay according to (Gay *et al.* 1999). H<sub>2</sub>O<sub>2</sub> was added exogenously to 10-day-old rose cell cultures to final concentrations of 0, 0.01, 0.1, 1.0 and 10 mM. Culture medium (100 µl) was assayed for H<sub>2</sub>O<sub>2</sub> content at time intervals.

## **2.13 Catabolism of ascorbate in response to oxidative stress**

### 2.13.1 [<sup>3</sup>H]Ascorbate catabolism–preparation of cell cultures

10-day-old rose cell culture was aliquotted into four cultures of 1ml under sterile conditions. Cell cultures were shaken at 120 rpm throughout the experiment under the same conditions in which the parent culture had been grown. 3 MBq of [<sup>3</sup>H]galactonolactone was dried in a speed vac and resuspended in 1.1ml spent medium from the parent cell culture. This was sterilised by passing through a sartorius Minisart filter unit (0.2 µm). Medium (200 µl) was removed from each culture and replaced with 200 µl of the sterilised filtrate. Culture medium (10 µl) was removed to assay the amount of radioactivity fed to each culture. Cell cultures were incubated overnight for 16 h. After 16 h incubation 10 µl of culture medium was removed from each culture. This was replaced with 10 µl of H<sub>2</sub>O<sub>2</sub> to a final concentration of either 0.1 or 1.0 mM, whilst 10 µl of water was added to a control culture.

### 2.13.2 [<sup>3</sup>H]Ascorbate catabolism–apoplastic and symplastic sampling

Samples of cell and culture medium (100 µl) were removed at time intervals. Culture medium was filtered off through a glass wool plug at the end of a pipette tip. Cells were separated from culture medium through a glass wool filter in a pipette tip. The uptake of radioactivity from the culture medium was assayed by liquid scintillation counting of 10 µl of culture medium symplastic contents was extracted by the addition of 80 µl of 40 % formic acid (v/v) in which cold ascorbate had been dissolved to a final concentration of 5 % (w/v). The symplasm was extracted for 5 minutes during which time the cells were mixed thoroughly with



the formic acid extractant. The symplastic extract (10 µl) was removed for scintillation counting and 30 µl was subjected to paper electrophoresis. Samples subjected to paper electrophoresis were frozen in liquid nitrogen immediately after sampling.

#### 2.13.3 [<sup>14</sup>C]Ascorbate catabolism–preparation of cell cultures

10-day-old rose cell cultures (750 µl) were incubated with 0.025 MBq [<sup>14</sup>C]ascorbate for 4 h, during which time approximately 90 % of the <sup>14</sup>C was taken up. Additional cold ascorbate was not added. Cell cultures were shaken at 120 rpm throughout the experiment under the same conditions in which the parent culture had been grown. After 4 h 10 µl H<sub>2</sub>O<sub>2</sub> was added to a final concentration of either 0.1 mM or 1.0 mM. 10 µl of water was added to control cultures.

#### 2.13.4 Fate of [<sup>14</sup>C]ascorbate–sampling of symplastic and apoplastic metabolites

Symplastic and apoplastic sampling of cells incubated in [<sup>14</sup>C]ascorbate was carried out as described for cells incubated with [<sup>3</sup>H]GalL with the following variation. 80 µl samples of cell and culture medium were removed at time intervals. Uptake of radioactivity from the culture medium was assayed by liquid scintillation counting of 5 µl of culture medium. The symplasm was extracted in 50µl of 40% formic acid. 5% non-radioactive ascorbate was used as a sacrificial protectant of the [<sup>14</sup>C]ascorbate. Of the resulting extract, 5µl was removed for scintillation counting whilst 10 µl was used for paper electrophoresis.

### **2.14 Transport of <sup>14</sup>C-labelled metabolites across the plasma membrane**

#### 2.14.1 Preparation of cell cultures and sampling of culture medium

Cell cultures were transferred into cell well plates and incubated with [<sup>14</sup>C]ascorbate for 5 h. During incubation and sampling cultures were shaken gently on a variomag monoshake shaker (H+P Labortechnik AG, Germany). Care was taken to insulate the bottom of cell well plates to prevent heat transfer from the shaker during incubation. After 5 h the cell culture

medium was replaced with non-radioactive culture medium from the parent culture from which the cells had originated. For 250µl cultures, 250µl of non-radioactive culture medium was added and then 250µl was removed from the culture. This process was repeated 6 times. For larger cultures, 1ml of non-radioactive culture medium was added and then 1ml was removed. This was repeated 5 times. In experiment 1, cells were allowed to sediment before the removal of medium. This process was speeded up in other experiment by use of a nylon mesh filter of 100µm pore size that was impenetrable to cells. The nylon mesh was secured over the end of a cut pipette tip with a segment of rubber tubing. The filter was kept in cultures for the duration of sampling. Culture medium was removed by pipetting from inside the cut pipette tip. Culture volumes were adjusted so that a minimum of 200µl cells and medium remained at the end of each experiment to prevent additional stress from drying out. Cultures were taken from the same parent culture in each experiment where possible (approx. 30 % settled cell volume) and maintained in a homogenous state by shaking during experiments.

#### 2.14.2 Experiment 1-response to 5.0 mM H<sub>2</sub>O<sub>2</sub>

650µl rose cell cultures were prepared as described and incubated with 0.1 MBq [<sup>14</sup>C]ascorbate per culture (0.37 – 1.1 MBq µmol<sup>-1</sup>)no non-radioactive ascorbate added) . After 5 h either water or H<sub>2</sub>O<sub>2</sub> (final concentration 5.0 mM) was added. Culture medium was sampled over a time period of 160 min after the addition of H<sub>2</sub>O<sub>2</sub>. Cells were filtered by pipetting 17µl culture medium up in a pipette tip with a glass wool plug. The plug was quickly cut off before culture medium was expelled into tubes containing 5µl 25 mM non-radioactive ascorbate and 0.05% catalase.

#### 2.14.3 Experiment 2-response to 5.0 mM H<sub>2</sub>O<sub>2</sub> again

650µl rose cell cultures were prepared as described and incubated with 0.1 MBq [<sup>14</sup>C]ascorbate per culture. After 5 h either water or H<sub>2</sub>O<sub>2</sub> (final concentration 5.0 mM) was

added. Culture medium was sampled at 5 s intervals for 30 s at 0 min, 1 min, 5 min and 10 min after the addition of H<sub>2</sub>O<sub>2</sub>. Cells were filtered and samples treated as described for expt. 1 (2.14.2)

#### 2.14.4 Experiment 3- response to 1.0 mM H<sub>2</sub>O<sub>2</sub>

650µl rose cell cultures were prepared as described (2.14.2) and incubated with 0.05 MBq of [<sup>14</sup>C]ascorbate. After 5 h 20 µl of either water or H<sub>2</sub>O<sub>2</sub> (final concentration 1.0 mM) was added. 7µl culture medium was sampled every 5s using a nylon mesh cell filter, of which 5 µl were counted.

#### 2.14.4 Experiment 4-addition of <sup>3</sup>H-marker compounds to control cell cultures

650µl rose cell cultures were prepared as described (2.14.2) and incubated with 0.05 MBq of [<sup>14</sup>C]ascorbate. After 5 h 20 µl of water and [<sup>3</sup>H]glucitol (dried under reduced pressure and resuspended in water) was added. 7µl culture medium was sampled at 5s intervals using a nylon mesh cell filter, of which 5 µl were counted.

#### 2.14.6 Experiment 5- addition of <sup>3</sup>H-marker compounds to 1 ml control cell cultures

1ml cell cultures were prepared as described and incubated with 0.05 MBq for 5 h. Aliquots of [<sup>3</sup>H]glucitol, [<sup>3</sup>H]xylitol and [<sup>3</sup>H]isoprimeveritol were dried under reduced pressure and resuspended in water. After replacement of the culture medium aliquots of <sup>3</sup>H were added, followed by either 20µl water or H<sub>2</sub>O<sub>2</sub> to a final concentration of 1.0 mM. Culture medium was sampled at 5s intervals using a nylon mesh cell filter.

#### 2.14.6 Experiment 6- addition of 0, 0.1 and 1 mM H<sub>2</sub>O<sub>2</sub> in the presence of a <sup>3</sup>H-marker

250µl Rose cultures were prepared as described and incubated with 0.025 KBq [<sup>14</sup>C]ascorbate. After 5 hours, [<sup>3</sup>H]glucitol was added followed by 20 µl of either µl water or H<sub>2</sub>O<sub>2</sub> (final concentrations 100 µM and 1.0 mM). Culture medium was sampled at 30 s intervals using a nylon mesh filter.

#### 2.14.6 Experiment 7- response of *Arabidopsis* cells to 1.0 mM H<sub>2</sub>O<sub>2</sub>

250µl *Arabidopsis thaliana* cells were incubated with 0.025 KBq [<sup>14</sup>C]ascorbate. After 5 h the culture medium was exchanged as described. Aliquots of [<sup>3</sup>H]glucitol (dried under reduced pressure and resuspended in water) were added, followed by 20 µl of either µl water or H<sub>2</sub>O<sub>2</sub> (final conc. 1 mM) . Culture medium was sampled at 5 s intervals using a nylon mesh filter.

#### 2.14.6 Experiment 8-the effect of addition of metabolites

250µl Rose cultures were prepared as described and incubated with 0.025 KBq [<sup>14</sup>C]ascorbate. After 5 hours, aliquots of [<sup>3</sup>H]glucitol (dried under reduced pressure and resuspended in water) were added followed by aliquots of either DKG, oxalate or threonate to a final concentration of 100 µM, 10 µl water or H<sub>2</sub>O<sub>2</sub> (final concentrations 100 µM and 1.0 mM). Culture medium was sampled at 10 s intervals using a nylon mesh filter.

### **2.15 Ascorbate catabolism *in planta***

#### 2.15.1 Growth conditions

Wild type and transgenic tobacco plants *Nicotiana tabacum* L., cv. Xanthi (expts. 1 and 2) and *Nicotiana tabacum* L., cv. Petite Havana (expt. 2) were grown in pots on John Innes no. 2 compost in controlled environment chambers at 22°C with a 16 h photoperiod and photosynthetic photon flux density of 250 µmol m<sup>-1</sup> s<sup>-1</sup>. Leaves (< 15 cm lamina length in *Nicotiana tabacum* L., cv. Xanthi plants, < 20 cm lamina length in *Nicotiana tabacum* L., cv. Petit Havana plants,) were harvested approximately 8 h in the light period.

#### 2.15.2 Experimental conditions

During experimentation leaves were kept in a fume hood under a light source (100 W). Leaves were protected from heat from the light source with a glass tank full of ice and water. Leaves were given 0.025 MBq in 20 µl water via the petiole followed by water when necessary. Position related to light source was changed over course of experiment.

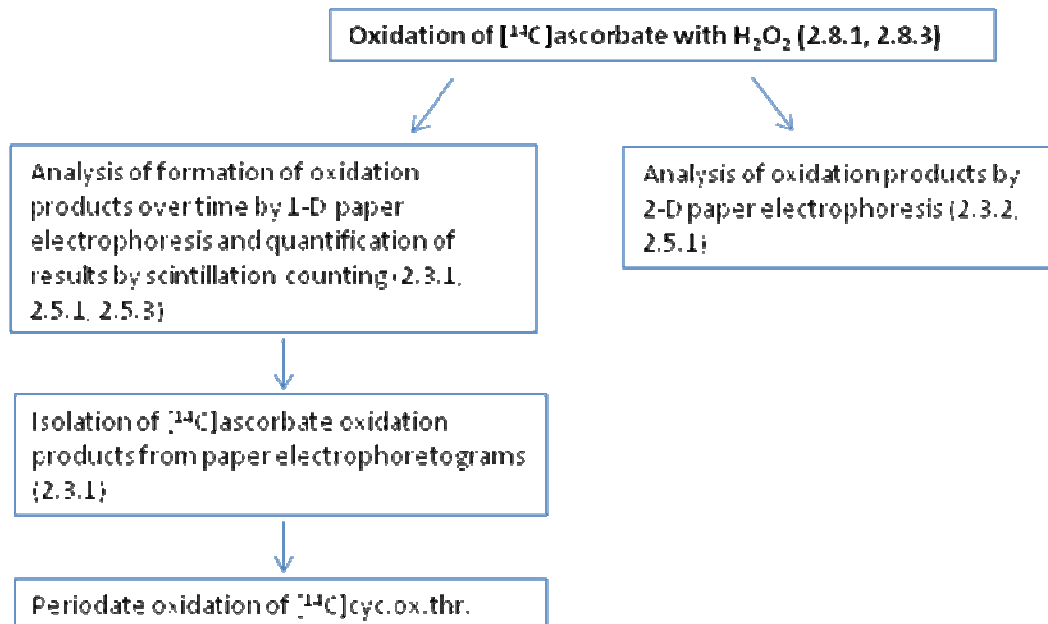
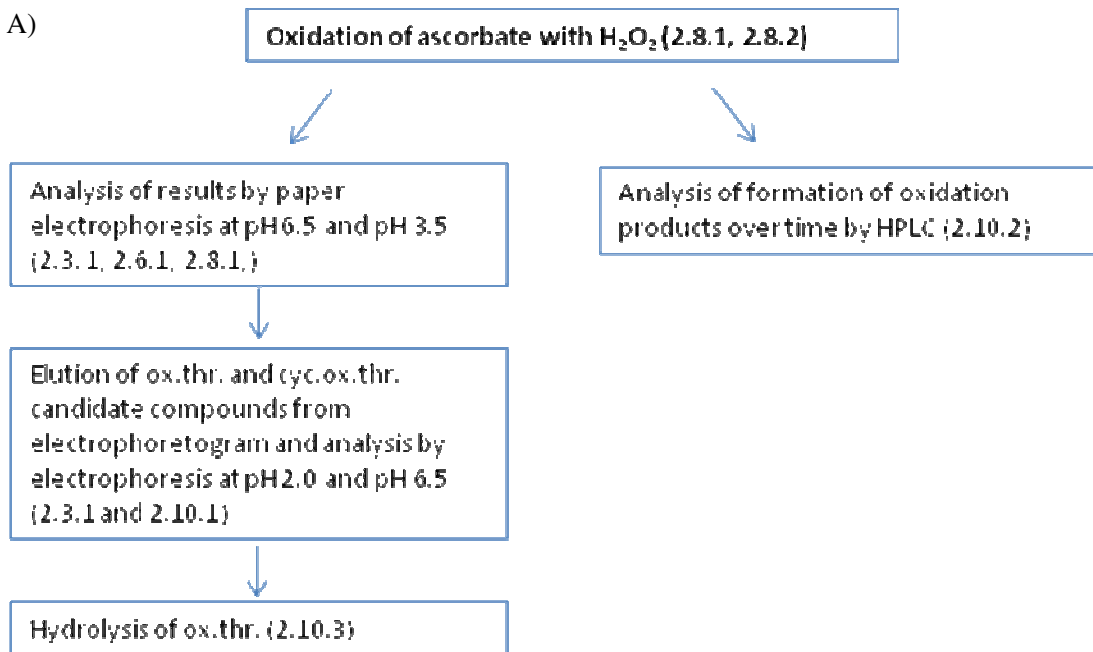
### 2.15.3 Vacuum infiltration

At time points the petiole of leaves were trimmed and then leaves were vacuum infiltrated. In expt. 1 the infiltration medium was water taken to pH 5.0 with  $\text{H}_3\text{PO}_4$ . In expt. 2 the infiltration medium was approximately 20  $\mu\text{l}$   $\text{H}_3\text{PO}_4$  with ascorbate (final concentration 10 mM) brought to pH 4.9 with NaOH at a temperature of 4 °C. Vacuum infiltration was performed at 0.1 atm absolute pressure for 20 s in 4 – 6 cycles in both experiments. After infiltration leaves were dipped through water (4 °C), blotted dry and the carefully rolled into 10 ml syringe chilled to 4 °C. Infiltration fluids were collected by centrifugation at 4 °C. Leaves were centrifuged for 400g (10 min) followed by 600 g (10 min) in experiment 1 and at 600g for 5 min in experiment 2. The infiltrate in experiment 2 was collected in chilled tubes containing 20  $\mu\text{l}$  chilled formic acid (20 % w/v) and ascorbate (25 mM).

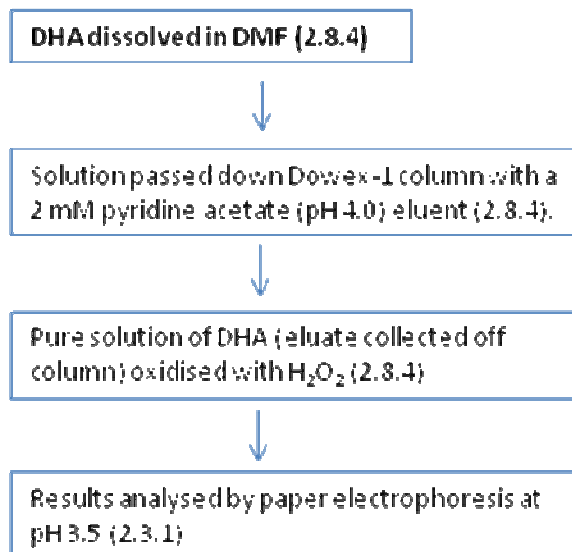
### 2.15.4 Leaf homogenisation

The midrib was excised and ground in 20 % formic acid (final concentration) supplemented with 5 mM ascorbate (final concentration, expt. 1) or 25 mM ascorbate (final concentration, expt. 2). The homogenate was centrifuged for 2 min at 3000 rpm and the supernatant collected.

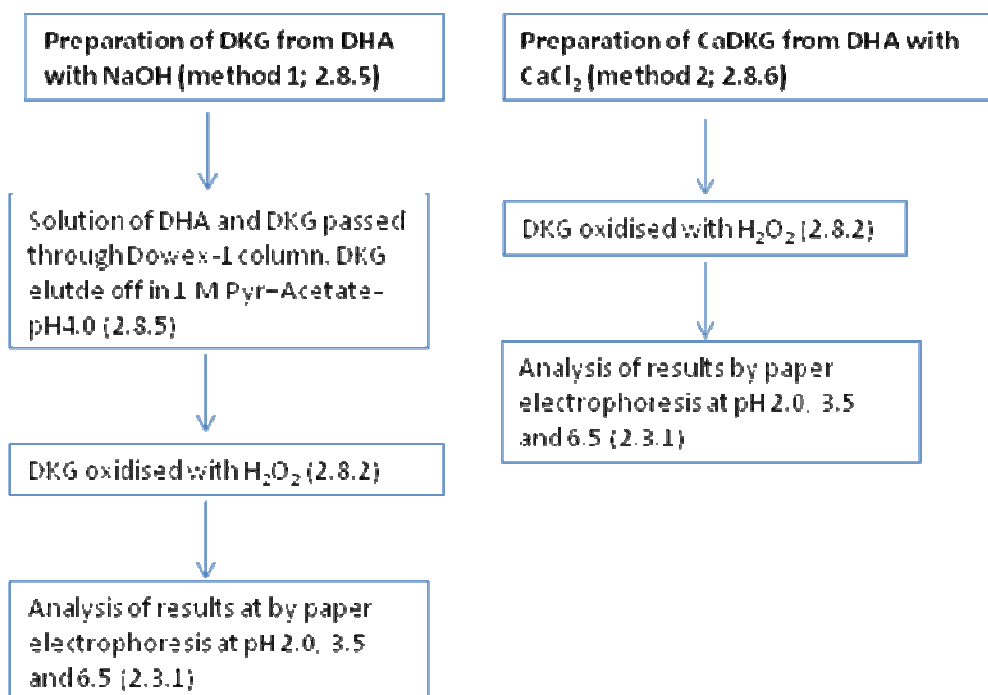
A)

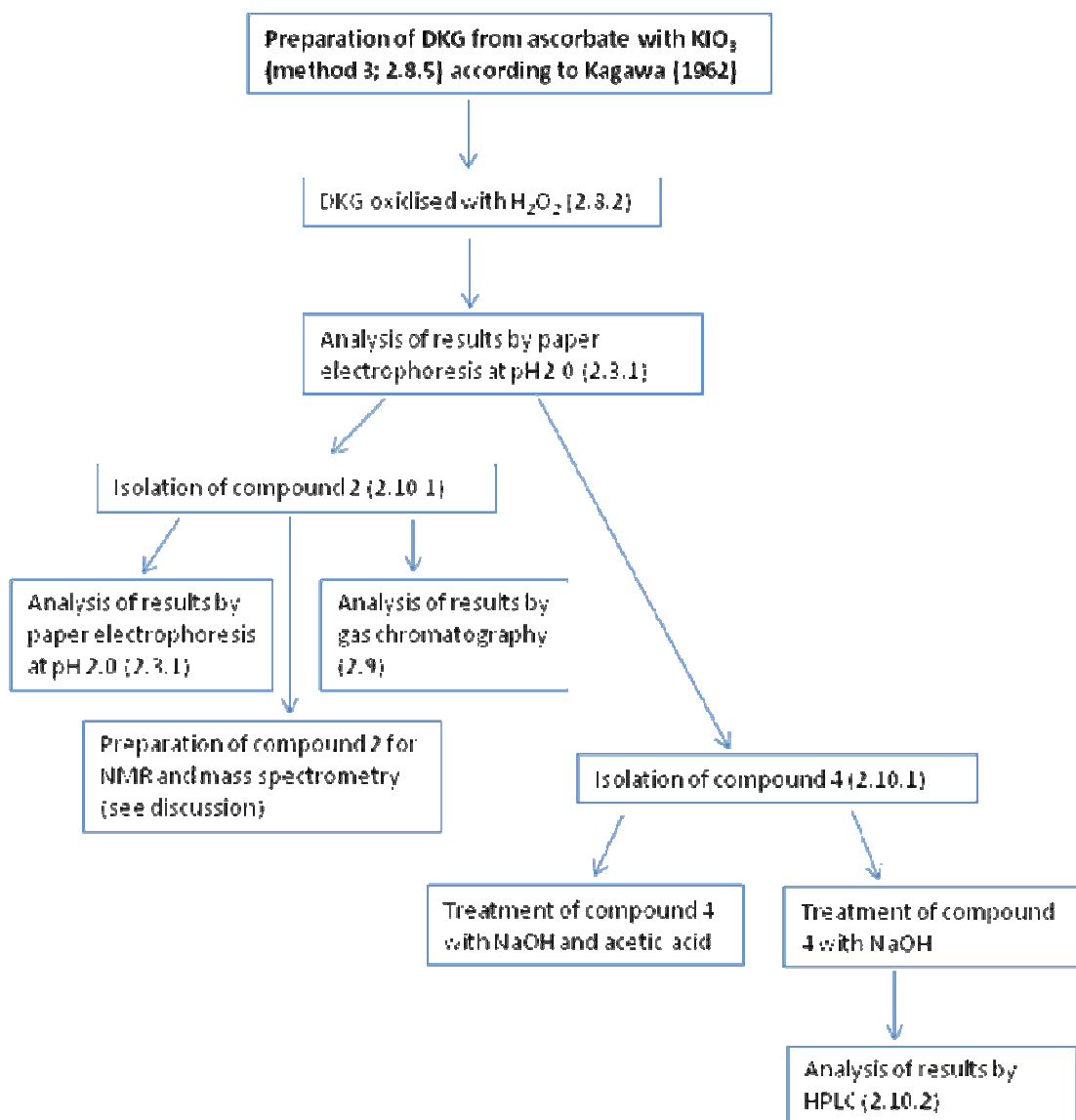


B)



C)





**Figure 2.1** Flow diagram showing an overview of in-vitro experiments on ascorbate and [<sup>14</sup>C]ascorbate (A), DHA (B) and DKG (C). The starting point of a set of experiments is highlighted in bold. Numbers in brackets refer to the relevant section of the materials and methods.



### 3. Results

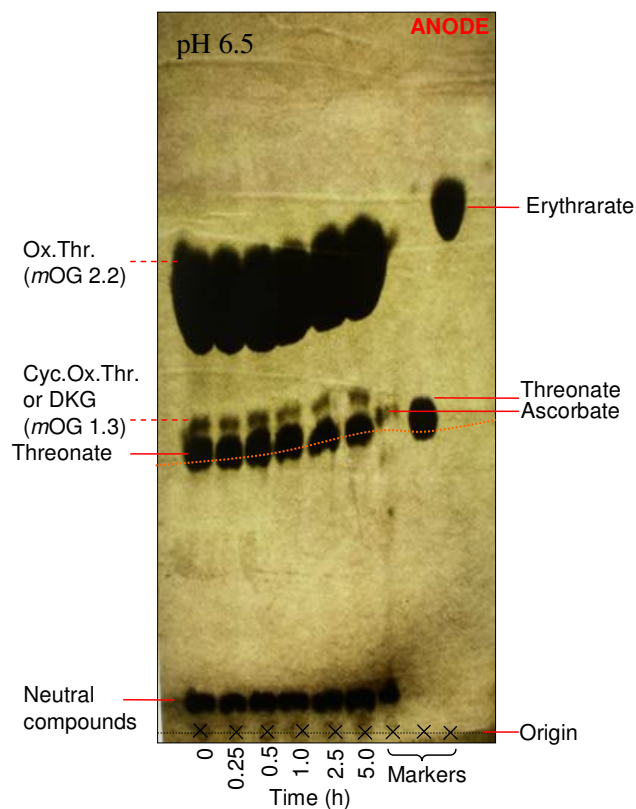
#### 3.1 The *in-vitro* oxidation of ascorbate

In a previous study, ascorbate was found to break down both enzymically and non-enzymically to oxalate via the novel intermediates cyclic-2,3-*O*-oxalyl-L-threonate, cyclic-3,4-*O*-oxalyl-L-threonate and 4-*O*-oxalyl-L-threonate (Green and Fry 2005). In 1979 Isbell and Frush reported that the oxidation of ascorbate yielded both oxalate and threonate and proposed a theoretical intermediate of an identical structure to the 2-*O*-oxalyl-L-threonate isomer. The *in-vitro* oxidative breakdown of ascorbate by H<sub>2</sub>O<sub>2</sub> therefore seemed to proceed by a pathway similar to that described by Green and Fry (2005) in cell culture medium. During the previous study (Green and Fry 2005), 4-*O*-oxalyl-L-threonate had been isolated and characterised but this had not been possible for either cyclic-2,3-*O*-oxalyl-L-threonate or cyclic-3,4-*O*-oxalyl-L-threonate, which could only partially be separated from compound C. The identity of compounds C and E was not known either (Green or Fry 2005).

The *in-vitro* oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub> was studied for two reasons. Firstly, this allowed intermediates in the pathway to be synthesised in sufficient quantities that they could be characterised further and identified. Secondly, as the pathway proposed by Green and Fry seemed to share some similarities with the H<sub>2</sub>O<sub>2</sub>-induced oxidation of ascorbate, it was felt that studying the oxidation of ascorbate *in vitro* could be a good way in which to explore further the dynamics of this pathway.

##### 3.1.1 Oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub> yields similar products to the breakdown of ascorbate in cell culture medium

Separation of ascorbate oxidation products by paper electrophoresis revealed four compounds that could be stained with silver nitrate (Fig. 3.01). One product was identified as threonate as it co-migrated with the threonate external marker. The main product observed had an *m*<sub>OG</sub> of 2.2, although this was difficult to ascertain accurately as so much of this compounds



**Figure 3.01 Oxidation of ascorbate by  $H_2O_2$  according to methods outlined by Isbell and Frush (1979)**

Ascorbate (10 mM) was oxidised with  $H_2O_2$  (1mol:90 mol, ascorbate: $H_2O_2$ ) at pH 7.0, room temperature. The solution was buffered with 1.0 M Na+lutidine-. Samples were loaded in two 40  $\mu$ l aliquots. Compounds were separated by paper electrophoresis buffered at pH 5.5. Labeled compounds on the right are external markers (0.5 % w/v). Labeled compounds on the left are reaction products identified by *mOG* values and the position of external marker compounds. Samples were analysed by paper electrophoresis at pH 5.5 (3.5 kV, 30 min). The electrophoretogram was stained with silver nitrate. Compounds of which the identity is uncertain are marked with a dashed line. The position of the orange G external marker is marked with a dotted line. The  $t = 0$  sample was taken immediately after the addition of  $H_2O_2$ .

was present that the electrophoretic mobility was slightly impeded (Fig. 3.01). The  $m_{OG}$  value of 2.2 was close to that of 2.32 assigned to ox.thr. (Green and Fry 2005) and so it was considered probable that this compound was ox.thr. Whilst threonate (or a compound with very similar electrophoretic mobility as threonate at pH 6.5) was indeed produced, as predicted by Isbell and Frush (1979), much greater quantities of the compound thought to be ox.thr. were present (Fig. 3.01). The rate-limiting step in this reaction sequence under these experimental conditions therefore appeared to be between ox.thr. and threonate. This was consistent with results from Green and Fry (2005) where the hydrolysis of ox. thr. to oxalate and threonate was proposed to be catalysed by an esterase. Also present was a compound ( $m_{OG}$ ) migrating just ahead of threonate which was thought to be DKG and/or one or both of the isomers of cyc. ox. thr. (Fig. 3.01).

Despite the first time point being taken almost instantly, all reaction products were present in the same proportions at 0 h and 5 h (Fig. 3.01). Ascorbate and threonate had similar migration distances and so both might have been present. Ascorbate rapidly reduces silver nitrate and so, at higher concentrations, is visible as a dark spot after the  $AgNO_3$  treatment (dip 1) whereas other compounds are not visible until after the NaOH treatment (dip 2). The quantities of ascorbate loaded at each time point (4  $\mu$ mol) should have been sufficient to show up after the  $AgNO_3$  stain yet no dark spot was noted at 0 h, implying extremely rapid oxidation of ascorbate. Although ascorbate is efficiently oxidised by  $H_2O_2$  (Deutsch 1998), complete oxidation of ascorbate had not been expected to be instantaneous. It was hypothesised that oxidation of ascorbate could have continued after samples had been loaded on to paper electrophoretograms and were being dried.

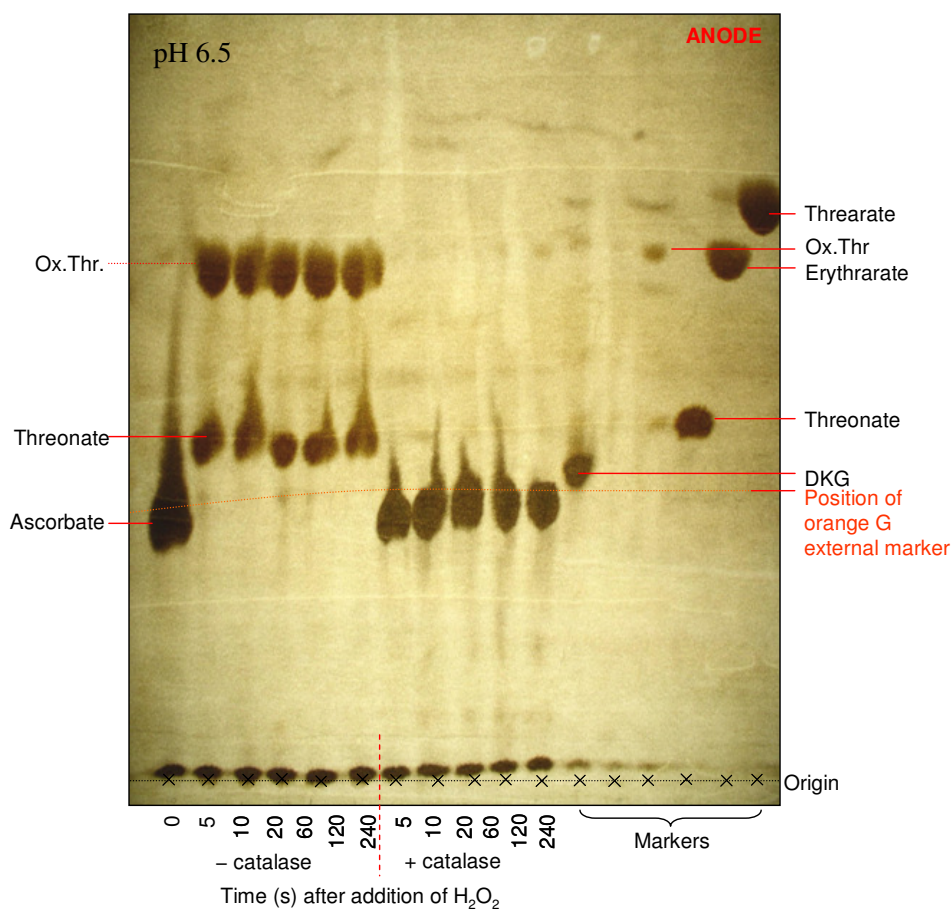
It was investigated whether ascorbate was genuinely oxidised as rapidly as this or whether rapid oxidation was caused by post-sampling oxidation was investigated. In the next experiment, after samples were removed at time points, either catalase or water was added to samples just before they were loaded on to the paper. Samples were removed at rapid time intervals from the solution as it was not known how fast the oxidation of ascorbate would be proceeding. The addition of catalase to samples had a profound effect on the apparent rate of oxidation of ascorbate (Fig. 3.02). As post-sampling oxidation did seem to be occurring, catalase was used to stop reactions in all subsequent experiments where ascorbate was oxidised with  $H_2O_2$  *in vitro*. When ascorbate was oxidised by  $H_2O_2$  for up to 1 h and samples were treated with catalase before loading on to the electrophoretogram, then ox. thr. took up to 30 min to show any appreciable increase (Fig. 3.03).

Cyc.ox.thr. and ox.thr. are highly mobile at pH 2.0 (Green 2003). At pH 2.0, only compounds with a low  $pK_a$  are appreciably ionised. At pH 6.5, most compounds with carboxyl groups are fully ionised. Ox.thr. had been found to have a low  $pK_a$  and possess two negative charges (Green and Fry 2005). The compounds thought to be cyc.ox.thr. and ox.thr. were eluted from a paper electrophoretogram and re-run at pH 6.5 (Fig 3.04) and pH 2.0 (Fig. 3.05) in the presence of a sample of freshly oxidised ascorbate. Approximately half the putative cyc.ox.thr. was present as ox.thr. after elution of cyc.ox.thr, indicating some non-enzymic breakdown (Fig. 3.04). This was consistent with the structure assigned to cyc.ox.thr. and its role as a precursor to ox.thr. (Green and Fry 2005). Ox.thr. was the only visible breakdown product of cyc.ox.thr. and did not itself seem to have broken down during elution (Fig. 3.04).

Cyc.ox.thr. and ox.thr. both showed high mobility at pH 2.0, co-migrating with the two faster-migrating ascorbate oxidation compounds in the ascorbate/ $H_2O_2$  reaction mixture (Fig. 3.05). It is noteworthy that the mobility of cyc.ox.thr. (1 -) is greater than of ox.thr. (2 -): this means that the  $pK_a$  of cyc.ox.thr. must be very low; lower than that of ox.thr.. As the compound

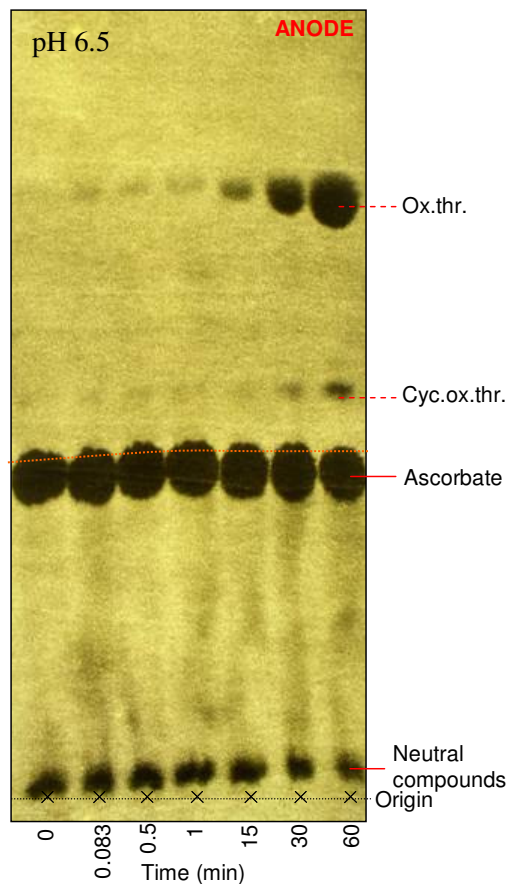
preliminarily identified as ox.thr. had a similar electrophoretic mobility at pH 6.5 and pH 2.0 to previous observations (Green 2003, Green and Fry 2005), the fastest migrating ascorbate oxidation product visible at pH 6.5 in figures 3.01 – 3.05 was identified as ox.thr. Green and Fry (2005) proposed that the ester bond in ox.thr. could be hydrolysed to yield threonate and oxalate. Alkali hydrolysis of the eluted putative ox.thr. yielded threonate (Fig. 3.06). Oxalate would not have been visible as it does not stain with silver nitrate. This confirmed the identity of the fastest migrating visible compound at pH 6.5 as that of ox.thr. Cyc.ox.thr. had been proposed to be a C<sub>6</sub> compound with a single negative charge and a low pK<sub>a</sub> (Green and Fry 2005). This corresponded to its mobility at pH 6.5 and pH 2.0 (Fig. 3.04, 3.05). As this compound yielded ox.thr. it was identified as cyc.ox.thr. Cyc.ox.thr. was found to co-migrate with threonate during electrophoresis at pH 6.5 (Fig. 3.04).

DKG was identified in the products of the ascorbate/H<sub>2</sub>O<sub>2</sub> reaction mixture (Fig. 3.04, 3.05) by its co-migration with the DKG external marker. The electrophoretic mobility of the marker and the oxidation product at pH 6.5 also corresponded to that which was observed previously for DKG (Green and Fry 2005). Proportionally, less DKG was formed during oxidation with H<sub>2</sub>O<sub>2</sub> than during the non-enzymic and enzymic breakdown of [<sup>14</sup>C]ascorbate in culture medium (Green and Fry 2005). Compounds C and E, described by Green and Fry (2005), were not observed either in figs 3.1 – 3.05. This may have been a result of the stronger oxidising conditions used here than in cell culture medium.



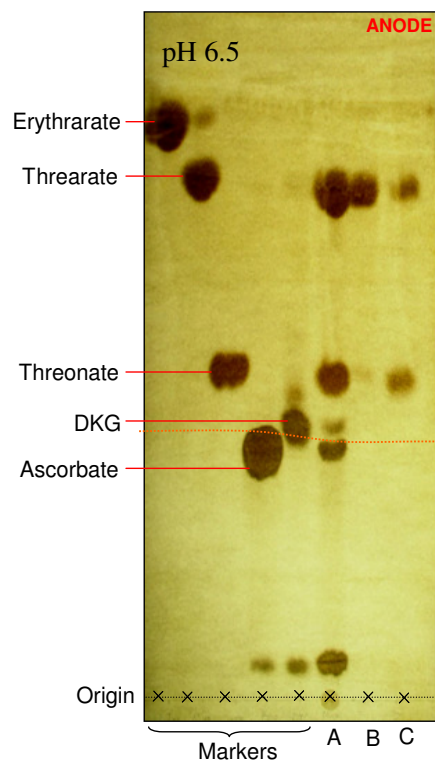
**Figure 3.02 The effect of catalase on the post-sampling oxidation of ascorbate by H<sub>2</sub>O<sub>2</sub>**

The absence of ascorbate had been noted in figure 3.01. The effect of the addition of catalase to samples was tested in case samples were being oxidized by H<sub>2</sub>O<sub>2</sub> as they were dried on to the paper. Ascorbate (50 mM) oxidised with a 1 : 2 molar ratio of ascorbate : H<sub>2</sub>O<sub>2</sub>. The solution was buffered at pH 7.0 with 1.0 M Na+lutidine-. Samples were removed in 20 µl aliquots at time points, added to 5 µl of either catalase (0.1 % w/v) for 1 min or water, then loaded on to the paper electrophoretogram. Products were analysed by electrophoresis at pH 6.5 (3.5 kV, 30 min). The DKG marker was synthesised by hydrolysis of DHA (0.1 M NaOH, 1 min). The identity of DKG was determined by the position relative to orange G and observations in previous studies (Green 2003, Green and Fry 2005). The electrophoretogram is shown here after staining with silver nitrate.



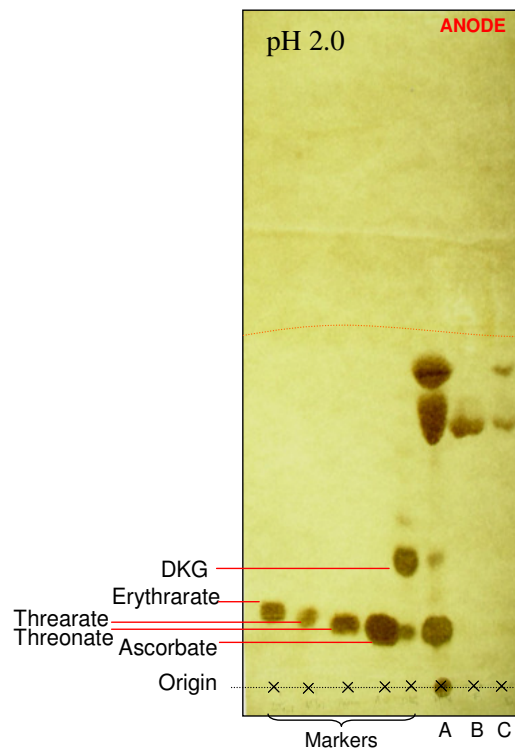
**Figure 3.03 The oxidation of ascorbate with catalase added to samples at time points**

The oxidation of ascorbate by  $\text{H}_2\text{O}_2$  was repeated with catalase being added to samples to prevent post-sampling oxidation. Ascorbate (50 mM) was oxidised with a 1 : 2 molar ratio of ascorbate :  $\text{H}_2\text{O}_2$ . The solution was buffered at pH 7.0 with 1.0 M Na+lutidine. Samples were removed in 20- $\mu\text{l}$  aliquots added to 5  $\mu\text{l}$  of 0.1 % (w/v) catalase for 1 min, then loaded on to the paper electrophoretogram. Products were analysed by electrophoresis at pH 6.5 (3.5 kV, 30 min). The silver nitrate stain is shown here. The external orange G marker is shown by a dotted line. Compounds whose identity had not been assigned at this stage are shown with a dashed line.



**Figure 3.04 The identification of cyc.ox.thr. and ox.thr. by electrophoresis at pH 6.5**

The compounds thought to be cyc.ox.thr.(C) and ox.thr. (B) were eluted from a paper electrophoretogram and then run alongside a sample of freshly oxidised ascorbate (A). Samples were analysed by electrophoresis at pH 6.5 (3.5 kV, 30 min) and stained with silver nitrate. A small amount of ox.thr. (B) has hydrolysed during elution to form threonate, which co-migrates with cyc.ox.thr. at pH 6.5. Orange G is marked with a dotted line.



**Figure 3.05 The identification of cyc.ox.thr. and ox.thr. by electrophoresis at pH 2.0.**

Experimental details were as in figure 3.04. Samples were analysed by electrophoresis at pH 2.0 (3.5 kV, 30 min) and then stained with silver nitrate.



To summarise, it can be said that all ascorbate breakdown products in the pathway proposed by Green and Fry (2005) with the exception of C and E were present when ascorbate was oxidised by H<sub>2</sub>O<sub>2</sub>. Oxalate does not stain with silver nitrate but was assumed to be present when threonate was formed by the hydrolysis of ox.thr. Oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub> was a good method by which to synthesise all metabolites identified by Green and Fry (2005) other than C and E for further study. Oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub> was a good way to study further the dynamics of this breakdown pathway but it was not identical to the breakdown pathway described by Green and Fry (2005). It has been noted, however, that threonate and cyc.ox.thr. co-migrated at pH 6.5. So far, analysis had been conducted at pH 6.5, therefore more investigations were carried out at pH 3.5 and pH 2.0.

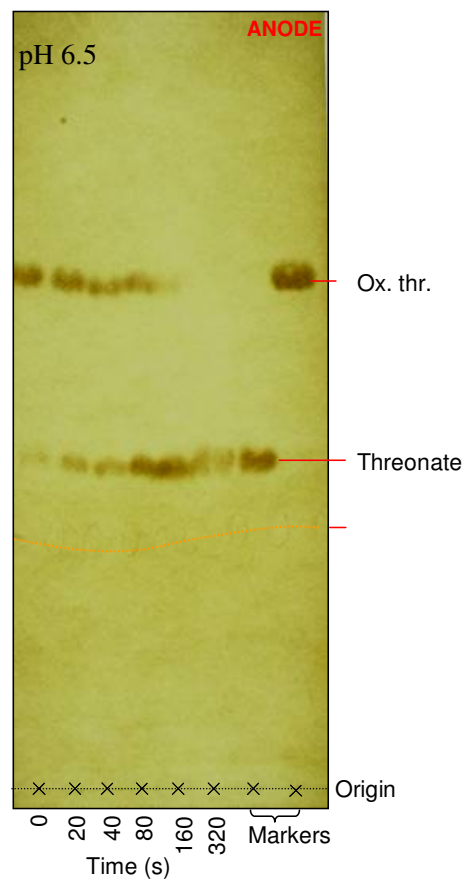
### **3.1.2 Analysis of cyc.ox.thr. and ox.thr. at pH 3.5 and pH 2.0**

Ascorbate oxidation products were separated by paper electrophoresis at pH 3.5 (Fig. 3.07). Interestingly, the compound identified as ox.thr. separated out into two compounds during electrophoresis at pH 3.5. Green and Fry (2005) discussed the theoretical existence of three isomers of ox.thr, 2-, 3- and 4-*O*-oxalyl-L-threonate and identified the presence of 4-*O*-oxalyl-L-threonate. Therefore the fastest-migrating compound detected in figure 3.07 was considered to be either 2- or 3-*O*-oxalyl threonate. The faint smear between the two isomers of ox.thr. suggested that either the faster migrating isomer (Fig. 3.07) was less stable, converting to the slower migrating isomer during electrophoresis, or that this smear represented interconversion between the two isomers.

Ox.thr. had not before been analysed at pH 3.5 (Green 2003, Green and Fry 2005). Two separate *in-vitro* preparations of ox.thr. were run alongside [<sup>14</sup>C]ox.thr. that had been synthesised *in vivo* in cell culture medium (Green 2003, [<sup>14</sup>C]ox.thr. provided by Dr. M.A.Green). This confirmed the electrophoretic mobility of ox.thr. at pH 3.5 and that [<sup>14</sup>C]ox.thr. synthesised *in*

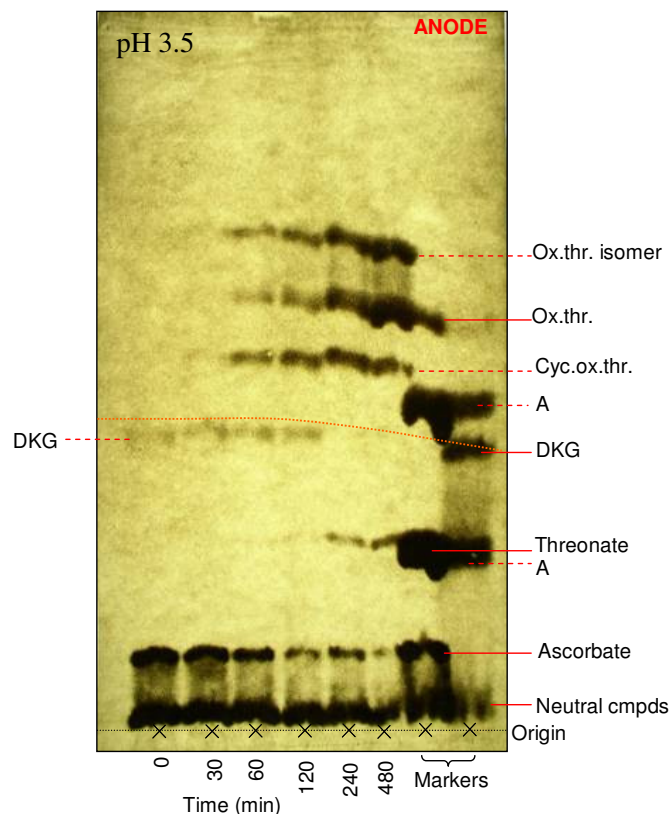
*in vivo* could be resolved into two isomers (Fig. 3.08). It was possible that all three theoretically possible isomers were present but with only two of the isomers resolved by electrophoresis at pH 3.5. The slower-migrating of the two isomers visible at pH 3.5 is referred to as ox.thr. and the faster as ox.thr.isomer. Although ox.thr had been reported to yield threonate (Green 2003), threonate was not visible in the [<sup>14</sup>C]ox.thr. sample (Fig. 3.08). Threonate is formed from C-3–C-6 of ascorbate and oxalate from C-1–C-2. Therefore, as the radiolabelled starting material was [1-<sup>14</sup>C]ascorbate, threonate would not have been labelled. [1-<sup>14</sup>C]Oxalate was not observed (Fig. 3.08). It was assumed to have migrated off the end of the paper. Threonate stains with silver nitrate and therefore was detected in the non-radiolabelled samples (Fig. 3.08).

Whether the isomers of ox.thr. interconverted or tended towards formation of the most stable of the isomers was tested with two-dimensional electrophoresis. Electrophoresis was carried out either twice at pH 3.5 (Fig. 3.09a) or at pH 3.5 followed by pH 2.0 (Fig. 3.09b), with the second separation at 90° from the first. In figures 3.09a and 3.09b, both ox.thr. and ox.thr. isomer were resolved into two isomers during the second separation by electrophoresis. This showed that the isomers of ox.thr. interconverted. After a second round of electrophoresis at pH 3.5 ox.thr, the slower migrating of the two isomers detected at pH 3.5 (ox.thr.), was present in greater proportions than the after faster migrating one (ox.thr. isomer), whether the starting compound had been ox.thr. or the isomer of ox.thr. (Fig. 3.09a). A similar phenomenon was seen when electrophoresis at pH 3.5 was followed by electrophoresis at pH 2.0 (Fig. 3.09b). On the assumption that ox.thr. rather than ox.thr. isomer was always formed in greater proportions, then at pH 2.0 ox.thr. migrated faster than ox.thr. isomer but at pH 3.5 this migration pattern was reversed.



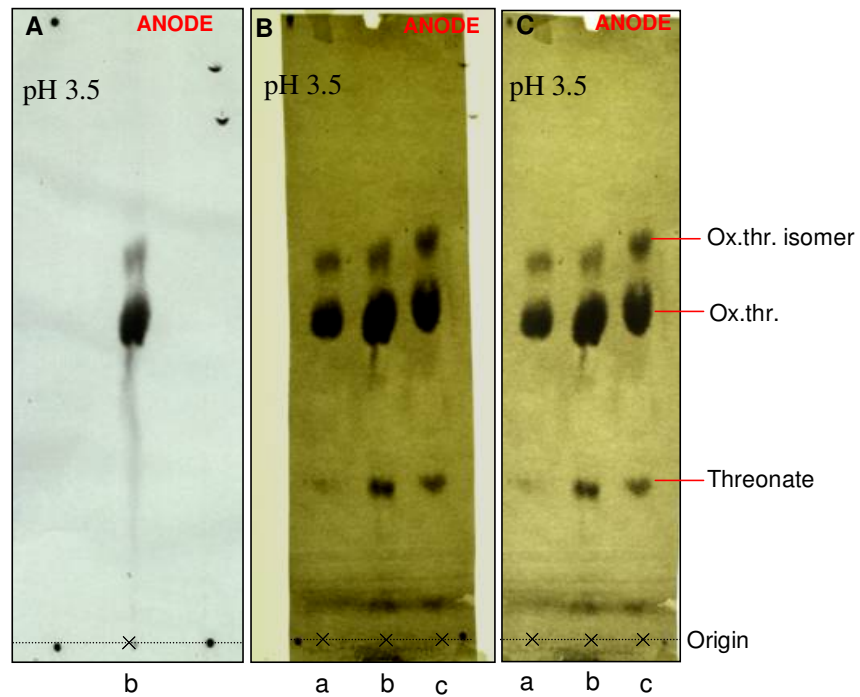
**Figure 3.06 Hydrolysis of ox.thr.**

Upon hydrolysis, ox.thr. was proposed to yield threonate and oxalate. The identity of ox.thr. was tested by hydrolysis of ox.thr. with NaOH. Putative ox.thr, isolated by elution from a paper electrophoretogram and hydrolysed with NaOH (final concentration 0.1 M). Samples were removed at time points, added to an equal volume of 0.2 M acetic acid and analysed by electrophoresis at pH 6.5 (3.5 kV, 30 min).



**Figure 3.07 Separation of ascorbate oxidation products by electrophoresis at pH 3.5**

As cyc.ox.thr. and threonate co-migrated at pH 6.5, ascorbate oxidation products were analysed at pH 3.5. An electrophoretogram run at pH 3.5 (3.5 kV, 30 min) and stained with silver nitrate is shown. Ascorbate (50 mM) was oxidised with H<sub>2</sub>O<sub>2</sub> in a 1 : 2 molar ratio. Solutions were buffered at pH 6.0 (1.0 M pyr+acetate<sup>-</sup>). Samples (20 µl) were loaded on to the paper. Another preparation of the DKG marker to that seen in previous figures was used. This preparation contained some impurities, marked 'A' here. The identity of DKG was determined by the position of compounds in the marker preparation relative to orange G and previous analysis by electrophoresis at pH 3.5 with more pure preparations of DKG (not shown). Compounds of which the identity was not certain are marked with dashed lines. Orange G is shown with a dotted line.



**Figure 3.08 Comparison of [ $^{14}\text{C}$ ]ox. thr. synthesised *in vivo* and ox.thr. synthesised *in vitro***

A. Autoradiogram of [ $^{14}\text{C}$ ]ox.thr. synthesised *in-vivo* (track 'B') supplied by M. A. Green (2003).

B. Autoradiogram shown in figure 3.08 A overlying an electrophoretogram stained with silver nitrate. The electrophoretogram shows two different *in-vitro* preparations of ox.thr. and ox.thr. isomer from ascorbate oxidised with  $\text{H}_2\text{O}_2$  (tracks 'A' and 'C').

C. Electrophoretogram stained with silver nitrate showing the two *in-vitro* preparations of ox.thr. and ox.thr. isomer (tracks 'A' and 'C') and the *in-vivo* preparation of [ $^{14}\text{C}$ ]ox.thr. (track 'B') supplied by M.A. Green (2003).

Electrophoresis was carried out at pH 3.5 (3.0 kV, 45 min).

Consistent with previous results (Fig 3.05), cyc.ox.thr. migrated faster than either ox.thr. or ox.thr.isomer at pH 2.0. No indications were observed of interconversion between ox.thr. and cyc.ox.thr (3.09a, 3.09b). Oxalate migrated most rapidly of all during electrophoresis at pH 3.5 (Fig. 3.09a) but was not observed in figure 3.09b. Reasons for this are discussed below (Fig. 3.10a, b, c). During the second round of electrophoresis both at pH 3.5 and pH 2.0, DHA broke down into cyc.ox.thr. and ox.thr. (Fig 3.09a,b). This is consistent with the pathway proposed by Green and Fry (2005) where both of these compounds are thought to stem from DHA.

Therefore it would appear that the isomers of ox.thr showed evidence of interconversion but an equilibrium existed between these isomers which favoured the formation of either one isomer or of two isomers which could not be resolved from each other.

### **3.1.3 The electrophoretic mobility of oxalate**

[<sup>14</sup>C]Oxalate was found to form streaks during electrophoresis at pH 2.0, pH 3.5 and pH 6.5 (Fig. 3.10a, b, c). Oxalate readily forms insoluble salts such as calcium oxalate (Nakata 1996). The streaks are thought to arise from the continued formation of insoluble oxalate salts as oxalate migrates across the paper. When non-radioactive oxalate was added to [<sup>14</sup>C]oxalate samples in high concentrations (20 mM) and chelating agents were added to electrophoresis buffers, the degree of streaking could be limited (Fig. 3.10 a, b, c). Streaking was most successfully prevented at pH 2.0 and pH 6.5 (Fig. 3.10 a, c) though the radioactive traces in lane B (Fig. 3.10 c) indicate some formation of insoluble calcium salts.

### **3.1.4 Dynamics of the ascorbate oxidative breakdown pathway**

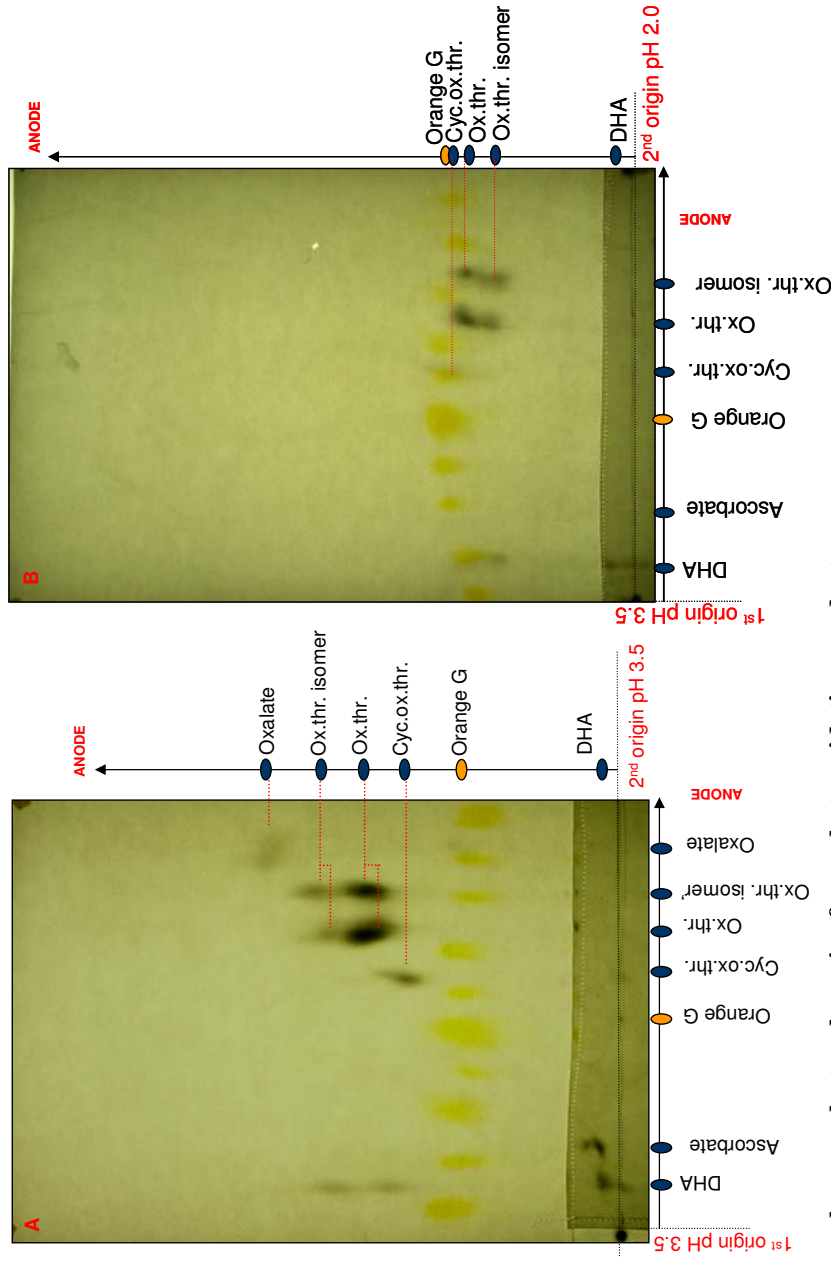
#### 3.1.4.1 Cyclic oxalyl threonate as a precursor of oxalyl threonate

In figure 3.03, an increase in the compound identified as cyc.ox.thr was first observed at 30 min. This was slightly after the noted increase in ox.thr at 15 min. The relative appearance of compounds could potentially have been masked by threonate which co-migrated with cyc.ox.thr

when separated by electrophoresis at pH 6.5, so oxidation products were separated at pH 3.5. When analysed by electrophoresis at pH 3.5, cyc.ox.thr, ox.thr. and ox.thr. isomer were all first observed at 60 min (Fig. 3.07). All three seemed to appear simultaneously. Silver nitrate is a relatively sensitive stain for many sugar-acids. However, it cannot be used for accurate quantification of compounds. Therefore a mixture of [<sup>14</sup>C]ascorbate and non-radioactive ascorbate was oxidised and oxidation products were quantified by scintillation counting.

When <sup>14</sup>C-labelled oxidation products were quantified, the reaction sequence of ascorbate → DHA → oxalyl threonate was apparent (Fig 3.11 a); ascorbate declined rapidly from 0 min, followed by a small peak in DHA at 30 min. Shortly after the proportion of DHA had started to increase, a steep rise in ox.thr. from 8 min onwards was observed. An increase in cyc.ox.thr. was not noted until 30 min, which suggested that although the precursor to cyc.ox.thr. was DHA and although cyc.ox.thr. was capable of yielding ox.thr, cyc.ox.thr. did not behave as if it were the obligatory precursor to ox.thr. If cyc.ox.thr. had been the precursor to ox.thr. in this pathway, cyc.ox.thr. could have been expected to peak in between the peak of DHA and the increase of ox.thr. A cyc.ox.thr. precursor might also have shown a decrease at 120 min after the rate of increase of ox.thr. started to decline. Errors derived from such sources as the excision of darkened spots from the electrophoretogram were assumed to be zero but it was borne in mind that small increases such as those in cyc.ox.thr. between 0 and 30 min had to be interpreted with caution. However, that an increase in ox.thr. should occur either simultaneously or shortly before the observed increase in cyc.ox.thr. was in agreement with results shown in figures 3.03, 3.07, 3.11b and analysis by HPLC (section 3.1.5).

Green and Fry (2005) proposed that DHA was oxidised to form cyc.ox.thr. which was then hydrolysed to form ox.thr. The rapid increase in yield in ox.thr. upon oxidation of ascorbate (Fig. 3.11a, b) is more compatible with the idea that ox.thr. is a direct oxidation product of DHA.



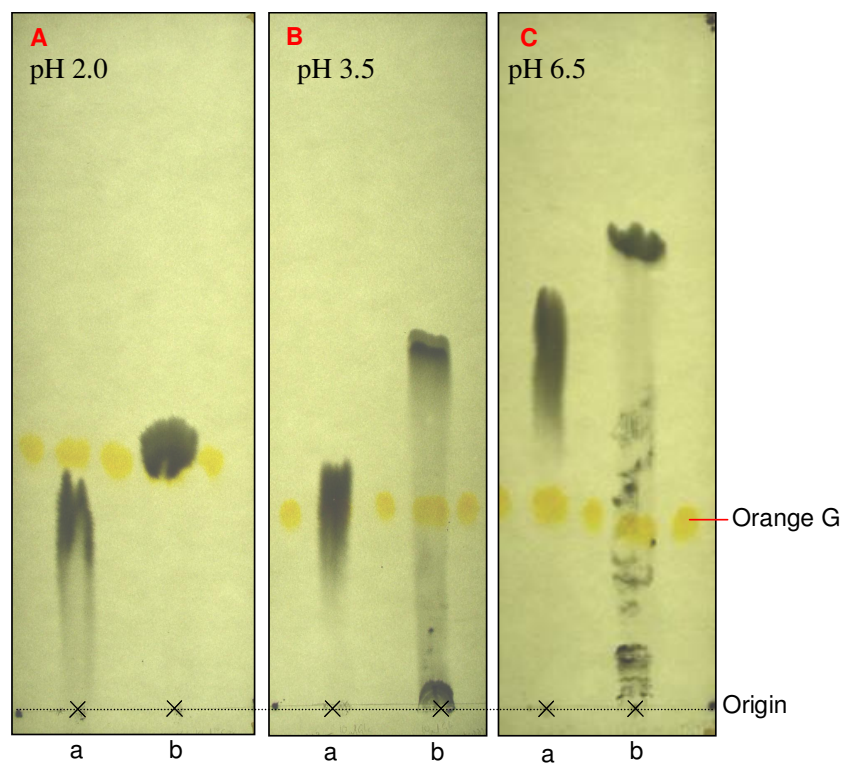
**Figure 3.09 Two-dimensional paper electrophoresis of ascorbate oxidation products**

[<sup>14</sup>C]Ascorbate and ascorbate (final concentration 10 mM) were oxidised with H<sub>2</sub>O<sub>2</sub> in a 1 : 2 molar ratio for 480 min. Solutions were buffered at pH 6.0 (1.0 M pyr+acetate-). Oxidation products were separated by paper electrophoresis at pH 3.5 (3.5 kV, 30 min), excised, reattached to a second paper then subjected to further electrophoresis (3.0 kV, 30 min). The autoradiogram is shown overlying the electrophoretogram (unstained) in both figures A and B.

**A.** Compounds separated by electrophoresis at pH 3.5 followed by electrophoresis at pH 3.5.

**B.** Compounds separated by electrophoresis at pH 3.5 followed by electrophoresis at pH 2.0.

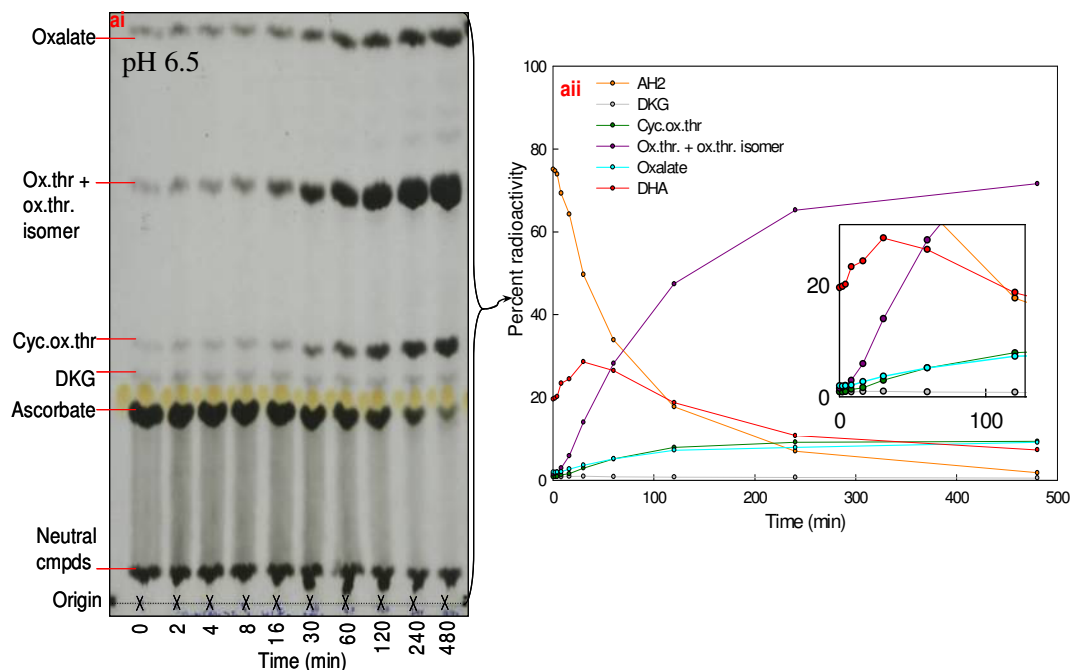




**Figure 3.10 The effect of non-radioactive oxalate electrophoretic mobility of [ $^{14}\text{C}$ ]oxalate**  
 Autoradiograms (overlying unstained electrophoretograms) showing [ $^{14}\text{C}$ ]oxalate (track 'a') or [ $^{14}\text{C}$ ]oxalate + non-radioactive oxalate at a final concentration of 20 mM (track 'b') were analysed by electrophoresis (3.0 kV, 30 min). Chelating agents (final concentration 5 mM) were added to electrophoresis buffers to minimise the formation of calcium[ $^{14}\text{C}$ ]oxalate during electrophoresis. Thiosulphate was added to the pH 2.0 electrophoresis buffer. EDTA was added to the pH 3.5 and pH 6.5 electrophoresis buffers. Formation of insoluble [ $^{14}\text{C}$ ]oxalate salts was considered responsible for the streaking and speckling visible here.  
 A. Electrophoretic mobility of [ $^{14}\text{C}$ ]oxalate at pH 2.0.  
 B. Electrophoretic mobility of [ $^{14}\text{C}$ ]oxalate at pH 3.5  
 C. Electrophoretic mobility of [ $^{14}\text{C}$ ]oxalate at pH 6.5

#### 3.1.4.2 Formation rates of oxalyl threonate and oxalyl threonate isomer

Oxidation products were separated at pH 3.5 (Fig. 3.11b) so that any differences between the two isomers of ox.thr. could be observed. A similar sequence of events in figure 3.11b as 3.11a was observed; ascorbate content declined steadily, DHA content peaked at 30 min, shortly before which levels of ox.thr. and ox.thr. isomer started to rise. Both isomers appeared simultaneously at 15 min and initial rates of increase were almost identical; the proportion of ox.thr. isomer increase at 10 % h<sup>-1</sup> and ox. thr. at 8 % h<sup>-1</sup> (Fig. 3.11b). This was approximately half the rate at which they had increased in figure 3.10a (21 % per hour) when the isomers were not separated. The rate of production of both isomers slowed at 120 min. This was expected as exactly the required amounts of H<sub>2</sub>O<sub>2</sub> had been added for ascorbate to be oxidised to ox.thr., but the decrease was greater in the faster migrating ox.thr.isomer. A faster decrease in ox.thr. isomer production is consistent with the hypothesis that an equilibrium exists between the two isomers in which the formation of ox.thr. is slightly favoured over the formation of ox.thr. isomer.

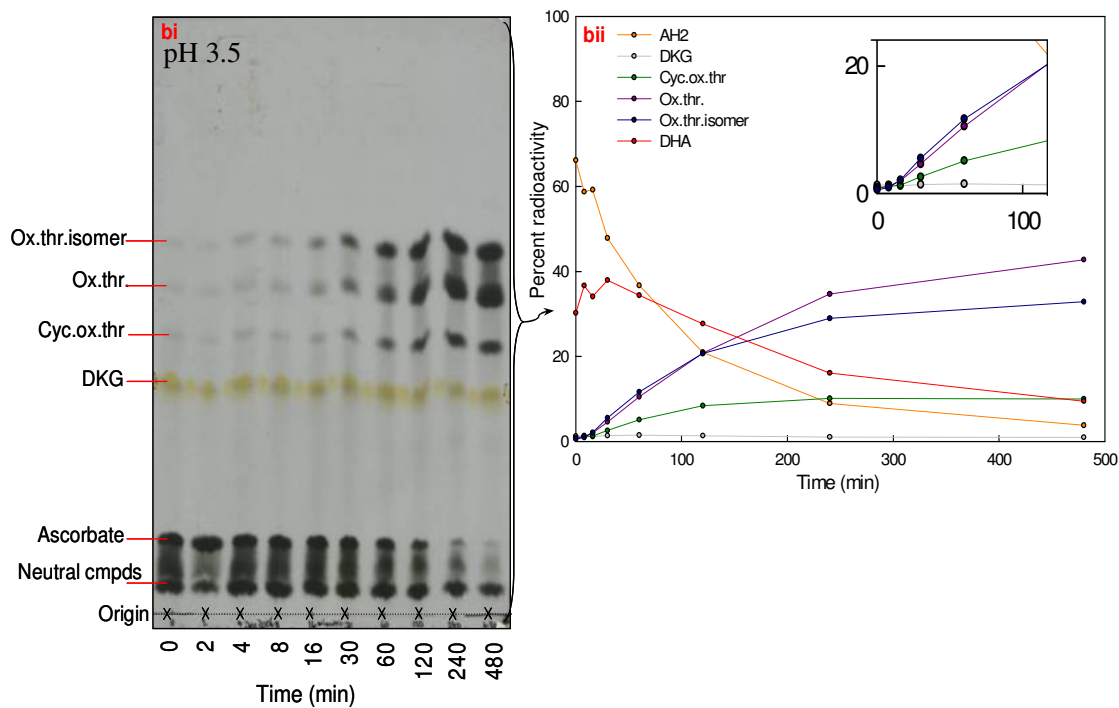


**Figure 3.11 Kinetic study of the *in-vitro* oxidation of [<sup>14</sup>C]ascorbate**

[<sup>14</sup>C]Ascorbate was oxidized with H<sub>2</sub>O<sub>2</sub> so that a more accurate, quantitative overview of the yield of ascorbate oxidation products over time could be formed. A solution of [<sup>14</sup>C]ascorbate (0.016 MBq) and non-radioactive ascorbate (final concentration 10 mM) was oxidised by H<sub>2</sub>O<sub>2</sub> in a 1 : 2 (ascorbate : H<sub>2</sub>O<sub>2</sub>) molar ratio. There was sufficient non-radioactive oxalate present for smearing of oxalate to have been prevented. Aliquots (5 μl) were loaded on to paper electrophoretograms. The reaction solution was buffered at pH 6.0 (pyridine+ acetate-); previous experiments (data not shown) had indicated that any variation in pH of the reaction solution between pH 4 and pH 7 did not affect the proportions of oxidation products in this experiment.

ai. [<sup>14</sup>C]Ascorbate and oxidation products separated by electrophoresis at pH 6.5. EDTA (final concentration 5 mM) was added to the electrophoresis buffer. The autoradiogram is pictured overlying the electrophoretogram (unstained). Streaks between [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA have been included as [<sup>14</sup>C]ascorbate in aii.

aii. Areas of the electrophoretogram that corresponded to darkened areas on the autoradiogram were excised and the radioactivity was quantified by scintillation counting. The radioactivity of each compound was expressed as a percentage of the total recovered radioactivity at each time point.



**Figure 3.11 Kinetic study of the in-vitro oxidation of [ $^{14}\text{C}$ ]ascorbate**

3.11 bi. [ $^{14}\text{C}$ ]Ascorbate and oxidation products separated by electrophoresis at pH 3.5. Thiosulphate (final concentration 5 mM) was added to the electrophoresis buffer. Other details as ai.

3.11 bii. See figure 3.11 aii for details.

#### 3.1.4.3 The *in-vitro* formation of oxalate from ox.thr.

The rate of oxalate formation from ascorbate in the presence of H<sub>2</sub>O<sub>2</sub> was slow; the maximum rate was less than 5 % h<sup>-1</sup> (Fig. 3.11a). At 480 min oxalate represented only 8 % of the radioactivity whereas 70 % was present as ox.thr. That relatively strong oxidative conditions should apparently not affect the yield of oxalate from ox.thr. is in agreement with Green and Fry (2005) who proposed that oxalate and threonate were formed by hydrolysis of the ester bond in ox.thr. There was strong evidence to support the idea that this reaction was catalysed by an esterase as the yield of oxalate was much higher in spent cell culture medium than in boiled spent cell culture medium (Green and Fry 2005). This too was consistent with the low rate of oxalate production observed *in vitro*.

Oxalate was not observed after products were separated at pH 3.5 (Fig. 3.11bi). As oxalate migrates approximately twice as far as the external orange G marker (Fig. 3.10bi), then migration off the end of the paper would not explain the absence of oxalate in figure 3.11bi. Non-radioactive oxalate was not added to samples before electrophoresis in either figure 3.10 a or b. The disparity in oxalate migration was greatest at pH 3.5 (Fig. 3.10bii) and streaking was prominent even when non-radioactive oxalate was added to [<sup>14</sup>C]oxalate as a sacrificial protectant. This suggests that oxalate was least stable and formed insoluble salt most readily during electrophoresis at pH 3.5 compared to pH 6.5 or pH 2.0. Therefore separation of compounds at pH 6.5 was preferred when [<sup>14</sup>C]oxalate was expected to be present. As [<sup>14</sup>C]oxalate was only present in low concentrations it was not thought that the reduced migration of [<sup>14</sup>C]oxalate would have interfered with quantification of the presence of other compounds such as [<sup>14</sup>C]ox.thr. or [<sup>14</sup>C]ox.thr. isomer.

#### 3.1.4.4 The formation of [<sup>14</sup>C]DKG during the oxidation of [<sup>14</sup>C]ascorbate

An important difference between the non-enzymic breakdown in freshly autoclaved culture medium (Green and Fry 2005) and the oxidation of [<sup>14</sup>C]ascorbate by H<sub>2</sub>O<sub>2</sub> was the formation of [<sup>14</sup>C]DKG. In a previous study, after 8 h [<sup>14</sup>C]DHA and [<sup>14</sup>C]DKG were the predominant products in freshly autoclaved cell culture medium (Green and Fry 2005). Conversely, in the presence of H<sub>2</sub>O<sub>2</sub>, the yield of [<sup>14</sup>C]DKG was low and did not appear to change over time. DKG is formed via the hydrolysis of DHA (Bánhegyi and Loewus 2004). It is possible that oxidation and hydrolysis are two competing reactions so the fate of DHA is largely determined by the conditions (strongly or mildly oxidising) in which it is formed from ascorbate. Again, compounds C and E were not observed, though yields in freshly autoclaved cell culture medium were also very low (Green and Fry 2005).

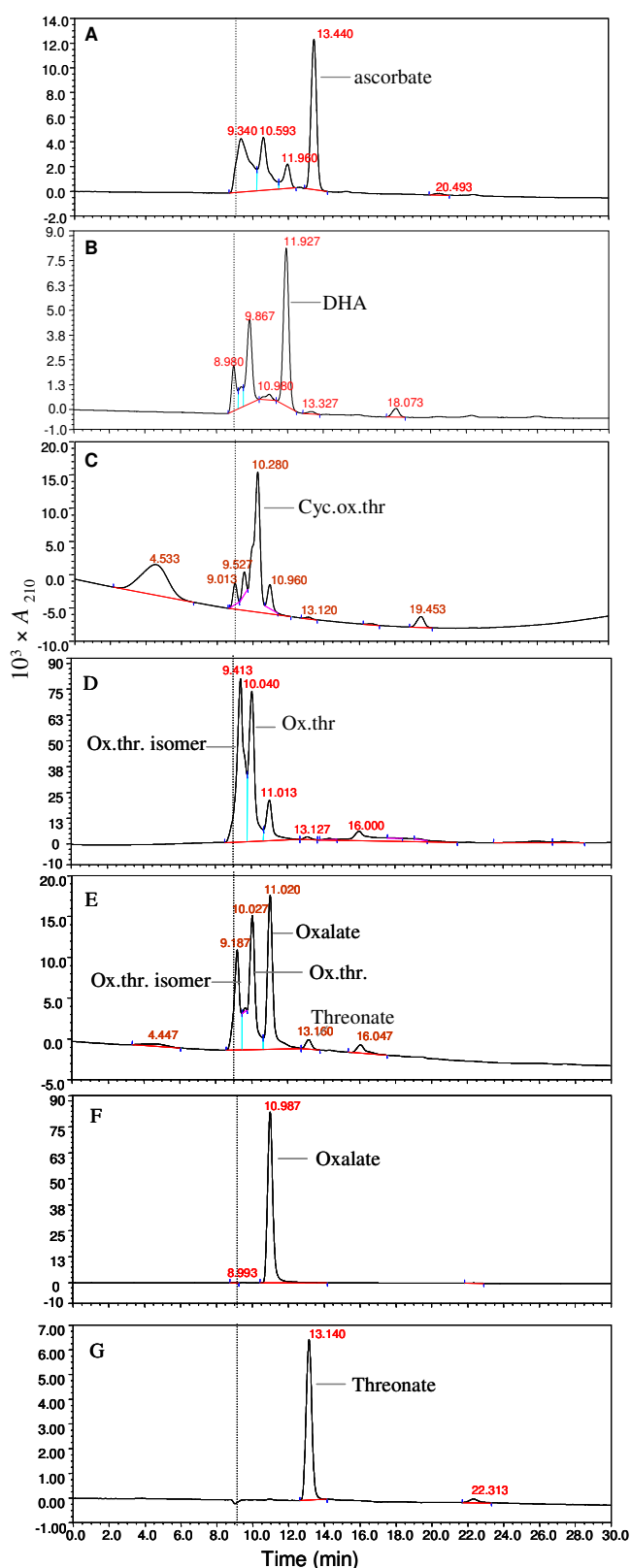
#### **3.1.5 HPLC analysis of the oxidative breakdown of ascorbate**

Although experiments with [<sup>14</sup>C]ascorbate had suggested cyc.ox.thr. was not the obligate precursor of ox.thr. it was desirable to demonstrate this with an alternative method. In addition, it had not been possible to detect compounds that no longer contained the former 1-<sup>14</sup>C moiety by scintillation counting, whilst compounds that were present in too low concentrations or did not reduce AgNO<sub>3</sub> would not have been detected by silver nitrate staining. Therefore, the oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub> was analysed with HPLC and UV detection at 210 and 250 nm.

A series of markers was produced for the identification of ascorbate oxidation products with HPLC. Where markers had to be produced for compounds that were not available commercially e.g. ox.thr, compounds were isolated by paper electrophoresis. Compounds that had been eluted off paper electrophoretograms invariably gave multiple peaks. As the molar absorbance for these compounds was not known, it was not immediately obvious which peaks represented the compounds of interest. A series of oxidation and hydrolysis reactions was carried

out on DHA, cyc.ox.thr and ox.thr. (results not shown). Results were compared before and after hydrolysis and the corresponding peaks for cyc.ox.thr. and ox.thr. were deduced. The resulting markers are shown in figure 3.12 a – h.

Ascorbate oxidation products were identified from the standards and compounds eluted from paper electrophoretograms (Fig. 3.12 a – h). Low levels of breakdown products, mostly DHA, were observed at 0 min (Fig. 3.13). This was expected as ascorbate does break down slowly in storage. Little change was noted over the first 15 min. This was consistent with previous observations (Figs. 3.03, 3.07, 3.11). It was possible that there was more DHA present that there initially appeared to be as the molar absorbance of DHA was lower at 210 nm than at 195 nm. However, analysis at 195 nm resulted in much background noise (results not shown). Ox.thr. was found to elute between 9.25 min and 9.35 min and was present as a single peak (Fig. 3.13). When ox.thr. and ox.thr. isomer had been isolated separately, elution times of 10.03 min and 9.41 min, respectively, had been found (Fig 3.12 e, f). Possibly a single peak was present rather than two peaks because of the equilibrium that was thought to exist between the two isomers. It was difficult to tell exactly when the ox.thr./ox.thr. isomer peak first appeared as a little was present at 0 min, though the first appreciable increase was seen at 60 min. A definite increase in the cyc.ox.thr. peak was not noted until 120 min, although a trace increase in cyc.ox.thr. content might have been occurring at 60 min. In either case, these results confirm the results of the [<sup>14</sup>C]ascorbate studies— that cyc.ox.thr. is not the obligate precursor of ox.thr. or ox.thr. isomer. Results from the [<sup>14</sup>C]ascorbate and HPLC studies do not show any decrease over time in cyc.ox.thr. content even after 480 min which suggests that neither is cyc.ox.thr. the obligate precursor of ox.thr. or ox.thr. isomer nor is it behaving as a precursor at all during the course of the H<sub>2</sub>O<sub>2</sub> induced oxidation of ascorbate.



**Figure 3.12 Ascorbate and ascorbate oxidation compounds prepared as markers for HPLC analysis**

A. Ascorbate (0.5 % w/v) in water. Retention times in this run were approximately 0.2 min longer than those in figure 3.15. It is not known why.

B. A 3M solution of DHA in 100 % DMF was diluted in water to 0.003 M before HPLC

C. Cyc.ox.thr. was passed down the HPLC column immediately after elution off a paper electrophoretogram. This was necessary to minimise breakdown of cyc.ox.thr. The baseline has been affected by other compounds e.g. remnants of pyridine or acetate that would have co-eluted off the paper.

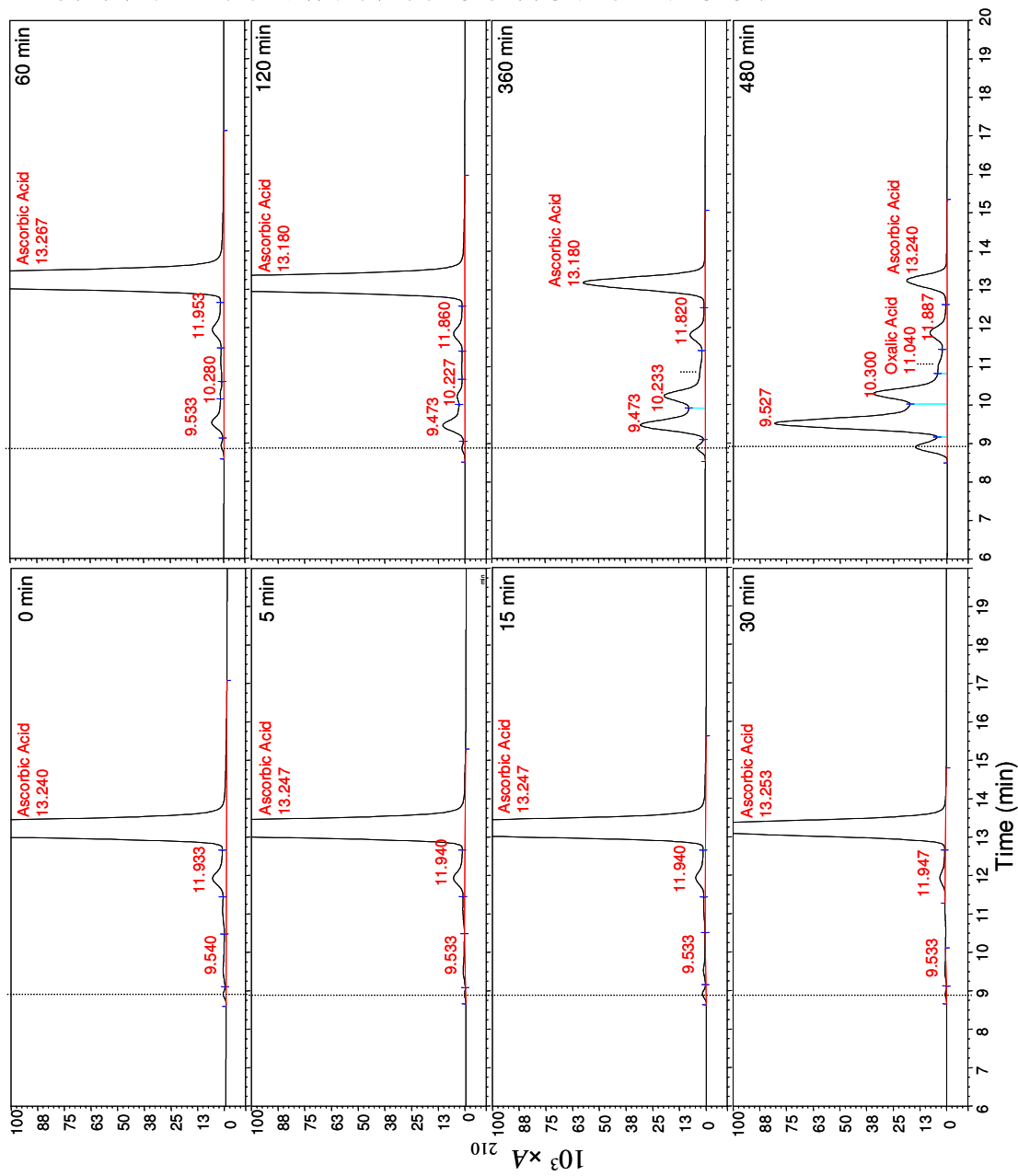
D. Ox.thr. isomer eluted from a paper electrophoretogram. Variation in the baseline was minimised by thorough washing of the column in between passing compounds eluted off paper electrophoretograms down the column.

E. Ox.thr. eluted from a paper electrophoretogram.

F. Oxalate (0.5 % w/v) in water

G. Threonate (0.5 % w/v) in water





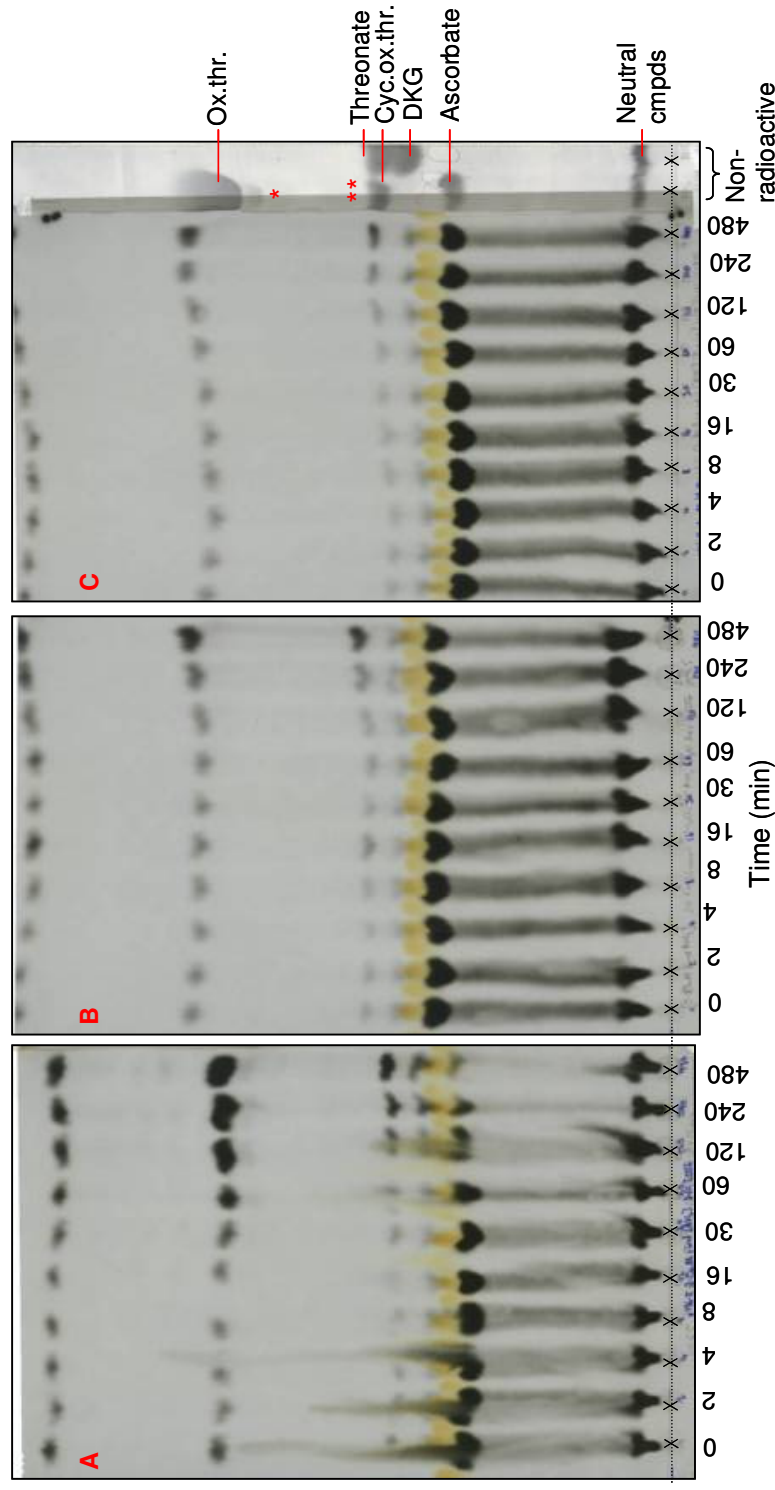
**Figure 3.13 HPLC**  
analysis of the kinetics of  
the oxidation of ascorbate  
with  $H_2O_2$ .

Ascorbate (50 mM) was  
oxidised with  $H_2O_2$  in a 1 :  
2 ascorbate :  $H_2O_2$  molar  
ratio. The reaction solution  
was buffered at pH 6.0 (1.0  
M pyridine+acetate-).  
Aliquots were removed at  
time points and added to  
catalase (0.1 % w/v).  
Catalase was removed  
prior to HPLC by a size  
exclusion column with a  
water eluent. Absorbance  
of the eluate was measured  
at 210 nm. The time at  
which the void volume  
eluted off the HPLC  
column is represented by  
the dotted line.

No evidence was seen for the formation of any compounds other than DHA, cyc.ox.thr, the isomers of ox.th, and oxalate. Threonate was not observed, though this may have been a result of its low molar absorbance at 210 nm (Fig. 3.13 h). A model for the in vitro oxidation pathway of ascorbate was generated in which DHA can be oxidised simultaneously to both cyc.ox.thr. and ox.thr (Fig. 4.1).

### **3.1.6 The effect of ascorbate concentration on the formation of oxidative breakdown products**

The formation of compounds C and E had been observed to vary, depending on the concentration of ascorbate present in the reaction solution. Formation of E had been observed to be inhibited by higher concentrations of ascorbate (S. C. Fry, unpublished results). As experiments thus far had involved ascorbate concentrations higher than those in experiment carried out by Green and Fry (2005), it was questioned whether the formation of C and E might have been inhibited. [<sup>14</sup>C]Ascorbate was oxidised in the presence 0 – 4 mM non-radioactive ascorbate (Fig. 3.14 a – c). H<sub>2</sub>O<sub>2</sub> concentration was changed in accordance with the total initial ascorbate concentration. Compound E was observed neither at low nor high concentrations of cold ascorbate. The concentration of ascorbate did not therefore appear to have an effect on the production of compound E, although this may have been masked if the highly oxidising experimental conditions did not favour the production of DKG, as DKG was proposed to be the precursor of compound E. H<sub>2</sub>O<sub>2</sub> was not added to experiments where C and E were observed (Green and Fry 2005).



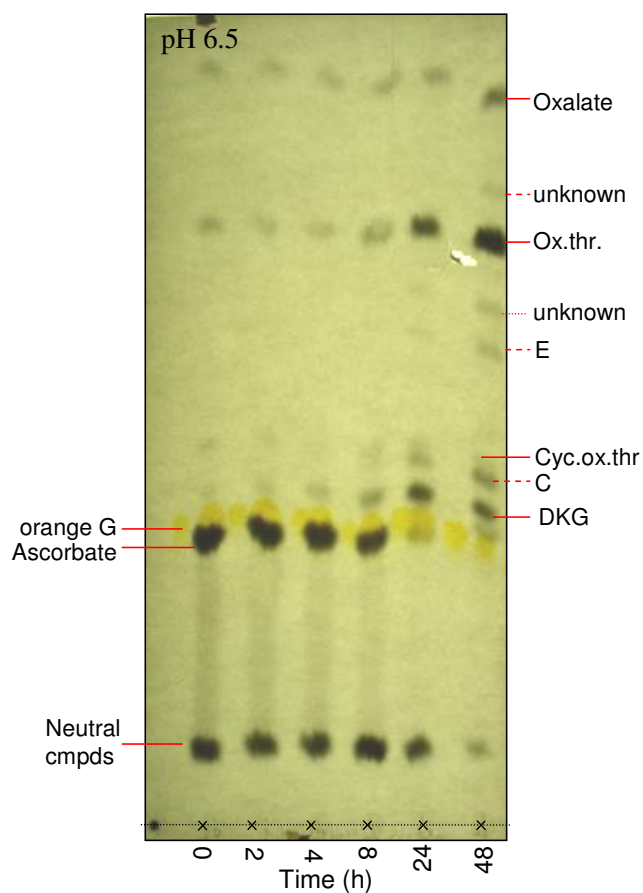
**Figure 3.14 The effect of ascorbate concentration on the formation of oxidative breakdown products**  
 $[^{14}\text{C}]$ Ascorbate (0.016 MBq) was mixed with varying concentrations of cold ascorbate and oxidised by  $\text{H}_2\text{O}_2$  in a 1 : 2 (ascorbate :  $\text{H}_2\text{O}_2$ ) molar ratio. Aliquots (5  $\mu\text{l}$ ) were loaded on to paper electrophoretograms. Oxidation products were separated by electrophoresis at pH 6.5 (3.5 kV, 35 min). EDTA to 5mM was added to the electrophoresis buffer. Non-radioactive markers were visualised by silver nitrate staining. The streaking in A is a result of running buffer being allowed to drip down the electrophoretogram after electrophoresis. The presence of some spots at 0 min indicates slight  $[^{14}\text{C}]$ ascorbate breakdown during storage. Expected positions of C (\*\*\*) and E (\*) are shown.

3.12 A. The oxidation of  $[^{14}\text{C}]$ ascorbate and cold ascorbate at a final concentration of 4 mM  
 3.12 B. The oxidation of  $[^{14}\text{C}]$ ascorbate and cold ascorbate at a final concentration of 0.2 mM  
 3.12 C. The oxidation of  $[^{14}\text{C}]$ ascorbate and cold ascorbate at a final concentration of 0.002 mM

### 3.1.7 Ascorbate breakdown under mildly oxidising conditions

Little oxidative breakdown of [ $^{14}\text{C}$ ]ascorbate was observed over time in the presence of low concentrations of non-radioactive ascorbate (Fig. 3.14b) or the absence of non-radioactive ascorbate (Fig. 3.14c). This is likely to reflect the instability of  $\text{H}_2\text{O}_2$  at such low concentrations and so this reaction can be considered to show the fate of ascorbate in much more mildly oxidising conditions than the results shown in all previous figures. The formation of [ $^{14}\text{C}$ ]DKG was still very low (3.14b, c). These solutions were un-buffered. The pH was found to be 4.4 at the start and end of the reaction. In experiments when ascorbate was oxidised in freshly autoclaved cell culture medium in the absence of deliberately added  $\text{H}_2\text{O}_2$  and higher concentrations of [ $^{14}\text{C}$ ]DKG were formed, the solutions had been buffered between pH 6.0 and 6.5 (Green 2003, Green and Fry 2005). It is therefore likely when oxidation is not the predominant process that pH is important in determining the fate of DHA.

Hydrolysis of [ $^{14}\text{C}$ ]DHA to [ $^{14}\text{C}$ ]DKG at pH 4.4 (pH of un-buffered solutions at start) did occur, only much more slowly than was observed by Green and Fry (2005); the concentration of [ $^{14}\text{C}$ ]DKG did not show an appreciable increase until 24 h (Fig. 3.15). At 24 h, breakdown products whose electrophoretic migration distances were similar to those reported for compounds C and E at pH 6.5 (Green and Fry 2005) were also visible. Visible too at 24 and 48 h were several other unidentified compounds. DKG is known to break down to over 50 different compounds (Deutsch 1995) which may explain the presence of multiple compounds at 24 and 48 h. Unless these compounds were to breakdown to yield  $^{14}\text{CO}_2$ , which would not have been observed by paper electrophoresis, then rapid turnover of [ $^{14}\text{C}$ ]DKG and  $^{14}\text{C}$ -labelled DKG breakdown products did not seem likely. Interestingly, the concentration of ox.thr. also appeared to rise at 24 h, which ran counter to the hypothesis that ox.thr. was formed directly from the oxidation of DHA. Ox.thr. was not proposed to be an oxidation product of DKG (Green and Fry 2005). The oxidative breakdown of DKG was investigated further (section 3.02).



**Figure 3.15 The breakdown of [ $^{14}\text{C}$ ]ascorbate under mildly oxidising conditions**

[ $^{14}\text{C}$ ]Ascorbate (0.025 MBq), in the absence of additional non-radioactive ascorbate, was diluted into 1 ml of water (unbuffered,  $\text{pH} \approx 4.5$ ) and sampled under sterile conditions. Aliquots (10  $\mu\text{l}$ ) were loaded on to paper electrophoretograms. Electrophoresis was carried out at  $\text{pH} 6.5$  (3.5 kV, 35 min). EDTA (final concentration 5mM) was added to the running buffer. Compounds whose identity is uncertain are marked with a dashed line. The autoradiogram overlying the unstained electrophoretogram is pictured here. Compounds whose identity is suspected are marked with a dashed line.

According to Kagawa (1962) the production of oxalate from DKG was accelerated in the presence of oxidising compounds such as  $H_2O_2$ . Possibly, what would have been required in this study for the production of C and E from ascorbate would have been a combination of a relatively high pH, so that DHA could yield DKG in the first instance, and the presence of  $H_2O_2$  so that DKG could have been oxidised. In the absence of exogenous  $H_2O_2$ , oxalate was not predicted to be a breakdown product of DKG (Green and Fry 2005). The oxidation of DHA and the oxidation and hydrolysis of DKG were therefore examined in more detail. These results are discussed in section 3.2.

### 3.2 The *in-vitro* oxidation of DHA and DKG

In the previous chapter the *in-vitro* oxidation of ascorbate to ox.thr. with H<sub>2</sub>O<sub>2</sub> had been investigated. It had been demonstrated, through HPLC and radioisotope studies, that cyc.ox.thr. was an oxidation product of DHA but did not act as an obligatory precursor to ox.thr ( Fig. 3.13, 3.11). During the oxidation of ascorbate, very low yields of DKG had been observed. The increased production of DKG observed after 24 h during the oxidation of ascorbate under mildly oxidising conditions (water and exposure to air, without added H<sub>2</sub>O<sub>2</sub>; Fig. 3.15) suggested that the strong oxidative conditions under which most of the oxidation experiments were carried out in section 3.1 might not have favoured the production of DKG. Ox.thr, regarded throughout section 3.1 as the main products of the oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub>, was, along with DKG and oxalate, observed to be one of the main breakdown products of ascorbate after 24 h mild oxidation (Fig. 3.15), whilst other breakdown products of mild ascorbate oxidation had electrophoretic mobilities at pH 6.5 similar to those reported for compounds C and E (Green 2003).

Compounds C and E had not been fully characterised in the study in which they were first observed (Green 2003). Compound C had been tentatively identified as a lactone (Green and Fry 2005), whilst DKG has been reported to convert into two lactones, 3,4-DKG lactone and 2,3-DKG lactone, which are thought to possess antioxidant properties (Li *et al.* 2001). Evidence for the uptake of compound E by cells had been observed (Green 2003). As compound E was charged (Green and Fry 2005) this would seem to imply some form of active uptake. Additionally, the formation of either DKG or ox.thr and cyc.ox.thr. from DHA represented a branch point in the ascorbate breakdown pathway proposed by Green and Fry (2005). Results in section 3.1 suggested that reaction conditions influenced which branch of the pathway was preferred. Further investigation into the formation and breakdown of DKG was therefore considered to be of interest.

Solutions of DHA and DKG were synthesised to high levels of purity so that the breakdown pathways of these compounds could be studied in more detail. Purity confirmed by paper electrophoresis and silver nitrate staining (data not shown but see  $t = 0$  in Fig. 3.16, 3.18 and 3.20)

### 3.2.1 The oxidation of DHA by $H_2O_2$

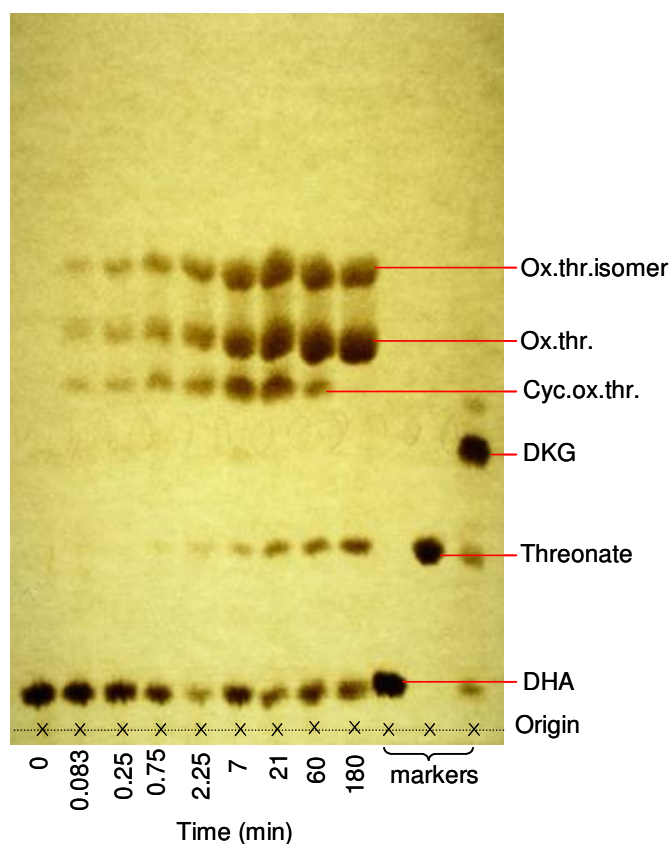
First, the hypothesis that cyc.ox.thr, ox.thr, threonate and oxalate were oxidation products of DHA and represented a separate branch of the ascorbate breakdown pathway was tested (Fig. 3.16). The solution of DHA that was oxidised with  $H_2O_2$  was sufficiently pure that no contaminants could be seen at 0 min in the electrophoretogram stained with silver nitrate (Fig. 3.16). Both ox.thr. and ox.thr. isomer were produced. Cyc.ox.thr. and both isomers of ox.thr. appeared simultaneously. Threonate was observed in trace quantities from 0.75 min onwards (Fig. 3.16) but a substantial increase in threonate was not seen until 7 min. These observations are consistent with the hypothesis that cyc.ox.thr, both isomers of ox.thr. and threonate (and, by implication, oxalate, although it could not be observed with a silver nitrate stain) derive from DHA and are not involved in the DKG branch of the pathway. No DKG was visible during this oxidation, except a very slight trace at 7 min. This was not a consistent feature of repeated DHA oxidation experiments and so was considered to be a post-sampling effect. These observations are also consistent with the hypothesis that cyc.ox.thr. is not the obligate precursor of ox.thr. and that yields of threonate (and therefore oxalate) from ox.thr. are low *in vitro*.

Cyc.ox.thr., ox.thr. and ox.thr. isomer appeared sooner in figure 3.16 than was observed during the oxidation of ascorbate with  $H_2O_2$ , being readily detectable at 0.083 min as opposed to 15 min (Fig. 3.3). The reasons for this are likely to be two-fold. Firstly, one less molecule of  $H_2O_2$  would have been required to oxidise DHA to ox.thr. than to oxidise ascorbate to ox.thr.



Secondly, and probably most importantly, the concentration of DHA in the solution post-purification was not known. It is possible that a high proportion of H<sub>2</sub>O<sub>2</sub> was used (thought to be approximately 20:1 H<sub>2</sub>O<sub>2</sub>:DHA) compared to the starting reagent in this experiment. Interestingly, although cyc. ox. thr. was not the obligatory precursor of ox. thr., only ox. thr. and thr. could be seen at 180 minutes. This suggested that although cyc.ox.thr. and ox.thr. were both produced directly from DHA, cyc. ox. thr. could yield ox. thr. under oxidising conditions so the cyc. ox. thr. pool became depleted prior to that of ox. thr., although ox.thr. is produced from cyc.ox.thr. by hydrolysis, not oxidation (Green and Fry 2005). Alternatively, if cyc.ox.thr. had been an acid anhydride, it could have been hydrolysed to yield threonate directly. The structure of cyc.ox.thr. is discussed in section 3.2.1.

These experiments showed that under strong oxidising conditions, DHA yielded cyc. ox. thr., ox. thr., thr. and, most probably, oxalate. Again, cyc. ox. thr. and ox. thr. were not produced in sequence although cyc. ox. thr. did seem to be capable of yielding ox. thr. The absence of DKG throughout the experiment was consistent with the hypothesis that strong oxidative conditions favoured the production of ox.thr. from DHA rather than the formation of DKG.



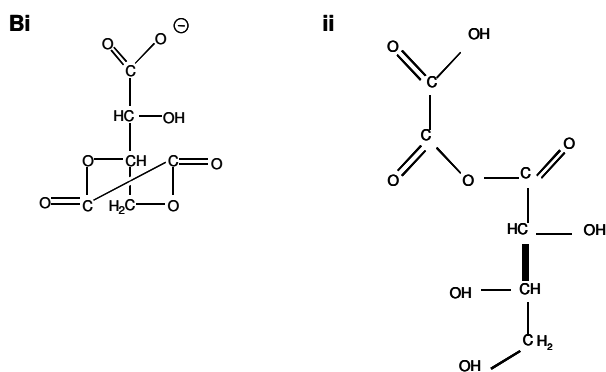
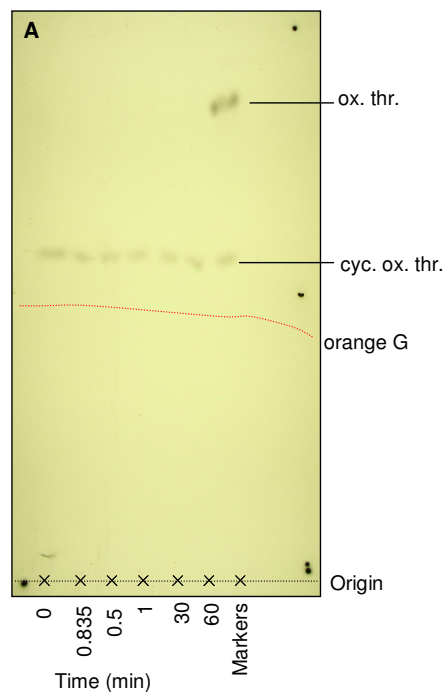
**Figure 3.16 The oxidation of pure DHA with  $H_2O_2$**

DHA was dissolved in 100% DMF. Negatively charged impurities that could have been formed during storage were removed by anion exchange chromatography (pyr<sup>+</sup> acetate<sup>-</sup> eluent 0.002 M pH 5). DHA was eluted off the column (estimated amount 0.001 mmol). This was oxidised with 0.02 mmoles of  $H_2O_2$  in a 0.5 ml reaction volume at pH 5. Samples were loaded in 20  $\mu$ l aliquots. The putative [<sup>14</sup>C]cyc.ox.thr. did not react with  $NaIO_4$  (Fig. 3.17a) and was therefore considered not to be an acid anhydride nor to break down directly to oxalate rather than to ox.thr. Had [<sup>14</sup>C]cyc.ox.thr. reacted with  $NaIO_4$ , a 2-C compound, [<sup>14</sup>C]glycollate, with a faster electrophoretic mobility than [<sup>14</sup>C]cyc.ox.thr. at pH 6.5, would have been observed.

### 3.2.2 The oxidative breakdown of DKG—experiment I

As compounds C and E had not been characterised, the mechanism by which they were produced (e.g. hydrolysis, oxidation) was not known. As they were believed to be produced non-enzymically from DKG, it was hoped that compounds C and E could have been synthesised from DKG and then isolated and identified. It had not been possible to determine the oxidative or hydrolytic breakdown products of DKG as very little DKG had been observed in experiments, possibly because experimental conditions had precluded the extensive formation of any DKG. The production of CO<sub>2</sub> during decarboxylation of DKG has been reported (Kagawa 1962) but no evidence for the generation of <sup>14</sup>CO<sub>2</sub> in rose cell cultures fed [1-<sup>14</sup>C]ascorbate had been reported (Green 2003). Analysis of the breakdown products of DKG by silver nitrate staining would also help determine what compounds could have been formed in the apoplast from DKG as previous studies involving the breakdown of [1-<sup>14</sup>C]ascorbate had only detected compounds that still contained the former C-1.

DKG, synthesised by hydrolysis of DHA and purified by anion-exchange chromatography, was incubated with H<sub>2</sub>O<sub>2</sub>. Products were analysed by electrophoresis at pH 2.0, pH 3.5 and pH 6.5. At pH 6.5 and at pH 3.5, silver nitrate staining revealed two compounds (Fig. 3.18b, c). These co-migrated with the threonate and DKG external markers, suggesting that threonate was the sole visible oxidation product. However, electrophoresis at pH 2.0 revealed three compounds (Fig. 3.18a). DKG was present at 0 min and a compound which migrated between DKG and un-charged compounds (electrophoresis at pH 2.0) was formed over time.



**Figure 3.17** Periodate oxidation of cyc.ox.thr.

A. [ $^{14}\text{C}$ ]Cyclic oxalyl threonate treated with  $\text{NaIO}_4$ . Samples were analysed by electrophoresis at pH 6.5 (35 min, 3.5 kV). The electrophoretogram was exposed to autoradiography film for 3 weeks. The autoradiogram is shown here overlying the electrophoretogram.

B. The present structure assigned to cyclic oxalyl threonate (i) and how the structure could be expected to appear if cyclic oxalyl threonate were an acid anhydride (ii). The bond that would be oxidised during the reaction of the acid anhydride is highlighted.

This compound seemed to peak in concentration at 20 – 60 min. An almost un-charged compound was observed to accumulate from 60 min onwards. This third compound was considered likely to be threonate, which is almost un-charged at pH 2.0. The maximum concentration attained by the second of these three compounds occurred as DKG showed a substantial decrease and threonate started to increase (Fig. 3.18a). This would be characteristic if this compound were an intermediate between DKG and threonate. In this study it is referred to as compound 2. Production of compound 2 was not dependent on the addition of H<sub>2</sub>O<sub>2</sub>. This is discussed in section 3.2.5.

The appearance of threonate seemed to occur earlier, at 5.20 min, at pH 6.5 (Fig. 3.18c) than at pH 3.5 (Fig. 3.18b) where it was not observed until 20 min. DKG seemed to persist until 120 min in the electrophoretogram run at pH 3.5 but only until 60 min in that run at pH 6.5. This could be explained if compound 2 were co-migrating with DKG at pH 3.5 and with threonate at pH 6.5, a hypothesis which would also explain why two compounds were observed at pH 3.5 and pH 6.5 but three were observed at pH 2.0. Compound 2 was isolated and electrophoresed at pH 6.5, pH 3.5 and pH 2.0 (Fig. 3.19), confirming the hypothesis. It was not known whether compound 2 would have been generated through the decarboxylation of DKG or would still retain C-1 and so, if it were produced *in vivo*, be radiolabelled. However, even if it were produced *in vivo*, the electrophoretic mobility would have meant that in previous studies it would have been missed as it co-migrated with DKG. Threonate was not proposed to be formed from DKG (Green and Fry 2005) but would not have shown up in radioactive products derived after feeding of [1-<sup>14</sup>C]ascorbate to cell suspension cultures.

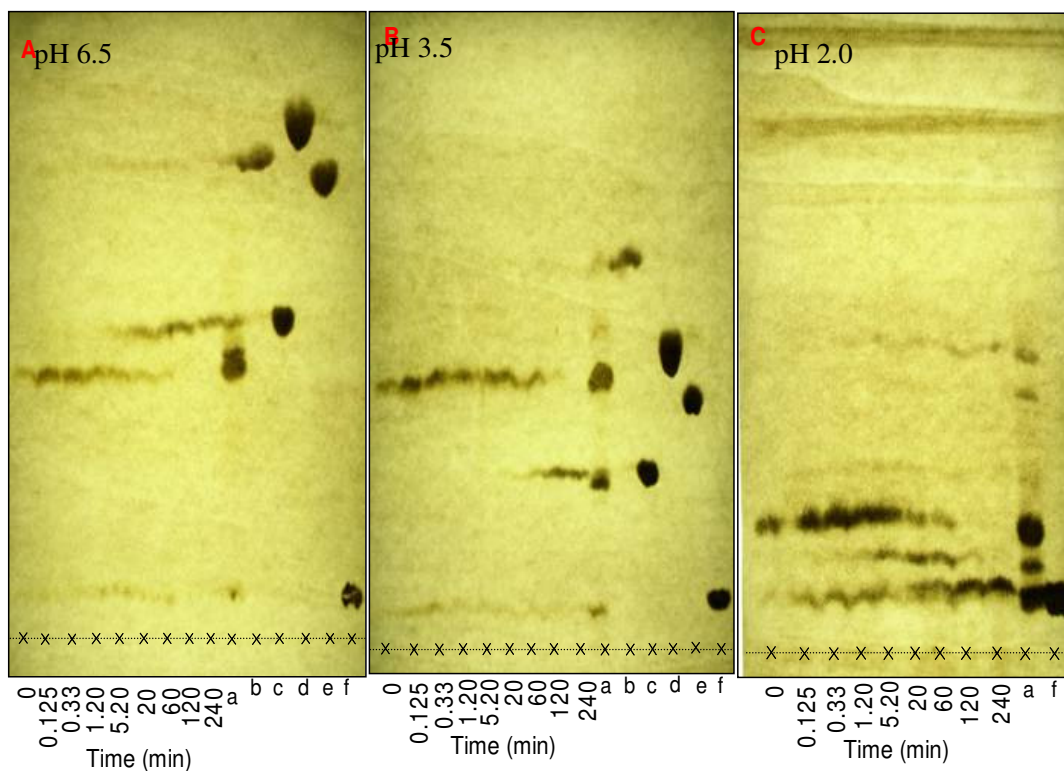
Neither compounds C nor E were observed during the incubation of DKG with H<sub>2</sub>O<sub>2</sub>. C and E were thought to be C<sub>6</sub> compounds (Green and Fry 2005), whilst threonate is a C<sub>4</sub> compound. The existence of threonate as an oxidation product of DKG would suggest that the formation of C/E and threonate are exclusive. It had been reported previously that certain

experimental conditions favoured different reaction products of DKG (Kagawa 1962). Alternatively, multiple C<sub>6</sub> and C<sub>4</sub> compounds could have been produced from DKG but C/E might not have stained with silver nitrate.

In figure 3.18 c, a very faintly staining mark was noticed that ran parallel with the ox.thr. marker but it was too faint for further analysis. DKG had not been proposed to yield ox.thr. but compound E migrated relatively close to ox.thr. at pH 6.5. So that the question of whether this was a genuine oxidation product of DKG could be addressed and so that compound 2 might be further analysed, solid DKG was synthesised to a high degree of purity following methods adapted from Kagawa (1962).

### **3.2.3 The oxidative breakdown of DKG—experiment II**

Following the successful synthesis of DKG to a high level of purity, DKG was once more oxidised with H<sub>2</sub>O<sub>2</sub> (Fig. 3.20). Under strong oxidative conditions, production of threonate was again observed. Compound 2 continued to show characteristics of being an intermediate compound, peaking in concentration at around 60 min. The starting concentration of DKG was sufficient to reveal that, as had been just discernable in figure 3.18c, a compound with a mobility at pH 2.0 similar to that of ox.thr. was detected with silver nitrate (Fig. 3.20). This compound was first seen at 30 min, shortly after the formation of the intermediate compound but before the appearance of threonate. Based on the reported mobility of compound E at pH 2.0 (Green 2003) the mobility of compound 4 was more characteristic of ox.thr. than of compound E at pH 2.0. This compound is referred to as compound 4. Threonate and compound 4 were the only two compounds visible at 480 min and so were considered to be end products of the reaction of DKG and H<sub>2</sub>O<sub>2</sub>.



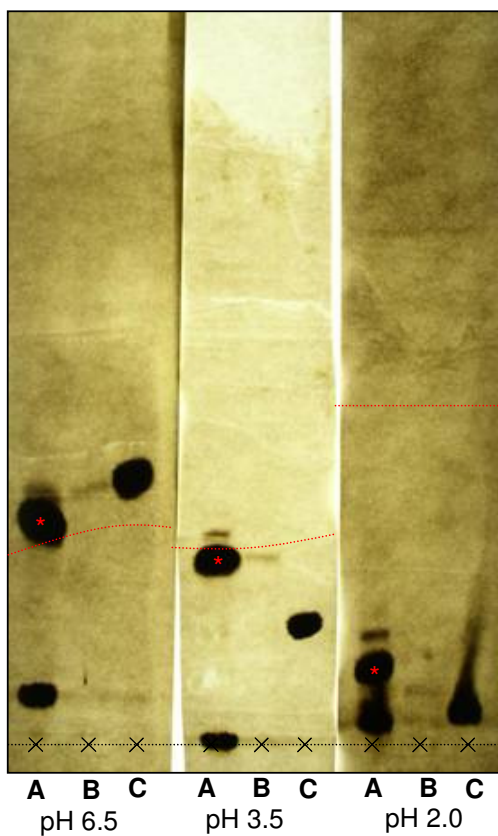
**Figure 3.18 The oxidation of DKG – experiment 1**

DHA was hydrolysed (0.75 M NaOH 1 min) to produce DKG. DKG was separated from residual DHA by anion exchange chromatography. Residual DHA was removed with  $\text{pyr}^+$  acetate $^-$  (0.002 M pH 5). DKG was then eluted with  $\text{pyr}^+$  acetate $^-$  (0.1 M pH 5). Eluted solutes (DKG approx. conc. 0.01 mM) were oxidised with 0.1 mM  $\text{H}_2\text{O}_2$ . Oxidation products (20  $\mu\text{l}$  aliquots) were analysed by electrophoresis. Silver nitrate stains of paper electrophoretograms are pictured here. External markers are to the right in each diagram (A, DKG; B, ox.thr; C, threonate; D, erythrate; E, threarate; F, glucose.) Only ox.thr. and not ox.thr. isomer in visible at pH 3.5 (B) as ox.thr. isomer, being less stable, is thought to convert to ox.thr. during elution from paper.

A. Analysis of oxidation products by electrophoresis at pH 6.5

B. Analysis of oxidation products at pH 3.5

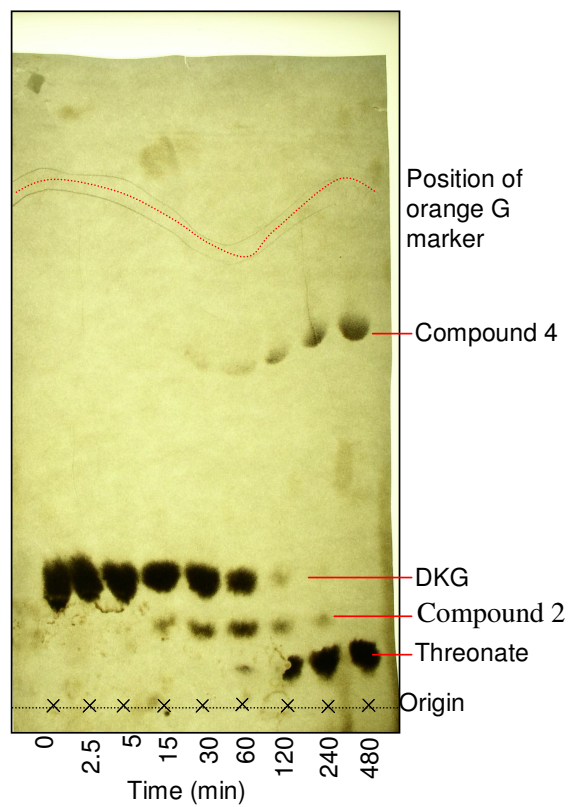
C. Analysis of oxidation products at pH 2.0



**Figure 3.19 The electrophoretic mobility of compound 2**

Compound 2 (B) was isolated by elution from paper following electrophoresis at pH 2.0, then subjected to electrophoresis at pH 6.5, pH 3.5 and pH 2.0 alongside a DKG marker (A) and a threonate marker (C). The DKG marker was synthesised from the hydrolysis of DHA. Of the three compounds in the preparation, DKG was identified by  $m_{OG}$  values at pH 6.5 reported in previous studies (Green 2003, Green and Fry 2005) and is asterisked here.





**Figure 3.20 The oxidation of purified DKG with H<sub>2</sub>O<sub>2</sub> –experiment 2**

DKG (final concentration 0.06 M, unbuffered, pH approx. 4.5) was oxidised with H<sub>2</sub>O<sub>2</sub> (final concentration 1 M). Oxidation products (20 µl aliquots) were analysed by paper electrophoresis at pH 2.0 (40 min, 3.5 kV).

### 3.2.4 Identification of compound 4

#### 3.2.4.1 Confirmation that compound 4 was neither compound C nor compound E

Compound 4 was tested for characteristics intrinsic to compound E. A diagnostic test for compound E is the lactonisation of E to form compound C upon treatment with acid and subsequent reversal of this process in the presence of alkali (Green and Fry 2005). Compound 4 was isolated and portions were treated with either NaOH or acetic acid. No charged compounds were observed after the treatment of compound 4/ E with alkali (Fig. 3.21). C and E had been shown to be charged at pH 2.0 (Green 2003). In theory, treatment with alkali should have resulted in no discernable change in migration at pH 2.0 had compound 4 been compound E. Acidification of compound 4 also resulted in the formation of compounds that were neutral at pH 2.0, though a small portion of compound 4 remained (Fig. 3.21). Even if neither compound C nor E stained with silver nitrate, these results suggest that compound 4 was neither C nor E because a neutral hydrolysis product, possibly threonate, had been formed. If compound 4 were ox.thr., then threonate (neutral at pH 2.0) would have been produced upon treatment with alkali.

#### 3.2.4.2 Compound 4 exhibits the characteristics of ox.thr.

A portion of the sample at 480 min in figure 3.20 was subjected to electrophoresis at pH 6.5, pH 3.5 and pH 2.0 (Fig. 3.22). At pH 6.5, compound E would be expected to run approximately  $\frac{2}{3}$  of the way between cyc.ox.thr. and ox.thr. markers. At pH 2.0, compound E should migrate slightly closer to the cathode than ox.thr. Notwithstanding the poor staining of markers at pH 3.5, the mobility of compound 4 compared to that of ox.thr. (Fig. 3.22) indicated that compound 4 was, in fact, ox.thr. It was also possible to identify the compound that was neutral at pH 2.0 in the 480 min sample as threonate. As the electrophoretogram at pH 3.5 had stained so poorly it was not possible to tell whether both isomers of ox.thr. were present.

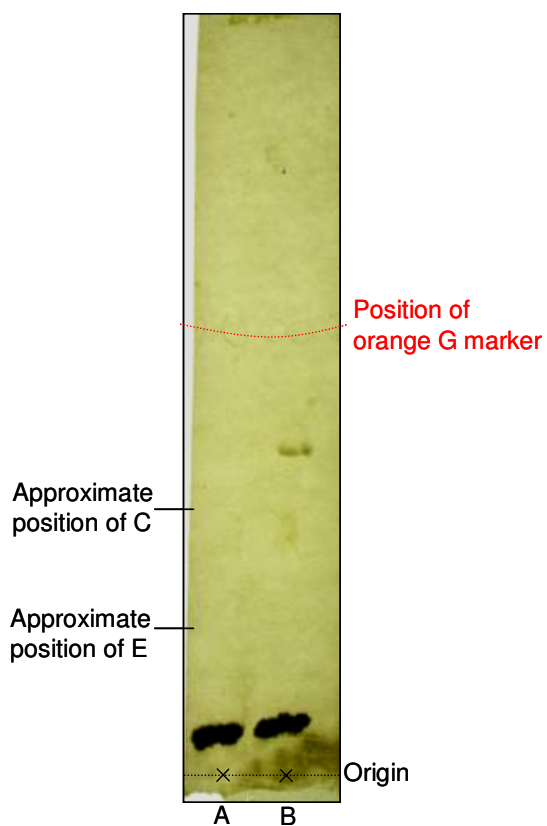


Figure 3.21 Treatment of compound 4 with either acid or alkali

Compound 4 was isolated and treated with either 1 M NaOH for 4 hours (track A) or 2 M acetic acid for 4 hours (track B). Samples were then neutralised with either 1 M NaOH or 2 M acetic acid. Products were analysed by paper electrophoresis at pH 2.0 (30 min, 3.5 kV). Positions of compounds C and E were estimated based on data from Green (2003).

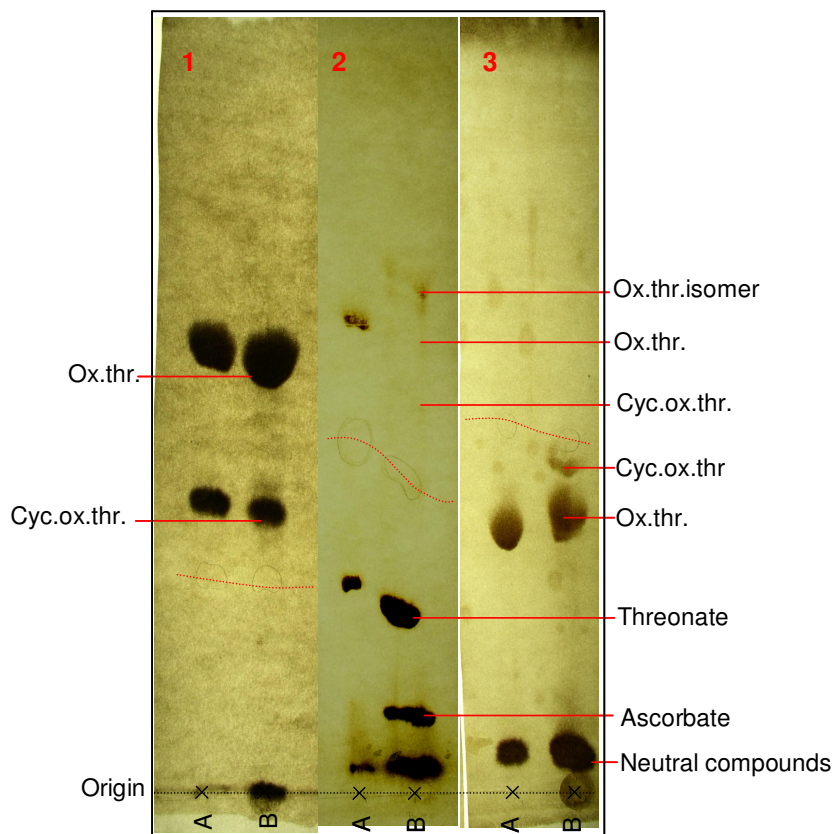


Figure 3.22 The electrophoretic mobility of compound 4  
 The oxidation products present at 480 minutes (threonate and compound 4) in figure 3.19 were subjected to paper electrophoresis buffered at pH 6.5 (1), pH 3.5 (2) and pH 2.0 (3). Oxidation products (track A) were run next to external makers (track B). Ox.thr. was not loaded in sufficient quantity in (2) and so stained very faintly.

#### 3.2.4.3 Analysis of the structure and identity of compound 4 by HPLC

The similarity in electrophoretic mobility at all three pH's was not considered a sufficient basis on which to conclude that compound 4 and ox.thr. were one and the same. Before accepting the identity of compound 4 as that of ox.thr, compound 4 was tested for its ability to be hydrolysed to oxalate and threonate. The formation of oxalate and threonate via hydrolysis is a good test for the presence of ox.thr. As oxalate does not stain with silver nitrate, it was necessary to analyse products of this test with HPLC.

Both compound 4 and ox.thr. were eluted off the same paper electrophoretogram so as to minimise any differences that might conceivably alter retention time on the column. The preparation of ox.thr. had a retention time of 9.97 min with a shoulder eluting at 9.59 min (Fig. 3.23a). Previously (Fig. 3.13), a retention time of 10.00 min had been found for ox.thr, with a shoulder at 9.63 min that was thought to be ox.thr. isomer. Some oxalate and threonate was present at the start from hydrolysis during the isolation procedure (Fig. 3.23 a). Compound 4 had a retention time of 10.01 minutes with traces of oxalate and threonate and a slight shoulder around 9.5 minutes (Fig. 3.23b). Although this was very similar to the results with oxalyl threonate, the peak had a slightly broader shape as if multiple compounds might have been eluting together. When a mixture of the two compounds was applied to the column (Fig. 3.23c) a retention time of 9.99 minutes was recorded, which suggested that the retention times of the individual compounds might have been very slightly altered.

Upon treatment of ox.thr. with NaOH, the main peak of ox.thr. decreased. This was accompanied by an increase in the shoulder peak before any increase in threonate or oxalate was measured (Fig. 3.24a), as if isomerisation took place to yield both ox.thr. and ox.thr. isomer prior to hydrolysis. In figure 3.01, ox.thr. had appeared to hydrolyse very readily. Between 0.5 and 1 h there was a marked decrease in ox.thr. and increase in oxalate and threonate. This was not so apparent in the case of threonate whose molar absorbance at 210 nm had been found to be low

(Fig. 3.12h). After 2 h of exposure to NaOH ox.thr. and ox.thr. isomer had been completely hydrolysed into threonate and oxalate, as predicted by previous findings (Fig. 3.06).

The retention time of compound 4 (Fig. 3.24b) was marginally longer than that of ox.thr. in figure 3.24a, at 10.15 min as opposed to 10.00 min. The main peak was also broader, as if it contained more than one co-eluting compound. Otherwise, the behaviour of compound 4 was similar to that of ox.thr. within the first hour of addition of NaOH. After the first hour the retention time of the broad, main peak became longer; 10.20 min after 1 h, 10.25 min after 2 h. Meanwhile, the peak became sharper, more typical of a single compound. During the hydrolysis of compound 4 (Fig. 3.24b) oxalate and threonate increased, though not as much as during the hydrolysis of ox.thr. (Fig. 3.24a).

The interpretation of these results is that compound 4 consisted of ox.thr. and a second, unidentified compound which does not contain an ester bond that can be hydrolysed by NaOH. The ox.thr. present in both samples was hydrolysed to produce threonate and oxalate. In figure 3.24b the unidentified compound, eluting close to ox.thr. distorted the retention time and shape of the ox.thr. peak and *vice versa*. As ox.thr. was hydrolysed, this distortion was resolved. It would seem then that ox.thr, under these experimental conditions, can therefore be produced from the oxidation of DKG. An important consideration is whether this will actually have any impact on the catabolism of DKG in the apoplast *per se*. Some time is given to this in section 3.2.6.

## **3.2.5 Analysis of compound 2**

### **3.2.5.1 Oxidation of compound 2**

Ox.thr. can be hydrolysed to yield oxalate and threonate. It was not possible therefore to be certain whether the threonate produced during the oxidation of DKG was a product of compound 2 or compound 4. If it were being produced by compound 2 this would represent two

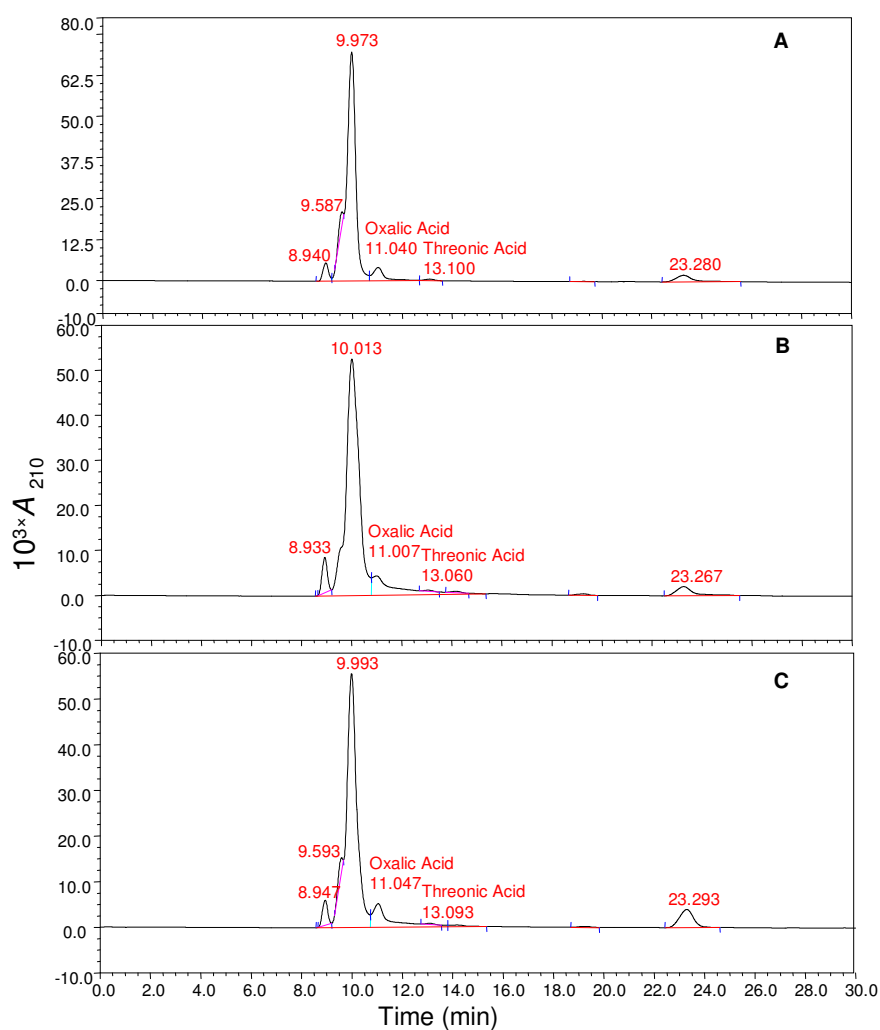


Figure 3.23 HPLC analysis of compound 4 compared to ox.thr.

Compound 4, ox.thr. and a mixture of compound 4 and ox.thr. were analysed on an anion exclusion column with a sulphuric acid eluent (47 mM ). Compounds were detected by their absorbance at 210 nm. The peak at 8.9 min represents column contents which eluted before the sample, containing larger compounds that will have been eluted off the paper electrophoretogram. The peak at 23.3 min was considered to be a contaminant from the electrophoresis buffer.

A. Ox.thr.

B. Compound 4

C. Ox.thr. + compound 4 (50:50)

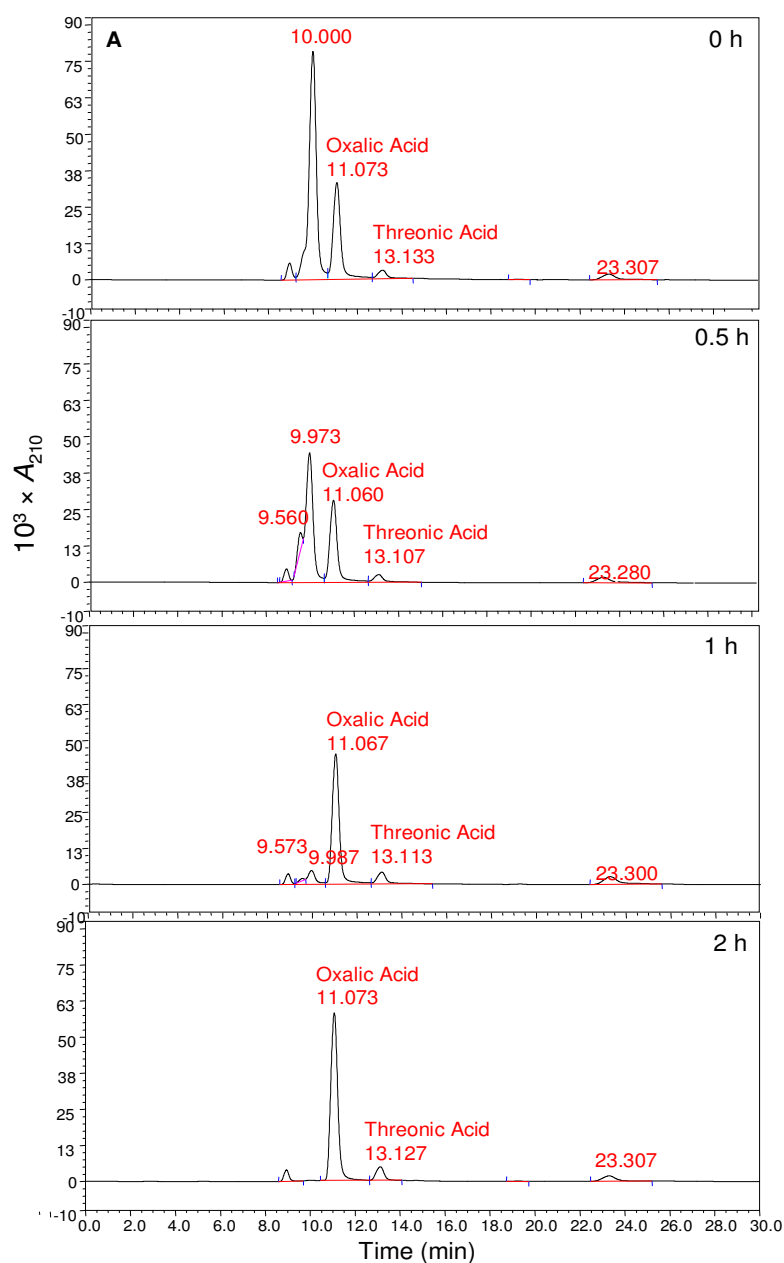


Figure 3.24 Hydrolysis of compound 4 and ox.thr.

Ox.thr. and compound 4 were isolated by elution off a paper electrophoretogram was treated with 0.1 M (initial concentration) NaOH. Samples were removed at time points and treated with acetic acid. HPLC analysis was as detailed as in figure 3.23. The peak at 8.9 minutes represented the void volume and the peak at 23.3 minutes was considered to be a contaminant from the electrophoresis buffer. In a previous hydrolysis of ox.thr. (Fig. 3.06), lower final concs. of ox.thr. and higher final concs. of NaOH were used. The half life of ox.thr. was shorter (Fig. 3.06) but the disparity in half life was greater than expected.

A. Hydrolysis of ox.thr.



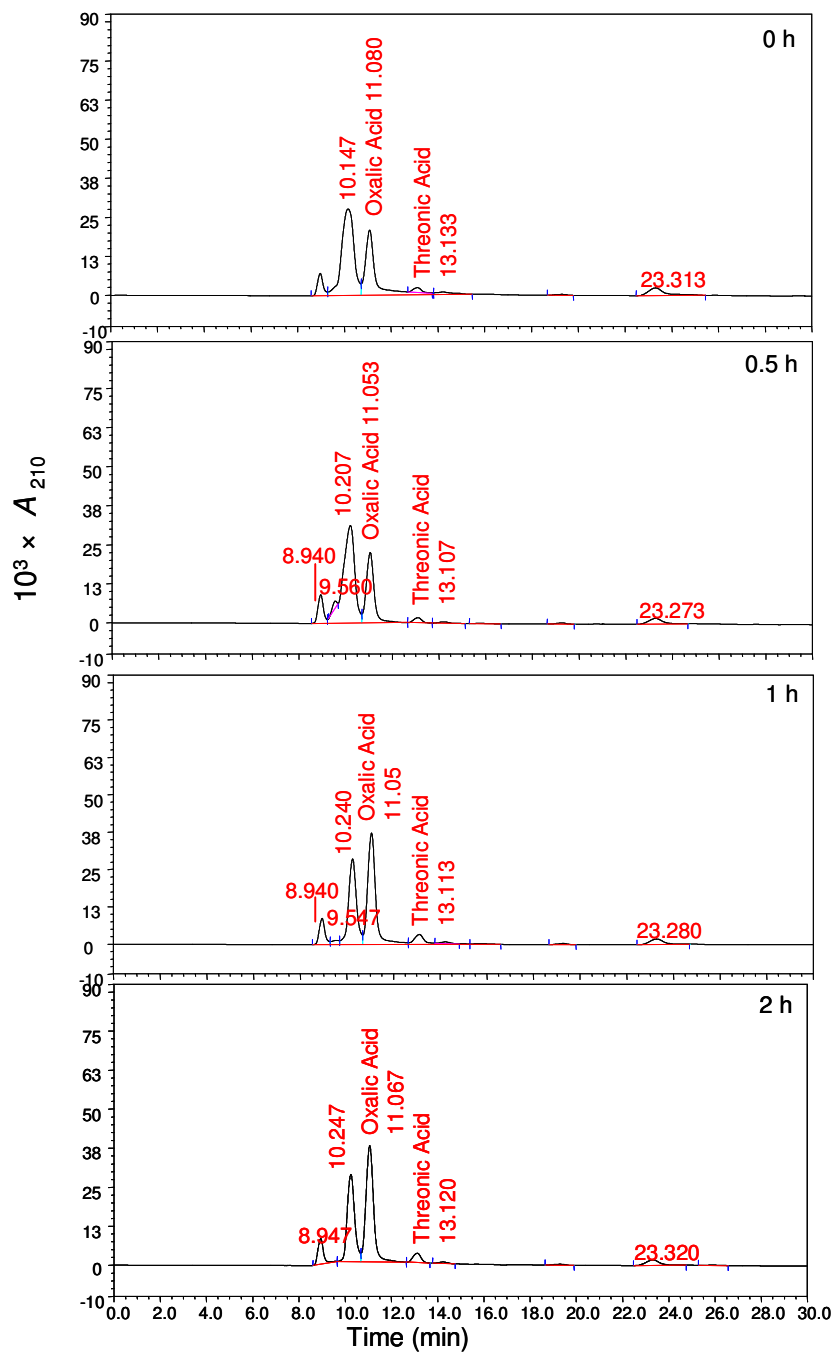


Figure 3.24 Hydrolysis of compound 4 and ox.thr.

B. Hydrolysis of compound 4

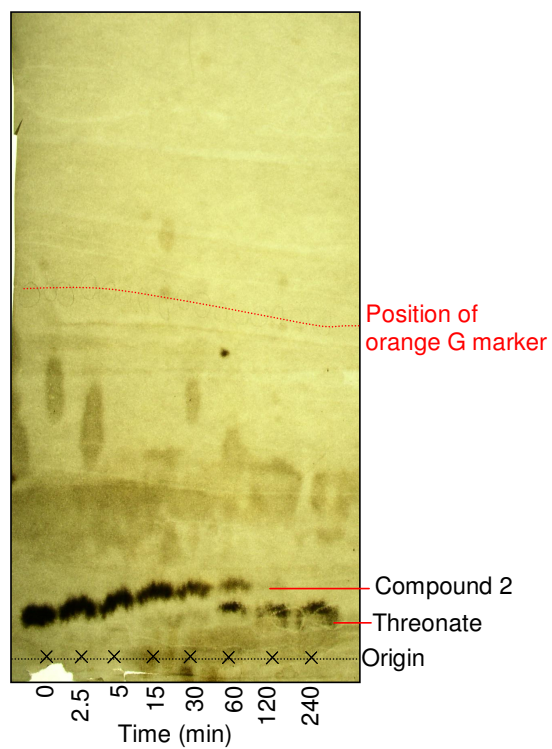


Figure 3.25 Oxidation of compound 2

Compound 2 was isolated by elution off a paper electrophoretogram (approx. final conc. 0.01M) and oxidised with  $\text{H}_2\text{O}_2$  (final concentration 0.5 M). The solution was unbuffered with a pH of approx. 4.5. Products were analysed by paper electrophoresis at pH 2.0 (30 min 3.5 kV).

possible routes by which DKG could yield threonate. If this were to occur *in vivo*, this would mean three routes by which threonate could potentially be formed from ascorbate (2 via DKG, 1 as described by Green and Fry 2005). When compound 2 was oxidised with H<sub>2</sub>O<sub>2</sub>, threonate was the only product detected (Fig. 3.25). The concentration of compound 2 was sufficient that all products staining with silver nitrate would have shown up. This demonstrated that ox.thr. was not a product of compound 2 and opened up the possibility that compound 2 was a C<sub>5</sub> or C<sub>4</sub> intermediate in between DKG (a C<sub>6</sub> compound) and threonate (a C<sub>4</sub> compound).

#### 3.2.5.2 Xylonate and 2-keto-xylonate as candidates for compound 2

Experimental conditions depending, threonate, xylonate and lyxonate have been suggested as possible C<sub>4</sub> or C<sub>5</sub> metabolites of DKG as well as oxalate (Bánhegyi and Loewus 2004). All these products bar oxalate would not have been labelled if [1-<sup>14</sup>C]ascorbate had been the starting compound as they are thought to be produced via decarboxylation of DKG at C-1. 2-Keto-L-xylonate had been suggested as a possible C<sub>5</sub> product of DKG that would have not been labelled from feeding [<sup>14</sup>C]ascorbate if decarboxylation had occurred (Green and Fry 2005).

#### 3.2.5.3 Synthesis of 2-keto-xylonate

2-Keto-xylonate is not available commercially and so had to be synthesised from xylulose- 5-phosphate. The details of this synthesis are outlined in section 2.10.5. This was essentially a 2-step synthesis. In the first step, D-xylulose-5-phosphate was partially oxidised to the 2-keto- D-xylonate- 5-phosphate (Fig. 3.26). The second step involved the removal of the phosphate group with phosphatase (Fig. 3.27) to yield 2-keto- D-xylonate, which would [<sup>14</sup>C]oxalate-migrate with the expected product, 2-keto- L-xylonate.

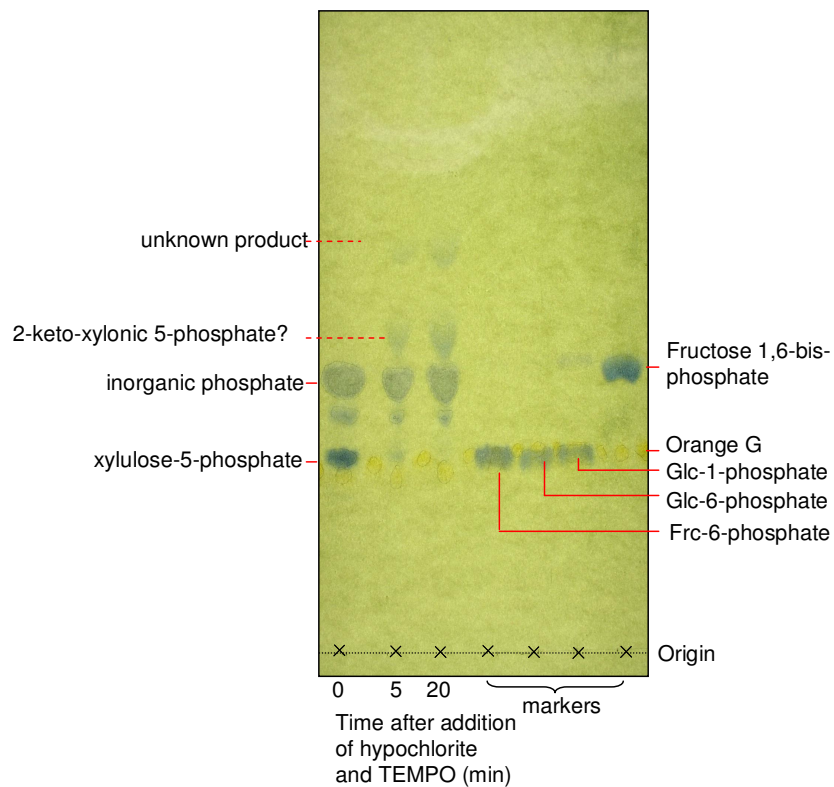


Figure 3.26 The synthesis of 2-keto-xylonic 5 phosphate  
 Xylulose- 5-phosphate was oxidised with Na hypochlorite + TEMPO (see section 2.15.2).  
 Products were analysed by paper electrophoresis at pH 6.5 (30 min, 3.5 kV) and molybdate  
 staining. Compounds whose identity is uncertain are marked with a dashed line.

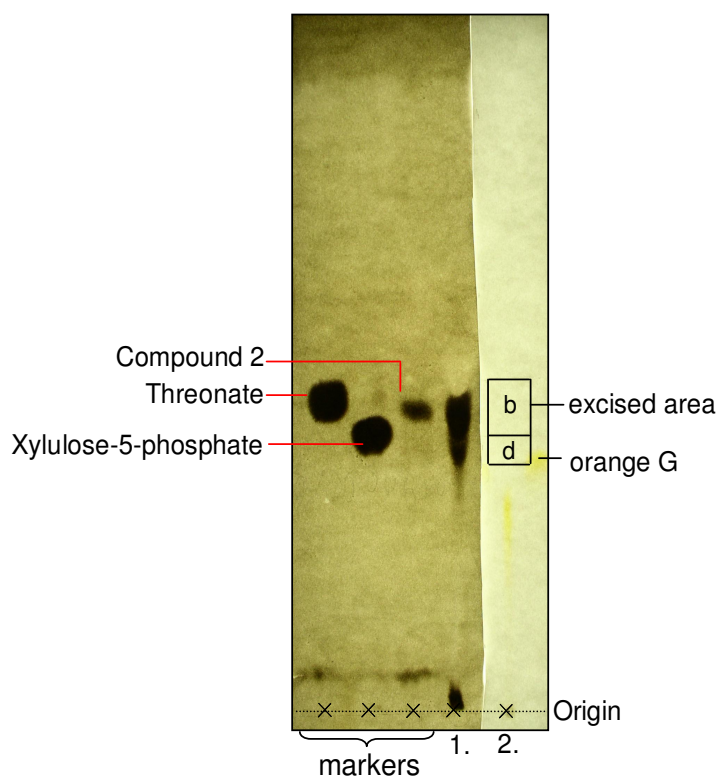


Figure 3.27 The synthesis of 2-keto-xylonate from 2-keto-xylonate 5-phosphate  
2-Keto-xylonate 5-phosphate was treated with acid phosphatase (final concentration 1% w/v) for 24 h. The positions of markers and a portion of the product (track 1) were determined by silver nitrate staining, following paper electrophoresis at pH 6.5. An unstained portion of the product (excised area and track 2) was eluted for use as markers in figure 3.28 (area 'b' used as marker 'b', area 'd' as marker 'd' Fig. 3.28)

#### 3.2.5.4 Compound 2 is neither xylonate, lyxonate or 2-keto-xylonate

Compound 2 was subjected to electrophoresis at pH 2.0, pH 3.5 and pH 6.5 alongside markers for xylonate and 2-keto-xylonate. Xylonate *and* lyxonate are isomers (Nelson and Cox 2000) and were expected to show similar electrophoretic mobility. After electrophoresis at pH 2.0, compound 2 was seen to migrate ahead of xylonate (Fig. 3.28a). Electrophoresis at pH 3.5 and pH 6.5 revealed a difference in the mobility of xylonate and compound 2 (Fig. 3.28b, c). It was surmised that compound 2 was neither xylonate, lyxonate nor 2-keto-xylonate.

Compound 2 was only slightly more mobile than DKG at pH 6.5. If it had two or more carboxyl groups it would have been expected to show much greater mobility than DKG as was the case with ox.thr, threarate and erythrate, which possess two negative charges (Green and Fry 2005). Compound 2 appeared to have a higher  $pK_a$  than DKG, as mobility was less than DKG at pH 2.0. It was hard to see therefore, how compound 2 could have been a (C<sub>6</sub>, 1-) compound and have a greater charge:mass ratio than DKG at pH 6.5, equally at pH 3.5 and yet have a higher  $pK_a$  than DKG. The most simple explanation for the electrophoretic mobility of compound 2 in relation to the markers compounds was that compound 2 was either a C<sub>4</sub> or a C<sub>5</sub> compound.

#### 3.2.5.5 The evolution of O<sub>2</sub> or CO<sub>2</sub> during the oxidation of DKG

During the oxidation of DKG with H<sub>2</sub>O<sub>2</sub> it had been noticed that bubbles of gas evolved. This could have been either O<sub>2</sub>, formed from the degradation of H<sub>2</sub>O<sub>2</sub>, or CO<sub>2</sub> produced during the decarboxylation of DKG. If the gas were CO<sub>2</sub>, this would point towards compound 2 being a C<sub>5</sub> compound. The gas evolved was analysed by gas chromatography (GC). Aqueous solutions of DKG, buffered at pH 6, were oxidised with H<sub>2</sub>O<sub>2</sub>. As a control for the effect of the instability of diluted H<sub>2</sub>O<sub>2</sub> at room temperature, air was gathered from a sealed vial containing H<sub>2</sub>O<sub>2</sub> and

water (Fig. 3.29b). Air was also sampled from a solution of DKG which had not been oxidised with  $\text{H}_2\text{O}_2$  so that the role of  $\text{H}_2\text{O}_2$  in decarboxylation of DKG could be assessed (Fig. 3.29c).

Analysis of air samples by  $\text{CO}_2$  GC showed that air from tubes containing DKG and  $\text{H}_2\text{O}_2$  were heavily enriched in  $\text{CO}_2$  (Fig. 3.29a). No evidence of  $\text{CO}_2$  evolution was seen when only  $\text{H}_2\text{O}_2$  and water were present (Fig. 3.29b). When GC specific for the detection of  $\text{O}_2$  was performed on all samples, no evidence for the generation of  $\text{O}_2$  was observed (results not shown). Interestingly,  $\text{CO}_2$  seemed to have been formed even in the absence of  $\text{H}_2\text{O}_2$  (Fig. 3.29c). Concentrations were slightly lower than in the presence of  $\text{H}_2\text{O}_2$  but were still far above the  $\text{CO}_2$  levels of ambient air. If compound 2 were present in this vial this would suggest that compound 2 was important in both the oxidative and non-oxidative breakdown of DKG.

### **3.2.6 The oxidation and hydrolysis of DKG at pH 6**

The generation of  $\text{CO}_2$  had been measured in solutions of DKG buffered at pH 6 in the presence and absence of  $\text{H}_2\text{O}_2$  (Fig. 3.29). However, the occurrence of compound 2 during these reactions had not been checked. Compound 2 had shown the characteristics of an intermediate compound between DKG and threonate, reaching maximal concentrations at around 60 min (Fig. 3.20). However, the solution in figure 3.20 was unbuffered. It was important to know whether production of compound 2 occurred at pH 6.0 as cell suspension cultures, in which compounds C and E had been observed, had been buffered between pH 6.0 and pH 6.5.

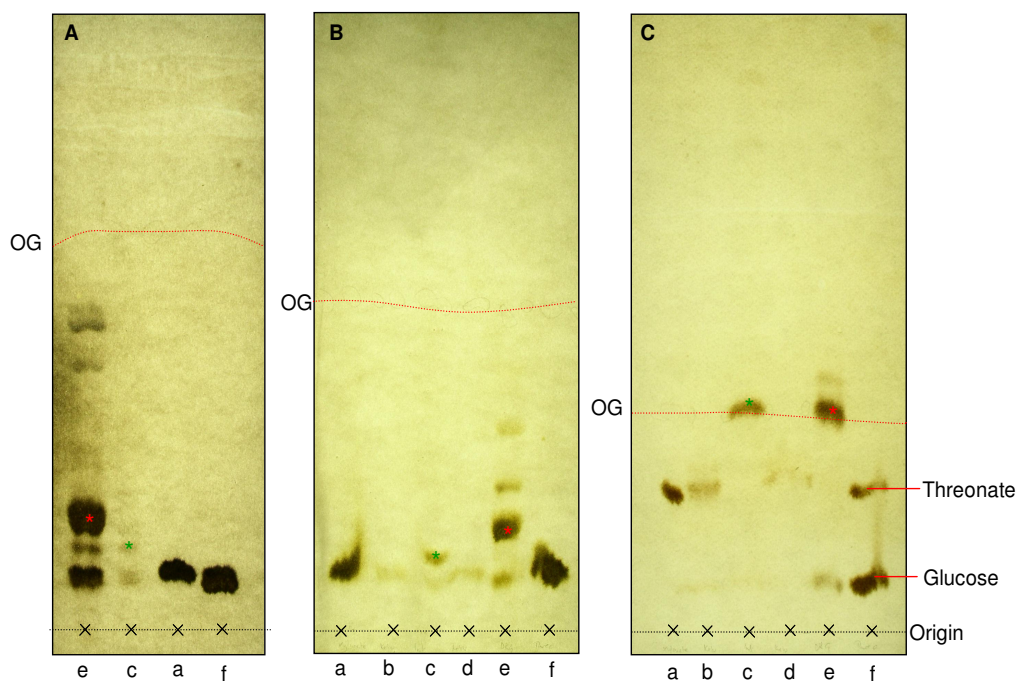


Figure 3.28 Electrophoretic mobility of the intermediate compound 2 compared to xylonate and 2-keto-xylonate

Compound 2 (c) was isolated by elution off a paper electrophoretogram and run alongside candidate compounds xylonate (a) and 2-keto-xylonate (b, d). DKG (e) and a threonate-glucose mixture (f) were included as additional markers. Some impurities were present in the DKG marker so DKG has been asterisked in red. The compound 2 has been asterisked in green. OG = orange G. Silver nitrate stains of electrophoretograms are pictured.

A. Electrophoresis at pH 2.0

B. Electrophoresis at pH 3.5

C. Electrophoresis at pH 6.5



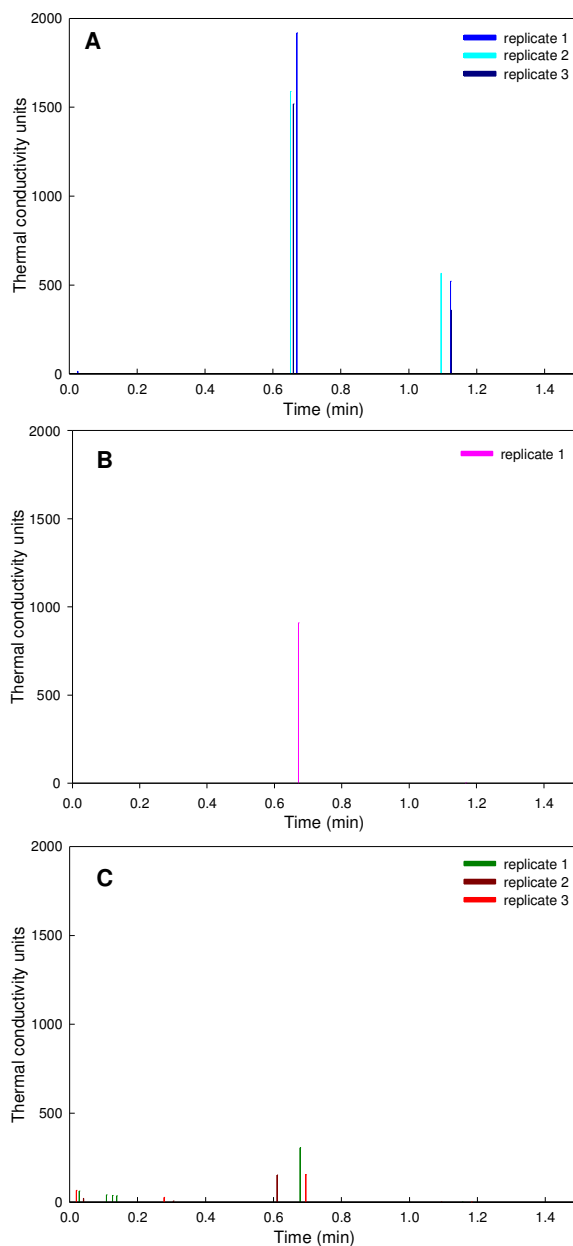


Figure 3.29 The evolution of CO<sub>2</sub> during the oxidation and hydrolysis of DKG

GC analysis of gas evolved during the oxidation or hydrolysis of DKG. The CO<sub>2</sub> peak elutes at approx. 0.7 min.

DKG (final concentration 150 mM) was either oxidised with H<sub>2</sub>O<sub>2</sub> (final concentration 300 mM) or had extra water added. Solutions were unbuffered, at approx. pH 4.5 in A, B and pH 6 in C. As a control, H<sub>2</sub>O<sub>2</sub> (final concentration 300 mM) was diluted with water. Solutions (final volume 16 ml) were sealed in air-tight vials with a rubber seal pierced with a hypodermic needle through which gas was collected after 1 h. Solutions of DKG + H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> + water were triplicated. Bars represent the maximum height of peaks which showed normal distribution curves.

A. DKG + H<sub>2</sub>O<sub>2</sub>. The identity of the peaks at 1.5 min are not known.

B. DKG + water

C. H<sub>2</sub>O<sub>2</sub> + water

DKG appeared to be less stable at pH 6.0 than lower pHs; several degradation products were seen at 0 min, just after the addition of H<sub>2</sub>O<sub>2</sub> and/or buffer (Fig. 3.30a). Compound 2 was present before the oxidation reaction (Fig. 3.30a) but was relatively short-lived, though whether this was a result of the increased proportion of H<sub>2</sub>O<sub>2</sub> to DKG or because the solution was buffered at pH 6.0 was not clear. DKG was also short lived during the oxidation reaction, being undetectable by 1 min (Fig. 3.30a). Compound 2 was present throughout the hydrolysis of DKG (Fig. 3.30b), as had been predicted from the evolution of CO<sub>2</sub> in the absence of H<sub>2</sub>O<sub>2</sub> (Fig. 3.29c). Other than the rapid disappearance of DKG in the presence and absence of H<sub>2</sub>O<sub>2</sub> and compound 2, the main difference between the behaviour of DKG was the observation of a compound which migrated ahead of DKG (towards the anode) but behind compound 4/ox.thr. This compound this showed a similar mobility to compound C (Green 2003) during electrophoresis at pH 2.0.

If this compound was C or E then this would imply that under conditions which favoured the formation of C and/or E from DKG before 0 min in figure 3.30, compound 2 and therefore probably threonate were also produced. This implies that compound 2 and threonate could be expected to have formed in 5-day-old cell culture medium where maximal production of compound E had been measured in the spent culture medium of 5-day-old cell suspension cultures after 8 h (Green and Fry 2005).

The model for the *in vitro* oxidation of ascorbate, in which DHA was oxidised to both cyc.ox.thr. and ox.thr. was expanded to include the hydrolysis of DHA to DKG (Fig. 4.1). In this model, the fate of DHA (either oxidation or hydrolysis) is largely determined by reaction conditions. Threonate was predicted to be formed whether hydrolysis or oxidation was the principal process by which reactions occurred.

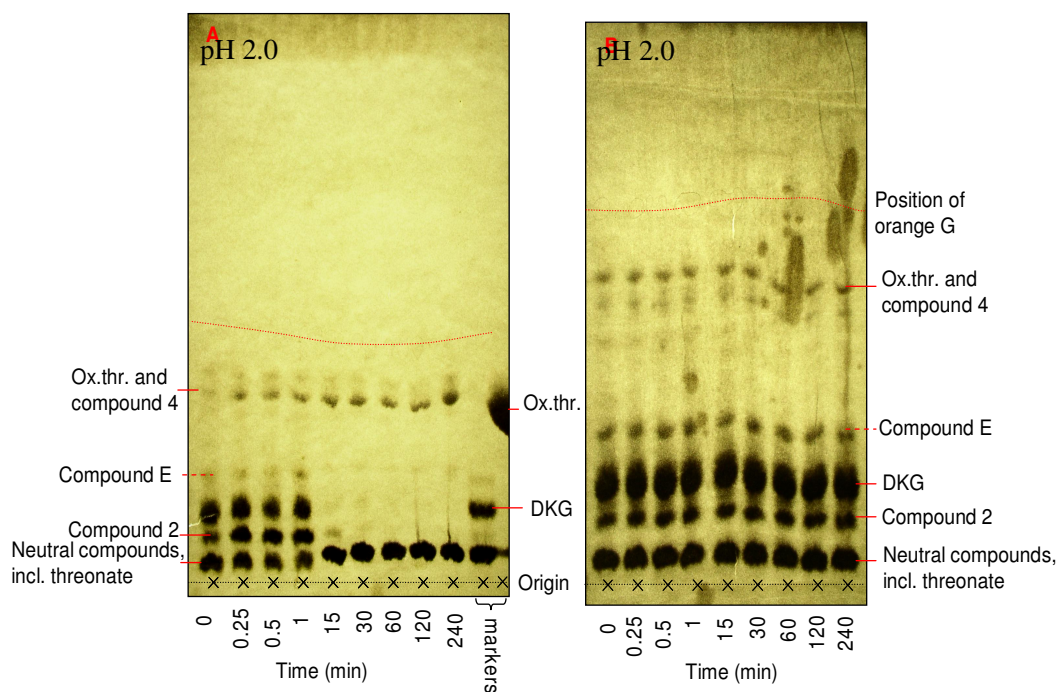


Figure 3.30 The oxidation and/or hydrolysis of DKG at pH 6.0

In the previous study (Green and Fry 2005) where DKG was hypothesized to breakdown to compounds C and E, cell culture medium was buffered at pH 6.0. The *in vitro* breakdown of DKG at pH 5.5 - 6.0 was therefore studied at pH 6.0.

A DKG (final concentration 0.09 M) solution ( $\text{pyr}^+ \text{acetate}^-$ , pH 6.0, final concentration 0.09 M) was either oxidised with  $\text{H}_2\text{O}_2$  (final concentration 0.16 M) or was given an equivalent volume of water. Compounds whose identity was uncertain are marked with a dashed line.

A. DKG +  $\text{H}_2\text{O}_2$ .

B. DKG + water

### 3.2.7 The catabolism of DKG in spent cell culture medium

Production of  $^{14}\text{CO}_2$  was not reported to have been a major part of apoplastic [ $^{14}\text{C}$ ]ascorbate catabolism in 5-day-old cell suspension cultures (Green 2003). This shows that extensive decarboxylation of DKG to yield compound 2 and threonate was not occurring. Spent 5-day-old cell culture medium was incubated with DKG for up to 6 h. Unless [ $^{14}\text{C}$ ]DKG was used, or compounds analysed by HPLC, results were best assayed by  $\text{AgNO}_3$  staining. For DKG to be visible by  $\text{AgNO}_3$  staining, high concentrations had to be used; culture medium was incubated with DKG at 25 mM. DKG catabolism was compared in water and spent, cell-free, culture medium. Samples did not run properly during electrophoresis at pH 3.5 and pH 6.5 (results not shown), so only results after electrophoresis at pH 2.0 are shown (Fig. 3.31).

DKG, incubated with both water and spent culture medium, stained clearly (Fig. 3.31). The heavily staining neutral compound was glucose, the carbon source used in, some of which would still have been present 5 days after subculturing. No other compounds over the 6 h time course were detected in spent cell culture medium (Fig. 3.31). During electrophoresis at pH 2.0, neither compound C nor E was expected to be uncharged and so if present would have still been visible even in the presence of glucose. As putative  $\text{C}_6$  organic acids, compounds C and E were considered likely to stain with silver nitrate. It is possible though, that they did not. Although only a qualitative analysis of results was possible, no decrease in the intensity of the DKG spots was noticed in either spent cell culture medium or the water control (Fig. 3.31).

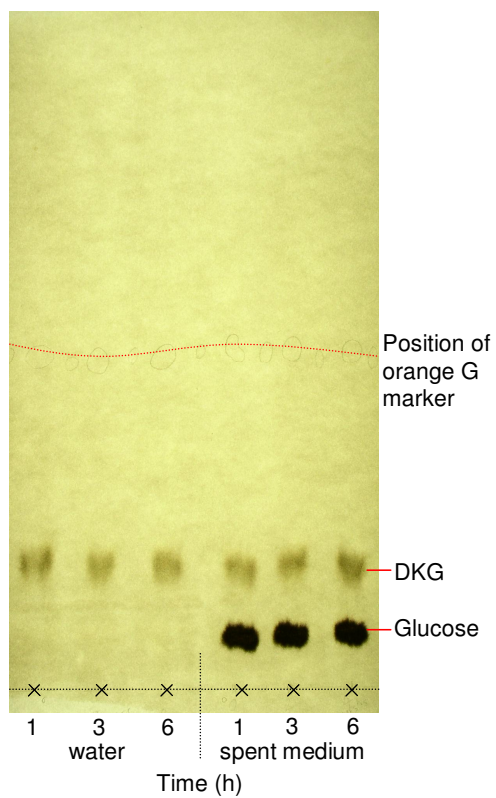


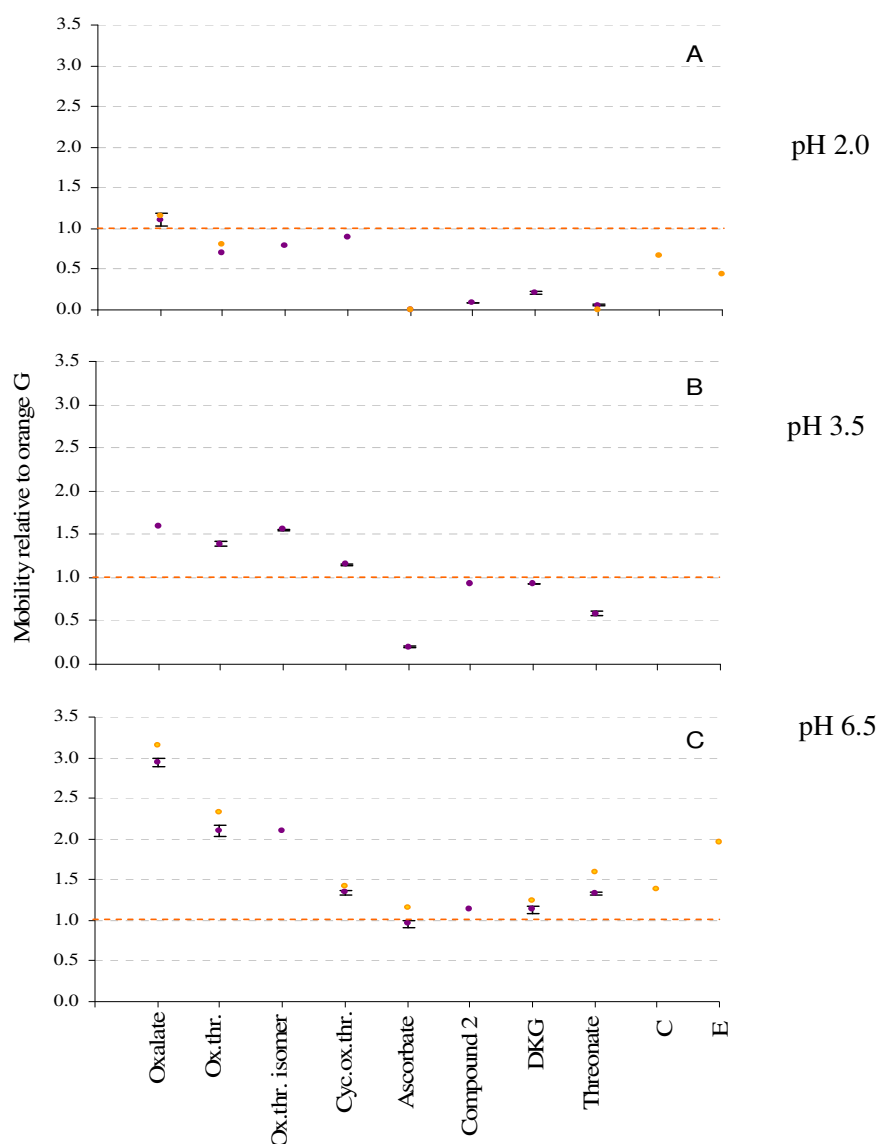
Figure 3.31 The catabolism of DKG in spent cell culture medium

DKG (final concentration 25 mM) was added to either water or spent 5-day-old cell culture medium. Solutions were buffered with MES (5 mM final concentration , pH 5.5). Samples were analysed by paper electrophoresis at pH 2.0 (30 min, 3.5 kV).

These results indicate that 25 mM DKG is not catabolised further in spent 5-day-old cell suspension culture medium. This is at odds with the findings in the above sections where compound 2 formed readily in a solution of DKG buffered at pH 6.0 (Fig. 3.30). Compound 2 would have separated from uncharged compounds during electrophoresis at pH 2.0 and therefore have been detectable if it were present. The formation of compound E from [<sup>14</sup>C]ascorbate has been found to be dependent on the starting concentration of ascorbate; formation of E was inhibited at higher concentrations (S. C. Fry, unpublished results). A comparison of DKG metabolism in figure 3.31 with results published by Green and Fry (2005) is difficult as concentrations of DKG were so different. The synthesis of DKG to high level of purity by the adaptation of methods published by Kagawa (1962) show that [<sup>14</sup>C]DKG could probably be synthesised from a mixture of [<sup>14</sup>C]ascorbate and cold ascorbate to a reasonable degree of purity. The catabolism of [<sup>14</sup>C]DKG in spent 5-day-old cell culture medium and concurrent analysis of <sup>14</sup>CO<sub>2</sub> production would answer many of the remaining questions as to the metabolism of DKG in the apoplast. It would not, however, answer questions concerning the formation of DKG in the apoplast in the first place. This matter is dealt with in subsequent chapters.

### **3.2.8 Summary of the electrophoretic mobility of ascorbate, DHA and DKG breakdown products**

The electrophoretic mobilities of the oxidation products of ascorbate, DHA and DKG at pH 2.0, pH 3.5 and pH 6.5 were combined and reproduced in figure 3.32. This was used for the identification of metabolites *in vivo* in the next chapters.



**Figure 3.32 Electrophoretic map of measured mobilities relative to orange G external marker**

Mobility was measured as the distance moved relative to orange G ( $m_{OG} = 1$ ) and to a neutral compound ( $m_{OG} = 0$ ) at pH 2.0, pH 3.5 and pH 6.5. Neutral compounds were found to migrate towards the anode as a result of a phenomenon known as endo-electro osmosis. Where no mobility is shown for a compound, the compound was not observed at that pH. Data were taken from this study (●) and a previous study (●) (Green 2003). Error bars (SE) represent samples sizes varying between 3 and 8. The position of the orange G markers is represented by the orange dashed line.

A. pH 2.0

B. pH 3.5

C. pH 6.5

### 3.3 The radiolabelling of endogenous ascorbate

#### 3.3.1 The choice of radiolabelled ascorbate precursor

Intracellular and extracellular ascorbate metabolism in response to oxidative stress was investigated in cell extracts and culture medium of cells that had been loaded with radiolabelled ascorbate.

A cell suspension culture in which the cells were loaded with radiolabelled ascorbate could, in theory, be achieved in two ways: either by feeding a radiolabelled ascorbate precursor or by feeding radiolabelled ascorbate itself. Ascorbate has been shown to be relatively unstable in cell culture medium (Green and Fry 2005). Endogenous synthesis of radiolabelled ascorbate was preferred over feeding of radiolabelled ascorbate as then the culture medium would not contain any radiolabelled breakdown products of exogenous [ $^{14}\text{C}$ ]ascorbate that could have interfered with analysis. The last step of ascorbate synthesis occurs on the inner mitochondrial membrane (Wheeler *et al.* 1998). Feeding a radiolabelled ascorbate precursor would mean that all radiolabelled ascorbate was synthesised endogenously. Radiolabelled ascorbate would therefore be subject to the regulatory processes acting over the ascorbate pool size such as the rate of diffusion of ascorbate through the somewhat porous outer mitochondrial membrane or coupling of GLDH activity to the mitochondrial electron transport chain (see section 1.6.3). Ascorbate metabolic responses to stress as measured by fate of endogenous radiolabelled ascorbate could therefore be considered to be more biologically representative than the fate of exogenous radiolabelled ascorbate that had been taken up by cells.

Only 10 % of D-[U- $^{14}\text{C}$ ]mannose was found to be incorporated into L-[U- $^{14}\text{C}$ ]ascorbate in peas (Wheeler *et al.* 1998). A more appropriate radiolabelled ascorbate precursor would be a metabolite in the biosynthetic pathway which occurred downstream of GDP-L-mannose and so was less likely to participate in other competing metabolic pathways. L-Galactono-1,4-lactone (GalL) is the immediate precursor to ascorbate (Wheeler *et al.* 1998). L-[6- $^3\text{H}$ ]GalL was readily



available and so was considered to be an appropriate radiolabelled ascorbate precursor to feed to cell suspension cultures.

The fate of extracellular [1-<sup>14</sup>C]ascorbate had been studied previously (Green and Fry 2005). Compounds such as threonate, which did not contain the former 1-<sup>14</sup>C were not labelled and therefore could not be detected by the methods employed in this study (autoradiography and scintillation counting). Feeding L-[6-<sup>3</sup>H]GalL would yield L-[6-<sup>3</sup>H]ascorbate. It would therefore be possible to detect L-[6-<sup>3</sup>H]ascorbate metabolites that would not have contained 1-<sup>14</sup>C if [1-<sup>14</sup>C]ascorbate had been fed to cells, which could potentially yield additional information on ascorbate breakdown in cell suspension cultures.

### **3.3.2 The effect of exogenous GalL on intracellular ascorbate concentration.**

GalL is synthesised intracellularly and so would not usually be taken up by cells. Synthesis of [<sup>3</sup>H]ascorbate would require that [<sup>3</sup>H]GalL was both taken up across the plasma membrane and then converted into [<sup>3</sup>H]ascorbate. Ascorbate concentration in cell extracts following treatment with exogenous GalL was measured spectrophotometrically via the reduction of DCPIP from blue to colourless by ascorbate. Whilst other compounds present in the spent culture medium and cell homogenate may have been capable of reducing DCPIP, reduction is thought to occur more rapidly by ascorbate than any other reducing compound that would have been present. Samples here were measured 20 s after addition to DCPIP.

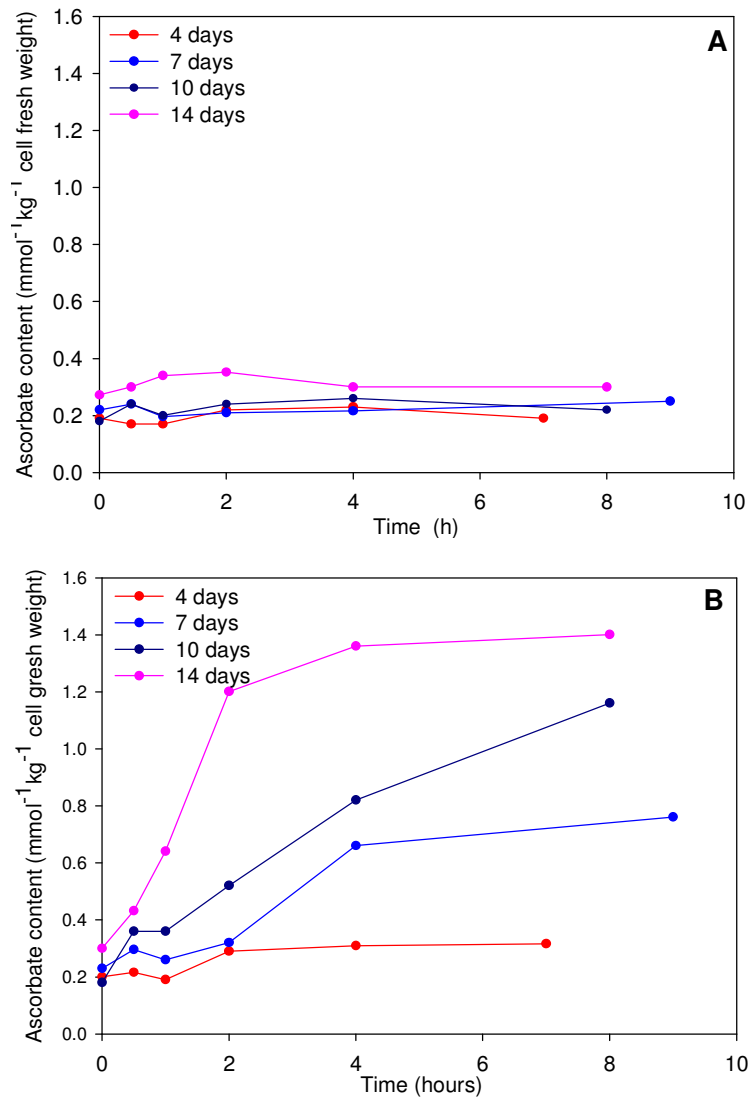
Rose cells showed an increase in ascorbate content in response to exogenous GalL (Fig. 3.33). When exogenous GalL was fed to cell cultures, the age of cell culture seemed to impact on the increase in ascorbate content in response to exogenous GalL. The rate of GalL-dependent ascorbate synthesis increased steadily with age from 4- to 14-day-old cell cultures (Fig. 3.33b), indicating that either the concentration or the activity of GLDH was increasing with age. However, GLDH activity has been found to decrease with age in plants (Bartoli *et al.* 2000). If

100 % uptake of GalL and 100 % conversion of GalL to ascorbate was assumed, then in cell cultures supplied with GalL to a final concentration 25 mM, the maximum theoretical yield of ascorbate was approximately 200 mmol<sup>-1</sup> kg<sup>-1</sup> fresh cell weight. Clearly the maximum yield of ascorbate measured (Fig. 3.33b) was much less than the theoretical yield.

Uptake of GalL may occur by diffusion across the plasma membrane. Given that GLDH activity could be expected to decline with cell culture age, it is possible that in older cells, membranes were more permeable to neutral compounds such as GalL. Variability in membrane structure is known to be an important component in diffusion of compounds into the cell (Antunes and Cadenas 2000). The rate of uptake of GalL in cell suspension cultures was investigated further with the use of [<sup>3</sup>H]GalL.

### **3.3.3 Assessment of the purity of [<sup>3</sup>H]GalL**

[<sup>3</sup>H]GalL was assessed for purity before being fed to cell suspension cultures. Analysis of a mixture of [<sup>3</sup>H]galactonate and [<sup>3</sup>H]GalL revealed that two large peaks were present that co-migrated with the external markers for GalL and galactonate (Fig. 3.34). Two other much smaller peaks were visible at 12 cm and 14 cm. An equilibrium exists between GalL and galactonate. This equilibrium was thought to explain the presence of two large peaks in figure 3.34.



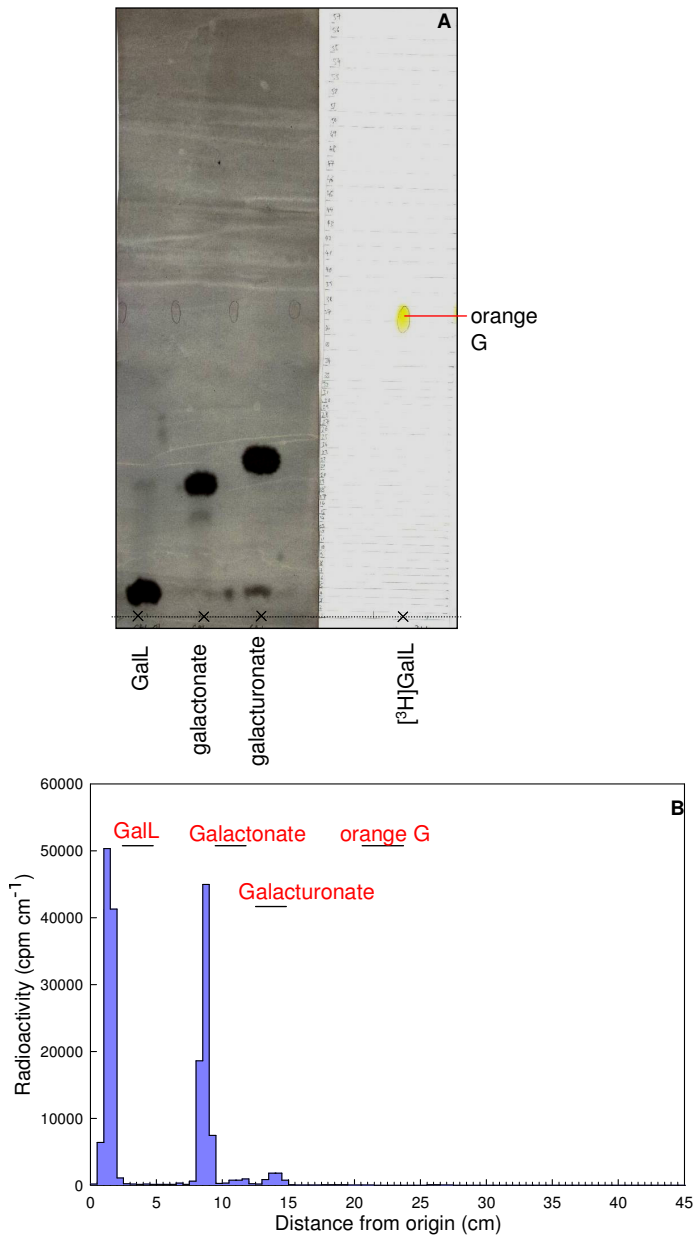
**Figure 3.33 Symplastic ascorbate content of cell suspension cultures**

Cell suspension cultures were fed GalL (final concentration of 25 mM) and buffered with MES (final concentration 5 mM). Washed cells were ground in 10 % MPA and centrifuged at 3000 rpm for 5 min. The supernatant was assayed for ascorbate by the reduction of DCPIP.

- A. Cell cultures (no exogenous GalL) with water added at 0 h
- B. Cell cultures with GalL added at 0 h.

Being neutral, GalL was thought to be more likely to diffuse through the plasma membrane. [ $^3\text{H}$ ]Galactonate was converted into [ $^3\text{H}$ ]GalL with a volatile acid (TFA), which could then be dried off before GalL was added to cell cultures (Fig. 3.35). The [ $^3\text{H}$ ]galactonate did not appear to be broken down by the acid treatment (Fig. 3.35). The rate of isomerisation, whilst never specifically calculated, was such that after storage overnight of a solution of GalL at 4°C in water, the ratio of acid:lactone was approximately 1:1. In future experiments in which [ $^3\text{H}$ ]GalL was fed to cells, [ $^3\text{H}$ ]GalL was resuspended in spent culture medium prior to addition to cell cultures. Spent culture medium was slightly acidic (pH 5.5 – 6.0) and [ $^3\text{H}$ ]GalL was added to cultures within a 5 minutes of resuspension so as to minimise formation of [ $^3\text{H}$ ]galactonate.

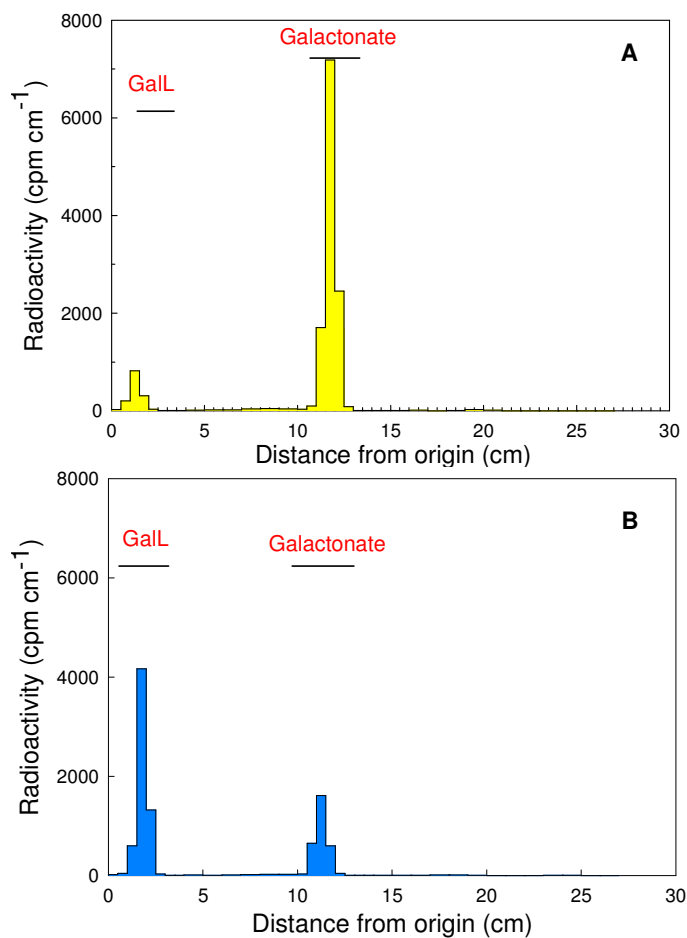
Neutral compounds cannot be separated from each other during paper electrophoresis. Neutral impurities may not therefore have been detected in figure 3.34. The equilibrated mixture of [ $^3\text{H}$ ]GalL and [ $^3\text{H}$ ]galactonate was separated by paper chromatography (Fig. 3.36). [ $^3\text{H}$ ]GalL was separated from potential neutral contaminants which did not contain a lactone ring; compounds with a lactone ring migrated faster than those without in this buffer system (Fig. 3.36). The two main peaks were identified as [ $^3\text{H}$ ]GalL and [ $^3\text{H}$ ]galactonate. A third peak was found to represent approximately 17 % of the total radioactivity in figure 3.36. It was possible to eliminate this peak by converting [ $^3\text{H}$ ]GalL to [ $^3\text{H}$ ]galactonate, separating [ $^3\text{H}$ ]galactonate from impurities by paper electrophoresis, eluting the [ $^3\text{H}$ ]galactonate off the paper and drying it under reduced pressure before converting it back to [ $^3\text{H}$ ]GalL. However, very low rates of uptake of thus treated [ $^3\text{H}$ ]GalL were consistently observed (data not shown) and so [ $^3\text{H}$ ]GalL was not further purified.



**Figure 3.34** Assessment of the purity of  $[^3\text{H}]\text{galactonate}$  by paper electrophoresis.

A. Paper electrophoretogram of  $[^3\text{H}]\text{galactonate}$  with external markers. Electrophoresis was carried out at pH 3.5 (40 min, 3.0 kV). External markers (GalL, galactonate and galacturonate) were stained with silver nitrate. The  $[^3\text{H}]\text{galactonate}$  track was divided into 0.5-cm and 1-cm-wide sections according to the position of external markers.

B. Scintillation counts of the  $[^3\text{H}]\text{GalL}$  sample.

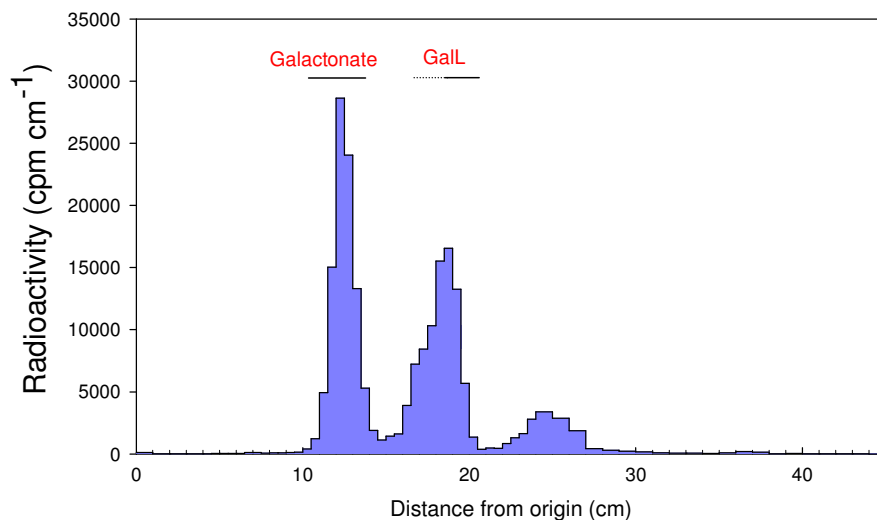


**Figure 3.35 The lactonisation of [<sup>3</sup>H]galactonate.**

Mixtures of [<sup>3</sup>H]GalL and [<sup>3</sup>H]galactonate were analysed by electrophoresis at pH 3.5 (3.5 kV, 30 min). Electrophoretograms were divided into strips. Strips were assayed for radioactivity by scintillation counting. Compounds named in red are external markers whose migration distance was determined by silver nitrate staining.

A. The acidic moiety in figure 3.35 A was eluted in water then analysed by electrophoresis.

B. A portion of the same eluate was treated with TFA (final concentration 1 M) for 30 min at room temperature, then dried under reduced pressure, re-dissolved in water and immediately re-analysed by electrophoresis.



**Figure 3.36 Analysis of the purity of  $[^3\text{H}]$ galactonate by paper chromatography**

Analysis of purity of a mixture of  $[^3\text{H}]$ GalL and  $[^3\text{H}]$ galactonic acid by paper chromatography. The chromatogram was run in butanol : acetic acid : water (12 : 3 : 5) ratio for 18 h. Markers shown refer to external markers stained with silver nitrate. The dotted line indicates smearing of the external marker.

### **3.3.4 Factors affecting the uptake of [<sup>3</sup>H]GalL by cell suspension cultures**

#### 3.3.4.1 Choice of species and age of cell suspension cultures

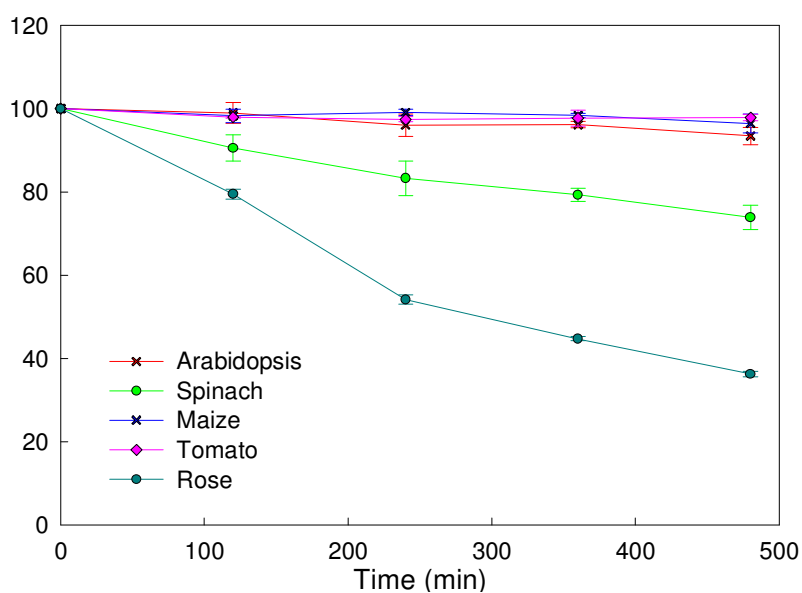
Initially, uptake of [<sup>3</sup>H]GalL was assayed in a range of 10-day-old cultures (Fig. 3.37) as a faster rate of uptake of DHA had been measured in 10-day-old cells compared to 5-day-old cells in a range of species (S. C. Fry, unpublished results). Once the optimal species of cell suspension culture had been chosen, the optimal age of the species was then checked in more detail.

The rate of uptake was greatest in rose cell suspension cultures (Fig. 3.37). This species was used for subsequent experiments involving [<sup>3</sup>H]GalL. Uptake of [<sup>3</sup>H]GalL was compared in a range of ages of rose cell suspension cultures. As predicted, uptake occurred more rapidly in older cells (Fig. 3.38). By 18 days post-subculturing, cells could be expected to be undergoing nutrient stress from depletion of resources. Although there was little difference between the rate of uptake in 9-day-old-cell cultures and 18 day-old cell cultures, 9 – 10-day-old cells were chosen for further experiments as this age was considered to represent a balance between [<sup>3</sup>H]GalL uptake, ascorbate synthesis and lack of nutrient stress. Glucose was still present in culture medium at in 10 day-old cell cultures but none was observed at 14 day-old cultures. Glucose levels were approximated by electrophoresis and silver nitrate staining (results not shown).

#### 3.3.4.2 Uptake of [<sup>3</sup>H]GalL and [<sup>3</sup>H]galactonate

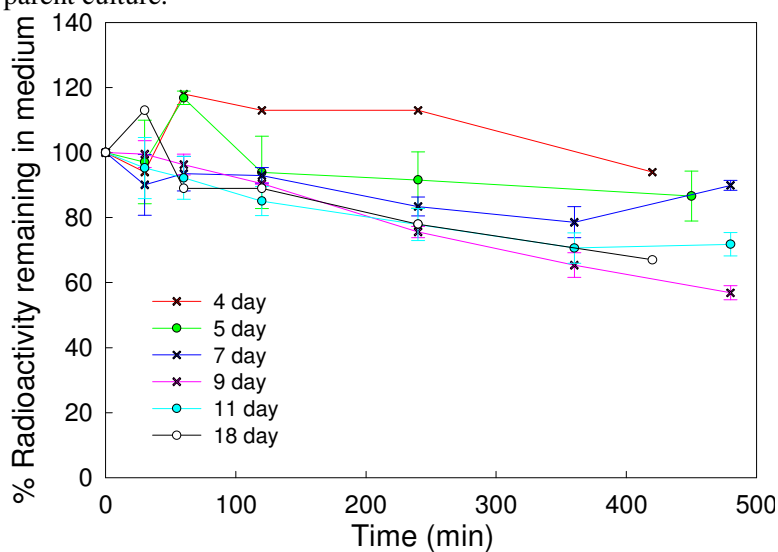
Even in 10-day-old rose cell cultures, the maximum uptake observed was little over 60 % after 8 hours of incubation with [<sup>3</sup>H]GalL (Fig. 3.37). If [<sup>3</sup>H]ascorbate metabolism were to be





**Figure 3.37 Uptake of [<sup>3</sup>H]GalL in cell suspension cultures**

10-day-old cell suspension cultures were adjusted to 20 % fresh weight (w/v) and buffered at pH 6.0 with MES (5 mM final concentration). [<sup>3</sup>H]Galactonate was treated with 2 M TFA, dried under reduced pressure and then re-dissolved in water immediately prior to addition to cultures. Culture medium was sampled in 100- $\mu$ l aliquots after cells had been allowed briefly to sediment at time points. Uptake of [<sup>3</sup>H]GalL (0.05 MBq ml<sup>-1</sup>) was assayed by liquid scintillation counting. Standard error (SE) bars represent cell cultures set up in triplicate but removed from the same parent culture.



**Figure 3.38 The effect of cell culture age on the uptake of [<sup>3</sup>H]GalL in rose cell suspension cultures**

Cell cultures prepared as described in figure 3.37. [<sup>3</sup>H]GalL was prepared and culture medium was sampled as described in figure 3.35

studied after feeding [ $^3\text{H}$ ]GalL to cells, a faster rate of uptake would be required. Charged compounds such as galactonate were expected to diffuse less readily through the plasma membrane. If uptake of GalL was diffusion-mediated, then equilibration of [ $^3\text{H}$ ]galactonate and [ $^3\text{H}$ ]GalL in the culture medium during the incubation period could have retarded the uptake of [ $^3\text{H}$ ]GalL.

The proportion of [ $^3\text{H}$ ]galactonate was assayed at the end of an 8-hour incubation with [ $^3\text{H}$ ]GalL (Fig. 3.39). In the absence of cells, a large peak in the position occupied by neutral compounds was recorded after 8 h. This peak was assumed to be [ $^3\text{H}$ ]GalL which would have meant that [ $^3\text{H}$ ]GalL was stable in 10-day-old spent culture medium and that only a small fraction of the  $^3\text{H}$  was present as [ $^3\text{H}$ ]galactonate. In the presence of cells, less [ $^3\text{H}$ ]galactonate was present after 8 hours than in the absence of cells, indicating the possible uptake or extracellular lactonisation of [ $^3\text{H}$ ]galactonate by cells (Fig. 3.39b). The major peak remaining after cells had been incubated with [ $^3\text{H}$ ]GalL was in the position occupied by neutral compounds. This was consistent with the idea that although [ $^3\text{H}$ ]GalL was stable in cell culture medium, uptake by cells was slow.

The rates of uptake of [ $^3\text{H}$ ]GalL and [ $^3\text{H}$ ]galactonate were compared. Sample experiments (expt.1 and 2) are shown (Fig. 3.40). In experiment 1 there was no difference in the total amount of [ $^3\text{H}$ ]galactonate or [ $^3\text{H}$ ]GalL taken up by cell after 8 hours, although the initial rate of uptake of [ $^3\text{H}$ ]GalL was slightly faster. In subsequent experiments [ $^3\text{H}$ ]GalL was supplied to cells as this was reported to be the direct precursor of [ $^3\text{H}$ ]ascorbate (Wheeler *et al.* 1998). Furthermore, studies feeding cold GalL had shown that exogenous GalL (Fig. 3.33) could increase the intracellular concentration of ascorbate, whilst this had not been ascertained in the case of galactonate.

The disparity in uptake between expts. 1 and 2 (Fig. 3.40) illustrates another important point. During a successful [ $^3\text{H}$ ]GalL uptake experiment, between approximately 60 % (Fig. 3.37,

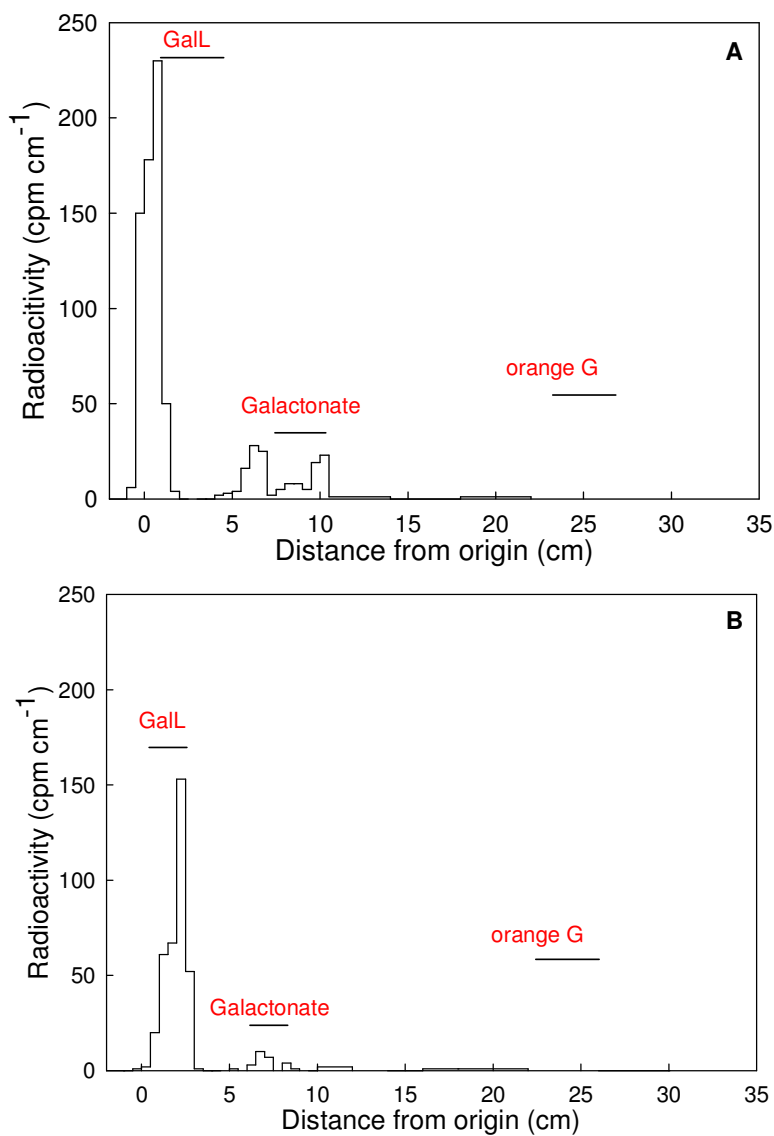
3.40) and 40 % (Fig. 3.37) uptake could occur. In expt. 2 less than 20 % uptake occurred (Fig. 3.40). All measurements in figure 3.40 were taken on the same day but cell cultures issued from different parent cultures. This showed that variability between cultures was likely to be one of the more important factors in determining the rate of [<sup>3</sup>H]GalL uptake.

#### 3.3.4.3 The effect of carbon source and buffer on the uptake of [<sup>3</sup>H]GalL

Uptake of [<sup>3</sup>H]GalL had been found to be highly variable but had never been found to be more than 70 % over 8 hours. The optimal conditions for the uptake of [<sup>3</sup>H]GalL by cells had to be found; if a 70 % uptake in 8 h were achieved consistently, this would be considered a satisfactory point from which to proceed with investigations into [<sup>3</sup>H]ascorbate metabolism. Several factors were investigated so that optimal conditions for the uptake of [<sup>3</sup>H]GalL could be found.

It was hypothesised that [<sup>3</sup>H]GalL might enter the cell via the same channels as glucose. [<sup>3</sup>H]GalL uptake could have been competing with the glucose carbon source fed to rose cell suspension cultures. Uptake was compared in cells grown with glycerol as the carbon source and cells grown with glucose as the carbon source (Fig. 3.41). A possible effect of the MES buffer on the uptake of [<sup>3</sup>H]GalL was also tested (Fig. 3.41).

Uptake was very slightly faster in the absence of the MES buffer (Fig. 3.41). The type of carbon source made a large difference, though not in the way postulated, as cells grown on glucose showed much better uptake (approximately 50 %) than those grown on glycerol (approximately 15 %) after 8 h. Glucose and GalL did not therefore appear to compete for transport across the plasma membrane. This was in agreement with results showing that uptake was similar in 9- and 18-day-old cell cultures (Fig. 3.38) despite glucose resources having been more depleted in 18-day-old cells compared with 9-day-old cell cultures. Increased uptake in cells fed glucose compared to glycerol could have reflected lower levels of stress in these cells.

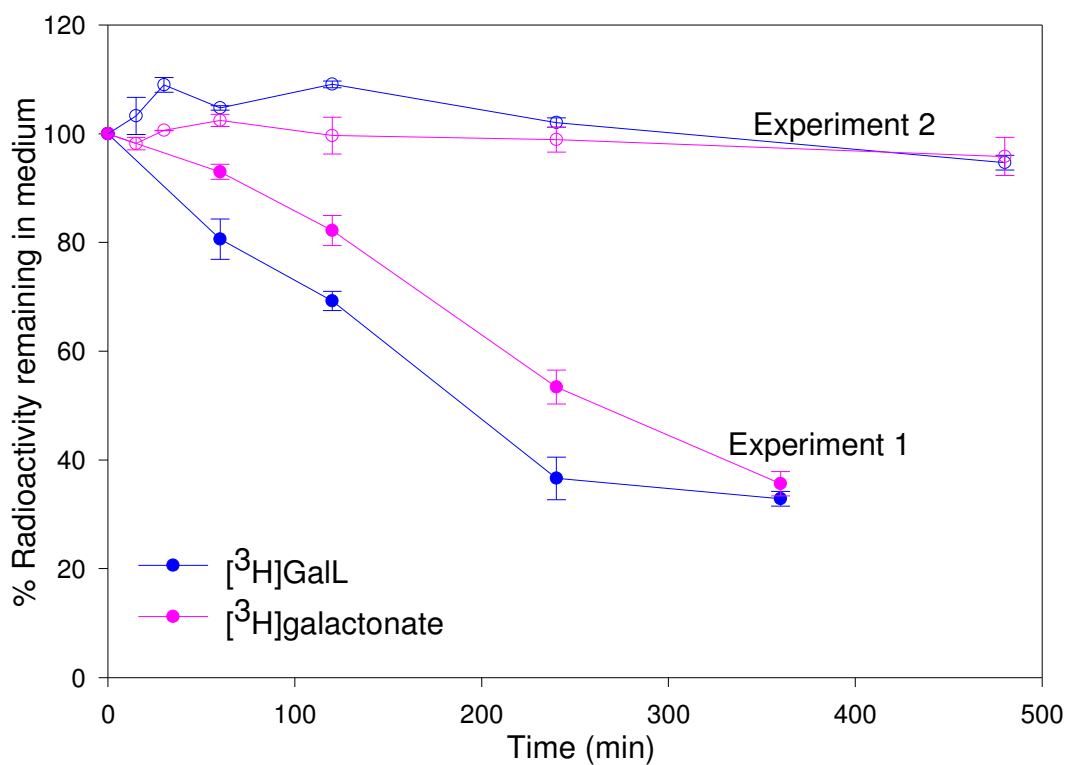


**Figure 3.39 The stability of  $[^3\text{H}]\text{GalL}$  in cell culture medium.**

After 8 h incubation of  $[^3\text{H}]\text{GalL}$  in cell cultures or in spent culture medium, samples of the medium were subjected to paper electrophoresis at pH 3.5 (30 min, 3.5 kV). The positions of external markers are shown in red.

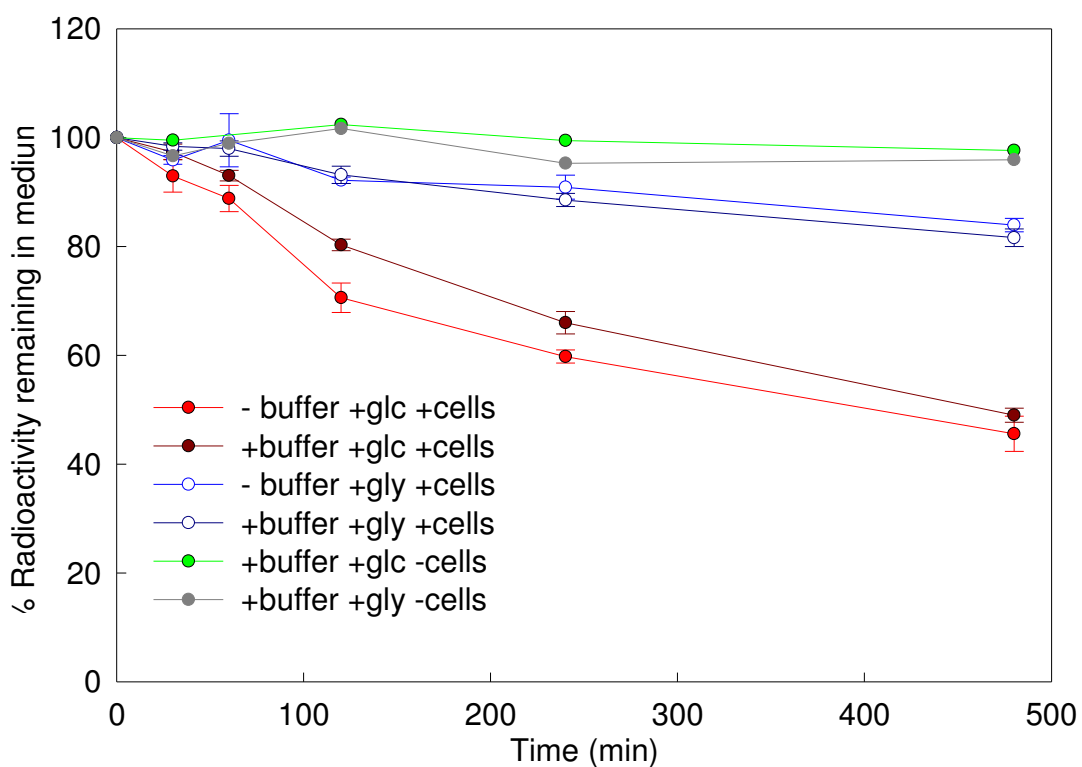
A.  $[^3\text{H}]\text{GalL}$  incubated in spent cell culture medium

B.  $[^3\text{H}]\text{GalL}$  incubated in the presence of cells



**Figure 3.40 Comparison of the uptake of  $[^3\text{H}]\text{GalL}$  and  $[^3\text{H}]\text{galactonate}$ .**

$[^3\text{H}]\text{Galactonate}$  was treated either with 0.1 M NaOH or 2 M TFA for 1 hour. Cell suspension cultures were adjusted to 20 % (w/v). Error bars (SE) represent cell cultures set up in triplicate from the same parent culture. Cell cultures used in 'experiment 1' and 'experiment 2' were derived from different parent cultures.



**Figure 3.41** The effect of carbon source and the presence of buffer on the uptake of [<sup>3</sup>H]GalL.

Cell suspension cultures were adjusted to 20 % fresh weight (w/v) grown in the presence of either glucose (glc) or glycerol (gly) carbon source were fed [<sup>3</sup>H]GalL (0.05 MBq ml<sup>-1</sup>). Cells grown on glycerol were acclimated to grow on glycerol. Cultures were either un-buffered or buffered with (final concentration 5 mM) MES. Each treatment was reproduced in triplicate except in spent culture medium where no cells were present. Culture medium was sampled at time points in 100- $\mu$ l aliquots after cultures had been allowed to sediment briefly.

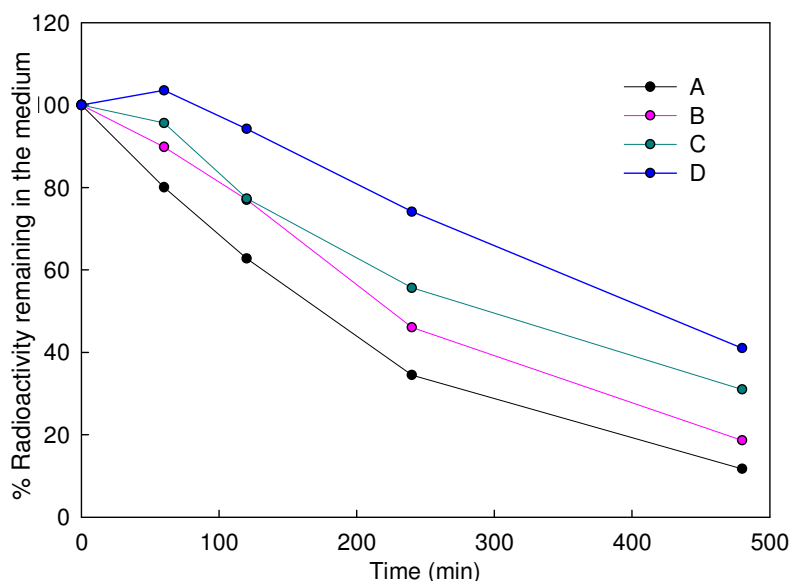
#### 3.3.4.4 Cell disturbance and uptake of [<sup>3</sup>H]GalL

In all uptake experiments thus far, cell density had been adjusted to 20 % fresh weight (w/v) shortly before the addition of [<sup>3</sup>H]GalL. It was hypothesised that this level of disturbance could have stressed cells, resulting in reduced uptake of [<sup>3</sup>H]GalL. Uptake of [<sup>3</sup>H]GalL was compared in cells adjusted to 20 % (w/v) and either allowed to acclimatise overnight after removal from the parent culture or not and cells which had not been adjusted to 20 % w/v and then either allowed to re-acclimatise or not.

Adjustment of cells to 20 % (w/v) had a more profound effect on uptake than the length of time cell cultures were given to re-equilibrate. After 8 hours, 85 % uptake was achieved by the cell culture in which both forms of disturbance had been eliminated (Fig. 3.42). In subsequent experiments cell density was not altered.

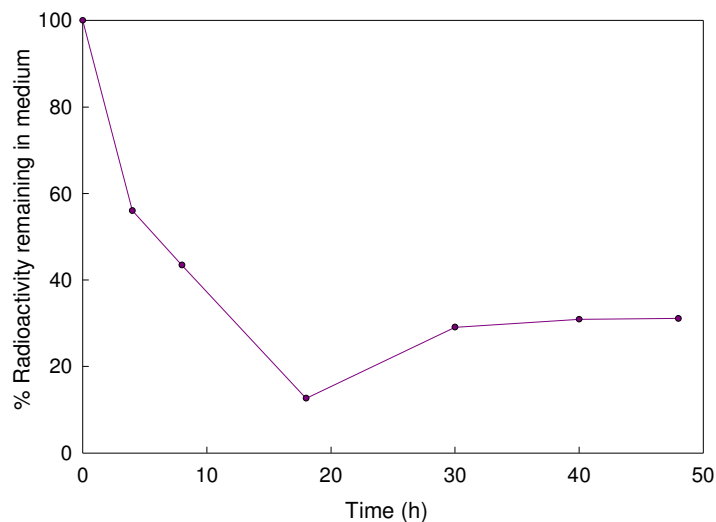
Although these experiments had helped find optimal experimental conditions for the uptake of [<sup>3</sup>H]GalL, variability in uptake rates between individual cell cultures had not been eliminated. Figure 3.43 shows uptake in a cell culture derived from a single parent culture to which the above optimal conditions of uptake had been applied. This cell culture did achieve just over 85 % uptake of [<sup>3</sup>H]GalL but only after 18 h (Fig 3.43). Between 8 h and 18 h, about 30 % of the <sup>3</sup>H remaining in the culture medium was taken up. For subsequent experiments cell cultures were incubated with [<sup>3</sup>H]GalL overnight for 16 h. Cultures were not incubated for longer, as after 18 h evidence was seen for the net export of <sup>3</sup>H-compounds from the symplast to the culture medium (Fig. 3.43).

The conditions for optimal [<sup>3</sup>H]GalL uptake were therefore set as a 16-h incubation with [<sup>3</sup>H]GalL under aseptic conditions, in 10-day-old rose cell suspension cultures, with cell density being the same as that of the parent culture. A buffer was not used and cells were grown on a glucose carbon source.



**Figure 3.42 The effect of cell culture disturbance on the uptake of [<sup>3</sup>H]GalL.**

Cell suspension cultures (10-day-old) were prepared from the same parent culture under aseptic conditions. Cultures A and B were not adjusted to 20 % fresh weight (w/v). The cell content was measured as 38 % fresh weight (w/v). The cell density was measured after the experiment and data was down-scaled accordingly so that it could be compared to the cultures at 20 % (w.v). Cultures C and D were adjusted to 20 % fresh weight (w/v). Prior to addition of [<sup>3</sup>H]GalL (0.05 MBq ml<sup>-1</sup>), cultures A and C were incubated overnight. Cultures B and D were aliquotted from the parent culture 10 minutes before addition of [<sup>3</sup>H]GalL (0.05 MBq ml<sup>-1</sup>). All cell cultures were un-buffered.



**Figure 3.43 The net uptake of [<sup>3</sup>H]GalL over 48 hours under optimal conditions**

A 10-day-old cell suspension culture (glc. grown, un-buffered) was sampled under sterile conditions for 48 hours. The cell cultures was not buffered nor adjusted for cell content. Culture medium was sampled in 100- $\mu$ l aliquots at time points after cultures had been allowed to sediment briefly. The increase between 18 h and 48 h could be the release of <sup>3</sup>H<sub>2</sub>O



### 3.3.5 Synthesis and identification of endogenous [<sup>3</sup>H]ascorbate and [<sup>3</sup>H]ascorbate metabolites

Cell suspension cultures were assayed for the presence of [<sup>3</sup>H]ascorbate after incubation with [<sup>3</sup>H]GalL. Ascorbate approximately co-migrated with galactonate during paper electrophoresis at pH 6.5 (Fig. 3.44), as expected for C<sub>6</sub> compounds with a single negative charge. Both ascorbate and galactonate are almost uncharged at pH 2.0. During electrophoresis at pH 3.5, galactonate migrated slightly further than ascorbate (Fig. 3.44). Therefore, for experiments in which [<sup>3</sup>H]galactonate had to be separated from [<sup>3</sup>H]ascorbate, paper electrophoresis was performed at pH 3.5, except in preliminary experiments (Fig. 3.45).

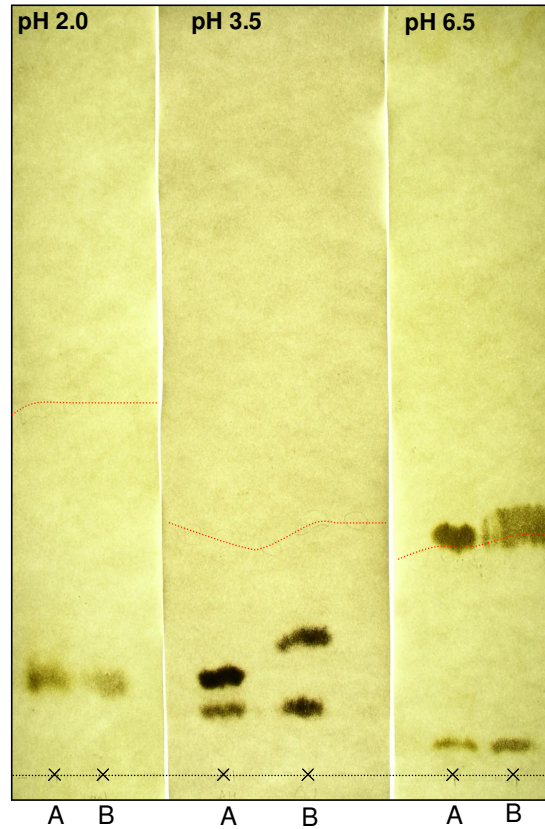
At pH 6.5, <sup>3</sup>H-labelled metabolites in cell extracts separated into three detectable bands (Fig. 3.45). Most of the <sup>3</sup>H in cell extracts was found in products 2 and 3 (Fig. 3.45). A strip of the electrophoretogram was assayed for <sup>3</sup>H-compounds present in trace amounts that might not have been visualised by fluorography (area 'C', Fig. 3.45). As products 1 and 2 were so close, product 1 appears as a shoulder to product 2. Importantly, no small peaks of radioactivity were seen that migrated further than the peak of products 1 and 2 (Fig. 3.46). Almost all the <sup>3</sup>H in the cell extract was contained in the three bands visible in figure 3.45. The cell extract shown in figure 2 had been sampled after 8 h. This suggests that these three products might have been quite stable in the symplast as no other breakdown products were visible.

At pH 6.5, galactonate and ascorbate both had a  $m_{OG}$  value of ~1.0. Product 2 had an  $m_{OG}$  value of 0.8 – 1.0 so the identity of product 2 was considered likely to be [<sup>3</sup>H]galactonate and/or [<sup>3</sup>H]ascorbate. Product 3, having a  $m_{OG}$  value of 0.0 was thought to be [<sup>3</sup>H]GalL, [<sup>3</sup>H]DHA or both. The identity of product 1 was less clear as the  $m_{OG}$  values were affected by samples not running straight (Fig. 3.45), possibly because of high levels of small, soluble salts in the sample. An estimation of its  $m_{OG}$  value at 1.1 – 1.3 suggested it was [<sup>3</sup>H]DKG. If cell extracts contained high concentrations of salts this could have interfered with migration of

samples during electrophoresis. A smaller volume of the cell extract (sample B, Fig. 3.45) was loaded but this was not sufficient to compensate for factors affecting the separation of compounds.

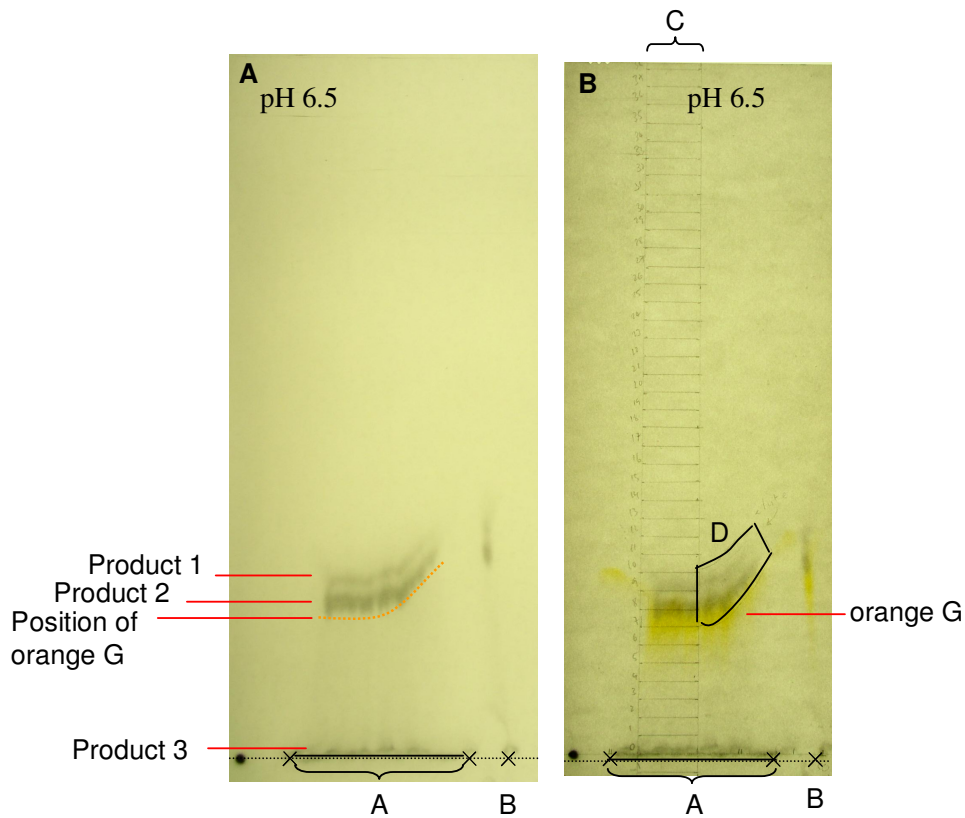
The identity of products 1 and 2 was tested further. A sample of both products eluted from area D (Fig. 3.45) was analysed at pH 3.5 (Fig. 3.47a). A portion of the eluate from area 'D' (Fig. 3.45) was treated with NaOH with the aim of testing for the presence of [<sup>3</sup>H]ascorbate or [<sup>3</sup>H]DHA (lane 'A', Fig. 3.47a). If either compound were present, they would be expected to break down into [<sup>3</sup>H]DKG, [<sup>3</sup>H]ox.thr, [<sup>3</sup>H]threonate and oxalate, although oxalate would no longer be radiolabelled and so would not be visible. Four <sup>3</sup>H-compounds could be seen in both lane A and lane B, two of which migrate with external markers for galactonate and neutral compounds. [<sup>3</sup>H]Ascorbate was absent (Fig. 3.47a). The two faster migrating compounds had *m*<sub>OG</sub> values of 0.6 and 1.5 and may this have been [<sup>3</sup>H]ascorbate degradation products. At pH 3.5 threonate has a *m*<sub>OG</sub> value of 0.6 and oxalyl threonate a *m*<sub>OG</sub> value of 1.4. The compounds with the *m*<sub>OG</sub> of 0.6 and 1.5 were therefore identified as ox.thr. and threonate, respectively. Lane B (Fig. 3.47a) was assayed for compounds present in concentration too low to be visualised on the fluorography film (Fig. 3.47a). Small peaks were detected at 22, 24 and 28 cm, characteristic of migration distances of [<sup>3</sup>H]cyc.ox.thr., [<sup>3</sup>H]ox.thr., and [<sup>3</sup>H]ox.thr. isomer. Breakdown of [<sup>3</sup>H]ascorbate was assumed to have occurred during elution from the paper electrophoretogram.

Although [<sup>3</sup>H]ascorbate was not observed (Fig. 3.47a) compounds which were thought to be the ascorbate breakdown products [<sup>3</sup>H]DKG, [<sup>3</sup>H]cyc.ox.thr., [<sup>3</sup>H]ox.thr., [<sup>3</sup>H]ox.thr. isomer and [<sup>3</sup>H]threonate were present in cell extracts following incubation with [<sup>3</sup>H]GalL. Ascorbate and DHA has been noted to be unstable, partially breaking down even during 2-D paper electrophoresis (Fig 3.9). It is likely then that [<sup>3</sup>H]ascorbate broke down during elution of area 'D' (Fig. 3.45) from the paper electrophoretogram. The presence of <sup>3</sup>H-labelled ascorbate



**Figure 3.44 Relative migration of ascorbate, DHA, GalL and galactonic acid**

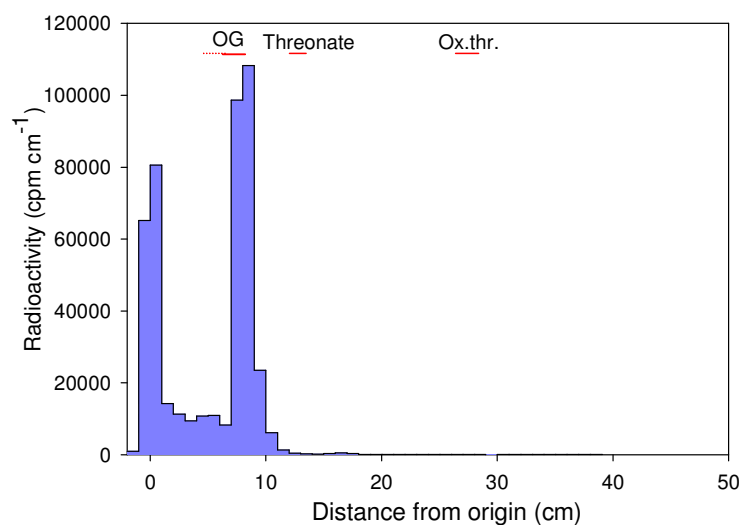
Marker solutions of ascorbate (containing small amounts of DHA) and galactonic acid (containing small amounts of GalL) were subjected to paper electrophoresis at pH 2.0, pH 3.5 and pH 6.5. The position of the orange G marker is marked in red. Lane A contains GalL and galactonate. Lane B contains ascorbate and DHA.



**Figure 3.45**  $^3\text{H}$ -Labelled symplastic products 8 h after feeding of  $[6\text{-}^3\text{H}]\text{GalL}$  to cell suspension cultures

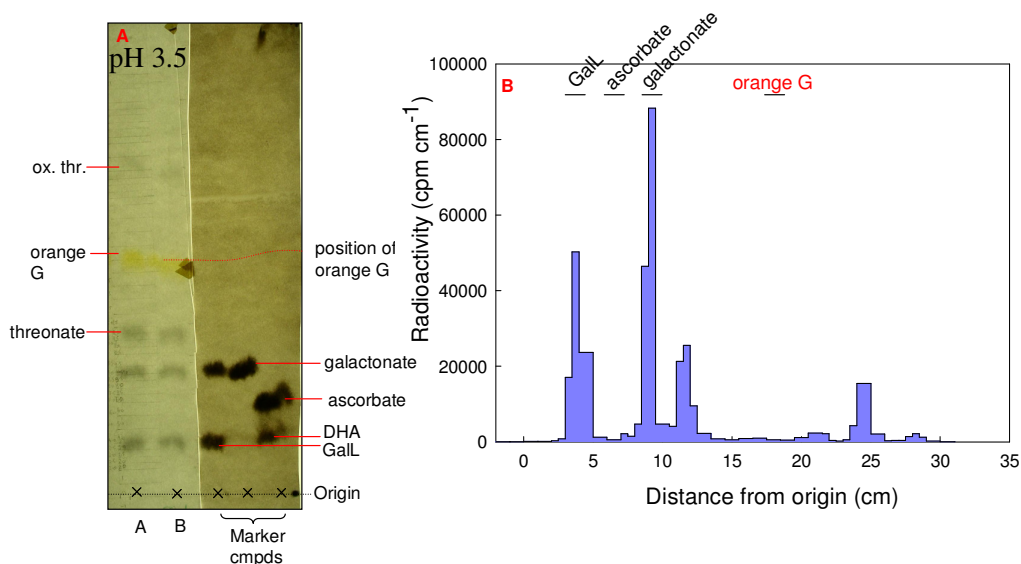
A. After 8 h incubation with  $[^3\text{H}]\text{GalL}$ , cells were rinsed in water and ground in 1 ml of 20% formic acid. The supernatant was subjected to electrophoresis at pH 6.5 (30 min, 3.5 kV). The supernatant was loaded on to the electrophoretogram as the streak marked A. A 50  $\mu\text{l}$  spot marked B was loaded in case large amounts of salts were present in the cell extract that prevented the electrophoretogram from running straight. The paper electrophoretogram was exposed to film for 4 weeks. The resulting fluorogram is shown.

B. The paper electrophoretogram overlaid with the fluorogram shown in 3.45A. Track C was assayed for radioactivity by scintillation counting.  $^3\text{H}$ -Labelled compounds were eluted from the area marked D for further analysis.



**Figure 3.46 Analysis of  $^3\text{H}$ -labelled symplastic compounds after an 8 h incubation with  $[6\text{-}^3\text{H}]\text{GalL}$ .**

The track marked 'C' in Fig. 3.45b was cut into 1-cm-wide strips. The presence of  $^3\text{H}$ -labelled compounds was assayed by paper scintillation counting. Positions of threonate and ox.thr. are estimates.



**Figure 3.47  $^3\text{H}$ -Labelled compounds extracted from the symplasm an 8 h incubation with  $[6\text{-}^3\text{H}]\text{GalL}$  after elution off a paper electrophoretogram**

A. Products 1 and 2 (not resolved) were eluted from the area marked D in Fig. 3.45 and treated with either 0.1 M NaOH (lane A) or water (lane B). Results were analysed by electrophoresis at pH 3.5 (35 min, 3.5 kV). The paper electrophoretogram was exposed to film for 5 weeks. External markers were stained with silver nitrate. The fluorogram overlying the paper electrophoretogram is shown here.

B. Lane B was cut into 1-cm-wide strips. The distribution of radioactivity in lane B was analysed by scintillation counting.

breakdown products was taken as strong evidence that [<sup>3</sup>H]ascorbate was present in figure 3.45 and therefore that endogenous [<sup>3</sup>H]ascorbate had been synthesised from the [<sup>3</sup>H]GalL precursor. The increase in total symplastic ascorbate content after incubation with [3H]GalL was not measured.

### **3.3.9 The generation of endogenous [<sup>3</sup>H]ascorbate vs. addition of exogenous [<sup>14</sup>C]ascorbate**

#### 3.3.9.1 The degradation of [<sup>3</sup>H]GalL during storage

The purity of [<sup>3</sup>H]GalL had been tested at the start of the present study (Fig. 3.34, 3.36). A few, small anomalous peaks of <sup>3</sup>H had been seen when [<sup>3</sup>H]GalL and [<sup>3</sup>H]galactonate were separated by chromatography and electrophoresis (Figs. 3.34, 3.36). Compared with the main peaks of acid and lactone, these were considered very minor impurities. Purification of the [<sup>3</sup>H]GalL would have been possible by elution of [<sup>3</sup>H]GalL from a paper electrophoretogram. However, very low rates of uptake by cell suspension cultures of [<sup>3</sup>H]GalL that had been thus purified were seen (results not shown). Given that the impurities were found to be so minor (Fig. 3.34, 3.36), purification was not carried out. The results shown in figures 3.45, 3.46 and 3.47 suggest that only [<sup>3</sup>H]GalL, [<sup>3</sup>H]galactonate, [<sup>3</sup>H]DHA, [<sup>3</sup>H]ascorbate and possibly [<sup>3</sup>H]DKG were present in the symplasm of cells which had been incubated with [<sup>3</sup>H]GalL. These results refer to an experiment carried out some three months earlier than the experiment shown in figure 3.48. In figure 3.48, even at the start of the 16 h incubation with [<sup>3</sup>H]GalL, at least 6 compounds were visible. Over the interceding months there appeared to have been extensive breakdown of the [<sup>3</sup>H]GalL stock. There did not appear to have been any discrimination between the uptake of [<sup>3</sup>H]GalL and the degradation products of [<sup>3</sup>H]GalL, as impurities were seen in cell extracts (Fig. 3.48).

[<sup>3</sup>H]ascorbate could be identified (Fig. 3.48) by  $m_{OG}$  values and external markers. Little [<sup>3</sup>H]ascorbate remained 8 h after treatment with exogenously added 0.1 mM H<sub>2</sub>O<sub>2</sub> and so it was

assumed that no impurities co-migrated with [<sup>3</sup>H]ascorbate. A band which co-migrated with the [<sup>3</sup>H]galactonate external marker was also seen. Some [<sup>3</sup>H]galactonate was expected in the symplasm of cells incubated with [<sup>3</sup>H]GalL. [<sup>3</sup>H]Threonate had only been observed after the breakdown of <sup>3</sup>H-labelled compounds during elution from the electrophoretogram. As [<sup>3</sup>H]threonate was not present in the original cell extract, ascorbate had been assumed to be quite stable in the symplasm. [<sup>3</sup>H]Threonate was therefore not expected to have been present at the start of the 16-h incubation; the band that can be seen co-migrating with the threonate external marker was considered to be an impurity. Only [<sup>3</sup>H]ascorbate, <sup>3</sup>H-labelled neutral compounds and [<sup>3</sup>H]galactonate could be identified with confidence and so only these were quantified and discussed (section 3.5).

#### 3.3.9.2 Incubation with [<sup>14</sup>C]ascorbate rather than [<sup>3</sup>H]GalL

As [<sup>3</sup>H]GalL had become extensively degraded since this study was started, the idea of synthesising endogenous [<sup>3</sup>H]ascorbate was abandoned in favour of working with [<sup>14</sup>C]ascorbate. Although this meant that radiolabelled ascorbate would not be endogenous, it was felt that this was outweighed by the advantages of working with [<sup>14</sup>C]ascorbate. Uptake of [<sup>14</sup>C]ascorbate by cells was less varied and more rapid than uptake of [<sup>3</sup>H]GalL. As <sup>14</sup>C is a stronger β-emitter than <sup>3</sup>H, autoradiography (as opposed to fluorography) could be carried out with much shorter exposure times; up to 2 weeks was generally sufficient for experiments involving [<sup>14</sup>C]ascorbate whereas up to 8 weeks exposure to fluorography film was required for visualisation of bands corresponding to <sup>3</sup>H-labelled compounds (Fig. 3.48). Purification of the degraded [<sup>3</sup>H]GalL stock would have been possible either by paper chromatography, paper electrophoresis or HPLC. However, as poor uptake rates of [<sup>3</sup>H]GalL eluted off paper electrophoretograms had been found and, moreover, that up to 8 weeks exposure was necessary

for adequate visualisation of bands on fluorograms, the decision was taken not to continue experimentation with [<sup>3</sup>H]GalL.

#### 3.3.9.3 Uptake of [<sup>14</sup>C]ascorbate by 10-day-old rose cell cultures.

As when [<sup>3</sup>H]GalL was fed to cell cultures, 10-day-old cultures had been used as a compromise between uptake, cell age and nutrient stress. In this study [<sup>14</sup>C]ascorbate uptake was found to be 65 % in the first 2 h compared with the maximum observed rate of 38% in the first two hours when [<sup>3</sup>H]GalL was fed to cells (Fig. 3.49). This resulted in a much lower amount of radioactivity remaining in the medium after the incubation period. Hence in experiments in section 3.5, 10-day-old rose cell were incubated with [<sup>14</sup>C]ascorbate for up to 5 h before stress treatment.

### **3.4 Selection of an oxidative stress for cell suspension cultures**

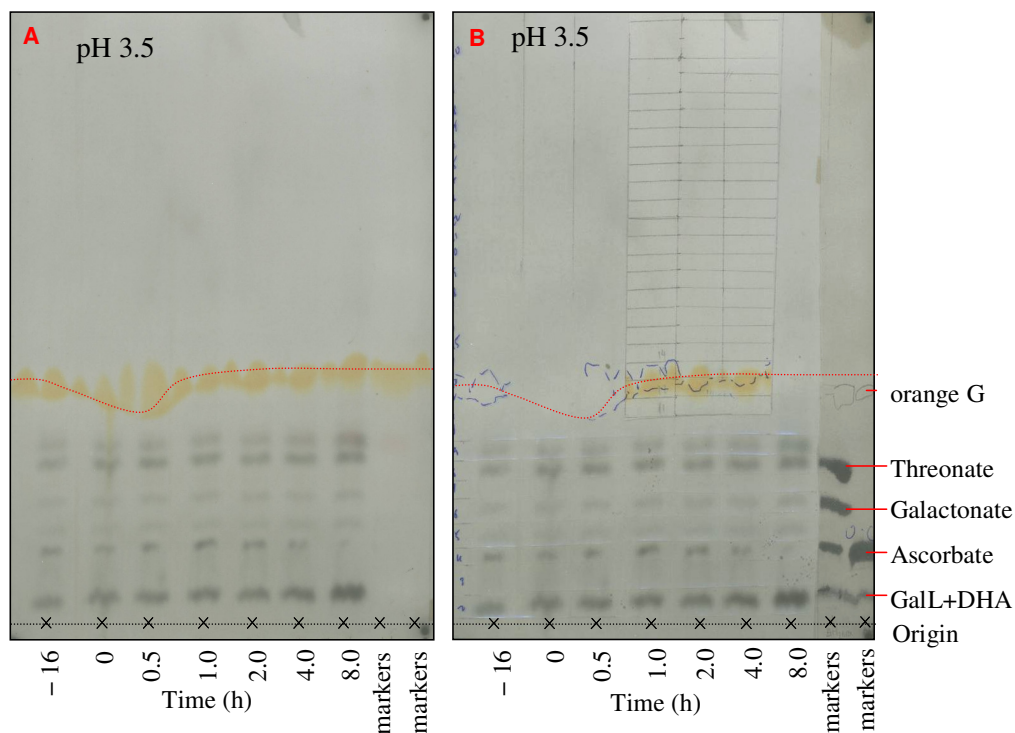
Oxidative stress was induced in cell suspension cultures in initial experiments by the addition of H<sub>2</sub>O<sub>2</sub> and methyl viologen (MV<sup>2+</sup>). MV<sup>2+</sup> accepts electrons in the photosynthetic electron transport chain between ferredoxin and NADP (Taiz and Zeiegr 1998). The methyl viologen radical can then reduce O<sub>2</sub> to O<sub>2</sub><sup>-</sup>, which then dismutates to form H<sub>2</sub>O<sub>2</sub>, hence MV<sup>2+</sup> is a source of intracellular O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. It is also thought to disrupt the mitochondrial electron transport (Frankart *et al.* 2002). The effect of H<sub>2</sub>O<sub>2</sub> on the irreversible breakdown of symplastic and apoplastic ascorbate and the effect of MV<sup>2+</sup> on the symplastic breakdown of ascorbate were measured (sections 3.5, 3.6). Ascorbate breakdown *in vivo* was then compared to the *in-vitro* oxidation of ascorbate that was investigated in sections 3.1 and 3.2, so that cellular metabolism could be separated from the direct oxidation of [<sup>14</sup>C]ascorbate by H<sub>2</sub>O<sub>2</sub>.

Generation of extracellular H<sub>2</sub>O<sub>2</sub> is a key feature of the stress-induced oxidative burst. It was hoped that examining ascorbate metabolism in response to exogenous H<sub>2</sub>O<sub>2</sub> would provide some insight into ascorbate metabolism in response to the oxidative burst. An essential



consideration was whether the concentration of exogenous  $\text{H}_2\text{O}_2$  supplied would simulate  $\text{H}_2\text{O}_2$  that might be generated during the oxidative burst. Too low, and  $\text{H}_2\text{O}_2$  might be metabolised in the culture medium without affecting cells, too high and unwanted additional stress could occur from excessive membrane damage, meaning that results would not be biologically relevant. Investigations were therefore carried out on the effect of  $\text{H}_2\text{O}_2$  on membrane integrity and the longevity of  $\text{H}_2\text{O}_2$  in cell cultures.

Some plants show enhanced resistance to  $\text{MV}^{2+}$ . This resistance appears to be closely tied to levels of ascorbate and antioxidant enzymes (Choi *et al.* 2004, Kwon *et al.* 2002). Plants showing increased tolerance of abiotic stress such as drought and salt stress also shown enhanced resistance to  $\text{MV}^{2+}$  (Rodrigues *et al.* 2006). Abiotic stress can result in increased oxidative stress and production of  $\text{O}_2^-$  (Buchanan *et al.* 2000). Whereas  $\text{H}_2\text{O}_2$  represented a short term stress which could be quickly metabolised, the  $\text{MV}^{2+}$  induced a more protracted stress which, in theory, would last until methyl viologen radicals had been reduced and all the resulting  $\text{O}_2^-$  reduced to  $\text{H}_2\text{O}_2$ . It was therefore of interest to study the effects of  $\text{MV}^{2+}$  on ascorbate metabolism as well as those of  $\text{H}_2\text{O}_2$  as this would show the response to a more protracted stress such as might be encountered during e.g. drought. It was not known at which concentration  $\text{MV}^{2+}$  would reproduce a biologically realistic level of  $\text{O}_2^-$ . Reported concentrations supplied to cell culture varied from 1  $\mu\text{M}$  (Pasternak *et al.* 2007) to 50  $\mu\text{M}$  (Tokunaga *et al.* 2004). Final concentrations of 1  $\mu\text{M}$  and 10  $\mu\text{M}$  were chosen for this study.

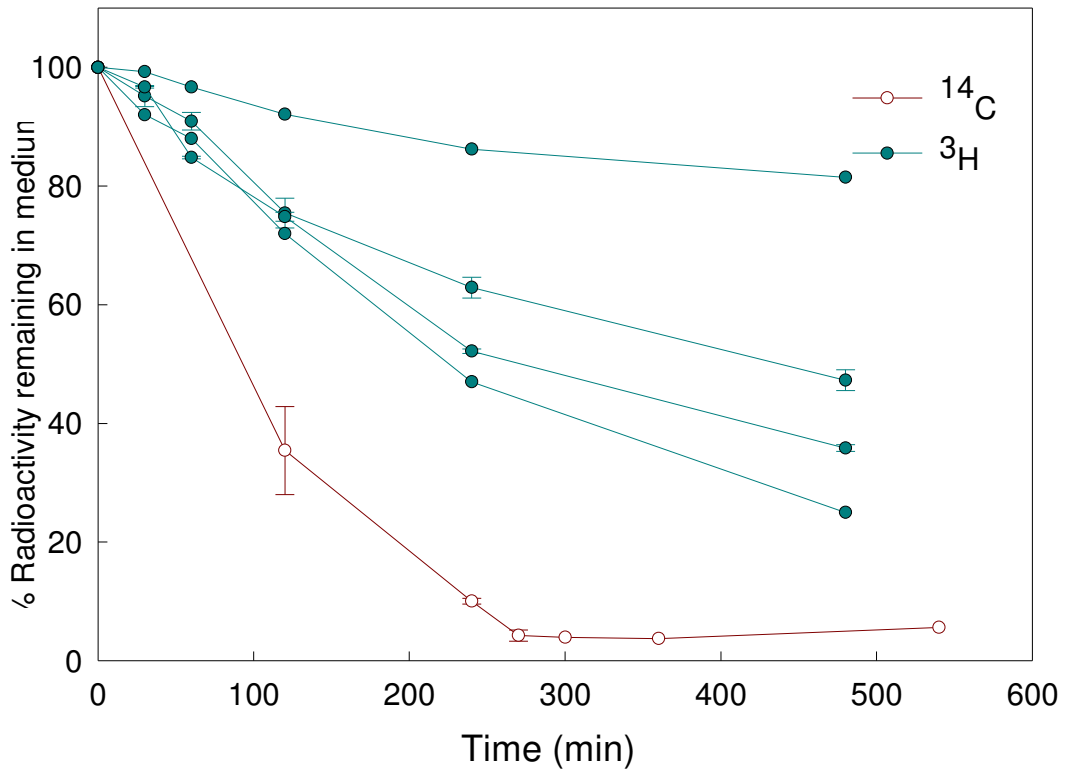


**Figure 3.48** Symplastic extracts of cell suspension cultures receiving  $\text{H}_2\text{O}_2$  to a final concentration of 0.1 mM

$\text{H}_2\text{O}_2$  was added (0 h) to 10-day-old cell suspension cultures after 16 h incubation with  $[\text{}^3\text{H}]\text{GalL}$  ( $1 \text{ MBq ml}^{-1}$ ). Symplastic extracts were subjected to paper electrophoresis at pH 3.5 (3.5kV, 30 minutes). Electrophoretograms fluorographed for 8 weeks. Cell extracts taken at the start of the 16-h incubation are marked as -16 h.

A. Fluorogram overlying paper electrophoretogram.

B. Fluorogram overlying paper electrophoretogram after staining of markers with silver nitrate. The electrophoretogram has been marked for scintillation counting.



**Figure 3.49 A comparison of the uptake of [ $^{14}\text{C}$ ]ascorbate and [ $^3\text{H}$ ]GalL**

Rose cells suspension cultures (10-day-old) were fed either [ $^{14}\text{C}$ ]ascorbate or [ $^3\text{H}$ ]GalL at 0 min. The 5 different experiments shown used cells taken from separate parent cell cultures but within a single experiment cells were taken from the same parent culture. Experiments from different parent cell cultures were conducted on different days. Error bars (SE,  $n = 3$ ) represent measurements from separate cell cultures but ones which derived from the same parent culture. The results of several experiments were chosen so that the variation in uptake of [ $^3\text{H}$ ]GalL could be demonstrated.

### **3.4.1 The effect of H<sub>2</sub>O<sub>2</sub> on membrane integrity longevity of H<sub>2</sub>O<sub>2</sub> in culture medium**

The conductivity of the cell bathing fluid (water, in the case of Fig. 3.50) was taken as an indicator of damage to cell membranes. The effect of 1 mM H<sub>2</sub>O<sub>2</sub> on the conductivity of bathing fluid (water) + cells was compared to the effect of the detergent Triton X-100 (Fig. 3.50). Triton X -100 is known to cause extensive damage to membranes (Langsfel *et al.* 1973).

Exposure of cells to Triton X -100 resulted in a rapid increase in conductivity of the cell suspension within 1 h (Fig 3.50). The curve of the increase in conductivity of the cell suspension was asymptotic; after 6 h of continued exposure to Triton X -100, membrane permeabilisation was assumed to be approaching a maximum. After 6 h the conductivity of the cell suspension treated with 1 mM H<sub>2</sub>O<sub>2</sub> was approximately a third of that when cells had been exposed to Triton X-100. Some of the increase in conductivity in response to 1 mM H<sub>2</sub>O<sub>2</sub> might have been from increased export of solutes into the bathing fluid in response to the stress but the results in figure 3.50 do seem to suggest that membrane permeabilisation increased in response to 1 mM H<sub>2</sub>O<sub>2</sub>. Some reports imply that H<sub>2</sub>O<sub>2</sub> at concentrations lower than 1 mM might not exert any effect at all on cells (Lamb and Dixon 1997, de Pinto *et al.* 2006). Concerns over whether 1 mM H<sub>2</sub>O<sub>2</sub> would be metabolised too quickly to induce a metabolic response or whether 1 mM H<sub>2</sub>O<sub>2</sub> might cause undue membrane damage were allayed by studying the effects of H<sub>2</sub>O<sub>2</sub> at final concentrations of both 0.1 mM and 1 mM.

### **3.4.2 Longevity of H<sub>2</sub>O<sub>2</sub> in culture medium**

The longevity of H<sub>2</sub>O<sub>2</sub> in cell culture medium increased with increasing H<sub>2</sub>O<sub>2</sub> concentration but even 10 mM H<sub>2</sub>O<sub>2</sub> was short lived, being undetectable after 40 minutes. 1 mM H<sub>2</sub>O<sub>2</sub> appeared to have been completely metabolised by 10 minutes (Fig 3.51). These findings are similar to those in previous studies where 20 mM H<sub>2</sub>O<sub>2</sub> had a half life of 2 – 5 min in Arabidopsis cell suspension cultures (Neill *et al.* 2002a). Rapid metabolism of H<sub>2</sub>O<sub>2</sub> in cell

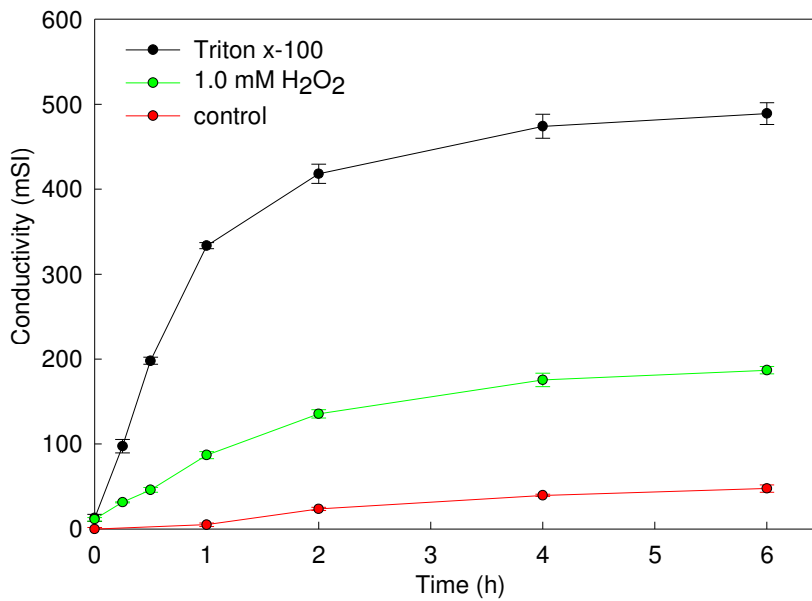
suspension cultures would argue against, but does not disprove, oxidative plasma membrane damage caused by H<sub>2</sub>O<sub>2</sub>. Values at 0 min for 10 mM H<sub>2</sub>O<sub>2</sub> were lower than expected. It is possible that at higher concentrations of H<sub>2</sub>O<sub>2</sub>, diffusion of H<sub>2</sub>O<sub>2</sub> across the plasma membrane occurred rapidly after addition of H<sub>2</sub>O<sub>2</sub> to the culture medium as the H<sub>2</sub>O<sub>2</sub> gradient across the membrane would have been steep at this point. As the culture medium only was sampled, this would not have accounted for H<sub>2</sub>O<sub>2</sub> that had entered the cells. The error on measurements for cultures given 1 mM H<sub>2</sub>O<sub>2</sub> was higher than other concentrations of H<sub>2</sub>O<sub>2</sub>. Possibly there was more variation in cell density in these cultures.

As H<sub>2</sub>O<sub>2</sub> was relatively short lived in cell suspension cultures, then any metabolic responses sustained beyond the time taken for H<sub>2</sub>O<sub>2</sub> to be metabolised could suggest a cell-mediated responses rather than direct effects of H<sub>2</sub>O<sub>2</sub> on ascorbate.

### **3.5 The effect of H<sub>2</sub>O<sub>2</sub> on intracellular and extracellular ascorbate metabolism**

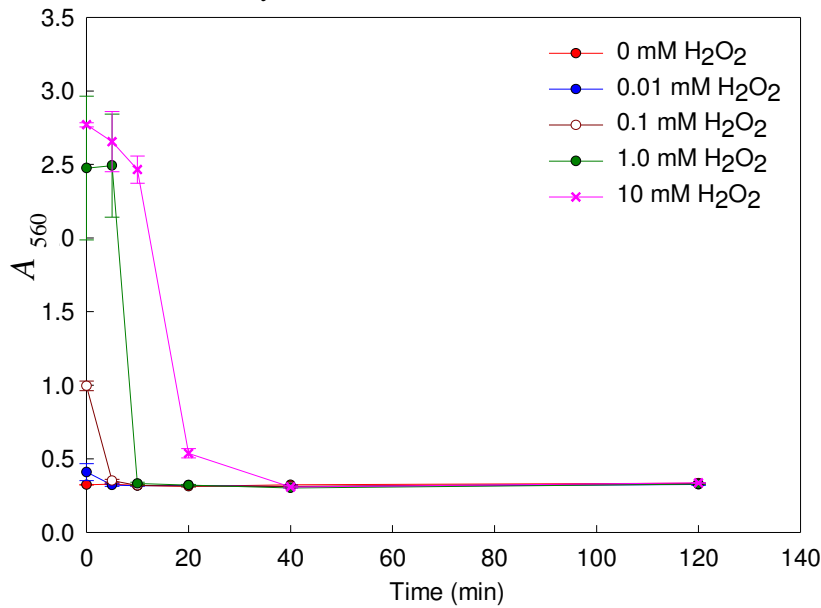
As discussed in section 3.3.9, identification of <sup>3</sup>H-labelled compounds in cell extracts, following incubation with [<sup>3</sup>H]GalL was problematic because of the extensive degradation of [<sup>3</sup>H]GalL. It is not possible to separate [<sup>3</sup>H]DHA from possible neutral contaminants by electrophoresis. Although a large portion of the <sup>3</sup>H-labelled neutral fraction was believed to be [<sup>3</sup>H]DHA based on responses to H<sub>2</sub>O<sub>2</sub> discussed below, it is here referred to as the <sup>3</sup>H-neutral fraction. Earlier work (Fig. 3.9) had shown that the <sup>14</sup>C-neutral fraction could be broken down to [<sup>14</sup>C]DHA breakdown products. It was therefore assumed to comprise only [<sup>14</sup>C]DHA and is here referred to as [<sup>14</sup>C]DHA.

Cell cultures used in a set of experiments (either the fate of [<sup>3</sup>H]ascorbate or [<sup>14</sup>C]ascorbate) were taken from the same parent culture and that set of experiments was carried out on the same day, with all other variables kept constant.



**Figure 3.50 The effect of H<sub>2</sub>O<sub>2</sub> on intracellular solute release..**

10-day-old rose cells were rinsed in water, resuspended in water (20 % fresh weight w/v) and treated with either water, H<sub>2</sub>O<sub>2</sub> or Triton x-100 (0.1 % w/v). The effect of other concentrations of H<sub>2</sub>O<sub>2</sub> on conductivity were not tested.



**Figure 3.51 The longevity of H<sub>2</sub>O<sub>2</sub> in cell cultures.**

Cell cultures were treated either with H<sub>2</sub>O<sub>2</sub> or with water. At time points cell-free medium was sampled. H<sub>2</sub>O<sub>2</sub> content was assayed with xylene orange.

### **3.5.1 Expt. 1a- the fate of endogenous [<sup>3</sup>H]ascorbate in control cell cultures**

The total radioactivity of the culture medium was found to be quite constant implying that little import or export occurred in this control culture (Fig. 3.52b). After 120 min the symplast appeared to become more oxidised as the proportion of <sup>3</sup>H-neutral compounds (thought to mostly consist of [<sup>3</sup>H]DHA) increased and the [<sup>3</sup>H]ascorbate content decreased (Fig 3.52a). As only 2 compounds and the neutral fraction were selected for analysis, it could not be said whether a more oxidised symplast resulted in the accumulation of ascorbate oxidation products such as [<sup>14</sup>C]ox.thr. or [<sup>14</sup>C]oxalate in the symplast. A comparison with control cultures incubated with [<sup>14</sup>C]ascorbate (Fig. 3.53a) suggested, however, that this did not occur. A sufficient excess of cell culture volume was present at 480 min so that cells did not experience stress from drying out. It was possible that the progressive change in cell number, density and/or culture volume comprised a stress which induced a change in the symplastic redox state.

That a similar phenomenon was seen in <sup>14</sup>C-labelled and <sup>3</sup>H-labelled cell cultures implies that, despite the multiple difficulties encountered in the analysis of cells incubated with [<sup>3</sup>H]GalL, some of the results selected for analysis from these cultures were still informative. In an earlier experiment cells were washed sequentially after incubation with the radiolabel; analysis of the radioactivity of these washes indicated that the method followed in these experiments successfully removed almost all extracellular radioactivity (data not shown).

### **3.5.2 Experiment 1b- the fate of [<sup>14</sup>C]ascorbate in control cell cultures**

Over 90 % of the [<sup>14</sup>C]ascorbate was taken up in 4 h. Before the addition of water to the control culture at 0 min, the majority of [<sup>14</sup>C]ascorbate was present in the symplasm in the reduced form, [<sup>14</sup>C]ascorbate, rather than [<sup>14</sup>C]DHA. Some [<sup>14</sup>C]oxalate, presumably a minor component of [<sup>14</sup>C]ascorbate stock, was present in the culture medium at the start of the

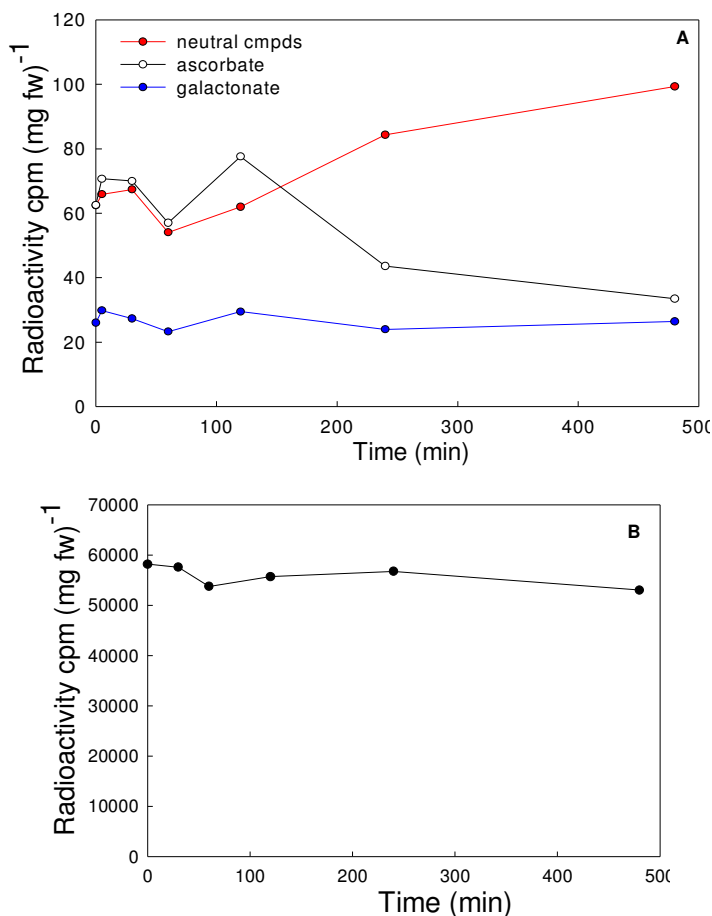
incubation period. During incubation, no evidence was seen for the uptake of this [ $^{14}\text{C}$ ]oxalate (Fig. 3.53c).

The addition of 20  $\mu\text{l}$  of water to the control culture was taken to represent a mild disturbance to cells as it only comprised approximately 2.5% of the initial culture volume and did not involve the addition of exogenous ROS. Despite the apparently mild nature of this treatment, the symplast did become more oxidised after the addition water; after 0 min, the majority of  $^{14}\text{C}$  was present as [ $^{14}\text{C}$ ]DHA (Fig. 3.53a). [ $^{14}\text{C}$ ]Ascorbate and [ $^{14}\text{C}$ ]DHA were present in equal proportions at 240 min; the symplastic redox state was returning to post-water-treatment levels. However, as sampling ceased at 240 min it was not possible to see if this was a genuine return to a pre-stress redox state. No increase was noted in symplastic [ $^{14}\text{C}$ ]ascorbate metabolites after 0 min despite the increased oxidation state of the symplast (Fig. 3.53a).

### **3.5.3 Experiment 2a- the fate of endogenous [ $^3\text{H}$ ]ascorbate in response to 0.1 mM $\text{H}_2\text{O}_2$**

The symplast appeared to become more oxidised over time but following a 0.1 mM  $\text{H}_2\text{O}_2$  stress, this effect was exaggerated compared to control cultures (Fig. 3.52a, 3.53a), with even lower levels of [ $^3\text{H}$ ]ascorbate at 480 min and higher level of  $^3\text{H}$ -labelled neutral compounds (Fig. 3.54a). Culture volumes were constant between experiments and so, in theory, levels of stress from a decreasing culture volume would have been constant, if this were indeed a source of stress. This suggests that the symplast seems to have become more oxidised in response to oxidative stress. Interestingly, 0.1 mM  $\text{H}_2\text{O}_2$  was not expected to last more than 5 min (Fig. 3.51) in the culture medium, implying this response was not direct consequences of the oxidation of [ $^3\text{H}$ ]ascorbate by  $\text{H}_2\text{O}_2$ .



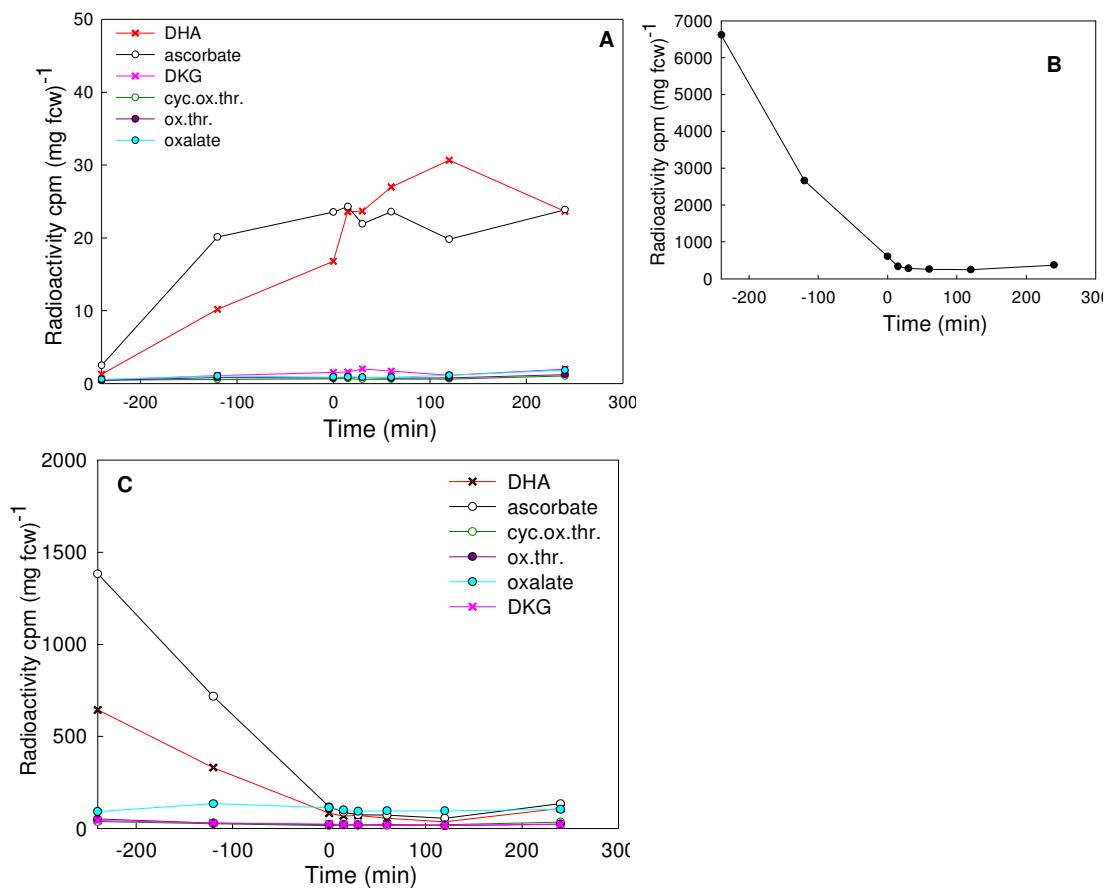


**Figure 3.52 Expt. 1a The fate of endogenous [<sup>3</sup>H]ascorbate in control cell cultures.**

Cell cultures (10-day-old) were incubated with [<sup>3</sup>H]GalL (1MBq in 1 ml culture) for 16 h under asptic conditions. Water (20  $\mu$ l ml<sup>-1</sup> culture) was added to the medium at 0 min. Cells were filtered, washed with water, weighed and bathed in a solution of 40% formic acid supplemented with 25 mM cold ascorbic acid for 5 min (1:1 cells:formic acid w/v). Cell extracts were analysed by paper electrophoresis at pH 3.5 (3.0 kV, 40 min). Electrophoretograms were fluorographed for 8 weeks.

A. The response of individual metabolites in the symplasm to the addition of water. Areas of the electrophoretogram that corresponded to bands on the fluorogram were excised and assayed for <sup>3</sup>H by scintillation counting. Compounds were identified with  $m_{OG}$  values of bands to those for external markers that had been stained with silver nitrate.

B. Total <sup>3</sup>H in the apoplast. Culture medium (10  $\mu$ l) was assayed for <sup>3</sup>H by scintillation counting after cells were removed by filtration.



**Figure 3.53 Expt. 1b—the fate of exogenous [ $^{14}\text{C}$ ]ascorbate in control cell cultures**

Cell cultures (750  $\mu\text{l}$  initial volume) were incubated with [ $^{14}\text{C}$ ]ascorbate for 4 h, from -240 – 0 min on graphs. Water was added immediately before 0 min. Cell extracts were made as in Fig. 3.52. Cell extracts and filtered culture medium were analysed by paper electrophoresis at pH 6.5 (3.0 kV, 30 min). Electrophoretograms were exposed to autoradiography film for 2 weeks. External markers were stained with silver nitrate. [ $^{14}\text{C}$ ]Ascorbate and [ $^{14}\text{C}$ ]ascorbate metabolites were identified by  $m_{\text{OG}}$  values and the positions of external markers. The areas of the electrophoretogram corresponding bands on the autoradiography film were assayed for  $^{14}\text{C}$  by scintillation counting.

A. The fate of individual [ $^{14}\text{C}$ ]ascorbate metabolites in the symplast.

B. Total  $^{14}\text{C}$  in the culture medium. Culture medium (10  $\mu\text{l}$ ) was assayed for  $^{14}\text{C}$  by scintillation counting after cells were removed by filtration.

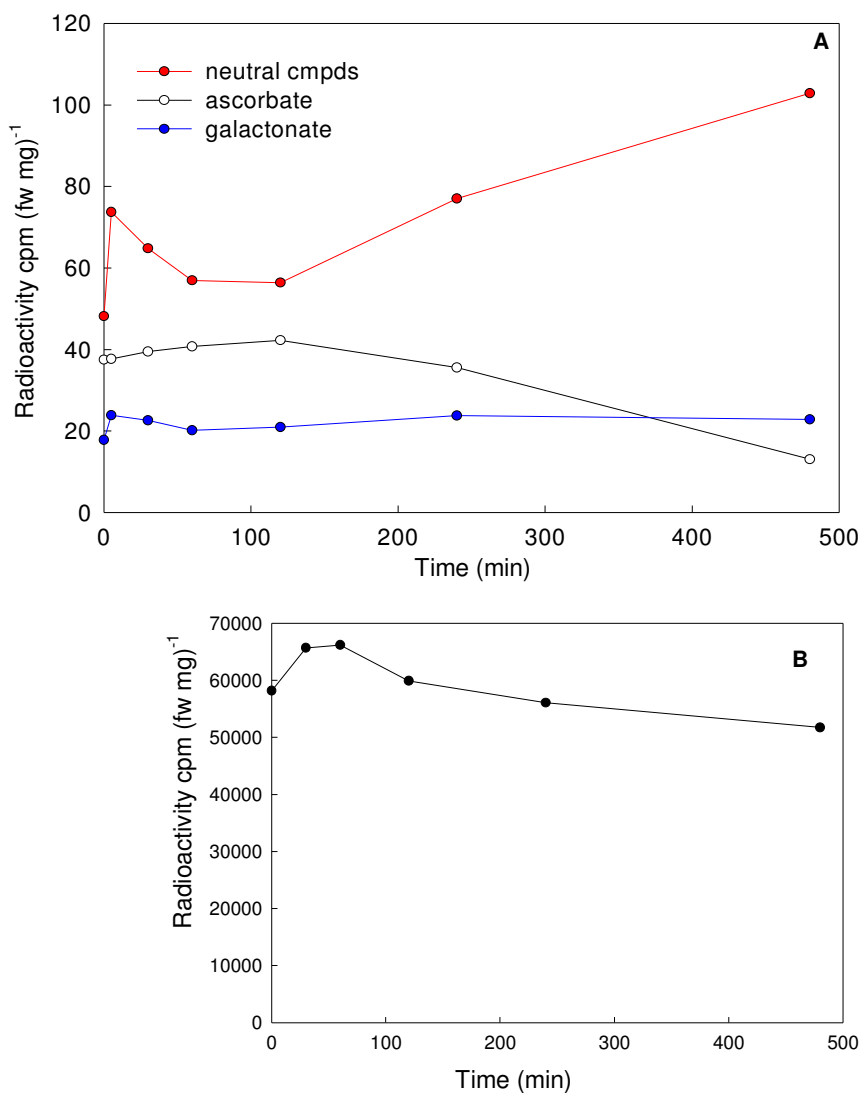
C. The fate of individual [ $^{14}\text{C}$ ]ascorbate metabolites in the culture medium.

An increase symplastic  $^3\text{H}$ -neutral compounds was observed immediately after the addition of 0.1 mM  $\text{H}_2\text{O}_2$  (Fig. 3.54a). This raises the possibility of import of [ $^3\text{H}$ ]DHA in response to a 0.1 mM  $\text{H}_2\text{O}_2$  stress but as this result was not repeated in cultures incubated with [ $^{14}\text{C}$ ]ascorbate and it was not possible to put error bars on results, such observations remain speculative.

### **3.5.4 Experiment 2b- the fate of [ $^{14}\text{C}$ ]ascorbate in response to 0.1 mM $\text{H}_2\text{O}_2$**

Uptake of [ $^{14}\text{C}$ ]ascorbate was rapid; over 90 % was taken up during the 4 h incubation period (Fig. 3.55b). Contrary to the control culture (Fig. 3.53a), pre-stress [ $^{14}\text{C}$ ]ascorbate levels were slightly lower than [ $^{14}\text{C}$ ]DHA levels during this time (Fig. 3.55a). Immediately after treatment with 0.1 mM  $\text{H}_2\text{O}_2$ , symplastic [ $^{14}\text{C}$ ]ascorbate levels were seen to almost double (Fig. 3.55a). Import of [ $^{14}\text{C}$ ]ascorbate (or [ $^{14}\text{C}$ ]DHA which was rapidly reduced to [ $^{14}\text{C}$ ]ascorbate) could have occurred in response to a 1 mM  $\text{H}_2\text{O}_2$  stress.

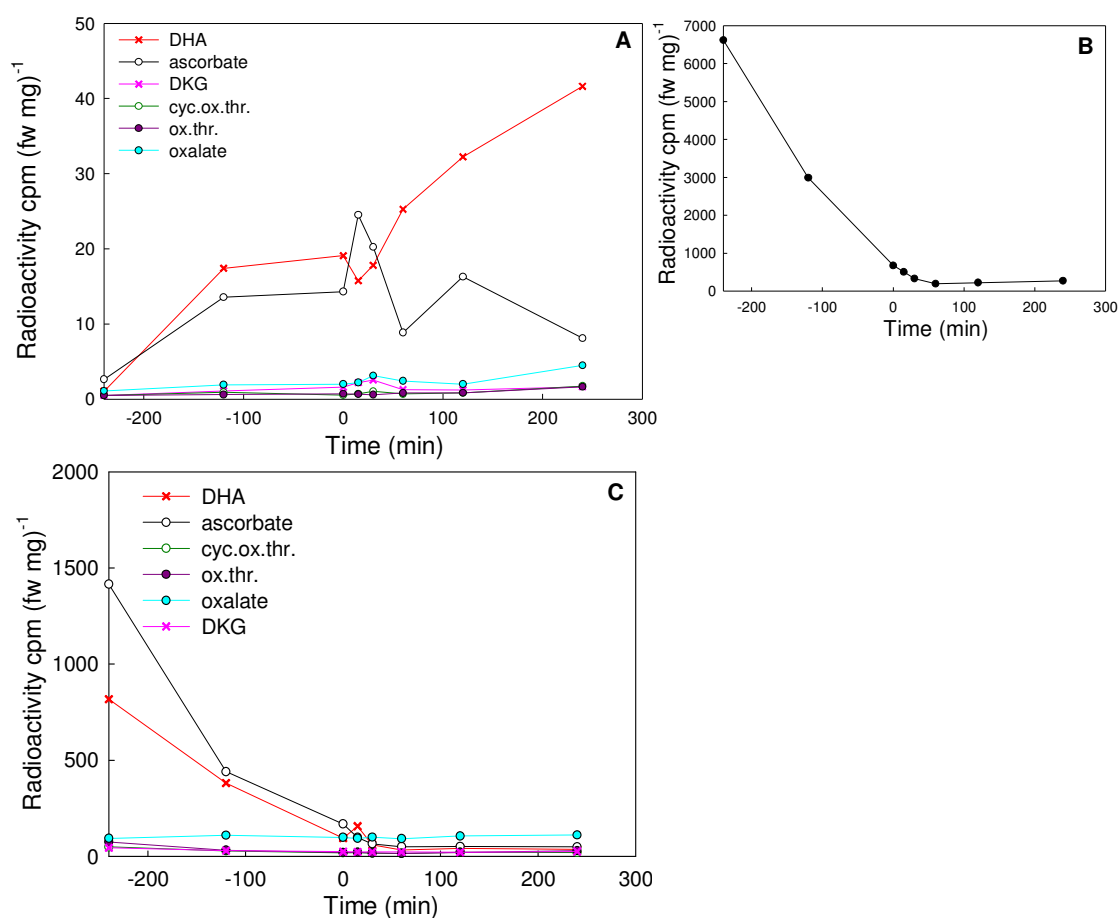
After 0.1 mM  $\text{H}_2\text{O}_2$  treatment the symplast became more oxidised. [ $^{14}\text{C}$ ]DHA increased steadily from 30 – 120 min whilst [ $^{14}\text{C}$ ]ascorbate showed an overall decrease during this time (Fig. 3.55a). Oxidation of the symplasm continued after 0.1 mM  $\text{H}_2\text{O}_2$  was predicted to have been metabolised in the culture medium (Fig. 3.51); the increased proportion of symplastic [ $^{14}\text{C}$ ]DHA did not seem to result solely from the oxidation of [ $^{14}\text{C}$ ]ascorbate by  $\text{H}_2\text{O}_2$ . Increased oxidation of the symplasm agreed with previous results where increased oxidation of symplasm was exaggerated in response to 0.1 mM  $\text{H}_2\text{O}_2$  compared to controls in cells incubated with [ $^3\text{H}$ ]GalL (Fig. 3.52a, 3.54a). A small but consistent increase in the symplastic concentration of [ $^{14}\text{C}$ ]oxalate was measured from 15 min onwards (Fig. 3.55a). Possibly, accumulation of [ $^{14}\text{C}$ ]oxalate was slightly promoted by the redox state becoming more oxidised, although with such low counts this was difficult to say with certainty.



**Figure 3.54 Expt. 2a –the fate of endogenous [<sup>3</sup>H]ascorbate in cell cultures receiving 0.1 mM H<sub>2</sub>O<sub>2</sub>.**

Methods were as detailed in figure 3.52. Immediately before ‘0 min’ H<sub>2</sub>O<sub>2</sub> was added to a final concentration of 0.1 mM.

- A. The response of individual metabolites in the symplasm to the addition of 0.1 mM H<sub>2</sub>O<sub>2</sub>.
- B. Total <sup>3</sup>H in the culture medium



**Figure 3.55 Expt. 2b The fate of endogenous [<sup>14</sup>C]ascorbate in cell cultures receiving 0.1 mM H<sub>2</sub>O<sub>2</sub>**

Methods were as detailed in figure 3.53. H<sub>2</sub>O<sub>2</sub> (final conc. 0.1 mM) was added to the medium at 0 min.

A. The response of individual metabolites in the symplasm to the addition of 0.1 mM H<sub>2</sub>O<sub>2</sub>.

B. Total <sup>14</sup>C in the culture medium.

C. The response of individual metabolites in culture medium to the addition of 0.1 mM H<sub>2</sub>O<sub>2</sub>.

### **3.5.5 Experiment 3a- the fate of endogenous [<sup>3</sup>H]ascorbate in response to 1 mM H<sub>2</sub>O<sub>2</sub>**

Immediately after cells were supplied with 1 mM H<sub>2</sub>O<sub>2</sub> a drop in symplastic [<sup>3</sup>H]ascorbate was measured (Fig. 3.56a). A drop in the <sup>3</sup>H-neutral fraction was also recorded a few minutes after the decrease in [<sup>3</sup>H]ascorbate (Fig. 3.56a). This could be a result of oxidation of [<sup>3</sup>H]ascorbate to [<sup>3</sup>H]DHA, conceivably from H<sub>2</sub>O<sub>2</sub> entering the cell. Concentrations of symplastic <sup>3</sup>H-neutral compounds and [<sup>3</sup>H]ascorbate were lowest at 60 min (Fig. 3.56a), whilst levels of <sup>3</sup>H were highest in the culture medium at 60 min (3.56b). This suggests that <sup>3</sup>H was exported following a 1 mM H<sub>2</sub>O<sub>2</sub> stress. It was not known in what form the <sup>3</sup>H had been exported.

In expts. 1a and 2a the proportion of [<sup>3</sup>H]DHA increased as [<sup>3</sup>H]ascorbate decreased, but in expt. 3a combined [<sup>3</sup>H]ascorbate and [<sup>3</sup>H]-neutral compounds decreased. Levels of both [<sup>3</sup>H]ascorbate and <sup>3</sup>H-neutral compounds showed some signs of recovery to initial values towards 480 min, with [<sup>3</sup>H]ascorbate recovering first, from 60 min onwards, but not to pre-stress-treatment levels (Fig. 3.56a). When [<sup>14</sup>C]ascorbate was fed to cells which were then stressed with 1 mM H<sub>2</sub>O<sub>2</sub>, a similar response was seen (Fig. 5.27a) Again, [<sup>14</sup>C]ascorbate levels recovered first but neither [<sup>14</sup>C]ascorbate nor [<sup>14</sup>C]DHA levels returned to pre-stress levels.

### **3.5.6 Experiment 3b- the fate of [<sup>14</sup>C]ascorbate in response to 1 mM H<sub>2</sub>O<sub>2</sub>**

Over 80 % of the [<sup>14</sup>C]ascorbate was taken up in 4 h (Fig. 3.57b), slightly less than in expts. 1b and 2b. During uptake, the majority of [<sup>14</sup>C]ascorbate was in the reduced form (Fig. 3.57a), as had been seen in expt. 1b but not in expt. 2b.

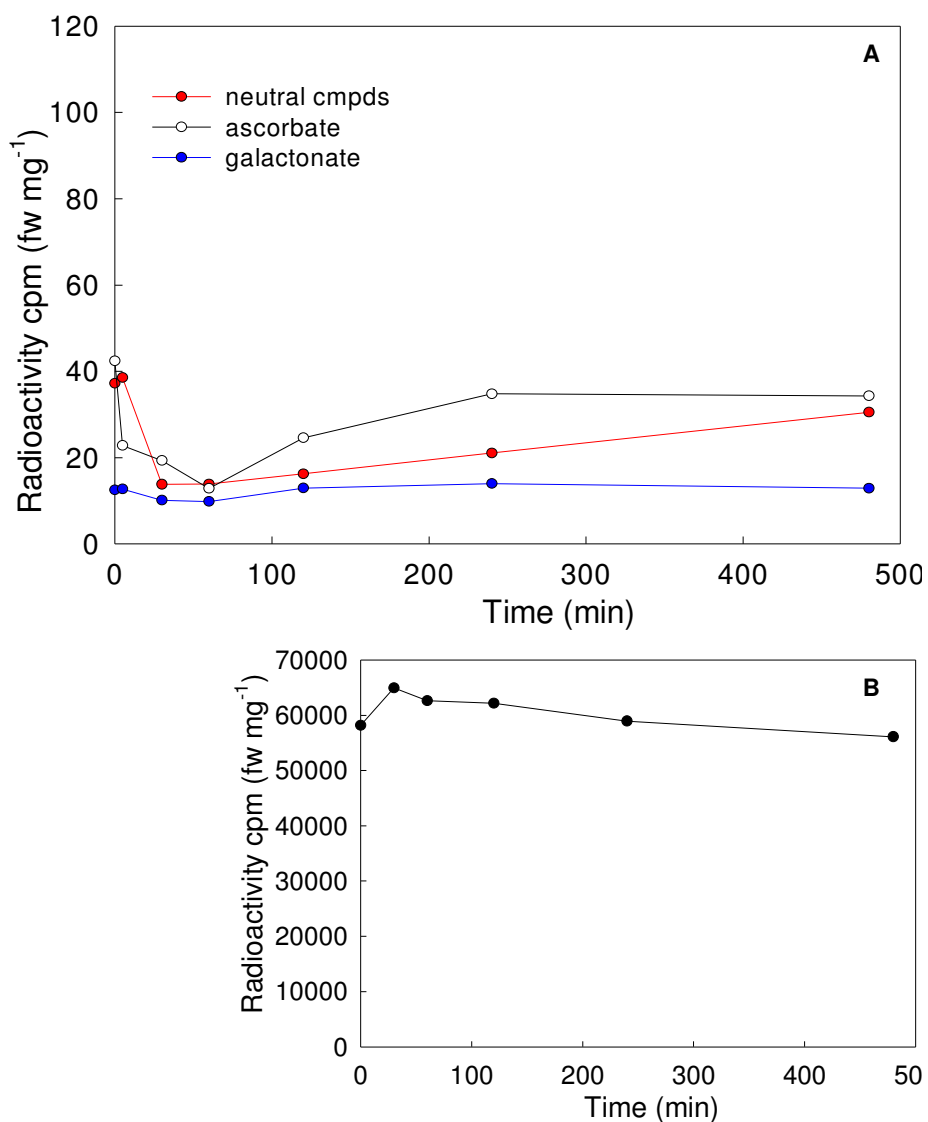
The symplastic [<sup>14</sup>C]ascorbate content dropped by approximately 75 % immediately after addition of H<sub>2</sub>O<sub>2</sub>, accompanied by a large increase in [<sup>14</sup>C]oxalate content and [<sup>14</sup>C]DHA (Fig. 3.57a). Analysis of individual metabolites present in the culture medium revealed an increase in [<sup>14</sup>C]DHA that reached a maximum at 30 min after H<sub>2</sub>O<sub>2</sub> addition, before decreasing

(Fig. 3.57c). Symplastic [ $^{14}\text{C}$ ]DHA also decreased from 15 – 30 min (Fig. 3.57a). This suggests that [ $^{14}\text{C}$ ]DHA was exported after treatment with 1 mM  $\text{H}_2\text{O}_2$ . It is important to note that this does not rule out export of [ $^{14}\text{C}$ ]ascorbate as [ $^{14}\text{C}$ ]DHA could have been reduced to [ $^{14}\text{C}$ ]ascorbate intracellularly and exported immediately afterwards. The increase in extracellular [ $^{14}\text{C}$ ]DHA (Fig. 3.57c) could also be attributed to exported [ $^{14}\text{C}$ ]ascorbate being oxidised to [ $^{14}\text{C}$ ]DHA in the culture medium. If [ $^{14}\text{C}$ ]ascorbate was exported in response to an oxidative stress, then it could be considered reasonable unlikely that once exported, [ $^{14}\text{C}$ ]ascorbate would be re-imported as [ $^{14}\text{C}$ ]ascorbate and not [ $^{14}\text{C}$ ]DHA. Therefore, the increase in [ $^{14}\text{C}$ ]ascorbate at 30 min (Fig. 3.57a) could be expected to be from ascorbate regeneration.

Extracellular [ $^{14}\text{C}$ ]oxalate concentrations increased from 15 min onwards (Fig. 3.57c). Again, this could have been due to oxidation of exported [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]DHA. As [ $^{14}\text{C}$ ]oxalate levels were stable after 60 min it was not considered likely that [ $^{14}\text{C}$ ]oxalate was readily transported across the plasma membrane, in agreement with previous studies (Green 2003). This then casts some doubt over the decrease in symplastic [ $^{14}\text{C}$ ]oxalate from 15 – 30 min (Fig. 3.57a); this was unlikely to be a result of [ $^{14}\text{C}$ ]oxalate export and if it were due to metabolism of [ $^{14}\text{C}$ ]oxalate, it seems odd that [ $^{14}\text{C}$ ]oxalate not metabolised further after 30 min.

[ $^{14}\text{C}$ ]Ox.thr. was detected in the symplasm. [ $^{14}\text{C}$ ]Ox.thr. levels increased after the addition of 1 mM  $\text{H}_2\text{O}_2$  but were always low (Fig. 3.57a) compared to [ $^{14}\text{C}$ ]oxalate.

*In-vitro* studies show yields of oxalate from ox.thr. are low; the [ $^{14}\text{C}$ ]oxalate levels in figure 3.57a imply that the esterase proposed by Green and Fry (2005) is present in the symplasm or



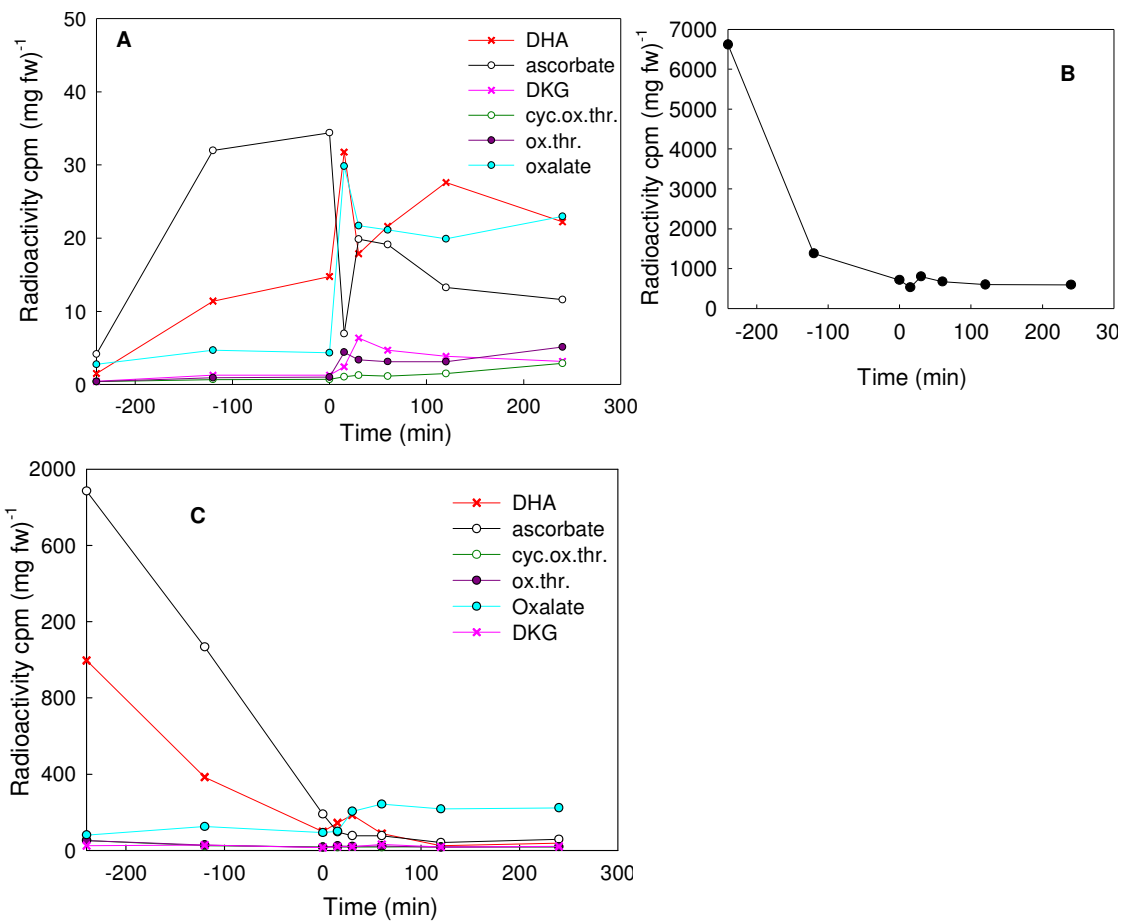
**Figure 3.56 Expt. 3a The fate of endogenous [<sup>3</sup>H]ascorbate in cell cultures receiving 1.0 mM H<sub>2</sub>O<sub>2</sub>**

Methods were as detailed in figure 3.52. H<sub>2</sub>O<sub>2</sub> (final concentration 1 mM) was added at 0 min.

A. The response of individual metabolites in the symplasm to the addition of 1.0 mM H<sub>2</sub>O<sub>2</sub>.

B. Total <sup>3</sup>H in the culture medium





**Figure 3.57 Expt. 3b** The fate of exogenous [<sup>14</sup>C]ascorbate in cell cultures receiving 1 mM H<sub>2</sub>O<sub>2</sub>

Methods were as detailed in figure 3.53. H<sub>2</sub>O<sub>2</sub> (final concentration 1 mM) was added to the cell culture medium at 0 min.

A. The response of individual metabolites in the symplasm to the addition of 1 mM H<sub>2</sub>O<sub>2</sub>.

B. Total <sup>14</sup>C in the culture medium

C. The response of individual metabolites in culture medium to the addition of 1 mM H<sub>2</sub>O<sub>2</sub>.

that symplastic conditions are more favourable for hydrolysis. However, [<sup>14</sup>C]DKG levels were low in the symplast:- if hydrolysis had been favoured, they would have been expected to be higher.

Similar to expts. 1b and 2b the symplast became more oxidised upon H<sub>2</sub>O<sub>2</sub> treatment; [<sup>14</sup>C]DHA increased compared to [<sup>14</sup>C]ascorbate (Fig. 3.57a). In contrast to expts. 1b and 2b, combined [<sup>14</sup>C]ascorbate + [<sup>14</sup>C]DHA levels after 1 mM H<sub>2</sub>O<sub>2</sub> treatment were lower than values prior to H<sub>2</sub>O<sub>2</sub> addition. A similar distinction was found to exist between expts. 1a, 2a and 3a. [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA levels could have become lowered after exposure to 1 mM H<sub>2</sub>O<sub>2</sub> as a result of continued export of either or both of these compounds. However it is also possible that oxidation of [<sup>14</sup>C]ascorbate to [<sup>14</sup>C]oxalate by H<sub>2</sub>O<sub>2</sub> meant that the total [<sup>14</sup>C]ascorbate + [<sup>14</sup>C]DHA pool had been irreversibly decreased and could only be replenished by biosynthesis *de novo*. As [<sup>14</sup>C]oxalate did not seem to be transported readily across the plasma membrane then irreversibly oxidised [<sup>14</sup>C]ascorbate could potentially be permanently compartmentalised on either side of the plasma membrane, depending on the site of oxidation.

### **3.5.7 Summary of the fate of endogenous [<sup>3</sup>H]ascorbate and [<sup>14</sup>C]ascorbate in cell suspension cultures in response to oxidative stress**

It was not possible to analyse a large amount of the data generated from cell suspension culture incubated with [<sup>3</sup>H]GalL. Nevertheless, similarities in the response of [<sup>3</sup>H]ascorbate and <sup>3</sup>H-labelled neutral compounds to responses of [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA suggest that these data are still informative. Cells seemed to be sensitive even to the addition of water as the symplast became more oxidised in control cells (Fig. 3.52a, 3.53a). Increasing proportions of [<sup>14</sup>C]DHA or <sup>3</sup>H-neutral compounds did not necessarily lead to an increase in irreversible breakdown of [<sup>3</sup>H]ascorbate or [<sup>14</sup>C]ascorbate (Fig. 3.53a, 3.55a).

In cell cultures treated with 0.1 mM H<sub>2</sub>O<sub>2</sub> the oxidation of the symplast was exaggerated compared to control cultures (Fig. 3.54a, 3.55a). This continued to happen after 0.1 mM H<sub>2</sub>O<sub>2</sub>

was expected to have been completely removed from the culture medium, suggesting that increasing proportions of [ $^{14}\text{C}$ ]DHA or  $^3\text{H}$ -neutral compounds was not a direct consequence of oxidation of [ $^{14}\text{C}$ ]ascorbate or [ $^3\text{H}$ ]ascorbate by  $\text{H}_2\text{O}_2$ . It is possible that some  $\text{H}_2\text{O}_2$  entered the cell. It was not possible to determine what concentrations of  $\text{H}_2\text{O}_2$  might have entered the cell. This raises an important question of the cellular concentration of  $\text{H}_2\text{O}_2$  at which irreversible ascorbate breakdown would occur.

In cultures stressed with 1 mM  $\text{H}_2\text{O}_2$ , evidence was seen for the export, and possibly import, of either [ $^{14}\text{C}$ ]ascorbate and [ $^3\text{H}$ ]ascorbate metabolites soon after the addition of  $\text{H}_2\text{O}_2$  to the culture medium. The peak in ascorbate at 30 min suggests that regeneration of ascorbate from DHA is likely to be happening. It was not possible to determine whether [ $^3\text{H}$ ]ascorbate or [ $^{14}\text{C}$ ]ascorbate was exported in the reduced or oxidised form.

Consistent with previous results (Green 2003), oxalate was not found to pass easily across the plasma membrane. [ $^{14}\text{C}$ ]Ascorbate that was oxidised to [ $^{14}\text{C}$ ]oxalate was thought to accumulate in either the symplasm or the culture medium, depending on the site of oxidation (Fig. 3.57a, c).

### **3.6 The effect of $\text{MV}^{2+}$ on intracellular ascorbate metabolism**

When cells pre-loaded with [ $^{14}\text{C}$ ]ascorbate were treated with  $\text{MV}^{2+}$  at final concentrations of both 1  $\mu\text{M}$  and 10  $\mu\text{M}$ , intracellular breakdown of [ $^{14}\text{C}$ ]ascorbate was observed. [ $^{14}\text{C}$ ]DHA, [ $^{14}\text{C}$ ]oxalate and [ $^{14}\text{C}$ ]ox.thr. were observed. [ $^{14}\text{C}$ ]DKG was present in trace amounts whilst neither [ $^{14}\text{C}$ ]cyc.ox.thr. nor [ $^{14}\text{C}$ ]C or [ $^{14}\text{C}$ ]E were observed (Fig. 3.58). The range of  $^{14}\text{C}$ -labelled metabolites is similar to that which was seen following treatment with 1 mM  $\text{H}_2\text{O}_2$  (Fig. 3.57a); [ $^{14}\text{C}$ ]DHA, [ $^{14}\text{C}$ ]ox.thr. and [ $^{14}\text{C}$ ]oxalate appear therefore to be the principal intracellular [ $^{14}\text{C}$ ]ascorbate breakdown products. More [ $^{14}\text{C}$ ]oxalate than [ $^{14}\text{C}$ ]ox.thr. was observed after 480 min (Fig. 3.58). Whilst this supports the idea of an esterase enzyme

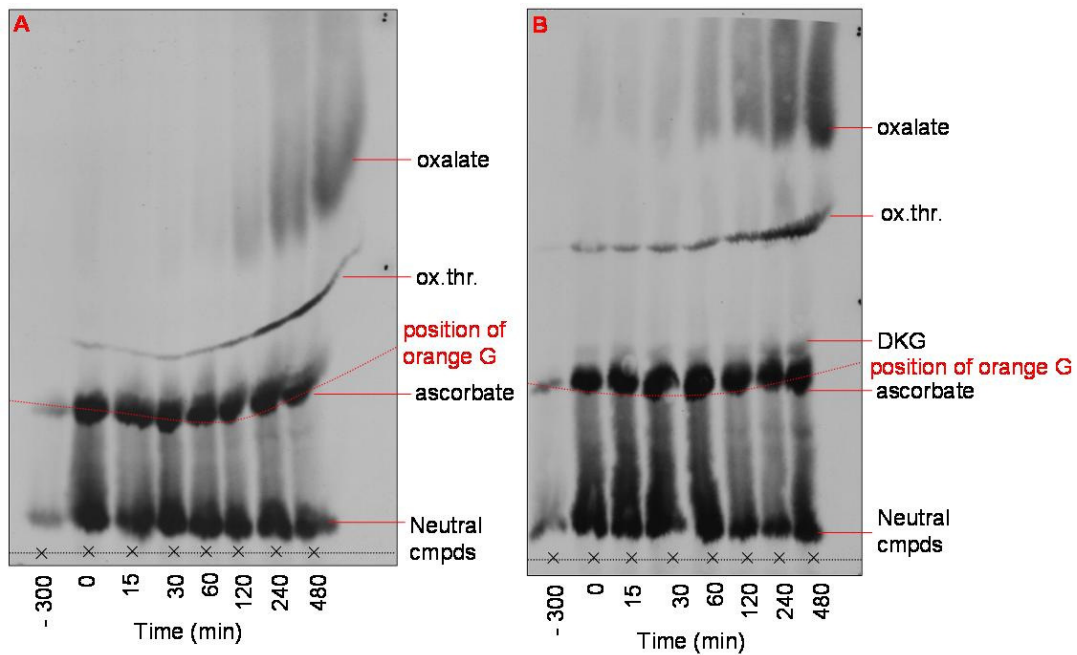
which catalyses the hydrolysis of ox.thr. to oxalate and threonate (Green and Fry 2005), a build up of [ $^{14}\text{C}$ ]ox.thr. can be seen 480 min after treatment with 10  $\mu\text{M}$   $\text{MV}^{2+}$  (Fig. 3.58b). This implies that the capacity of this proposed enzyme is not excessively high, an idea which is consistent with observations in figure 3.57a in which [ $^{14}\text{C}$ ]ox.thr. levels were lower than [ $^{14}\text{C}$ ]oxalate but were still easily detectable.

The gradual increase in [ $^{14}\text{C}$ ]ox.thr. and [ $^{14}\text{C}$ ]oxalate over time suggests that [ $^{14}\text{C}$ ]ascorbate breakdown is a direct result of increasing  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  in the cell. This shows that generation of [ $^{14}\text{C}$ ]ox.thr. from [ $^{14}\text{C}$ ]DHA is not a process which is actively increased as part of an oxidative stress response.

Without quantification of results, it is hard to gauge differences in intensity of darkened spots. In response to 10  $\mu\text{M}$   $\text{MV}^{2+}$ , there did seem to be an increased rate of [ $^{14}\text{C}$ ]ascorbate oxidation to [ $^{14}\text{C}$ ]DHA as judged by the amount of [ $^{14}\text{C}$ ]DHA present in samples between 0 min and 60 min and by the intensity of the smear between [ $^{14}\text{C}$ ]ascorbate and [ $^{14}\text{C}$ ]DHA. This smear was thought to represent interconversion between [ $^{14}\text{C}$ ]ascorbate and [ $^{14}\text{C}$ ]DHA. Intensity of the smear and of [ $^{14}\text{C}$ ]DHA appeared to peak at 30 min (Fig. 3.58b). The peak of breakdown of [ $^{14}\text{C}$ ]ascorbate to [ $^{14}\text{C}$ ]DHA at 30 min did not seem to coincide with an increase in [ $^{14}\text{C}$ ]ox.thr. or [ $^{14}\text{C}$ ]oxalate. [ $^{14}\text{C}$ ]Ox.thr. and [ $^{14}\text{C}$ ]oxalate only showed an appreciable increase from 120 min onwards (Fig. 3.58a), possibly from 60 min in figure 3.58b. This suggests that until a certain threshold of oxidative stress, production of [ $^{14}\text{C}$ ]ox.thr. and [ $^{14}\text{C}$ ]oxalate could be avoided, presumably from regeneration of [ $^{14}\text{C}$ ]DHA, as was inferred from figures 3.52 – 3.55 where increased proportions of [ $^{14}\text{C}$ ]DHA in the cell also did not coincide with an appreciable increase in [ $^{14}\text{C}$ ]oxalate.

Similar to many experiments where stress is simulated under artificial conditions, it was hard to know how intracellular concentrations of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  at 480 min after treatment with either 1  $\mu\text{M}$  or 10  $\mu\text{M}$   $\text{MV}^{2+}$  would compare with  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  generated during e.g. high light

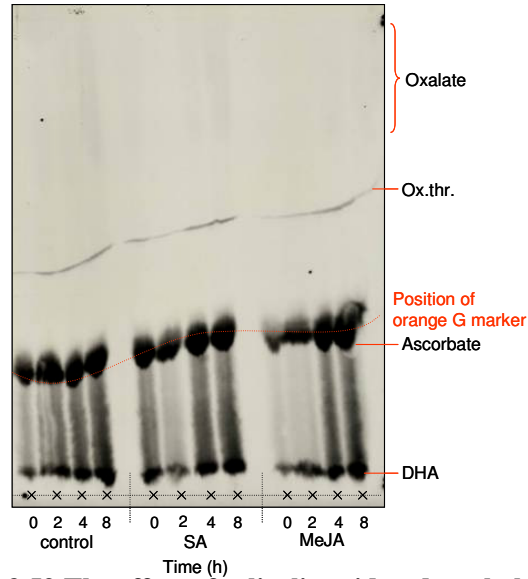
stress or drought stress and therefore whether oxalate would genuinely accumulate in the cell. It is also worth considering whether oxalate might be formed in some cell compartments in response to oxidative stress but not in others and what this might mean in terms of signalling and subsequent metabolic responses.



**Figure 3.58 The fate of symplastic ascorbate in response to MV<sup>2+</sup>**

Cell contents was extracted as detailed in figure 3.53. Samples were analysed by paper electrophoresis at pH 6.5 for either 30 min (A) or 35 min (B), 3.5 kV. MV<sup>2+</sup> was added at 0 min. Incubation with [<sup>14</sup>C]ascorbate was started at - 300 min.

- A. Symplastic <sup>14</sup>C-labelled metabolites in cell extracts following the addition of MV<sup>2+</sup> to 1 μM  
 B. Symplastic <sup>14</sup>C-labelled metabolites in cell extracts following the addition of MV<sup>2+</sup> to 10 μM



**Figure 3.59 The effect of salicylic acid and methyl jasmonate on [<sup>14</sup>C]ascorbate breakdown**  
 Either 20  $\mu$ l of salicylic acid or methyl jasmonate were added to the culture medium of 10-day-old rose cell cultures to a final concentration of 100  $\mu$ M. The control culture received 20 $\mu$ l water. Samples of cell extracts were taken as detailed in figure 3.53. Results were analysed by electrophoresis at pH 6.5 (3.5 kV, 30 min). SA, salicylic acid; MeJA, methyl jasmonate.

### 3.7 The effect of salicylic acid and methyl jasmonate on intracellular ascorbate metabolism

Salicylic acid and methyl jasmonate are known to be key compounds in co-ordinating responses to wounding or pathogen attack, including the oxidative burst (Buchanan *et al.* 2000). If irreversible ascorbate breakdown were to form part of a controlled response to a stress-induced oxidative burst, it was conceivable that these compounds might be involved in regulating the breakdown. The effect of these two compounds on ascorbate breakdown was therefore investigated. The addition of either salicylic acid or methyl jasmonate did not appear to have any effect on the rate of [<sup>14</sup>C]ascorbate breakdown (Fig. 3.59). As was seen in control cells, a small amount of [<sup>14</sup>C]ascorbate turnover appeared to be taking place as low levels of [<sup>14</sup>C]ox.thr. and [<sup>14</sup>C]oxalate were present (Fig. 3.59). As had been noted in previous experiments (Fig.3.58, Fig. 3.57), [<sup>14</sup>C]cyc.ox.thr. and [<sup>14</sup>C]C or [<sup>14</sup>C]E were not observed, indicating that they were not important intracellular [<sup>14</sup>C]ascorbate breakdown products. [<sup>14</sup>C]DKG had been observed in trace quantities in other experiments (Fig. 3.56 – 3.58) but was not seen at all in these cell extracts. This would seem to suggest that DKG is not normally an important compound in intracellular ascorbate breakdown either. The amount of [<sup>14</sup>C]DHA does seem to increase over time in all three treatments (Fig. 3.59) but without an appreciable increase in either [<sup>14</sup>C]ox.thr. or [<sup>14</sup>C]oxalate, similar to what had been observed in previous experiments (Fig.3.52 – 3.55). These results show that if controlled ascorbate breakdown was part of a stress response (in addition to oxidative breakdown that might result from increased ROS), breakdown was not elicited by either salicylic acid or methyl jasmonate.

### **3.8 Transport of [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]ascorbate metabolites across the plasma membrane**

#### **3.8.1 Transport of [<sup>14</sup>C]ascorbate and/or [<sup>14</sup>C]ascorbate metabolites across the plasma membrane in response to 5 mM H<sub>2</sub>O<sub>2</sub> (expts. 1 and 2)**

Results in section 3.5 suggested that one of the important responses to oxidative stress in cell suspension cultures was the transport of ascorbate and/or DHA across the plasma membrane. This response was investigated further in multiple experiments in which cells, incubated with [<sup>14</sup>C]ascorbate, were transferred to non-radioactive medium and stressed with exogenously added H<sub>2</sub>O<sub>2</sub>. In all experiments cell cultures of a settled cell volume of 30% were used and were maintained as homogenous cultures by shaking.

Initially it was not known whether sufficient radioactivity would be released from cells for accurate detection of radioactivity by scintillation counting or autoradiography. Cell cultures were therefore supplied with a more severe stress, 5 mM H<sub>2</sub>O<sub>2</sub>, rather than 1 mM H<sub>2</sub>O<sub>2</sub> as in previous experiments. Experiments using a 5 mM H<sub>2</sub>O<sub>2</sub> stress were repeated and then repeated again with 1 mM H<sub>2</sub>O<sub>2</sub>. So that exported compounds could be sampled before they were oxidised in the medium, samples were taken at rapid intervals. Analysis by electrophoresis revealed the presence of [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA (Fig. 3.60, 3.61). These were not present at 0 min, after the exchange of radioactive for non-radioactive medium, and so were believed to have been exported. Some of the intracellular radioactivity was exported followed by import of an almost equivalent quantity of radioactivity. This produced a pulse like pattern of radioactivity in the culture medium and was noted in response to both 5 mM H<sub>2</sub>O<sub>2</sub> and in the control culture (Fig. 3.60, 3.61). In the culture receiving 5 mM H<sub>2</sub>O<sub>2</sub>, pulses seemed to consist mainly of [<sup>14</sup>C]DHA (Fig. 3.61b). In the control culture pulses consisted mainly of [<sup>14</sup>C]ascorbate. Given the strong oxidising conditions of the culture with H<sub>2</sub>O<sub>2</sub> added, it was considered likely that radioactivity was exported as [<sup>14</sup>C]ascorbate and then, in the case of figure 3.61, had been



oxidised to [ $^{14}\text{C}$ ]DHA. Although pulses of radioactivity were seen in the absence of 5 mM  $\text{H}_2\text{O}_2$ , this does not necessarily mean that import and export of [ $^{14}\text{C}$ ]ascorbate is a continuous process, as exchange of the culture medium after the incubation with [ $^{14}\text{C}$ ]ascorbate may have induced some stress responses in cells.

Early (0 – 5 min) responses to 5 mM  $\text{H}_2\text{O}_2$  seemed distinct from later (5 – 160 min) responses. Pulses of [ $^{14}\text{C}$ ]DHA were seen before 5 min (Fig. 3.61a). After 5 min, sustained [ $^{14}\text{C}$ ]DHA export was observed, as well as the presence of [ $^{14}\text{C}$ ]DKG and [ $^{14}\text{C}$ ]oxalate (Fig. 3.61a, b). Intracellular formation of [ $^{14}\text{C}$ ]ascorbate oxidation products would have been expected in response to  $\text{H}_2\text{O}_2$  concentrations in excess of 1 mM, based on results in figure 3. 57a. It was considered possible that after 5 min membrane damage from 5 mM  $\text{H}_2\text{O}_2$  resulted in the indiscriminate export of  $^{14}\text{C}$ -labelled material, hence the observation of [ $^{14}\text{C}$ ]oxalate and [ $^{14}\text{C}$ ]DKG at later time points in figure 3.61b.

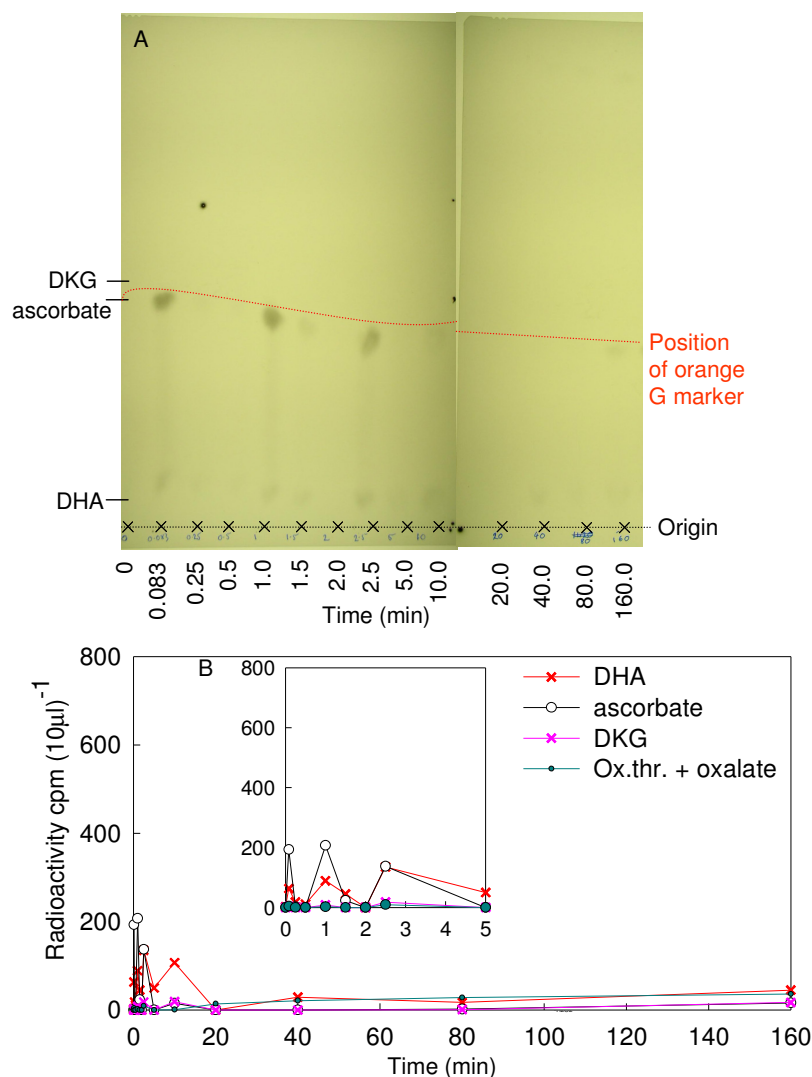
Interestingly, at early time points in the control culture [ $^{14}\text{C}$ ]ascorbate appeared to be re-taken up from the medium before it was oxidised further than [ $^{14}\text{C}$ ]DHA at early time points (Fig. 3.60b). Even in the presence of 5 mM  $\text{H}_2\text{O}_2$ , [ $^{14}\text{C}$ ]DHA mostly seemed to be re-imported from the medium before extensive oxidation to [ $^{14}\text{C}$ ]ox.thr. and [ $^{14}\text{C}$ ]oxalate (Fig. 3.61b). However, the presence of even small peaks of [ $^{14}\text{C}$ ]oxalate suggest that either indiscriminate export of the  $^{14}\text{C}$ -labelled material or post-sampling oxidation was occurring because if [ $^{14}\text{C}$ ]oxalate were exported it would not be re-imported from the medium based on previous data (Green 2003, Fig. 3.57d).

Peaks of radioactivity at early time points appeared to be larger in the oxidatively stressed culture compared to the control (Fig. 3.60b, 3.61b). Mean values for [ $^{14}\text{C}$ ]ascorbate and [ $^{14}\text{C}$ ]DHA in the control culture were similar but more variation was seen in [ $^{14}\text{C}$ ]ascorbate values than in [ $^{14}\text{C}$ ]DHA; [ $^{14}\text{C}$ ]DHA levels in the culture medium were more constant than

[<sup>14</sup>C]ascorbate levels (table 1a). It was not possible to compare this phenomenon to the stressed culture as [<sup>14</sup>C]ascorbate was thought to have been oxidised by exogenous H<sub>2</sub>O<sub>2</sub>.

Several interesting observations had been made from data from this expt. 1 but uncertainties remained over the discriminate or indiscriminate export of material at both early and later time points. The experiment was therefore repeated (expt. 2). The logarithmic time course (expt. 1) was abandoned in favour of a linear one where time points were removed every 5 s for 30 s intervals (expt. 2). The export of radioactivity in a pulse-like fashion was observed again in both control and oxidatively stressed cells (Fig. 3.62, 3.63). Excepting data from 600 – 630 s, pulses were larger particularly at early time points in cells treated with 5 mM H<sub>2</sub>O<sub>2</sub> compared to control cells (Fig. 3.62a,b and 3.63a,b). In both treatments the amount of <sup>14</sup>C in the medium and the base level of <sup>14</sup>C between pulses increased over time. If [<sup>14</sup>C]ascorbate was exported in response to stress, an increase in export of radioactivity over time in control cells was not unexpected as these cells would have experienced some stress through the exchange of radioactive for non-radioactive culture medium, even though this was taken from the original parent culture. Again, the average value for [<sup>14</sup>C]DHA in expt. 2 was lower than [<sup>14</sup>C]ascorbate and showed less variation around the mean than that of [<sup>14</sup>C]ascorbate (table 1b).

A similar experiment in which the exchange of radioactive and non-radioactive medium was not attempted as it was not thought that an increase in radioactivity in the culture medium would have been detected against the background level of radioactivity from e.g. [<sup>14</sup>C]oxalate and [<sup>14</sup>C]ox.thr. which would not have been taken up during the incubation period. However, if this series of experiments were to be continued, this would perhaps be an important control.

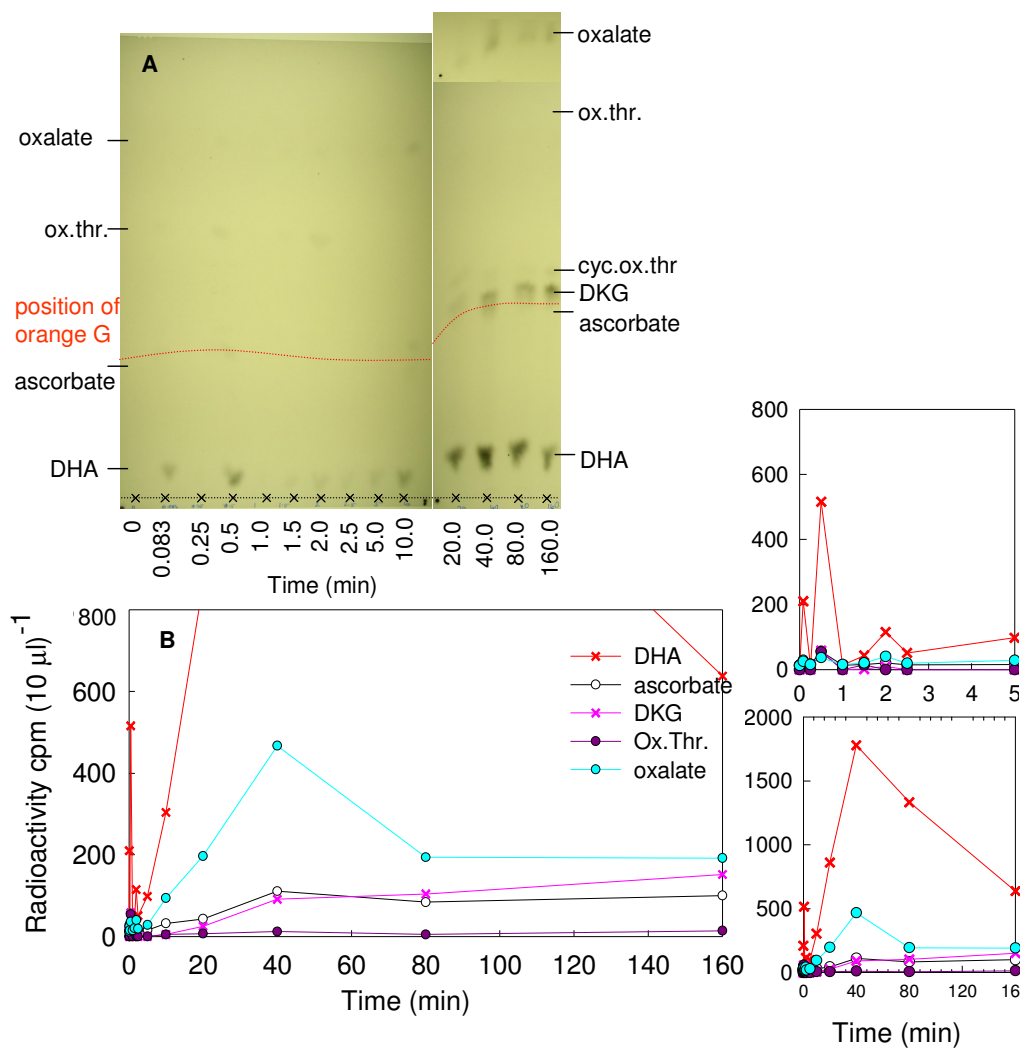


**Figure 3.60 Expt 1; export of symplastic  $[^{14}\text{C}]$ ascorbate metabolites in control cells.**

A 650  $\mu\text{l}$  cell culture (settled cell volume approx. 30%) was incubated with  $[^{14}\text{C}]$ ascorbate for 5 h. Radioactive culture medium was exchanged with non-radioactive culture medium and cells were then treated with 20  $\mu\text{l}$  of either water (control cells) or  $\text{H}_2\text{O}_2$  (final conc. 5 mM).

**A.** Autoradiogram of culture medium samples after electrophoresis. samples of culture medium (10  $\mu\text{l}$ ) were subjected to paper electrophoresis at pH 6.5, 3.5 kV for 30 min. Papers were exposed to autoradiography film for 2 weeks. Indicated positions of compounds are based on  $m_{\text{OG}}$  values rather than external markers.

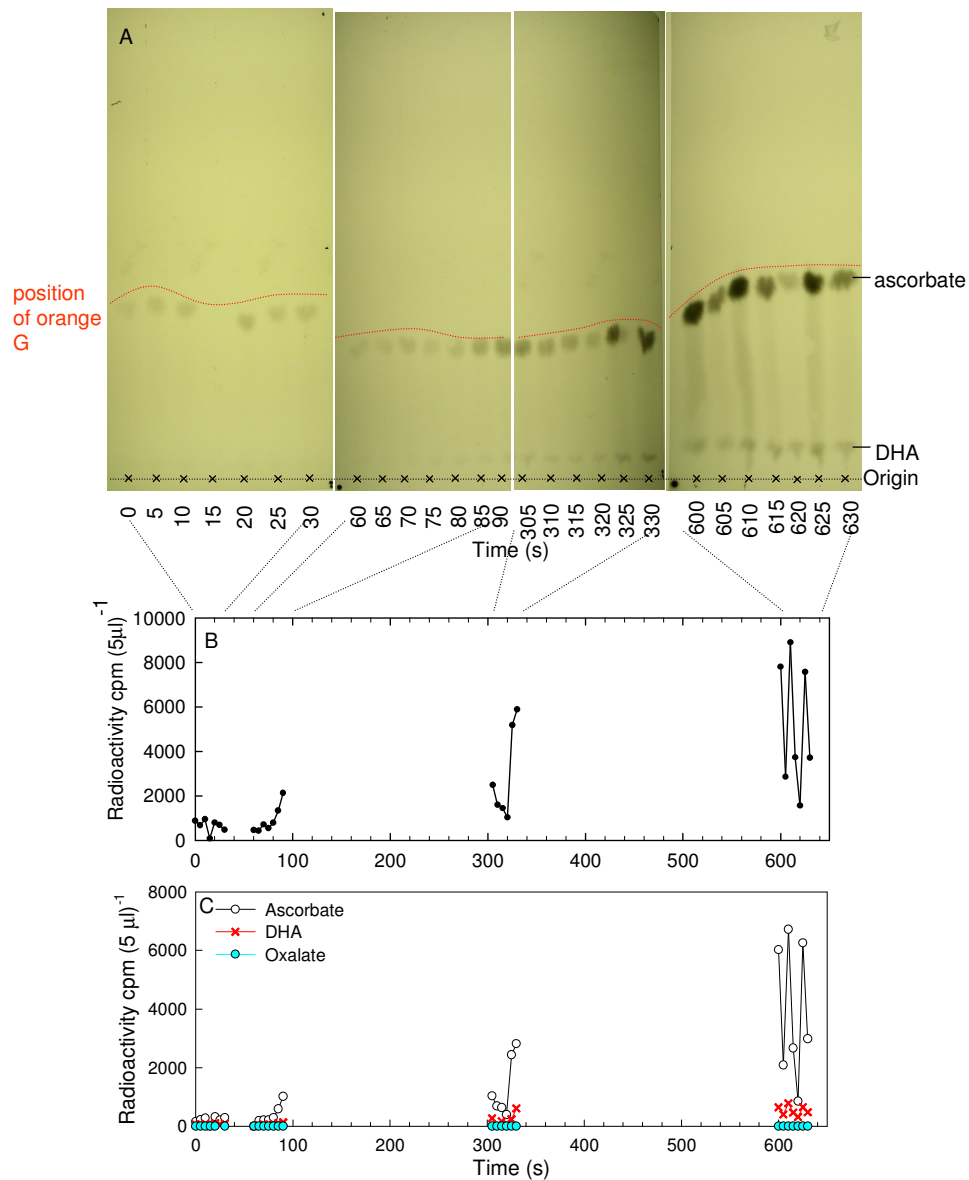
**B.** Analysis of individual metabolites exported into the culture medium. Areas of the paper electrophoretogram corresponding to darkened spots were excised and quantified by scintillation counting. If spots could not easily be seen, the area corresponding to the  $m_{\text{OG}}$  values for compounds were excised. Separation of  $[^{14}\text{C}]$ oxalyl threonate and  $[^{14}\text{C}]$ oxalate was not possible owing to streaking of low concentrations of  $[^{14}\text{C}]$ oxalate during electrophoresis.



**Figure 3.61 Expt 1; export of symplastic [<sup>14</sup>C]ascorbate metabolites in response to 5 mM H<sub>2</sub>O<sub>2</sub>**

**A.** Other details as Fig. 3.60

**B** Other details as Fig. 3.60

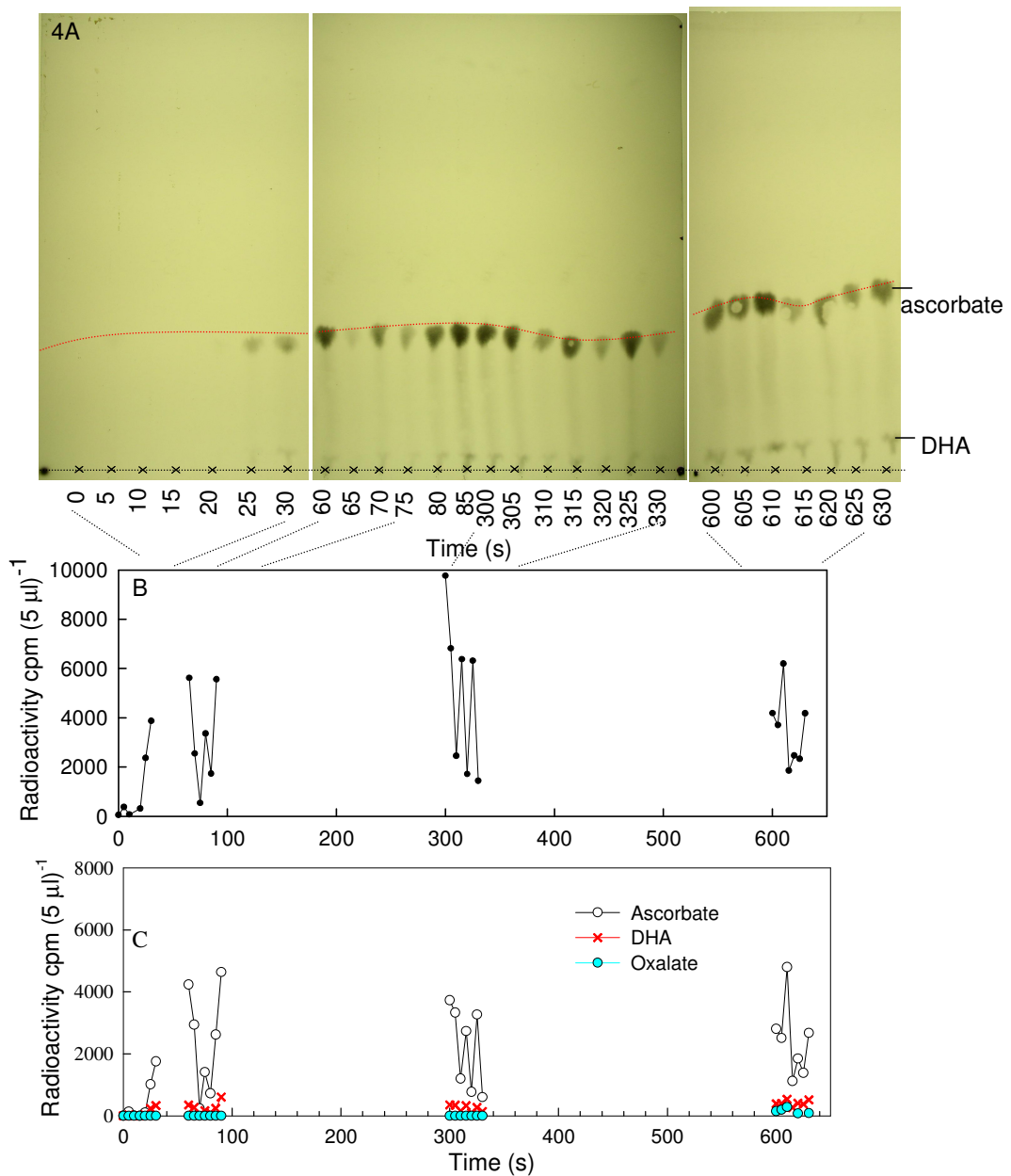


**Figure 3.62 Expt 2; export of symplastic [<sup>14</sup>C]ascorbate metabolites in control cells. For experiment overview, see figure 3.60.**

A. Autoradiogram of culture medium samples after electrophoresis. Samples (10 µl were subjected to paper electrophoresis at pH 6.5, 3.5 kV for 30 min. Papers were exposed to autoradiography film for 10 days.

B. Total <sup>14</sup>C in samples of culture medium. Aliquots (5µl) of samples of culture medium were assayed for radioactivity by liquid scintillation counting.

C. Analysis of individual exported metabolites. Areas of the paper electrophoretogram corresponding to darkened spots were excised and assayed for radioactivity by scintillation counting



**Figure 3.63 Expt. 2; export of symplastic [<sup>14</sup>C]ascorbate metabolites in cells receiving a 5 mM H<sub>2</sub>O<sub>2</sub> oxidative stress.**

A. H<sub>2</sub>O<sub>2</sub> (final conc. 5 mM) was added at 0 s. For other details see Fig. 3.62a.

B. Total <sup>14</sup>C in culture medium samples. Aliquots (5  $\mu$ l) of samples of culture medium were assayed for radioactivity by liquid scintillation counting.

C. Analysis of individual exported metabolites. For details see Fig. 3.62c.

**Table 1 Variation in [<sup>14</sup>C]DHA and [<sup>14</sup>C]ascorbate concentrations in the medium of cells in response to H<sub>2</sub>O<sub>2</sub> (expt. 1)**

A. The mean and standard deviation (n = 14) of [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA values shown in figures 3.60b and 3.61b.

0 mM H <sub>2</sub> O <sub>2</sub> (Fig. 3.60)	[ <sup>14</sup> C]DHA cpm (10 μl) <sup>-1</sup>	[ <sup>14</sup> C]ascorbate cpm (10 μl) <sup>-1</sup>
mean	54.87	48.20
st.dev.	59.45	76.26
5 mM H <sub>2</sub> O <sub>2</sub> (Fig. 3.61)	[ <sup>14</sup> C]DHA cpm (10 μl) <sup>-1</sup>	[ <sup>14</sup> C]ascorbate cpm (10 μl) <sup>-1</sup>
mean	426.47	50.67
st.dev.	532.37	52.98

B. Details as table 1a with data from figures 3.62b and 3.63b

0 mM H <sub>2</sub> O <sub>2</sub> (Fig. 3.62)	[ <sup>14</sup> C]DHA cpm (10 μl) <sup>-1</sup>	[ <sup>14</sup> C]ascorbate cpm (10 μl) <sup>-1</sup>
mean	288.74	1532.39
st.dev	292.16	1948.01
5 mM H <sub>2</sub> O <sub>2</sub> (Fig. 3.63)	[ <sup>14</sup> C]DHA cpm (10 μl) <sup>-1</sup>	[ <sup>14</sup> C]ascorbate cpm (10 μl) <sup>-1</sup>
mean	335.48	1871.65
st.dev	324.64	1438.25

As had been noted in expt. 1, few oxidation products other than [<sup>14</sup>C]DHA were detected in expt. 2 (Fig. 3.62c, 3.63c). This could be a result of less post-sampling oxidation in expt. 2 compared to expt. 1 but also argues against the indiscriminate export of radioactive material e.g. as a result of membrane damage from H<sub>2</sub>O<sub>2</sub> exposure. In figure 3.57a, it could be seen how within 15 min of exposure to 1 mM H<sub>2</sub>O<sub>2</sub>, more [<sup>14</sup>C]oxalate was present in the symplasm than [<sup>14</sup>C]ascorbate. If cells had been exposed to higher concentrations of H<sub>2</sub>O<sub>2</sub> (e.g. 5 mM rather than 1 mM H<sub>2</sub>O<sub>2</sub>) it is likely that symplastic concentrations of [<sup>14</sup>C]oxalate would have increased yet faster than was observed in response to 1 mM H<sub>2</sub>O<sub>2</sub>. Therefore, if the contents of the symplasm were being exported indiscriminately in response to 5 mM H<sub>2</sub>O<sub>2</sub>, appreciable amounts [<sup>14</sup>C]oxalate would have been detected in the medium.

#### 3.8.1.1 Summary of expts. 1 and 2 (Fig. 1 – 4)

In expts. 1 and 2 [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]DHA were exported in control cultures and cultures receiving a 5 mM  $\text{H}_2\text{O}_2$  stress. Some degree of stress was expected in control cultures as a result of the exchange of culture medium. Export of radioactivity was followed by rapid import, so radioactivity appeared to be exported in pulses. The size of pulses and the base level of radioactivity in between pulses increased over time. In response to  $\text{H}_2\text{O}_2$ , radioactivity was exported earlier and in larger pulses earlier than in control cultures. The range of  $^{14}\text{C}$ -labelled metabolites exported ([ $^{14}\text{C}$ ]ascorbate and [ $^{14}\text{C}$ ]DHA) did not reflect the expected range of symplastic metabolites in cells stressed with 1 mM  $\text{H}_2\text{O}_2$ ; export was therefore not thought to be indiscriminate. [ $^{14}\text{C}$ ]DHA concentrations in the culture medium were lower and more constant than [ $^{14}\text{C}$ ]ascorbate concentrations. One possible explanation for this was that radioactivity was exported as [ $^{14}\text{C}$ ]ascorbate, then converted to [ $^{14}\text{C}$ ]DHA in the medium and taken up by cells as [ $^{14}\text{C}$ ]DHA. This would be in agreement with reported results showing that ascorbate was taken up preferentially in its oxidised form (Horemans *et al.* 1998). If uptake occurred continuously as [ $^{14}\text{C}$ ]ascorbate was converted to [ $^{14}\text{C}$ ]DHA in the medium, this could explain the lower and more constant concentrations of [ $^{14}\text{C}$ ]DHA observed in the medium.

#### **3.8.2 Transport of [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]ascorbate metabolites across the plasma membrane in response to 1 mM $\text{H}_2\text{O}_2$ (expt. 3)**

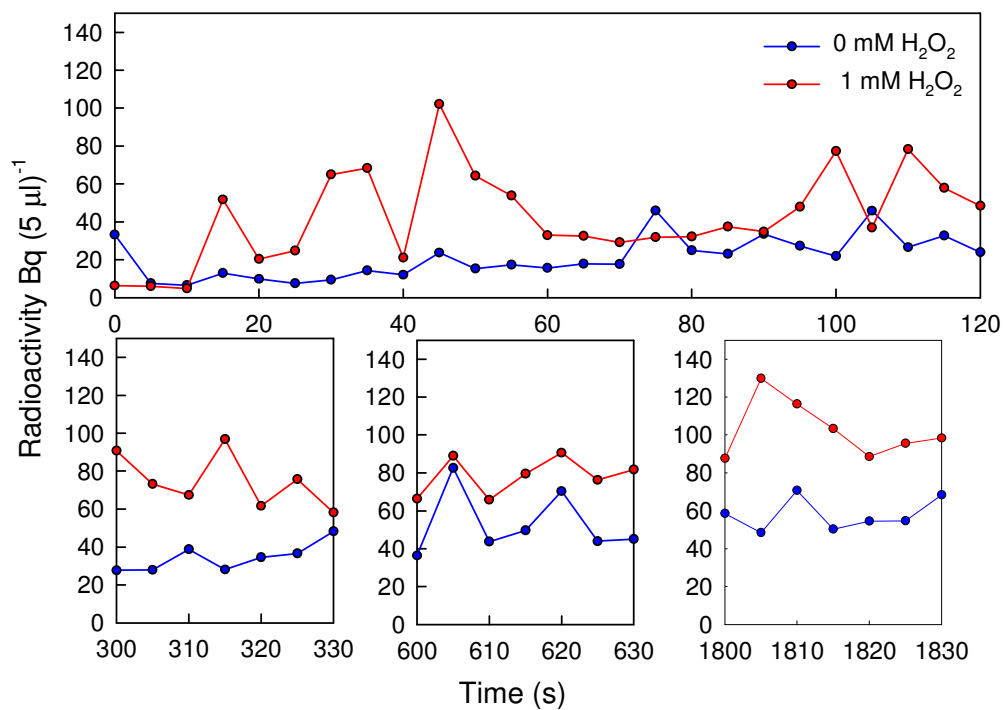
Expts. 1 and 2 had established that sufficient radioactivity was exported in response to 5 mM  $\text{H}_2\text{O}_2$  so as to be easily detected in the culture medium. The concentration of  $\text{H}_2\text{O}_2$  was lowered to 1 mM so that any possibility of undue membrane damage occurring could be minimised. Improvements were made to the sampling strategy; samples were removed at 5 s intervals continuously for the first 2 min (Fig. 3.64). It was hoped that this would allow for more detailed observations on the size and frequency of pulses; in expts. 1 and 2, few peaks had been



seen to have shoulders (Fig. 3.60b – 3.63b). It was therefore not possible to be certain that the ‘pulsing’ phenomenon had not been an artefact or had represented genuine peaks and troughs.

In expt. 3 export and import of radioactivity in pulses was observed once more (Fig. 3.64). Pulses of radioactivity were found to be larger in cultures receiving 1 mM H<sub>2</sub>O<sub>2</sub> compared to the control, particularly within the first minute. Peaks of radioactivity were lower in response to 1 mM H<sub>2</sub>O<sub>2</sub> (e.g. 56 bq and 75 bq 5 µl<sup>-1</sup>) than to 5 mM H<sub>2</sub>O<sub>2</sub> (e.g. 163 bq and 103 bq 5 µl<sup>-1</sup>). Radioactivity values of the troughs between peaks increased slowly in both 1 mM H<sub>2</sub>O<sub>2</sub> and in control cultures.

The results in figure 3.64 suggest that the duration of a pulse was typically 10 – 20 s and that pulses occurred every 20 – 40 s. It was important to bear in mind that, as sampling was not continuous, export and import of radioactivity could have been more frequent but this would not have been detected. Unlike the data shown in figures 3.60 – 3.63, in figure 3.64 peaks and troughs of radioactivity comprised several samples. This was less typical of random scatter than data in expts. 1 and 2 and more typical of genuine peaks and troughs. The descending series of data points between 45 s and 60 s in response to 1 mM H<sub>2</sub>O<sub>2</sub> was considered to be particularly characteristic of a pulse. As a <sup>3</sup>H marker was not included in expt. 3, changes in the <sup>14</sup>C content of the culture medium could not be proved not to be the result of sampling error. Average pipetting error in figures 3.66 and 3.67 was found to be less than 15 %. Assuming a 15 % error on all samples in figure 3.64, maxima and minima of radioactivity were still clearly significantly different in both control and oxidatively stressed cultures.



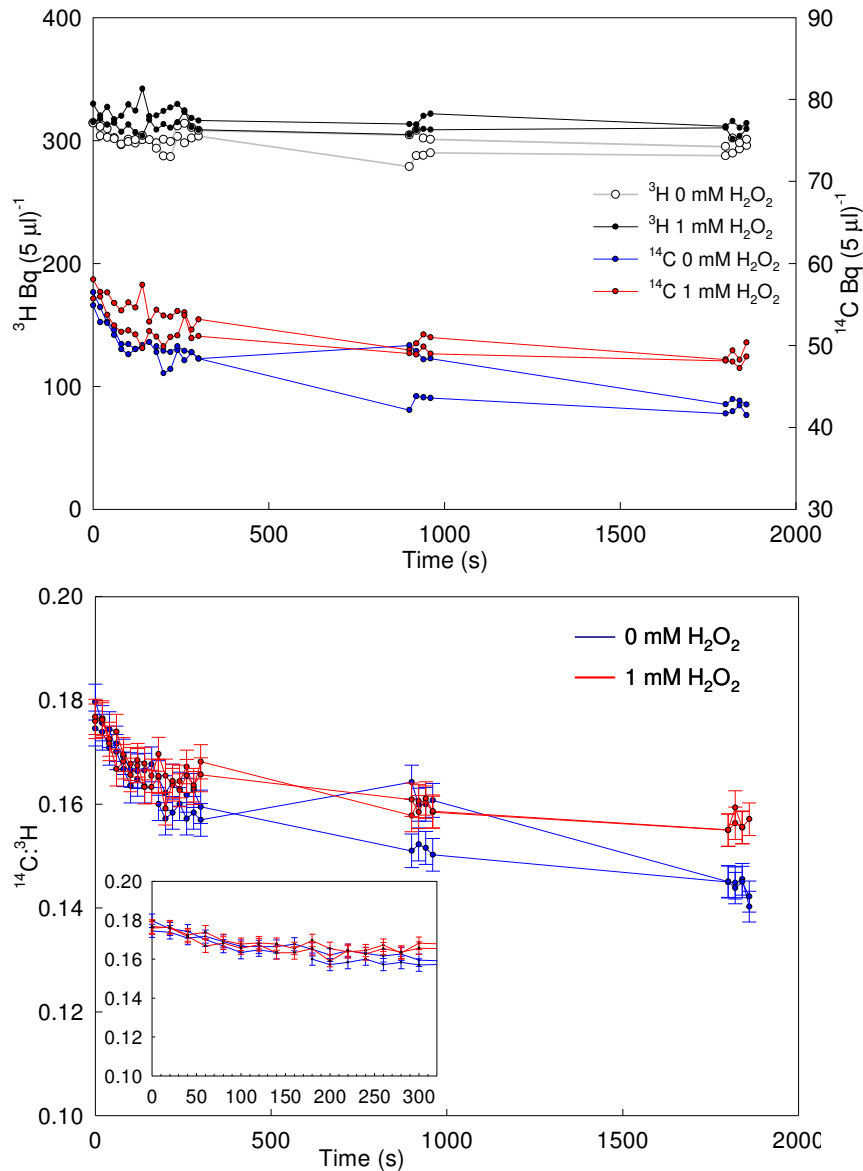
**Figure 3.64** Export of <sup>14</sup>C in response to 1 mM H<sub>2</sub>O<sub>2</sub>

A 650 μl rose cell culture was incubated with 0.05 MBq of [<sup>14</sup>C]ascorbate. Radioactive culture medium was then exchanged for non-radioactive culture medium and the medium was sampled through nylon mesh which successfully filtered out cells. Control cultures received 20 μl water and oxidatively stressed cells received 20 μl H<sub>2</sub>O<sub>2</sub> to a final conc. of 1 mM. Radioactivity in samples was assayed by scintillation counting.

### 3.8.3 Assessment of membrane damage by glucose transport during oxidative stress

It is notoriously difficult to accurately gauge extracellular and intracellular  $\text{H}_2\text{O}_2$  concentrations in plants in response to stress (Queval *et al.* 2008). A concentration of 1 mM  $\text{H}_2\text{O}_2$  had been chosen after careful consideration (see section 3.4) but it was not known what effect 1mM  $\text{H}_2\text{O}_2$  would have on the membrane during the first 5 min of exposure to  $\text{H}_2\text{O}_2$ . Such an early response was of interest because results from figures 3.60 – 3.64 showed a large disparity in pulse size between control and  $\text{H}_2\text{O}_2$  treatments in the first few minutes after  $\text{H}_2\text{O}_2$  addition. Rapid export of radioactivity followed by rapid import was not considered likely to be characteristic of membranes which had simply been damaged by 1mM  $\text{H}_2\text{O}_2$  but early effects on the plasma membrane were nevertheless tested. The effect of 1 mM  $\text{H}_2\text{O}_2$  on [ $^{14}\text{C}$ ]glucose uptake was measured cells in the presence of a [ $^3\text{H}$ ]glucitol marker which was not expected to be taken up by cells. If the membrane was damaged in response to 1 mM  $\text{H}_2\text{O}_2$  a difference between [ $^{14}\text{C}$ ]glucose uptake in  $\text{H}_2\text{O}_2$ -stressed and control cultures was expected. The [ $^3\text{H}$ ]glucitol marker was included so that any pipetting error could be accounted for; data was presented as the  $^{14}\text{C}:^3\text{H}$  ratio so that any artificial peaks of  $^{14}\text{C}$  that could possibly have arisen through sampling error would be negated.

There were no detectable pulses in [ $^{14}\text{C}$ ]glucose uptake such as had been seen during the uptake of [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]DHA in expts. 1 – 3. There did not appear to be any significant difference in the [ $^{14}\text{C}$ ]glucose uptake in control cell or  $\text{H}_2\text{O}_2$ -treated cells, as shown by the  $^{14}\text{C}:^3\text{H}$  ratio during the first 200 s. After 300 s a slight decrease in uptake was seen in  $\text{H}_2\text{O}_2$ -treated cells which became progressively more apparent towards 1800 s (Fig. 3.65).



**Figure 3.65 The effect of H<sub>2</sub>O<sub>2</sub> on [<sup>14</sup>C]glucose uptake.**

Rose cell cultures were shaken gently for 5 h under the same conditions as those cell cultures which had been incubated with [<sup>14</sup>C]ascorbate. At 0 s rose cells were fed either water or H<sub>2</sub>O<sub>2</sub>, an extracellular [<sup>3</sup>H]glucitol marker and [<sup>14</sup>C]glucose. Radioactivity was assayed by scintillation counting.

A Amounts of <sup>3</sup>H and <sup>14</sup>C present in the cell culture medium.

B Ratio of <sup>14</sup>C:<sup>3</sup>H present in the cell culture medium.

Previous work has shown that there is likely to be complex interplay between  $\text{Ca}^{2+}$ , ROS production and glucose uptake (Azevedo *et al.* 2006, Bourque *et al.* 2002). Therefore it was not possible to say whether the decreased rate of [ $^{14}\text{C}$ ]glucose uptake in  $\text{H}_2\text{O}_2$ -treated cells between 300 s and 1800 s was due to oxidative membrane disruption or not. However, this control does highlight two important points; that glucose uptake is under distinct control from ascorbate and/or DHA uptake and that membrane disruption was unlikely to have affected cells within the first 5 min. Despite the influence of ROS and  $\text{Ca}^{2+}$  on glucose uptake, if the plasma membrane had been oxidatively damaged before 300 s, it was expected that some difference in [ $^{14}\text{C}$ ]glucose uptake would still have been manifest.

#### **3.8.4 Export and import of [ $^{14}\text{C}$ ]ascorbate and/or $^{14}\text{C}$ -labelled metabolites in the absence of exogenous $\text{H}_2\text{O}_2$ and presence of [ $^3\text{H}$ ]glucitol (expts. 4 and 5)**

The rapid export and import of [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]DHA had been observed in control cultures (expts. 1 – 3) and was thought to be a response to the stress of the exchange of culture medium immediately prior to the start of sampling. The generation of pulses of radioactivity was investigated further in the absence of  $\text{H}_2\text{O}_2$  and in the presence of a [ $^3\text{H}$ ]glucitol marker (expt. 4, Fig. 3.66). As in figure 3.65, data was then presented as the  $^{14}\text{C}:^3\text{H}$  ratio. The possibility of a specific effect of the [ $^3\text{H}$ ]glucitol marker on the export and import of radioactivity was investigated by a repetition of expt. 4 with [ $^3\text{H}$ ]glucitol, [ $^3\text{H}$ ]isoprimeveritol and [ $^3\text{H}$ ]xylitol as markers (expt. 5).

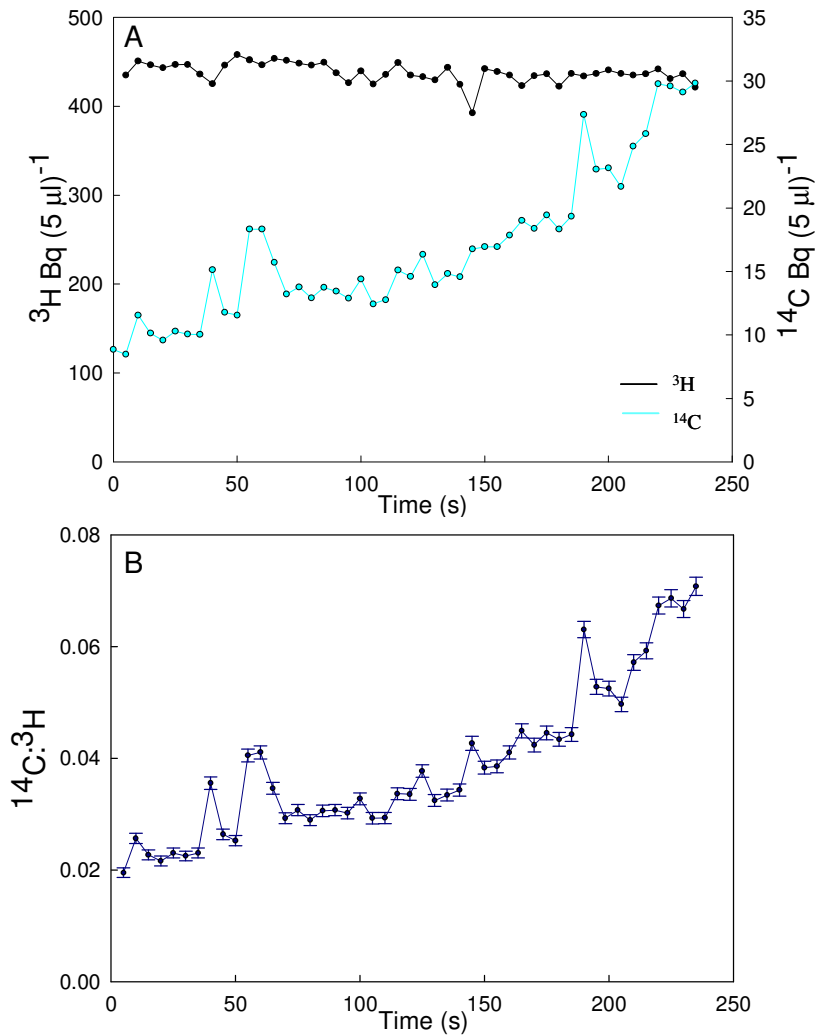
[ $^3\text{H}$ ]Glucitol was not taken up by cells whilst export and import of  $^{14}\text{C}$  in pulses was observed (Fig. 3.66). The pulses were still present after the  $^{14}\text{C}:^3\text{H}$  ratio had been calculated; it was possible to say that pulses were not an artefact of pipetting error. Calculation of the  $^{14}\text{C}:^3\text{H}$  ratio did not rule out the possibility that pulses were a result of a small number of cells contaminating certain samples. However, the use of a nylon mesh cell filter during sampling rendered this possibility unlikely. In addition, it had been discussed how symplastic

contamination of samples did not seem to have occurred in early (0 – 5 min) time points in expt. 2 (Fig. 3.61) as sufficient levels of [<sup>14</sup>C]ascorbate oxidation products were not observed in the culture medium compared to levels of [<sup>14</sup>C]ascorbate oxidation products found in the symplasm after a 1 mM H<sub>2</sub>O<sub>2</sub> stress (Fig. 3.57a).

As with the control culture in figure 3.64, pulses seemed to last for 10 – 20 s (Fig. 3.66). Assessing the frequency of pulses was difficult as no definite pulse was detected from 75 s to 175 s. At 125 s and 150 s, fainter pulses were detected. With these included, the pulsing frequency became every 50 s, comparable to the control culture in expt. 3 (Fig. 3.64). Pulses were marginally smaller than in the expt. 3 control, but as it was not possible to know whether peaks represented maxima of <sup>14</sup>C export, pulse size could not be accurately determined. The base level of <sup>14</sup>C in the control cultures in expts. 3 and 4 rose at a similar rate, both having reached 30 bq (5 μl)<sup>-1</sup> by 300 s (Fig. 3.64) and 240 s (Fig. 3.66).

Culture volumes had been chosen so that cells remained in a minimum of 250 μl at the end of each experiment but the reduction in culture volume could still be greater than 50 % after sampling was completed. One concern therefore was that cells might be responding to the stress of a progressively changing culture environment, even if an adequate volume remained to prevent cells drying out. Also, pulses of an equivalent size at the start and end of an experiment could have appeared to have increased with time as exported [<sup>14</sup>C]ascorbate became more concentrated. An important difference between expt. 4 and expt. 5 was that the 650 μl culture volume (expt. 4) was increased to 1 ml in expt. 5. This meant that after sampling, 650 μl remained in cell cultures in expt. 5.

The export of <sup>14</sup>C in pulses and the overall increase in <sup>14</sup>C concentrations in the medium in expt. 5 was again verified by the use of extracellular <sup>3</sup>H-markers (Fig. 3.67). In the absence of H<sub>2</sub>O<sub>2</sub>, pulsing was still seen. Pulses were still seen after 600 s (Fig. 3.67), as had been noted in



**Figure 3.66 Expt 4; export of symplastic radioactivity from cells in control cells**

650  $\mu\text{l}$  cell cultures were incubated with 0.05 MBq [ $^{14}\text{C}$ ]ascorbate for 5 h. Radioactive medium was then exchanged for non-radioactive medium and a [ $^3\text{H}$ ]glucitol marker was added.  $^3\text{H}$  and  $^{14}\text{C}$  in the culture medium were assayed by scintillation counting.

A. [ $^3\text{H}$ ]Glucitol and  $^{14}\text{C}$  (exported from cells) in the culture medium.

B. Ratio of  $^{14}\text{C} : ^3\text{H}$  in the culture medium.

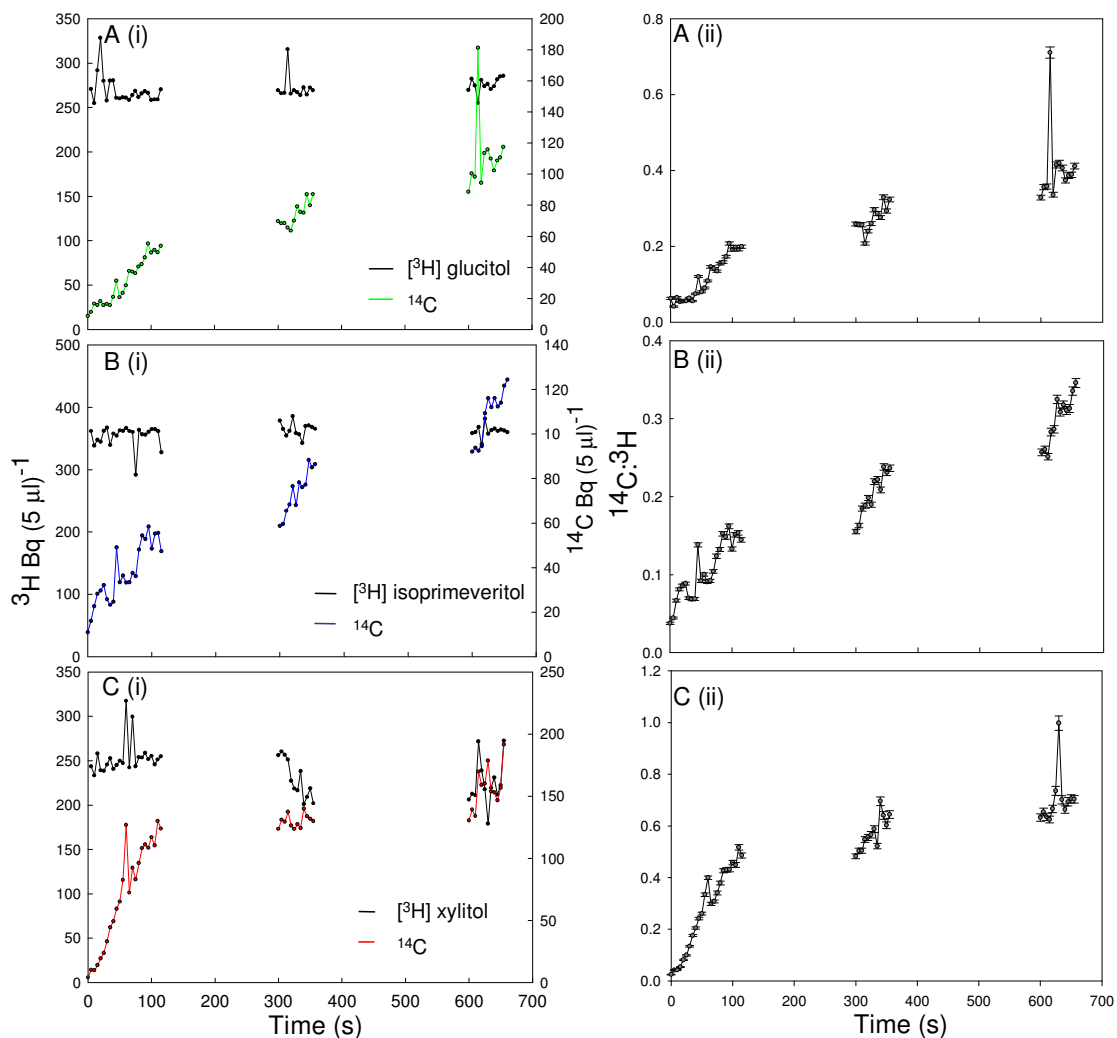


Figure 3.67 Expt 5; export of  $^{14}\text{C}$  from  $[^{14}\text{C}]$ ascorbate-fed cells in the absence of exogenous  $\text{H}_2\text{O}_2$  and presence of various extracellular  $^3\text{H}$  markers

$^3\text{H}$ -Labelled markers were added to cultures at 0 s. Final culture volumes were 1 ml.  $^3\text{H}$  and  $^{14}\text{C}$  in the culture medium were assayed by scintillation counting.

- A. (i)  $[^{14}\text{C}]$ Ascorbate and  $[^3\text{H}]$ glucitol. (ii) Ratio of  $^{14}\text{C}:^3\text{H}$  in the culture medium
- B. (i)  $[^{14}\text{C}]$ Ascorbate and  $[^3\text{H}]$ isoprimeveritol. (ii) Ratio of  $^{14}\text{C}:^3\text{H}$  in the culture medium
- C. (i)  $[^{14}\text{C}]$ Ascorbate and  $[^3\text{H}]$ xylitol. (ii) Ratio of  $^{14}\text{C}:^3\text{H}$  in the culture medium



the control culture in expt. 3 (Fig. 3.64). In expt. 5, as in expt. 3 and 4 pulses were detected but the rate at which pulses seemed to occur shows a slight variation between experiments; pulses were detected approximately every 20 – 40 s in all three repeats of the experiment (Figs. 3.64, 3.66, 3.67).  $^{14}\text{C}$  values at 0 s were equivalent to expts. 1 – 4 but the base level of radioactivity increased faster, reaching approximately  $50 \text{ bq } (5 \mu\text{l})^{-1}$  at 115 s (Fig. 3.67) compared to 24 and  $15 \text{ bq } (5 \mu\text{l})^{-1}$  (Fig. 3.64, 3.66). This highlights an important difference in figure 3.67: whilst rapid export and import of  $^{14}\text{C}$  was undoubtedly detected, ensuing import of  $^{14}\text{C}$  relative to export was less than had been seen before (expts. 1 – 4). Pulses seemed to be slightly larger, around  $15 - 20 \text{ bq } (5 \mu\text{l})^{-1}$  compared to figure 5 and 6. However, as shown by the peak at 615 s (Fig. 3.67b), the amplitude and frequency of peaks had to be treated with caution. The large size of this peak also raises the question of whether more peaks of that size existed but were not sampled at their maximum or whether some unaccounted for factor was affecting results. Rates of  $^{14}\text{C}$  export slowed slightly over time in figures 3.67a and 3.67c but this was not detected in figure 3.67b. Although at least 650  $\mu\text{l}$  of culture medium remained at the end of all these experiments (Fig. 3.67), it was still not certain how much a decrease in exported  $^{14}\text{C}$  over time might be masked by removal of culture medium and the progressive concentration of exported  $^{14}\text{C}$ .

When [ $^3\text{H}$ ]xylitol was used as marker some signs of uptake of [ $^3\text{H}$ ]xylitol were seen from 300s onwards (Fig. 3.67ci). It was therefore not clear whether the  $^{14}\text{C}:^3\text{H}$  ratio represented genuine peaks at 600 s onwards. [ $^3\text{H}$ ]Xylitol was not used as a marker in subsequent experiments.

### 3.8.5 Export and import of $^{14}\text{C}$ -labelled metabolites in the presence of exogenous $\text{H}_2\text{O}_2$ and [ $^3\text{H}$ ]glucitol (expt. 6)

The export and import of [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]DHA in response to 1 mM  $\text{H}_2\text{O}_2$  was examined in the presence of an extracellular [ $^3\text{H}$ ]glucitol marker (Fig. 3.68a, c). The experimental design was altered slightly so that the culture volume remained constant over time. As in previous experiments, the culture medium in which cells had been incubated with [ $^{14}\text{C}$ ]ascorbate was replaced with non-radioactive medium from the parent culture prior to sampling. In a departure from the methods followed in expts. 1 – 5, as samples were removed from the medium (now containing the extracellular [ $^3\text{H}$ ]glucitol markers and any exported  $^{14}\text{C}$ ) an equivalent volume of non-radioactive medium from the parent culture was added. This meant that the culture volume remained constant but the concentration of [ $^3\text{H}$ ]glucitol in the experimental culture decreased over time. When the raw  $^3\text{H}$  data was plotted, a linear decay was observed, as was predicted by the progressive dilution of  $^3\text{H}$  with non-radioactive medium. There were therefore two distinct elements to the  $^3\text{H}$  data that needed to be separated before a  $^{14}\text{C}:$  $^3\text{H}$  ratio could be calculated: the linear decay and the ‘noise’ in the data that resulted from sampling error. If the  $^{14}\text{C}:$  $^3\text{H}$  ratio was calculated from the raw  $^3\text{H}$  data, the increase over time would have been artificially large due to the ever decreasing denominator. For each experimental data point, the difference between the concentration of  $^3\text{H}$  given by the linear decay equation at a particular time point and the concentration given by the equation at  $t = 0$  was added to the measured concentration of  $^3\text{H}$  at that particular time point.

During the first 120 s, when it had been observed that the pulses of exported and imported  $^{14}\text{C}$  were of greater amplitude in cultures given  $\text{H}_2\text{O}_2$  compared to control cultures, no

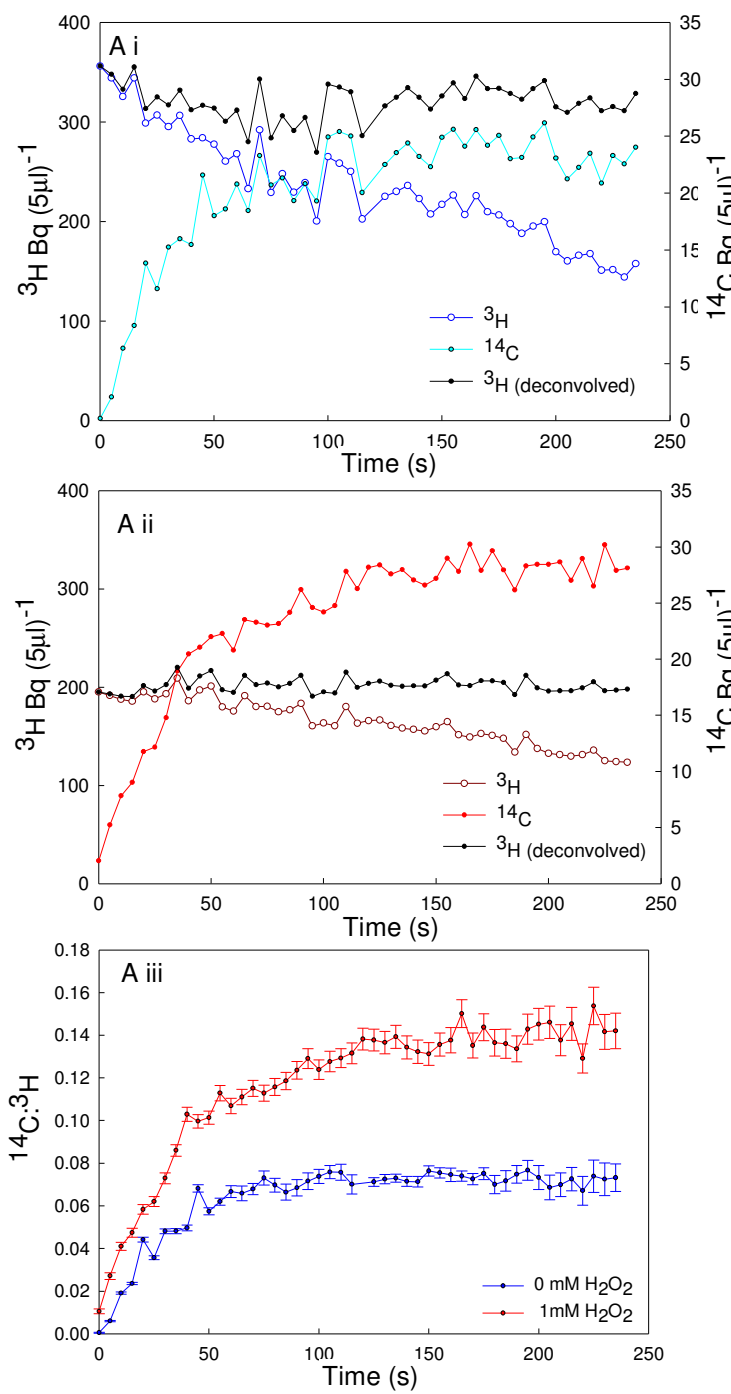
evidence for pulsing was seen at all in expt. 6a (Fig. 3.68a). Some evidence for pulsing was seen in the control culture in expt. 6a at 20 s and 45 s (Fig. 3.68a iii) but otherwise was not noted.

Although no pulsing was seen in cells treated with  $\text{H}_2\text{O}_2$  during the first 120 s after addition of  $\text{H}_2\text{O}_2$ , patterns of  $^{14}\text{C}$  export in treated and control cultures as expressed by the  $^{14}\text{C}:^3\text{H}$  ratio, were nevertheless distinct (Fig. 3.68a iii). Raw data for  $^{14}\text{C}$  do not appear very different in expt. 6a (Fig. 3.68a i, 3.68a ii). However, as the volume of these experiments was only 250  $\mu\text{l}$ , it is important to bear in mind that both  $^3\text{H}$  and  $^{14}\text{C}$  data was sensitive to errors in culture volume that could have arisen during the exchange of radioactive culture medium for non-radioactive medium. The same amount of  $^3\text{H}$  added to each culture in expt. 6a; differences in the activity of  $^3\text{H}$  reflect differences in culture volume. These differences were accounted for by the calculation of the  $^{14}\text{C}:^3\text{H}$  ratio.

Export in both cultures followed asymptotic curves, when expressed as  $^{14}\text{C}:^3\text{H}$  (Fig. 3.68a iii), implying a decrease in  $^{14}\text{C}$  export at later time points. This suggests that increasing concentrations of exported  $^{14}\text{C}$  in previous experiments, where medium was not replaced as it was removed, may have masked a decrease in  $^{14}\text{C}$  export over time. Individual metabolites were not analysed in expt. 6, so it could not be said whether the plateau from 75 s to 240 s in the control culture, or from 120 s to 240 s in the  $\text{H}_2\text{O}_2$  treated culture (Fig. 3.68a iii), represented import and export of [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]DHA or whether it represented oxidation products such as [ $^{14}\text{C}$ ]oxalate that could not be re-imported from the medium. It must be stressed also that because  $^{14}\text{C}$  was continually being removed from the medium and replaced with non-radioactive medium, the plateau area of the export curve represented a steady state between removal of  $^{14}\text{C}$  from sampling and export into the medium. The plateau of the  $^{14}\text{C}:^3\text{H}$  curve was higher for  $\text{H}_2\text{O}_2$ -stressed cells ( $\approx 0.13$ ) than control cells ( $\approx 0.06$ ) yet initial rates of export appeared similar (Fig. 3.68a iii). Rates of  $^{14}\text{C}$  export appeared to diverge at about 30 s; the rate in control cells decreased, reaching a plateau at about 75 s whereas in cells receiving  $\text{H}_2\text{O}_2$ ,

$^{14}\text{C}$  was exported at the initial rate until 40 s and then at a slower rate until approximately 120 s. Therefore, although no pulsing was seen, it still seemed to be the case that, in terms of export of  $^{14}\text{C}$ -labelled material, a distinct response to  $\text{H}_2\text{O}_2$  could still be identified in the first 2 min of cells experiencing stress.

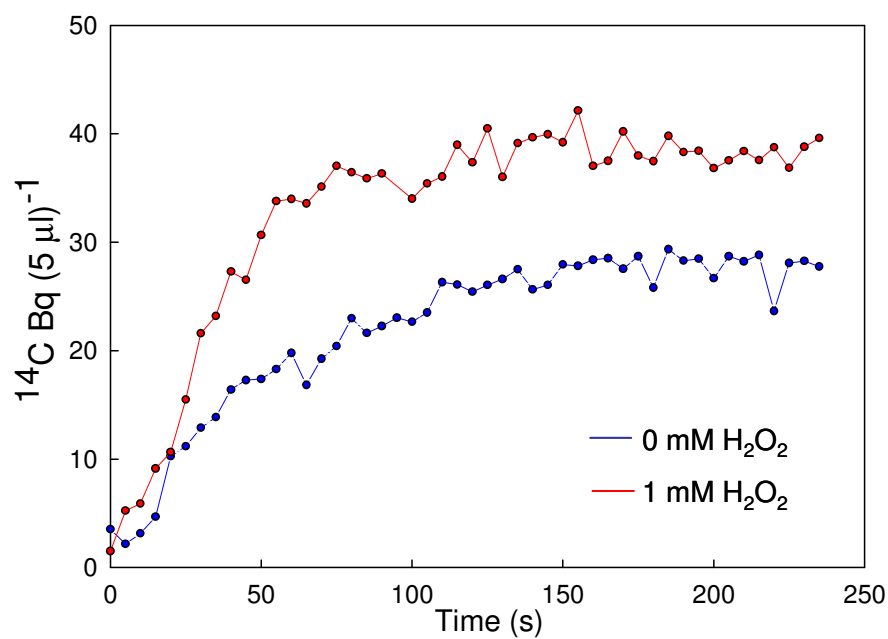
The 2 min window in which export appeared to be upregulated even in the absence of pulsing was investigated further (Fig. 3.68b, c). A remarkably similar pattern of export of  $^{14}\text{C}$  was noted between expts. 6a (Fig. 3.68a) and 6b (Fig. 3.68b). Export rates were almost identical in  $\text{H}_2\text{O}_2$ -treated cells and control cells until 20 s. At this point, the rate of  $^{14}\text{C}$  export decreased in



**Figure 3.68 Expt 6;**  
**export of [<sup>14</sup>C]ascorbate**  
**and/ or <sup>14</sup>C-ascorbate**  
**metabolites in response to**  
**H<sub>2</sub>O<sub>2</sub>.**

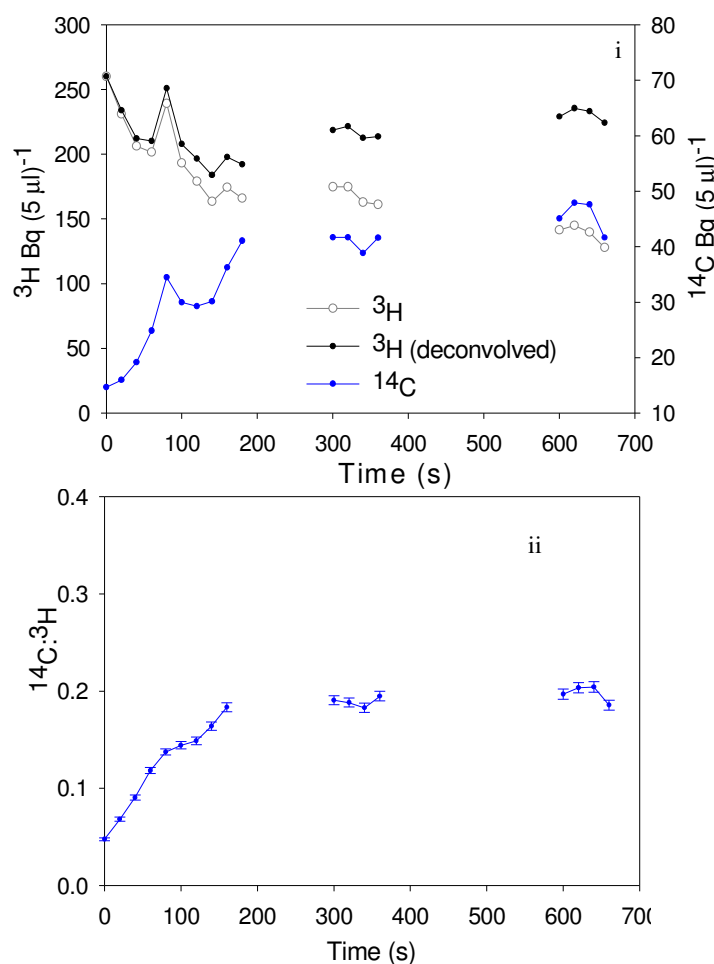
**A. Expt. 6a** 250 µl cell cultures were incubated with 0.025 MBq of [<sup>14</sup>C]ascorbate for 5 h. Radioactive cell culture medium was then replaced with non-radioactive medium. A [<sup>3</sup>H]glucitol marker was added. Either 20 µl of water (control cells) or H<sub>2</sub>O<sub>2</sub> (final conc. 1 mM) was added just before 0 s. 10 µl samples were removed through a nylon mesh. For every 4<sup>th</sup> sampled removed 40 µl of non-radioactive medium from the parent culture was added.

A i. <sup>3</sup>H (as measured and deconvolved data) and <sup>14</sup>C in the control culture  
 A ii. <sup>3</sup>H (as measured and deconvolved data) and <sup>14</sup>C in the culture receiving 1 mM H<sub>2</sub>O<sub>2</sub>  
 A iii. Ratio of <sup>14</sup>C:<sup>3</sup>H in medium after the addition of either water or H<sub>2</sub>O<sub>2</sub>.



**Figure 3.68 Expt 6;** export of [ $^{14}\text{C}$ ]ascorbate and/ or  $^{14}\text{C}$ -ascorbate metabolites in response to  $\text{H}_2\text{O}_2$ .

**B. Expt. 6b.** Export of  $^{14}\text{C}$  from cells given either additional water or  $\text{H}_2\text{O}_2$  (final concentration 1mM). An extracellular  $^3\text{H}$ -labelled marker was not used in expt. 6b, otherwise the experimental set up was identical. Cells did not come from the same parent culture as was used in expt. 6a.



**Figure 3.68 Expt 6; export of [ $^{14}\text{C}$ ]ascorbate and/ or  $^{14}\text{C}$ -ascorbate metabolites in response to  $\text{H}_2\text{O}_2$**

**C. Expt. 6c.** Export of  $^{14}\text{C}$  from cells given either additional water or  $\text{H}_2\text{O}_2$  (final concentrations 0.1mM and 1mM). See fig. 3.68a for other details.

i) The control culture, receiving only additional water at 0 s. Data as measured for  $^{14}\text{C}$  and  $^3\text{H}$  are shown, plus deconvolved  $^3\text{H}$  data.

ii)  $^{14}\text{C}:^3\text{H}$  Ratio (calculated from deconvolved  $^3\text{H}$  data) showing the export of  $^{14}\text{C}$  in control cells.

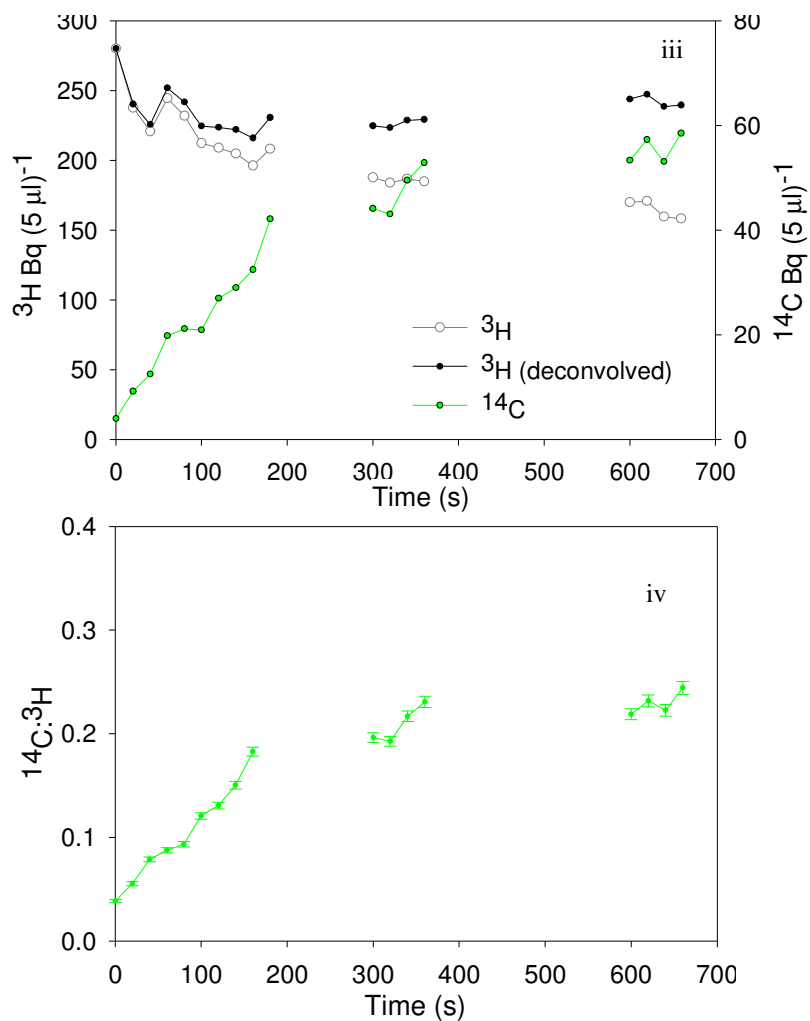


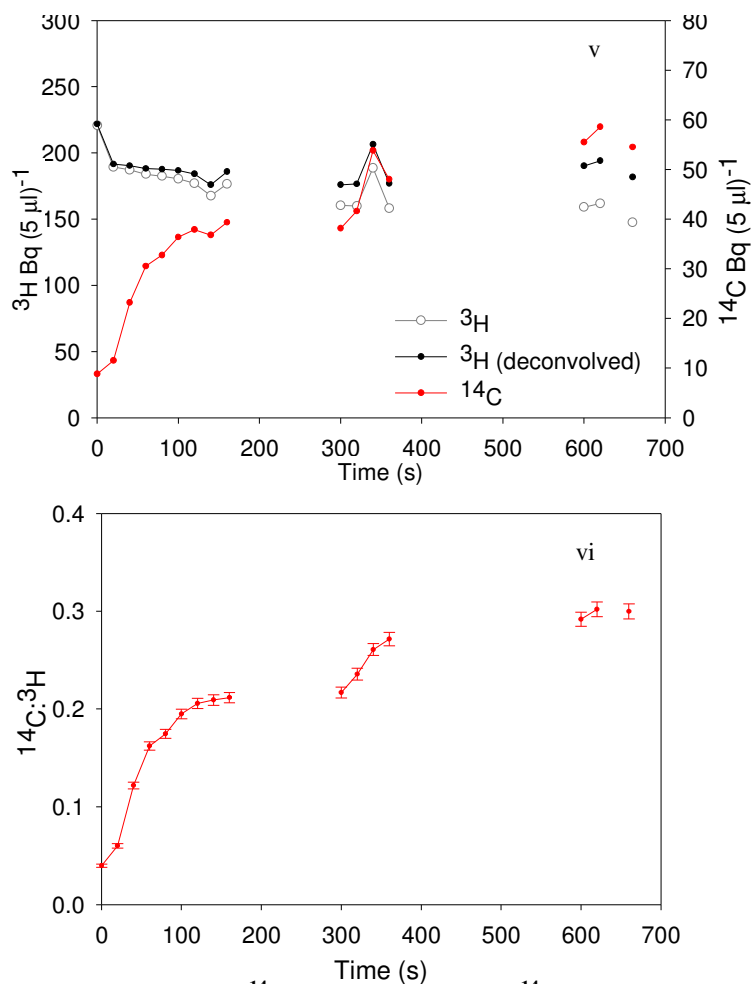
Figure 3.68 Expt 6; export of [ $^{14}\text{C}$ ]ascorbate and/ or  $^{14}\text{C}$ -ascorbate metabolites in response to  $\text{H}_2\text{O}_2$

**C. Expt. 6c.** Export of  $^{14}\text{C}$  from cells given either additional water or  $\text{H}_2\text{O}_2$  (final concentrations 0.1mM and 1mM). See fig. 3.68a for other details.

iii) Cell culture given  $\text{H}_2\text{O}_2$  to a final concentration of 0.1 mM at 0 s. Data shown as in Fig. 3.68 ci.

iv)  $^{14}\text{C}:^3\text{H}$  Ratio (calculated from deconvolved  $^3\text{H}$  data) showing the export of  $^{14}\text{C}$  in response to 0.1 mM  $\text{H}_2\text{O}_2$ .





**Figure 3.68 Expt 6; export of [ $^{14}\text{C}$ ]ascorbate and/ or  $^{14}\text{C}$ -ascorbate metabolites in response to  $\text{H}_2\text{O}_2$**

**C. Expt. 6c.** Export of  $^{14}\text{C}$  from cells given either additional water or  $\text{H}_2\text{O}_2$  (final concentrations 0.1mM and 1mM). See fig. 3.68a for other details.

v) The culture receiving  $\text{H}_2\text{O}_2$  (final concentration of 1 mM) at 0 s. Data shown as in Fig. 3.68 Ci.

vi)  $^{14}\text{C}:^3\text{H}$  Ratio (calculated from deconvolved  $^3\text{H}$  data) showing export of  $^{14}\text{C}$  in response to 1 mM  $\text{H}_2\text{O}_2$ .

control cultures but was maintained in H<sub>2</sub>O<sub>2</sub>-treated cultures until 40 – 55 s. Export was sustained at a slower rate until approximately 115 s (Fig. 3.68b). Unlike the control culture in expt. 6a, where a distinct plateau was observed from 75 s onwards, export continues slowly in the control in expt. 6b until around 175 s.

The experiment was repeated once more (expt. 6c) with several additions: a [<sup>3</sup>H]glucitol marker was included, sampling was continued for up to 660 s and the effect of 0.1 mM H<sub>2</sub>O<sub>2</sub> was compared to the addition both water and 1 mM H<sub>2</sub>O<sub>2</sub>. Export rates were fastest before 60 s in cells treated with 1 mM H<sub>2</sub>O<sub>2</sub> (Fig. 3.68c v, vi). As predicted from expts 6a and 6b, export then slowed and appeared to have reached a plateau by 160 s. However, data from 300 s to 600 s suggests that despite the apparent plateau at 120 – 160 s (Fig. 3.68c vi) export of <sup>14</sup>C was sustained until 600 s, albeit slowly relative to initial rates. Export was very similar in response to 0.1 mM H<sub>2</sub>O<sub>2</sub> as to additional water given to control cells, showing neither increased export within the first 2 min nor sustained export afterwards (Fig. 3.68c ii, iv). It would seem that a response is triggered only above a certain threshold of H<sub>2</sub>O<sub>2</sub> concentration. Expts. 6b and 6c confirm observations made in expt. 6a that export of [<sup>14</sup>C]ascorbate and/or [<sup>14</sup>C]ascorbate metabolites in response to 1 mM H<sub>2</sub>O<sub>2</sub> is characterised by an increase in export in approximately a 30 – 100 s window after the addition of H<sub>2</sub>O<sub>2</sub>.

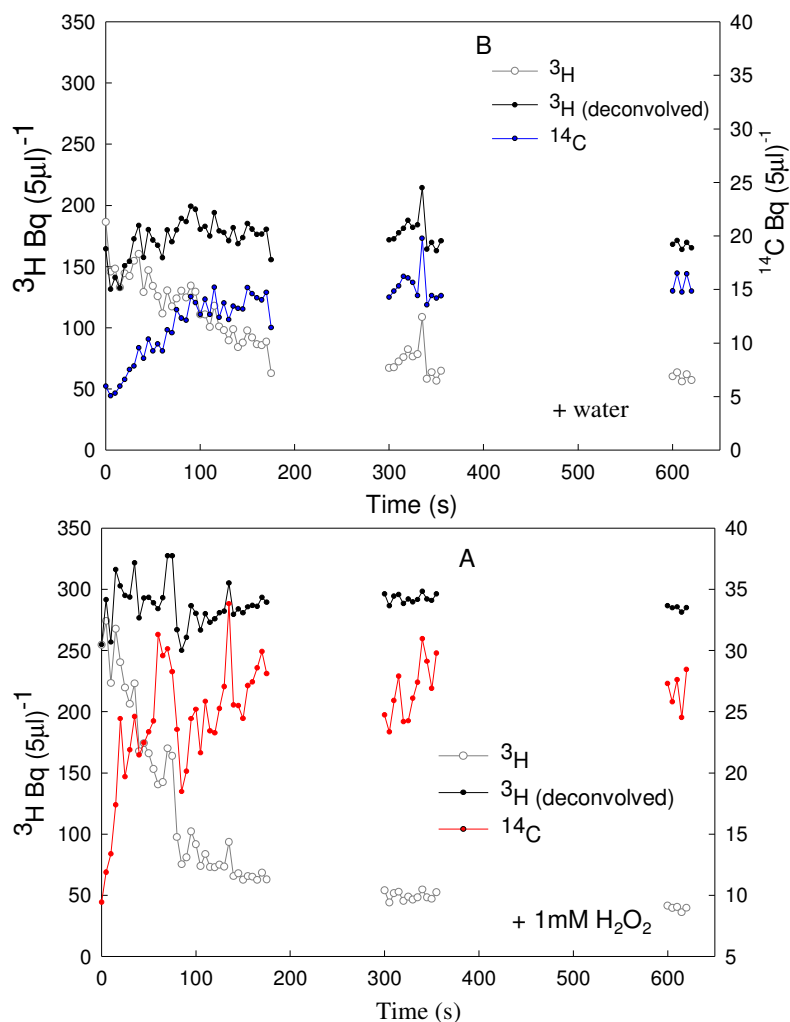
### **3.8.6 Export and import of <sup>14</sup>C-labelled metabolites in response to a 1 mM H<sub>2</sub>O<sub>2</sub> stress in *Arabidopsis* cell suspension cultures (expt. 7)**

As less evidence for export and import of <sup>14</sup>C in pulses had been seen in later experiments on rose cells cultures in expt. 6, the existence of this phenomenon was examined in *Arabidopsis* cell suspension cultures (expt. 7, Fig. 3.69). The methodology followed was as in expt. 6. The decrease in concentration of [<sup>3</sup>H]glucitol over time followed an exponential rather than the linear decay which had been seen in rose cultures (Fig. 3.69a, b). This implied that the *Arabidopsis* cells were taking up the extracellular [<sup>3</sup>H]glucitol marker. As in experiments with

rose cell suspension cultures, the background ‘noise’ of pipetting error was deconvolved from the exponential decay trend. The exponential decay in figures 3.69a and b was fitted to the model  $y = y_0 + a^{(-bx)}$  with  $R^2$  values of 0.95 and 0.88, respectively. As with linear decay, the difference between  $^3\text{H}$  concentration at 0 s and other time points was calculated and added to the measured  $^3\text{H}$  concentration at appropriate time points. The  $^{14}\text{C}:^3\text{H}$  ratio was calculated from the deconvolved  $^3\text{H}$  data (Fig. 3.69ci, ii), otherwise decreases in  $^{14}\text{C}$  from rapid import were not seen clearly as the  $^3\text{H}$  denominator decreased. For comparison, the  $^{14}\text{C}:^3\text{H}$  ratio as calculated with the raw  $^3\text{H}$  data has been included (Fig. 3.69d).

Similar to the response seen in rose cells,  $^{14}\text{C}$  was exported most rapidly for 30 s following addition of 1 mM  $\text{H}_2\text{O}_2$ . Unlike in rose cells, initial rates of  $^{14}\text{C}$  export were not similar in both treatments, but were much faster in cells treated with 1 mM  $\text{H}_2\text{O}_2$  than in control cells (Fig. 3.69a, ci). After 30 s, the rate of  $^{14}\text{C}$  export in  $\text{H}_2\text{O}_2$  treated cells became comparable to that of control cells. A steady state between export to the medium and removal of  $^{14}\text{C}$  from the medium in samples was reached at around 100 s in the control culture and between 150 s and 300 s in the  $\text{H}_2\text{O}_2$ -stressed culture (Fig. 3.69c). In rose cultures, although it had appeared that this state had been reached at around 75 s in control cells and 120 s in  $\text{H}_2\text{O}_2$ -treated cells in expt. 6a, it was shown in expt. 6b that  $^{14}\text{C}$  levels in the medium increased slowly until sampling was ceased at 660 s. In the *Arabidopsis* cultures, sampling was continued until 660 s. There did not appear to be an appreciable increase in  $^{14}\text{C}$  content of the medium of *Arabidopsis* cell cultures between 300 s and 660 s (Fig. 3.69a, b, c).

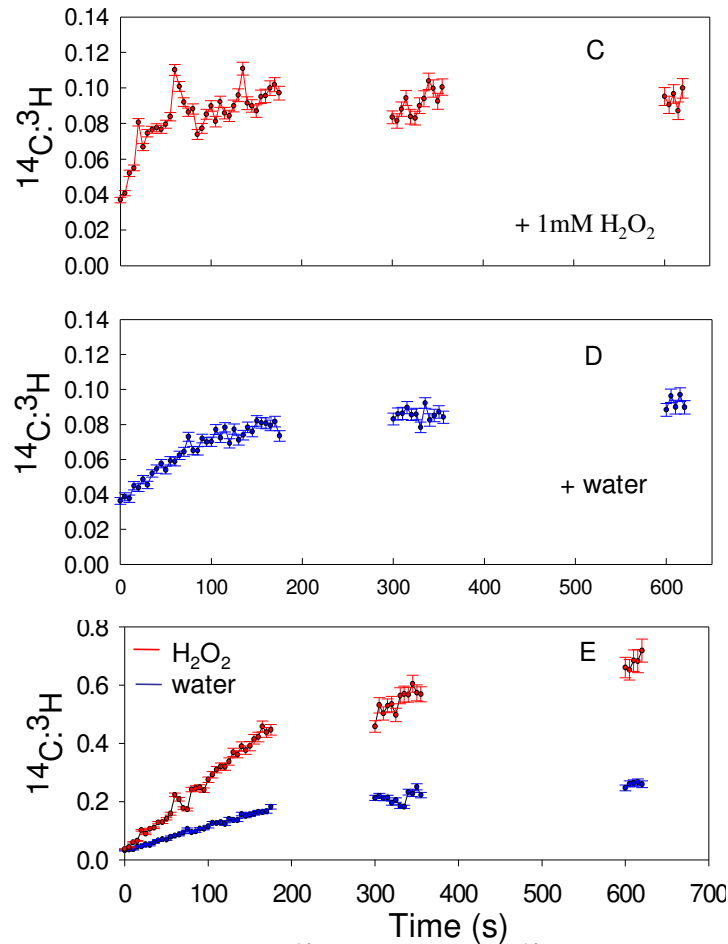
Rapid export and import of  $^{14}\text{C}$  in pulses was seen in *Arabidopsis* cultures (Fig. 3.69c). Pulses were distinct when the  $^{14}\text{C}:^3\text{H}$  ratio was calculated from the raw  $^3\text{H}$  data (Fig. 3.69d) but as levels of  $^3\text{H}$  decreased so quickly during the first 120 s, the ‘import stage’ of a pulse was masked by the exaggerated decrease of the ratio denominator at the next time point. When the



**Figure 3.69 Expt 7; export of [<sup>14</sup>C]ascorbate and <sup>14</sup>C-ascorbate metabolites in response to 1 mM H<sub>2</sub>O<sub>2</sub> in *Arabidopsis* cell suspension cultures**

The experiments investigating the H<sub>2</sub>O<sub>2</sub>-induced export of intracellular <sup>14</sup>C was performed once using *Arabidopsis thaliana* cells. [<sup>3</sup>H]Glucitol was used as an extracellular marker in the culture medium. The experimental set up was as described in figure 3.68a. Radioactivity was assayed by scintillation counting. Data for [<sup>3</sup>H]glucitol concentrations were deconvolved from the exponential decay trend (see main text for details).

- A. <sup>3</sup>H and <sup>14</sup>C in medium after addition of H<sub>2</sub>O<sub>2</sub>.
- B. <sup>3</sup>H and <sup>14</sup>C in medium after addition of 1 mM water.



**Figure 3.69 Expt 8; export of [ $^{14}\text{C}$ ]ascorbate and  $^{14}\text{C}$ -ascorbate metabolites in response to 1 mM  $\text{H}_2\text{O}_2$  in *Arabidopsis* cell suspension cultures**

- C. Ratio of  $^{14}\text{C}:^3\text{H}$  in medium after the addition of  $\text{H}_2\text{O}_2$ , calculated with deconvolved  $^3\text{H}$  data .
- D. Ratio of  $^{14}\text{C}:^3\text{H}$  in medium after the addition of water, calculated with deconvolved  $^3\text{H}$  data.
- E. Ratio of  $^{14}\text{C}:^3\text{H}$  calculated with raw  $^3\text{H}$  data.

$^{14}\text{C}:$  $^3\text{H}$  ratio was calculated from the deconvolved data, pulsing was evident in the culture receiving  $\text{H}_2\text{O}_2$  (Fig. 3.69ci) and was occasionally detected in the control culture (Fig. 3.69cii). As had been seen in rose cell cultures (Expts. 1 – 5), the amplitude of pulses was increased in response to 1 mM  $\text{H}_2\text{O}_2$  and this was particularly apparent during the first 150 s following  $\text{H}_2\text{O}_2$  addition (Fig. 3.69ci).

#### 3.8.6.1 Summary of experiments 3 – 7

Rose and *Arabidopsis* cell suspension cultures were loaded with [ $^{14}\text{C}$ ]ascorbate and then subjected to either a mild stress (exchange of radioactive culture medium for non-radioactive culture medium) or a more severe oxidative stress (addition of either 0.1 mM  $\text{H}_2\text{O}_2$  or a 1 mM  $\text{H}_2\text{O}_2$ ). Following either a mild or a more severe stress,  $^{14}\text{C}$  was exported from cells. The rate of export was greater in cells exposed to 1 mM than control cells or those receiving 0.1 mM  $\text{H}_2\text{O}_2$ . Export of  $^{14}\text{C}$  was often followed by rapid import of  $^{14}\text{C}$ , appearing to be exported in pulses. Pulses of  $^{14}\text{C}$  were noted in both rose and *Arabidopsis* cells. In cells challenged with 1 mM  $\text{H}_2\text{O}_2$ , these pulses were observed to be larger, particularly so in the first 2 min following addition of  $\text{H}_2\text{O}_2$ . Export of  $^{14}\text{C}$  was not always observed to occur in pulses in rose cells. When pulses were not observed, an increased rate of export in 1 mM  $\text{H}_2\text{O}_2$ -treated cells was observed during a 30 – 120 s window compared to control cells. An early increase in the rate of  $^{14}\text{C}$  export seemed to be dependent on the strength of the  $\text{H}_2\text{O}_2$  signal, as no increase in  $^{14}\text{C}$  export relative to control cells was seen in cells treated with 0.1mM  $\text{H}_2\text{O}_2$ .

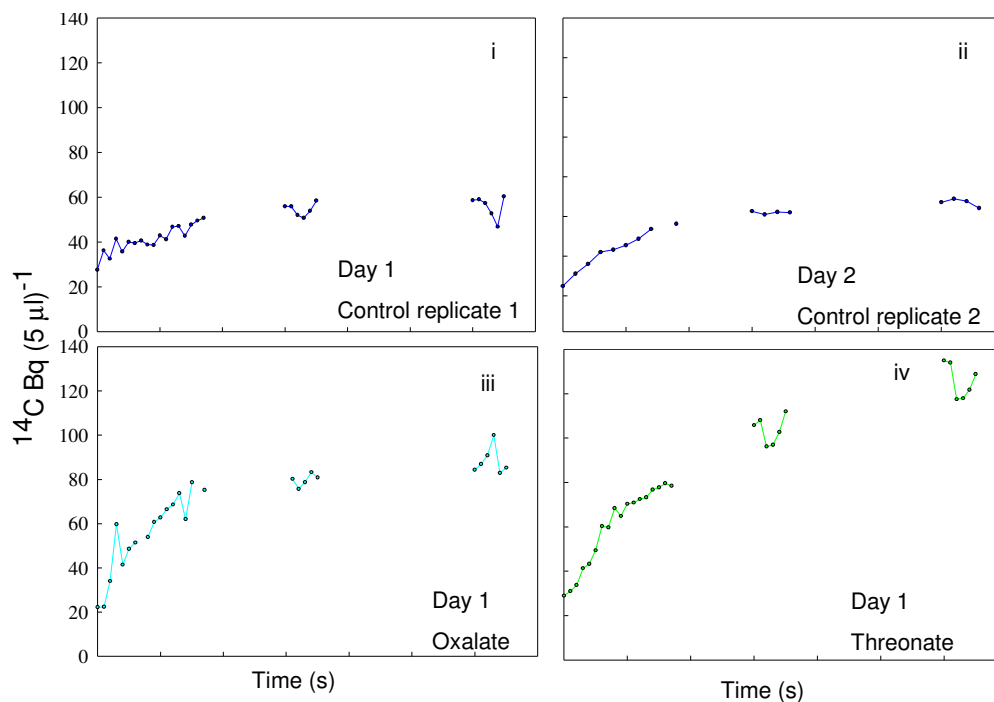
### 3.8.7 The effect of ascorbate metabolites of the export and import of [<sup>14</sup>C]ascorbate and/or [<sup>14</sup>C]ascorbate metabolites

It had been noted in expts. 1 and 2 that [<sup>14</sup>C]ascorbate and/or [<sup>14</sup>C]DHA seemed to be exported and imported, as opposed to any other oxidation product such as [<sup>14</sup>C]ox.thr. or [<sup>14</sup>C]oxalate. At early time points (0 – 5 min), there seemed to be little build up of either [<sup>14</sup>C]ox.thr., [<sup>14</sup>C]oxalate or any other [<sup>14</sup>C]ascorbate breakdown products other than [<sup>14</sup>C]DHA. Depending on how oxidising the apoplastic environment becomes when exposed to certain stresses e.g. ozone, ascorbate oxidation products might accumulate in the apoplast. The question was therefore posed whether a build up of oxidation products, other than [<sup>14</sup>C]DHA, in the apoplast could act as a stress signal and stimulate the export of [<sup>14</sup>C]ascorbate from [<sup>14</sup>C]ascorbate-loaded cells.

Increased export of <sup>14</sup>C from cells was observed in response to oxalate and threonate (final concentration 100 μM) and ox.thr. (estimated final concentration 100 μM) compared to control cells, although neither showed the rapid increase in export of <sup>14</sup>C before 120 s that was characteristic of the response to 1 mM H<sub>2</sub>O<sub>2</sub>. Rather, export seemed to be sustained until sampling ceased at 660 min. Ox.thr. was given to cells in two separate experiments, replicates 1 and 2. The method used to exchange the medium in which cells had been incubated with [<sup>14</sup>C]ascorbate for non-radioactive medium differed subtly between day 1 and day 2, meaning the medium was exchanged more efficiently day 2 (Fig. 3.70 v and vi). Otherwise, methods were identical. It was considered possible that the more efficient exchange of medium had affected the measured levels of <sup>14</sup>C at later time points, as these were greater in figure 3.70 i, iii, iv and v, which were carried out on day 1 with the original method for culture exchange. As ox.thr. had been isolated by elution off a paper electrophoretogram, the preparation of ox.thr. was likely to

have contained traces of the pyridine/ acetic acid buffer as well as material from the paper. A control was included where the contents of a strip of the same electrophoretogram, of an equivalent size as the strip off which ox.thr. had been eluted, was added to the medium. Export of  $^{14}\text{C}$  from these cells was also increased. This would suggest that although rates of export of  $^{14}\text{C}$ ascorbate and/or  $^{14}\text{C}$ ascorbate metabolites (likely to be  $^{14}\text{C}$ DHA) do seem to respond to changes in the cell culture medium other than  $\text{H}_2\text{O}_2$ , this response was not specific to either ox.thr., threonate or oxalate.

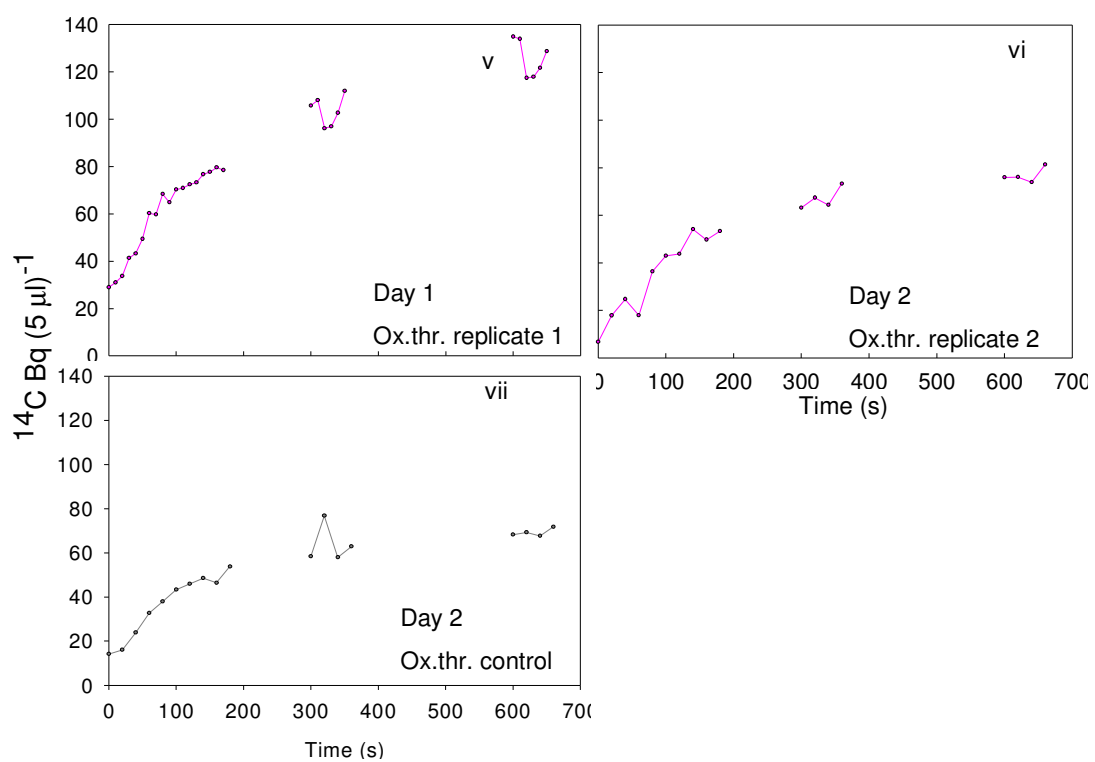




**Figure 3.70 Export of [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]ascorbate metabolites in response to ascorbate oxidation products**

The effect of ascorbate oxidation products on the export of intracellular  $^{14}\text{C}$  was investigated. The experimental set-up was as described in Fig. 3.68a, except that either oxalate, threonate (final conc.  $100\ \mu\text{M}$ ) or ox.thr. (estimated final conc.  $100\ \mu\text{M}$ ) were added, in  $20\ \mu\text{l}$  aliquots, to cells instead of  $\text{H}_2\text{O}_2$ . Experiments were carried out on two separate days (labelled 1 or 2). All experimental cultures were taken from the same culture. Differences in  $^{14}\text{C}$  in the medium at 0 s are thought to be a result of more thorough removal of residual  $^{14}\text{C}$  from the medium after the 5 h incubation with [ $^{14}\text{C}$ ]ascorbate. Control cultures received  $20\ \mu\text{l}$  water at 0 s. The ox.thr. control refers to cells given  $20\ \mu\text{l}$  of the eluate from a strip excised from the same paper electrophoretogram from which the ox.thr. was isolated.

- i) Cells + additional water, day 1
- ii) Cells + additional water, day 2
- iii) Cells + oxalate, day 1
- iv) Cells + threonate, day 1



**Figure 3.70 Export of [ $^{14}\text{C}$ ]ascorbate and/or [ $^{14}\text{C}$ ]ascorbate metabolites in response to ascorbate oxidation products**

- v) Cells + ox.thr., day 1
- vi) Cells + ox.thr., day 2
- vii) Cells + water eluted off the same paper electrophoretogram as ox.thr., day 1

### 3.9 The role of ascorbate oxidase (AO) in ascorbate catabolism

Ascorbate oxidase is a cell wall located enzyme which catalyses the oxidation of ascorbate to MDHA (Pignocchi *et al.* 2003); MDHA then disproportionates to form MDHA and ascorbate (Buettner and Schafer 2004). Ascorbate breakdown was investigated in excised leaves of transgenic tobacco plants with enhanced expression of a cucumber ascorbate oxidase (AO sense plants). It has been reported that AO sense plants have a more oxidised apoplast than wild type (WT) plants; a greater proportion of the ascorbate pool was found to be present as DHA rather than ascorbate (Pignocchi *et al.* 2003). It was hypothesised that if an increase in AO expression resulted in a more oxidised ascorbate:DHA ratio in the apoplast, then this would increase flux through the apoplastic ascorbate breakdown pathway that had been described by Green and Fry (2005). Higher levels of ascorbate metabolites such as ox.thr. and oxalate were therefore considered likely in the apoplast of AO sense plants compared to WT controls.

Two experiments were carried out with AO sense and WT excised leaves of *Nicotiana tabacum* L., cv. Xanthi (expt. 1 and 2) and one on excised leaves of *Nicotiana tabacum* L., cv. Petite Havana (expt. 3) in which [<sup>14</sup>C]ascorbate was fed via the petiole. Apoplastic fluids were sampled by vacuum infiltration. Leaves containing the remains of the apoplastic fluids and the cell contents were homogenised and <sup>14</sup>C-labelled metabolites in the supernatant were analysed. A problem encountered in both expts. 1 and 2 was that very low volumes of infiltrant buffer and apoplastic fluids were recovered from leaves. Based on trials with non-radiolabelled leaves, around 50 – 100 µl of fluid was expected to have been recovered from leaves. In expt. 1 less than 10 µl was recovered from each leaf. In expt. 2, 2 – 40 µl was recovered but even in these samples radioactivity was too low to yield meaningful data. Low recovery of apoplastic fluids may have been a result lower stomatal conductance of leaves under the experimental conditions

described in section 2.15.2, meaning that less fluid was infiltrated into leaves under vacuum. Analysis was therefore confined to samples of leaf homogenate, except in expt. 3 where sufficient volumes (30 – 70 µl) of infiltrated buffer + apoplastic fluids were recovered for analysis. <sup>14</sup>C-Labelled metabolites in leaves would have been mostly symplastic as only about 10 % of the total ascorbate pool is thought to be found in the apoplast (Noctor and Foyer 1998). This could have affected results because if the effect of increased AO activity of [<sup>14</sup>C]ascorbate metabolism was confined solely to the apoplast then large differences between the apoplast of WT and AO sense leaves could have appeared slight when apoplastic <sup>14</sup>C-labelled metabolites were mixed with symplastic <sup>14</sup>C-labelled metabolites.

[<sup>14</sup>C]Ascorbate was taken up by *Nicotiana* leaves. After 8 h [<sup>14</sup>C]ascorbate had localised towards the lamina tip. Whilst veins were strongly labelled it was also possible to see radioactivity in the mesophyll (Fig. 3.71). The presence of radioactivity in the mesophyll was desirable as this would have increased the likelihood of extraction of radiolabelled apoplastic fluids.

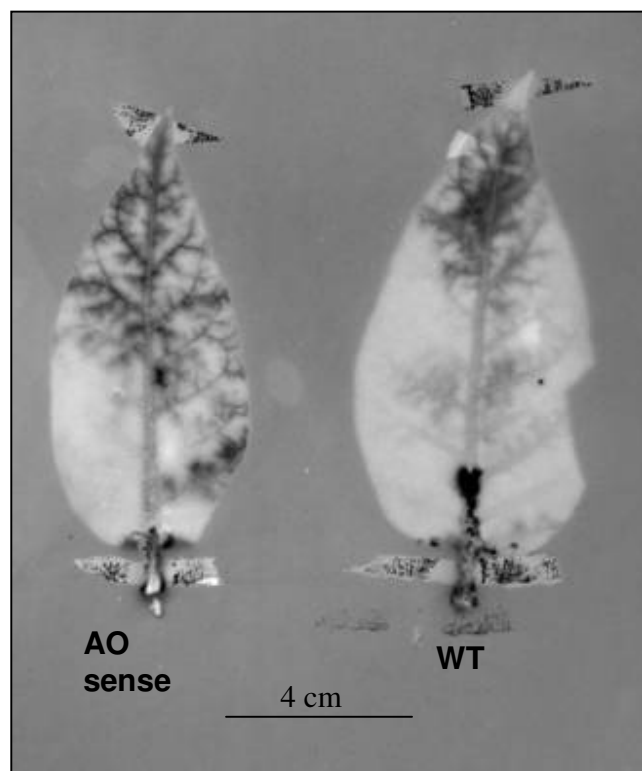
### 3.9.1 [<sup>14</sup>C]Ascorbate catabolism in AO sense and WT leaves — expt. 1

In expt. 1 it was observed, as predicted from previous studies (Pignocchi *et al.* 2003), that levels of [<sup>14</sup>C]DHA were higher in AO sense leaves and that levels of [<sup>14</sup>C]ascorbate were lower compared to WT controls. After 6 h there was an almost 20 % difference in [<sup>14</sup>C]DHA levels and almost a 30 % difference in [<sup>14</sup>C]ascorbate compared to WT leaves (Fig. 3.71a, b). [<sup>14</sup>C]Ox.thr. were very low in both WT and AO sense leaves. This was initially interpreted as a sign of high activity of the apoplastic esterase that was proposed by Green and Fry (2005). However, results from expt. 2 (discussed later) cast some doubt over the [<sup>14</sup>C]ox.thr. data (Fig. 3.71c). More [<sup>14</sup>C]oxalate was seen in leaf homogenate samples in AO sense plants compared to WT plants (Fig. 3.71c). From this observation it was preliminarily concluded that increased AO expression did result in greater flux through the described pathway. [<sup>14</sup>C]Oxalate was either metabolised very slowly or acted as an end product in this pathway, as only a very marginal decrease in [<sup>14</sup>C]oxalate content of leaves was seen between 1.5 and 6 h. The increased [<sup>14</sup>C]oxalate content of AO sense leaves was apparent even at 0.5 and 1.5 h, although the differences in [<sup>14</sup>C]DHA content compared to WT leaves were not obvious until 6 h (Fig. 3.71a). If [<sup>14</sup>C]oxalate were an end product of this pathway and greater flux through the pathway happened in AO sense plants, then it would follow that differences between [<sup>14</sup>C]oxalate accumulation in mutant and WT leave should become manifest at quite an early stage of the expt., as onwards metabolism of [<sup>14</sup>C]oxalate would not mask any differences in [<sup>14</sup>C]oxalate levels between genotypes.

Therefore, the conclusions from results in expt. 1 were that increased expression of AO resulted in greater flux through the ascorbate breakdown pathway, probably as more [<sup>14</sup>C]DHA was present. [<sup>14</sup>C]Oxalate appeared to be an end product of the pathway and the esterase seemed to be present and highly active. The effect of AO expression on [<sup>14</sup>C]ascorbate metabolism in this experiment would seem to suggest that the step between ascorbate and DHA was critical in

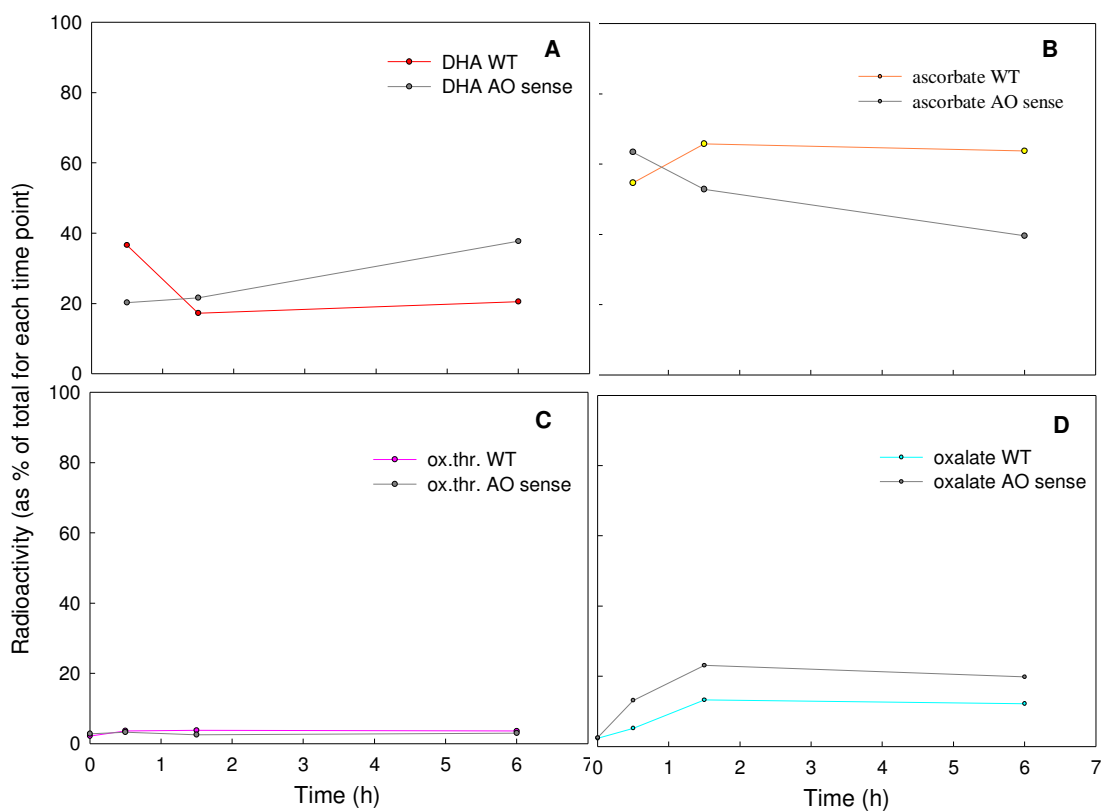
determining flux through the ascorbate breakdown pathway and the extent of oxalate accumulation. Interestingly, when the apoplastic ascorbate and DHA content of AO sense plants was examined (Pignocchi *et al.* 2003), the total ascorbate content (ascorbate + DHA) of the apoplast of the sense plants was found to be less than both WT and AO antisense plants. A plausible explanation is that more irreversible loss from the total apoplastic ascorbate pool was occurring as DHA was catabolised to oxalate in AO sense plants, hence the lower total apoplastic ascorbate content than in WT plants.

At pH 6.5, DKG migrated ahead (closer to the anode) of ascorbate (see sections 3.1 and 3.2 for details on the electrophoretic mobility of DKG). In expt. 1 a compound was observed with a similar electrophoretic mobility to DKG at pH 6.5 (Fig. 3.73a). At pH 3.5 DKG is known to co-migrate with the external orange G marker (see sections 3.1, 3.2). A spot of equivalent intensity to that of the compound thought to be DKG at pH 6.5 was seen at pH 3.5 (Fig. 3.73b) but this did not co-migrate with orange G and so was not thought to be DKG. The compound which co-migrated with DKG at pH 6.5 was not identified and was not noted in expt. 2 (Fig. 3.73).



**Figure 3.71** Autoradiogram of *Nicotiana tabacum* L. cv. Xanthi leaves fed [ $^{14}\text{C}$ ]ascorbate

[ $^{14}\text{C}$ ]Ascorbate (0.025 kBq) was fed via the petiole, followed by water. Leaves were sampled 8 h after [ $^{14}\text{C}$ ]ascorbate had been fed and were exposed to autoradiography film overnight. The darker strips at either end of the leaf are tape, along which radioactivity from the leaf seems to have accumulated whilst leaves were packed into the autoradiography cassette. It is interesting to note that [ $^{14}\text{C}$ ]ascorbate seems to have accumulated at the tips of leaves.

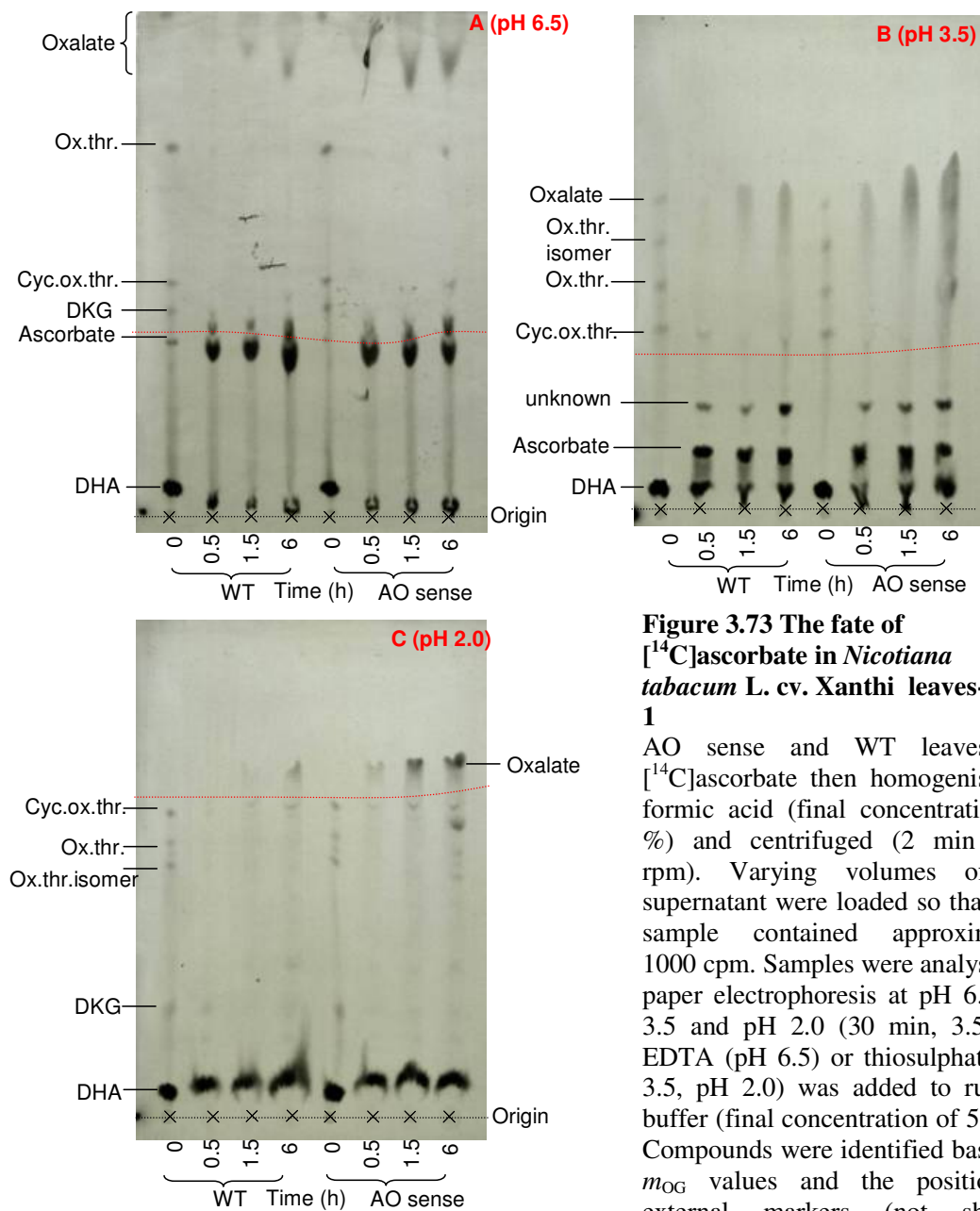


**Figure 3.72 Quantification of [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]ascorbate metabolites in *Nicotiana tabacum* L., cv. Xanthi leaves in expt. 1**

Compounds that corresponded to darkened spots on the autoradiogram (Fig. 3.73 a) were excised and assayed for radioactivity by scintillation counting. Values for [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA at 0 h have been omitted. At 0 h [<sup>14</sup>C]ascorbate was added to apoplastic fluids and leaf homogenates post-sampling. The amount added was over estimated compared to the total radioactivity at other time points (Fig. 3.73). A comparison of 0 h values to results at other time points was therefore felt to misleading for [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA. Values at 0 h were included for [<sup>14</sup>C]ox.thr. and [<sup>14</sup>C]oxalate as, being low, they demonstrated that no oxidation of [<sup>14</sup>C]ascorbate beyond [<sup>14</sup>C]DHA had occurred during storage.

- A. [<sup>14</sup>C]DHA
- B. [<sup>14</sup>C]Ascorbate
- C. [<sup>14</sup>C]Oxalate
- D. [<sup>14</sup>C]Ox.thr.





**Figure 3.73 The fate of  $[^{14}\text{C}]$ ascorbate in *Nicotiana tabacum* L. cv. Xanthi leaves-expt. 1**

AO sense and WT leaves fed  $[^{14}\text{C}]$ ascorbate then homogenised in formic acid (final concentration 20 %) and centrifuged (2 min 3000 rpm). Varying volumes of the supernatant were loaded so that each sample contained approximately 1000 cpm. Samples were analysed by paper electrophoresis at pH 6.5, pH 3.5 and pH 2.0 (30 min, 3.5 kV). EDTA (pH 6.5) or thiosulphate (pH 3.5, pH 2.0) was added to running buffer (final concentration of 5 mM). Compounds were identified based on  $m_{\text{OG}}$  values and the position of external markers (not shown). Oxidation of  $[^{14}\text{C}]$ ascorbate to  $[^{14}\text{C}]$ DHA seems to have occurred at 0 h. This is thought to have occurred during the experiment rather than during storage. Oxidation of  $[^{14}\text{C}]$ DHA does not seem to have occurred.

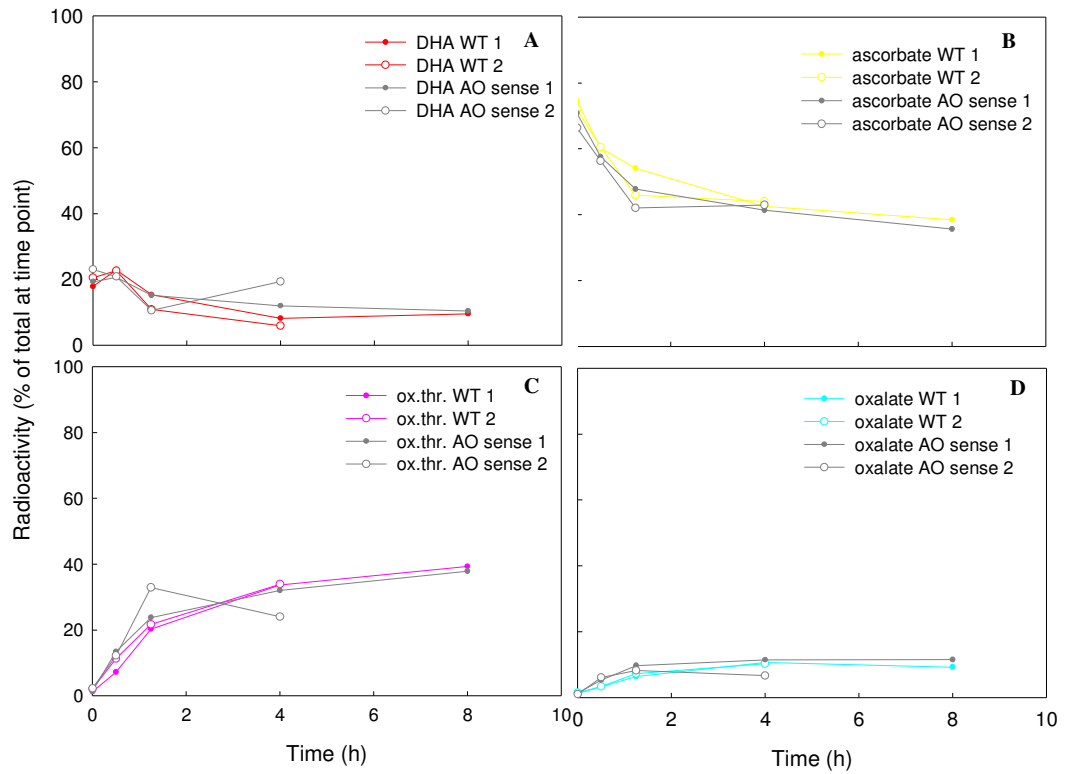
### 3.9.2 [<sup>14</sup>C]Ascorbate catabolism in AO sense and WT leaves — expt. 2

When expt. 1 was repeated, no difference was seen in [<sup>14</sup>C]ascorbate metabolism between AO sense and WT plants (Fig. 3.74). At 0.5 h, values for the compounds analysed (excepting [<sup>14</sup>C]ox.thr.) are similar in both expts. 1 and 2; differences between expts. are unlikely to be a result of extensive post-sampling oxidation of [<sup>14</sup>C]ascorbate or major differences in the capacity of leaves to metabolise [<sup>14</sup>C]ascorbate between expts. 1 and 2. The age of leaves used in expts. 1 and 2 was not controlled; in expt. 1 plants were at a slightly later stage of development than in expt. 2 and so very small, young leaves had to be used, as the only other leaves present were too large to fit into syringe barrels. In expt. 2 slightly older leaves than those used in expt. 1 were chosen so that sufficient volumes of apoplastic fluid could be extracted. AO is reported to be expressed most highly in young tissue (Esaka *et al.* 1992, Kato and Esaka 1996). Sufficient volumes of apoplastic fluids for analysis were not collected from the leaves chosen in expt. 2 but differences in leaf age could have impacted on results. A slight increase in [<sup>14</sup>C]DHA content over time in expt. 2 was seen in AO sense plants (Fig. 3.74a) and at 0.5 h and 1.5 h [<sup>14</sup>C]oxalate content was higher (3.74d). These differences were marginal, though, as might have been expected if differences in the expression of AO were not so great between AO sense and WT leaves.

[<sup>14</sup>C]Ascorbate decreased with time in both WT and AO sense leaves (Fig. 3.74b). This is contrary to WT leaves in expt. 1 and 10-day-old rose cell cultures where [<sup>14</sup>C]ascorbate seemed relatively stable over time. Although the symplasm was expected to comprise much more of the volume of leaf homogenate samples than the apoplast, it is difficult to come to conclusions on the symplastic stability of [<sup>14</sup>C]ascorbate in expt. 2, without knowing the proportion of apoplastic fluids in the samples or the distribution of <sup>14</sup>C between apoplast and symplast. [<sup>14</sup>C]DHA levels were low but quite constant over time (Fig. 3.74a), whilst [<sup>14</sup>C]ox.thr.

and [ $^{14}\text{C}$ ]oxalate increased (Fig. 3.74c, d). This would suggest a relatively rapid turnover of [ $^{14}\text{C}$ ]DHA to [ $^{14}\text{C}$ ]ox.thr. or [ $^{14}\text{C}$ ]oxalate. Contrary to expt. 1, [ $^{14}\text{C}$ ]ox.thr. was observed to accumulate over time in both genotypes (Fig. 3.74d). Ox.thr. is relatively stable in solutions at acidic pH. Final concentrations of formic acid (which would have denatured the esterase) were the same in leaf homogenates in expt. 1 and expt. 2 so post-sampling hydrolysis of [ $^{14}\text{C}$ ]ox.thr. should not have decreased in expt. 2 compared to expt. 1. It is conceivable that this enzyme shows developmental regulation as well as AO and was more highly expressed in younger leaves, although the differences in [ $^{14}\text{C}$ ]ox.thr. accumulation in expt. 1 and 2 were quite extreme; some expression could still have been expected in older leaves. [ $^{14}\text{C}$ ]Oxalate accumulated in all leaves in expt. 2 (Fig. 3.74c) but to a slightly lower extent that was seen in expt. 1 (Fig. 3.72d), as would have been expected if esterase activity had been lower in leaves in expt. 2. The difference between [ $^{14}\text{C}$ ]ox.thr. in expts. 1 and 2 is greater than the difference in [ $^{14}\text{C}$ ]oxalate, implying that flux to [ $^{14}\text{C}$ ]ox.thr. was less in expt. 1. This may explain in part why lower levels of [ $^{14}\text{C}$ ]ox.thr. were seen in expt. 1 than in expt. 2.

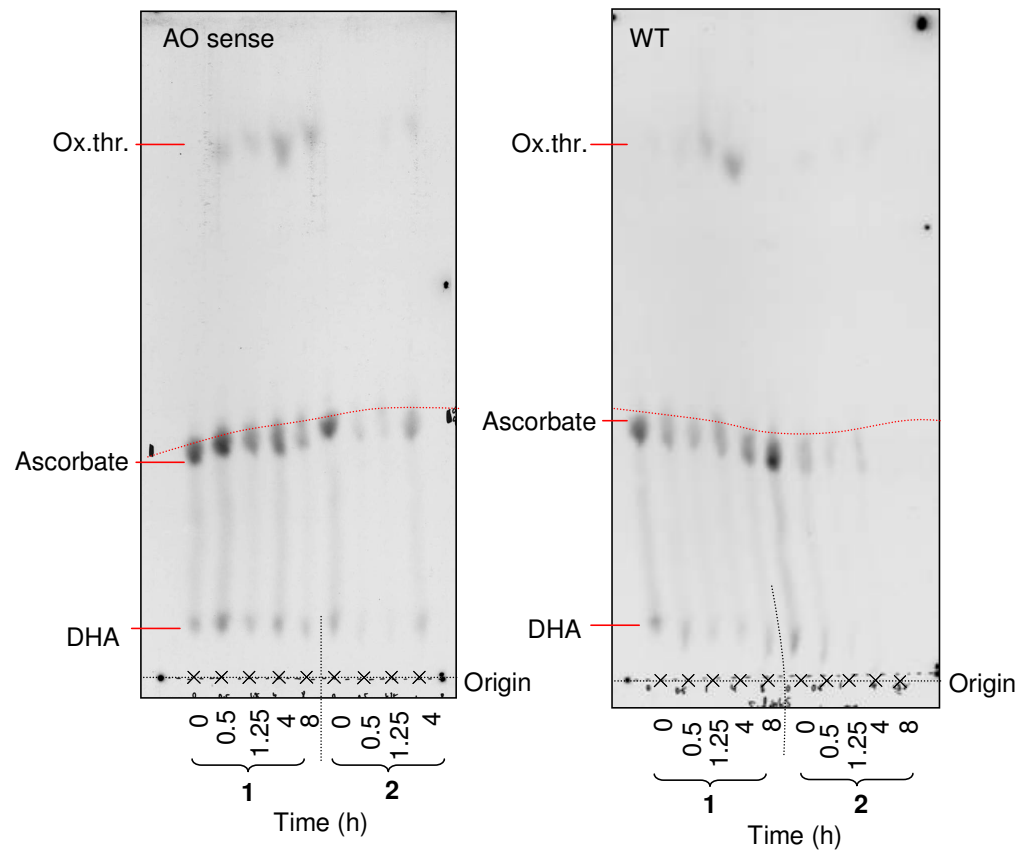
Data in expt. 2 does provide some useful information on the catabolism of [ $^{14}\text{C}$ ]ascorbate *in planta*, as the pathway described by Green and Fry (2005) had hitherto only been investigated in rose cell suspension cultures. The formation of [ $^{14}\text{C}$ ]ox.thr. and [ $^{14}\text{C}$ ]oxalate in expt. 2 shows that ox.thr. is an intermediate in the catabolism of [ $^{14}\text{C}$ ]ascorbate *in planta*. The apparent absence of [ $^{14}\text{C}$ ]DKG and [ $^{14}\text{C}$ ]cyc.ox.thr. in both expts. 1 and 2 suggests that these compounds may not be important intermediates of ascorbate catabolism *in planta*. Whilst no differences were seen between genotypes in expt. 2, the proportions of each metabolite at time points are very similar in all 4 leaves suggesting these data are reliable.



**Figure 3.74** Quantification of [ $^{14}\text{C}$ ]ascorbate and [ $^{14}\text{C}$ ]ascorbate metabolites in *Nicotiana tabacum* L., cv. Xanthi leaves in expt. 2

For details see figure 3.72.

- A. [ $^{14}\text{C}$ ]DHA
- B. [ $^{14}\text{C}$ ]Ascorbate
- C. [ $^{14}\text{C}$ ]Ox.thr.
- D. [ $^{14}\text{C}$ ]Oxalate

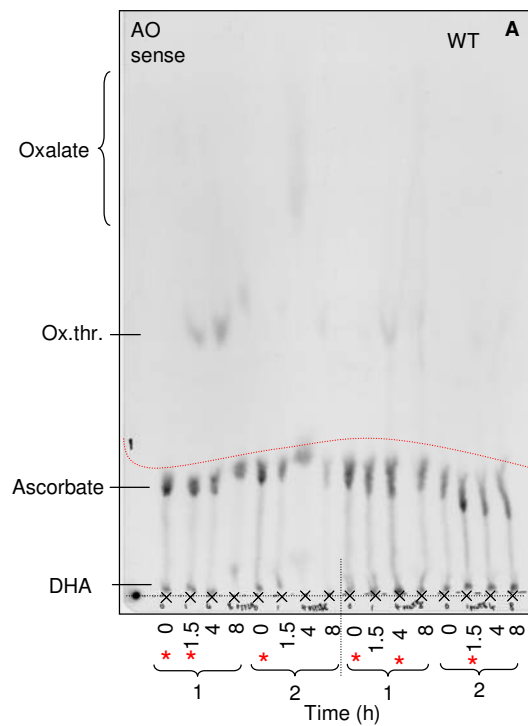


**Figure 3.75** The fate of [ $^{14}\text{C}$ ]ascorbate in *Nicotiana tabacum* L. cv. Xanthi leaves-expt. 2.

AO sense and WT leaves were homogenised in 20 % formic acid and 25 mM ascorbic acid (final concentrations) and centrifuged (2 min 3000 rpm). Samples of the supernatant (5  $\mu\text{l}$ ) were analysed by paper electrophoresis at pH 6.5 (35 min, 3.5 kV). EDTA (final concentration 5 mM) was added to the electrophoresis buffer. 1 and 2 refer to 2 replicate samples of excised leaf. Autoradiograms are pictured. Compounds that corresponded to darkened spots were excised. The data from these samples is shown in figure 3.74.

### 3.9.3 [<sup>14</sup>C]Ascorbate catabolism in AO sense and WT leaves of *Nicotiana tabacum* L., cv. Petite Havana —expt. 3

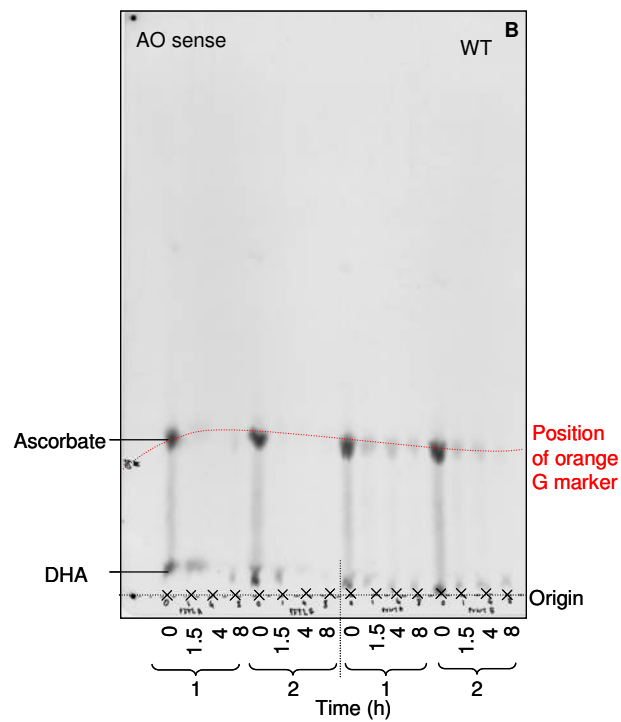
[<sup>14</sup>C]ascorbate catabolism was investigated in the leaves of a second cultivar, *Nicotiana tabacum* L., cv. Petite Havana. Larger volumes of apoplastic fluids were collected from these leaves ( $\approx 30 - 70 \mu\text{l}$ ) though apoplastic samples often showed signs of symplastic contamination (visible green colouring in samples A at 0 h, 1.5 h and B at 0 h in AO sense leaves and samples A at 0 h, 4 h and B at 1.5 h). There was not sufficient time to quantify these results by scintillation counting. A qualitative assessment of the autoradiogram of apoplastic samples suggests that differences between genotypes would not have been very large. However, it is worth drawing attention to one important point. Figure 3.76b shows samples containing symplasm + apoplast whereas figure 3.76a shows apoplastic samples. Symplastic + apoplastic samples seem to have consisted mainly of [<sup>14</sup>C]ascorbate and [<sup>14</sup>C]DHA. [<sup>14</sup>C]Ox.thr. and [<sup>14</sup>C]oxalate present in figure 3.76a are therefore unlikely to be a result of symplastic contamination of apoplastic fluids. This would then imply that the [<sup>14</sup>C]ox.thr. and [<sup>14</sup>C]oxalate present in figure 3.76a were formed in the apoplast rather than the symplasm, supporting the apoplastic location of ascorbate catabolism (Green and Fry 2005). Although samples in figure 3.76b were known to be a mixture of apoplastic + symplastic fluids, <sup>14</sup>C-labelled metabolites located to the apoplast will have been more concentrated in figure 3.76a. [<sup>14</sup>C]Ox.thr. and [<sup>14</sup>C]oxalate may have appeared to be absent in figure 3.76b as they were present in relatively low quantities in the symplastic + apoplastic samples. A greater proportion of [<sup>14</sup>C]ascorbate relative to [<sup>14</sup>C]DHA can be seen in WT samples compared to AO sense samples (Fig. 3.76b). This agrees with previous reports (Pignocchi *et al.* 2003) although it is not possible to say whether this represents <sup>14</sup>C in the symplasm or the apoplast.



**Figure 3.76 The fate of  $[^{14}\text{C}]$ ascorbate in *Nicotiana tabacum* L., cv. Petite Havana-expt. 3.**  
 Details as Fig. 3.75

A. Apoplastic samples.  
 Symplastic contamination is thought to have occurred in asterisked samples

B. Symplasm + apoplast



#### **3.9.4 Summary of [<sup>14</sup>C]ascorbate breakdown in tobacco leaves**

Overexpression of AO does appear to increase flux through the ascorbate breakdown pathway although this may only occur in younger tissues where AO is thought to be more highly expressed. Results suggest that the breakdown pathway of ascorbate in plants is similar to that described by Green and Fry (2005) in rose culture medium but that breakdown proceeds mainly through the intermediate ox.thr. and that cyc.ox.thr. and DKG are not produced. Oxalate does not appear to be metabolised further in tobacco leaves. There are some indications that [<sup>14</sup>C]ascorbate breakdown may have occurred apoplastically but it is difficult to reach a firm conclusion on this point.



## 4.0 Discussion

### 4.1. The *in-vitro* analysis of ascorbate breakdown

#### 4.1.1 The production of cyc.ox.thr. and ox.thr. from DHA

The early part of this study concerned the *in-vitro* oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub>. The oxidation of ascorbate with H<sub>2</sub>O<sub>2</sub> has also been found to provide a useful way in which some of the compounds downstream of ascorbate, particularly cyc.ox.thr. and ox.thr., in the pathway proposed by Green and Fry (2005) can be synthesised. The results have revealed some important points which have revealed important differences between the *in-vitro* and *in-vivo* breakdown of ascorbate.

Cyc.ox.thr. was believed to be the precursor to ox.thr. (Green and Fry 2005). This study has shown that whilst cyc.ox.thr. does yield ox.thr. it does not appear to act as this obligate precursor during *in-vitro* oxidation of ascorbate. The structure of cyc.ox.thr. was tested; it was found not to be an acid anhydride and so the structure assigned by Green and Fry (2005) is favoured. Green and Fry (2005) suggested that only the 4-*O*-oxalyl-L-threonate isomer, of the theoretical 2-, 3- and 4-*O*-oxalyl-L-threonate isomers, was present in cell culture medium fed exogenous ascorbate. This study has demonstrated the presence of at least two isomers of ox.thr. It is not known which two of the three isomers were present and so have been referred to as ox.thr. and ox.thr. isomer throughout the present study. These isomers were seen to interconvert. The equilibrium between them slightly favoured the formation of ox.thr. (the apparently more stable of the two) over ox.thr. isomer. Under non-enzymic, *in-vitro* conditions ox.thr. was found to accumulate during the oxidation of ascorbate, emphasising the potential importance of the esterase that was proposed to catalyse the hydrolysis of ox.thr. to oxalate (Green and Fry 2005).

A method was developed in which solutions of DHA could be made to a high degree of purity. Oxidation of solutions of DHA showed that cyc.ox.thr., ox.thr. and threonate were oxidation products of DHA which supported the pathway described by Green and Fry (2005).

#### 4.1.2 The breakdown of DKG

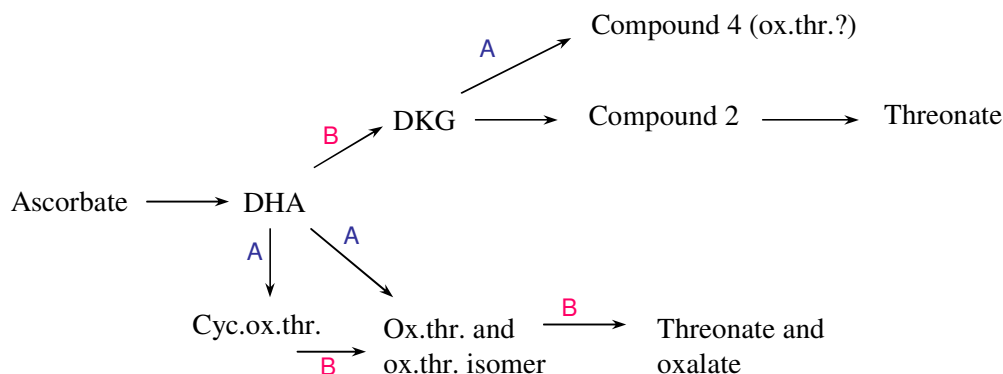
DKG is formed by the hydrolysis of DHA (Bánhegyi and Loewus 2004). Results in this study showed that the formation of DKG from ascorbate seemed to be inhibited by strongly oxidising conditions or by acidic ( $\approx$  pH 4.5) conditions. Threonate was found to be formed from DKG under oxidative and non-oxidative conditions. Threonate was formed via an unidentified intermediate referred to as compound 2. This was accompanied by the production of  $\text{CO}_2$ , suggesting that this intermediate compound was a C-5 compound or possibly a C-4 compound. Threonate as a product of DKG metabolism has been reported on several occasions (Banhegyi and Loewus 2004, Kagawa 1962, Kagawa et al. 1961) but no reference has been found to an interemediate compound between DKG and threonate. L-Erythrulose, L-threose, L-xylose, L-lyxose, L-xylonate and L-lyxonate have been reported as products of DKG metabolism or of DHA decarboxylation (Chan et al. 1957, Kagawa et al. 1961, Bánhegyi and Loewus 2004). 2-Keto-L-xylonate was considered a possible product of DKG decarboxylation (Green and Fry 2005). Based on electrophoretic mobilities compound 2 has been shown not to be any of these compounds but neither has its identity been ascertained.

NMR analysis on compounds 2 that had been eluted from paper electrophoretograms was attempted but it was not possible to interpret results as there was so much background noise from other compounds which had come from the paper. It was not possible to eliminate these contaminants on a biogel P-2 column so HPLC was used to prepare a solution of compound 2 in  $\text{H}_2\text{SO}_4$ , which was then precipitated with  $\text{BaCO}_3$ . An important next step will be mass spectrometry analysis of this compound.

Evidence was seen for the operation of two oxidative breakdown pathways of DKG; the one described above in which threonate seemed to be the end product and one in which a compound (compound 4) with very similar properties to ox.thr. seemed to be an end product. The production of compound 4 appeared to be favoured under oxidative conditions. The possible

production of ox.thr., threonate or compound 2 was not considered to be of relevance to DKG breakdown *in vivo* as discussed below.

Based on the findings of this study, the *in-vitro* ascorbate breakdown pathway can be represented as shown in figure 4.1:



**Figure 4.1 The proposed *in-vitro* breakdown pathways of ascorbate**

Where the type of reaction impacted in the products that were yielded, the reaction has been marked as either an oxidation (A) or a hydrolysis (B).

## 4.2 The *in-vivo* analysis of ascorbate breakdown

### 4.2.1 *In-vivo* analysis of individual pathway components

#### 4.2.1.1 Intracellular ascorbate breakdown occurs slowly

Results show that intracellular ascorbate is largely stable over time in rose cell suspension cultures; only small amounts of [ $^{14}\text{C}$ ]ox.thr. and [ $^{14}\text{C}$ ]oxalate were produced over the course of 8 h in the absence of any exogenous stress. Ascorbate could be expected to have been stable in the symplasm, as ascorbate is recycled intracellularly in the ascorbate-glutathione cycle (Foyer et al. 1994). A figure of 13 % h<sup>-1</sup> was put on ascorbate turnover in pea embryonic axes (Smirnoff 2000). Ascorbate turnover seemed to be slower than 13 % h<sup>-1</sup> in 10-day-old rose cell

cultures, as the majority as  $^{14}\text{C}$  was still present in the symplasm as [ $^{14}\text{C}$ ]ascorbate after 4 h in the absence of exogenous stress.

#### 4.2.1.2 DKG is not a major component of ascorbate breakdown in cell cultures or *in-planta*

Results from cell suspension cultures fed 25 mM DKG suggested that DKG was not broken down further in the culture medium. This was in contrast to results by Green and Fry (2005) where DKG was proposed to be broken down to two unidentified compounds, C and E, in the culture medium. Final concentrations of DKG were much higher (25 mM) compared to concentrations of [ $^{14}\text{C}$ ]ascorbate added to cell cultures (0.5 mM) by Green and Fry (2005) so a direct comparison of results is difficult. Relative to the concentrations of other compounds present in the symplasm of rose cells or in tobacco leaves, levels of [ $^{14}\text{C}$ ]DKG were low. Compounds C and E were not observed either. This implies that [ $^{14}\text{C}$ ]DKG, C and E were not important intracellular breakdown products of [ $^{14}\text{C}$ ]ascorbate in 10-day-old rose cell cultures or in tobacco leaves but their presence in the apoplast in low quantities has not been ruled out. This ambiguity could be resolved by the adaptation of the method outlining the synthesis of DKG (Kagawa 1962) for the synthesis of [ $^{14}\text{C}$ ]DKG. This would allow the fate of a range of concentrations of DKG to be followed in the culture medium as detection of [ $^{14}\text{C}$ ]DKG is much more sensitive than silver nitrate staining of non-radioactive DKG.

#### 4.2.1.3 Cyc.ox.thr. and ox.thr. in rose cell extracts and *in-planta*

Compared to [ $^{14}\text{C}$ ]ox.thr. and [ $^{14}\text{C}$ ]oxalate, low levels of [ $^{14}\text{C}$ ]cyc.ox.thr. were detected in tobacco leaf homogenates. Low levels of [ $^{14}\text{C}$ ]cyc.ox.thr. were detected when rose cells were challenged with 1 mM  $\text{H}_2\text{O}_2$ , otherwise [ $^{14}\text{C}$ ]cyc.ox.thr. was not detected in rose cell extracts. *In-vitro* results indicated that cyc.ox.thr. was not the obligated precursor to ox.thr. It has not been possible to distinguish definitely between a rapid rate of [ $^{14}\text{C}$ ]cyc.ox.thr. turnover as an intermediate or whether [ $^{14}\text{C}$ ]cyc.ox.thr. was not present in the symplasm of rose cells and *in*

*planta* in tobacco leaves. However, it seems possible from these results that cyc.ox.thr. did not act as a precursor to ox.thr. *in planta*.

In the absence of exogenous stress, [<sup>14</sup>C]ox.thr. was observed at low levels in rose cell extracts. It was also present in tobacco leaves. Whilst it is not certain that cyc.ox.thr. acts as a precursor to ox.thr., ox.thr. was frequently detected. Green and Fry (2005) first reported its existence and its role as an intermediate in apoplastic breakdown of ascorbate to oxalate. To this it can be added that ox.thr. appears to act as an intermediate in ascorbate breakdown *in planta* and in symplastic ascorbate breakdown in cultured rose cells.

#### 4.2.1.5 The role of the proposed esterase enzyme

Green and Fry (2005) proposed that the hydrolysis of the ester bond ox.thr. was catalysed by an esterase. This was emphasised in *in-vitro* experiments in the early parts of this study. When challenged with 1 mM H<sub>2</sub>O<sub>2</sub> or 1 μM and 10 μM MV<sup>2+</sup>, symplastic [<sup>14</sup>C]oxalate levels were greater than those of [<sup>14</sup>C]ox.thr. This supports the existence of the esterase enzyme in the symplasm. In response to this it could be argued that the intracellular environment might promote non-enzymic hydrolysis of ox.thr. However, this does not seem to be likely given the low levels of [<sup>14</sup>C]DKG that were formed from the hydrolysis of [<sup>14</sup>C]DHA.

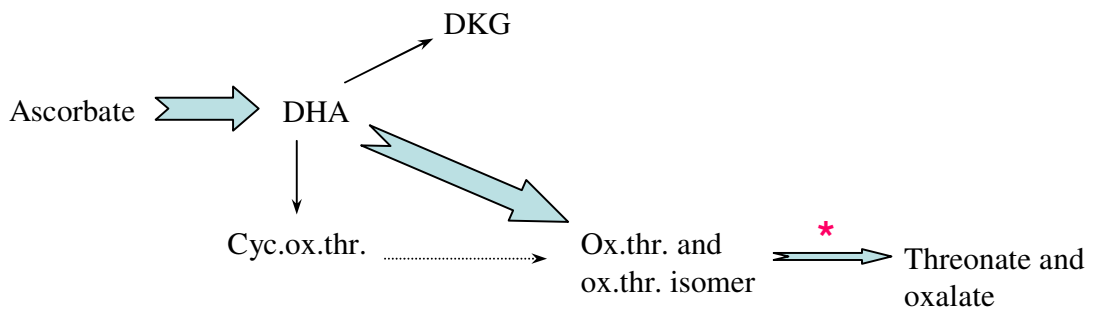
Although lower amounts of [<sup>14</sup>C]ox.thr. compared to [<sup>14</sup>C]oxalate were observed in response to 1 mM H<sub>2</sub>O<sub>2</sub>, the level of [<sup>14</sup>C]ox.thr. increased over time in response to MV<sup>2+</sup>. The observed accumulation of ox.thr. suggested that if symplastic hydrolysis of ox.thr. was determined by the esterase, its capacity was saturated by the level of oxidative stress induced by MV<sup>2+</sup> at 1 μM and 10 μM. In tobacco leaves (expt. 1 Fig. 3.72) more [<sup>14</sup>C]oxalate relative to [<sup>14</sup>C]ox.thr. was measured. This supports the existence of the esterase *in planta*. As it was not possible to separate symplastic and apoplastic moieties in experiments on tobacco leaves, no information could be gleaned on the relative importance of this enzyme in either compartment.

Both intracellular and cell wall located esterases exist in plants; pectin methyl esterases catalyse the demethylesterification of pectins and are known to be important in cell elongation and cell-cell adhesion (Bosch et al. 2005). There exist a range of intracellular esterases; the often-used fluorescein diacetate cell viability test depends on their activity (Steward et al. 1999). Possible roles of several intracellular esterases have been identified; methyl salicylate esterase and methyl jasmonate esterase are thought to be involved in the regulation of their respective substrates in defence signalling (Forouhar et al. 2005). It would be very interesting to see whether any of these esterases could catalyse the formation of threonate and oxalate from oxalyl threonate.

#### 4.2.1.6 Oxalate as an end product of the pathway

Green and Fry (2005) suggested that further metabolism of oxalate could occur in the apoplast, thereby acting as a source of  $H_2O_2$ . In this study [ $^{14}C$ ]Oxalate was seen to accumulate in cells which had not been challenged with an oxidative stress and in tobacco leaves. In response to oxidative stress, concentrations of [ $^{14}C$ ]oxalate increased in cell extracts and in the culture medium. In none of these cases was any sign of a decrease in [ $^{14}C$ ]oxalate detected. Even 1 mM  $H_2O_2$  was metabolised within minutes in the culture medium and so represented a pulse of oxidative stress, after which further [ $^{14}C$ ]oxalate metabolism would not have been masked by the continued oxidation of [ $^{14}C$ ]ascorbate. No decrease in [ $^{14}C$ ]oxalate was measured 4 h after the addition of 1 mM  $H_2O_2$  to cell cultures. Together, these results do not support the idea of further metabolism of oxalate.

In light of the above results and the *in-vitro* results the ascorbate breakdown pathway *in-planta* could be represented as shown in figure 4.2:



**Figure 4.2 A proposed pathway for ascorbate breakdown *in-planta***

The proposed pathway is based on ascorbate breakdown in rose cell culture medium as described by Green and Fry (2005). Arrow weight indicates relative importance of reaction, whilst a dotted arrow shows indicates uncertainty as to whether or not a reaction takes place. The esterase proposed by Green and Fry (2005) is shown by as asterisk.

#### 4.2.2 Oxalate accumulation and the role of oxalate oxidases

The accumulation of intra- and extracellular oxalate seen in this study would seem to imply that oxalate oxidases are either absent or inactive in rose cell suspension cultures and tobacco leaves. The levels of oxalate that had accumulated in tobacco leaves after several hours were relatively low but if these results are to be taken as an indication of normal ascorbate metabolism, then over the lifetime of a plant, considerable levels of oxalate would accumulate in leaves. Oxalate can be stored as calcium oxalate in plants and has been found in high levels in some plants (Nakata and McConn 2007). An important question following these observations would concern the relationship between ascorbate turnover and the accumulation of calcium oxalate. *Medicago truncata* mutants with elevated intracellular calcium oxalate levels showed a decrease in ascorbate content compared to controls, whilst exogenously supplied ascorbate raised the calcium oxalate content (Franceschi and Nakata 2007). It would be particularly interesting to see whether the apparent increase in flux through the ascorbate breakdown pathway in AO sense plants could be related to an increase in calcium oxalate content.

Germin like proteins have been found in many plant species. Members of this protein family located to the apoplast of monocots have been shown to have oxalate oxidase activity

(Lane et al. 1993) but oxalate oxidase activity has not been reported yet in dicots. Either tobacco plants in this study did not possess an oxalate oxidase or that under the experimental conditions it was not active. In rose cells [ $^{14}\text{C}$ ]oxalate levels remained elevated after addition of  $\text{MV}^{2+}$  or 1 mM  $\text{H}_2\text{O}_2$ . This does not support the existence of oxalate oxidase activity in rose cells nor does it support the possible upregulation of activity in response to  $\text{H}_2\text{O}_2$ .

#### **4.2.3 The role of AO in ascorbate breakdown and oxalate accumulation**

A moderate increase in accumulation of [ $^{14}\text{C}$ ]oxalate was observed in AO sense plants compared to WT plants in the present study, as well as a decrease in [ $^{14}\text{C}$ ]ascorbate content of AO sense leaves compared to WT leaves at later time points (expt. 1 Fig. 3.72). AO is a cell wall located enzyme (Pignocchi et al. 2003). This would seem to suggest that increasing the MDHA content and (assuming a constant rate of disproportionation to DHA) the DHA content of the apoplast, does affect the rate of ascorbate breakdown.

The apoplast is widely believed to be a more oxidising environment than the symplast (Pignocchi and Foyer 2003). When DHA is taken up by the cell it all seems to be reduced back to ascorbate (Potters et al. 2000). DHA is therefore more likely to have been oxidised in the apoplast than in the symplast; the increase in [ $^{14}\text{C}$ ]oxalate accumulation in the leaf homogenates was therefore most likely to be representative of the apoplast. The results in figure 3.76 are inconclusive but show that further study in this area could be worthwhile.

### **4.3 Ascorbate breakdown in response to oxidative stress**

#### **4.3.1 Accumulation of extracellular and intracellular [ $^{14}\text{C}$ ]ascorbate metabolites**

Intracellular ascorbate breakdown was examined in response to a short-lived oxidative stress,  $\text{H}_2\text{O}_2$ , and a more protracted oxidative stress generated by  $\text{MV}^{2+}$ . The intracellular redox state appeared to be sensitive to a mild, short-lived oxidative stress (0.1 mM  $\text{H}_2\text{O}_2$ ). A lesser response was also seen in control cells implying sensitivity to the addition of water to control



cells or to sampling; [ $^{14}\text{C}$ ]ascorbate levels decreased whilst [ $^{14}\text{C}$ ]DHA levels increased. It is conceivable that one or both of these processes was sufficient to induce a stress response which resulted in a change in the intracellular redox state. Despite the relatively short longevity of 0.1 mM  $\text{H}_2\text{O}_2$  in the medium, the redox state became more oxidised in response to 0.1 mM  $\text{H}_2\text{O}_2$ , implying indirect effects of  $\text{H}_2\text{O}_2$  on the ascorbate content of cells. This did not seem to affect the irreversible breakdown of [ $^{14}\text{C}$ ]ascorbate.

A protracted change in redox state of the symplasm as indicated by increasing DHA contents and decreasing ascorbate contents after a pulse of  $\text{H}_2\text{O}_2$  would seem to indicate a signalling response that was maintained after the initial  $\text{H}_2\text{O}_2$  stress had dissipated. Several authors point to the stability of the redox state of the cytosol and its ability to readjust quickly to non-oxidative external changes (Pignocchi and Foyer 2003, Potters et al. 2000). The results from these experiments suggest that the symplasm is less stable in response to low, transient concentrations of  $\text{H}_2\text{O}_2$ . The activity of APX and DHAR has been shown to decrease in older cell suspension cultures (de Pinto et al. 2000). As the cell cultures used in these experiments were 10 days old it is possible that recovery of the intracellular redox status in response to an external oxidative stress was not very efficient. Rapid uptake of ascorbate in younger cells has been reported by other groups (Potters et al. 2002). Further experimentation could include a repeat of these experiments in cells which take up [ $^{14}\text{C}$ ]ascorbate at an earlier age so that the possibility of inefficient regeneration of ascorbate would be minimised. It is also possible, though, that this was a controlled change in redox state in response to an  $\text{H}_2\text{O}_2$  signal.

In response to either a more severe oxidative stress (1 mM  $\text{H}_2\text{O}_2$ ) or a more protracted oxidative stress (1  $\mu\text{M}$  or 10  $\mu\text{M}$   $\text{MV}^{2+}$ ) irreversible [ $^{14}\text{C}$ ]ascorbate breakdown occurred. [ $^{14}\text{C}$ ]Oxalate accumulated rapidly in response to 1 mM  $\text{H}_2\text{O}_2$  and more progressively in response to  $\text{MV}^{2+}$ . One reason for the rapid  $\text{H}_2\text{O}_2$ -induced response could have been the uptake of  $\text{H}_2\text{O}_2$  into the cytosol via aquaporins (Neill et al. 2002). A pertinent question becomes: what, if uptake

occurred, was the final concentration of H<sub>2</sub>O<sub>2</sub> in the cell? Also, if H<sub>2</sub>O<sub>2</sub> is taken up from the apoplast under normal cellular conditions or if H<sub>2</sub>O<sub>2</sub> is concentrated at certain locations in the cell, does this result in formation of oxalate from ascorbate? Under conditions of more protracted generation of intracellular oxidative stress, e.g. combined high light and drought stress, is a threshold limit reached at which the ascorbate regeneration system can no longer prevent irreversible breakdown? In these sets of experiments the longer term responses of cells were not followed, however, one future study based on these results could be to see whether a shift in intracellular redox state preceded cell death, or whether dying cells, e.g. during the hypersensitive response, showed an increase in oxalate levels. It would also be interesting to record the fate of ascorbate and oxalate in cells of mutants known to be more resistant to e.g. MV<sup>2+</sup>.

Concentrations of H<sub>2</sub>O<sub>2</sub> *in vivo* are notoriously hard to measure accurately; variation in published concentrations has merited a paper dedicated to this topic alone (Queval et al. 2008). The concentrations of exogenous H<sub>2</sub>O<sub>2</sub> used in this experiment (0.1 mM and 1 mM) are thought to be within the bracket of expected extracellular concentrations in response to stress (Queval et al. 2008). This implies that formation of oxalate from ascorbate could occur in response to an oxidative burst e.g. from ozone exposure or pathogen attack. This could be an interesting basis for further study. If the irreversible breakdown of ascorbate occurs on a regular basis, this would mean the permanent removal of carbon from the ascorbate pool and so some coordination with ascorbate biosynthesis would seem likely. Plausibly, such a situation could arise through the metabolism of calcium oxalate, as Ca<sup>2+</sup> is an essential element in coordinating many cellular responses (Buchanan et al. 2000).

### 4.3.2 Transport of ascorbate and DHA across the plasma membrane

The export of ascorbate in response to oxidative stress has been previously studied with respect to ozone (Luwe and Heber 1995, Veljovic-Jovanovic *et al.* 2001, Padu *et al.* 2005). The study focussed on the export of radioactivity from cells loaded with [<sup>14</sup>C]ascorbate in response to either a H<sub>2</sub>O<sub>2</sub> stress or the stress encountered by disturbance from the exchange of cell culture medium. Radioactivity was exported in response to the exchange of medium. More radioactivity was exported in response to 1mM and it seemed that yet more was exported by cells immediately after treatment with 5 mM H<sub>2</sub>O<sub>2</sub>. Export in response to H<sub>2</sub>O<sub>2</sub> appeared to be characterised by an early increase in the rate of export between approximately 30 s and 120 s in rose cells. The above phenomena were also observed in *Arabidopsis* cell suspension cultures. In both rose and *Arabidopsis* cells, export of radioactivity was followed by rapid import; radioactivity appeared to be exported in pulses. The amplitude of pulses within the first 2 min increased in response to 1 mM or 5 mM H<sub>2</sub>O<sub>2</sub>. Pulsing was not always detected in rose cell cultures; no pulsing was observed after cell had recovered from a 6 week period where growth and division in this cell line appeared to be notably decreased. A sustained search for evidence of pulsed [<sup>14</sup>C]ascorbate export in a range of cell suspension cultures would be necessary to appreciate how transitory this particular response was. An increased rate of export was observed consistently in repeated experiments in response to 1 mM or 5mM H<sub>2</sub>O<sub>2</sub>. No increase compared to control cells was seen in response to 0.1 mM H<sub>2</sub>O<sub>2</sub>. When the exported compounds were identified, they were found to be predominantly [<sup>14</sup>C]ascorbate with some [<sup>14</sup>C]DHA. [<sup>14</sup>C]Ascorbate concentrations were more varied than [<sup>14</sup>C]DHA, leading to the speculation that radioactivity was exported as [<sup>14</sup>C]ascorbate in pulses and then taken up as [<sup>14</sup>C]DHA; the combination of oxidation of [<sup>14</sup>C]ascorbate to [<sup>14</sup>C]DHA and uptake of [<sup>14</sup>C]DHA could have resulted in the observed lower and more constant concentrations of [<sup>14</sup>C]DHA in the medium compared to [<sup>14</sup>C]ascorbate. This interpretation would be consistent with observations made by

Horemans et al. (1998a, b) and Potters et al. (2000) where DHA was taken up preferentially to ascorbate by a carrier with a high affinity for DHA and then re-reduced inside the cell.

The apoplast is thought to be the site of generation of the oxidative burst as NADPH oxidase, which catalyses the production of  $O_2^-$  is located on the outer side of the plasma membrane (Lamb and Dixon 1997). The apoplast is also where potentially damaging compounds, such as ozone, are first encountered. Based on the current understanding, ascorbate export to the apoplast is thought to be increased in response to an e.g. ozone stress so that the damaging effects of ROS can be negated (Noctor and Foyer 1998). This leads on the question of what might regulate the export of [ $^{14}C$ ]ascorbate and possible import of [ $^{14}C$ ]DHA that was observed in the present study. The existence of pulses of [ $^{14}C$ ]ascorbate could possibly implicate the involvement of  $Ca^{2+}$ . The case for  $Ca^{2+}$  regulation of ascorbate export and DHA import is discussed below. It has been proposed that changes in the redox state of the apoplast could instigate important changes in receptors at the plasma membrane. This way signals regarding the extracellular environment could be transmitted across the plasma membrane (Foyer and Noctor 2005). As discussed, the intracellular redox state of cells was sensitive to apparently minor changes in the external environment, such as the addition water to control cultures. As these responses were most evident within the first 2 min of a stress being administered, it is possible that subtle, rapid responses in ascorbate transport and/or redox state are easily overlooked.

It is interesting to note that addition of organic acids seemed to induce an increase in the rate of [ $^{14}C$ ]ascorbate export. The pH of the apoplast has been found to be highly regulated, at least in part by the plasma membrane bound H<sup>+</sup>-ATPase (Savchenko et al. 2000). If highly regulated, changes in apoplastic (or in this case, the culture medium) pH could be a stress signal. Alkalisiation of the apoplast is associated with pathogen attack and  $H_2O_2$  production (Bolwell et al. 1995). It is perhaps not surprising then that addition of 100  $\mu$ M organic acids should result in

increased export of [ $^{14}\text{C}$ ]ascorbate. It would be interesting to see whether an equivalent export could be induced by alkalisation of the medium.

Discussion of the possible mechanisms by which increased ascorbate export in the form of pulses might be regulated still circumvents a central question: why, as does seem to be the case, is ascorbate exported in pulses? If this process is primarily  $\text{Ca}^{2+}$ -regulated, then pulses in activation of ascorbate or DHA membrane transporters could simply reflect oscillations in intracellular or extracellular  $\text{Ca}^{2+}$  concentration. A relationship between relative concentrations of intracellular ascorbate and DHA is known to be closely linked to progression through the cell cycle (Arrigoni 1994, Kato and Esaka 1999). Potters et al. (2000) found that exogenous DHA could delay progression through the cell cycle for the amount of time required for uptake of this DHA from the medium. This lead to the hypothesis that during this time the cell might be receiving signals pertaining to the state of the extracellular environment based on the redox state of extracellular ascorbate before committing to the next stage of the cell cycle. Therefore an alternative answer to the above question could be that the amount of DHA imported after a rapid burst of ascorbate export represents a similar mechanism by which the cell might deduce the state of the extracellular environment without exporting too large a proportion of the total ascorbate. In this study, maximum pulses size was not known. An important next step if these experiments were continued would be to determine absolute pulse maxima and relate this to the total ascorbate content. The total ascorbate content could be easily determined by scintillation counting of a certain volume of cells after a 5 h incubation with [ $^{14}\text{C}$ ]ascorbate. Maximum pulse size would be difficult to know without a continuous sampling system though if enough data could be gathered from sufficient repeats of the methods employed in this study, e.g. with the use of multi-pipettors, it might be possible to infer maximum peak height reasonably accurately.

Results were analysed carefully with respect to possible membrane disruption in the presence of 1 mM and 5 mM  $\text{H}_2\text{O}_2$ .  $^{14}\text{C}$  export in [ $^{14}\text{C}$ ]ascorbate loaded cells was not thought to

be a result of increased membrane permeabilisation from H<sub>2</sub>O<sub>2</sub>, although the extent of structural damage was not measured directly. Increased export of <sup>14</sup>C in [<sup>14</sup>C]ascorbate loaded cells and export in pulses was seen in control cells not receiving H<sub>2</sub>O<sub>2</sub>. <sup>14</sup>C was exported slower and the amplitude of pulses appeared to be lower compared to H<sub>2</sub>O<sub>2</sub>-stressed cells. No uptake of an extracellular [<sup>3</sup>H]glucitol marker was seen in rose cells challenged with 1 mM H<sub>2</sub>O<sub>2</sub>. These are considered to be important pieces of evidence against the observed export in response to H<sub>2</sub>O<sub>2</sub> being solely a result of H<sub>2</sub>O<sub>2</sub>-induced membrane disruption. [<sup>3</sup>H]Glucitol seemed to be taken up by *Arabidopsis* cells (Fig. 3.69), as was [<sup>3</sup>H]isoprimeveritol (results not shown). A useful further experiment would involve the use of an extracellular <sup>3</sup>H-marker which was not taken up by *Arabidopsis* cells. [<sup>3</sup>H]Mannitol could be a candidate for such as marker. An assessment of cell viability after <sup>14</sup>C export experiments would also be useful such as the Evan's blue cell viability test (Horemans et al 2007).

#### **4.3.3 Could ascorbate export pulses be Ca<sup>2+</sup> regulated?**

Ca<sup>2+</sup> is known to show oscillations in plants cells, both from an influx of external Ca<sup>2+</sup> and from the release of intracellular stored (Buchanan et al. 2000). The release of Ca<sup>2+</sup> from intracellular stores has been shown to be a prerequisite for an elicitor induced oxidative burst and H<sub>2</sub>O<sub>2</sub> accumulation in tobacco cells (Cessna and Low 2001). NADPH oxidase, which generates superoxide, is known to be Ca<sup>2+</sup> regulated (Neill et al. 2002). The uptake of DHA may well be linked to Ca<sup>2+</sup> channel activity as DHA uptake can be inhibited by Cd<sup>2+</sup> and La<sup>3+</sup> (Horemans et al. 2007). La<sup>3+</sup> is an inhibitor of Ca<sup>2+</sup> channels and Cd<sup>2+</sup> is thought to compete with Ca<sup>2+</sup> for receptor binding sites. Results from Horemans et al. (2007) seem to suggest that if Ca<sup>2+</sup> is involved with transport of DHA across the plasma membrane this would depend on the uptake of external Ca<sup>2+</sup> sources. Whether or not there exists a link between Ca<sup>2+</sup> oscillations and the [<sup>14</sup>C]ascorbate/ [<sup>14</sup>C]DHA pulsing phenomenon observed in this study is not clear although it

seems likely given the above observations. The elicited release of internal  $\text{Ca}^{2+}$  stores seemed to peak at around 2 min in tobacco cells, whilst in animal cells, the frequency and amplitude of  $\text{Ca}^{2+}$  pulses is thought to help specify the appropriate response (Cessna and Low 2001). In plants, a plasma-membrane-located receptor for extracellular  $\text{Ca}^{2+}$  has been shown to affect the amplitude of intracellular  $\text{Ca}^{2+}$  pulses (Tang et al. 2007). In this study an increased amplitude of [ $^{14}\text{C}$ ]ascorbate/[ $^{14}\text{C}$ ]DHA pulses was seen in response to exogenous  $\text{H}_2\text{O}_2$ . A relationship between  $\text{Ca}^{2+}$  and ascorbate/ DHA membrane transport could be investigated by measuring the rates of [ $^{14}\text{C}$ ]ascorbate/ [ $^{14}\text{C}$ ]DHA export in response to  $\text{H}_2\text{O}_2$  in the presence of  $\text{Ca}^{2+}$  chelating agents and inhibitors of internal  $\text{Ca}^{2+}$  unloading. It would be very interesting to study ascorbate transport in response to the addition of  $\text{Ca}^{2+}$  to the medium but care would be needed interpreting results as it is known that the addition of  $\text{Ca}^{2+}$  can increase the size of CaOxalate crystals in plant cells through increased formation of oxalate (Franceschi and Nakata 2005), presumably through increased ascorbate breakdown.

These results also imply that cells were showing synchrony in their response to  $\text{H}_2\text{O}_2$ , particularly so when pulsing was observed. Oscillations in external  $\text{Ca}^{2+}$  concentrations have been shown to influence oscillations in intracellular  $\text{Ca}^{2+}$  concentrations (Berridge et al. 2003, Tang et al. 2007) whilst diurnal, circadian  $\text{Ca}^{2+}$  fluxes operating at the whole tissue level in plants are thought to synchronise oscillations in intracellular  $\text{Ca}^{2+}$  (Tang et al. 2007). Work exploring the relationship between single cell and cell culture responses has shown a good level of correlation between the two levels when  $\text{Ca}^{2+}$  pulses increased to most (but not all) types of stress tested (Cessna et al. 2001). Whilst much of this evidence is highly circumstantial with regards to the possibility of ascorbate oscillations, it does show that the exploration of a link between ascorbate transport and  $\text{Ca}^{2+}$  has the potential to be an interesting area of future research.

#### 4.4. Summary

This thesis has improved understanding of the ascorbate breakdown pathway *in vitro*, in cell suspension cultures and *in planta*. Findings *in vitro* can be largely corroborated with those in cell suspension cultures and *in planta*. The overall objective was to study the response of the ascorbate breakdown pathway, as described by Green and Fry (2005) to external stimuli and to examine transport of the metabolites described in this pathway between the apoplast and the symplast. It has been shown that the intracellular ascorbate redox state may be sensitive to mild oxidative stress and that a more severe or prolonged exposure to oxidative stress may result in intracellular irreversible ascorbate breakdown. Several experiments concerning whether a shift in redox state or increased oxalate accumulation were characteristic of cells programmed to die have been outlined. The activity of the cell-wall-located enzyme ascorbate oxidase may well influence the amount of ascorbate that is broken down in the apoplast and the extent to which oxalate accumulates there. It is also possible that the proposed esterase enzyme (Green and Fry 2005) may affect the amounts of oxalate that accumulates in the apoplast. Transport across the plasma membrane was thought to be confined to ascorbate and DHA. Export of radioactivity from [<sup>14</sup>C]ascorbate loaded cells increased almost instantly in response to H<sub>2</sub>O<sub>2</sub> and was maintained for up to 2 min. Radioactivity was thought to be transported across the plasma membrane as [<sup>14</sup>C]ascorbate and/or [<sup>14</sup>C]DHA. Radioactivity was often observed to be exported and imported in pulses in both rose and *Arabidopsis* cells. The amplitude of these pulses appeared to increase in response to H<sub>2</sub>O<sub>2</sub>. Several interesting areas for further study, such as a link between ascorbate transport and Ca<sup>2+</sup> and the possibility of oxalate accumulation in response to stress, have been highlighted by these results.



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