Apoplastic Ascorbate Metabolism in Rose Cell Suspension Cultures

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This thesis has been composed by myself and the work, of which it 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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Martha Alexandra Green

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In the rest of this short section I will endeavour to thank all the people who have supported me throughout the course of this degree. This, I hasten to add, will be done in no particular order.

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To Mum and Dad,

With my love.

Abstract

L-Ascorbate is reported to be present in plant cell walls and is thought to inhibit oxidative coupling and lignification in the wall. It may also promote wall expansion. Ascorbate oxidase is active in growing cell walls and results in the formation of monodehydroascorbate radicals. The regeneration of ascorbate from monodehydroascorbate radicals results in electron flow and proton extrusion into the apoplast. This could lead to increased wall expansion according to the acid growth hypothesis. Ascorbate non-enzymically reduces O_2 and Cu^{2+} to produce H_2O_2 and Cu^+, which can participate in a Fenton reaction to produce hydroxyl radicals. Thus, ascorbate can act as a pro-oxidant and generate hydroxyl radicals, which may mediate wall polysaccharide scission. Other roles of apoplastic ascorbate include defence against the harmful effects of atmospheric oxidants such as ozone and SO_2. Despite its importance in the apoplast, little is known about apoplastic ascorbate metabolism.

Intraprotoplasmic ascorbate is a precursor for synthesis of oxalate and other organic acids such as tartrate. However, the metabolism of apoplastic ascorbate to products other than monodehydroascorbate radicals and dehydroascorbate has not been described. It would be interesting to identify metabolic fates of apoplastic ascorbate and to compare these with the fate of intraprotoplasmic ascorbate.

Endogenous intraprotoplasmic ascorbate in rose cell suspension cultures as a model system ranged from 0.05 mmol kg^{-1} in 0-d-old cultures to 1.1 mmol kg^{-1} in 5-d-old cultures. Apoplastic ascorbate was estimated as 0.5 and 8 μM in 0- and 5-d-old cultures respectively, indicating that ascorbate is endogenous to, and may be metabolised within, the apoplast.

Exogenous (apoplastic) 1 mM L-[1-^{14}C]ascorbate was almost completely consumed (metabolised and/or taken up) by rose cultures within 8 hours of administration. Total ^{14}C was removed from medium but slower than ascorbate. The calculated concentration of metabolites of ascorbate showed that metabolites were formed in the medium and then removed from the medium in 5-d-old cultures. Removal of metabolites could be due to either uptake by or binding to cells.

The nature of the metabolites of 0.5 mM [1-^{14}C]ascorbate was examined in 5-d-old rose culture and spent medium by electrophoresis at pH 6.5. Ascorbate was metabolised both enzymically in spent medium and non-enzymically in boiled spent medium. Three ^{14}C-metabolites were identified as dehydroascorbate, diketogulonate and oxalate. Other acidic ^{14}C-metabolites (C, D, E and F) have not as yet been identified. F is highly mobile during electrophoresis at pH 2.0, showing that it has a low pK. C, D and E are also mobile at pH 2.0 but less so than F. E and C are interconvertible non-enzymically during storage and can E be regenerated by treatment with NaOH, suggesting that C is a lactone of E. ^{14}C-F was converted to [^{14}C]oxalate by whole culture and by spent medium but not by boiled spent medium, indicating an enzyme-catalysed reaction. The enzyme was partially inhibited by 100 mM azide but not by antioxidants. [^{14}C]Oxalate was produced from ^{14}C-F by alkali hydrolysis indicating the presence of an oxalyl ester group. The metabolism of apoplastic ascorbate, described in this thesis, is very different from its intraprotoplasmic metabolism. Threarate and threonate, metabolites of intraprotoplasmic ascorbate were not detected. I have identified novel metabolites and propose a novel pathway for the metabolism of apoplastic ascorbate.

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1 Introduction

1.1 Introduction to ascorbate

1.1.1 History

Sailors on the long sea voyages that became more common during the middle ages were plagued by a mysterious, incredibly painful and ultimately fatal disease. This illness would flare up for no apparent reason after a few months at sea. The consequences of this disease—called scurvy—were devastating. It was not uncommon to lose half the crew or more to the ravages of scurvy on long voyages (Davies et al. 1991, Carpenter 1986). Scurvy existed throughout history but its effects were obvious amongst seafaring adventurers. It was perceived to be a disease of only a narrow section of the population and thought not to affect the rich and famous, although there is evidence to suggest its appearance, especially during winter, amongst the upper echelons of society including Henry VIII and James I (Davies et al. 1991, Carpenter 1986).

Over the centuries it was suggested that the consumption of various fruit juices and herbs was effective for prevention or treatment of the disease. James Lind tested this suggestion while surgeon on H.M.S. Salisbury between 1746 and 1747 (Stewart and Guthrie 1953, Carpenter 1986). He found oranges and lemons to be an effective treatment for the disease but he remained sceptical of the preventative role of fresh fruit and vegetables (Carpenter 1986, Stewart and Guthrie 1953). The British Navy was eventually persuaded to carry lemon juice and, from 1795, scurvy was no longer a problem (Carpenter 1986). It was not until the middle of the 19th century that scurvy was recognised as a deficiency disease (Carpenter 1986)—there

was some substance which, if absent from the diet, resulted in scurvy (Harden and Zilva 1918).

The role of substance(s) present in fresh fruit and vegetables in prevention of scurvy was examined further during the early part of the 20th century. In 1907 Holst and Frölich published their results of an investigation into the cause of scurvy in Guinea Pigs. Further research carried out at the Lister Institute during the First World War was able to suggest the effectiveness of milk and fruit juice in prevention of scurvy (Chick et al. 1918a, b, Chick and Rhodes 1918). In 1919 the antiscorbutic factor was described as water soluble C (Drummond 1919) and quickly became known as vitamin C.

1.1.2 Discovery of ascorbate

In the late 1920's Albert Szent-Györgyi extracted a 'reducing factor' from adrenal cortex, orange juice and cabbage water (Szent-Györgyi 1928). This reducing agent was believed by Szent-Györgyi to be a new hormone with the nature of a sugar. It had the molecular formula $C_6H_8O_6$ and was named 'hexuronic acid.' In 1932, amid some controversy, vitamin C and 'hexuronic acid' were suggested to be identical (Waugh and King 1932, Svirbely and Szent-Györgyi 1932, Szent-Györgyi and Haworth 1933). In 1933 Szent-Györgyi extracted enough 'hexuronic acid' from Hungarian paprika to allow elucidation of its structure and properties (Herbert et al. 1933, Birch et al. 1933, Hirst et al. 1933). The structure suggested by Haworth et al. (1934) was confirmed by the development of various synthetic routes to production (Haworth et al. 1934) and hexuronic acid was renamed ascorbic acid.

The term vitamin C refers to a mixture of two compounds—ascorbic acid and dehyroascorbic acid. The antiscorbutic properties of vitamin C are conferred by ascorbic acid. Dehydroascorbate does have some antiscorbutic activity but it is less effective in prevention of scurvy than ascorbic acid. Ascorbic acid is chemically the simplest of all the vitamins and was among the first to be isolated, purified and characterised. The trivial name ascorbic acid refers to *threo*-hex-2-enono-1,4-lactone (Loewus 1980). Ascorbic acid has a chemical structure comprising a five-member lactone ring with a carbonyl group at C1 and an ene-diol group at C2 and C3. The ene-diol group provides ascorbic acid with complex redox chemistry. The acidic properties of ascorbic acid are conferred by the ionisation of the enolic OH at C3 and C2 with pK values of pH 4.25 and 11.8 respectively (Fig. 1). Ascorbic acid is a highly reactive reducing agent, although it has the relatively low reduction potential of +282 mV (Buettner 1993, Buettner and Jurkiewicz 1996).

Ascorbate is readily oxidised by O_2 in the presence of trace amounts of catalytic metal ions (Buettner and Jurkiewicz 1996) or the enzyme ascorbate oxidase, and by H_2O_2 in the presence of ascorbate peroxidase (Loewus 1980, 1988). Oxidation of ascorbate by O_2 occurs by two sequential one-electron oxidation reactions (Fig. 2). Loss of the first electron results in the formation of the monodehydroascorbate radical (MDHA) (Bielski and Richter 1975). The MDHA disproportionates forming ascorbate and dehydroascorbate. Oxidation of ascorbate is readily reversible. MDHA is reduced to ascorbate by the action of the enzyme MDHA reductase and NAD(P)H or reduced ferredoxin. Arrigoni et al. (1981) examined the presence of MDHA reductase and dehydroascorbate reductase in

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tissue homogenates obtained from onion roots, pea seedlings broad beans and many other plants. They suggested that MDHA reductase is ubiquitious in plants and is found in several compartments of the plant cells while dehydroascorbate reductase is only found in some plant species and in some subcellular compartments. MDHA reductase has been purified from both the cytosol (Borraccino et al. 1986) and the mitochondria of potato tubers (De Leonardis et al. 1995), and from spinach leaf chloroplasts (Hossain et al. 1984b). Dehydroascorbate, formed by disproportionation of MDHA, is reduced to ascorbate by the action of dehydroascorbate reductase. This enzyme is not found in all plants but has been extracted from spinach leaves (Hossain and Asada 1984a) and potato tubers (Arrigoni et al. 1981, Dipierro and Borraccino 1991, Foyer and Halliwell 1977). Dehydroascorbate reductase was found in only the cytosol of potato tubers (Arrigoni et al. 1981, Dipierro and Borraccino 1991) but has been detected in the stroma of spinach leaf chloroplasts (Miyake and Asada 1992). Dehydroascorbate reductase uses glutathione (GSH) as a reductant and the glutathione disulphide (GSSG) that is formed is re-reduced by the action of glutathione reductase and NADPH. This forms part of the ascorbate–glutathione cycle (Asada 1999, Noctor and Foyer 1998, Foyer and Halliwell 1976). MDHA reductase is ubiquitous in plant cells while dehydroascorbate reductase has only been detected in the cytosol and chloroplasts (Arrigoni et al. 1981, Miyake and Asada 1992).

At neutral pH the lactone ring of dehydroascorbate is hydrolysed resulting in the formation of diketogulonate (Fig. 3) at a rate of approximately 0.5%/min (Bode et al 1990). Below pH 5 dehydroascorbate is more stable and hydrolysis of the lactone ring occurs more slowly, with approximately 3% hydrolysis of

dehydroascorbate in 4 hours (Bode et al. 1990). This reaction is generally accepted to be irreversible *in vivo* but chemically reversible *in vitro* by treatment with hydroiodic acid (Penny and Zilva 1945, Deutsch 1998).
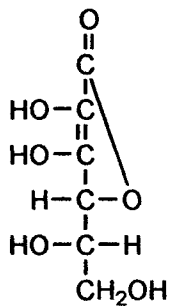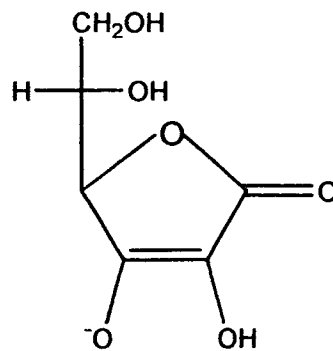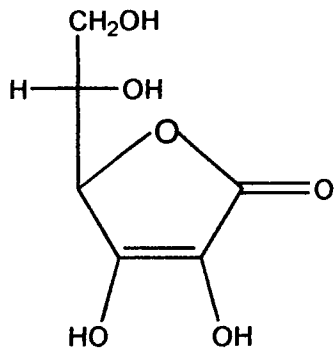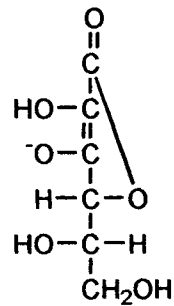
Figure 1. Structure of ascorbic acid

Ascorbic acid has a pK of 4.25 at C_3 and therefore is at least 50% ionised above pH 4.25.
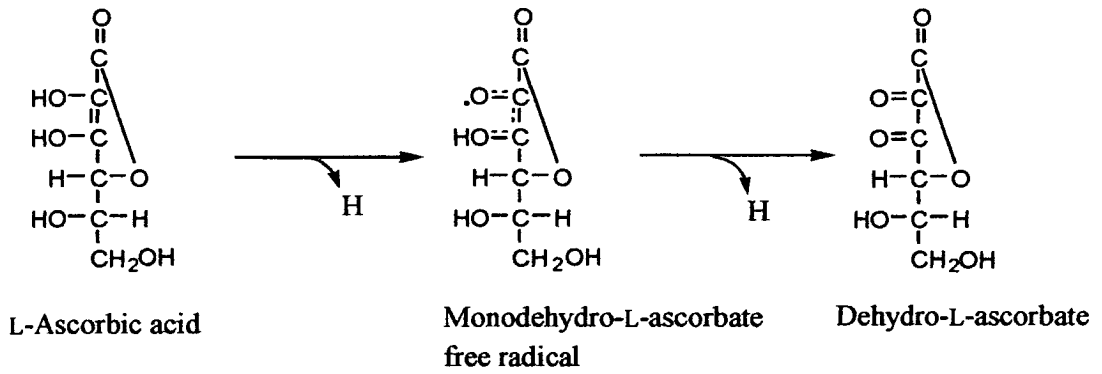
Figure 2. Oxidation of ascorbate
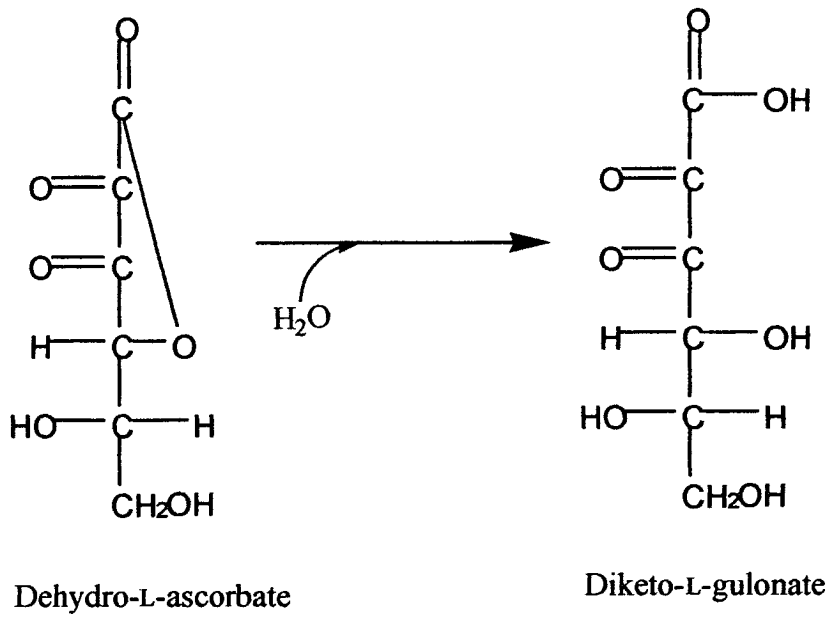
Figure 3. Hydrolysis of dehydroascorbate

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1.2 Ascorbate in mammals

1.2.1 Roles of ascorbate in mammals

Ascorbate (and/or dehydroascorbate) is an essential component of the human diet. Most animals are able to synthesise their own vitamin C but a few such as Guinea pigs, some fish, birds and invertebrates and primates—including humans— are unable to synthesise vitamin C. These animals rely on their diet as their sole source of vitamin C (Chatterjee 1973).

Ascorbic acid is required for post-translational hydroxylation of proline and lysine to allow the quarternary structure of collagen to form correctly (Peterkofsky 1991). Collagen formed in the absence of ascorbic acid is unable to form proper fibres and the symptoms of scurvy result. In addition to this, ascorbic acid is required for the formation of some hormones including nor-adrenaline and serotonin (Davies et al. 1991, Diliberto et al. 1991, Englard and Seifter 1986).

Ascorbic acid can function as a scavenger of reactive oxygen species such as superoxide and hydroxyl radicals (Niki 1991). These are capable of inflicting oxidative damage on biological membranes, enzymes and genetic material. Ascorbic acid protects aqueous parts of the cell while vitamin E and essential fatty acids protect the cell membrane (Davies et al. 1991, Frei 1991).

1.2.2 Metabolism and degradation of ascorbate in animals

A common feature of ascorbate metabolism is the reversible oxidation of ascorbate and formation of dehydroascorbate and MDHA radicals. In animals dehydroascorbate is reduced to ascorbate by glutathione (GSH) and other enyzmic systems. Metabolism of ascorbate proceeds *via* dehydroascorbate which is non-

enzymically hydrolysed to diketogulonate. Diketogulonate *is* decarboxylated releasing CO_2 and a 5-carbon compound or it is cleaved between carbons 2 and 3 to form oxalate and threonate. Diketogulonate has been detected as a urinary metabolite of ascorbate while almost half of urinary oxalate is derived from ascorbate (Brown and Jones 1996). This section aimed to provide and overview of ascorbate metabolism and degradation in animals and has not discussed the differences in metabolism of ascorbate by different species. Some of the metabolites of ascorbate described in animals are also formed in some plant species although other metabolites of ascorbate in plants, such as tartrate, have not been described in animals.

1.3 Roles of ascorbate in plants

Ascorbate has biochemical functions as an antioxidant (Bodannes and Chan 1979, Nishikimi 1975) and enzyme co-factor (Davies et al. 1991, Carpita and Gibeaut 1993), in electron transport (Miyake and Asada 1992, Asard et al. 1993) and as a precursor for tartrate and oxalate. In these capacities ascorbate has a multitude of roles in plants (Smirnoff 1996) from photosynthesis to cell wall metabolism and defence against oxidative stress.

1.3.1 Photosynthesis

Ascorbate is found in high concentrations in chloroplasts, 10 mM in those of pea leaves (Foyer and Lelandais 1996) or 30–40% of the total ascorbate in spinach leaf protoplasts (Foyer et al., 1983). Ascorbate in chloroplasts protects photosynthetic pigments from the potentially harmful effects of light and thus maintains the photosynthetic capacity of the chloroplast. Ascorbate has several roles in

photoprotection. These include the removal of hydrogen peroxide and formation of the photoprotectant zeaxanthin.

Superoxide radicals are produced in illuminated chloroplasts, especially when carbon fixation is limited. Superoxide is disproportionated to hydrogen peroxide and O_2 by the action of superoxide dismutases in the thylakoid membranes and lumen (Hayakawa et al. 1984) and non-enzymically (Gross 1976). Hydrogen peroxide is inhibitory to photosynthetic carbon fixation (Kaiser 1976) and must be removed. Chloropasts lack catalase (Tolbert et al. 1968, Frederich and Newcomb 1969)—the enzyme usually responsible for removal of hydrogen peroxide. Instead, intact chloroplasts reduce hydrogen peroxide in the stroma (Nakano and Asada 1980) in a reaction involving ascorbate peroxidase and ascorbate as the electron donor (Nakano and Asada 1981, Jablonski and Anderson 1982). The ascorbate peroxidase-catalysed removal of hydrogen peroxide results in the formation of monodehydroascorbate radicals and dehydroascorbate. Ascorbate is regenerated by the action of MDHA reductase and dehydroascorbate reductase. The electron donors required for MDHA and dehydroascorbate reductase catalysed regeneration of ascorbate are derived from photosystem I of photosynthesis. In conditions of high light intensity and when carbon fixation is limited, O_2 in the thylakoids of spinach leaf chloroplasts is reduced to water *via* superoxide and H_2O_2, mediated by ascorbate (Miyake and Asada 1992). This allows dissipation of light energy without carbon assimilation. Monodehydroascorbate radicals can be photoreduced to ascorbate without the participation of MDHA reductase. Monodehydroascorbate radicals accept electrons from electron carriers in photosystem I or the cytochrome *b/f* complex (Miyake and Asada 1992).

The xanthophyll cycle is an important photoprotective system in plants required for the dissipation of excess excitation energy. Xanthophylls are located in thylakoid membranes and undergo light-dependent reactions. Excess excitation energy within the light-harvesting complexes is dissipated as heat by the conversion of violaxanthin to zeaxanthin. Violaxanthin de-epoxidase catalyses the de-epoxidation of violaxanthin to form zeaxanthin. The enzyme is activated in excess light by acidifiction of the thylakoid lumen as a result of rapid photosynthetic electron transport. Excess acidification of the thylakoid lumen occurs as a result of the reduction of O_2 to H_2O_2 by photosystem I. The reduction of O_2 to H_2O_2 coupled with the removal of H_2O_2 by ascorbate peroxidase is referred to as the Mehler peroxidase reaction (Neubauer and Yamamoto 1979, 1992). Below pH 6.6, violaxanthin de-epoxidase is associated with the thylakoid membrane and capable of catalysing the de-epoxidation of violaxanthin to zeaxanthin providing ascorbate is present. Thus, ascorbate provides protection against the harmful effects of excess light. Ascorbic acid, predominant over the ascorbate anion at low pH, is the preferred substrate for violaxanthin de-epoxidase (Eskling 1997, Niyogi 1999).

Ascorbate in chloroplasts functions to protect the photosynthetic pigments from damage when exposed to high light intensity and prevents the inhibition of carbon fixation. The presence of ascorbate maintains the photosynthetic capacity of chloroplasts, allowing photosynthesis to continue to function in conditions which would otherwise be inhibitory to carbon fixation or result in destruction of photosynthetic pigments.

1.3.2 Defence against stress

Reactive oxygen species, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$), are formed as part of many metabolic processes. They are also formed in response to pathogens and as a result of stresses, both natural, such as low temperature or mineral deficiencies, and man-made, such as herbicides and pollutants (Foyer and Lelandais 1994).

Ascorbate is present in the apoplast—the extraprotoplasmic component of plant tissues—and has an important role there in protection against the harmful effects of reactive oxygen species. Anti-oxidant systems function to remove reactive oxygen species and other free radicals. This system comprises anti-oxidant enzymes including dehydroascorbate reductase and MDHA reductase and low molecular weight compounds such as ascorbate and glutathione which function as electron donors.

Ozone (O_3) is one of the most prevalent and damaging photochemically produced air pollutants. In some heavily polluted areas the powerful oxidising properties of ozone and the oxidative damage it causes are responsible for reductions in crop yields and damage to forest environments.

Ozone enters the leaves through open stomata (Kerstiens and Lendzian 1989) and dissolves in the apoplastic fluid of mesophyll cells. In aqueous solution ozone decomposes and forms the hydroxyl radical ($^{\cdot}OH$). This is enhanced by the presence of phenolics such as ferulic acid— a component of the cell wall— caffeic acid, and cinnamic acid (Grimes et al. 1983). Damage inflicted by ozone can cause major alterations to membrane function (Pauls and Thompson 1981) and can have

detrimental effects on components of the cytosol (Heath 1980). Ozone must be detoxified in the apoplast to minimise damage to the plant.

Ascorbate is a highly reactive anti-oxidant present in the apoplast (Sánchez et al. 1997, Hove et al. 2001, Luwe and Heber 1995, Luwe et al. 1993). It reacts rapidly with ozone and the potentially toxic degradation products of ozone to remove them from the apoplast before any damage is inflicted on the cell membrane. Ascorbate is important in determining resistance to ozone (Zheng et al. 2000, Lyons et al. 1999) and is a scavenger of ozone (Luwe et al. 1993). Plants with increased leaf total ascorbate concentrations appear to have increased resistance to ozone (Maddison et al. 2002). An ascorbate deficient *Arabidopsis* mtuant (*soz1*) only accumulates 30% of the normal ascorbate concentration. This mutant shows increased sensitivity to ozone, sulphur dioxide and ultraviolet B irradiation indicating that ascorbate is involved in defence against air pollutants and environmental stresses (Conklin et al. 1996).

Sulphur dioxide, produced by burning of fossil fuels, enters the leaves of plants through open stomata and dissolves in apoplastic fluid forming sulphurous acid (H_2SO_3). This is neutralised forming sulphite and bisulphite (Takahama et al. 1992). The presence of sulphite (SO_3^{2-}) and/or bisulphite (HSO_3^-) leads to increased formation of superoxide (Asada and Kiso 1973, Asada et al. 1974). SO_3^{2-} and/or HSO_3^- are oxidised to sulphate (SO_4^{2-}) by apoplastic peroxidases (Takahama et al. 1992). Oxidation of SO_4^{2-} and/or HSO_3^- also results in the formation of sulphite radicals. In the absence of ascorbate, sulphite radicals are capable of initiating uncontrolled radical reactions (Takahama et al. 1992). The cytotoxic effects of SO_2 are caused by the formation of superoxide and sulphite radicals. The removal of

these compounds by apoplastic peroxidases which use ascorbate as a co-factor (Takahama et al. 1992), and by superoxide dismutases which convert superoxide to hydrogen peroxide and O_2 (Tanaka and Sugahara 1980) minimises cytotoxic effects.

1.3.3 Transport and regeneration of ascorbate

The oxidation of apoplastic ascorbate to the monodehydroascorbate radical and/or dehydroascorbate provides a first line of defence against the harmful effects of reactive oxygen species and other free radicals. Apoplastic ascorbate must be regenerated to maintain the apoplastic ascorbate concentration and the redox status of the apoplast.

Enzymes such as dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase have not been detected in the apoplast as represented by the extraprotoplasmic washing fluid (Luwe 1996, Luwe and Heber 1995, Polle et al. 1990). The absence of these enzymes in solution in the apoplast indicates that an alternative mechanism for the reduction of dehydroascorbate and regeneration of ascorbate exists. These enzymes could be bound to the cell wall or cell membrane and therefore would be undetectable in the extraprotoplasmic washing fluid. If enzymes such as dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase were bound to the cell wall or cell membrane they could act to regenerate apoplastic ascorbate.

The regeneration of apoplastic ascorbate may occur as a result of carrier mediated diffusion (Horemans 1996) of dehydroascorbate (Horemans et al. 1997, Rautenkrantz 1994) into the cytosol in exchange for cytosolic ascorbate (Horemans et al. 1998). Diffusion of ascorbate into the apoplast results in the regeneration of

apoplastic ascorbate. Once inside the cytosol, dehydroascorbate is reduced to ascorbate by the ascorbate–glutathione cycle as described in section 1.1.3.

Reduction of apoplastic monodehydroascorbate radicals to ascorbate occurs as a result of electron transfer from intraprotoplasmic ascorbate to apoplastic monodehydroascorbate radicals *via* a transmembrane *b*-type cytochrome (Horemans et al. 1994, Asard 1995). Dehydroascorbate was not reduced as a result of this *b*-type cytochrome-mediated electron transport (Horemans et al. 1994). A plasma membrane-associated protein, differing from cyt *b* but possessing the ability to reduce monodehydroascorbate radicals, has also been identified (Bérczi and Møller 1998). This is located on the cytosolic surface of the plasma membrane and may be involved in the regeneration of cytosolic ascorbate from MDHA radicals formed as a result of cytochrome *b*-mediated electron transfer from cytosolic ascorbate to apoplastic monodehydroascorbate radicals.

Apoplastic ascorbate could be formed from monodehydroascorbate radicals as a result of the disproportionation (described in section 1.1.3) of MDHA radicals to ascorbate and dehydroascorbate. The dehydroascorbate produced was not reduced by the cytochrome system (Horemans et al. 1994).

1.3.4 The effect of ascorbate on plant cell division

Treatment of non-dividing plant cells, arrested in the G1 phase of the cell cycle, with ascorbate sometimes results in the progression of the cell cycle from G1 phase to S phase (Innocenti et al. 1990, Liso et al. 1988, Liso et al. 1984). Ascorbate is required to enable competent cells to progress through the cell cycle but does not induce the proliferation of non-competent cells (Citterio et al. 1994). The physiological role of ascorbate in cell cycle progression is not well documented but

Liso et al. (1984) suggested it may be related to the control of hydroxyproline-rich protein synthesis.

The exogenous application of a mixture of ascorbate and dehydroascorbate, calculated to contain MDHA radicals (0.1 µM), was capable of stimulating cell proliferation by shortening the G1 phase. Ascorbate and dehydroascorbate applied separately had no stimulatory effect (De Cabo et al. 1993). MDHA radicals were proposed to stimulate the trans-plasmamembrane redox system which would stimulate general metabolism and hence cell growth (De Cabo et al. 1993).

1.3.5 Roles of ascorbate in cell wall expansion

Ascorbate may have an important role in cell wall metabolism and expansion. Ascorbate oxidase catalyses the aerobic oxidation of ascorbate to monodehydroascorbate radicals and ultimately to dehydroascorbate. Ascorbate oxidase is found in the cell wall and has been associated with rapidly growing cells (Mertz 1961, Lin and Varner 1991). The activity of ascorbate oxidase has been shown to increase when callus begins to grow after transfer to fresh culture medium and following sub-culture of suspension cultured pumpkin cells (Esaka et al. 1988). Ascorbate and ascorbate oxidase have been detected in the extraprotoplastic washing fluid extracted from several plant species (Takahama and Oniki 1993). The extraprotoplasmic washing fluid represents the apoplastic fluid.

Ascorbate oxidase generates MDA, from which ascorbate may be regenerated by donation of electrons from cytochrome *b* (Horemans et al. 1994). The net result is transport of electrons across the plasma membrane into the apoplast. Electron transfer may stimulate plasma membrane H^+-ATPases (Carrasco-Luna et al. 1995) and consequently promote proton extrusion into the apoplast.

According to the acid growth hypothesis (Rayle and Cleland 1992) this would lead to increased cell expansion.

Tobacco protoplasts transformed with pumpkin ascorbate oxidase expand more rapidly than wild-type tobacco protoplasts (Kato and Esaka 2000). This indicates that the effect of ascorbate oxidase on cell expansion is not related to the cell wall. Kato and Esaka (2000) propose that ascorbate oxidase stimulates trans-plasma-membrane redox systems and hence uptake of nutrients and ions. The resultant increase in osmotic pressure would then stimulate protoplast expansion.

Many plant 2-oxoglutarate-dependent dioxygenases (2-ODD) also require ascorbate as a co-factor. These enzymes are involved in the biosynthesis of signalling compounds and secondary metabolites such as flavonoids and alkaloids (Prescott 1996). Prolyl 4-hydroxylase (P4H) is a 2-ODD and is responsible for the post-translational hydroxylation of proline in plant cell wall hydroxyproline-rich glycoproteins. Inhibition of this enzyme by 3,4,-DL-dehydroproline in onion root causes an increase in ascorbate and ascorbate peroxidase activity which inhibits cell division and stimulates cell expansion (De Tullio et al. 1999, De Gara et al. 1991).

1.3.6 Lignification and other oxidative coupling reactions

Lignification occurs by polymerisation of monolignol radicals produced from monolignols by the action of cell wall peroxidases (Ros Barceló 1997). Ascorbate inhibits peroxidase-mediated monolignol oxidation by reducing the radical products to their original state and thus preventing their polymerisation. If ascorbate is absent the monolignol radicals are not reduced and instead polymerise to form lignin (Otter and Polle 1994).

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In a similar manner, ascorbate influences cross-linking of cell wall polysaccharides. Phenolic compounds, other than those involved in lignification, undergo oxidative coupling reactions and, if bound themselves to polysaccharides, cause the cross-linking of polysaccharides. Such coupling reactions may be catalysed by peroxidases in the cell wall (Fry 1983, Fry et al. 2000). In the presence of ascorbate, oxidised phenolic compounds are reduced preventing oxidative coupling reactions. When ascorbate is absent, oxidised phenolic compounds are not reduced and can undergo oxidative coupling, to form cell wall cross-links. The presence of ascorbate inhibits such cross-linking in the apoplastic extract of spinach leaves (Takahama and Oniki 1992).

1.3.7 Polysaccharide scission

Ascorbate is a reducing agent that provides protection against damage by free radicals. Ascorbate may, however, in certain circumstances function to generate hydroxyl radicals ($^{\bullet}$OH) which could have a useful role in loosening the cell wall.

Hydroxyl radicals are generated by reaction of hydrogen peroxide with a reduced transition metal ion such as Cu^+ or Fe^{2+}. The apoplast contains O_2, ascorbate and Cu^{2+}. Ascorbate mediates reduction of O_2 and Cu^{2+} to produce hydrogen peroxide and Cu^+ (Equations 1 and 2). Hydrogen peroxide is also produced as a result of the superoxide dismutase catalysed removal of superoxide. These products can participate in a Fenton reaction to produce hydroxyl radicals and regenerate Cu^{2+} (Equation 3). Overall ascorbate mediates the reduction of O_2 to $^{\bullet}$OH (Equation 4; Halliwell and Gutteridge 1990).

Equation 1:

$$AH_2 + O_2 \longrightarrow A + H_2O_2$$

Equation 2:

$$AH_2 + 2Cu^{2+} \longrightarrow A + 2H + 2Cu^+$$

Equation 3:

$$Cu^+ + H_2O_2 \longrightarrow {}^\bullet OH + OH^+ + Cu^{2+}$$

Equation 4:

$$3AH_2 + 2O_2 \longrightarrow 3A + 2^\bullet OH + 2H_2O$$
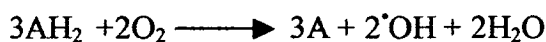
(AH_2, ascorbate; A, dehydroascorbate)

Ascorbate, Cu^{2+} and O_2 are all present in the cell wall and generate hydroxyl radicals in simple *in vitro* reaction mixtures (Fry, 1998). The hydroxyl radicals generated are capable of causing scission of cell wall polysaccharides *in vitro* (Fry, 1998). Kuchitsu et al. (1995) used EPR spectroscopy to detect hydroxyl radicals formed in the extracellular medium of cell suspension cultured rice cells and suggested that the hydroxyl radicals could be formed by the Fenton reaction. Fry et al. (2002) developed a technique to detect hydroxyl radical-induced scission of cell wall polysaccharides *in vivo*. Fry et al. (2002) used NaB^3H_4 to recognise hydroxyl radical-attacked cell wall polysaccharides and thus indicated that pear fruit cell wall polysaccharides undergo hydroxyl radical-induced scission during fruit ripening. Ascorbate-induced production of hydroxyl radicals does cause polysaccharide scission and could, therefore, have a useful role in loosening of the cell wall to enable cell expansion (Schopfer, 2001) and fruit softening (Dumville and Fry 2003).

1.3.8 Ascorbate oxidase

Ascorbate oxidase is a copper-containing enzyme localised in plant cell walls (Esaka et al. 1989, Takahama and Oniki 1994). Ascorbate oxidase catalyses the oxidation of ascorbate to dehydroascorbate *via* MDHA radicals. The precise

physiological role of ascorbate oxidase is unclear but it is involved in the regulation of the redox state of ascorbate in the apoplast (Pignocchi and Foyer 2003). High ascorbate oxidase activity is associated with rapidly growing cells including cultured cells on transfer to fresh medium (Lin and Varner 1990). Ascorbate oxidase promotes cell growth by enhancing cell wall expansion (Pignocchi and Foyer 2003) and might also promote cell growth by increasing vacuolisation (Lin and Varner 1990). The formation of dehydroascorbate and MDHA radicals as a result of ascorbate oxidase activity results in the stimulation of transmembrane redox systems and leads to increased cell expansion (Smirnoff and Wheeler 2000). Ascorbate oxidase is unlikely to be involved in detoxification reactions since it utilises O_2 and not H_2O_2.

1.4 Synthesis of ascorbic acid

1.4.1 Chemical synthesis of ascorbic acid

In the early 1930's, Haworth and Hirst used the six-carbon sugar D-galactose as the starting material for preparation of ascorbic acid. An eight-stage synthetic route was used to prepare L-xylosone (L-xylos-2-ulose) from D-galactose. L-Xylosone was utilised in a three-stage reaction sequence to produce a small quantity of L-ascorbic acid (Ault et al. 1933).

Methods for synthesis of ascorbic acid from several five-carbon sugars including xylose, lyxose and arabinose are now well established (Davies et al. 1991).

The most important commercial method for ascorbic acid synthesis was devised about a year after Haworth and Hirst first produced ascorbic acid from L-

xylosone. This process forms the basis for commercial manufacture of vitamin C and uses a six-carbon sugar, D-glucose as the starting substance. Several methods for L-ascorbic acid synthesis from glucose now exist. Some involve inversion of the carbon chain, while others do not (Davies et al. 1991).

Methods for chemical synthesis of vitamin C can fall into three categories: those using a six-carbon precursor, with or without chain inversion; those involving combination of one- and five-carbon compounds and those involving combination of two- and four-carbon units (Davies et al., 1991).

1.4.2 L-Ascorbate biosynthesis

L-Ascorbate is synthesised by all plants and by most members of the animal kingdom. Some animals cannot synthesise ascorbate and rely on their diet as the sole source of ascorbate.

Biosynthesis of L-ascorbate in animals is well understood. D-Glucuronate is oxidised to L-gulono-1,4-lactone, which is oxidised to ascorbate. The carbon chain is inverted i.e. C-1 of glucose becomes C-6 of ascorbic acid (Burns 1967).

Despite the requirement of ascorbate in human nutrition and its importance in plants, the major pathway for ascorbate biosynthesis in plants has only recently been elucidated (Wheeler et al. 1998). Two pathways had previously been suggested. One, based on the animal biosynthetic pathway, is referred to as the inversion pathway. The other involved unusual intermediates D-glucosulose and L-gulosulose, with no inversion of the carbon chain, and is referred to as the non-inversion pathway.

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1.4.3 Inversion pathways

L-Galactono-1,4-lactone was identified as an effective precursor of ascorbate in plants (Mapson 1954). Similar to the formation of L-gulono-1,4-lactone in the animal biosynthetic pathway, the formation of L-galactono-1,4-lactone from glucose *via* D-galacturonic acid in plants would entail inversion of the carbon chain (Isherwood et al. 1954). D-Glucose and D-galactose were suggested to be converted to L-ascorbate *via* L-gulonolactone (pathway 1) or L-galactonolactone (pathway 2).

Pathway 1

D-glucose ⟶ D-glucuronolactone ⟶ L-gulonolactone ⟶ L-ascorbate

Pathway 2

D-galactose ⟶ D-galacturonic acid methyl ester ⟶ L-galactonolactone ⟶ L-ascorbate

The carbon chain is not broken but both reaction series involve inversion of the carbon chain (Isherwood et al., 1954). Synthesis of L-ascorbate from D-glucuronolactone and D-galacturonic acid methyl ester has been shown to occur in *Arabidopsis* cell suspension culture (Davey et al., 1999). L-Ascorbate biosynthesis in ripe strawberry fruit has been shown to proceed from D-galacturonic acid *via* L-galactonic acid (Mapson and Isherwood 1956). The conversion of D-galacturonic acid to L-galactonic acid is catalysed by the NADPH-dependent enzyme D-galacturonic acid reductase (Aguis et al. 2003) in ripe strawberry fruit.

The synthesis of ascorbate from D-galacturonic acid does occur in strawberry fruit, and possibly in other plants, although the ascorbate biosynthetic pathway proposed by Wheeler et al. (1998) described in section 1.4.4, appears to be the major route for ascorbate biosynthesis.

1.4.4 Non-inversion pathways

Tracer studies (Loewus and Stafford, 1958) have shown that the carbon chain is not inverted during synthesis of L-ascorbic acid from D-glucose in excised grape leaves. L-Galactono-1,4-lactone is an effective precursor of ascorbic acid (Mapson et al., 1954) but until recently there had been no evidence to support a biosynthetic process for D-glucose to L-galactono-1,4-lactone. Ascorbate biosynthesis from glucose (without chain inversion) requires oxidation of C1 of glucose with subsequent 1,4 lactonisation, oxidation of C2 or C3 to generate the ene-diol group and epimerisation at C5 to change from D to L configuration.

A pathway from D-glucose to L-ascorbic acid *via* D-glucos-2-ulose (D-glucosone) and L-gulos-2-ulose (L-sorbosone) was proposed to involve oxidation of C2 of glucose, then epimerisation of C5 and then oxidation at C1 (Loewus et al. 1990). Exogenous glucosulose and gulosulose have been shown to be incorporated into ascorbate (Pallanca and Smirnoff 1999, Saito et al. 1990, Loewus et al. 1990, Loewus 1988). However, a pathway resulting in the conversion of glucosulose and gulosulose to ascorbate is unlikely to be a major ascorbate biosynthetic pathway (Pallanca and Smirnoff 1999) because the enzymes required for conversion of glucose or glucose-6-phosphate to glucosulose and gulosulose have not been detected in plants (Pallanca and Smirnoff 1999). A dehydrogenase capable of converting gulosulose to L-ascorbate has been detected but has low affinity for gulosulose and would therefore require a high concentration of gulosulose to facilitate formation of ascorbate (Loewus et al. 1990, Pallanca and Smirnoff 1999). Neither glucosulose nor gulosulose has been detected in plants and, if present, they are so at a low concentration (Pallanca and Smirnoff 1999).

Wheeler et al. (1998) re-examined the possible source of L-galactono-1,4-lactone, an effective precursor of ascorbic acid (Fig. 4). Feeding L-galactose resulted in a rapid increase in ascorbate in barley leaf slices, *Arabidopsis* leaves and pea seedlings, suggesting that L-galactose is converted to L-galactono-1,4-lactone. A novel enzyme that catalysed the conversion of L-galactose to L-galactono-1,4-lactone was detected in, and extracted from, cell-free extracts of *Arabidopsis* leaves and pea seedlings. This novel enzyme, referred to as L-Galactose dehydrogenase, oxidises L-galactose at C1 using NAD^+ and thus produces L-galactono-1,4-lactone without inversion of the carbon chain.

L-Galactose residues in the cell wall are derived from GDP-L-galactose. The enzyme GDP-mannose 3",5"-epimerase is responsible for the 3,5-epimerisation of the mannose residue of GDP-D-mannose resulting in formation of GDP-L-galactose (Wolucka et al. 2001, Wheeler et al., 1998). GDP-L-Galactose is hydrolysed to L-galactose in extracts of pea seedlings and *Arabidopsis* leaves but it is unclear if this hydrolysis proceeds *via* L-galactose-1-P (Wheeler et al. 1998).

The pathway proposed by Wheeler et al. (Fig. 4) produces L-galactono-1,4-lactone from D-glucose or D-mannose via GDP-D-mannose and L-galactose. The novel enzyme L-galactose dehydrogenase is responsible for the oxidation of L-galactose to L-galactono-1,4-lactone. L-Galactono-1,4-lactone is then oxidised to ascorbic acid by L-galactono-1,4-lactone dehydrogenase in mitochondria using cytochrome *c* as an electron acceptor (Mapson et al. 1954, Mapson and Breslow 1958).

The work of Wheeler et al. (1998) provides an explanation for the lack of inversion of the carbon skeleton of glucose during ascorbate biosynthesis. L-

Galactonolactone has for many years been believed to be the immediate precursor of ascorbate but it was unclear how galactonolactone could be formed from glucose without chain inversion. The identification of L-galactose as a likely precursor of L-galactonolactone and hence ascorbate, and the enzyme L-galactose dehydrogenase provided the missing links in ascorbate biosynthesis in plants.
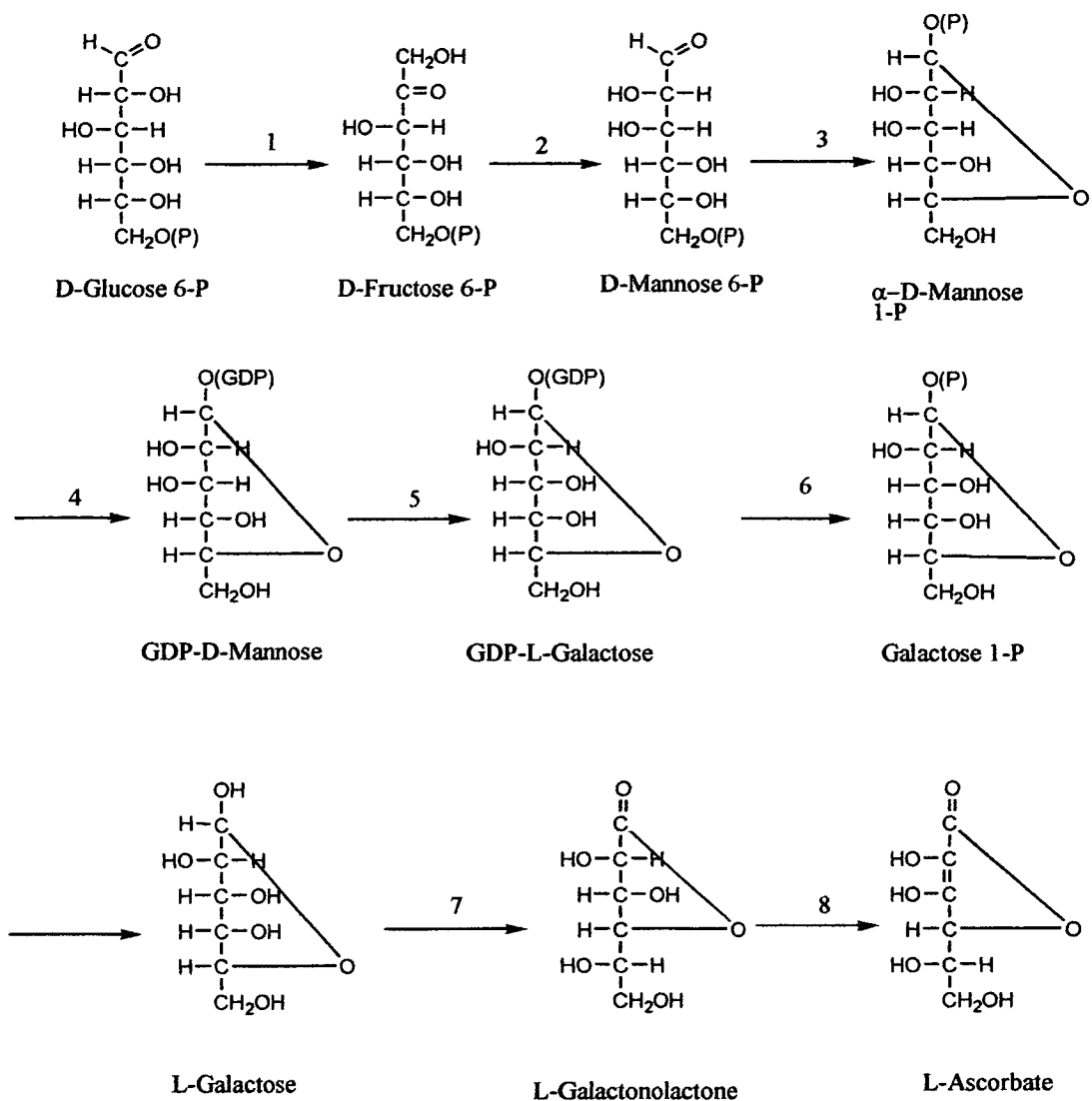
Figure 4. Biosynthesis of ascorbate in plants according to Wheeler et al. (1998)

Ascorbate synthesis in plants proceeds from glucose without inversion of the carbon chain. Enzymes are 1, hexose phosphate isomerase; 2, phosphomannose isomerase; 3, phosphomannose mutase; 4, GDP-mannose pyrophosphorylase; 5, GDP-mannose 3",5"-epimerase; 6, phosphodiesterase; 7, L-galactose dehydrogenase; 8, L-galactono-1,4-lactone dehydrogenase

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1.5 Breakdown products of ascorbate

1.5.1 Tartrate accumulation in the Vitaceae

Ascorbate and tartrate commonly occur together in plants. This observation by Hough and Jones in 1956 led to the suggestion by Loewus and Stafford (1958) that ascorbic acid may be a direct precursor of tartrate.

Grapes, *Vitis vinifera*, belong to the family Vitaceae and accumulate tartrate. Immature grape berries are highly active in synthesising tartrate during the early stages of ripening (Saito and Kasai 1968). Tartrate and malic acid comprise 80–90% of the total organic acid fraction in leaves and fruit of the grape *Vitis vinifera*. Tartrate is most abundant and is actively synthesised in grape berries in the early stages of ripening. As ripening proceeds tartrate is converted to an insoluble salt (Saito and Kasai 1968). The metabolic origin of tartrate has been examined in grapes owing to its importance in wine making. It has also been studied in *Parthenocissus quinquefolia*, another vitaceous plant capable of being grown throughout the year in a controlled environment.

1.5.2 Tartrate biosynthesis in the Vitaceae

Loewus and Stafford (1958) suggested· that cleavage of the C2–C3 bond of ascorbate and oxidation of the terminal group(s) of the resulting 4-carbon compound could give rise to tartrate. The amount of ^{14}C found in tartrate after feeding of [6-^{14}C]ascorbate to grape leaves was too low to add significance to the suggestion that tartrate may be derived directly from ascorbate. The small amount of radioactivity detected in tartrate suggested that ascorbate might be an indirect precursor for tartrate *via* the leaf sugar pool. Organic acids such as malic acid or

other Krebs cycle acids were also considered unlikely to be preferential precursors for tartrate because ^{14}C derived from these acids was incorporated into sugars before being gradually incorporated into [^{14}C]tartrate (Stafford and Loewus 1958, Saito and Kasai 1969).

Feeding of ^{14}CO$_2$ to young grape berries in light conditions resulted in the almost immediate formation of ^{14}C-labelled tartrate. In dark conditions the accumulation of labelled tartrate was much slower, suggesting that tartrate is formed from sugars derived from CO$_2$ fixation (Saito and Kasai 1969). [U-^{14}C]Glucose, [U-^{14}C]fructose, [U-^{14}C]sucrose and D-[6-^{14}C]glucuronate were equivalent to ^{14}CO$_2$ as precursors for tartrate and were not considered preferential precursors since the ratio of incorporation of ^{14}C into tartrate compared to that into other organic acids such as malic acid was low (Saito and Kasai 1969). Feeding of D-[6-^{14}C]glucuronolactone and [1-^{14}C]ascorbate resulted in high incorporation of ^{14}C into tartrate in grape berries. This indicates that ascorbate is an effective precursor of tartrate and that the tartrate formed contains the C-1 of ascorbate (Saito and Kasai 1969), with tartrate presumably derived from C1 to C4 of ascorbate. In agreement, [6-^{14}C]ascorbate does not result in the formation of [^{14}C]tartrate in grape berries (Loewus and Stafford 1958), indicating that tartrate formed by metabolism of ascorbate does not contain C-6 of ascorbate. [4-^{14}C]Ascorbate fed to grape berries was equivalent to [1-^{14}C]ascorbate as a precursor for carboxyl labelled tartrate (Williams and Loewus 1978). This indicates that metabolism of ascorbate in grape berries results in the formation of tartrate from carbons 1–4 of ascorbate (Williams and Loewus 1978).

Having established that tartrate is derived from carbons 1–4 of ascorbate in vitaceous plants, researchers continued to examine the metabolic pathway leading

from ascorbate to tartrate. [1-^{14}C]Ascorbate vacuum-infiltrated into grape berry slices was incorporated into L-idonate and 2-keto-L-idonate then into tartrate over a 5-hour period (Saito and Kasai 1982). Feeding of specifically labelled L-idonate and 2-keto-L-idonate and the identification of an intermediate as 5-keto-D-gluconate (= 5-keto-L-idonate) showed that tartrate is synthesised from ascorbate via 2-keto-L-idonate then idonate and 5-keto-D-gluconate in grapes (Fig. 5; Saito and Kasai 1984). Hydrolysis of the lactone ring of ascorbate forms 2-keto-L-idonate, which is then enzymically reduced to idonate, which is enzymically oxidised to 5-keto-L-idoniate. 5-Keto-L-idonate is then cleaved between C4 and C5 to yield tartrate (Saito et al. 1997). The number of ^{18}O atoms incorporated into tartrate and oxalate in the presence of ^{18}O$_2$ or H$_2$^{18}O suggests that this pathway involves the action of a hydrolase (Saito et al. 1997). If glycollaldehyde was the C$_2$ by-product (Williams et al. 1979, Kotera et al. 1972) then the conversion of 5-keto-L-idonate to tartrate would also involve oxidation (Saito and Kasai 1984, Saito et al. 1997).

Cleavage of the C4–C5 bond of 5-keto-L-idonate results in the formation of tartrate from carbons 1–4 of ascorbate. Glycollaldehyde is a product of tartrate biosynthesis from 5-keto-L-idonate in the bacterium *Gluconobacter suboxydans* (Kotera et al. 1972). In this organism glycollaldehyde was then oxidised to glycollate. The 2-carbon compound derived from carbons 5–6 of ascorbate during tartrate biosynthesis in grape was suggested to be glycollaldehyde (Williams et al. 1979, Helsper 1981). A radiolabelled product of tartrate synthesis from [5-- ^{14}C]ascorbate or [6-^{14}C]ascorbate extracted from detached leaves of *Parthenocissus*
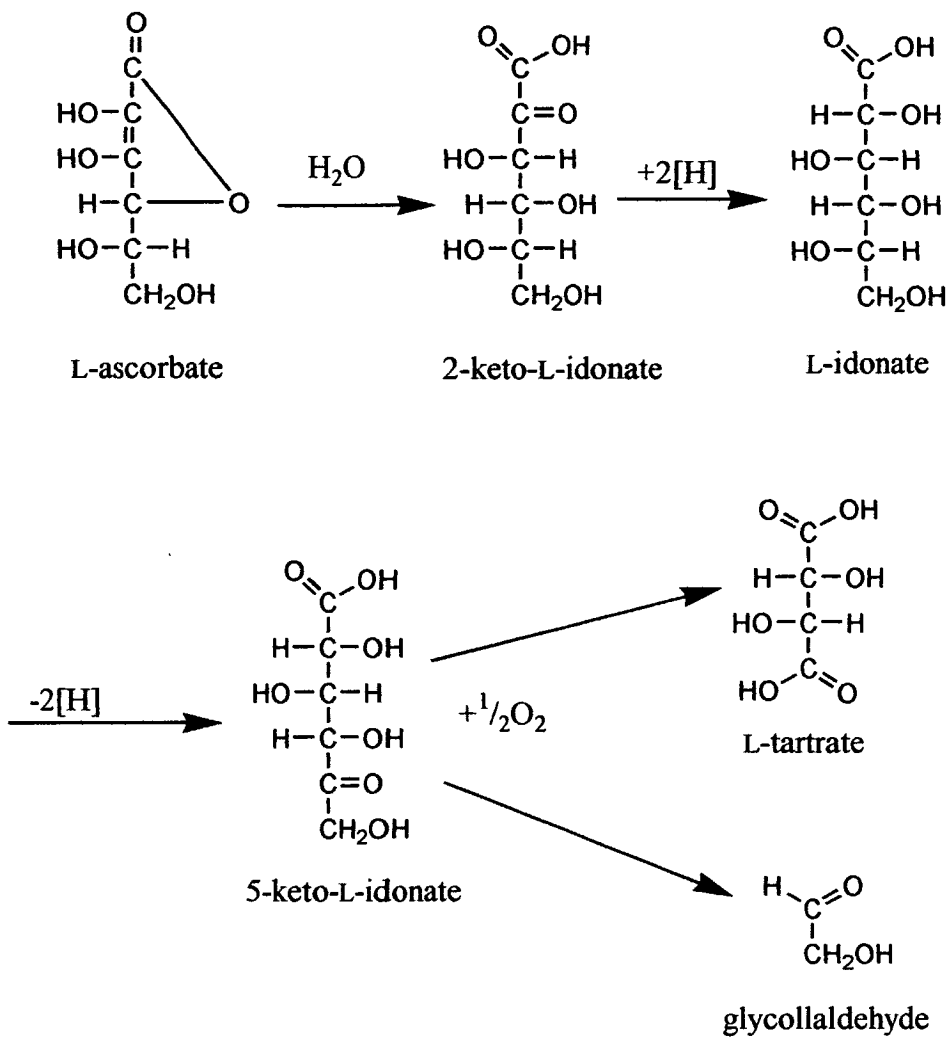
Figure 5. Proposed conversion of ascorbate to tartrate in vitaceous plants (Saito and Kasai 1984)

was thought to be glycollaldehyde. HPLC analysis indicated that the radiolabelled product had a retention time identical to that of authentic glycollaldehyde. The effect of chemical oxidation, by sodium periodate and dimedone reagent, with and without prior reduction by $NaBH_4$ on the radiolabelled product was characteristic of the effect of such treatment on authentic glycollaldehyde (Helsper et al. 1981). These results indicate that glycollaldehyde is likely to be a product of ascorbate metabolism and tartrate biosynthesis in *Parthenocissus*. Loewus and Stafford (1958) and Williams et al. (1979) suggested that glycollaldehyde could be oxidised to glycollate and recycled into hexose metabolism. However, this was thought by Helsper et al. (1981) to be unlikely since the inhibition of glycollate oxidase by 2-pyridylhydroxymethane sulphonate did not affect incorporation of radiolabel from the C_2 product of tartrate synthesis into sugars.

Biosynthesis of tartrate from ascorbate via idonate has been examined in protoplasts but has not been reported in the apoplast.

1.5.3 Tartrate accumulation in the Geraniaceae

Quantitative analysis of tartrate as a taxonomic characteristic of the family Geraniaceae was performed by Stafford (1961). A range of species within each of the three genera, *Geranium*, *Erodium* and *Pelargonium* were examined.

The plants examined were divided into groups depending on their level of tartrate accumulation. Group A accumulates high levels of tartrate—greater than 0.1 μmol per gram fresh weight. Group B are low accumulators of tartrate, amassing less than 0.1 μmol tartrate per gram fresh weight. The third group, C, are non-accumulators.

Stafford examined 20 species of *Geranium* and *Erodium* and 32 species of *Pelargonium*. All species of *Geranium* and *Erodium* belonged to group C, non-accumulators. Of the 32 species of *Pelargonium*, most were high accumulators while others were low- or non-tartrate accumulating species (Stafford 1961).

1.5.4 Biosynthesis of tartrate and oxalate in the Geraniaceae

The nature of tartrate biosynthesis has been examined in *Pelargonium*. Wagner and Loewus (1973) examined the synthesis of tartrate from L-ascorbate and L-galactono-1,4-lactone, the immediate precursor of L-ascorbate. ^{14}C, supplied as L-[U-^{14}C]galactono-1,4-lactone, was recovered in ascorbate, tartrate and oxalate. When L-[6-^{14}C]galactono-1,4-lactone or L-[6-^{14}C]ascorbate was supplied as the labelled precursor, tartrate was the predominantly labelled product with negligible ^{14}C found in oxalate. When [1-^{14}C]ascorbate was administered as the labelled precursor, oxalate was the predominantly labelled product with almost no label detected in tartrate (Wagner and Loewus 1973). These results indicate that tartrate biosynthesis proceeds via a different pathway in *Pelargonium* compared with the Vitaceae. In *Pelargonium*, it is likely that a carboxyl carbon of tartrate is derived from C6 of ascorbate and oxalate is derived from C1 of ascorbate (Wagner and Loewus 1973). Ascorbate may be cleaved between carbons 2 and 3 to form oxalate from C1–2 and tartrate from C3–6. As predicted by this supposition, administration of [4-^{14}C]ascorbate to detached leaves of *Pelargonium* resulted in the formation of ^{14}C-labelled tartrate (Williams and Loewus 1978).

Degradation of [^{14}C]tartrate, formed by metabolism of [4-^{14}C]ascorbate in detached leaves of *Pelargonium*, indicated that most of the ^{14}C was located in internal carbons, either C2 or C3. ^{14}C-Labelled tartrate derived from [6-

[14C]ascorbate was labelled in carboxyl carbons, either C1 or C4 of tartrate (Williams and Loewus 1978). ^{14}C-Labelled oxalate was not produced when [4-^{14}C]ascorbate was administered. A mixture of [4-^{3}H]ascorbate and [6-^{14}C]ascorbate fed to *Pelargonium* was converted to tartrate labelled with ^{14}C and ^{3}H in a ratio almost identical to that of the original labelled ascorbate (Williams et al. 1979). These results indicate that in *Pelargonium* oxalate is derived from C1–2 of ascorbate and tartrate from C3–6.

Williams et al. (1979) also demonstrated the concurrent formation of tartrate and oxalate from ascorbate in *Pelargonium*. When [U-^{14}C]ascorbate was fed both ^{14}C-labelled tartrate and ^{14}C-labelled oxalate increased during a 24-hour period. This highlights another major difference in ascorbate metabolism between the Geraniaceae and the Vitaceae. In the Vitaceae, oxalate is not a product of ascorbate metabolism (see section 1.5.2).

1.5.5 Threonate is an intermediate of tartrate synthesis in Pelargonium

Oxidative cleavage of the C2–C3 bond of ascorbate is catalysed by the enzyme ascorbate oxygenase in the fungus *Myrothecium verrucaria* and results in the formation of oxalate and threonate (Fig 6; White and Krupka 1965). The enzymic cleavage of ascorbate between C2 and C3 in *Myrothecium verrucaria* is superficially similar to the C2–C3 cleavage described in *Pelargonium* (Williams et al. 1979, Helsper and Loewus 1982, Saito et al. 1984). However, ascorbate oxygenase has not been detected in plants. Chemical treatment of ascorbate with alkaline hydrogen peroxide also yields oxalate and L-threonate in a scheme proposed by Isbell and Frush (1979) and presented in Fig. 7. This information
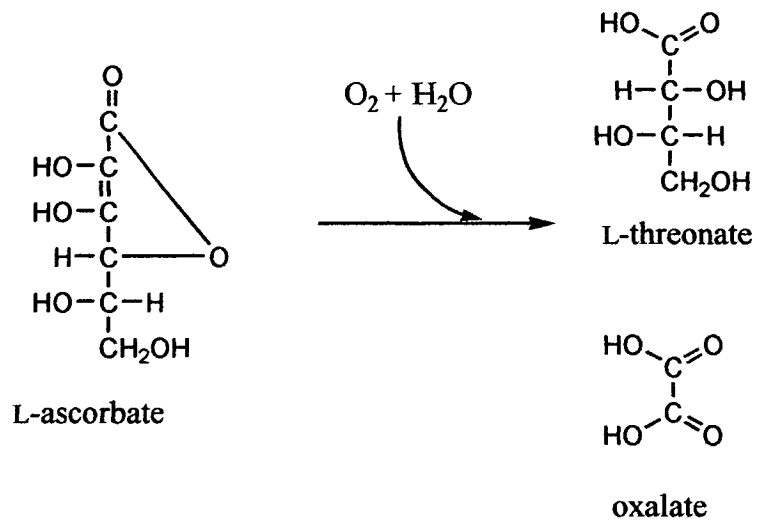
Figure 6. Formation of threonate and oxalate from ascorbate in *Myrothecium verrucaria* (White and Krupka 1965)
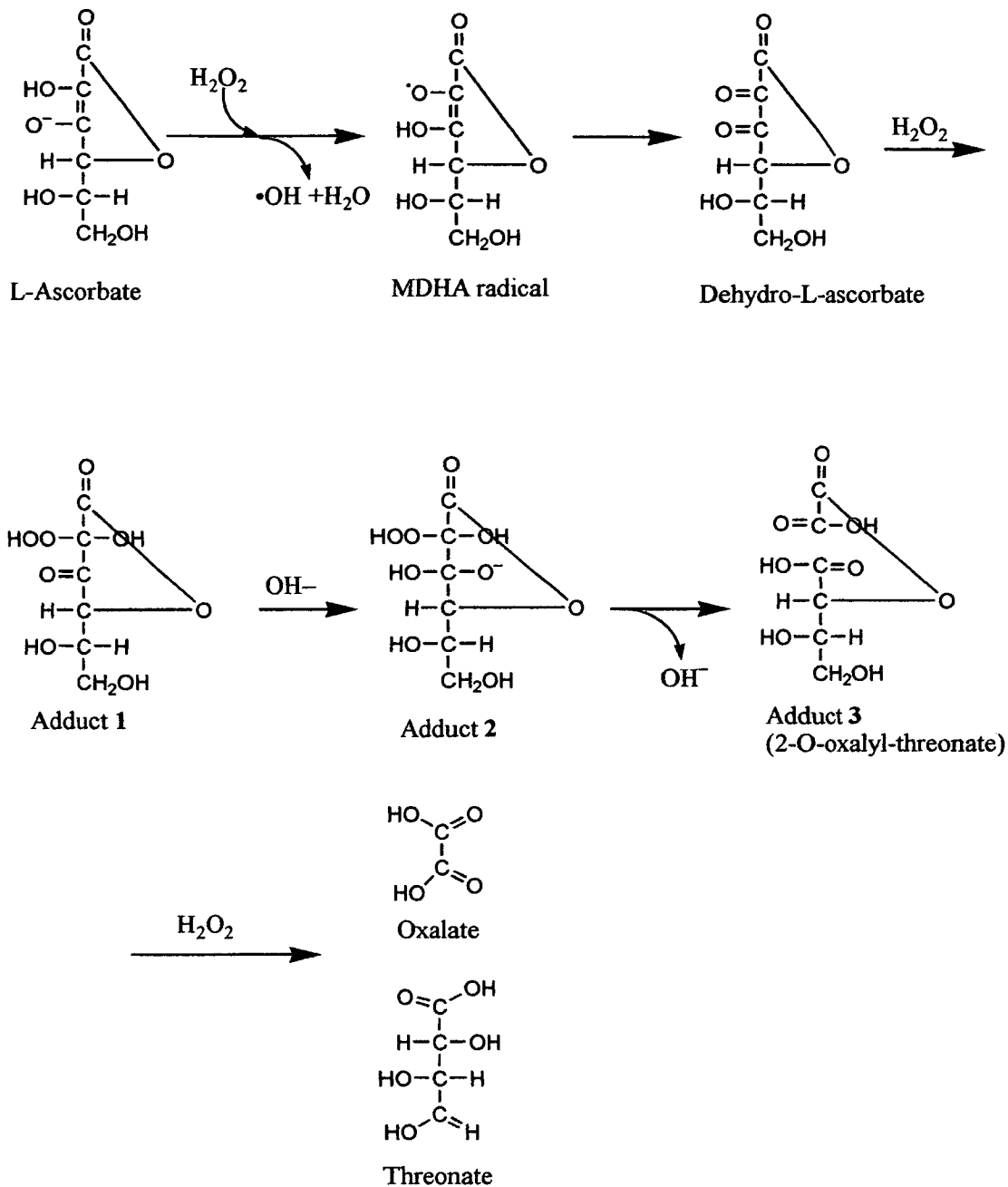
Figure 7. Representation of the preparation of threonate by oxidation of ascorbate.

Threonate and oxalate proposed to be formed by oxidation of ascorbate via MDHA radical to dehydroascorbate. Addition of H_2O_2 to dehydroascorbate yields adduct **1** or its equivalent; addition of the OH^- to gives rise to adduct **2**. Elimination of OH^- from adduct **2** yields adduct **3**. Hydrolysis of adduct **3** results in the formation of threonate and oxalate (Isbell and Frush 1979).

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suggests that threonate may be an intermediate of the conversion of ascorbate to tartrate in *Pelargonium*.

L-Threonate, a natural constituent of *Pelargonium crispum* (450 µg/g fresh weight; Helsper and Loewus 1982) is formed from ascorbate (Helsper and Loewus 1982, Saito et al. 1984). [U-^{14}C]Ascorbate fed to detached apices, containing three expanded leaves, of *Pelargonium* was converted to L-[^{14}C]threonate, L-[^{14}C]tartrate and [^{14}C]oxalate, indicating that L-threonate is a metabolite of ascorbate. Tartrate formed by feeding ^{14}C-labelled ascorbate to *Pelargonium crispum*, *Vitis labrusca*, and *Parthenocissus inserta* was found, by chemical analysis, to be in the L-(+) configuration (Wagner et al. 1975).

Analysis of metabolism of [4-^{3}H]ascorbate in another oxalate-accumulating species, *Rumex* × *acutus* (Polygonaceae), showed that the threonate formed was labelled internally but that when [6-^{3}H]ascorbate was administered label was found in the hydroxymethyl group. These results indicate that threonate is formed from carbons 3–6 of ascorbate (Helsper and Loewus 1982). When [1-^{14}C]ascorbate was supplied, [^{14}C]oxalate was formed and virtually no label was found in threonate (Helsper and Loewus 1982). Tartrate was not formed in *Rumex* × *acutus*; instead the [^{14}C]threonate was converted to ^{14}CO$_2$.

The major synthetic route for tartrate formation in *Pelargonium* is from ascorbate via threonate (Fig. 8). This pathway involves the hydrolysis and oxidation of ascorbate to form threonate and oxalate. This is followed by oxidation of the C4 hydroxymethyl group of threonate to form tartrate (Helsper and Loewus 1982, Saito et al. 1997). Synthesis of tartrate from ascorbate via 2-keto-L-idonate, idonate and 5-keto-L-idonate— the major synthetic pathway for tartrate in grapes— does not
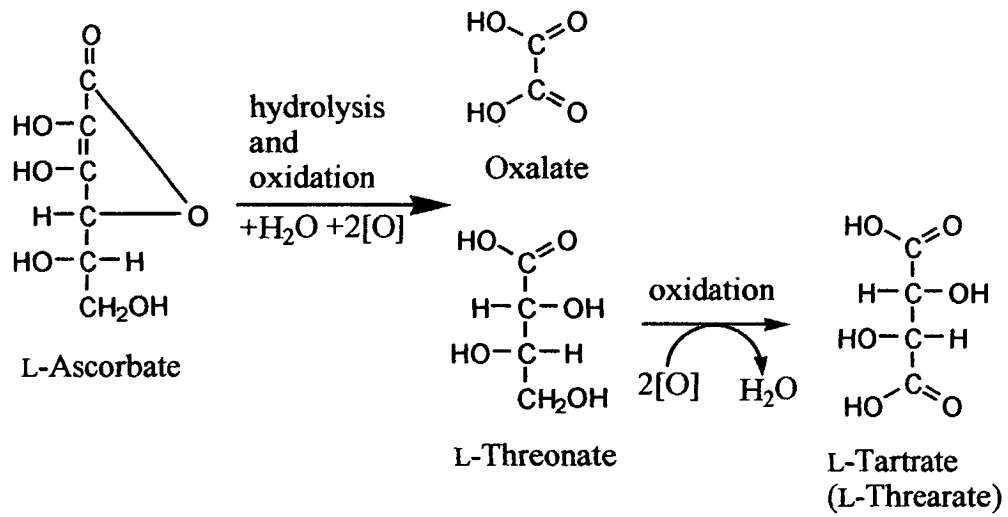
Figure 8. Biosynthesis of tartrate in oxalate-accumulating species such as *Pelargonium* (Helsper et al. 1982, Saito et al. 1984)

contribute to tartrate synthesis in *Pelargonium* (Saito et al. 1984). 5-Keto-L-idonate is an intermediate of tartrate biosynthesis from D-glucose in a minor synthetic pathway for tartrate in *Pelargonium*. This pathway does not involve ascorbate (Saito et al. 1984).

1.5.6 Summary of ascorbate metabolism and tartrate biosynthesis

The major biosynthetic pathways for tartrate differ in different species of plants. The different biosynthetic pathways are summarised in Figure 9.

In the Vitaceae, ascorbate is cleaved between C4 and C5 and carbons 1 and 4 of ascorbate become carboxyl carbons of tartrate. This occurs via three intermediates and involves hydrolysis, reduction and oxidation. The remaining 2-carbon portion, derived from carbons 5 and 6 of ascorbate, possibly forms glycollaldehyde and then glycollate.

In the Geraniaceae, ascorbate is metabolised to threonate and oxalate by hydrolysis and oxidation. The C1–C2 portion of ascorbate forms oxalate and the C3–C6 portion forms threonate. Threonate can then be oxidised to tartrate in *Pelargonium* or converted to CO_2 in *Rumex*.

The metabolism of ascorbate proceeds via different pathways in different plants. Neither of the two pathways described are necessarily representative of the predominant metabolic pathway for ascorbate breakdown in plants in general.

1.5.7 Oxalate Biosynthesis

Oxalate is derived from ascorbate in oxalate-accumulating plants other than than the Geraniaciae (Loewus and Yang 1975). Oxalate in plants can be formed by oxidation of glycollate produced during photorespiration (Richardson and Tolbert 1961) or
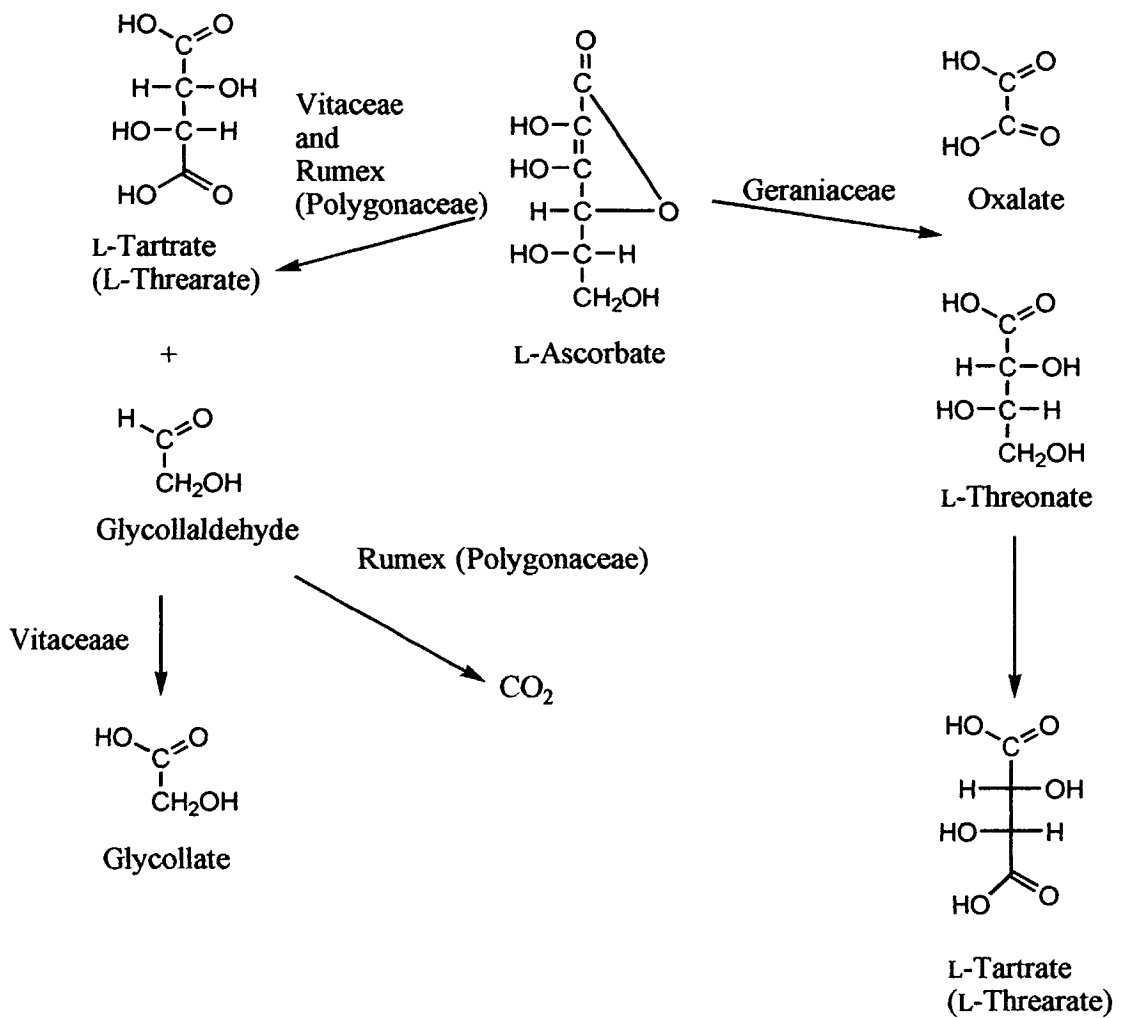
Figure 9. Summary of the major biosynthetic pathways of tartrate in plants

during tartrate synthesis from ascorbate (Williams et al. 1979, Helsper and Loewus 1982). Many oxalate-accumulating plants form calcium oxalate in crystal idioblast cells.

Calcium oxalate formation from free oxalate in crystal idioblast cells of *Lemna minor* is as a result of ascorbate metabolism, not glycollate oxidase-catalysed oxidation of glycollate to glyoxylate with further oxidation to oxalate (Li and Franceschi 1990). Previous work by Franceschi (1987) had suggested that calcium oxalate could be deposited in crystal idioblast cells by oxidation of glycollate and/or glyoxylate dervied from ascorbate. Calcium oxalate formed in crystal idioblast cells of *Yucca torreyi* (Agavaceae) is also derived from ascorbate and not from glycollate or glyoxylate (Horner et al. 2000). Radiolabelled oxalate was formed in *Pistia stratiotes* (Araceae) after feeding [1-^{14}C]ascorbate but not after feeding [6-^{14}C]ascorbate (Keates et al. 2000), indicating that free oxalate and calcium oxalate are derived from carbons 1–2 of ascorbate. This is in agreement with the suggested C2–C3 cleavage of ascorbate in the Vitaceae, the Geraniaceae and the Agavaceae. Glycollate was a poor substrate for oxalate formation and hence it is concluded that glycollate is not a major precursor of oxalate in crystal idioblast cells of *P. stratiotes* (Kostman et al. 2001).

1.5.8 Oxalate metabolism

Oxalate, often considered to be an end-product, may undergo further metabolism in some tissues. Oxalate is oxidised to CO_2 and H_2O_2 by oxalate oxidase (E.C. 1.2.3.4) and O_2. Germin, a developmentally regulated protein found in wheat (Lane et al. 1993), is an oxalate oxidase. This enzyme, when bound to the cell wall as in maize (Vulaković and Šukalović 2000), could result in generation of ˙OH radicals as a

result of H_2O_2 generation. Increased formation of ˙OH radicals could cause non-enzymic scission of cell wall polysaccharides. On the other hand, increased amounts of H_2O_2 could promote peroxidase-mediated oxidative coupling reactions. Label derived from [^{14}C]oxalate or from labelled precursors of oxalate fed to whole *Lemna minor* plants in light conditions, was incorporated into starch (Franceschi 1987), suggesting that the $^{14}CO_2$ formed may function as an internal reservoir for photosynthesis in oxalate-accumulating plants (Loewus 1999, Nuss and Loewus 1978).

Oxalate decarboxylase (EC 4.1.1.2) catalyses the formation of formic acid and CO_2 from oxalate in fungi (Shimanazono 1955, Mäkelä et al. 2002, Kathiara et al. 2000). Oxalate decarboxylase has not been described in plants.

1.6 Ascorbate and plant cell walls

Some ascorbate is found in the apoplast of plant cells (Sánchez et al. 1997, Hove et al. 2001, Luwe and Heber 1995, Luwe et al. 1993, Takahama and Oniki 1993). Apoplastic ascorbate is involved in the defence against the harmful effects of atmospheric pollutants (Takhama et al. 1992, Zheng et al. 2000, Lyons et al. 1999), and in cell wall expansion (Lin and Varner 1991, Carrasco-Luna et al. 1995, De Tullio et al. 1999, Fry et al. 2002) and cell division (Innocenti et al. 1990, Liso et al. 1988, Liso et al. 1984). Many of these processes involve the oxidation of ascorbate to MDHA radicals and/or dehydroascorbate. Apoplastic ascorbate may be regenerated by uptake of dehydroascorbate in exchange for cytosolic ascorbate or by electron transfer from cytosolic ascorbate to apoplastic MDHA radicals (Horemans et al. 1994, Horemans 1996, 1997, Asard 1995).

The intraprotoplasmic metabolism of ascorbate to tartrate, threonate and oxalatehas been described. However, apoplastic ascorbate metabolism and its implications for cell wall metabolism are not well documented.

In order to appreciate fully the documented roles of apoplastic ascorbate and to speculate on the potential effects of novel roles or metabolic pathways, it is necessary to have a general understanding of the primary cell wall of plants.

1.7 Introduction to plant cell walls

Plant cells, unlike animal cells, possess a cell wall. This feature has roles in structure, defence, growth and transport.

The first functions of the cell wall to be recognised were structural— the provision of strength and shape. The cell wall is a major factor in the achievement of large size and structural strength by plants.

Despite the semi-inextensible nature of the cell wall, growth still occurs. Cell wall expansion is achieved by selective, closely-controlled weakening of the cell wall. The weakening permits cell expansion without compromising the overall integrity of the cell wall (Brett and Waldron 1996).

The cell wall is formed from cross-linked macromolecules that present an obstacle to the movement of large molecules. Its negative charge can also retard the progress of positively charged molecules. This feature of the cell wall has advantages for cell defence against potentially pathogenic organisms including viruses. The deposition of structural polymers and enrichment of defence proteins in response to attack is an active mechanism of defence (Esquerré-Tugayé et al. 2000). Small molecules and ions are capable of moving through the cell wall matrix. The

walls of neighbouring cells are in contact and the movement of small molecules forms a major transport mechanism referred to as apoplastic transport.

Some fragments of primary cell wall polysaccharides have biological activity as growth regulators, in control of flowering and in activation of resistance mechanisms (Albersheim and Darvill 1985, Dumville and Fry 2000, Albersheim and Anderson-Prouty 1975).

1.7.1 Cell wall formation

The cell wall forms after cell plate formation. In the majority of cell-types new cell wall material is deposited along the entire expanding wall. The middle lamella is formed first and is found at the interface where neighbouring cell walls are in contact. The next major layer is the primary cell wall. Material continues to be deposited in the primary cell wall as long as the cell is increasing in surface area. A third layer, the secondary cell wall, is formed by some specialised cells (Brett and Waldron 1996, Carpita and Gibeaut 1993).

1.7.2 Primary cell wall structure

Primary cell walls are composed of three phases. Approximately 50% of cell wall mass is the microfibrillar phase—cellulose and hemicellulose. This is embedded in the second domain, a matrix of pectic polysaccharides, constituting approximately 30% of cell wall mass. The third domain consists of structural proteins such as extensin (Carpita and Gibeaut 1993, McNeil et al. 1984).

1.7.3 The microfibrillar phase

The primary cell wall may be referred to as type I or type II depending its composition. Type I cell walls are found in dicots, gymnosperms and non-

graminaceous monocots. Type II cell walls are found in graminaceous moncots which include cereals and grasses.

The microfibrillar phase of type I cell walls is a network of cellulose microfibrils linked by a network of xyloglucans as the major hemicellulose. In type II cell walls, glucuronoarabinoxylan (GAX) is the major hemicellulose and very little xyloglucan is present (Brett and Waldron 1996, Bacic et al. 1988).

Cellulose is a linear β-(1→4)-linked glucan. In the primary cell wall, cellulose aggregates and forms microfibrils. Each microfibril is approximately 5–15 nm wide and spaced 20–40 nm from other microfibrils. They are usually orientated perpendicular to the principal axis of growth with cellulose molecules aligned parallel to the long axis of the microfibril (McCann et al. 1990, Carpita and Gibeaut 1993).

Xyloglucan hydrogen-bonds tightly to cellulose, either to the surface of individual microfibrils or between different microfibrils. Xyloglucan is a linear polymer of β-(1→4)-linked D-glucose substituted with α-(1→6)-linked D-xylose residues. Some of the xylose side chains are terminated by a β-(1→2)-linked galactose residue. Xyloglucan may also contain L-fucose and a small amount of arabinose residues (Hayashi 1989).

1.7.4 The pectin matrix

The fundamental constituents of pectin are homogalacturonan, α-(1→4)-linked polymers of D-galacturonate and rhamnogalacturonan I, a polymer of the disaccharide unit [(1→2)-α-L-rhamnose-(1→4)-α-D-galacturonate]. These pectic polysaccharides are characteristic components of the matrix of the primary cell wall

of dicots. They are present in graminaceous monocots, although to a lesser extent than in dicots (McNeil et al. 1984, Brett and Waldron 1996).

1.7.5 Homogalacturonans

These may be partially methyl esterified. There is a tendency for homogalacturonans to be insoluble resulting in formation of rigid gels in the presence of calcium. Homogalacturonans are found in considerable quantities in some fruits and the primary cell wall of dicots. They are thought to have an important structural role in the cell wall and the ability of homogalacturonan fragments to elicit a defence response suggests a possible regulatory role (Carpita and Gibeaut 1993, Brett and Waldron 1996, McNeil et al. 1985).

1.7.6 Rhamnogalacturonan I (RG-I)

RG-I is a major component of the middle lamella and the primary cell wall of dicots. It is composed of a backbone of galacturonic acid linked by an α-(1→4)-linkage to rhamnose which in turn is α-(1→2)-linked to galacturonic acid. Galacturonic acid and rhamnose probably form an alternating sequence. The galacturonic acid residues may be methyl esterified (Bacic et al 1988) and neutral side chains are attached to the 4 position of rhamnose. Side chains average about 7 residues, principally arabinose and galactose (Brett and Waldron 1996, MacNeil et al. 1975, Lau et al. 1985).

1.7.7 Cell wall proteins

The primary cell wall contains a variety of proteins, most of which are glycoproteins; only the glycine-rich proteins are non-glycosylated. Many cell wall

proteins contain the unusual amino acid hydroxyproline (Brett and Waldron 1996, MacNeil et al. 1975). The three major classes of cell wall proteins are structural proteins and mostly covalently cross-linked to other components of the cell wall. Extensin for example, is considered to be covalently linked to RG-I in the pectin matrix (Qi et al. 1995). The structural proteins include hydroxyproline-rich glycoproteins e.g. extensins, glycine-rich proteins and proline-rich proteins. Arabinogalactan-proteins are soluble and can diffuse in the cell wall. This property could allow them to act as messengers and enable them to influence cell development during differentiation. (Krueger and Holst 1993, Cassab 1998).

Enzymes present in the cell wall are involved in cross-linking structural proteins, alteration of wall structure and modification of nutrients before absorption by the cell. These have an important role in cell wall expansion (Bacic 1988, Cassab 1998).

Other cell wall proteins include cysteine-rich thionins, calmodulin-binding protein, leucine-rich repeat proteins and histidine–tryptophan-rich proteins (Cassab 1998).

1.7.8 Phenolic compounds of the primary cell wall

Compared with the secondary cell wall, the primary cell wall contains only small amounts of phenolic compounds. The primary cell wall contains two types of polymer-bound phenols: derivatives of cinnamic acid, associated with cell wall polysaccharides; and tyrosine residues found in cell wall proteins.

Ferulic acid is a major phenolic compound found esterified to primary cell wall polysaccharides of graminaceous plants (Smith and Hartley 1983, Harris and Hartley 1980) and dicots (Fry 1982a). In monocots, ferulic acid is esterified to

glucuronoarabinoxylan (Kato and Nevins 1985) while in some dicots, such as spinach, ferulic acid is attached to galactose- and/or arabinose-rich regions of pectic polysaccharides (Fry 1983).

Ferulic acid residues attached to cell wall polysaccharides are subject to oxidation by extraprotoplasmic peroxidases (Fry 1982a) and intraprotoplasmic peroxidases (Fry et al. 2000). Dimerisation of ferulate by oxidative coupling may form cross-links between cell wall polymers (Markwalder and Neukom 1976) that could reduce cell wall extensibility and prevent cell elongation (Fry 1983). Oxidative coupling of polymer-bound phenolics strengthens the cell wall and protects cell wall polysaccharides from enzymic degradation (Hartley and Jones 1976).

The hydroxyproline-rich cell wall glycoprotein extensin is cross-linked by aromatic cross-links. The protein contains a phenolic amino acid called isodityrosine. This is formed by the oxidative coupling of two tyrosine residues (Fry 1982b, Epstein and Lamport 1984). The isodityrosine cross-links are probably responsible for holding extensin in the cell wall and may limit cell wall extensibility (Fry 1982b). Isodityrosine is subject to oxidative coupling and forms pulcherosine— biphenyl-linked isodityrosine and tyrosine (Brady et al. 1998)— and di-isodityrosine in cell walls (Brady and Fry 1997).

1.7.9 Phenolic compounds in the secondary cell wall

In some secondary cell walls and in the primary cell walls of cells with secondary cell walls, large amounts of phenolic compounds link to form large polymers such as lignin. Lignin is usually deposited in specialised cells such as xylem vessels and

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tracheids after cell elongation has ceased. Once cells have become lignified, nutrient transfer ceases and no elongation can occur. Lignified cells provide the support that plants require to be able to grow in a terrestrial environment, they are involved in water transport and they also protect the plant against pathogen attack (Brett and Waldron 1996, Vance et al. 1980) and provide a mechanism for the immobilisation of harmful metabolites (Trench and Sandermann 1981). Lignins vary in composition depending on the type of plant, the tissue within the plant and its physiological role (Gross 1979).

Lignin biosynthesis is the product of a long sequence of reactions. The shikimate pathway is responsible for the formation of aromatic amino acids (Hermann 1995) including phenylalanine and tyrosine. These enter the common phenylpropanoid pathway where they are converted to hydroxycinnamoyl CoA thioesters—the precursors of lignins and other phenolic compounds including flavonoids (Ros Barceló 1997). The lignin-specific pathway converts hydroxycinnamoyl CoA esters into hydroxycinnamoyl alcohols via two reductive steps catalysed by the enzymes cinnamyl CoA reductase and cinnamyl alcohol dehydrogenase (Whetten and Sederoff 1995). The last stage of lignin biosynthesis involves the enzymic oxidation of cinnamyl alcohols—coumaryl, coniferyl and sinapyl alcohols—to form phenoxy radicals (monolignol radicals) that non-enzymically polymerise forming oligomers which eventually grow into lignin polymers (Hapiot et al. 1994). The oxidation of coumaryl, coniferyl and sinapyl alcohols is catalysed by peroxidases and/or by O_2-dependent oxidases known as laccases.

Secreted peroxidases are glycoproteins and are either acidic or basic. Basic peroxidases are found in both the cell wall and vacuoles while acidic peroxidases, which have a higher affinity for cinnamyl alcohols, are associated only with the cell wall (Ros Barceló 1997). The hydrogen peroxide required for these preoxidase-catalysed reactions may be formed by the action of other peroxidases located in the cell walls of lignifying tissues (Elstner and Heupel 1976, Goldberg et al. 1985).

The role of laccase in lignification has received little attention compared with that of peroxidases. Laccases may be involved in lignification (Bao et al. 1993, Sterjiades et al. 1992, Driouich et al. 1992) as hydrogen peroxide-independent phenoloxidases capable of oxidising hydroxycinnamyl alcohols using O_2. The phenoxy radicals can then polymerise in the same way as those formed by peroxidase action. Plant laccases are copper-containing enzymes belonging to the blue copper oxidase group, which also includes ascorbate oxidase (Ros Barceló 1997). Apoplastic ascorbate could prevent the polymerisation of monolignols and thus prevent lignification. The apoplastic metabolism of ascorbate is, therefore, relevant to lignification.

1.8 Summary

Ascorbate has many roles in plants. It functions as an anti-oxidant providing defence against oxidative stress. Apoplastic reactions that result in the formation of hydrogen peroxide could promote the ascorbate-mediated generation of hydroxyl radicals ($^{\bullet}$OH). An increase in the formation of $^{\bullet}$OH could promote non-enzymic polysaccharide scission and thus increase cell wall expansion. Ascorbate in the apoplast can also influence cell wall expansion by preventing oxidative coupling

reactions in the primary cell wall and also in the formation of the secondary cell wall.

Intraprotoplasmic ascorbate is metabolised to threonate, tartrates (threarate and erythrarate) and oxalate via different species-specific pathways. In this thesis I have examined the metabolism of apoplastic ascorbate with the aim of identifying novel metabolites or any reactions that may result in hydrogen peroxide formation or increased ·OH.

The aim of the work described in this thesis was to examine the apoplastic metabolism of ascorbate beyond the well-documented redox system comprised of ascorbate, MDHA radicals and dehydroascorbate. The hydrolysis of dehydroascorbate to diketogulonate has also been well documented. The metabolism of ascorbate to compounds other than MDHA radicals, dehydroascorbate and diketogulonate has been described in animals and in the symplast of plant cells but has not, until now, been reported in the apoplast of plant cells. Comparison of intraprotoplasmic and apoplastic ascorbate metabolism indicated major differences between the two systems. With the exception of dehydroascorbate, diketogulonate and oxalate, the metabolites of apoplastic ascorbate do not correspond to the metabolites of intraprotoplasmic ascorbate. It was hoped that characterisation of apoplastic ascorbate metabolism would provide a link with cell wall expansion. Generation of H_2O_2 as a metabolic by-product could promote hydroxyl radical formation and hence result in cell wall expansion as a consequence of polysaccharide scission. Alternatively, an increased amount of H_2O_2 could promote peroxidase-mediated oxidative coupling and inhibit cell wall expansion. Thus, the metabolism of apoplastic ascorbate could have far reaching

consequences for the cell wall and this was what provided the inspiration for the work described in this thesis.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, United Kingdom), Fisher Chemicals (Fisher Scientific, Loughborough, U.K.) or BDH AnalaR Chemicals Ltd. (Poole, United Kingdom). L-[1-^{14}C]Ascorbic acid and [^{14}C]oxalate were purchased from Amersham Pharmacia Biotech U.K. Limited. Poly-Prep chromatography columns (conical polypropylene columns with a polyethylene bed support to retain fine particles) and Bio-Gel P-2 were obtained from Bio-Rad Laboratories, Hemel Hempstead, U.K. All water used was deionised unless otherwise stated.

2.2 Tissue culture

2.2.1 Culture medium

Rose cell suspension culture medium was prepared in 1-l batches containing the following: $CaCl_2$ (74 mg/l), KH_2PO_4 (140 mg/l), KCl (750 mg/l), $NaNO_3$ (850 mg/l), $MgSO_4.7H_2O$ (250 m/l), $MnSO_4.4H_2O$ (1 mg/l), H_3BO_3 (0.2 mg/l), $ZnSO_4.7H_2O$ (0.5 mg/l), KI (0.1 mg/l), $CuSO_4.5H_2O$ (0.02 mg/l), $CoCl_2.6H_2O$ (0.01 mg/l), $NaMoO4.2H_2O$ (0.02 mg/l), $FeCl_3.6H_2O$ (5.4 mg/l), $NaEDTA.2H_2O$ (7.4 mg/l), 2,4-D (1.0 mg/l), kinetin (0.5 mg/l) and sucrose (20g/l). The pH was adjusted to 6.0 using NaOH (1 M). Culture medium was divided among 250-ml conical

flasks (50 ml in each), stoppered with a foam bung and covered with a double layer of aluminium foil before being sterilised by autoclave for 15 minutes at 121°C.

Maize culture medium was prepared in 2-l batches containing Murashige and Skoog (Sigma cat. no. M5519) basal medium (9.4 g), sucrose (40 g), 2,4-D (20 ml) made up to 2 l with distilled water. The pH was adjusted to 5.6–5.8 with NaOH (1 M). Medium (200 ml) was dispensed into 500-ml conical flasks and autoclaved as above.

Spinach cell suspension culture medium was prepared in 2-l batches from Murashige and Skoog (Sigma cat. no. M5524) basal medium (8.8 g) and glucose (20 g) made up to 2 l with distilled water. The pH was set to 4.4 by gradual addition of NaOH (1 M). Medium was sterilised as described above.

2.2.2 Maintenance of cell suspension cultures

Cell suspension cultures were sub-cultured fortnightly under aseptic conditions. Cells in 14-day-old cultures were allowed to settle and excess medium was discarded if necessary. The remaining cells were resuspended and divided among five flasks of fresh medium.

2.2.3 Selection of plant cultures

In preliminary experiments (data not shown), the fate of non-radioactive ascorbate was examined in maize, spinach and rose cultures. Other model systems such as *Arabidopsis* cell suspension cultures were not available for use until preliminary work was complete and an experimental system established. Ascorbate was oxidised rapidly in fresh, unused, maize and spinach media. Ascorbate was oxidised in fresh rose medium but much more slowly and to a lesser extent than in maize or

spinach medium. Rose cell suspension cultures were therefore used for all further experiments.

2.3 Assays for ascorbate

2.3.1 Measurement of ascorbic acid concentration

2,6-Dicholorophenolindophenol (DCPIP) is pink in acid solution and is decolourised rapidly by ascorbic acid. A standard dilution series of ascorbic acid in metaphosphoric acid (5% w/v) was prepared and titrated with DCPIP. Two methods were used depending on the sensitivity required.

Ascorbic acid concentrations in the range of 0 to 1.0 mM were assayed by volumetric titration. DCPIP (0.1% w/v) was added to aliquots of ascorbic acid (0–1.0 mM, 1 ml) in 10-µl shots until it was no longer decolourised and a slight pink colour persisted for approximately 10 seconds.

Ascorbic acid concentrations in the range of 0 to 0.1 mM were assayed by colourimetric titration. DCPIP (0.01% w/v, 0.4 ml) was added to ascorbic acid (0–0.1 mM, 0.5 ml). Absorbance was measured at 519 nm 20 seconds after the addition of DCPIP. This method was capable of detecting the presence of ascorbic acid at concentrations of 10 µM or above.

2.3.2 Measurement of endogoenous, symplastic ascorbate

Cell suspension cultures of various ages were filtered through a double thickness of muslin to remove spent culture medium. Cells, approximately 2–15 g fresh weight, were resuspended in metaphosphoric acid (5%, 100 ml) to release and stabilise ascorbate. After approximately 1 hour of stirring with a magnetic flea, samples (5

ml) were removed, filtered through a Poly-Prep column and assayed for ascorbate by timed colourimetric titration with DCPIP.

2.3.3 Measurement of endogenous, apoplastic ascorbate

Cell suspension cultures were allowed to settle before spent culture medium (5 ml) was removed and filtered through a Poly-Prep column. The filtrate (0.8 ml) was collected and stabilised in metaphosphoric acid (20%, 0.2 ml). Ascorbate was assayed by timed colourimetric titration with DCPIP. This assay (limit of detection = 10 μM) did not detect ascorbate.

Cell-free spent medium (50 ml) was obtained by filtration of rose cell suspension culture through a double layer of nylon mesh (50 μm pore size). The filtrate was divided into 25-ml aliquots and freeze dried. Each aliquot was redissolved in metaphosphoric acid (5% w/v, 2 ml) and then aliquots were combined. Concentrated spent medium (0.5 ml) was mixed with DCPIP (0.01% w/v, 0.4 ml) and the absorbance at 519 nm measured in quartz cuvettes compared to concentrated spent medium. The concentration of ascorbate was calculated from the change in absorbance at 519 nm from 20 to 60 seconds.

2.4 Separation of apoplastic metabolites

2.4.1 High voltage paper electrophoresis

Paper electrophoresis was used to separate charged molecules on the basis of their charge to mass ratio. Samples were dried on to Whatman 3MM paper along an origin drawn 11 cm from the cathodic end of the paper. External markers were

loaded at a distance of 1.5 cm centre to centre along the origin. Orange G (1 μl, 0.5%) was loaded in the gaps between markers and samples and used to monitor the progress of electrophoresis visually.

The paper was laid on a glass loading plate with the origin raised between two glass rods and wetted with buffer. The origin was wetted last. Excess buffer was removed by blotting.

The paper was suspended in a large tank filled with an immiscible coolant. The end of the paper closest to the origin was held in a trough containing approximately 250 ml of buffer and a platinum cathode. The bottom end of the paper dipped into another layer of the same buffer containing the platinum anode. The coolant was water-cooled to below 30°C during electrophoresis.

The buffer for electrophoresis at pH 6.5 was acetic acid:pyridine:water (1:33:300 v/v/v) and the coolant toluene. Typical running conditions for Whatman 3MM paper of width 28 cm were 3 kV (approximately 120 mA), for 30 minutes. For pH 2.0 electrophoresis the buffer was formic acid:acetic acid:water (1:35:355 v/v/v) and the coolant was white spirit. Again typical running conditions were 3 kV (approximately 120 mA), for 30 minutes for samples loaded on Whatman 3MM paper of width 30 cm. [^{14}C]Oxalate forms a radioactive streak during electrophoresis. This is likely to be due to formation of radiolabelled calcium oxalate. Formation of the radioactive streak is minimised, but not entirely prevented, by the addition of a chelating agent to the running buffer: EDTA (to 5 mM) was added to pH 6.5 buffer and thiosulphate (to 5 mM) added to pH 2.0 buffer. Low concentrations [^{14}C]oxalate are detected as a grey streak on the autoradiogram while high concentrations of [^{14}C]oxalate migrate further and as a

more discrete spot because there is more oxalate than contaminating Ca^{2+}. Electrophoresis at pH 3.5 used a buffer of acetic acid:pyridine:water 10:1:189 v/v/v. The buffer used for wetting the paper was half the concentration of the trough buffer. Typical electrophoresis conditions were 3 kV (approximately 120 mA) for 1 hour unless otherwise stated.

For purification of radiolabelled metabolites, samples of 1 ml were loaded over 30 cm on Whatman 3MM prior to electrophoresis in either pH 3.5 or pH 6.5 buffer.

The mobility of compounds separated by 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 the reference point for calculation of the mobility of acidic compounds. Relative mobility (m_{OG}) was calculated as the distance migrated from a neutral marker divided by the distance migrated by orange G from a neutral marker.

2.4.2 Paper chromatography

Paper chromatography was used to resolve compounds that were immobile during electrophoresis and for purification of radiolabelled compound F. A series of standards including ascorbate, dehydroascorbate, diketogulonate, organic acids and glucose were loaded on to Whatman No. 1 paper and subjected to chromatography in a number of different solvent systems. Resolution of the markers by descending paper chromatography was most effective when a solvent consisting of butanol:acetic acid:water (12:3:5 v/v/v) was used over a period of 24 hours. For purification of radiolabelled compound F, 1 ml of solution was loaded over 30 cm

on to Whatman 3MM paper. Three replicate chromatograms were loaded and run in butanol:acetic acid:water (12:3:5 v/v/v) for 20 hours.

2.4.3 Gel-permeation chromatography

Gel-permeation chromatography was used to separate large molecules such as polysaccharides and enzymes from small molecules such as monosaccharides and organic acids present in culture medium. Large molecules travel relatively fast through the gel matrix as they travel between the gel beads. Small molecules travel more slowly as they enter pores in the beads and therefore travel further.

A Bio-Gel P-2 column (Bio-Gel P-2 fractionation range 100–1800 Da, Bio-Rad Laboratories, Hemel Hempstead U.K.) with a bed volume of 10 ml was calibrated with blue dextran to mark the void and cobalt chloride to mark the included volume. This column was used to separate culture medium solutes into a high-M_r fraction and a low-M_r fraction. Samples (1 ml) of spent medium and boiled spent medium were applied to a Bio-Gel P-2 column and eluted with pyridine:acetic acid:water (1:1:98). The void volume, containing high-M_r compounds, and the included volume, containing low-M_r compounds, were collected. These were vacuum dried for 12 hours then redissolved in the original sample volume of 1 ml. Fractions were collected manually.

A Bio-Gel P-2 column with 80-ml bed volume was used to purify radiolabelled metabolites. Blue dextran was used to mark the void and ^{3}H-labelled water to mark the included volume. The sample (3 ml) was applied to the column and eluted with water. Fractions were collected using an automated fraction collector (Bio-Rad model 2110 fraction collector).

2.4.4 Anion-exchange chromatography

Anion-exchange chromatography separates compounds on the basis of charge. Anion-exchange resins bind negatively charged compounds while cation-exchange resins bind positively charged molecules.

Dowex 1, a strongly basic anion-exchanger was washed twice in MES⁻ buffer (0.5 M, pH 7.5, Na⁺) for 1 hour each. It was then rinsed 3 times in MES (10 mM, pH 5.5, Na⁺). The prepared Dowex 1 resin (20 μl) was added to a solution of [^{14}C]dehydroascorbate (100 μl, section 2.5.1) and shaken gently for 5 minutes. The resin was sedimented by centrifugation at 3000 rpm for approximately 10 seconds. The supernatant was used as a solution of compounds with no affinity for Dowex 1 such as [^{14}C]dehydroascorbate. Acidic compounds such as [^{14}C]diketogulonate and [^{14}C]ascorbate remained bound to the anion-exchange resin. Any [^{14}C]diketogulonate or other acidic compounds in the supernatant were either present as a result of incomplete removal or through rapid regeneration of these compounds on removal of the anion-exchange resin.

2.5 Detection of standards and metabolites

2.5.1 Preparation of standards for electrophoresis and chromatography

Ascorbic acid (10 mM) was stored in 25-μl aliquots at –20°C until required as a marker for paper electrophoresis or chromatography.

Ascorbate oxidase-impregnated spatulas (Roche Diagnostics Ltd, Lewes U.K.), were used to prepare dehydroascorbic acid from ascorbic acid. Spatulas were immersed in a solution of ascorbic acid (10 mM, 14 ml, pH 3.13) and stirred rapidly

at room temperature for 1 hour. The solution was divided into 0.5-ml aliquots and stored at –20°C.

Diketogulonate was prepared by alkaline hydrolysis of dehydroascorbic acid (White and Krupka 1965). Dehydroascorbic acid solution (0.5 ml, 10 mM) was incubated at room temperature for 5 minutes with NaOH (0.1 M) then neutralised with acetic acid (0.2 M) and used immediately.

Oxalic acid marker was prepared as a 1% w/v solution. All other markers used were 0.5% w/v solutions. A mixture of markers was prepared as a mixture of 0.5 ml of each individual marker: glucose (0.08% w/v), 2-keto-D-gluconic acid (0.08% w/v), threonic acid (0.08% w/v), meso-tartaric acid (0.08% w/v), L-tartaric acid (0.08% w/v) and oxalic acid (0.17% w/v).

2.5.2 Detection of standards by staining with silver nitrate

A series of three solutions was used to stain organic acids such as ascorbic acid, its potential breakdown products and non-acidic compounds including dehydroascorbate (Trevelyan et al. 1950).

Chromatograms or electrophoretograms were dipped once through a solution of silver nitrate (5 mM in acetone) and allowed to dry for 15 minutes. The papers were then twice dipped through NaOH (0.125 mM in 96% ethanol) with 15 minutes' drying following each dip. Papers were then drawn steadily through the final solution, an aqueous solution of sodium thiosulphate (10% w/v) and immediately transferred to a basin of running tap-water and washed for approximately 1 hour.

2.5.3 Detection of standards by staining with bromophenol blue

Bromophenol blue changes from yellow at pH 2.8 to violet at pH 4.5. It was used to detect oxalate and other organic acids after electrophoresis (Jork et al. 1990, Fry 1988). Bromophenol blue (40 mg) was dissolved in NaOH (10 mM, 100 ml). The paper was dipped three times through a solution methanol (25%) in diethyl ether (75%) with 5 minutes' drying following each dip to remove any residual acetic or formic acid. The paper was then gently and evenly sprayed with bromophenol blue. Any yellow spots appearing on the blue background were marked with a soft pencil. Bromophenol blue stain was preferred for staining electrophoretograms run in pH 2.0 buffer because the detection of oxalate was better than when silver nitrate stain was used.

2.5.4 Detection of standards by staining with bromothymol blue

Bromothymol blue was used to detect acidic compounds separated by electrophoresis in pH 6.5 buffer. As a pH indicator, bromothymol blue changes colour from yellow at pH 6 to blue at pH 8. Bromothymol blue was prepared and used as for bromophenol blue.

2.5.5 Detection of radiolabelled metabolites by autoradiography

Radiolabelled metabolites present in culture medium and separated by either paper chromatography or paper electrophoresis were detected by autoradiography using Kodak BioMax MR-1 film. Exposure times ranged from 60 hours to 2 weeks.

2.5.6 Quantification of radioactivity by scintillation counting

Scintillation counting was performed on a Beckman LS 6500 multi-purpose scintillation counter. 'OptiPhase Hisafe' (Fisher Chemicals) scintillation fluid (10 volumes) was added to liquid samples. Radiolabelled compounds separated by electrophoresis were located by autoradiography. The area of the electrophoretogram corresponding to each radiolabelled compound was removed and radioactivity quantified by scintillation counting in 'OptiScint Hisafe 3' scintillation fluid (2 ml).

2.5.7 Elution of compounds from paper electrophoretograms and chromatograms

The area of paper encompassing each radiolabelled compound located by autoradiography was cut out and placed in a 5-ml syringe barrel. The syringe barrel was suspended in a 15-ml centrifuge tube and the paper moistened with water. The compound of interest was collected in the centrifuge tube after centrifugation for 5 minutes at 3000 g. The paper was re-moistened and re-centrifuged up to 6 times to ensure that elution was essentially complete (Eshdat and Mirelman 1972).

2.6 Fate of exogenous ascorbate added to rose cultures

2.6.1 Preparation of experimental cultures

The fate of radioactive ascorbate was examined in buffered, experimental rose cell suspension cultures of different ages. Experimental cultures (45 ml) were prepared by the addition of MES buffer (Na^+, 50 mM, 5 ml) with pH adjusted to that of the spent medium of the culture in question (pH 6.0 to 7.0).

2.6.2 Addition of ascorbate to experimental cultures

[1-^{14}C]Ascorbate (0.1 μCi, 13 Ci/mol) mixed with non-radioactive ascorbate (100 mM) was added to experimental cultures to give a final concentration of 1 mM (specific radioactivity = 0.079 μCi/mol). The cultures were stoppered with foam bungs and shaken at 125 rpm at room temperature for up to 8 hours.

2.6.3 Analysis of culture medium

Samples of culture medium were obtained at intervals throughout this period as follows. Cultures were removed from shaking and the cells allowed to settle; duplicate 0.5-ml samples of clear medium were then removed. The concentration of ascorbate was measured by volumetric titration with DCPIP (see section 2.3.1) and total radioactivity was measured by scintillation counting.

2.7 Analysis of radiolabelled metabolites of ascorbate

2.7.1 Purification of radiolabelled metabolites from electrophoretograms

Radiolabelled metabolites of [1-^{14}C]ascorbate were eluted from electrophoretograms using the syringe barrel method (section 2.5.7) and vacuum dried overnight in a Savant AES1010 automatic environment Speed Vac system on low heat with no radiant cover. Radiolabelled metabolites were re-dissolved in water to a total volume of 480 μl.

2.7.2 Identification of compound G

Replicate samples of partially purified compound G (20 μl; 19 000 cpm) were mixed with non-radioactive oxalate (100 μg) and each applied to Whatman 3MM paper for electrophoresis in pH 2.0 buffer containing thiosulphate (5 mM) at 3 kV

for 30 minutes. Radiolabelled compound G was detected autoradiography for 1 week. Non-radioactive oxalate was detected by staining with silver nitrate, bromophenol blue or bromothymol blue.

2.7.3 Analysis of radiolabelled compound F

The mobility of radiolabelled compound F (~8 300 cpm) was compared with the mobilities of internal markers of threarate and erythrarate (0.1 mg) and ketomalonic acid by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes. The mobility of radiolabelled compound F after electrophoresis in pH 3.5 buffer at 3 kV for 30 minutes was compared with the mobilities of non-radiolabelled internal markers (0.1 mg) of glyoxylate, glycollate and glycerate. Radiolabelled compound F was subjected to electrophoresis in pH 2.0 buffer containing thiosulphate (5 mM) at 3 kV for 30 minutes to allow comparison of its mobility with various non-radiolabelled external markers (0.1 mg) including dihydroxyfumarate, oxaloacetate, malate, fumarate, succinate, threonate, and 2-ketogluconate. Radiolabelled compounds were detected by autoradiography for 1 week. Non-radiolabelled markers were detected by staining with silver nitrate or bromophenol blue.

2.7.4 Analysis of radiolabelled compound E

Radiolabelled compound E (50 μl; ~11 400 cpm) was treated with NaOH (1 M, 5 μl) overnight at room temperature then neutralised with acetic acid (2 M, 5.5 μl). A control sample (50 μl) was treated with acetic acid (2 M, 5 μl) overnight at room temperature then neutralised with NaOH (1 M, 5.5 μl). A sample of each (20 μl) was mixed with either threonate or oxalate as a non-radiolabelled internal marker then subjected to electrophoresis in pH 6.5 buffer containing EDTA at 3 kV for 30

minutes. Radiolabelled compounds were detected by autoradiography for 1 week. Non-radiolabelled markers were detected by staining with silver nitrate.

2.7.5. Analysis of radiolabelled compounds C and D

Radiolabelled compounds C and D were eluted from electrophoretograms as a mixture of C and D.

A sample of radiolabelled compounds C and D (20 μl; 9 000 cpm) was subjected to electrophoresis in pH 6.5 buffer at 3 kV for 45 minutes to resolve radiolabelled compounds C and D and compare their mobility to the mobilities of various non-radiolabelled external markers (0.1 mg) including threarate, erythrarate, glyoxylate and glycerate. Radiolabelled compounds were detected by autoradiography for 1 week. Non-radiolabelled compounds were detected by staining with silver nitrate or bromophenol blue.

2.7.6 Alkali treatment of radiolabelled compounds C, D, E and F

Individual samples of radiolabelled compounds C (and/or D), E and F (each 50 μl) were treated with NaOH (1 M, 5 μl) overnight at room temperature. Samples were neutralised by the addition of acetic acid (0.2 M). A portion (20 μl) of each neutralised sample was loaded on to Whatman 3MM paper for electrophoresis in either pH 6.5 buffer containing EDTA or pH 2.0 buffer containing thiosulphate. A control sample of each radiolabelled compound (50 μl) was treated with acetic acid (0.2 M) overnight under the same temperature conditions as the NaOH-treated samples. The control samples were neutralised by the addition of NaOH (0.1 M) then subjected to electrophoresis alongside NaOH-treated samples.

2.8 Examination of the fate of [1-^{14}C]ascorbate in rose culture medium

The fate of a mixture of [1-^{14}C]ascorbate and non-radiolabelled ascorbate was examined as described in section 2.6.2. The following method details the analysis of [1-^{14}C]ascorbate and its products which was not mixed with non-radiolabelled ascorbate in rose culture medium.

2.8.1 Preparation of experimental cultures

Rose cell suspension cultures and fresh medium (45 ml) were prepared as described in section 2.6.1. Complete cultures were filtered through a double layer of muslin to remove large cell aggregates. The settled cell volume of the filtrate was adjusted to 10% of the total volume by removal of some medium. Spent culture medium was obtained by filtration of the whole culture through a double thickness of nylon mesh (pore size 100 μm) to remove all cells. Spent medium was heated on a hot plate from cold and boiled for 5 minutes then cooled to room temperature before use as boiled spent medium. Small portions of culture or medium (approximately 133–160 μl) were maintained in glass tubes (12 mm × 75 mm) at room temperature and shaken at 125 rpm.

2.8.2 Addition of exogenous [1-^{14}C]ascorbate

The fate of [1-^{14}C]ascorbate in over an 8-hour period was examined in fresh rose culture medium, complete rose culture, spent medium and boiled spent medium on three occasions.

In experiment I, dry [1-^{14}C]ascorbate (17 Ci/mol) was re-dissolved to a final concentration of 0.5 mM in buffered, 5-d-old whole culture, spent medium, boiled spent medium or fresh medium (160 μl).

In experiment II, [1-^{14}C]ascorbic acid (14 Ci/mol, 10 μl of aqueous solution) was added to buffered, 5-d-old culture medium (133 μl) to a final concentration of 0.5 mM. The potential role of enzymes in ascorbate metabolism was investigated by examination of the fate of radioactive ascorbate in high and low molecular weight fractions of culture medium. [1-^{14}C]Ascorbate (14 Ci/mol, 10μl) was added to a final concentration of 0.5 mM to high-M_r and low-M_r fractions (133 μl). Samples were removed and analysed as described in section 2.8.3.

In experiment III, the fate of [1-^{14}C]ascorbate (14 Ci/mol, 10 μl of aqueous solution) giving a final concentration of 0.5 mM was examined in buffered, 5-d-old culture medium of rose cell suspension cultures (143 μl).

2.8.3 Sampling and analysis of radioactive apoplastic metabolites

Samples of 20 μl of whole culture were filtered through glass wool packed into a yellow pipette tip to remove cells. In experiment I, the filtrate was stored on liquid nitrogen until further analysis. In experiments II and III, non-radioactive ascorbic acid (25 mM, 4 μl) was added to the filtrate in an attempt to stabilise any radioactive ascorbate remaining in the sample. Samples of fresh medium, spent culture medium and boiled spent medium (20 μl) were added directly to non-radioactive ascorbic acid (25 mM, 4μl) without filtration. All samples were stored on liquid nitrogen for the rest of the 8-hour time course prior to further analysis.

For analysis of metabolites, samples of culture medium were thawed, loaded on to Whatman 3MM paper and subjected to high-voltage electrophoresis in pH 6.5 buffer containing EDTA for 30 minutes at 3 kV. Metabolites were detected by

autoradiography for 1 week. Metabolites were quantified by scintillation counting of the areas of the electrophoretogram corresponding to metabolites.

2.9 Feeding radiolabelled metabolites of ascorbate

2.9.1 Preparation and feeding of [1-^{14}C]dehydroascorbate—experiment I

[^{14}C]Dehydroascorbate was prepared by treatment of [1-^{14}C]ascorbate (5 μCi, 50 μl) with an ascorbate oxidase-impregnated spatula for 5 minutes. [^{14}C]Dehydroascorbate (1 μCi in 10 μl) was added to a final concentration of 0.5 mM to whole rose culture, spent medium, boiled spent medium and fresh medium (133 μl) prepared as described in section 2.8.1.

2.9.2 Metabolism of ^{14}C-labelled dehydroascorbate—experiment II

[^{14}C]Dehydroascorbate was prepared by treatment of unbuffered [1-^{14}C]ascorbate (14 Ci/mol, 50 μl) with an ascorbate oxidase-impregnated spatula for 20 minutes. [^{14}C]Dehydroascorbate was fed to rose culture as described in section 2.9.1.

2.9.3 Metabolism of ^{14}C-labelled dehydroascorbate—experiment III

Dowex 1 (MES☐) anion-exchange resin (20 μl) was added to a prepared solution of [^{14}C]dehydroascorbate (100 μl, 7.14 mM) and gently shaken for 5 minutes in an attempt to remove [^{14}C]diketogulonate and any remaining ascorbate. The ion-exchange resin was sedimented by centrifugation and the supernatant (10 μl) immediately added to rose culture or culture medium (133 μl) as described in section 2.8.1.

2.9.4 Metabolism of ^{14}C-labelled diketogulonate—experiment I

[^{14}C]Diketogulonate formed by addition of [1-^{14}C]ascorbate to rose culture and culture medium (section 3.4.3) was purified from electrophoretograms, dried, then redissolved in water (60 μl). [^{14}C]Diketogulonate (0.002 μCi in 10 μl; 0.143 mM) was added to rose culture and culture medium (133 μl) prepared as described in section 2.8.1. Samples of culture medium (20 μl) taken at intervals over the 8-hour time course were stored in liquid nitrogen then analysed as described in section 2.8.3.

2.9.5 Metabolism of ^{14}C -labelled diketogulonate—experiment II

[^{14}C]Diketogulonate (5 μCi) was purified from stock [1-^{14}C]ascorbate (13 Ci/mol) that had been stored at –20°C for 2 years. Stock [1-^{14}C]ascorbate had broken down forming [^{14}C]diketogulonate and trace amounts of other radiolabelled compounds including [^{14}C]oxalate and compounds with similar mobility to compounds C/D and F. The old stock [1-^{14}C]ascorbate (5 μCi in 50 μl) was subjected to electrophoresis in pH 6.5 buffer containing EDTA to 5 mM, at 3 kV for 30 minutes and [^{14}C]diketogulonate located by autoradiography. [^{14}C]Diketogulonate was eluted from the electrophoretogram, dried and redissolved in water (50 μl) using the method described in section 2.5.7. [^{14}C]Diketogulonate (1 μCi in 10 μl, 7.7 mM) was added to rose culture and culture medium (133 μl) prepared according to the method described in section 2.8.1. Samples of culture and culture medium (20 μl) were removed at intervals over the 8-hour time course then analysed following the method described in section 2.8.3.

2.9.6 Metabolism of ^{14}C-labelled compound F

Radiolabelled compound F, formed by metabolism of [1-^{14}C]ascorbate in rose culture and culture medium (see section 3.6.5), was eluted from the electrophoretograms using the method described in section 2.5.7 then dried and redissolved in water (60 μl). Radiolabelled compound F (0.0023 μCi in 10 μl, 16.4 μM) was added to rose culture and culture medium (133 μl) prepared according to the method described in section 2.8.1. Samples of culture and culture medium (20 μl) were removed at intervals and stored on liquid nitrogen prior to analysis as described in section 2.8.3.

2.9.7 Effect of inhibitors on metabolism of radiolabelled compound F

Radiolabelled compound F (0.0046 μCi in 20 μl, 16.4 μM) was added to spent medium (120 μl), prepared as described in section 2.8.1, containing either sodium azide or dithiothreitol (10 mM). Samples of medium (20 μl) were removed to storage in liquid nitrogen at intervals over an 8-hour period. Samples were thawed at the end of the time course then analysed as described in section 2.8.3.

2.9.8 Metabolism of [^{14}C]oxalate

Experimental rose culture and culture medium (200 μl), prepared according to the method described in chapter 2.8.1, was added to a commercial preparation (Amersham Pharmacia Biotech U.K. Ltd.) of dried [^{14}C]oxalate (10 Ci/mol) to give a final concentration of 0.5 mM. At intervals over an 8-hour period samples of culture medium (20 μl) were removed to storage on liquid nitrogen until further analysis by the method described in section 2.8.3.

3. Results

3.1 Measurement of endogenous ascorbate

The concentration of ascorbate naturally occuring in the symplast of maize, rose and spinach cell suspension cultures and in the apoplast of rose cell suspension cultures was measured to compare the amounts of ascorbate present in different ages of cells and cell cultures.

3.1.1 Endogenous symplastic ascorbate

The symplastic concentration of endogenous ascorbate was measured in maize, rose and spinach cell suspension cultures, from 0-d- to 14-d-old, by titration with DCPIP.

The concentration of symplastic endogenous ascorbate in rose cultures ranged from 0.05 mmol kg^{-1} fresh weight in 0-d-old culture to a maximum of 1.06 mmol kg^{-1} fresh weight in 5-d-old cultures. The concentration of symplastic ascorbate increased rapidly in the 3 hours following innoculation (Fig. 10). Endogenous symplastic ascorbate in 14-d-old cultures dropped to 0.23 mmol kg^{-1} fresh weight, a level similar to that of 0-d-old cultures (Fig. 10).

A similar pattern was observed in maize cultures but the initial symplastic ascorbate concentration was higher, 0.47 mmol kg^{-1} fresh weight. The concentration of ascorbate was highest in 4-d-old cultures, 0.84 mmol kg^{-1} fresh weight (Fig. 10), then decreased with culture age. In 14-d-old cultures the concentration of ascorbate was the same as in 0-d-old cultures (Fig. 10).

Endogenous symplastic ascorbate in spinach cell suspension culture increased rapidly from 0.19 mmol kg^{-1} fresh weight in 0-d-old cultures to 0.62 mmol kg^{-1} fresh weight in 3-hour-old cultures (Fig. 10). The concentration of

endogenous symplastic ascorbate decreased with age from 3-hour-old cultures (Fig. 10). After 14 days the concentration of ascorbate had decreased to 0.15 mmol kg^{-1} fresh weight, similar to its original level in 0-d-old cultures (Fig. 10).
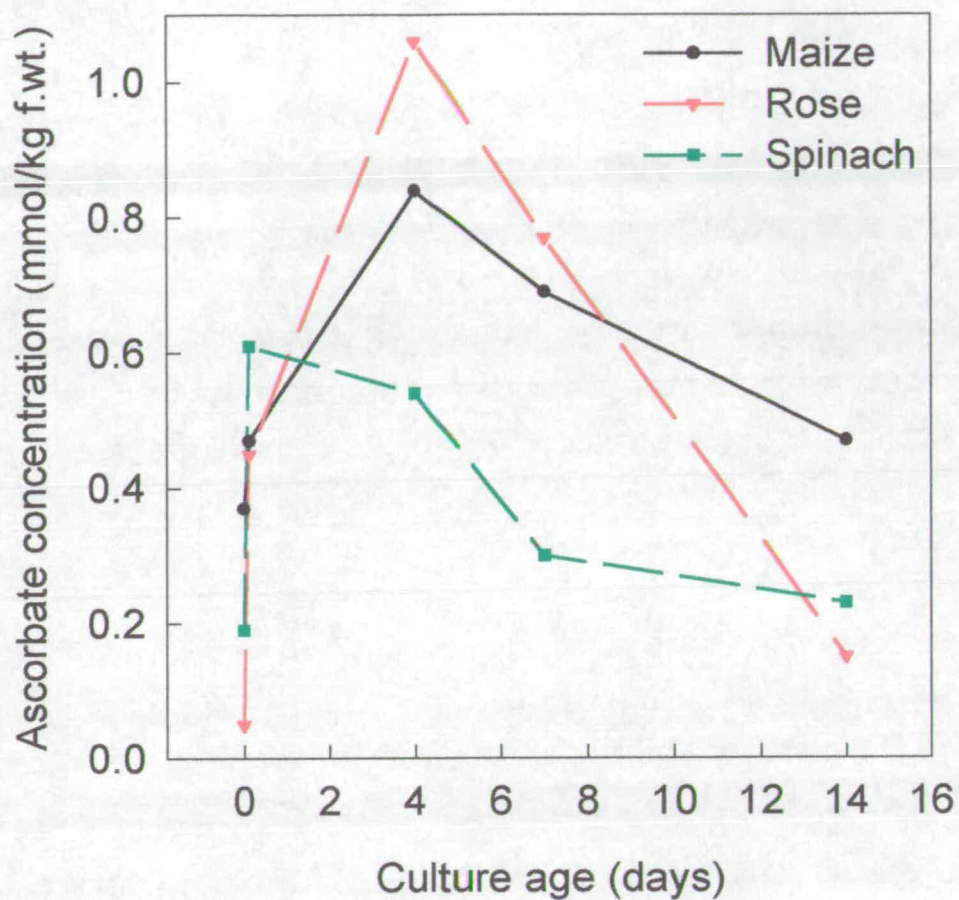
Figure 10. Concentration of endogenous, symplastic ascorbate in maize, rose and spinach cell suspension cultures.

Ascorbate concentration was measured by timed, colourimetric titration with DCPIP (0.01% w/v). Cells were filtered from suspension cultures ranging in age from 0 d to 14 d and suspended in HPO_3 (5% w/v, 50 ml).

3.1.2 Endogenous apoplastic ascorbate

Ascorbate was not detected in the medium of cell suspension cultures at concentrations greater than 10 μM. Ascorbate may be present in the apoplast at concentrations of less than 10 μM. Titration with DCPIP detects a minimum concentration of 10 μM ascorbate.

The apoplast is the fluid that bathes the plant cell wall and into which compounds from within the cell are secreted. The medium of cell suspension cultures represents the apoplast, in very dilute form. Many substances are secreted into the apoplast are present in very small amounts and when diluted in culture medium their detection is difficult.

Cell-free spent medium (50 ml) from 0-d-old to 7-d-old rose cell suspension cultures was concentrated by freeze drying then redissolved in metaphosphoric acid (5% w/v, 2 ml). The concentration of ascorbate in concentrated spent medium (0.5 ml) was measured by spectrophotometric titration with DCPIP and calculated by measuring the change in absorbance at 519 nm from 20 to 60 seconds after addition of DCPIP (0.01%, 0.4 ml).

The concentration of endogenous ascorbate in the apoplast of 0-d-old rose culture 0.5 hours after innoculation was thereby estimated at 0.52 μM (Fig. 11). This increased to 7.8 μM in 3- and 5-d-old cultures and then decreased (Fig. 11).

It was not possible to use this method the measure the concentration of endogenous apoplastic ascorbate in rose cultures older than 7 days old. The high concentration of other components of the apoplast in older cultures made it cloudy and viscous and therefore difficult to concentrate for measurements of absorbance. Ascorbate is present in the apoplast of rose cell suspension cultures. This allows the apoplastic metabolism of ascorbate to be studied using rose cell suspension cultures.
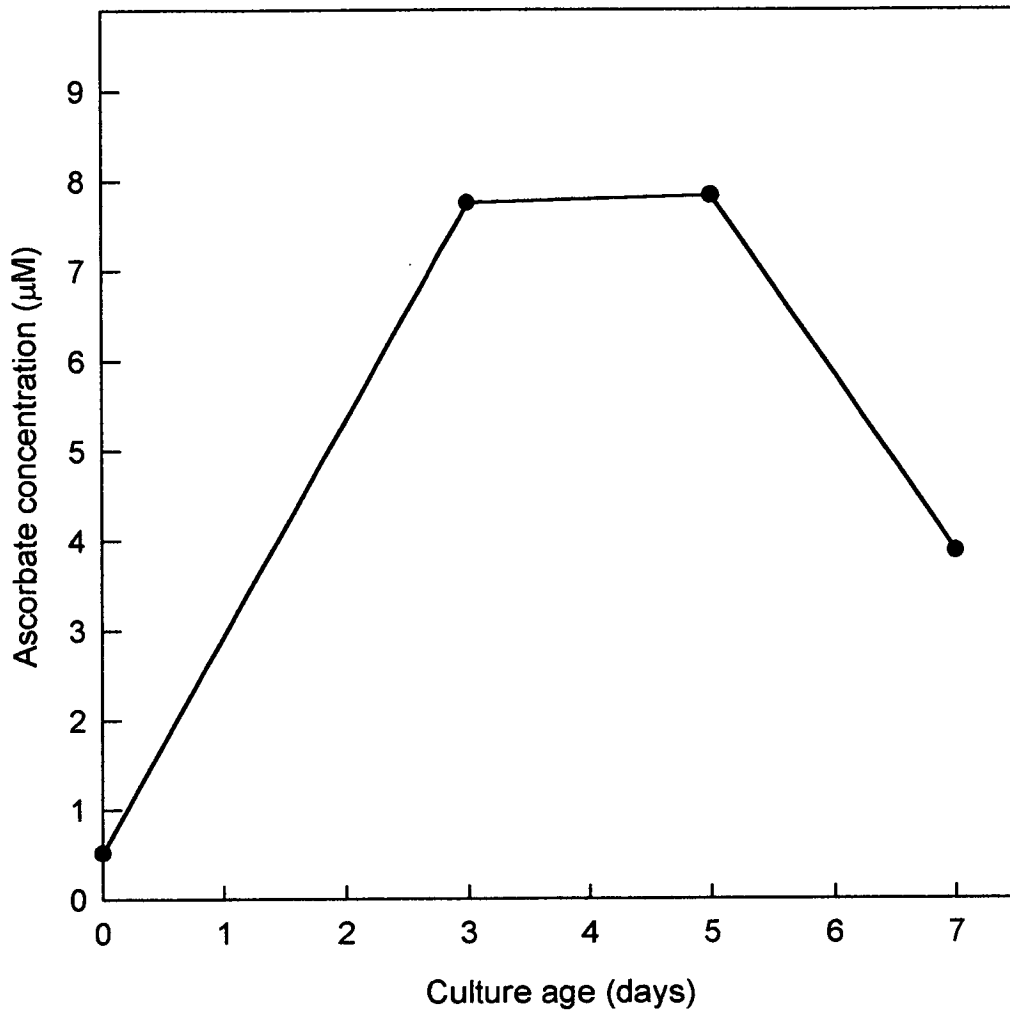
Figure 11. Concentration of endogenous apoplastic ascorbate

Concentration of ascorbate in concentrated spent medium measured by colourimetric titration with DCPIP

3.2 Removal of [1-^{14}C]ascorbate from rose culture medium

The fate of ascorbate and its metabolites were examined in rose culture medium. [1-^{14}C]Ascorbate (0.1 μCi) mixed with non-radioactive ascorbate was fed to experimental rose cultures (50 ml) to give 1 mM. At intervals samples of medium were removed and assayed for ascorbate by titration with DCPIP and for total ^{14}C by scintillation counting. This allowed the fate of ascorbate to be compared with the fate of radiolabelled compounds derived from and including ascorbate. The results obtained gave an indication of the formation and fate of metabolites of ascorbate.

Non-radioactive ascorbate was essentially completely removed from all ages of rose culture within 8 hours (Fig. 12A). Ascorbate was removed from the medium of 3- and 5-d-old cultures with a half-life of approximately 75 minutes and was not detected in the culture medium after about 4 hours. The removal of ascorbate from 0-, 9- and 14-d-old cultures was much slower, with a half-life of approximately 140 minutes. Ascorbate was removed from 1- and 7-d-old cultures with an approximate half-life of 105 minutes.

The uptake of ^{14}C-labelled compounds was calculated from the percentage remaining in the culture medium (Fig 12 B). The rate of uptake over an 8-hour period increased with age. Approximately 80% of ^{14}C remained in the culture medium of 0- and 1-d-old cultures after 8 hours. This decreased to less than 60% in 7- and 9-d-old cultures. Uptake of radiolabelled compounds was almost complete, only 15% remaining, in 14-d-old culture (Fig. 12 B). These results indicate that [1-^{14}C]ascorbate and/or its ^{14}C-labelled metabolites are taken up by rose cells.

The concentration of apoplastic radiolabelled metabolites of [1-^{14}C]ascorbate was calculated by subtraction of ascorbate concentration (DCPIP assay) from the total (starting material plus ^{14}C-labelled metabolites; calculated by

conversion of percentage of radiolabel remaining in medium to a molarity taking the specific activity of the initial 1 mM ascorbate).

Radiolabelled metabolites of ascorbate accumulated in the culture medium of 0-d-old culture over 8 hours (Fig. 12 C). Formation of radiolabelled metabolites in 1-d-old culture occurred during the first 4 hours but then the concentration of radiolabelled metabolites was constant for the remainder of the time course. A similar pattern was observed in 9-d-old culture. Radiolabelled metabolites accumulated in 3-d-old culture during the first 2 hours; some uptake by cells then occurred from 2 to 8 hours (Fig. 12 C). A high concentration of apoplastic metabolites accumulated in 5-d-old rose cultures during the first 3 hours. The concentration of radiolabelled metabolites then decreased during the last 4 hours of the time course by over 50% (Fig. 12 C). This shows that ascorbate is metabolised in the apoplast of 5-d-old rose cultures and uptake of metabolites by cells occurs. Radiolabelled metabolites accumulated in the culture medium of 7-d-old cultures during the first 5 hours (Fig. 12 C). During the last 3 hours of the time course the concentration of radiolabelled metabolites decreased, indicating that some uptake of metabolites occurred (Fig. 12 C). The concentration of radiolabelled metabolites present in the culture medium of 14-d-old culture was very low throughout the 8-hour time course (Fig. 12 C). This is due either to immediate uptake of any metabolites formed or to uptake of ascorbate.
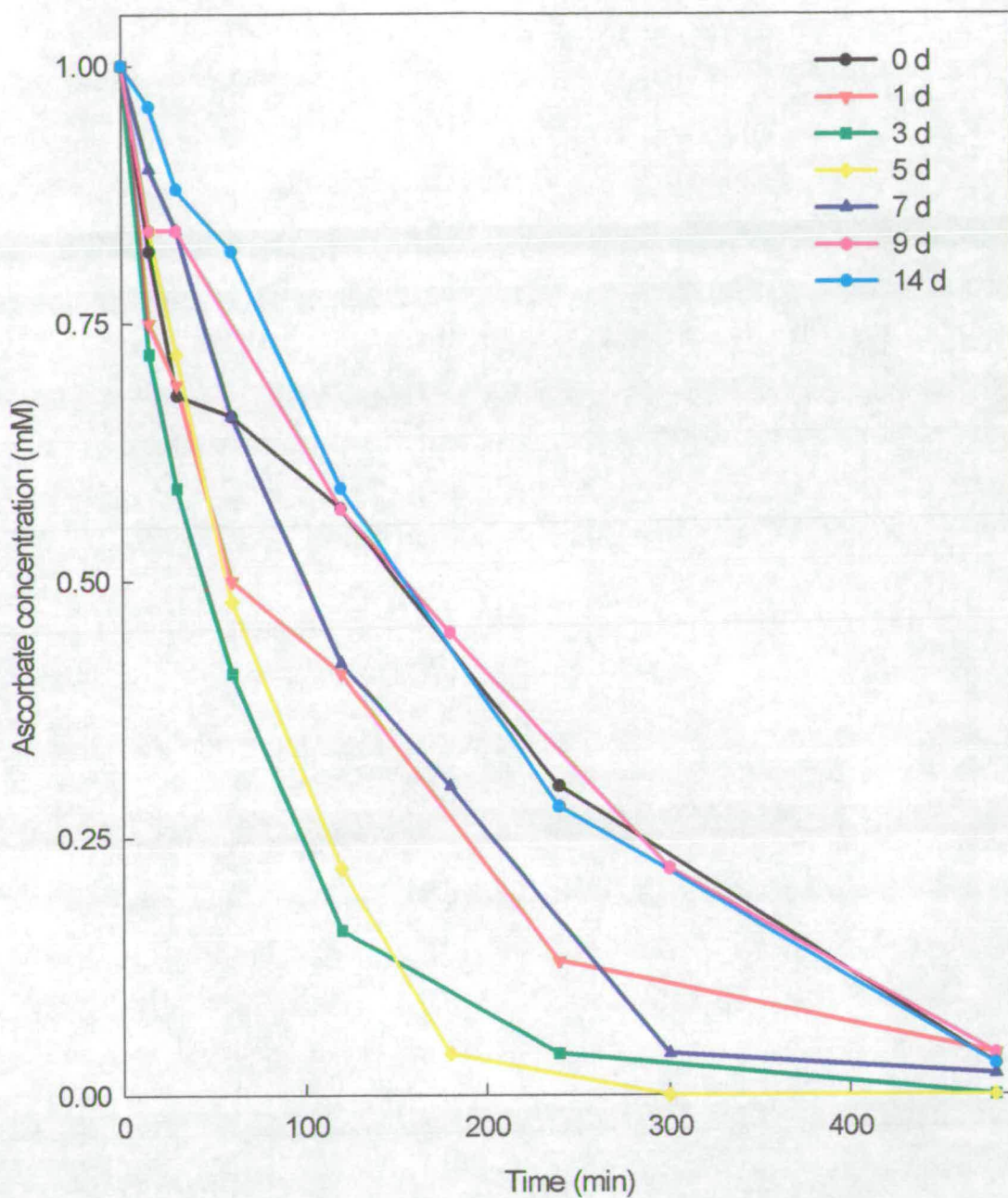
Figure 12. Removal of [1-^{14}C]ascorbate from medium of rose cultures.

A. Concentration of ascorbate remaining in culture medium measured by volumetric titration with DCPIP.
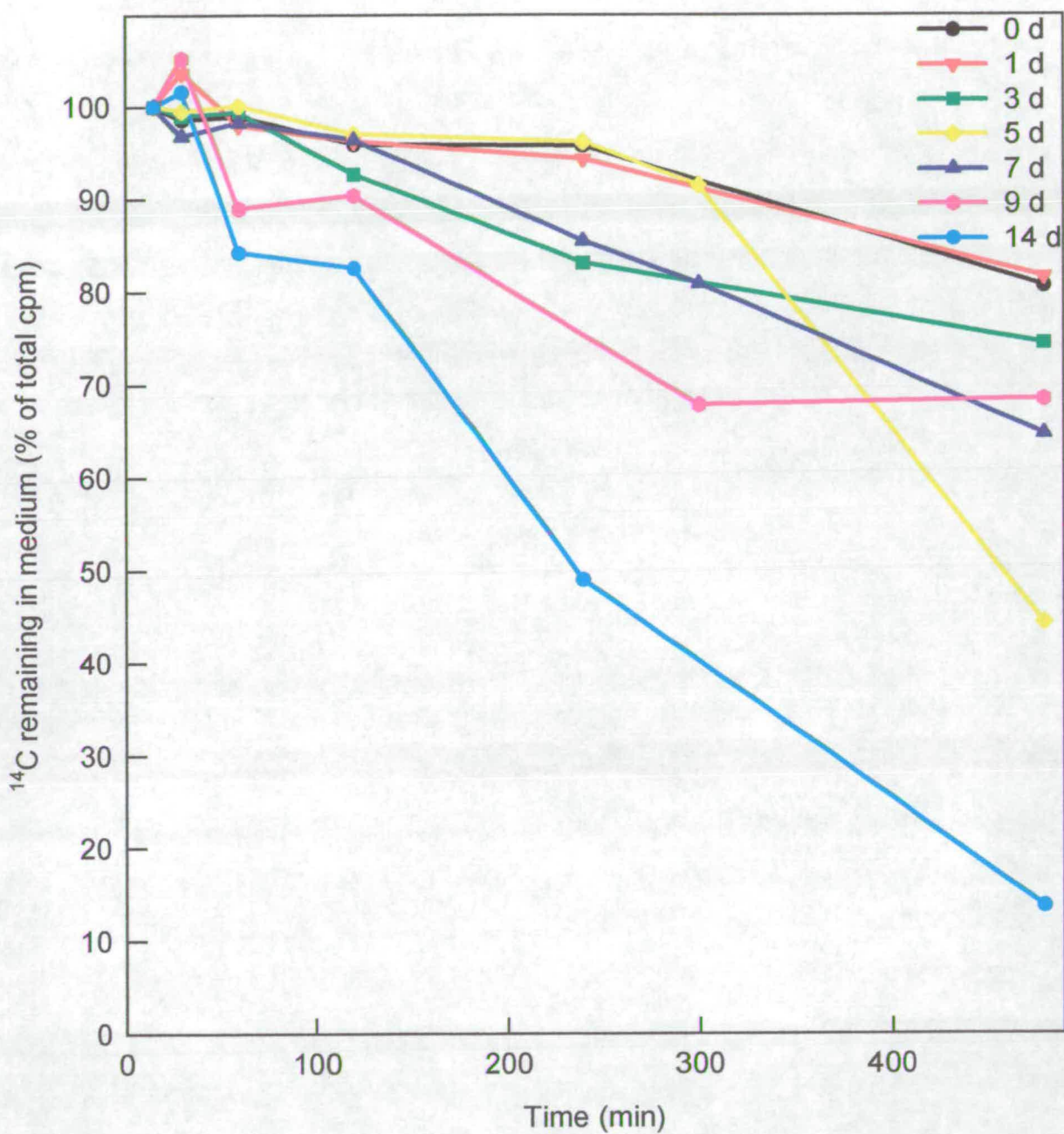
Figure 12.

B. Uptake of [1-^{14}C]ascorbate and/or its radiolabelled metabolites by rose cultures measured by scintillation counting the percentage of radiolabel remaining in culture medium.
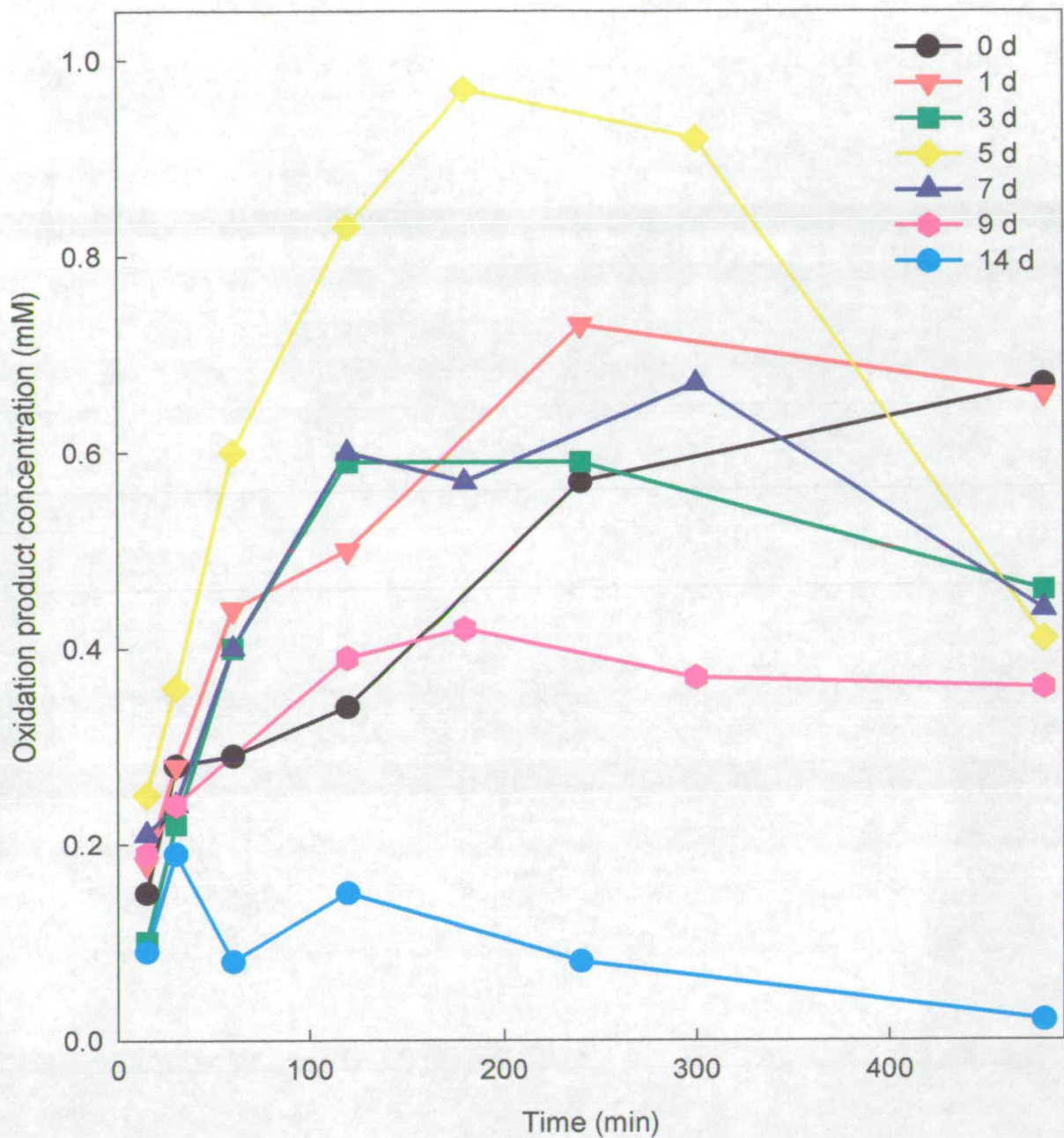
Figure 12.

C. Concentration of apoplastic metabolites of [1-^{14}C]ascorbate calculated by conversion of cpm/0.5 ml to molarity and subtraction of the molar concentration of ascorbate measured by titration with DCPIP.

The time taken for the concentration of ascorbate to decrease by half was used to estimate the rate of removal of ascorbate from whole rose cultures of age 0 to 14 d (Fig. 13 A). The rate at which ascorbate was removed from the medium reached a maximum of 0.011 mM/min in 3-d-old cultures. The rate of uptake decreased in 5- and 7-d-old culture and reached a minimum of 0.0019 mM/min in 9-d-old cultures. The rate of ascorbate removal in 9- and 14-d-old cultures was approximately equivalent to its rate in 0-d-old culture. The rapid removal of ascorbate from the medium of a 5-d-old culture (Fig. 12 A, 13 A) and the apparent formation and uptake of radiolabelled metabolites (Fig. 12 C) made 5-d-old rose cultures an interesting subject for the study of ascorbate breakdown.

The rate of uptake of $[1-^{14}C]$ascorbate and/or its metabolites was estimated by measurement of the time taken for the concentration of ^{14}C present in the culture medium to decrease by 25% (Fig. 13 B). The rate of change of concentration of radiolabelled compounds was similar in 0- and 1-d-old cultures, 0.57 and 0.52 μM/min. It increased with culture age and reached a maximum of 2.5 μM/min in 14-d-old culture (Fig. 13 B) The increase in rate of uptake of $[1-^{14}C]$ascorbate and/or its metabolites with culture age could be due to the five-fold growth of cells during the culture period. Growth phase analysis of the rose cell cultures was not performed for this work. However, most growth occurred during the first 7 days of the culture period which suggests that the increase in rate of uptake of $[1-^{14}C]$ascorbate and/or its metabolites between 7- and 14-d-old cultures was not due to continued culture growth.
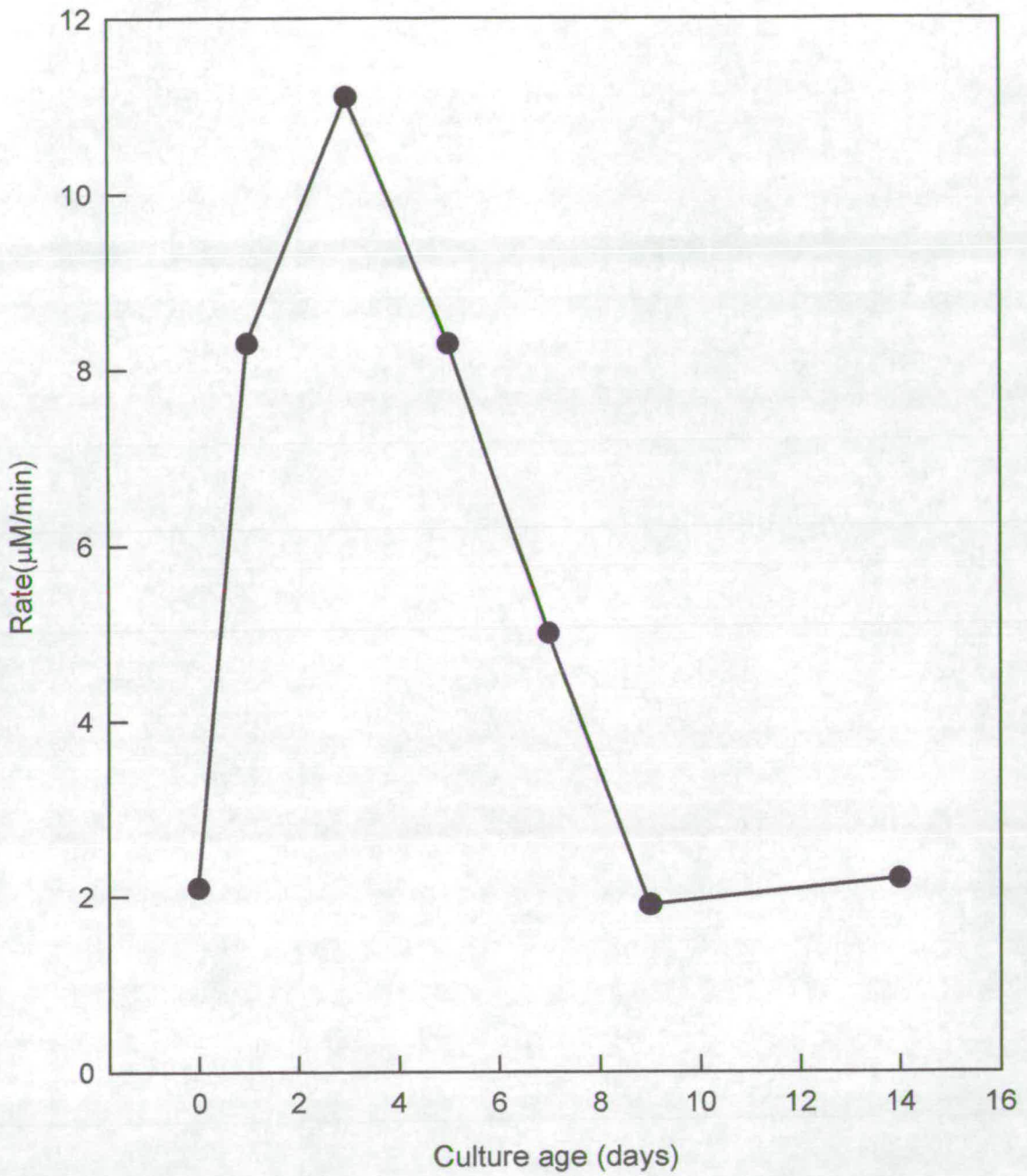
Figure 13. Rate of removal of [1-^{14}C]ascorbate and metabolites of [1-^{14}C]ascorbate in rose cultures

A. Rate of removal (uptake and/or metabolism) of ascorbate measured by titration with DCPIP.
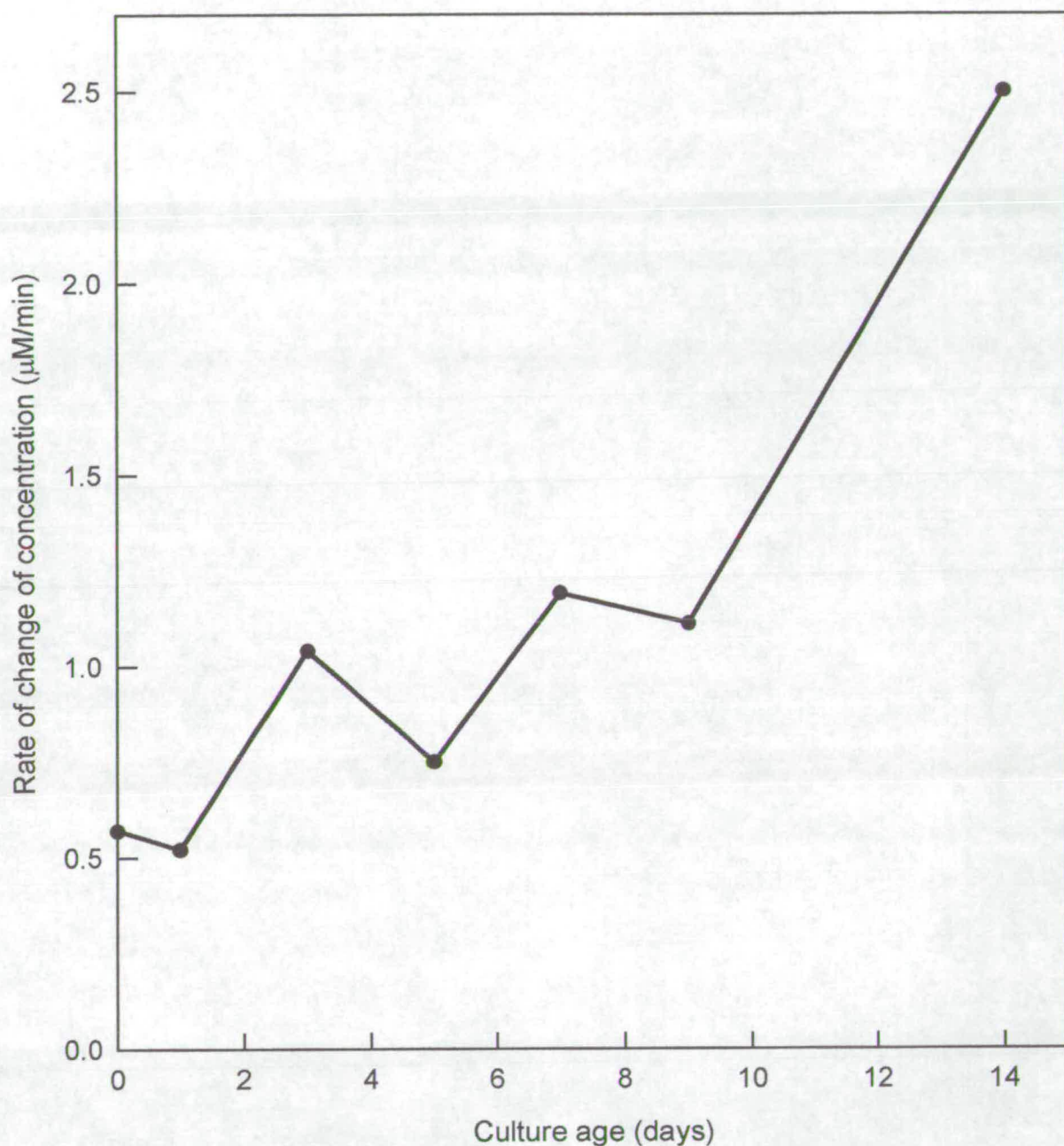
Figure 13.

B. Rate of uptake of [1-^{14}C]ascorbate and/or its metabolites calculated by measurement the change in concentration of radiolabelled compounds in culture medium. Concentration of radiolabelled compounds was calculated from the percentage of radioactivity remaining in medium (Fig. 12 B) and then converted to a molarity taking into account the specific activity of the initial 1 mM ascorbate.

3.3 Properties of metabolites of [1-^{14}C]ascorbate

3.3.1 Introduction

Addition of [1-^{14}C]ascorbate to rose culture resulted in the formation of at least eight radiolabelled metabolites (Fig. 14). These compounds were eluted from electrophoretograms, dried and redissolved in water prior to further analysis. The identity of radiolabelled metabolites was examined by comparison with internal and external non-radiolabelled markers after electrophoresis. Properties of unidentified radiolabelled metabolites were also examined. Structures of putative radiolabelled metabolites are shown in Figure 16.

At pH 6.5 most carboxyl groups are almost fully ionised and compounds carrying such groups are mobile during electrophoresis. At pH 3.5, close to the pK of many carboxyl groups, electrophoresis can result in resolution of compounds with similar m_{OG} values at pH 6.5. Compounds with low pK values, such as oxalate, ketomalonate and compound F, are ionised at pH 2.0 and can be resolved by electrophoresis at pH 2.0. Other compounds such as ascorbate are not ionised at such a low pH and are immobile during electrophoresis at pH 2.0. The mobility of compounds during electrophoresis is related their charge to area ratio (Offord 1966), calculated as charge/$M_r^{2/3}$. The predicted and measured mobilities of various markers are shown in figure 15.

The metabolism of ascorbate in fruits of grape has been shown to result in the formation of tartrate from carbons 1 to 4. Glycollaldehyde is suggested to be formed from carbons 5 and 6 of ascorbate (Williams et al. 1979). In geranium leaves, ascorbate metabolism results in the formation of oxalate from carbons 1 and 2, and threonate and subsequently tartrate (Helsper and Loewus 1982) from carbon

3 to 6 (Williams et al. 1979). The stoichiometry of possible reactions by which ascorbate and/or dehydroascorbate could result in the formation of glycollaldehyde from carbons 5 and 6 and various 4-carbon compounds is considered in Figure 17 A. Potential stoichiometries such as cleavage of the C2–C3 bond of ascorbate and/or dehydroascorbate resulting in the formation of a 2-carbon compound, derived from carbons 1 and 2, and threonate (Williams et al. 1979), derived from C3–6 have also been suggested (Fig. 17 B). Other potential stoichiometries include the cleavage of ascorbate and/or dehydroascorbate between carbons 3 and 4 yielding various compounds including glycerate, glyceraldehyde, hydroxymalonate and ketomalonate (Fig. 17 C). Stoichiometries provide an indication of the type of reaction and the likelihood of its occurrence. Hydrolysis and oxidation reactions may occur in culture medium but reduction (addition of 2[H]) is unlikely to occur in cell-free culture medium since no suitable reductant is present. Cytochrome present in the plasma membrane of living cells might facilitate reduction of compounds present medium of whole cultures.
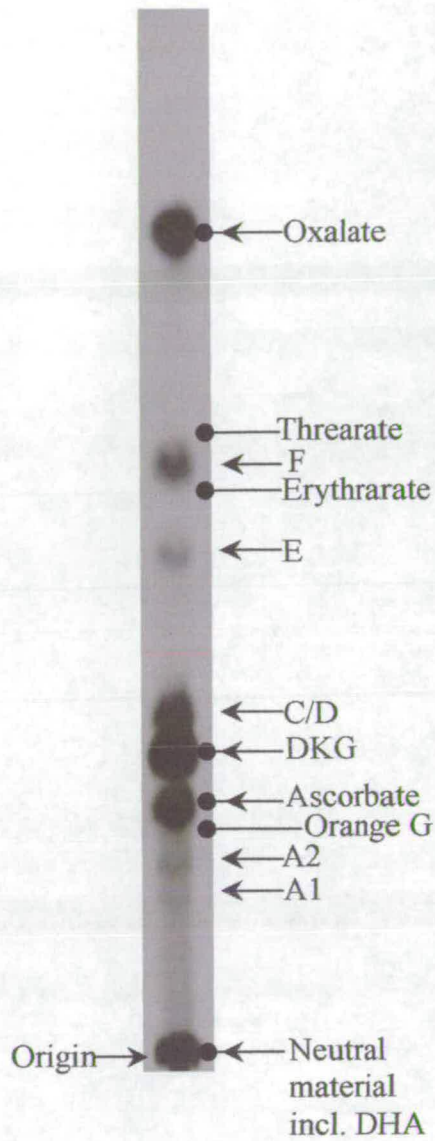
Figure 14. Radiolabelled metabolites of [1-^{14}C]ascorbate

Metabolites of [1-^{14}C]ascorbate 2 hours after addition to whole, 5-d-old rose culture. Soluble extracellular compounds were analysed by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes followed by autoradiography.

●——represents the approximate mobility of non-radiolabelled external markers
←——represents radiolabelled metabolites of [1-^{14}C]ascorbate
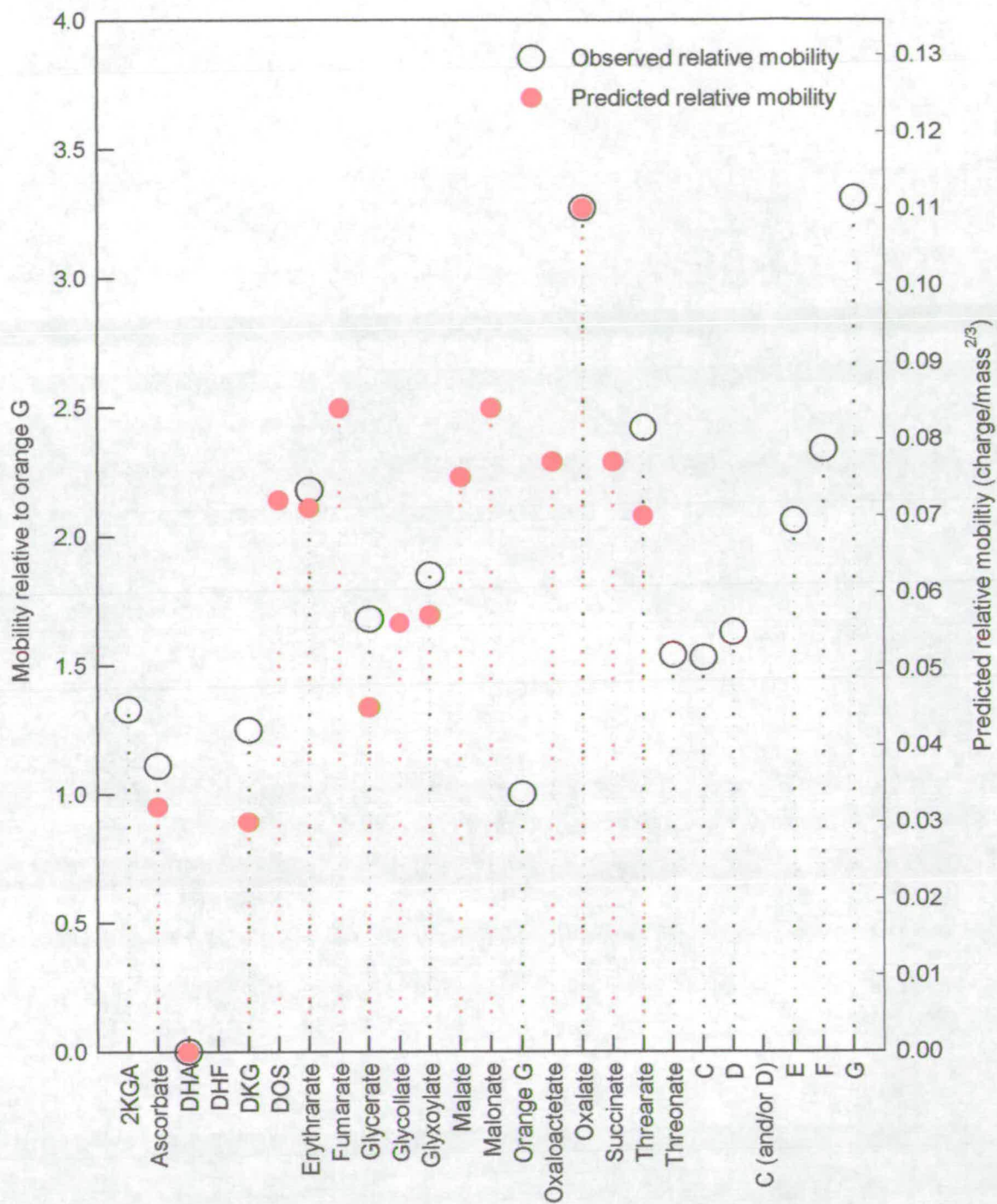
Figure 15. Electrophoretic map of measured and calculated mobilities

A. Mobility was measured as the distance moved by the compound (relative to orange G and a neutral compound) after electrophoresis at pH 6.5. Neutral compounds may migrate towards the positive electrode as a result of endo-electro osmosis. Predicted relative mobility was calculated as the charge at pH 6.5 to area ratio (charge/$M_r^{2/3}$). pK values were obtained from the suppliers chemical data. Data for observed relative mobility is absent if the compound could not be detected. Data for predicted relative mobility is absent if a pK value was unknown.
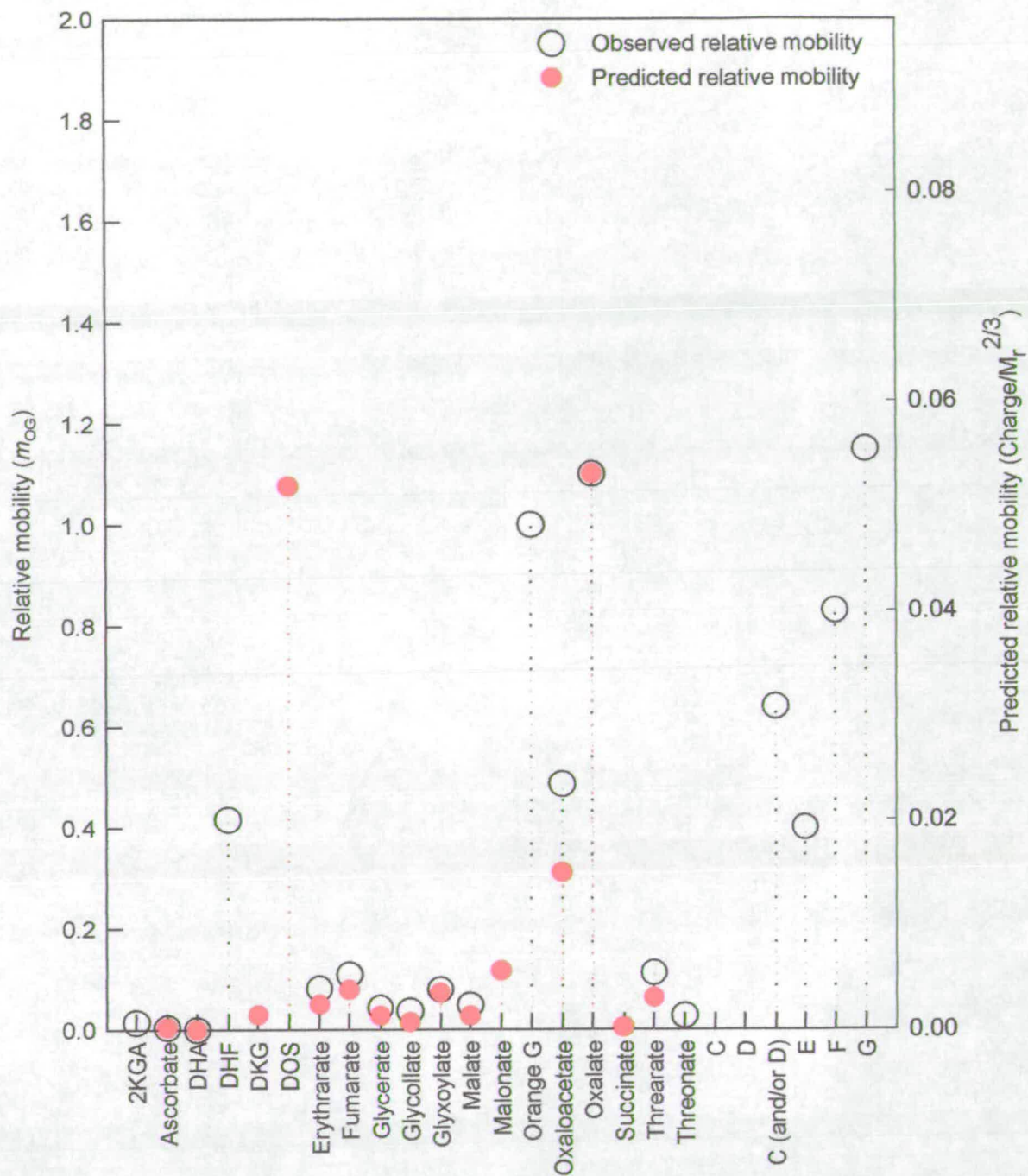
Figure 15.

B. Electrophoretic mobility measured and predicted for compounds at pH 2.0
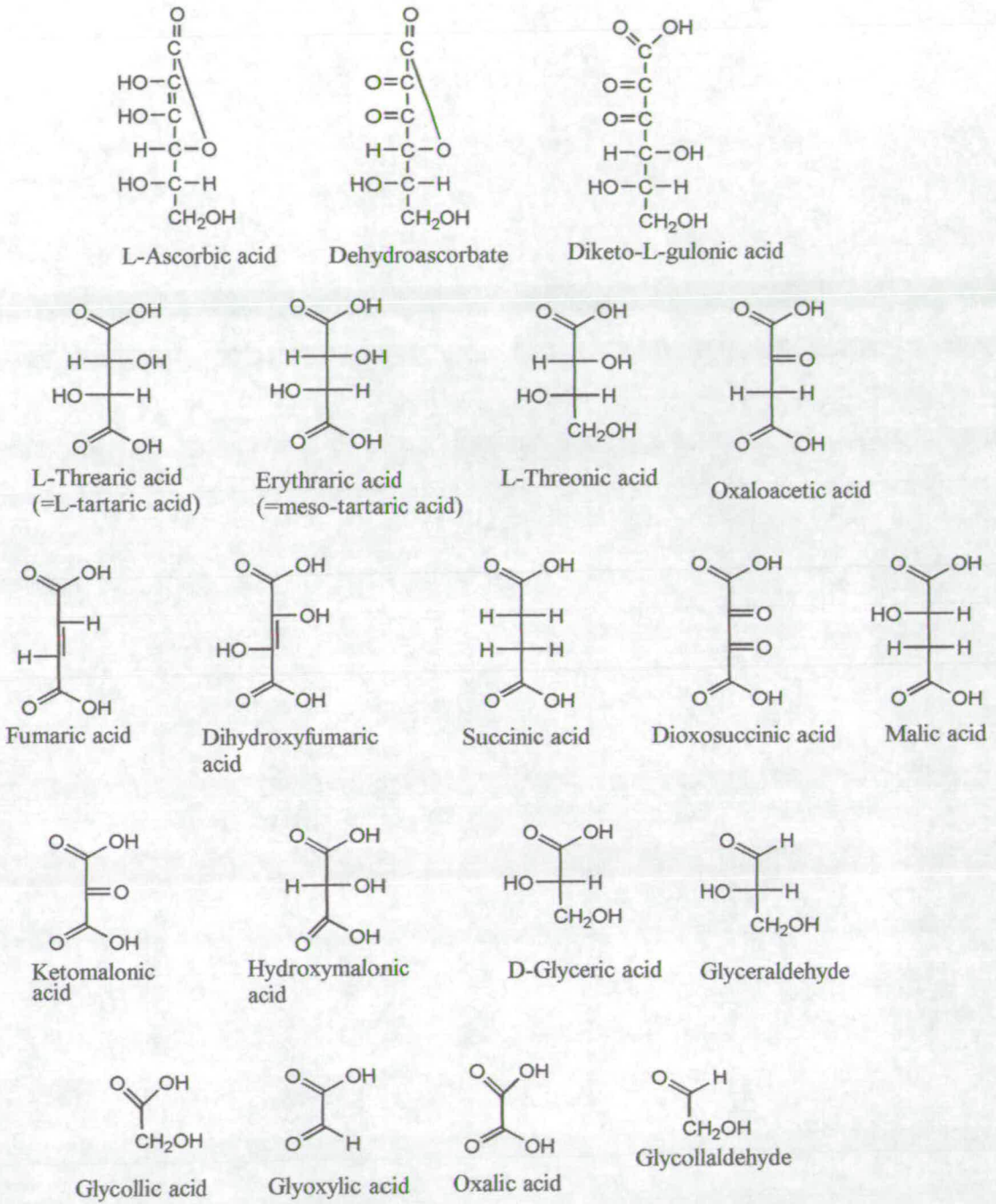
Figure 16. Structures of putative radiolabelled metabolites of ascorbate

L-Ascorbic acid $\xrightarrow{+O_2}$ Dihydroxyfumaric acid $C_4H_4O_6$
$C_6H_8O_6$

$\xrightarrow{+\frac{1}{2}O_2}$ Oxaloacetic acid $C_4H_4O_5$

$\xrightarrow{+H_2O +\frac{1}{2}O_2}$ L-Threaric acid/ Erythraric acid $C_4H_6O_6$

$\xrightarrow{}$ Fumaric acid $C_4H_4O_4$

$\xrightarrow{H_2O}$ Malic acid $C_4H_6O_5$

$\xrightarrow{H_2O +2[H]}$ L-Threonic acid $C_4H_8O_5$

$\xrightarrow{+2[H]}$ Succinic acid $C_4H_6O_4$

} + Glycollaldehyde $C_2H_4O_2$

Dehydroascorbate $\xrightarrow{+H_2O +\frac{1}{2}O_2}$ Dihydroxyfumaric acid $C_4H_4O_6$
$C_6H_6O_6$

$\xrightarrow{+2H_2O}$ L-Threaric acid/ Erythraric acid $C_4H_6O_6$

$\xrightarrow{+H_2O}$ Oxaloacetic acid $C_4H_5O_4$

$\xrightarrow{+2[H]}$ Fumaric acid $C_4H_4O_4$

$\xrightarrow{H_2O +2[H]}$ Malic acid $C_4H_6O_5$

$\xrightarrow{+4[H]}$ Succinate $C_4H_6O_4$

$\xrightarrow{+H_2O +4[H]}$ L-Threonic acid $C_4H_8O_5$

} + Glycollaldehyde $C_2H_4O_2$

Figure 17. Stoichiometry of putative reactions for metabolism of ascorbate and dehydroascorbate

A. Cleavage of ascorbate or dehydroascorbate between carbons 4 and 5 to yield glycollaldehyde (C5 and C6) and various 4-carbon compounds.

L-Ascorbic acid $\xrightarrow{\text{H}_2\text{O} + \text{O}_2}$ Oxalic acid
$C_6H_8O_6$ $\qquad\qquad\qquad\qquad\qquad$ $C_2H_2O_4$

$\xrightarrow{\text{H}_2\text{O} + \frac{1}{2}\text{O}_2}$ Glyoxylic acid $\left.\right\}$ + L-Threonic acid
$\qquad\qquad\qquad\qquad\qquad$ $C_2H_2O_3$ $\qquad\qquad$ $C_4H_8O_5$

$\xrightarrow{2\text{H}_2\text{O}}$ Glycollic acid
$\qquad\qquad\qquad\qquad\qquad$ $C_2H_4O_3$

Dehydrascorbate $\xrightarrow{\text{H}_2\text{O} + 1\frac{1}{2}\text{O}_2}$ Oxalic acid
$C_6H_6O_6$ $\qquad\qquad\qquad\qquad\qquad$ $C_2H_2O_4$

$\xrightarrow{\text{H}_2\text{O} + \text{O}_2}$ Glyoxylic acid $\left.\right\}$ + L-Threonic
$\qquad\qquad\qquad\qquad\qquad$ $C_2H_2O_3$ $\qquad\qquad$ acid $C_4H_8O_5$

$\xrightarrow{2\text{H}_2\text{O} + \frac{1}{2}\text{O}_2}$ Glycollic acid
$\qquad\qquad\qquad\qquad\qquad$ $C_2H_4O_3$
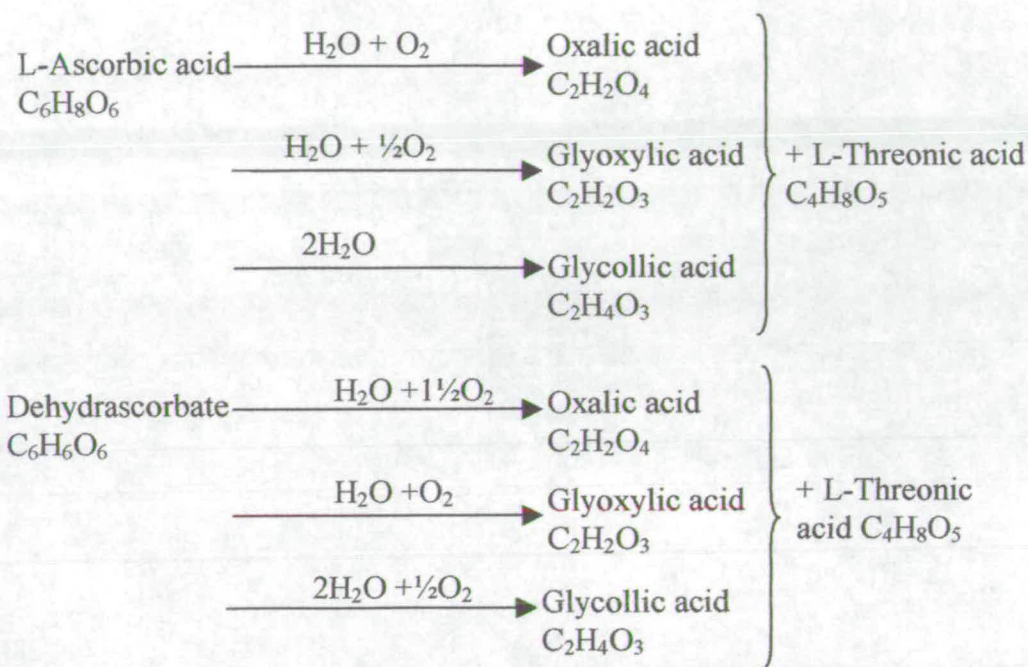
Figure 17.

B. Cleavage of ascorbate or dehydroascorbate between carbons 1 and 2 to yield threonate (C3 to C6) and glyoxylate, glycollate or oxalate

L-Ascorbic acid ——— $1\frac{1}{2}O_2$ ——→ Ketomalonic acid
$C_6H_8O_6$ $C_3H_2O_5$

——— $H_2O + O_2$ ——→ Hydroxymalonic acid $C_3H_4O_5$

+ L-Glyceric acid $C_3H_6O_4$

Dehydroascorbate ——— $H_2O + O_2$ ——→ Ketomalonic acid
$C_6H_6O_6$ $C_3H_2O_5$

——— $2H_2O + \frac{1}{2}O_2$ ——→ Hydroxymalonic acid $C_3H_4O_5$

+ L-Glyceric acid $C_3H_6O_4$

L-Ascorbic acid ——— O_2 ——→ Ketomalonic acid
$C_6H_8O_6$ $C_3H_2O_5$

——— $H_2O + \frac{1}{2}O_2$ ——→ Hydroxyketomalonic acid $C_3H_4O_5$

+ L-Glyceraldehyde $C_3H_6O_3$

Dehydroascorbate ——— $H_2O + \frac{1}{2}O_2$ ——→ Ketomalonic acid
$C_6H_6O_6$ $C_3H_2O_5$

——— $2H_2O$ ——→ Hydroxyketomalonic acid $C_3H_4O_5$

+ L-Glyceraldehyde $C_3H_6O_3$

Figure 17.

C. Cleavage of ascorbate between carbons 3 and 4 to yield L-glycerate or L-glyceraldehyde and hydroxymalonate or ketomalonate.

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3.3.2. Radiolabelled compound G is oxalate

The mobility of ^{14}C-labelled compound G (m_{OG} = 3.67, Fig. 14) derived from [1-^{14}C]ascorbate was similar to that of [^{14}C]oxalate (m_{OG} = 3.27, Fig. 18) during electrophoresis at pH 6.5. This led to the suggestion that radiolabelled compound G might be oxalate.

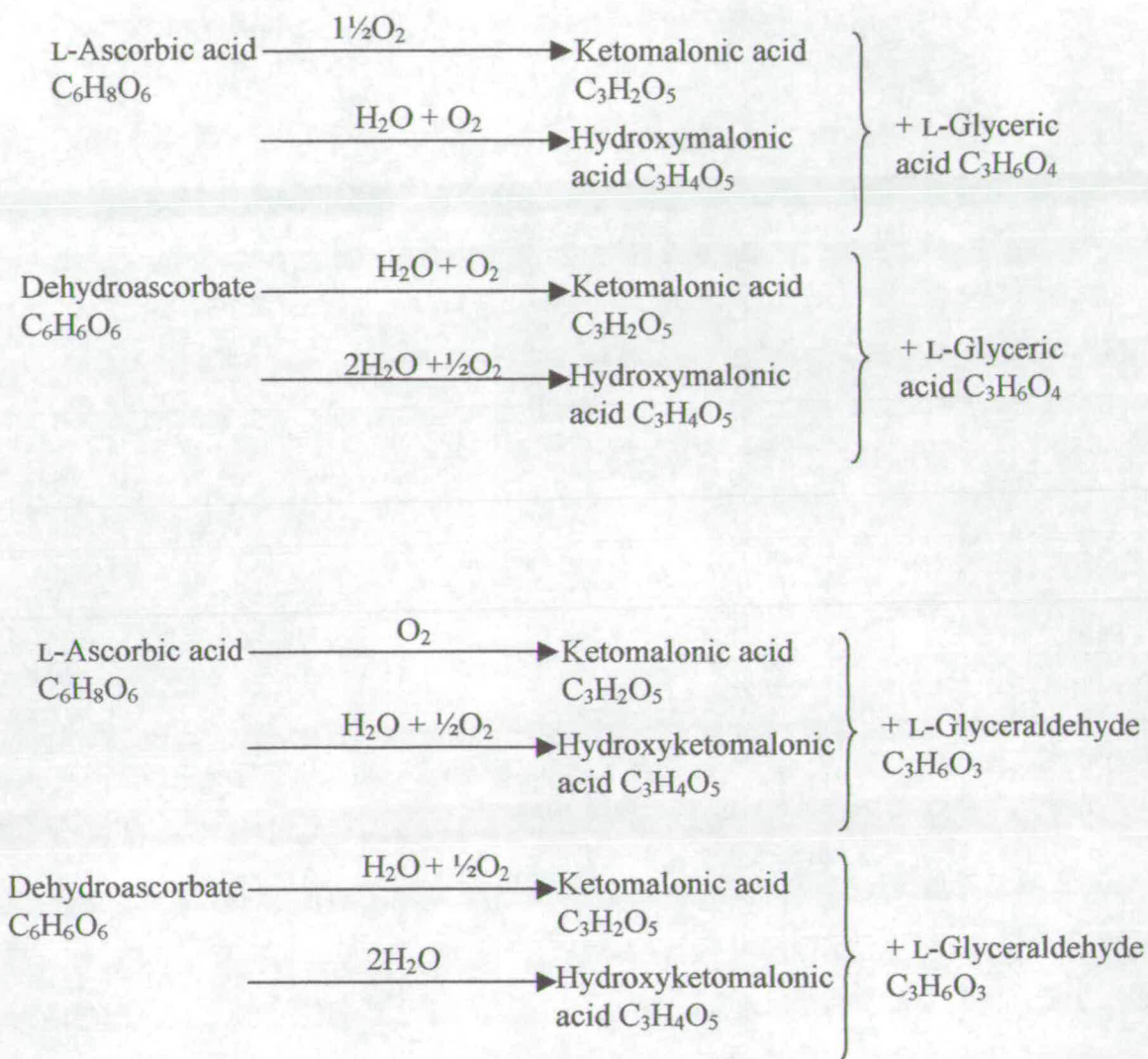[^{14}C]Oxalate readily forms a streak, possibly of insoluble calcium oxalate, during paper electrophoresis. This was observed as a radioactive streak from the origin when authentic [^{14}C]oxalate was detected by autoradiography. Formation of the streak was minimised but not prevented by the addition of a chelating agent to the electrophoresis buffer.

When radiolabelled compound G was subjected to electrophoresis in pH 2.0 buffer containing thiosulphate, a single radioactive spot (approximate m_{OG} = 1.15) was detected (Fig. 19 A) within a larger, more diffuse spot. Radiolabelled compound G co-migrated with non-radiolabelled oxalate present as an internal marker (approximate m_{OG} = 1.17, Fig. 19 B, C). Oxalate is one of the few organic acids that migrates rapidly at pH 2.0. The high electrophoretic mobility of G compared with oxalate at pH 2.0 helped to identify G as oxalate.

The metabolite referred to as radiolabelled compound G is likely to be oxalate and is derived from a portion of [1-^{14}C]ascorbate containing the radiolabelled carbon atom. [^{14}C]Oxalate is therefore most likely to be derived from carbons 1 and 2 of [1-^{14}C]ascorbate.
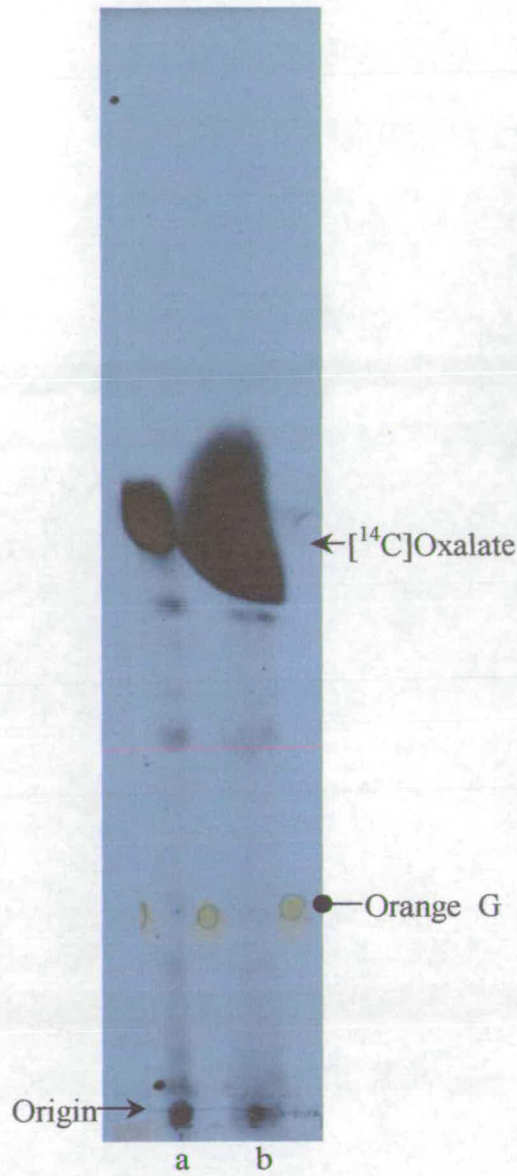
Figure 18. Mobility of authentic [^{14}C]oxalic acid.

Electrophoresis of [^{14}C]oxalic acid in pH 6.5 buffer containing EDTA at 3 kV for 30 minutes followed by autoradiography. a, [^{14}C]oxalate (0.1 µCi, 2 µl); b, [^{14}C]oxalate (0.1 µCi, 2 µl) premixed with unlabelled oxalate (0.5%, 8 µl). The unstained electrophoretogram was overlayed with the autoradiograph to allow comparison of the mobility of orange G, a non-radiolabelled external marker, with that of [^{14}C]oxalate.
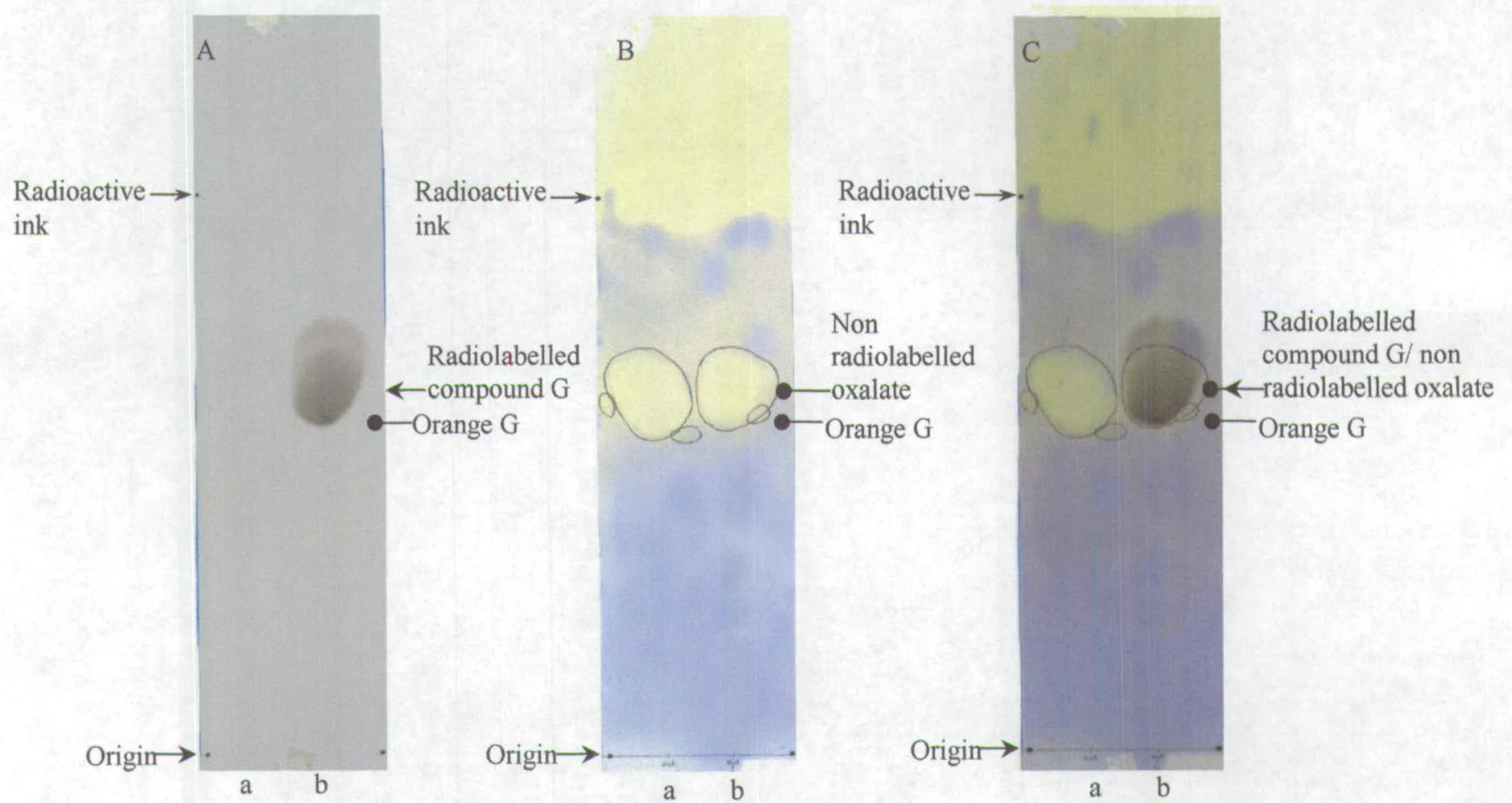
Figure 19. Radiolabelled compound G co-migrates with oxalate detected with bromophenol blue stain.

Radiolabelled compound G plus non-radiolabelled oxalate (2 mg) were subjected to electrophoresis at pH 2.0 with thiosulphate, at 3 kV for 30 minutes. Track a contained only non-radioactive oxalate, track b contained a mixture of G and non-radiolabelled oxalate Radiolabelled G was detected by autoradiography (A), non-radiolabelled oxalate by staining with bromophenol blue (B). The mobility was compared by overlaying the stained electrophoretogram with the autoradiogram (C). Radioactive ink was used to confirm the alignment of the electrophoretogram with the autoradiogram

●—— represents the mobility of external standards   ←— indicates radiolabelled compounds

3.3.3 Radiolabelled compound F

The electrophoretic mobility of radiolabelled compound F was compared with that of unlabelled standards added as internal markers in an attempt to identify compound F.

The mobility of radiolabelled compound F on electrophoresis in pH 6.5 buffer (Fig. 14, section 3.3.1) was suggestive of a compound comprising 4 carbon atoms and carrying two negatively charged groups, such as threarate or erythrarate. Such a compound would have a charge:carbon ratio of 2:4. Radiolabelled compound F was mixed with non-radiolabelled threarate or erythrarate and subjected to electrophoresis in pH 6.5 buffer. Radiolabelled compound F migrated further than erythrarate but not as far as threarate although radiolabelled compound F did overlap with both internal non-radiolabelled markers (Fig. 20). These results show that F is neither threarate nor erythrarate.

Alternatively, the mobility was suggestive of a compound with a charge:carbon ratio of 1:2, that is, comprising 2 carbon atoms with only one negatively charged group such as glycollate or glyoxylate. Radiolabelled compound F was mixed with glycollate, glyoxylate or glycerate and subjected to electrophoresis in pH 3.5 buffer. Radiolabelled compound F was highly mobile and did not co-migrate with glycollate, glyoxylate or glycerate (Fig. 21), indicating that F may have an unusually low pK.

Compound F is highly mobile during electrophoresis at pH 2.0. Its mobility was compored to another carboxylic acid—ketomalonic acid—which is also highly mobile during electrophoresis at pH 2.0 (Fig. 15). Therefore, radiolabelled compound F was mixed with non-radiolabelled ketomalonic acid (0.2 mg) and subjected to electrophoresis. At pH 6.5, radiolabelled compound F ($m_{OG} = 2.54$) did

not migrate as far as ketomalonic acid (m_{OG} = 3.11; charge:carbon = 2:3) (Fig. 22). At pH 2.0, radiolabelled compound F (m_{OG} = 0.80) was more mobile than ketomalonic acid (m_{OG} = 0.38; Fig 23), indicating that F does indeed have a very low pK and that it is clearly not ketomalonic acid.
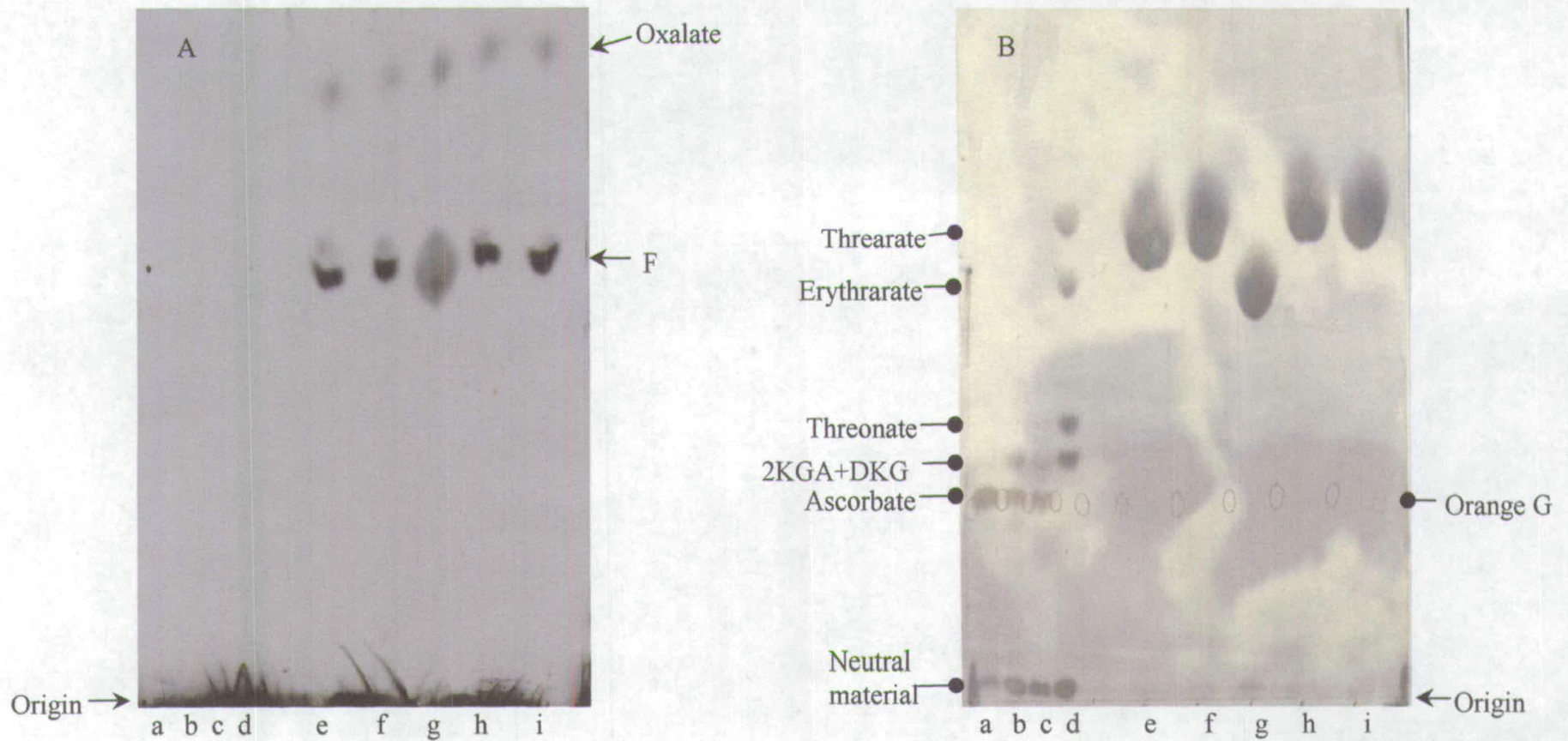
Figure 20. Comparison of the electrophoretic mobility of radiolabelled compound F, threarate and erythrarate.

Radiolabelled compound F (tracks e–i) mixed with internal markers (0.1 mg) e, f, D/L-threarate; g, erythrarate; h, D-threarate; i, L-threarate. External markers: a, ascorbate; b, DHA; c, DKG; d, mix of markers of glucose, 2KGA, threonate, erythrarate and threarate. Electrophoresis was performed in pH 6.5 buffer with EDTA at 3 kV for 30 minutes. Radiolabelled compounds were detected by autoradiography (A). Non-radiolabelled compounds were detected by silver staining (B).

●——— represents non-radiolabelled markers
←——— represents radiolabelled compounds

Figure 20, continued.

C. The silver-stained electrophoretogram (Fig. 20 B) was overlayed with the autoradiogram (Fig. 20 A). Radiolabelled compound F is highlighted in red.
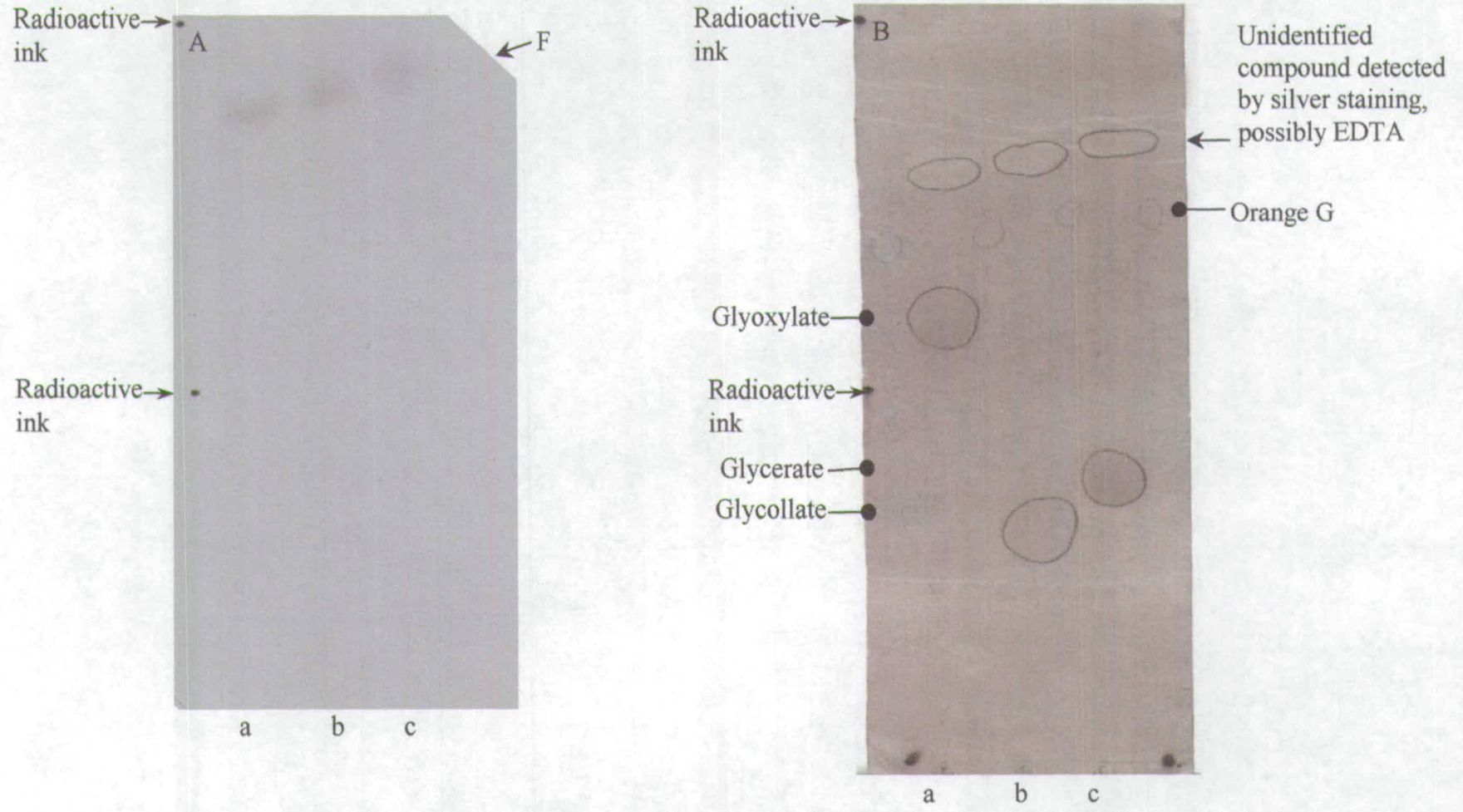
Figure 21 Comparison of radiolabelled compound F with glycollate, glyoxylate and glycerate

Radiolabelled compound F (tracks a–c) mixed with internal standards a, glyoxylate; b, glycollate; c, glycerate was subjected to electrophoresis in pH 3.5 buffer, 3 kV for 30 minutes. Oxalate would have migrated off the paper under these conditions of electrophoresis. Radioactive ink was used to align the autoradiogram with the stained electrophoretogram.

A. Radiolabelled compound F detected by autoradiography   ●—— represents non-radiolabelled marker
B. Non-radiolabelled internal markers detected by silver staining   ←—— represents radiolabelled compounds
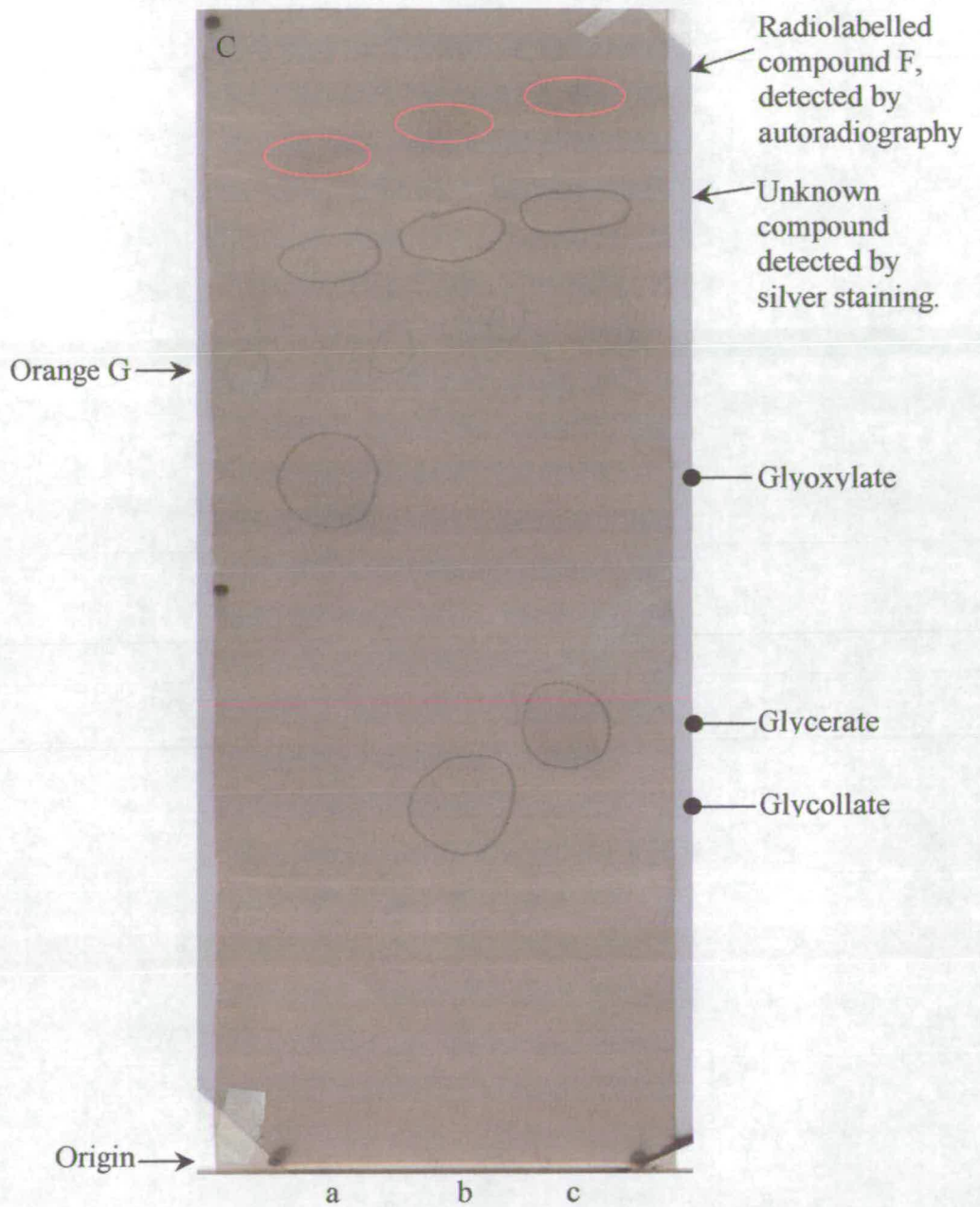
Figure 21, continued.

C. The silver nitrate-stained electrophoretogram (Fig. 21 B) was overlayed with the autoradiogram (Fig. 21 A).
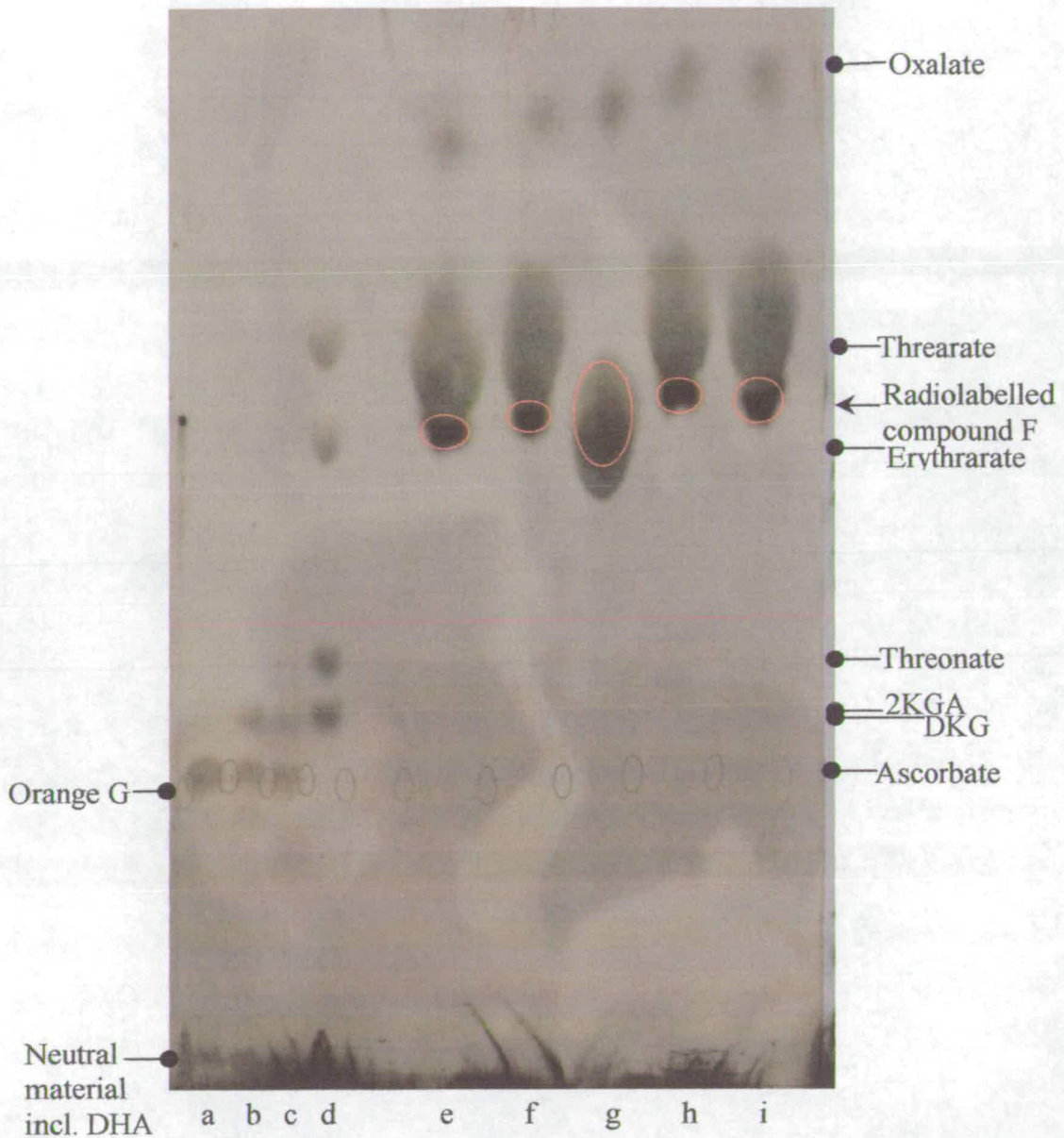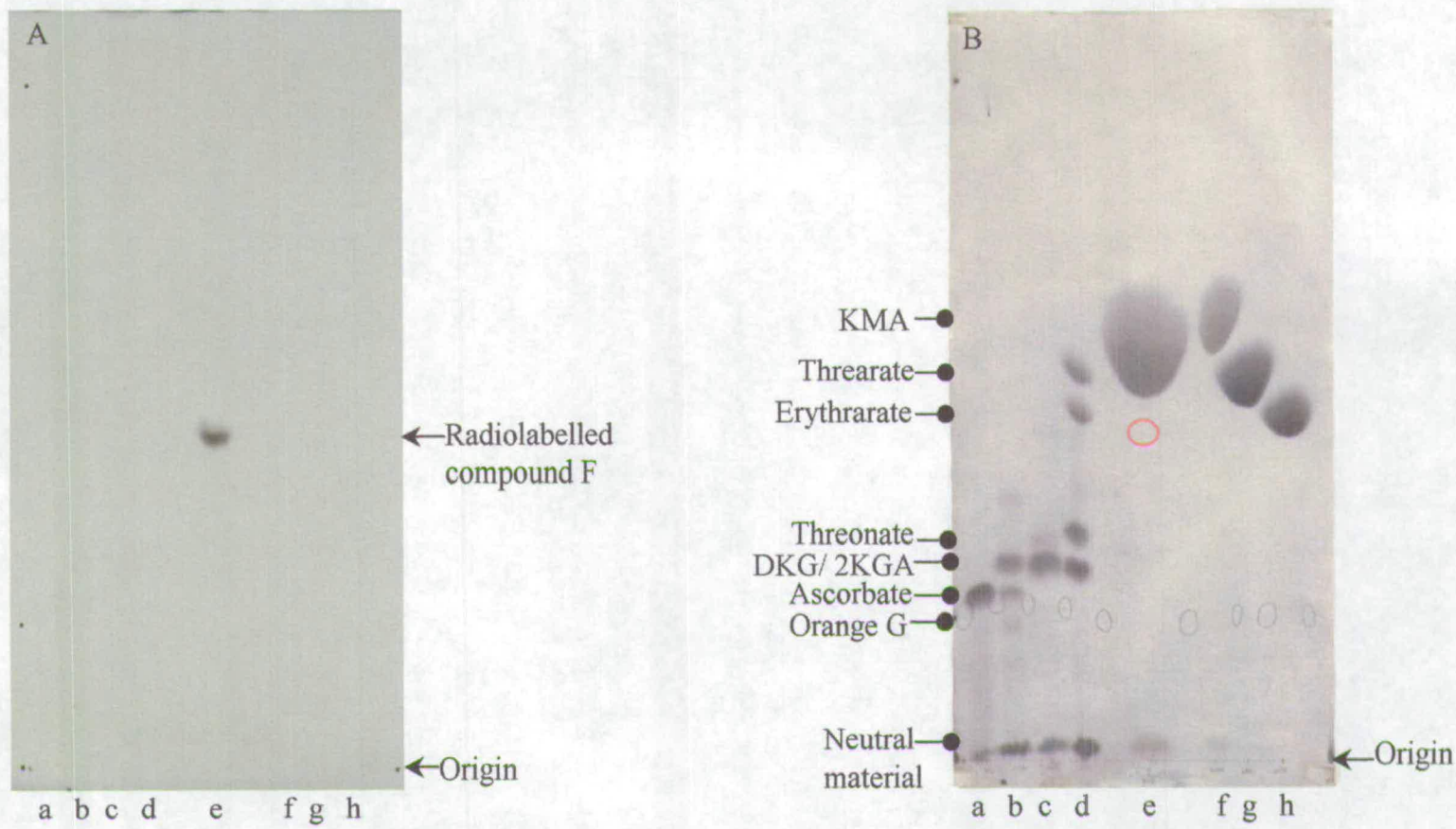
Figure 22. Comparison of the electrophoretic mobility of radiolabelled compound F, ketomalonic acid and various external markers

Radiolabelled compound F mixed with ketomalonic acid (KMA; track e): external markers a, ascorbate; b, DHA; c, DKG; d, glucose, 2KGA, threonate, erythrarate, threarate; f, ketomalonic acid; g, threarate; h, erythrarate. Each sample was analysed by electrophoresis in pH 6.5 buffer with EDTA, 3 kV for 30 minutes. A, Radiolabelled compound F and radioactive ink dots detected by autoradiography. B, Non-radiolabelled markers detected by silver nitrate staining. The position of radiolabelled compounds is outlined in red. The lower m_{OG} value of F compared to erythrarate is likely to be an artefact due to the excesive loading of ketomalonic acid.

●— represents non-radiolabelled markers
←— represents radiolabelled compounds
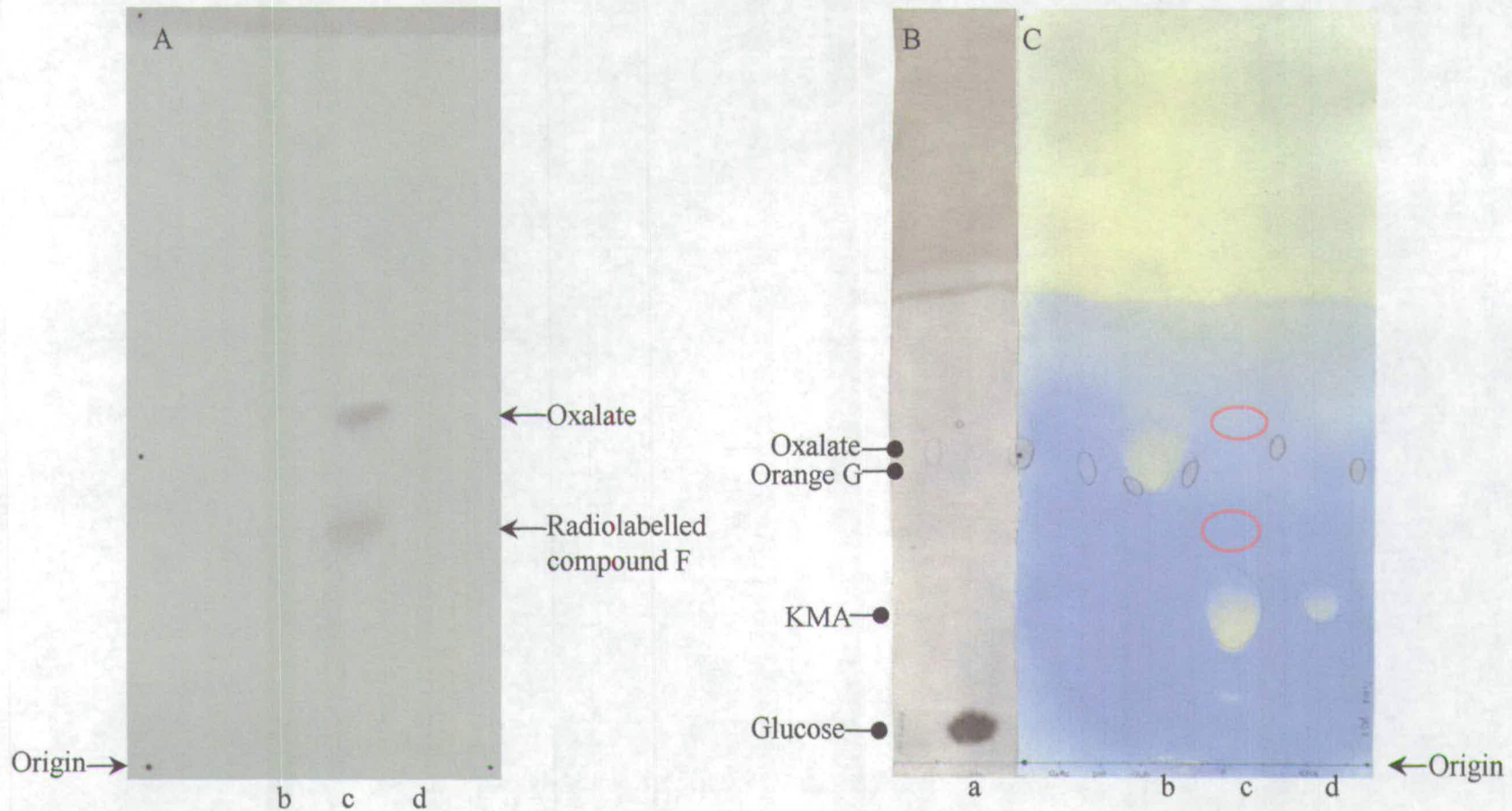
Figure 23. Mobility of radiolabelled compound F compared with glucose, oxalate and ketomalonic acid after electrophresis in pH 2.0 buffer

Radiolabelled compound F (with [^{14}C]oxalate contaminant) mixed with ketomalonic acid (track c) and external standards a, glucose; b, oxalate; c, d, ketomalonic acid (0.2 mg) were analysed by electrophoresis in pH 2.0 buffer with thiosulphate at 3 kV for 30 minutes. A. Radiolabelled compounds detected by autoradiography. B. Non-radiolabelled markers detected by silver staining. C. Non-radiolabelled markers detected by staining with bromophenol blue. The position of radiolabelled compounds is outlined in red.

●— represents non-radiolabelled markers
←— represents radiolabelled compounds

The mobility of radiolabelled compound F ($m_{OG} = 0.83$; Fig. 24 A) during electrophoresis in pH 2.0 buffer was compared with those of various external markers (Fig. 24 B), few of which were appreciably mobile.

Commercial oxaloacetate (Fig. 24 B, C, track m) and DHF (Fig. 24 B, C, track n) were each contaminated with at least two other acidic compounds. The acidic spot in each track that stained most strongly with silver nitrate was assumed to be oxaloacetate or DHF. Oxaloacetate ($m_{OG} = 0.49$), DHF ($m_{OG} = 0.42$) and oxalate ($m_{OG} = 1.10$) were electrophoretically mobile at pH 2.0 (Fig. 24). However, none of these compounds co-migrated with radiolabelled compound F (Fig. 22, 23, 24). These results show that F is highly charged at pH 2.0 and, therefore, has unusually low pK value(s).
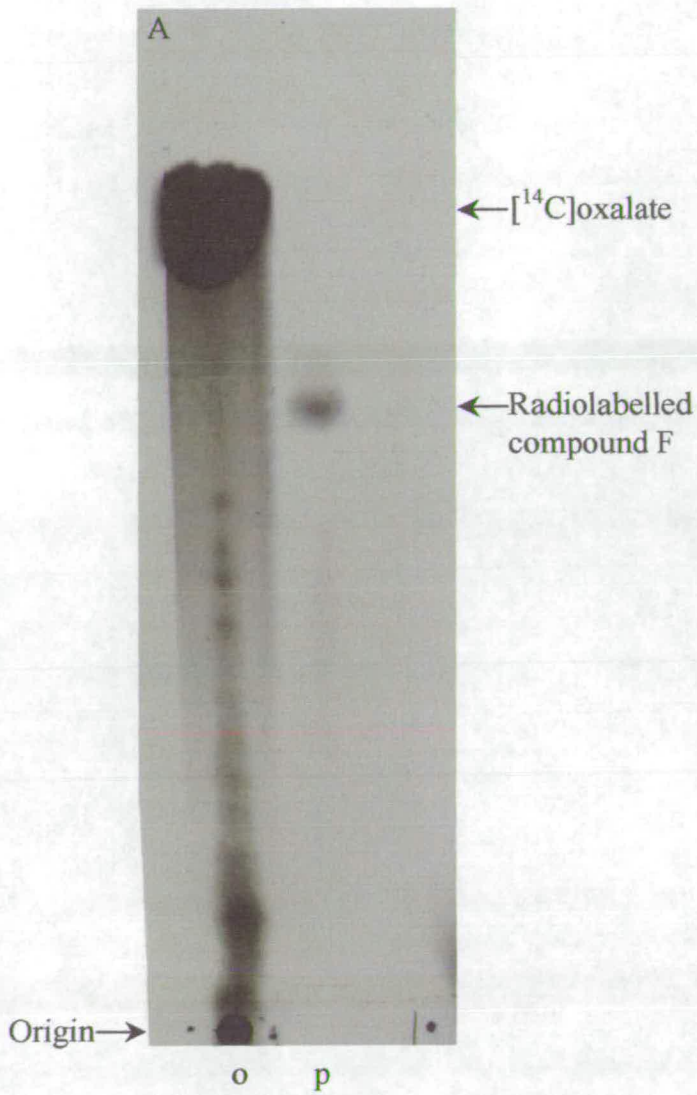
Figure 24. Mobility of radiolabelled compound F from a replicate preparation after electrophoresis in pH 2.0 buffer containing thiosulphate

A. Radiolabelled compound F (track p) and radiolabelled oxalate (track o) subjected to electrophoresis in pH 2.0 buffer with thiosulphate, 3 kV for 30 minutes. Radiolabelled compounds were detected by autoradiography

●——represents non-radiolabelled markers
←——represents radiolabelled compounds
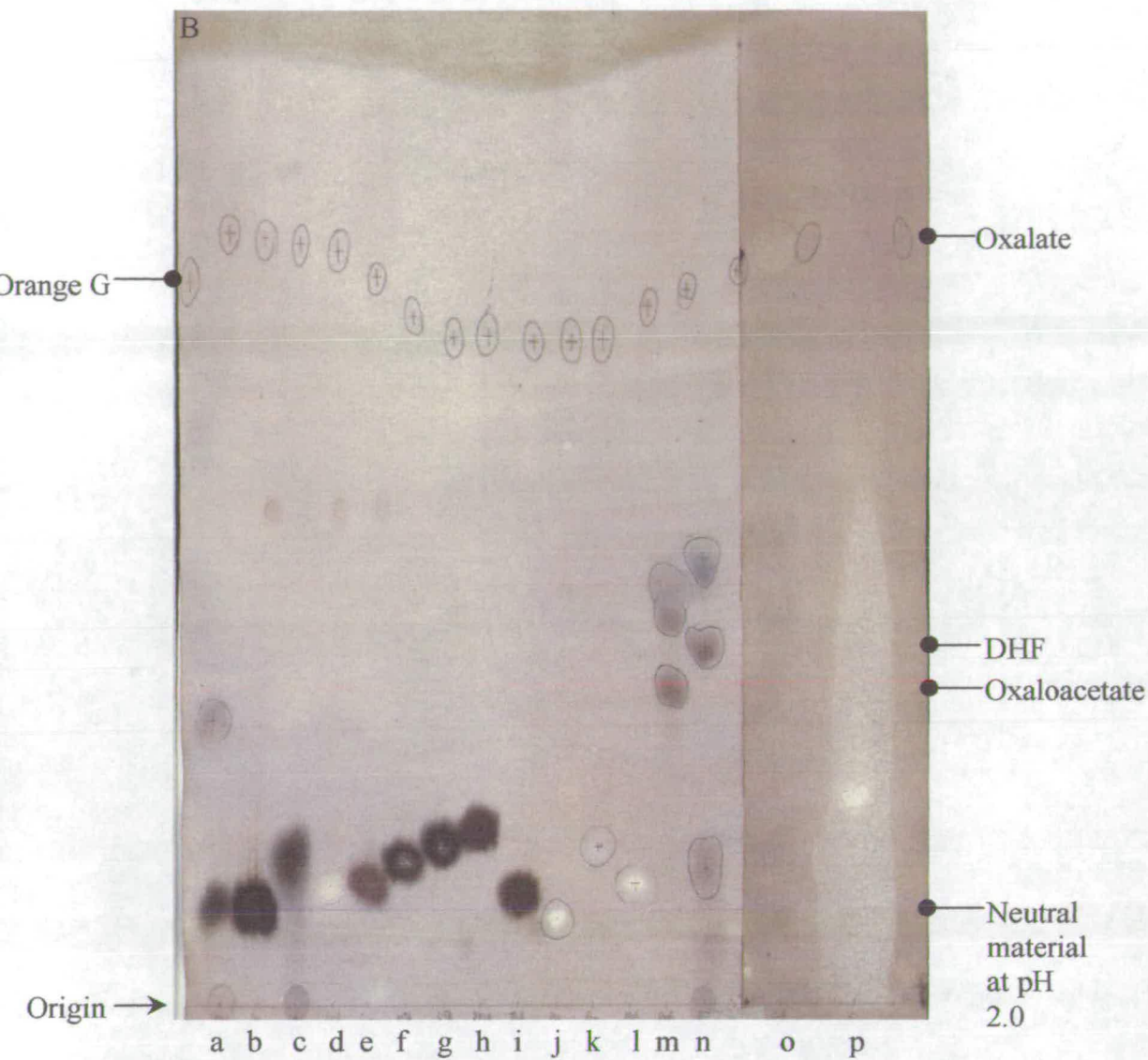
Figure 24.

B. Mobility of external markers (a, ascorbate; b, glucose; c, glyoxylate; d, glycollate; e, glycerate; f, threarate; g, erythrarate; h, 2-ketogluconate; i, threonate; j, succinate; k, fumarate; l, malate; m, oxaloacetate; n, dihydroxyfumarate) and internal marker o, oxalate stained with silver nitrate
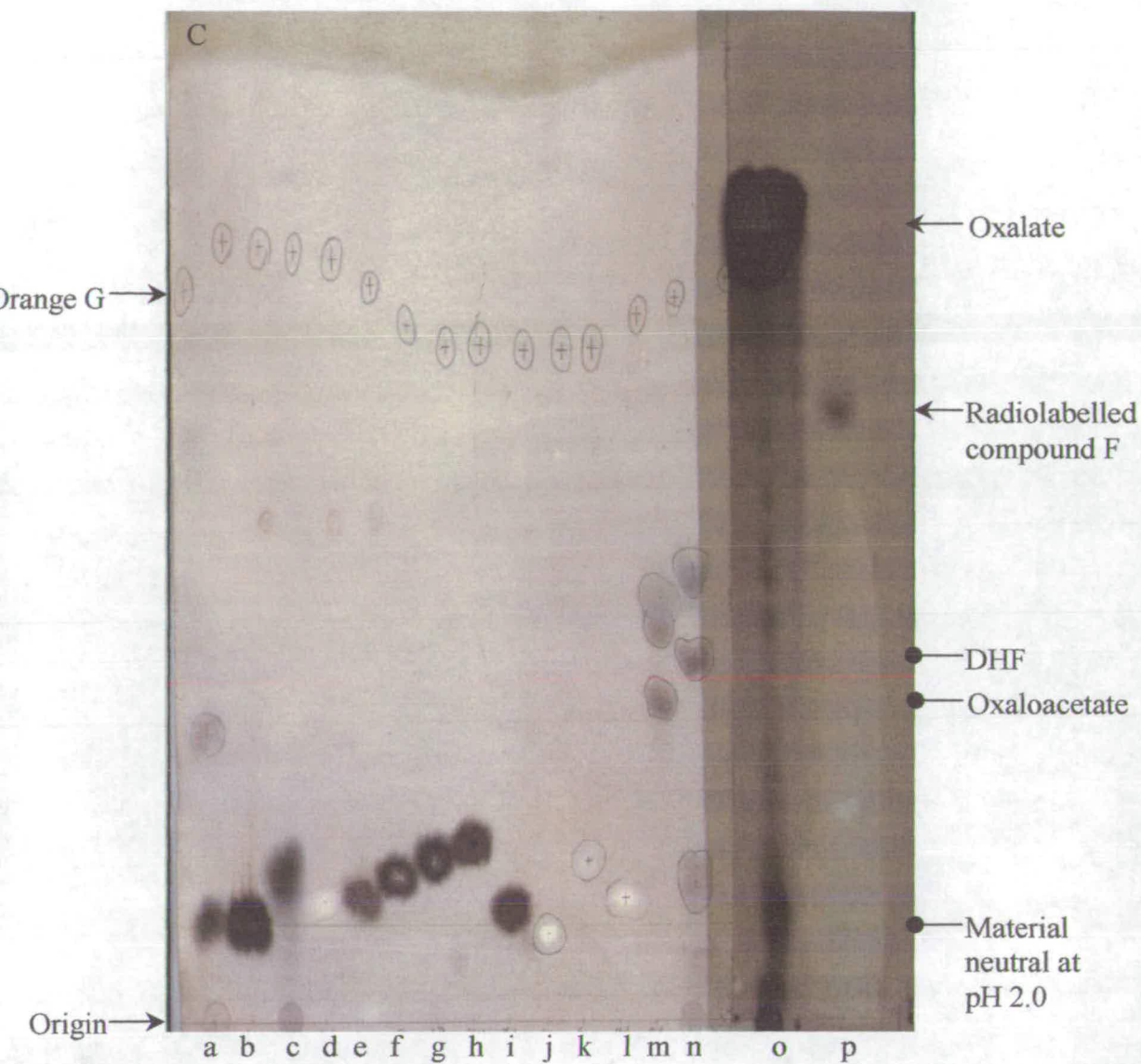
Figure 24.

C. The silver-stained electrophoretogram (Fig. 24 B) was overlayed with the autoradiogram presented in Figure 24 A.

●—— represents non-radiolabelled external standards
←—— represents radiolabelled compounds

3.3.4 Susceptibility of radiolabelled compound F to alkaline hydrolysis

Radiolabelled compound F is enzymically converted to oxalate (section 3.5.6). The susceptibiltiy of F to alkaline hydrolysis was analysed to examine the possible presence of an alkali-labile bond such as an ester bond. An ester bond could undergo hydrolysis in culture medium and result in the formation of oxalate from F. The effect of enzyme inhibitors on conversion of F to oxalate is described in section 3.3.5.

Radiolabelled compound F was treated with either NaOH or acetic acid for 24 hours at room temperature. After 24 hours the sample treated with NaOH was brought to pH 4.7 with acetic acid while the sample treated with acetic acid was brought to pH 4.7 with NaOH. A sample of each reaction mixture was subjected, along with a sample of untreated radiolabelled compound F, to electrophoresis in either pH 6.5 or pH 2.0 buffer.

The radiolabelled compound F used was not pure and contained a small amount of $[^{14}C]$oxalate (Fig. 25). When treated with NaOH, radiolabelled compound F was completely removed and the amount of $[^{14}C]$oxalate present increased compared with untreated samples or samples treated with acetic acid (Fig. 25). Low concentrations of $[^{14}C]$oxalate are likely to form radiolabelled calcium oxalate during electrophoresis in pH 2.0 buffer. This appears as a grey smear when detected by autoradiography and does not migrate as far as higher concentrations of oxalate (Fig. 25 B).

These results indicate that radiolabelled compound F possesses an alkali-labile bond such as an ester bond that is non-enzymically hydrolysed to release radiolabelled oxalate by treatment with NaOH. Any other products are not radiolabelled and therefore have not been detected.
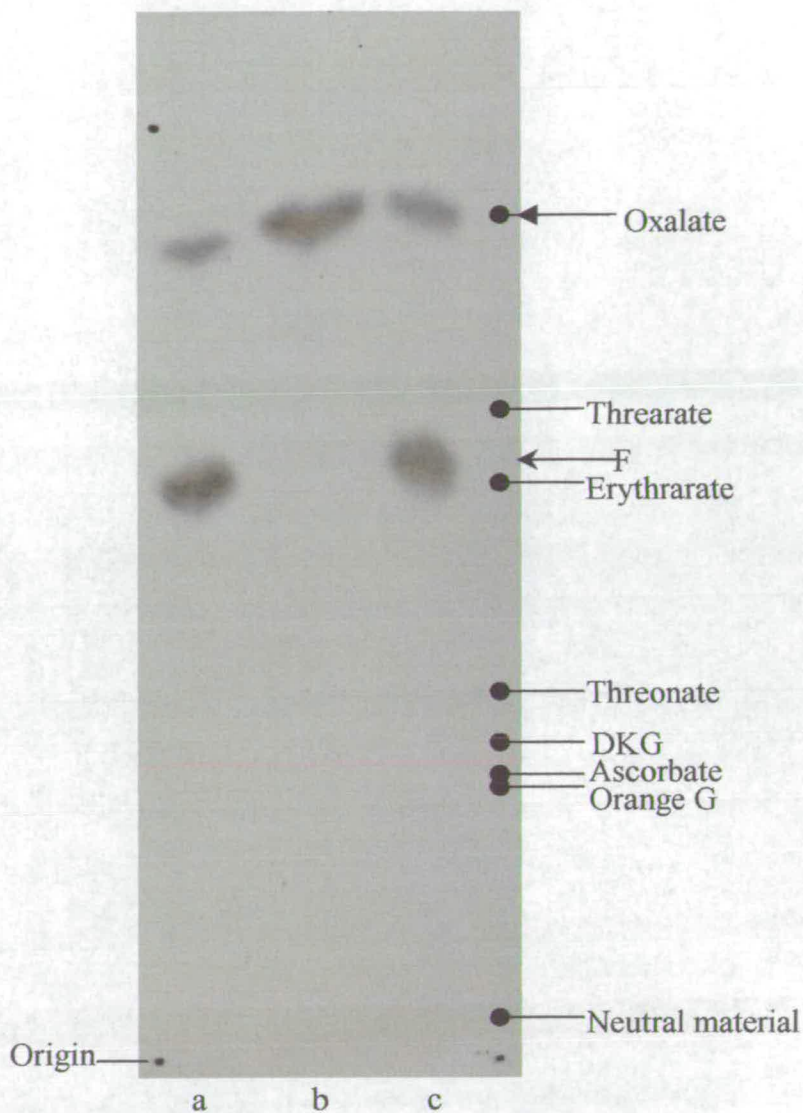
Figure 25. Alkaline hydrolysis of radiolabelled compound F

A. Effect of NaOH or acetic acid on radiolabelled compound F. a, Radiolabelled compound F; b, radiolabelled compound F treated with NaOH; c, radiolabelled compound F treated with acetic acid. Soluble products were analysed by electrophoresis in pH 6.5 buffer, 3 kV for 30 minutes. Radiolabelled compounds were detected by autoradiography.

●── represents the approximate mobiltiy of external standards
←── represents radiolabelled compounds
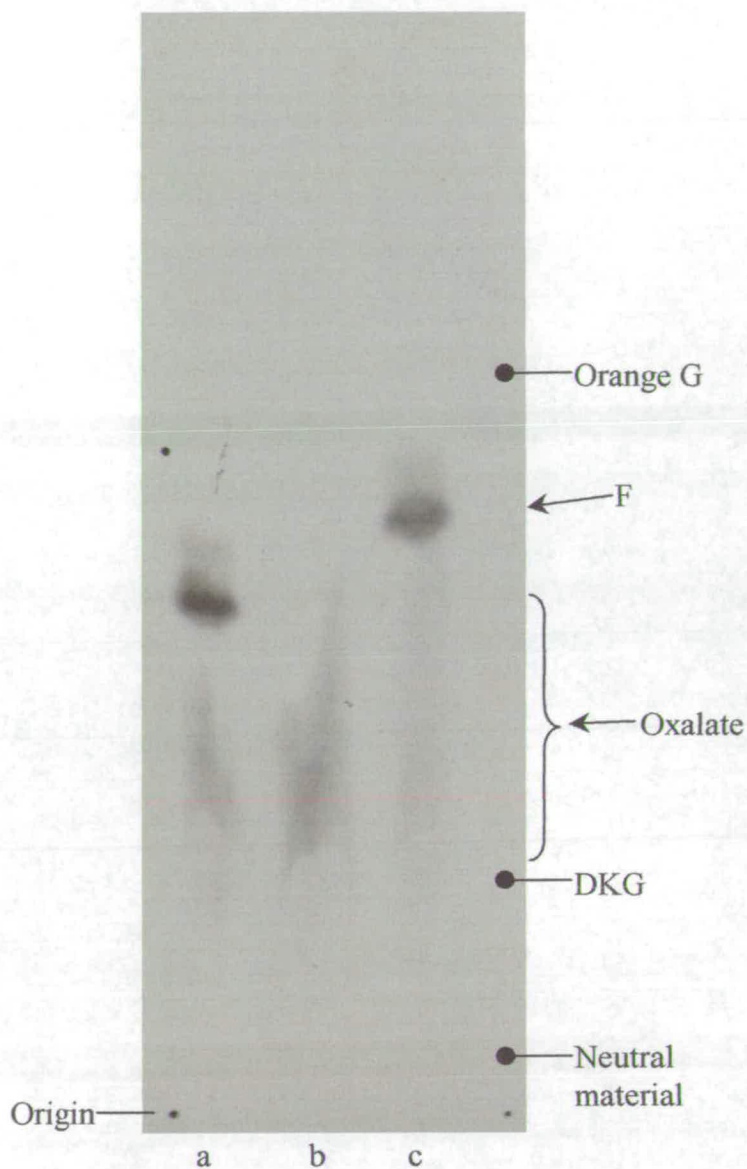
Figure 25.

B. Tracks a–c as described in Fig. 25 A. Soluble products were analysed by electrophoresis in pH 2.0 buffer with thiosulphate at 3 kV for 30 minutes. Radiolabelled compounds were detected by autoradiography.

3.3.5 Effect of inhibitors on metabolism of radiolabelled compound F

Radiolabelled compound F is converted to oxalate by the action of an apoplastic enzyme (section 3.5.6) and non-enzymically in the presence of NaOH (section 3.3.4). The nature of the enzymic conversion of radiolabelled compound F to oxalate in spent medium was examined by the use enzyme inhibitors. Radiolabelled compound F was added to spent medium from 5-d-old rose cultures containing either of the enzyme inhibitors sodium azide or dithiothreitol. Samples of medium were removed to storage in liquid nitrogen at intervals over an 8-hour period. They were then subjected to paper electrophoresis in pH 6.5 buffer.

Radiolabelled compound F, present in spent medium, was almost completely converted to [^{14}C]oxalate after 2 hours (Fig 26 A, D). When sodium azide was present the conversion of radiolabelled compound F to [^{14}C]oxalate was slower. Some radiolabelled compound F remained after 4 hours but was almost completely removed after 8 hours (Fig. 26 B, D). The presence of the reducing agent dithiothreitol in spent medium did not inhibit conversion of radiolabelled compound F to [^{14}C]oxalate (Fig 26 C, D).

Azide inhibits the activity of enzymes that have copper of iron as part of the catalytic mechanism. Azide also inhibits other enzymes including decarboxylases (Dixon and Webb 1958). The ability of sodium azide to inhibit conversion of radiolabelled compound F to [^{14}C]oxalate could suggest that an oxidation reaction is being inhibited. However, dithiothreitol, a reducing agent, did not inhibit conversion of radiolabelled compound F to [^{14}C]oxalate, suggesting that the reaction is not an oxidation reaction (section 3.3.4).
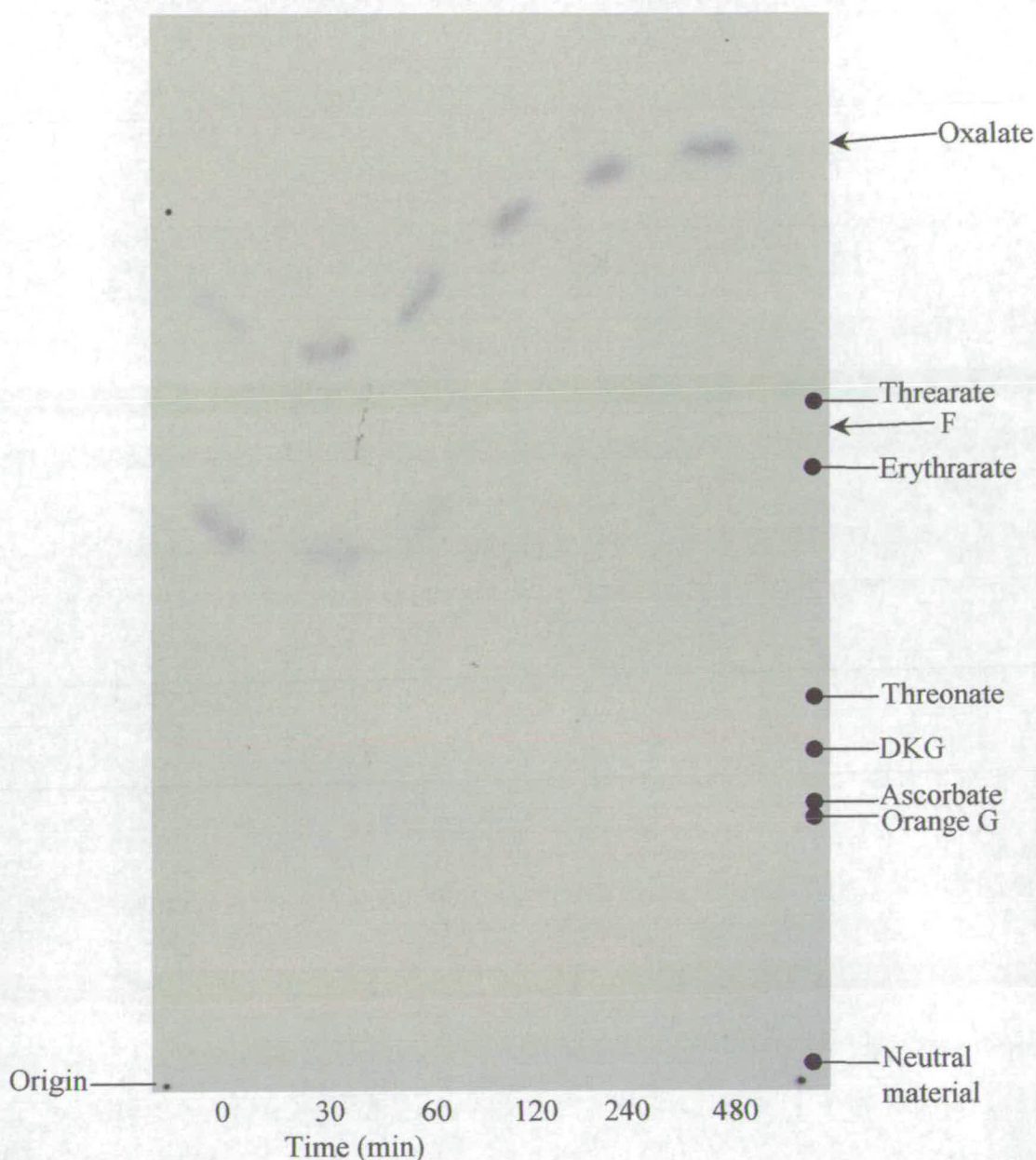
Figure 26. Effect of inhibitors on metabolism of radiolabelled compound F

A. Conversion of radiolabelled compound F to [^{14}C]oxalate in spent medium. Spent medium was analysed by electrophoresis in pH 6.5 buffer and radiolabelled compounds were detected by autoradiography. Differences in the mobilites were due to uneven removal of buffer from the wet paper.

●———— represents the approximate mobility of external standards
←———— represents radiolabelled compounds
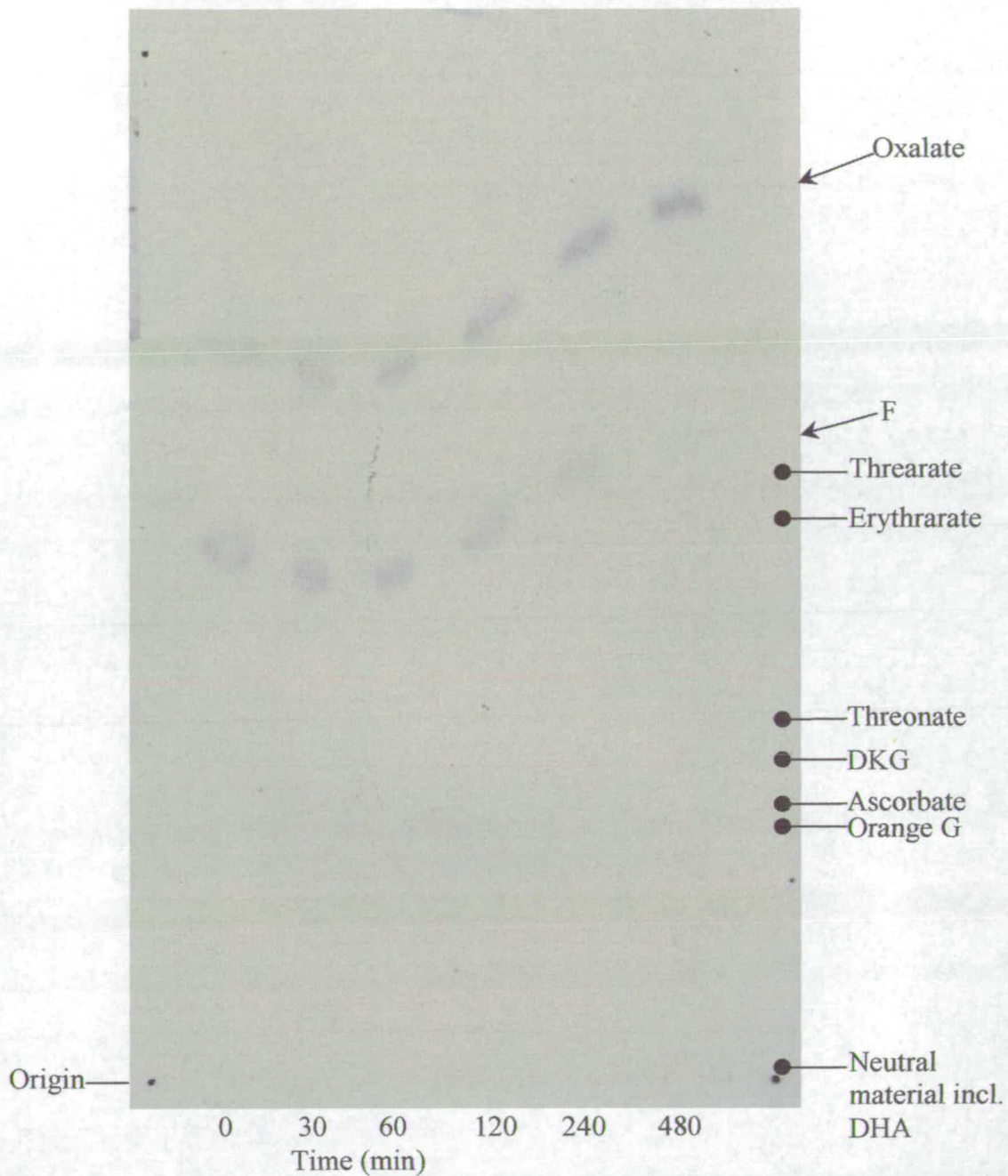
Figure 26.

B. Conversion of radiolabelled compound F to [^{14}C]oxalate in the presence of sodium azide (10 mM). Samples were incubated and analysed as described in Fig. 26 A.
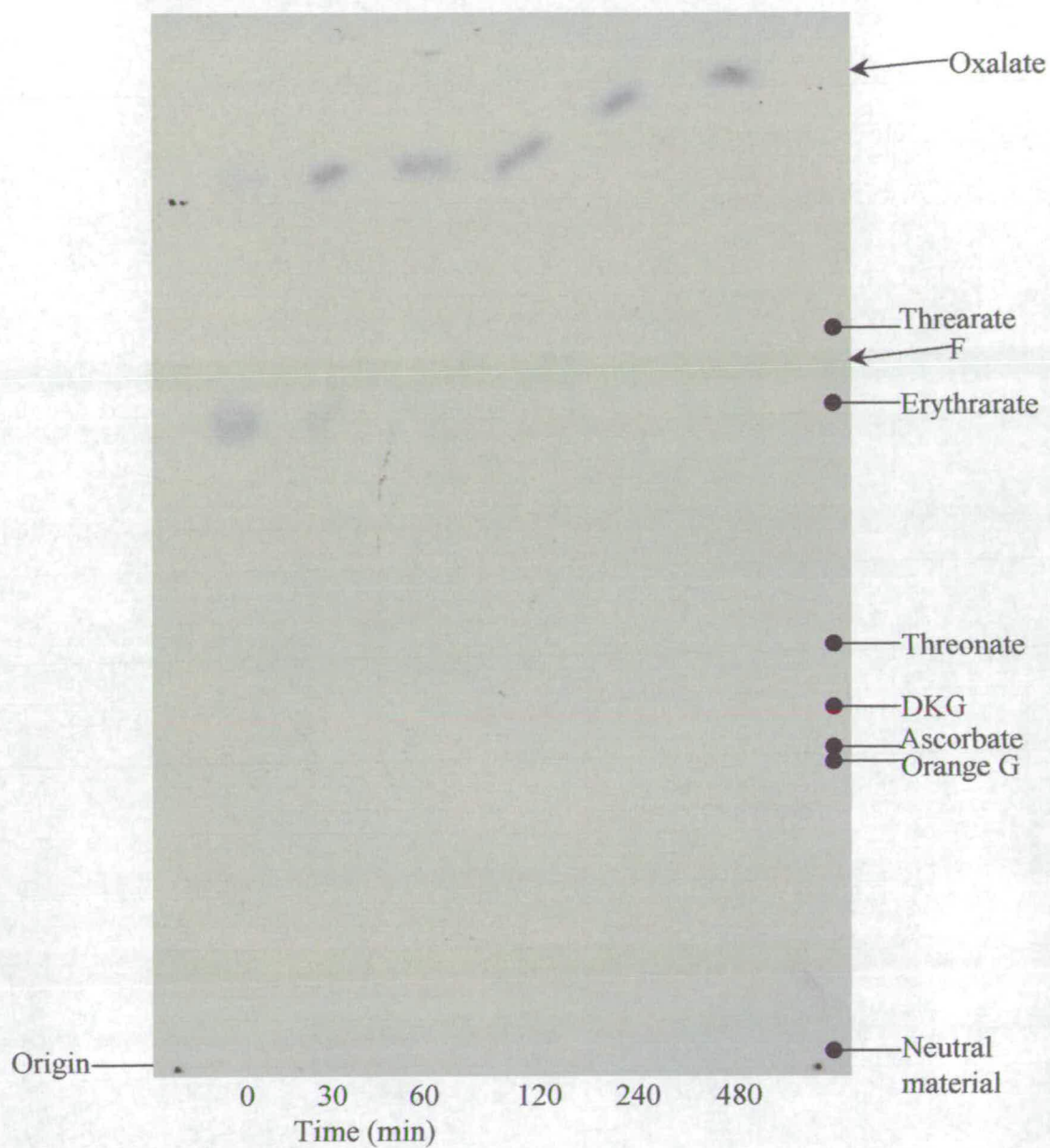
Figure 26.

C. Conversion of radiolabelled compound F to [^{14}C]oxalate in the presence of dithiothreitol (10 mM). Samples were incubated and analysed as described in Fig. 26 A.
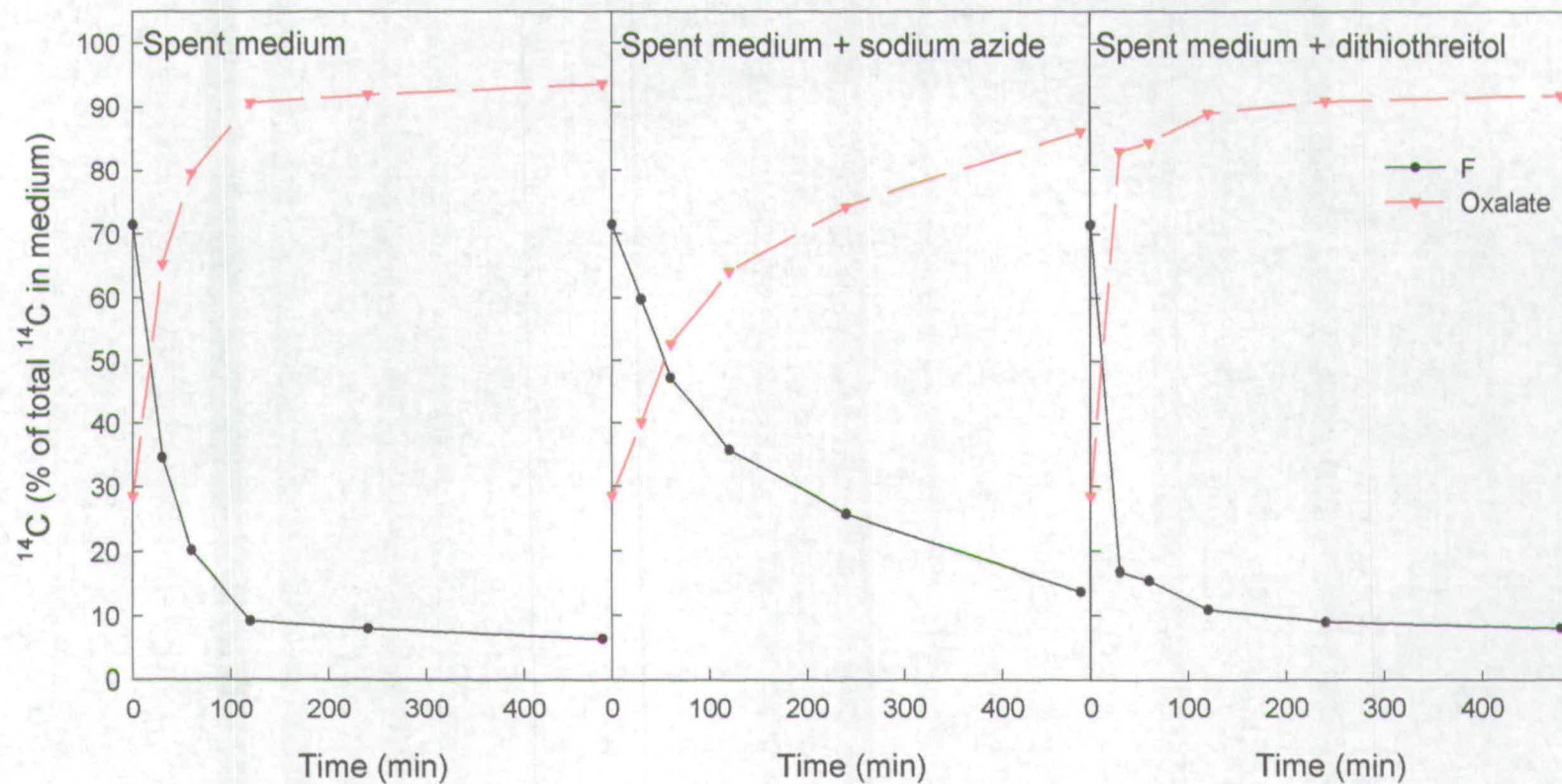
Figure 26.

D. Effect of sodium azide and dithiothreitol on metabolism of radiolabelled compound F in spent medium. The areas of paper corresponding to metabolites detected in Fig. 26 A–C were scintillation counted to quantify radioactivity. The initial amount of radioactivity presented was calculated as the average of the amount of radioactivity present at time-0 in spent medium, spent medium containing sodium azide and spent medium containing dithiothreitol.

3.3.6 Radiolabelled compounds C and D

Two of the radiolabelled compounds formed when $[1\text{-}^{14}C]$ascorbate was fed to rose culture and culture medium (Chapter 3.4) had very similar mobility after electrophoresis in pH 6.5 buffer. The slower-migrating compound is referred to as C and the faster migrating compound as D. Radiolabelled compounds C and D were eluted from electrophoretograms, dried and redissolved in water.

A mixture of radiolabelled compounds C and D was subjected to electrophoresis at pH 6.5 for 45 minutes instead of the usual 30 minutes. Compound D ($m_{OG} = 1.15$) migrated further than compound C ($m_{OG} = 1.12$) (Fig. 27 A, track r), and small amounts of radiolabelled compounds E ($m_{OG} = 1.43$) and F ($m_{OG} = 1.54$) were also formed. Compound C was predominant. These results indicate that one of the two compounds C or D, more likely C since it is predominant in the sample, can be converted to E and F. It is unclear if C is converted to E which is converted to then F or whether C is converted directly to and F.

Radiolabelled compounds C and D had similar mobility to 2-ketogulonate ($m_{OG} = 1.14$) and threonate ($m_{OG} = 1.25$) on electrophoresis in pH 6.5 buffer but neither standard exactly co-migrated with radiolabelled compound C or D (Fig. 27 A).

A sample of C and D was found to contain small amounts of E suggesting that C may be converted to E. A sample of radiolabelled compound E purified from pH 6.5 electrophoretograms was subjected to repeat electrophoresis at pH 6.5 and found to contain two radiolabelled compounds. One had similar mobility to C, the other to E (Fig. 27 A). This led to the suggestion that radiolabelled compound E may break down to compound C.

C (containing a small amount of D) and E were treated with NaOH to test for the presence of an ester bond within these compounds and to examine the nature of their interconversion. The compounds were treated with NaOH or acetic acid then neutralised and the products analysed by electrophoresis (Fig. 27 B).

Compound E, which is partially converted to C during storage (Fig. 27 A track, s; Fig 27 B, track d), was restored to essentially pure E when treated with NaOH (Fig. 27 B, track e). A sample of C (containing a small amount of D) was subjected to repeat electrophoresis (Fig. 27 B, track a) and found to contain 3 other radiolabelled compounds—E, F and oxalate—in addition to C and/or D which were not resolved but were predominant. When the mixture of C and D was treated with NaOH (Fig. 27 B, track b) only radiolabelled E and oxalate were detected. These results show that C (and/or D) is converted to E by treatment with NaOH and C (and/or D) and E are interconvertable. Previous results (see section 3.3.4) have shown that F is converted to oxalate by NaOH.

A mixture of radiolabelled compounds C and D and a sample of E were analysed by electrophoresis at pH 2.0 (Fig. 28). Four radiolabelled compounds were detected in what began as a mixture of C and D (Fig. 28 A, track a). The most mobile compound had $m_{OG} = 0.92$, similar to that of oxalate (section 3.3.2; $m_{OG} = 1.15$). Low concentrations of [^{14}C]oxalate usually appear as a grey smear when detected by autoradiography. The [^{14}C]oxalate present in the mixture of C and D appears as a discrete spot indicating that the concentration of oxalate must be relatively high. The m_{OG} value of the second most mobile compound ($m_{OG} = 0.74$) was indicative of compound F (section 3.3.3; $m_{OG} = 0.88$). Two radiolabelled compounds were detected in the sample of E (Fig. 28 A, track b). These
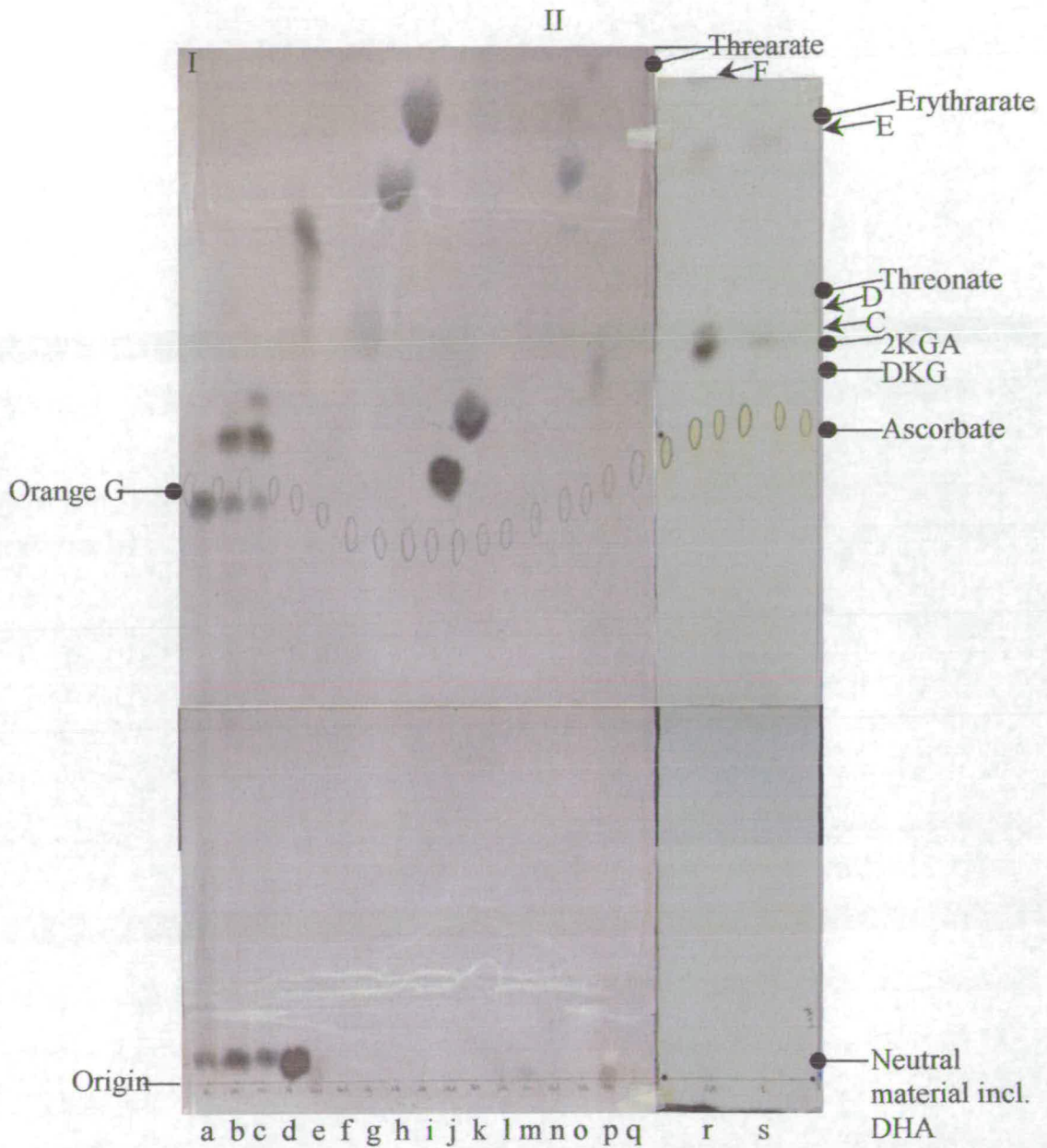
Figure 27. Resolution of radiolabelled compounds C, D and E, and evidence for their interconversion.

A. Mobility of radiolabelled compounds C and D (track r) and E (track s) separated by electrophoresis in pH 6.5 buffer, 3 kV for 45 minutes and detected by autoradiography (II) compared with silver stained (I) external markers a, ascorbate; b, DHA; c, DKG; d, glucose; e, glyoxylate; f, glycollate; g, glycerate; h, erythrarate; i, threarate; j, 2-ketogluconate; k, threonate; l, succinate*; m, fumarate*; n, malate ; o, oxaloacetate; p, dihydroxyfumarate; q, oxalate[†]. The unstained electrophoretogram was overlayed with the autoradiogram (II).

●——represents the mobility of external markers
←——represents radiolabelled compounds

* Not stained by silver nitrate. [†] Off the end of the electrophoretogram
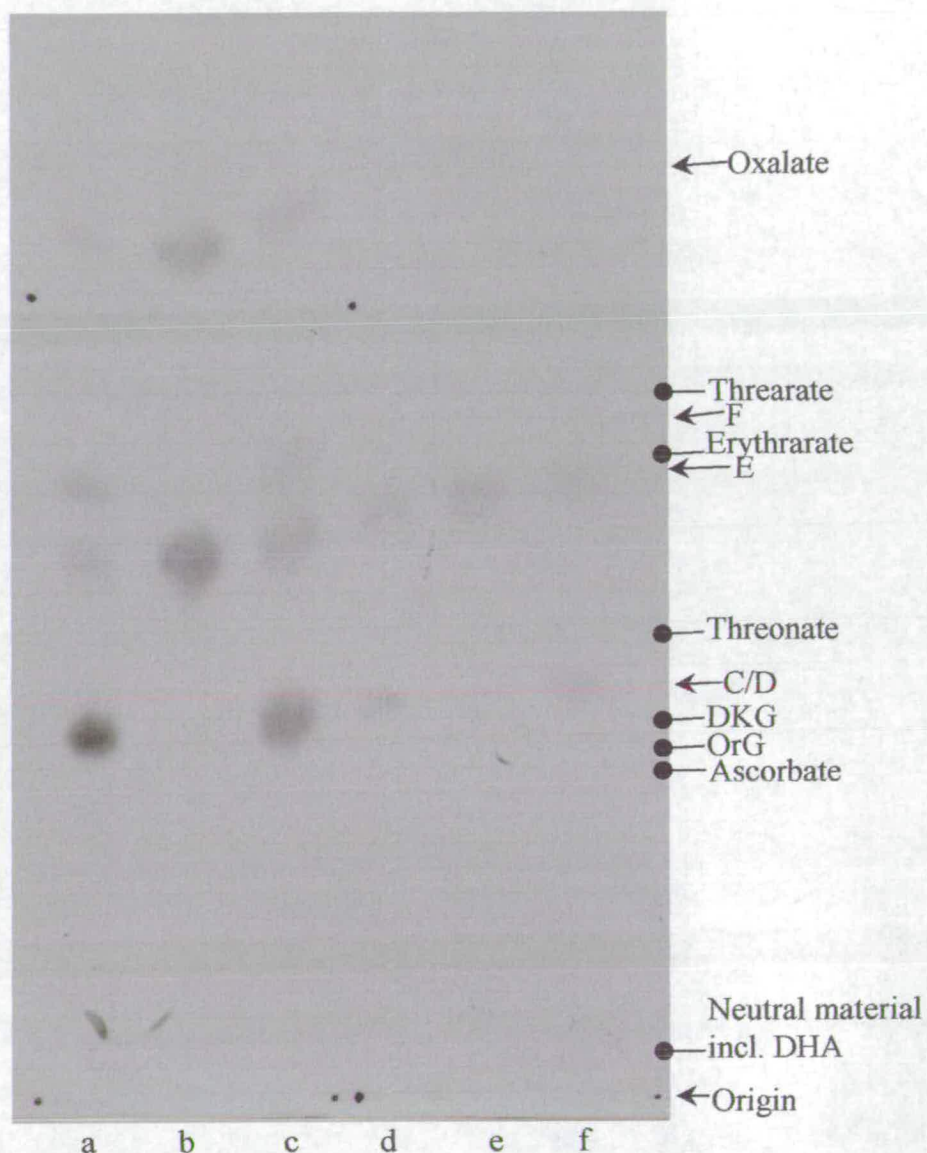
Figure 27.

B. Radiolabelled compound C and D— predominantly C as observed in Fig 27 A— untreated (track a) and treated with NaOH (track b) or acetic acid (track c) and radiolabelled compound E untreated (track d) and treated with NaOH (track e) or acetic acid (track f). Products analysed by electrophoresis in pH 6.5 buffer followed by autoradiography.

●—— represents the mobility of external markers
←—— represents radiolabelled compounds

corresponded to the two least mobile components of the sample of C and/or D) and were assumed to be E and C.

Samples of C (and/or D) (Fig. 28 B, track d) and E (Fig. 28 B, track a) were subjected to repeat electrophoresis at pH 2.0 and treatment with NaOH followed by electrophoresis to analyse products (Fig. 28 B). Treatment of C (and/or D) with NaOH (Fig. 28 B, track e) resulted in the removal of the most mobile compound which is therefore believed to be C since this is removed by treatment with NaOH. The results also indicated that compound E is less mobile ($m_{OG} = 0.41$) than compound C (and/or D) ($m_{OG} = 0.66$) during electrophoresis at pH 2.0 in contrast to the order of mobilities of these compounds at pH 6.5.

The mobiltiy of E during electrophoresis at pH 6.5 is suggestive of a compound with four or more carbons and two negative charges while the mobiiltiy of C (and/or D) is suggestive of a similarly sized compound but with only 1 negative charge. The formation of C during storage of E may be due to lactonisation of E. Treatment with NaOH would hydrolyse the intramolecular ester bond in compound C and produce E. The acid group of C which is not involved in formation of the ester bond is suggested to have a low pK which would make compound C highly mobile during electrophoresis at pH 2.0. The pK of this group when present in E, the non-lactone form, may be higher than the same group in C, making E less mobile than C during electrophoresis at pH 2.0. At pH 6.5 both the acid groups in E are likely to be almost fully ionised which makes E more mobile than C which only has one free acid group. Thus the reversal of the order of mobility of compounds C and E during electrophoresis in pH 6.5 and pH 2.0 buffer may be explained. Compound C is predominant over E after electrophoresis at pH 6.5 while after
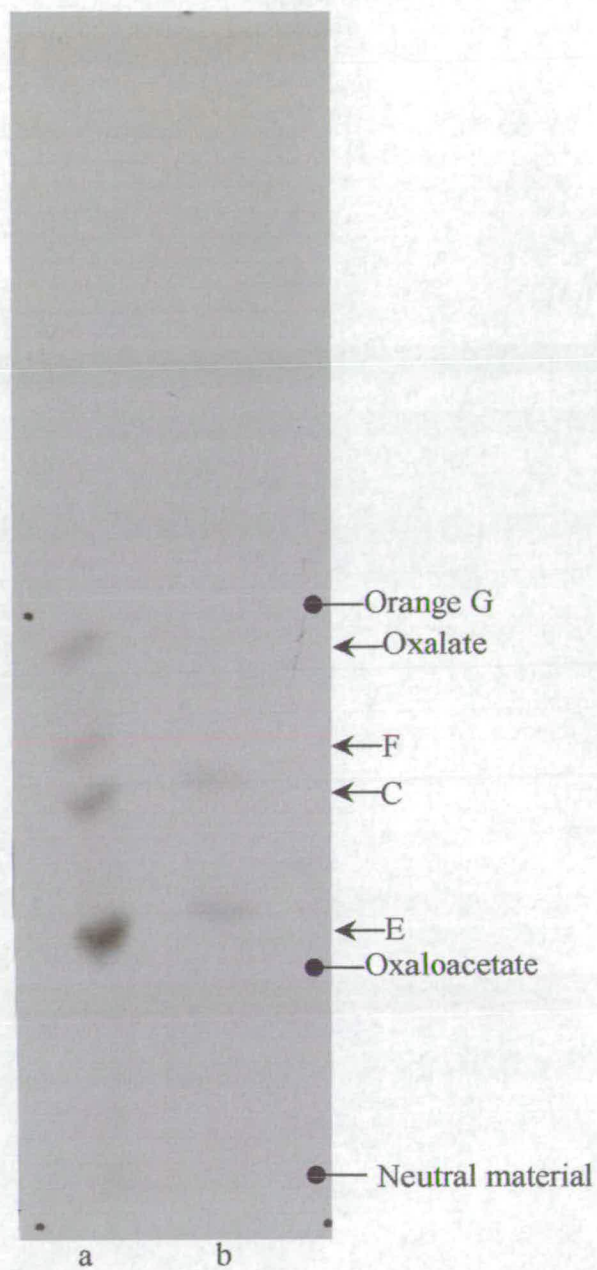
Figure 28. Resolution and interconversion of C, D and E analysed by electrophoresis at pH 2.0.

A. Radiolabelled compound C (and/or D) (track a) and E (track b)— as observed in Fig. 27 A— analysed by electrophoresis in pH 2.0 buffer followed by autoradiography.
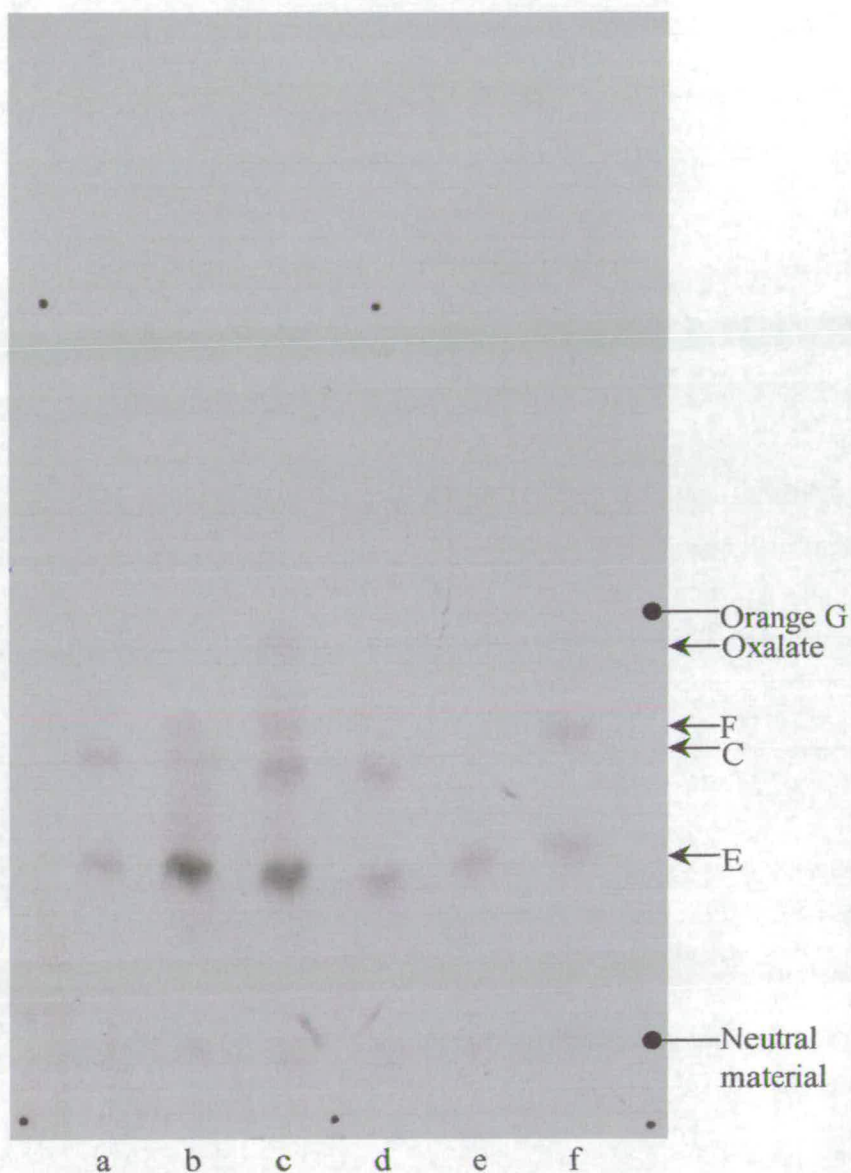
Figure 28.

B. Radiolabelled compounds C and D (track d) treated with NaOH (track b) or acetic acid (track c) and radiolabelled compound E (track a) treated with NaOH (track e) or acetic acid (track f). Products were analysed by electrophoresis in pH 2.0 buffer followed by autoradiography.

electrophoresis at pH 2.0, E is predominant. Lactonisation of E to form C may be inhibited at pH 2.0 if the acid groups involved in lactonisation are not ionised.

Treatment of E with NaOH or acetic acid was repeated to allow comparison of products with internal markers (Fig. 29). The products of each treatment were mixed with either threonate or oxalate as a non-radiolabelled internal marker then analysed by electrophoresis at pH 6.5. As before, untreated E contained an equivalent amount of C (and/or D) and both these compounds were unaffected by treatment with acetic acid (Fig. 29 A). Treatment with NaOH resulted in the removal of C (and/ or D) and formation of E (Fig. 29 A). These results confirm that compound E is converted to C (and/or D) during storage and can be regenerated by treatment of C (and/or D) with NaOH.

Radiolabelled compound C (m_{OG} = 1.30) had a lower mobility than non-radiolabelled internal marker threonate detected by silver nitrate (m_{OG} = 1.41) (Fig. 29); therefore compound C is not threonate. Radiolabelled oxalate was not detected in any of the samples (Fig. 29 A) and non-radiolabelled oxalate was not detected by staining with silver nitrate (Fig. 29 B).

Compounds C and E, two metabolites of apoplastic ascorbate, undergo interconversion possibly by the formation or hydrolysis of an intramolecular ester. Lactonisation, formation of C from E, occurs non-enzymically during storage of E. Hydrolysis of the lactone can occur spontaneously as observed in the mixture of C (and/or D) but is promoted by the presence of NaOH.
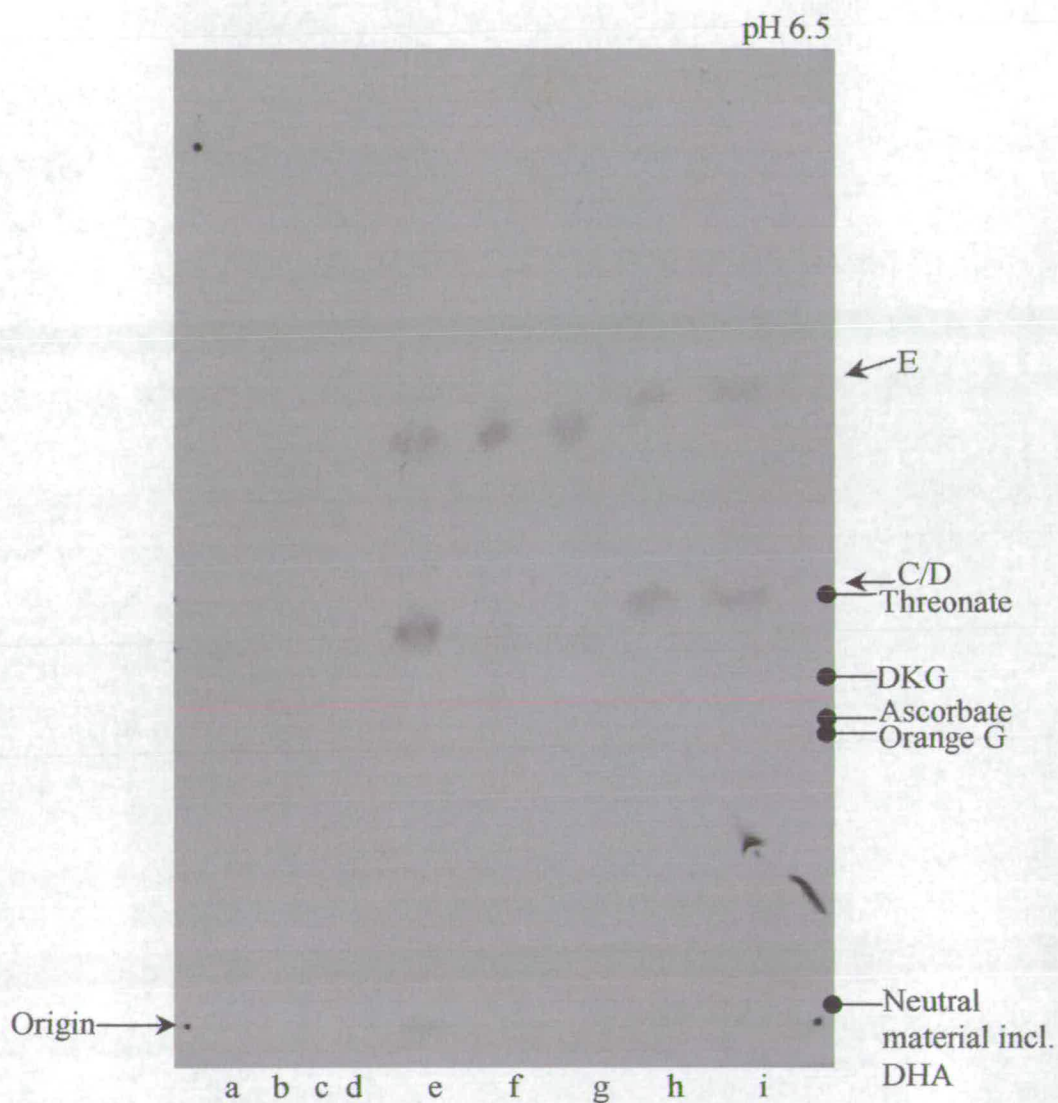
Figure 29. Comparison of E and alkali hydrolysis products of E by electrophoresis at pH 6.5

A. Radiolabelled compound E untreated (track e) and treated with NaOH and mixed with oxalate (track f) or threonate (track g) or treated with acetic acid and mixed with oxalate or threonate (tracks h, i). Tracks a–d contain external standards a, ascorbate; b, DHA; c, DKG; d, glucose. Products were analysed by electrophoresis pH 6.5 buffer and detected by autoradiography.

●——represents the mobility of external markers
←——represents radiolabelled compounds

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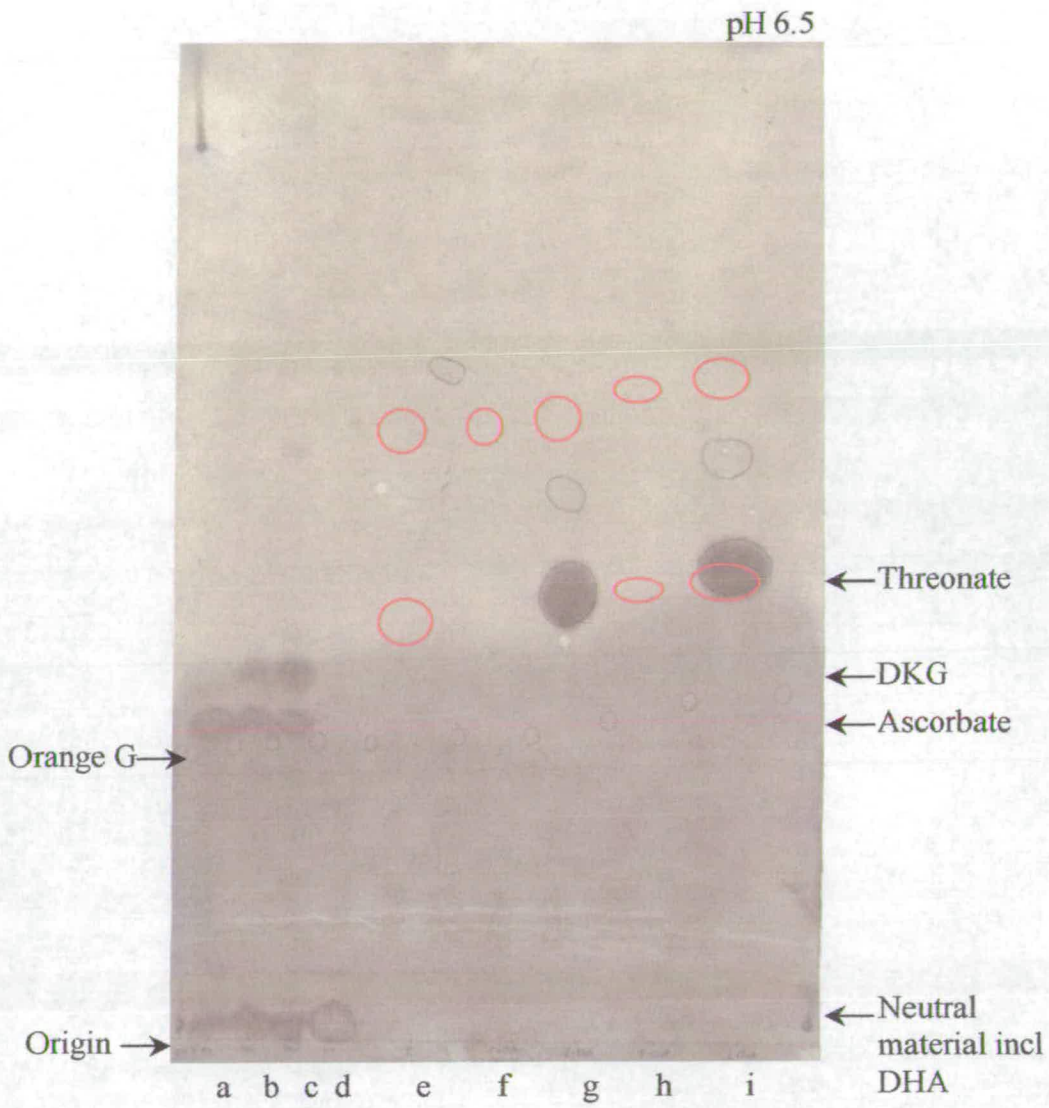
Figure 29.

B. Internal and external markers stained with silver nitrate. Non-radiolabelled oxalate was not detected. Tracks are labelled as described in Fig. 29 A. Red outlines indicate radiolabelled compounds as identified by autoradiography in Fig. 29 A

3.4 Fate of [1-^{14}C]ascorbate in rose culture medium

The fate of exogenous L-[1-^{14}C]ascorbate added to the culture medium of rose cell suspension cultures was examined in a series of three experiments. [1-^{14}C]Ascorbate was added to fresh medium, whole culture, spent medium and boiled spent medium. Overall the results indicate that [1-^{14}C]ascorbate was degraded non-enzymically in fresh medium and boiled spent medium with metabolism occurring in whole and cell-free cultures. The same compounds were produced by metabolism and degradation and only differed in the amount produced in each system. The same basic experiment was performed three times to ensure the labelling pattern was consistent between replicate experiments. The first experiment (section 3.4.1) examined the fate of [1-^{14}C]ascorbate in fresh rose medium, whole culture, cell-free spent medium and boiled spent medium. The second experiment (section 3.4.2) included the examination of the fate of [1-^{14}C]ascorbate in high and low M_r fractions of cell-free spent and boiled spent medium. Finally, the third experiment (section 3.4.3) included quantitative analysis of [1-^{14}C]ascorbate and its radiolabelled metabolites.

3.4.1 Fate of [1-^{14}C]ascorbate—experiment I

[1-^{14}C]Ascorbate (17 Ci/mol) was dissolved to a final concentration of 0.5 mM in rose culture or culture medium (160 μl). Samples of medium, taken at intervals, were subjected to electrophoresis and autoradiography to separate and visualise metabolites.

[1-^{14}C]Ascorbate (m_{OG} □ 1.00) appeared to be unstable and break down during drying on to Whatman 3MM paper. This was seen clearly in the 0-minute time point (Fig. 30A). However, the apparent breakdown was likely to be due to

impurity of the stock [1-^{14}C]ascorbate used in this exeriment. Although other neutral compounds such as glucose would have been present in samples of culture medium only radiolabelled compounds were detected by autoradiography. Radiolabelled neutral material was likely to be primarily [^{14}C]dehydroascorbate. Oxidiation of [1-^{14}C]ascorbate to [^{14}C]dehydroascorbate occurred throughout electrophoresis as seen by the dark streak between these compounds on autoradiograms of electrophoretograms (Fig. 30.).

[1-^{14}C]Ascorbate gradually broke down in fresh culture medium (Fig. 30.A, Table 1). After 480 minutes very little [1-^{14}C]ascorbate was present. The amount of neutral material, likely to include [^{14}C]dehydroascorbate, remained relatively constant throughout the time course with only a slight decrease. [^{14}C]Diketogulonate (m_{OG} = 1.19) increased as did the almost inseparable radiolabelled compounds C and D (m_{OG} = 1.31). [^{14}C]Diketogulonate was identified by comparison of its mobility with non-radiolabelled diketogulonate produced by hydrolysis of dehydroascorbate. Radiolabelled compound F (m_{OG} = 2.06) increased with time, while [^{14}C]oxalate (m_{OG} = 2.76) remained constant (Fig. 30 A, Table 1). A small amount of radiolabelled compound E (m_{OG} = 1.76) appeared later in the course of the experiment, after 240 minutes. Other radiolabelled compounds A1 and A2 (m_{OG} = 0.65 and 0.78 respectively) were also present after 240 minutes (Fig. 30 A, Table 1). The results showed that [^{14}C]ascorbate was broken down non-enzymically by the components of culture medium. Copper and iron ions present at 0.6–20 µM or 1–10 µM respectively are, to different extents, capable of promoting ascorbate oxidation (Winterbourn 1979, 1981, Fry 1998).

The fate of $[1-^{14}C]$ascorbate in whole culture (Fig. 30 B, Table 1) was broadly similar to that of $[1-^{14}C]$ascorbate in fresh medium (Fig. 30 A, Table 1). $[1-^{14}C]$Ascorbate was removed more rapidly— perhaps owing to uptake as well as metabolism. $[^{14}C]$Dehydroascorbate decreased while the increase in $[^{14}C]$diketogulonate followed a pattern similar to its increase in fresh medium (Fig. 30 A, B, Table 1). Radiolabelled compound F decreased from 30 to 240 minutes but there appeared to be a slight increase in radiolabelled compound F from 240 to 480 minutes and a more substantial increase in the amount of $[^{14}C]$oxalate present after 8 hours (Fig. 30 B, Table 1). Radiolabelled compound E appeared in small quantities after 240 minutes. Two radiolabelled compounds, C and D, with similar mobility were present throughout the 8-hour time course. The faster moving compound, radiolabelled compound D, was visible after 30 minutes and increased until 120 minutes. It then decreased during the remainder of the time course. The slower moving compound, radiolabelled compound C, appeared after 60 minutes and was approximately equal to the faster migrating compound at 120 minutes. After this time the slower running compound was predominant (Fig. 30 B, Table 1).Radiolabelled compounds A1 and A2 seen in Fig. 30 A were absent, perhaps owing to uptake or to inhibition of their formation by the cells. The presence of cells could facilitate the uptake of metabolites and/or secretion of compounds that interfere with apoplastic metabolism, and/or could secrete enzymes that further modify ^{14}C-products.

In spent culture medium cells had been removed and therefore neither uptake nor secretion was possible although any compounds secreted before cells were removed would still be present. $[1-^{14}C]$Ascorbate was broken down more

rapidly in spent medium (Fig. 30 C, Table 1) than in fresh medium (Fig. 30 A, Table 1) and completely degraded by about 240 minutes (Fig. 30 C). [^{14}C]Dehydroascorbate also decreased although its removal was incomplete. [^{14}C]Diketogulonate was abundant throughout the time course. The two radiolabelled compounds, C and D, were present in increasing quantity although were difficult to differentiate. Radiolabelled compound F decreased with time but [^{14}C]oxalate increased and became a major product. Radiolabelled compound E was present at an early stage, 30 minutes, and increased in quantity over the remainder of the time course (Fig. 30 C, Table 1). These results suggest that the formation of compound E was dependent on the presence of enzymes and comparison with Fig. 30 B suggests that compound E, or a precursor of this compound, may be taken up by cells. Radiolabelled compounds A1 and A2 were present throughout the course of the experiment (Fig. 30 C, Table 1) suggesting their absence from whole cultures (Fig 30 B, Table 1) may be due to uptake by cells.

The fate of [1-^{14}C]ascorbate in boiled culture medium (Fig. 30 D, Table 1) was similar to its fate in fresh medium (Fig 30 A, Table 1) since both fresh medium and boiled medium lack active enzymes. [1-^{14}C]Ascorbate was almost completely degraded after 8 hours while [^{14}C]dehydroascorbate decreased gradually and [^{14}C]diketogulonate increased strongly. Radiolabelled compound F increased slightly while [^{14}C]oxalate remained constant. After 480 minutes radiolabelled compounds C and D were present as two partially resolved spots of approximately equal quantity. The radiolabelled compounds A1 and A2 were detected at 120 minutes and were present for the remainder of the time course (Fig. 30 D. Table 1). This suggests that their absence from whole culture is due to uptake by cells (Fig.

30 B). Some radiolabelled compound E was produced in boiled spent culture medium (Fig. 30 D, Table 1).

[^{14}C]Diketogulonate, when produced in this experiment, appeared relatively stable. It did not itself appear to promote or prevent the formation of other metabolites. This suggests that [^{14}C]diketogulonate was not itself further metabolised. Instead, [^{14}C]dehydroascorbate and/or [^{14}C]ascorbate are metabolised to form both [^{14}C]diketogulonate and the range of other metabolites.

The fact that compound E was produced in spent medium (Fig. 30 C, Table 1) but almost absent from whole culture (Fig 30 B, Table 1) suggests that E or its precursor was taken up by cells. Its near absence in boiled culture medium (Fig. 30 D) suggests that it was the product of enzyme action.

Table 1. Summary of results describing the fate of ^{14}C from [1-^{14}C]ascorbate in 5-d-old rose cultures and medium.

Compound	Fresh Medium	Whole Culture	Spent medium	Boiled Spent Medium
DHA	OOOOOO	OOOOOo	–OOOoo	OOOOoo
A1	????..	??.	–?....	???...
A2	????..	??.	–?....	???...
AH$_2$	OOOOO .	Ooo ..	–oo ..	Ooo .
DKG	oooOOO	oooOOO	–OOOOO	ooOO
C	...	..	–. ???	O
D	.. OO	?.. ..	–Ooooo	...OOO
E	. .	...	– . oO	...
F	OOOOO	OOOOOO	–OOOO	OOOOO
Oxalate	OOOOOO	OOOOOO	–OOOOO	OOOOOO

Each symbol represents the relative amount present at a time point (O > o > · > .).

? indicates a compound that may be present but is unclear owing to streaking or is obscured by another compound. Blank indicates a compound that may be present but was undetectable. ▌ Represents missing data. The information in this table was derived from autoradiographs of electrophoretograms shown in Figure 30.
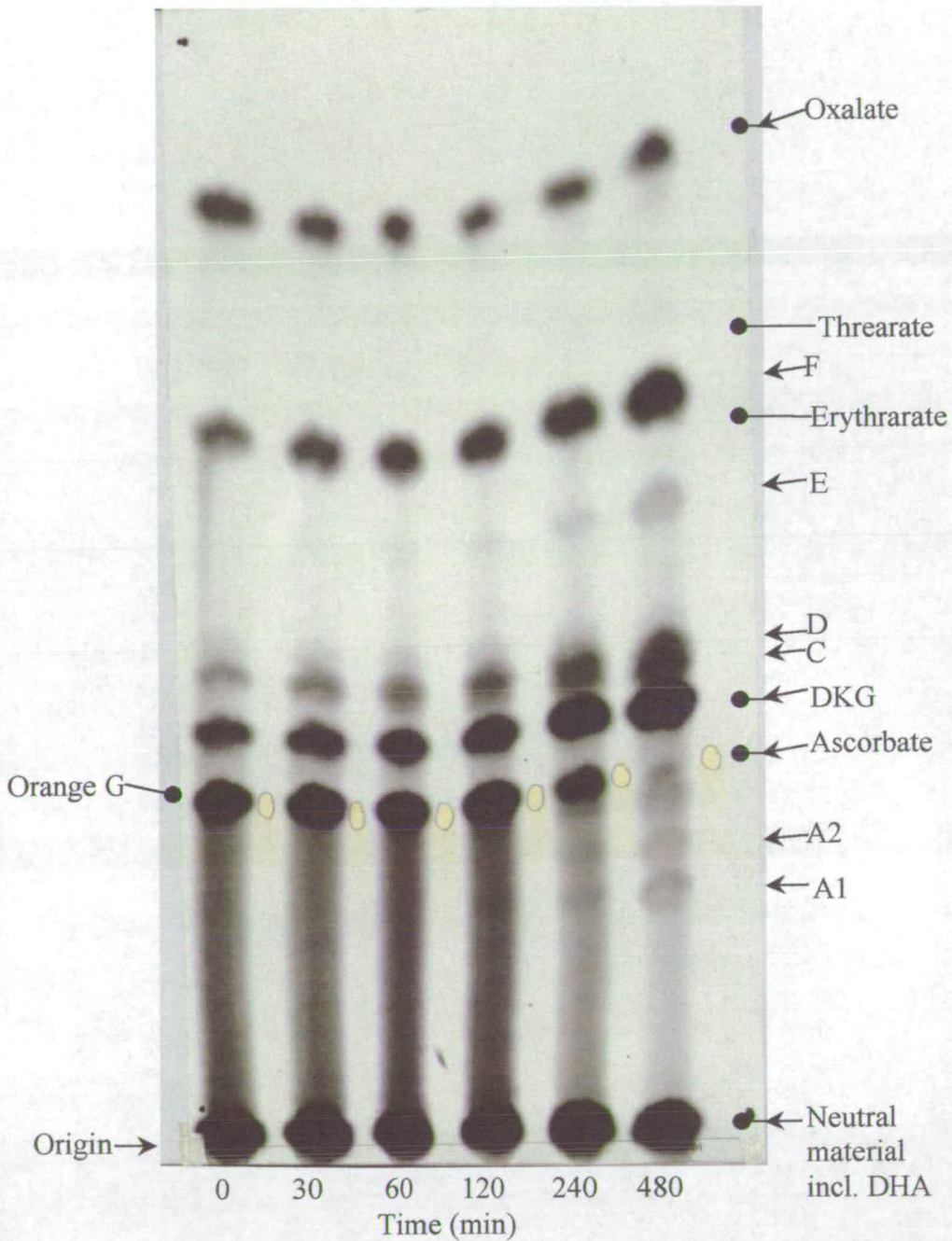
Figure 30. Fate of [1-^{14}C]ascorbate in rose culture and culture medium—experiment I

A. Non-enzymic breakdown of ascorbate in fresh rose culture medium. Soluble products were analysed by electrophoresis in pH 6.5 buffer, 3 kV for 30 minutes followed by autoradiography. The electrophoretogram was overlayed with the autoradiogram allowing comparison of the external orange G marker with radiolabelled metabolites

●——— represents the approximate mobility of external standards

←——— represents radioactive metabolites of ascorbate

134
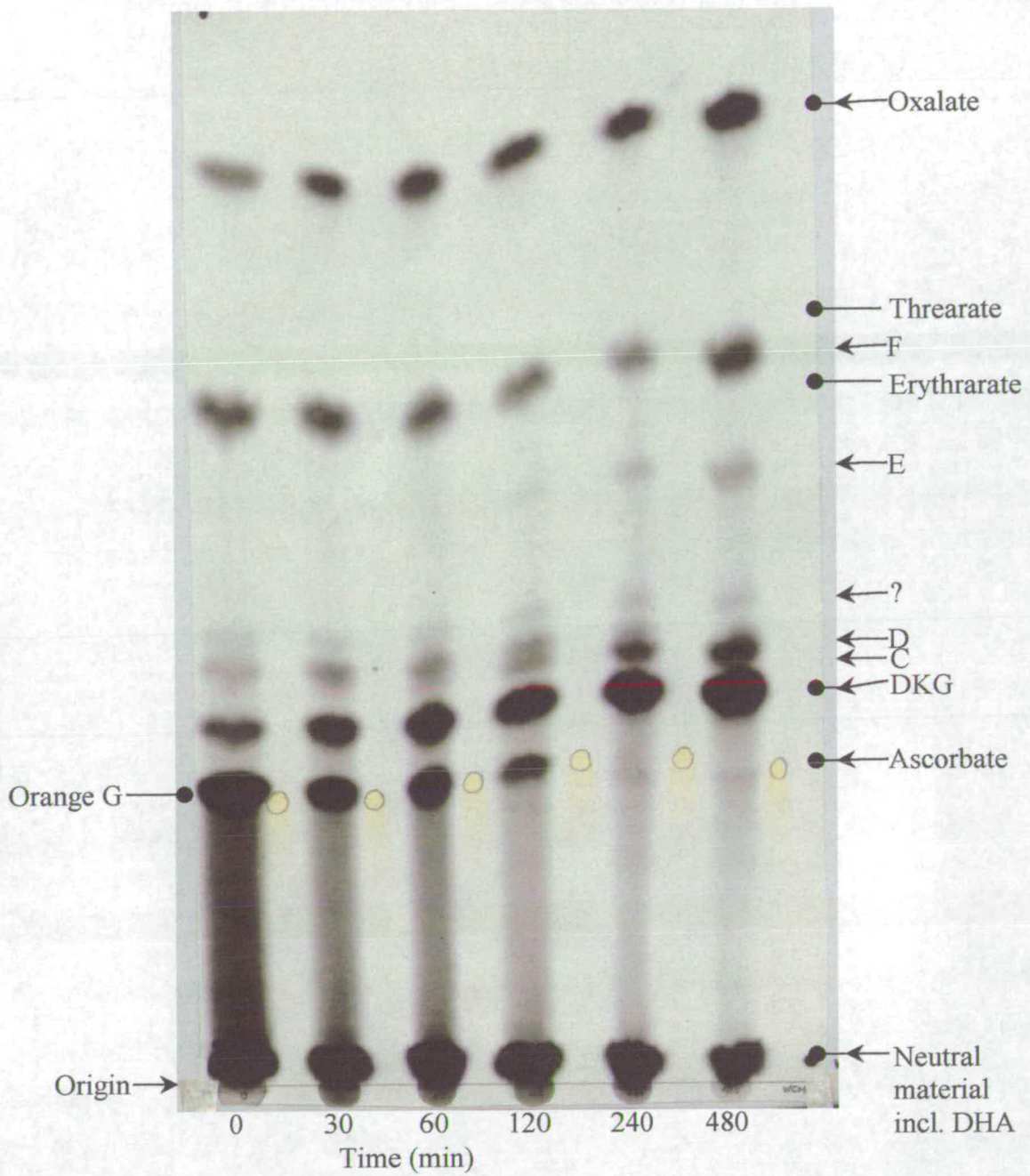
Figure 30.

B. Metabolism of ascorbate in whole, 5- d-old rose cell culture. Soluble, extracellular products were analysed as described in Figure 30 A.
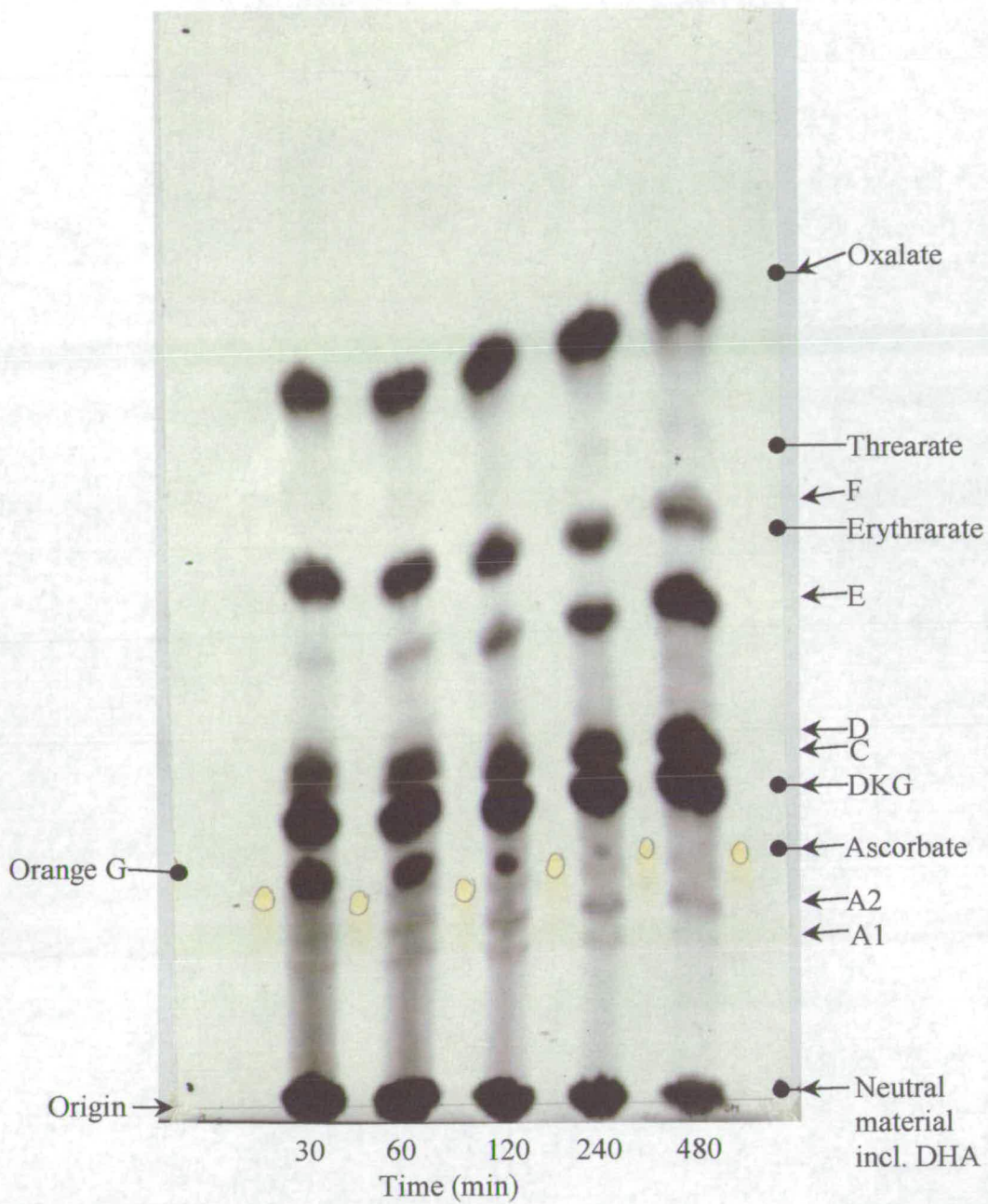
Figure 30

C. Breakdown of [1^{14}-C]ascorbate in spent medium obtained from 5-d-old rose cell suspension cultures. Soluble products were analysed as described in Figure 30 A.

136
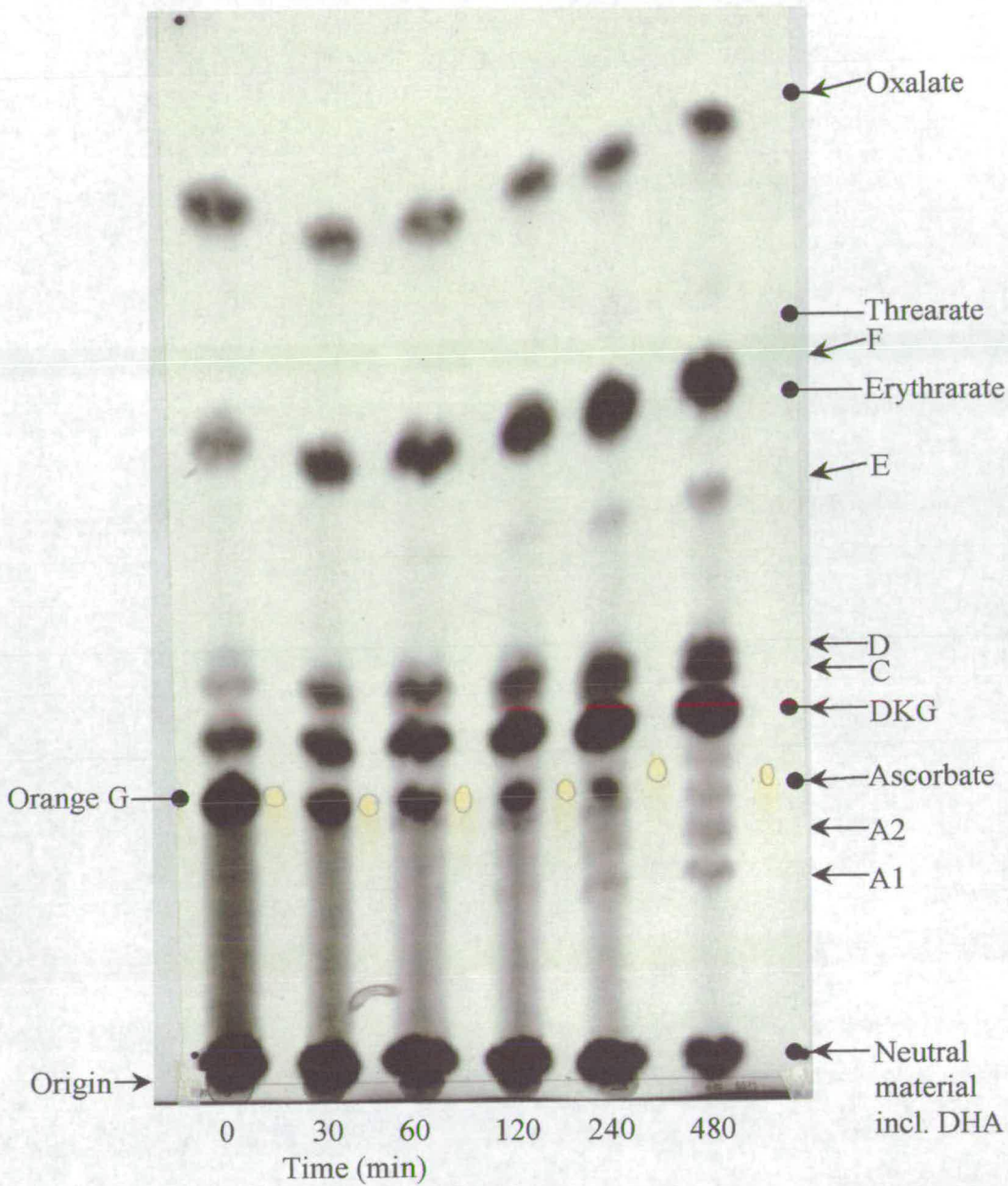
Figure 30

D. Fate of [^{14}C]ascorbate added to boiled, spent medium obtained from 5-d-old rose cell suspension cultures. Soluble products were analysed as described in Figure 30 A.

3.4.2 Fate of [1-^{14}C]ascorbate—experiment II

The fate of [1-^{14}C]ascorbate (14 Ci/mol) added to rose culture and culture medium (133 µl) at a final concentration of 0.5 mM was examined. The potential role of enzymes was investigated by examination of the fate of [1-^{14}C]ascorbate in high-M_r and low-M_r fractions of spent and boiled spent culture medium.

The breakdown of stock solutions of [1-^{14}C]ascorbate (50 µl, 5 µCi) for the present experiment was minimised by storage in liquid nitrogen. The '0-minute' time points (Fig. 31 A–H, Table 2.) still showed some oxidation of [1-^{14}C]ascorbate to [^{14}C]dehydroascorbate but the formation of other compounds such as [^{14}C]diketogulonate, [^{14}C]F and [^{14}C]oxlate was minimal compared to previous experiments (Fig. 31 A–D, Table 3.4.1).

[1-^{14}C]Ascorbate broke down in fresh medium (Fig. 31 A) forming considerable amounts of [^{14}C]dehydroascorbate. Streaks of ^{14}C between [^{14}C]dehydroascorbate and [1-^{14}C]ascorbate were present throughout the time course but diminished in intensity at later time points as the quantity of remaining [1-^{14}C]ascorbate decreased. These streaks obscured the possible presence of radiolabelled compounds A1 and A2. [^{14}C]Diketogulonate was present (trace amount) after 30 min and increased over the remainder of the time course. The radiolabelled compounds C and/or D were present in trace amount until 240 minutes and in moderate quantity by 480 minutes. These appeared to be two separate compounds but poor resolution made them difficult to distinguish. After 480 minutes radiolabelled compound E was present in a very small amount. Radiolabelled compound F increased slightly over the 8 hours but remained small in

amount. [^{14}C]Oxalate, was present in a small but consistent amount. The breakdown of [1-^{14}C]ascorbate in fresh culture medium was entirely non-enzymic.

In whole culture (Fig. 31 B) [1-^{14}C]ascorbate broke down more rapidly and was absent after 240 minutes. Very little [^{14}C]dehydroascorbate was present after 240 minutes and was absent by 480 minutes. The removal of [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate could be due to uptake by cells or to metabolism in the culture medium. Streaks between [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate were present until 60 minutes. After 120 minutes decreasing amounts of radiolabelled compound A1 and an accumulation of radiolabelled compound A2 were observed. [^{14}C]Diketogulonate rapidly accumulated in the culture medium and remained present in large amounts. A small quantity of radiolabelled compounds C and/or D appeared after 30 minutes, increased slightly by 60 minutes and then remained approximately constant. Radiolabelled compound E appeared at 60 minutes and gradually increased during the following 7 hours. The amount of radiolabelled compound F was roughly constant from 30 to 480 minutes; more was present than in fresh medium (Fig. 31 A, Table 2). [^{14}C]Oxalate gradually accumulated in the medium of whole cultures. These results show that metabolism of ascorbate was more extensive in medium of whole cultures (Fig. 31 B) than in fresh medium (Fig. 31 A) and comparison with metabolism in spent medium (Fig. 31 C) suggests that uptake of metabolites occurred when cells were present (Fig. 31 B).

[1-^{14}C]Ascorbate and [^{14}C]dehydroascorbate disappeared from cell-free spent medium by 480 minutes (Fig. 31 C, Table 2). Their removal from spent medium was slower than from whole culture (Fig. 31 B, Table 2). This suggested that uptake and metabolism occurred simultaneously or that cell wall bound

enzymes were responsible for metabolism. After 60 minutes radiolabelled compounds A1 and A2 were visible despite dark streaks. Radiolabelled compound A1 decreased and was absent from spent medium by 480 minutes. Radiolabelled compound A2 accumulated over the remainder of the time course and is suggested to be subject to uptake in the presence of cells (Fig. 31 B, C). [^{14}C]Diketogulonate remained relatively constant in culture medium throughout the time course while radiolabelled compounds C and/or D increased gradually in amount (Fig. 31 C). Radiolabelled compound E was present in greater quantity than in whole culture (Fig. 31 B, C, Table 2), after 30 minutes, and continued to increase. The accumulation of radiolabelled compound E in spent medium (Fig. 31 C. Table 2) compared with whole culture (Fig. 31 B, Table 2) suggests that compound E, or a precursor of this compound, is taken up by cells or metabolised by wall bound enzymes. A similar suggestion could be made for radiolabelled compound F, which was present in much larger amounts in spent medium (Fig. 31 C) than whole culture (Fig. 31 B). [^{14}C]Oxalate steadily accumulated in spent medium (Fig. 31 C) following a pattern similar to its accumulation in whole culture (Fig. 31 B) indicating that it was not extensively taken up, or further metabolised, by the cells.

The removal of [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate was slower from boiled spent medium (Fig. 31 D, Table 2) than from medium of whole cultures (Fig. 31 B, Table 2) or spent medium (Fig. 31 C, Table 2). [1-^{14}C]Ascorbate decreased from 120 to 480 minutes while [^{14}C]dehydroascorbate increased slightly during the first 2 hours but then gradually decreased. Small amounts of [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate remained after 8 hours in boiled spent medium (Fig. 31 D). This suggests that enzymes released by cells could have a role

in ascorbate and dehydroascorbate metabolism. Radiolabelled compound A1 decreased while radiolabelled compound [^{14}C]A2 increased during the last 6 hours of the time course. Both these radiolabelled compounds were obscured during the first two hours. Their formation does not rely on enzymes secreted by cells but does rely on some heat-stable compound(s) released from cells since radiolabelled compounds A1 and A2 were not formed in fresh culture medium (Fig 31 A, D, Table 2). The formation of radiolabelled compounds C and/or D in boiled spent medium was similar to their formation in unboiled spent medium and whole culture (Fig. 31 B–D) but more extensive than in fresh medium (Fig. 31 A). This indicates that although they can be formed non-enzymically by components of fresh medium, their formation is enhanced by the presence of heat-stable cellular metabolites. Alternatively, the formation of these compounds may be inhibited by a component of fresh medium that is removed by 5 days' growth of cells. Radiolabelled compound E was formed after 60 minutes and, although it increased over the next 7 hours, it did not accumulate as much as in unboiled spent medium (Fig. 31C, D, Table 2). Therefore, formation of this metabolite was promoted by the presence of enzymes but also occurred non-enzymically. Radiolabelled compound F accumulated in boiled spent medium (Fig. 31 D) in large quantities. Less [^{14}C]oxalate accumulated in boiled spent medium (Fig 31 D, Table 2) than in unboiled spent medium (Fig. 31 C, Table 2) or whole culture (Fig. 31 B, Table 2). Its formation therefore appears to be promoted by, but not dependent on, the presence of enzymes in medium.

The role of enzymes in apoplastic ascorbate metabolism was examined by addition of [1-^{14}C]ascorbate to the high molecular weight fraction of spent medium

(Fig. 31 E) and of boiled spent medium (Fig. 31 F). The amounts of [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate present remained reasonably constant in the high-M_r fractions of both spent (Fig. 31 E, Table 2) and boiled spent medium (Fig. 31 F, Table 2) although formation of [^{14}C]diketogulonate was slightly more rapid in the high-M_r fraction of unboiled spent medium (Fig. 31 E, Table 2), which shows that although the formation of [^{14}C]diketogulonate is promoted in the presence of enzymes it also occurs in their absence. The possible formation of radiolabelled compounds A1 and A2 in the high-M_r fraction of spent medium (Fig. 31 E) was obscured until 120 minutes but after this time neither radiolabelled compound was present. The density of streaks between [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate (Fig. 31 F) obscured the presence of radiolabelled compounds A1 and A2 throughout the time course. This prevented any conclusions as to the nature of A1 and A2 formation being drawn. Radiolabelled compounds C and/or D were present in larger amounts after 480 minutes in the high-M_r fraction from boiled spent medium (Fig. 31 F) than in that from unboiled spent medium (Fig. 31.E). A very small amount of radiolabelled compound E was present after 480 minutes in the high-M_r fractions of spent medium and boiled spent medium (Fig. 31 E, F, Table 2)—much less than in whole culture, spent medium and boiled spent medium (Fig. 31 B, C, D, Table 2). Accumulation of radiolabelled compound F in the high-M_r (Fig. 31 E, F, Table 2) fractions of culture medium showed a similar pattern to that in fresh medium (Fig 31 A, Table 2). Similar observations were made for [^{14}C]oxalate.

The effect of low-M_r compounds on [1-^{14}C]ascorbate metabolism was examined by addition of [1-^{14}C]ascorbate to the low-M_r fraction of spent medium

and of boiled spent medium. The amount of [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate remained relatively constant throughout the time course in the low-M_r fraction from both spent medium and boiled spent medium (Fig. 31 G, H, Table 2). A gradual increase in [^{14}C]diketogulonate was observed. No radiolabelled compound E was formed and radiolabelled compound F and [^{14}C]oxalate remained constant over the 8-hour period. The density of ^{14}C streaks between [^{14}C]dehydroascorbate and [1-^{14}C]ascorbate obscured the possible presence of radiolabelled compounds A1 and A2 throughout the time course and were the most consistent and dense ^{14}C streaks observed in any of the condition examined.

Table 2 Summary of the fate of [1-^{14}C]ascorbate in rose culture, culture medium and fractions of culture medium—experiment II

Cmpd	Fresh medium	Whole culture	Spent medium	Boiled spent medium	High-M_r fraction of spent medium	High-M_r fraction of boiled spent medium	Low-M_r fraction of spent medium	Low-M_r fraction of boiled spent medium
DHA	OOOOOO	OOOOo∘	OOOOo∘	OOOO∘.	OOOOOO	OOOOOO	−OOOOO	OOOOOO
A1	?????	???...	??...	???∘∘O	????	????	−?????	?????
A2	?????	???...	??∘∘∘∘	???∘∘.	????	????	−?????	??????
AH$_2$	OOOOOO	OOOO	OOOOo	OOOOOo	OOOOOO	OOOOOO	−OOOOO	OOOOOO
DKG	..∘∘OO	.OOOOO	∘OOOOO	.∘∘OOO	??∘∘OO	..∘∘∘O	−..∘OO	..∘∘∘∘
C	∘∘∘∘OO	∘∘OOO	.∘∘OOO	.∘∘OOO	.....∘∘	......∘	−...∘∘	∘∘∘
D	.....∘	∘∘OOO	.∘∘OOO	.∘∘OOO	......∘∘	......∘	−...∘∘	...∘∘∘
E	.∘	..∘∘	.∘∘OO	.∘OO	.	.		
F	∘∘∘∘OO	.OOOOO	.∘OOOO	.∘∘OOO	.....∘O	.....∘∘	−..∘∘∘	∘∘∘∘∘∘
Oxalate	∘∘∘∘∘∘	.∘∘OOO	.∘∘OOO	.∘∘∘∘O	...∘OO	∘∘∘∘∘O	−OOOOO	OOOOOO

Each symbol represents a time point (0, 30, 60...minutes). − indicates a missing sample, ? indicates a compound that may be present but is obscured by streaking or is indistinguishable from another darker spot. Spots C and D were difficult to differentiate.
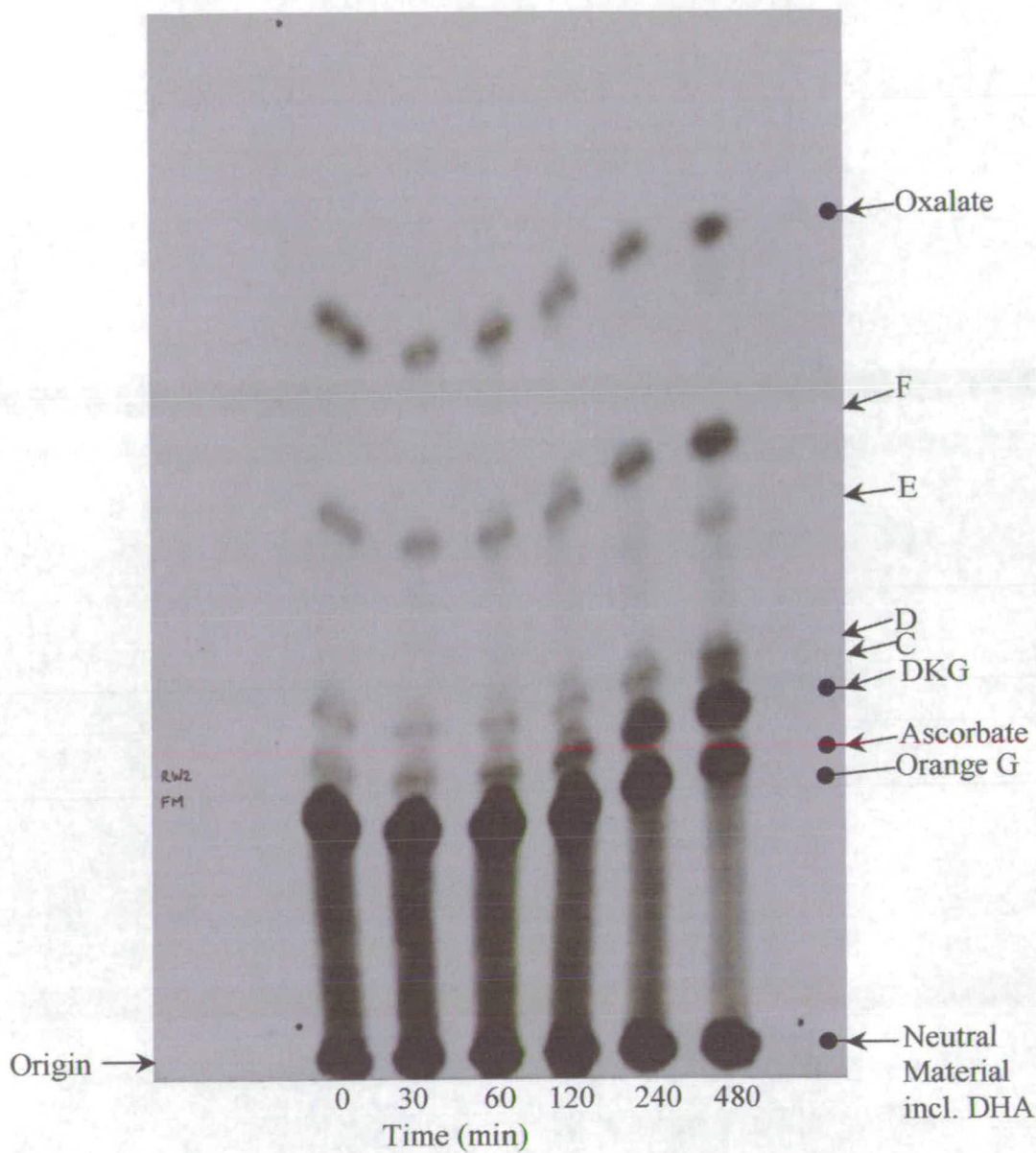
Figure 31. Fate of [1-^{14}C]ascorbate in rose cultures, culture medium and fractions of culture medium—experiment II

A. Non-enzymic breakdown of [1-^{14}C]ascorbate in fresh, unused culture medium. Soluble products were analaysed by electrophoresis in pH 6.5 buffer, 3 kV for 30 minutes followed by autoradiography.

●— represents the mobility of external standards
◀— represents radioactive metabolites of [1-^{14}C]ascorbate

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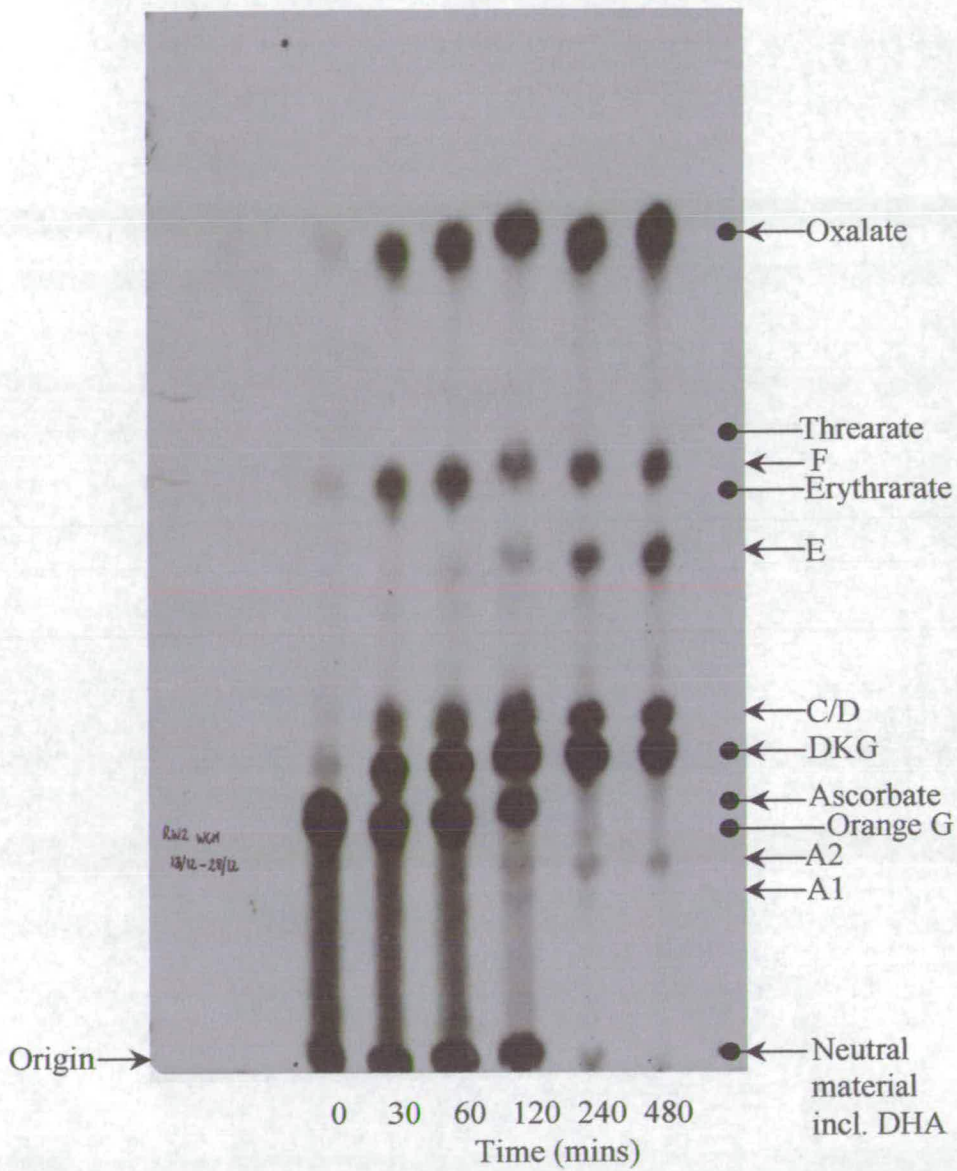
Figure 31.

B. Metabolism of [1-^{14}C]ascorbate in whole 5-d-old rose cultures. Soluble, extracellular products were analysed as in Fig. 31 A.
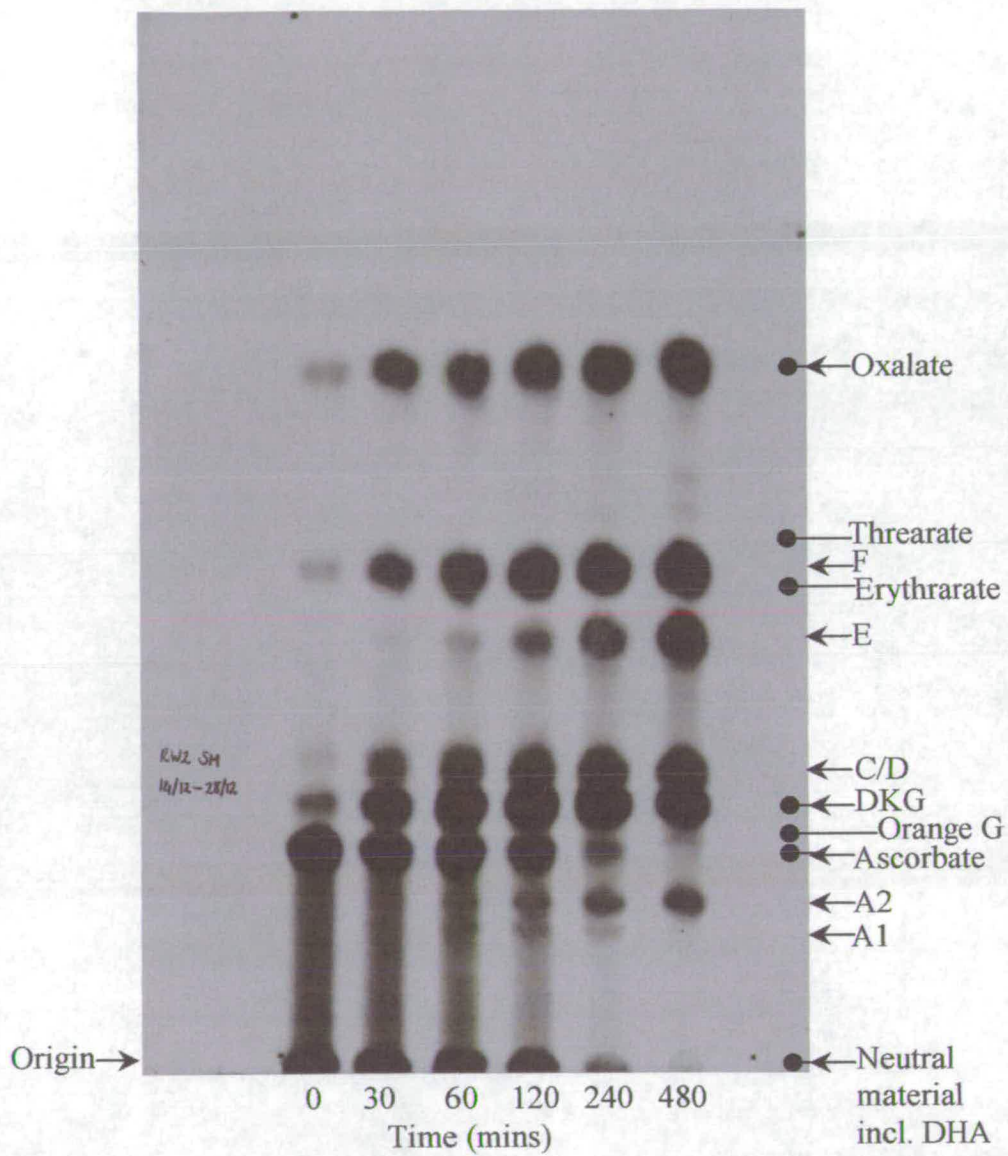
Figure 31

C. Breakdown of [1-^{14}C]ascorbate in spent medium from 5-d-old rose cell suspension culture. Soluble products were analysed as described in Fig 31 A.
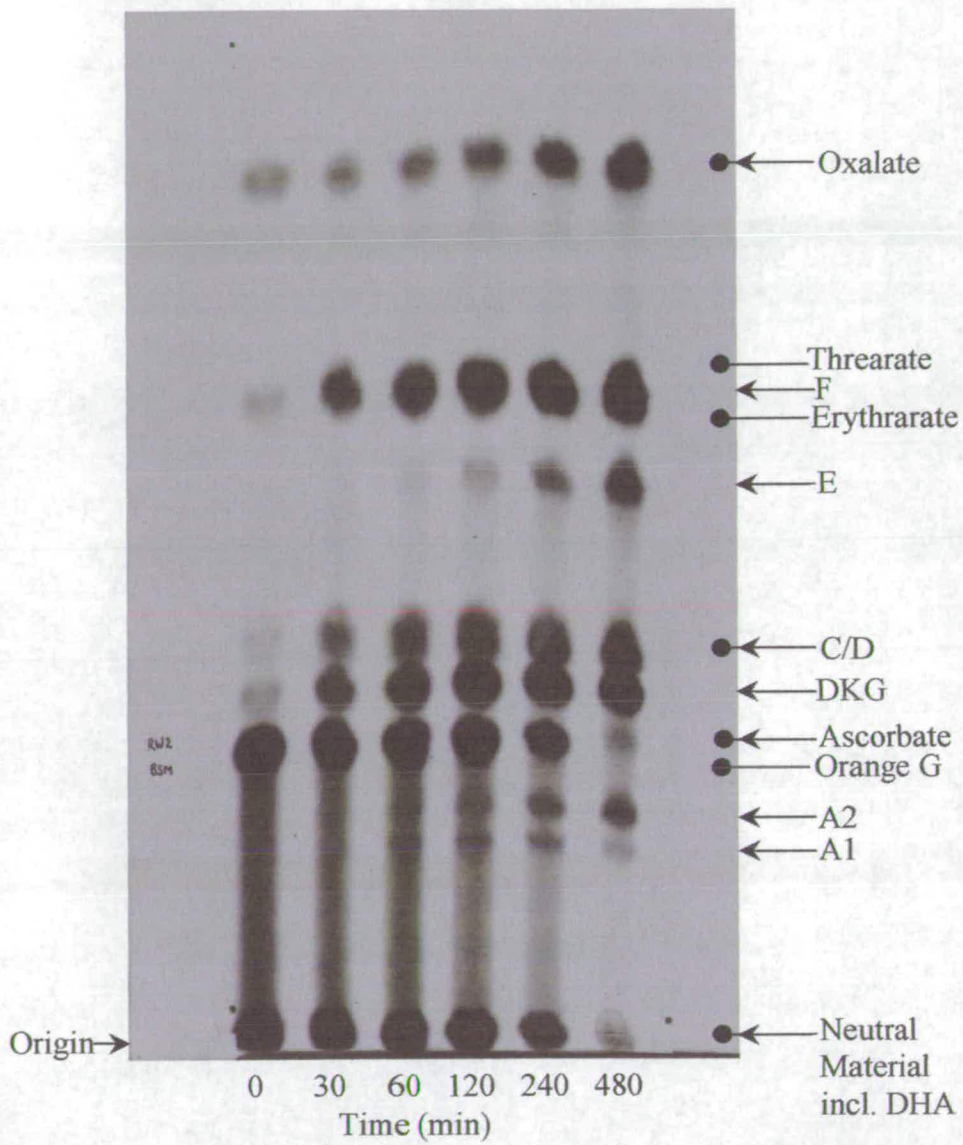
Figure 31

D. Fate of [1-^{14}C]ascorbate in boiled spent culture medium. Soluble products were analysed as described in Figure 31 A.
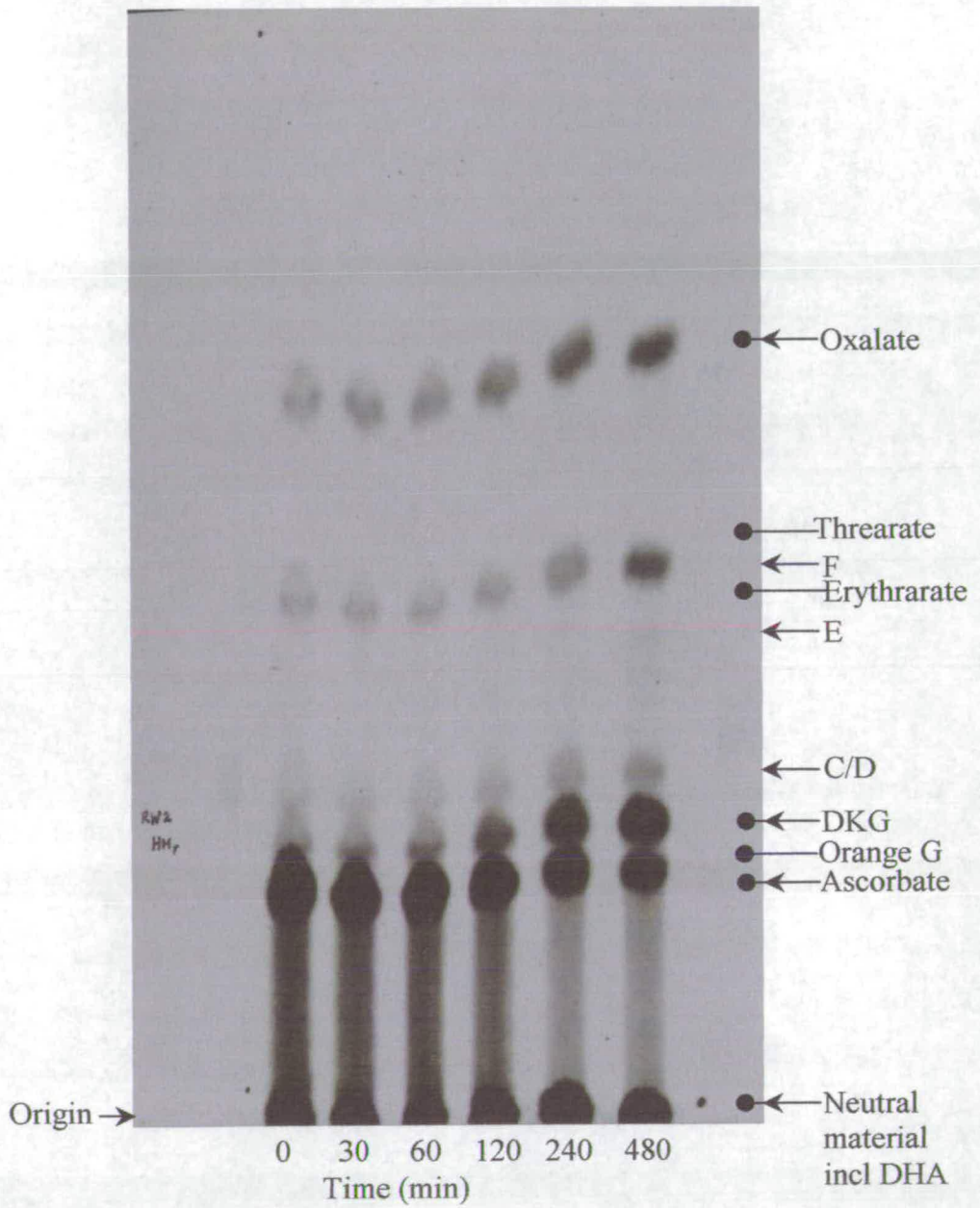
Figure 31

E. Breakdown of [1-^{14}C]ascorbate in the high molecular weight fraction of spent medium from 5-d-old rose culture. Soluble products were analysed as described in Fig. 31 A.
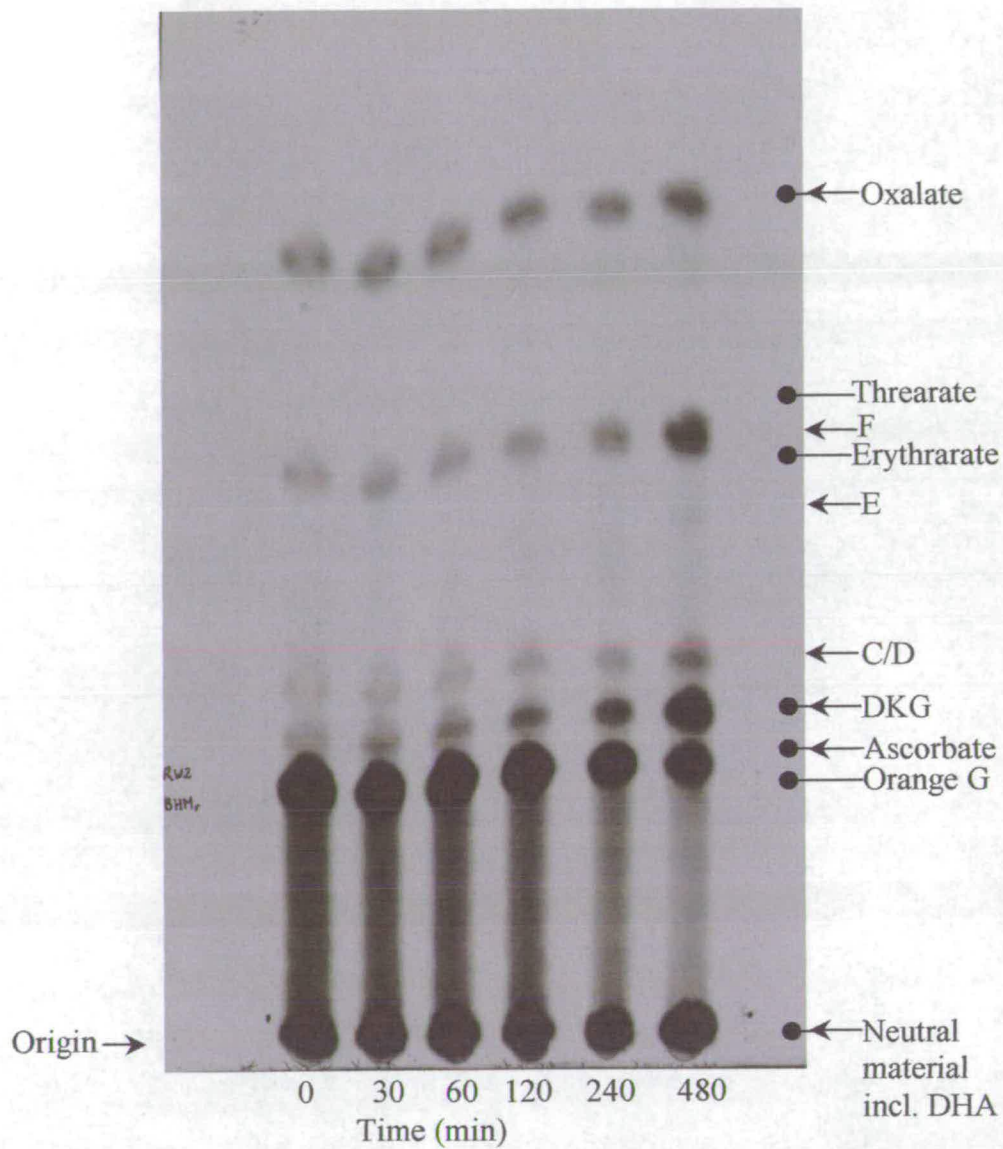
Figure 31

F. Breakdown of [1-^{14}C]ascorbate in the high molecular weight fraction of boiled spent medium from 5-d-old rose cultures. Soluble products were analysed as described in Figure 31 A.
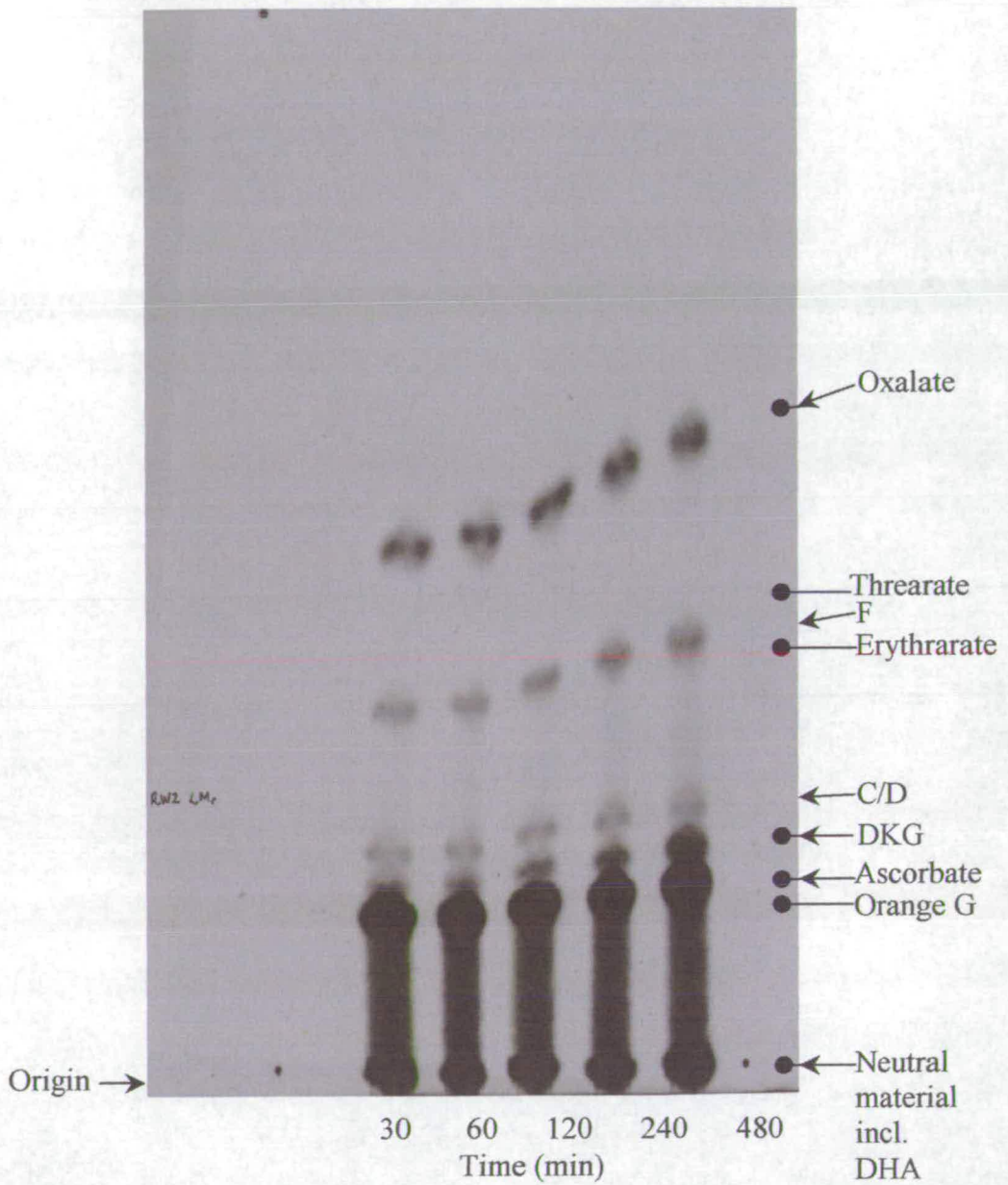
Figure 31

G. Breakdown of [1-^{14}C]ascorbate in the low molecular weight fraction of spent culture medium from 5-d-old rose cultures. Soluble products analysed as described in Figure 31 A.
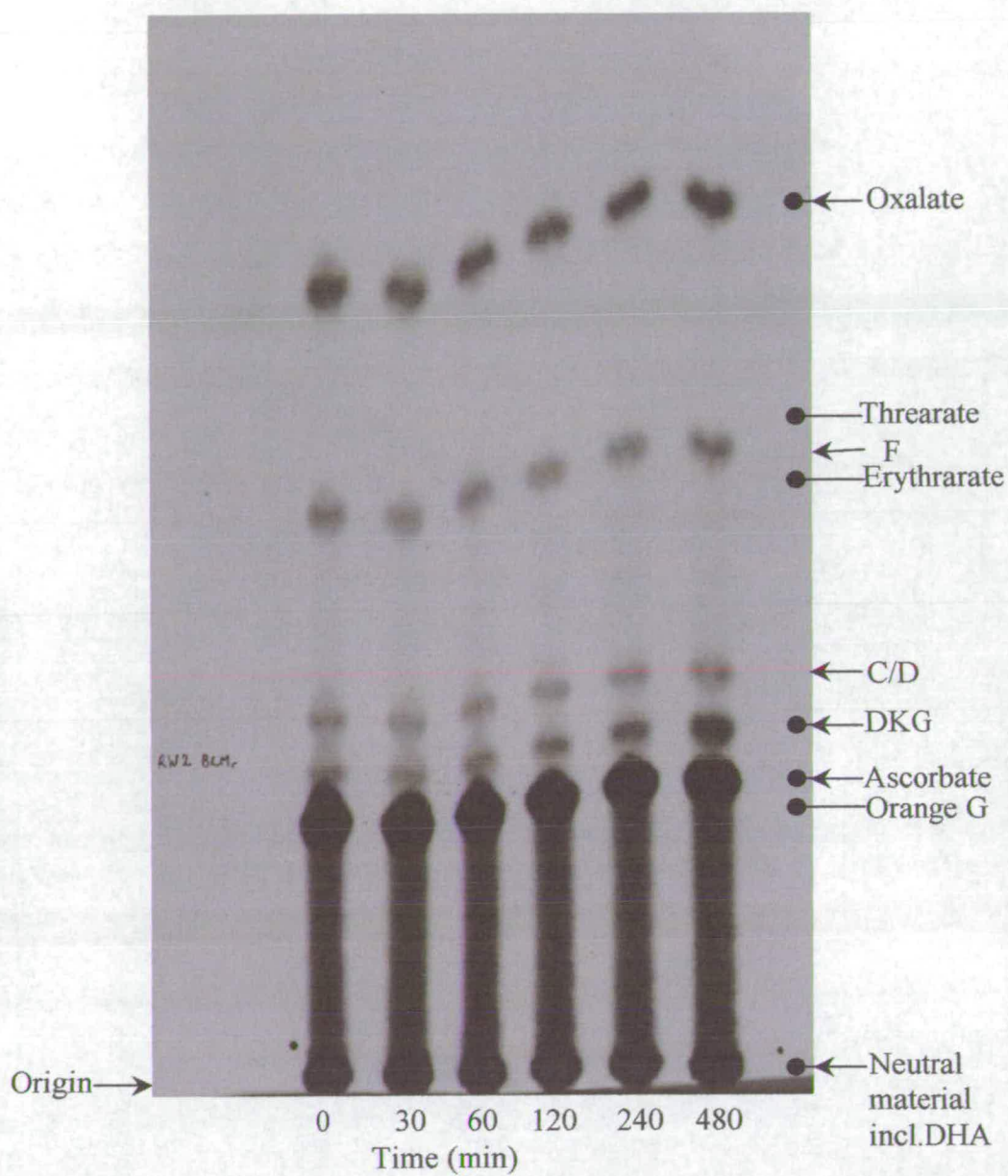
Figure 31

H. Fate of [1-^{14}C]ascorbate in the low molecular weight fraction of boiled spent medium from 5-d-old rose cultures. Soluble products were analysed as described in Figure 31 A.

3.4.3. Fate of [1-^{14}C]ascorbate—experiment III

The fate of [1-^{14}C]ascorbate (14 Ci/mol) at a final concentration of 0.5 mM was again examined in rose culture and culture medium (143 µl). Soluble, extracellular products were analysed by electrophoresis in pH 6.5 buffer followed by autoradiography to detect metabolites. Radiolabelled metabolites detected by autradiography were excised from electrophoretograms and quantified.

Breakdown of [1-^{14}C]ascorbate during storage on liquid nitrogen was minimal. The 0-minute samples showed some oxidation of [1-^{14}C]ascorbate to [^{14}C]dehydroascorbate but other breakdown products were limited (Fig. 32 A–D).

In fresh medium (Fig. 32 A, Table 3) [1-^{14}C]ascorbate decreased gradually over the 8-hour time course as it broke down non-enzymically. [^{14}C]Dehydroascorbate was formed during the first 60 minutes but then decreased for the rest of the time course (Fig. 32 A, E). Increasing amounts of [^{14}C]diketogulonate were formed over the time course (Fig 32 A, E). Radiolabelled C/D were present in very small quantities by 30 minutes. The amounts of these compounds increased slightly over the next 7.5 hours (Fig. 32 A, E). A small amount of radiolabelled E was present after 8 hours. Radiolabelled compound F was present at 30 minutes and increased during the next 7.5 hours (Fig. 32 A, E). [^{14}C]Oxalate was present in a small quantity at 30 minutes and increased slightly over the rest of the time course (Fig. 32 A, E). These results show that oxidation of [1-^{14}C]ascorbate and hydrolysis of [^{14}C]dehydroascorbate occur non-enzymically and, although some further non-enzymic breakdown occurred, it was limited.

[1-^{14}C]Ascorbate was rapidly removed from the medium of a whole culture and had completely disappeaed by 4 hours (Fig. 32 B). [^{14}C]Dehydroascorbate was

also completely removed after 4 hours (Fig. 32 B, E). The amount of [^{14}C]diketogulonate increased during the first hour but declined slightly over the remainder of the time course (Fig. 32 B, E). A small amount of radiolabelled A2 was detected from 2 to 8 hours. Radiolabelled compounds C and/or D increased during the first 60 minutes and remained constant over the following 7 hours (Fig 32 B, E). A small amount of radioactive compound E appeared at 2 hours and increased slightly over the remainder of the time course (Fig. 32 B, E). Radiolabelled compound F was present in culture medium in a small quantity after 30 minutes (Fig. 32 B, E). The amount of radiolabelled compound F remained roughly constant throughout the time course (Fig. 32 B, E). [^{14}C]Oxalate accumulated to considerable amounts in the medium of whole cultures (Fig. 32 B). It was not clear from these results which metabolites were produced in the culture medium or whether metabolism was intracellular with metabolites secreted into the culture medium.

In cell-free spent medium [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate were rapidly removed and were absent by 4 hours suggesting that these compounds were subject to degradation by apoplastic enzymes even in the absence of any uptake by cells (Fig. 32 B, C, E). [^{14}C]Diketogulonate, detected by autoradiography (Fig. 32 C), appeared to accumulate in the first 2 hours then remain constant for the rest of the time course (Fig. 32 C). However, when quantified, the amount of [^{14}C]diketogulonate present was found to decrease during the last 6 hours of the time course (Fig. 32 E). Radiolabelled compounds C and/or D increased during the first half of the time course then remained roughly constant for the final 4 hours (Fig. 32 C, E). Radiolabelled compound E was detected after 30 minutes and

increased considerably throughout the remainder of the time course. This suggests that either compound E or a precursor of compound E is usually taken up by cells. A small amount of radiolabelled compound F was detected at time 0. This appeared to decrease slightly but was roughly constant for the final 4 hours (Fig. 32 C, E). $[^{14}C]$Oxalate accumulated over the 8-hour time course.

The breakdown of $[1-^{14}C]$ascorbate in boiled spent medium (Fig. 32 D, Table 3) was slower than in whole culture (Fig. 32 B, Table 3) and in unboiled spent medium (Fig. 32 C, Table 3) but similar to fresh medium (Fig. 32 A, Table 3). $[^{14}C]$Dehydroascorbate was not completely removed from boiled spent medium after 8 hours although it was greatly reduced in amount compared to fresh medium (Fig. 32 A, Table 3). $[^{14}C]$Diketogulonate accumulated during the first 2 hours and was roughly constant for the remainder of the time course (Fig. 32 D, E). The accumulation of $[^{14}C]$diketogulonate in fresh medium (Fig. 32 A, E, Table 3) was slower but continued throughout the time course compared to boiled spent medium (Fig. 32 D, E Table 3). A small amount of radiolabelled compound A1 was present at 2 hours but was absent for the rest of the time course. Radiolabelled compound A2 was present in a small quantity throughout the time course. Radiolablelled C/D increased gradually throughout the time course (Fig. 32 D, E). Radiolabelled compound E was detected after 1 hour and increased gradually during the following 7 hours (Fig. 32 D, E); itfollowed a similar pattern to its formation in native spent medium (Fig. 32 C, E, Table 3). Radiolabelled compound F accumulated considerably during the first 2 hours in boiled spent medium, decreasing only slightly during the final 4 hours (Fig. 32 D, E), suggesting that it is broken down by enzymes present in the culture medium (Fig. 32 C, E). Accumulation of

[^{14}C]oxalate was much slower than in whole culture (Fig. 32 B, Table 3) and in unboiled spent medium (Fig. 32 C, Table 3) but faster than in fresh medium (Fig. 32 A, Table 3) supporting the suggestion that enzymic breakdown of radiolabelled compound F results in formation of oxalate.

The results suggest that ascorbate, dehydroascorbate and diketogulonate are not extensively taken up by cells but that ascorbate and dehydroascorbate are metabolised both enzymically and non-enzymically in culture medium. Compound E is formed in large quantities in spent medium and boiled spent medium. It does not accumulate as much in whole culture suggesting that this compound or a precursor of it is taken up by cells present in whole culture. Compound F is broken down by the action of enzymes. Denaturation of enzymes in spent medium allowed this compound to accumulate.

Table 3 Fate of [1-^{14}C]ascorbate in rose culture and culture medium—experiment III

Compound	Fresh medium	Whole culture	Spent medium	Boiled spent medium
DHA	○○○○○○	○○○	◯○○	○○○ .
A1	. .	? ? ?	? ? ?	? ? ? .
A2	. .	? ? ? . . .	? ? ? . . .	? ? ? . . .
Ascorbate	◯◯◯○○	◯○○	◯○○	◯◯○○○ .
DKG	. . ○◯	. ○○◯	. ○○◯	. ○◯◯
C/D	. . .	○○	○○	○○○
E	.	. .	. . ○◯	○○
F	. . ○	. .	. .	○○◯
Oxalate	. . . .	○○◯	○○◯	. . ○

Summary of results describing the fate of ^{14}C from [1-^{14}C]ascorbate in 5-d-old rose culture and culture medium. The information was derived from the autoradiographs shown in Figure 32. Each symbol represents the relative amount of each radioactive compound present at a time point (◯>○> > .). ? indicates a compound that may be obscured by another radiolabelled compound or by streaking. A blank indicates an undetected compound.
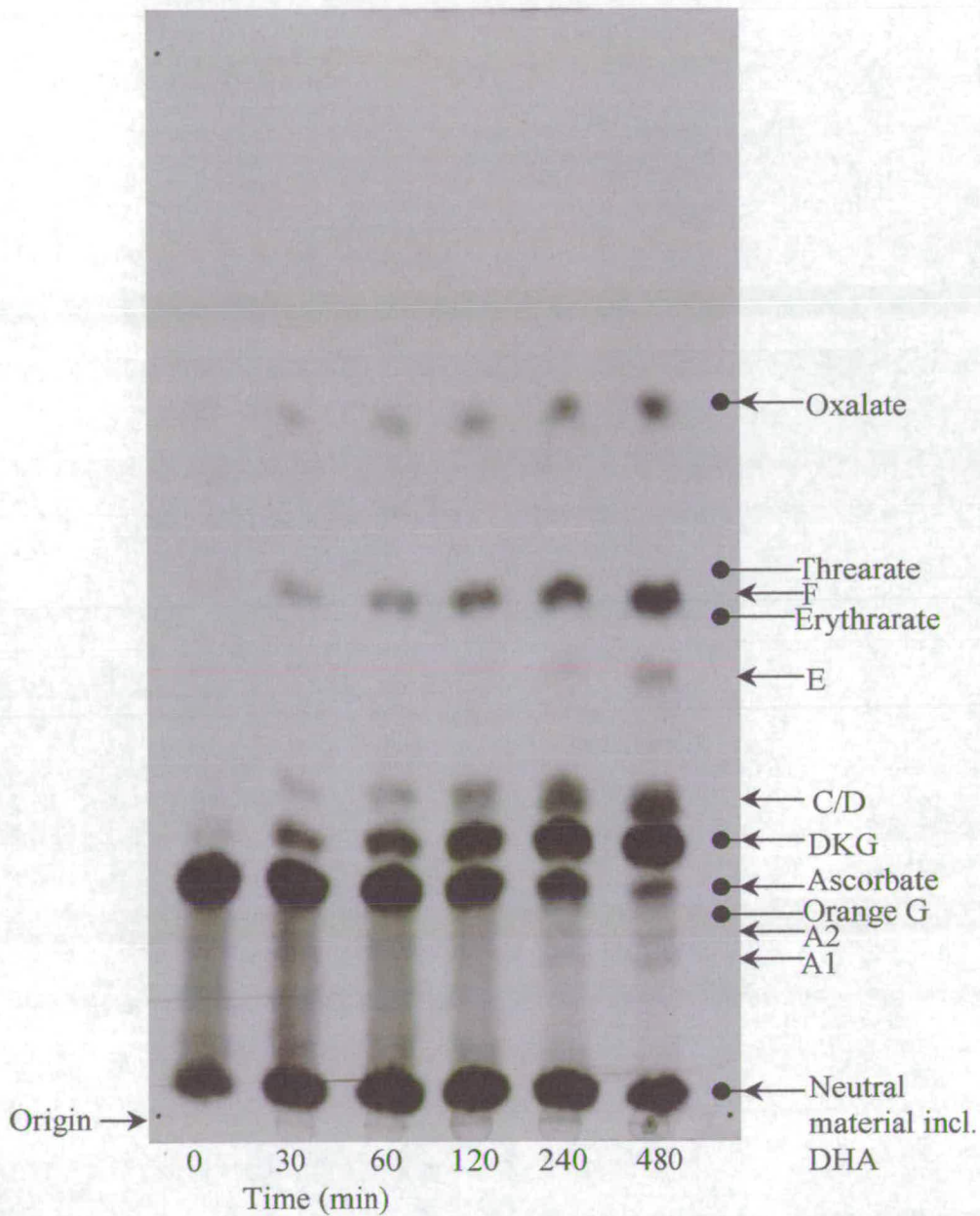
Figure 32. Fate of [1-^{14}C]ascorbate in rose cultures and culture medium

A. Non-enzymic breakdown of [1-^{14}C]ascorbate in fresh, unused rose culture medium. Soluble products were analysed by electrophoresis pH 6.5 buffer, 3 kV for 30 minutes followed by autoradiography.

●——represents the approximate mobility of external standards
←——represents radioactive metabolites of ascorbate
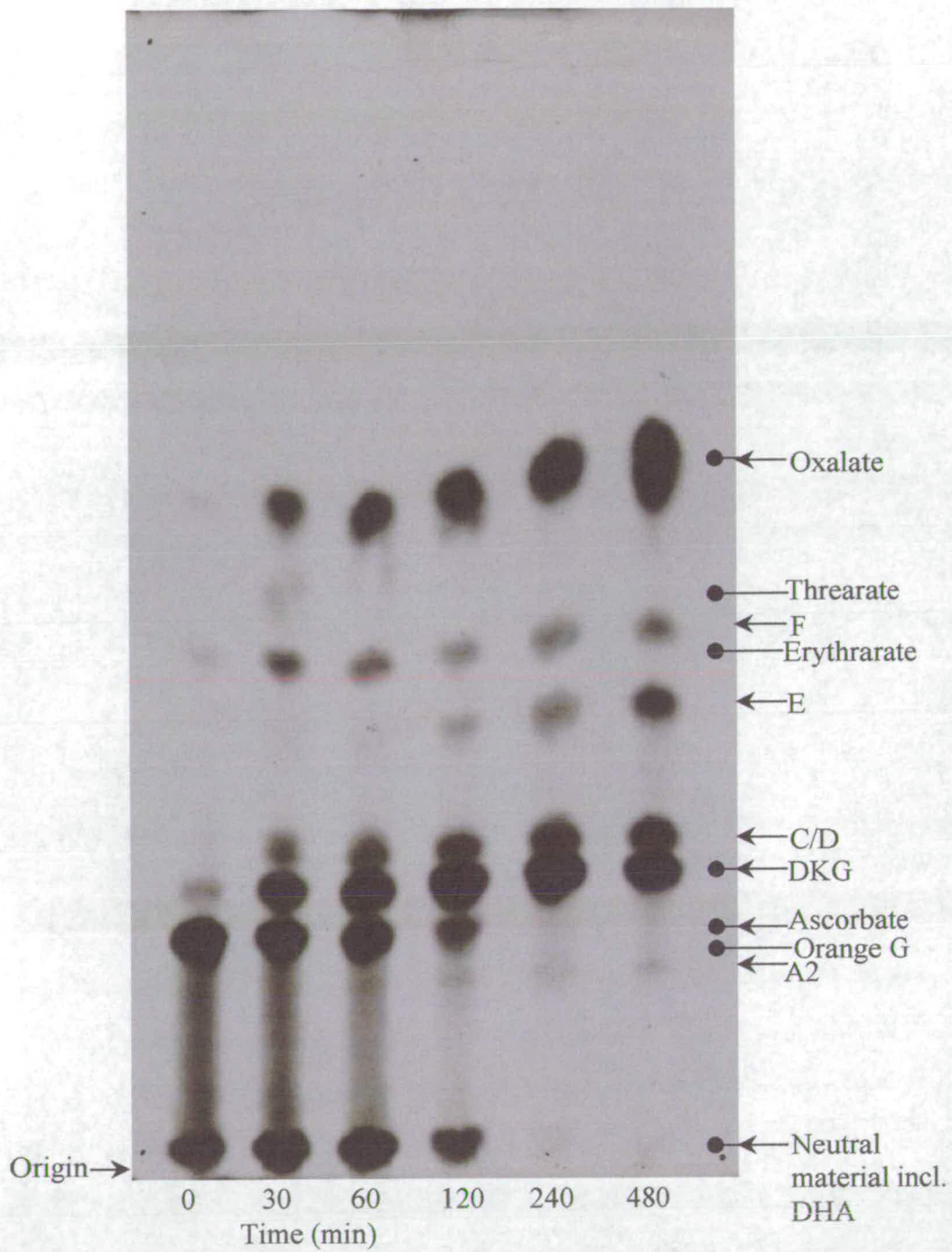
Fig. 32

B. Metabolism of [1-^{14}C]ascorbate in a whole 5-d-old rose culture. Analysis of soluble, extracellular products as described in Figure 32 A.

Labels on figure:
Oxalate
Threarate
F
Erythrarate
E
C/D
DKG
Ascorbate
Orange G
A2
A1
Neutral material incl. DHA

Origin →

Time (min): 0 30 60 120 240 480
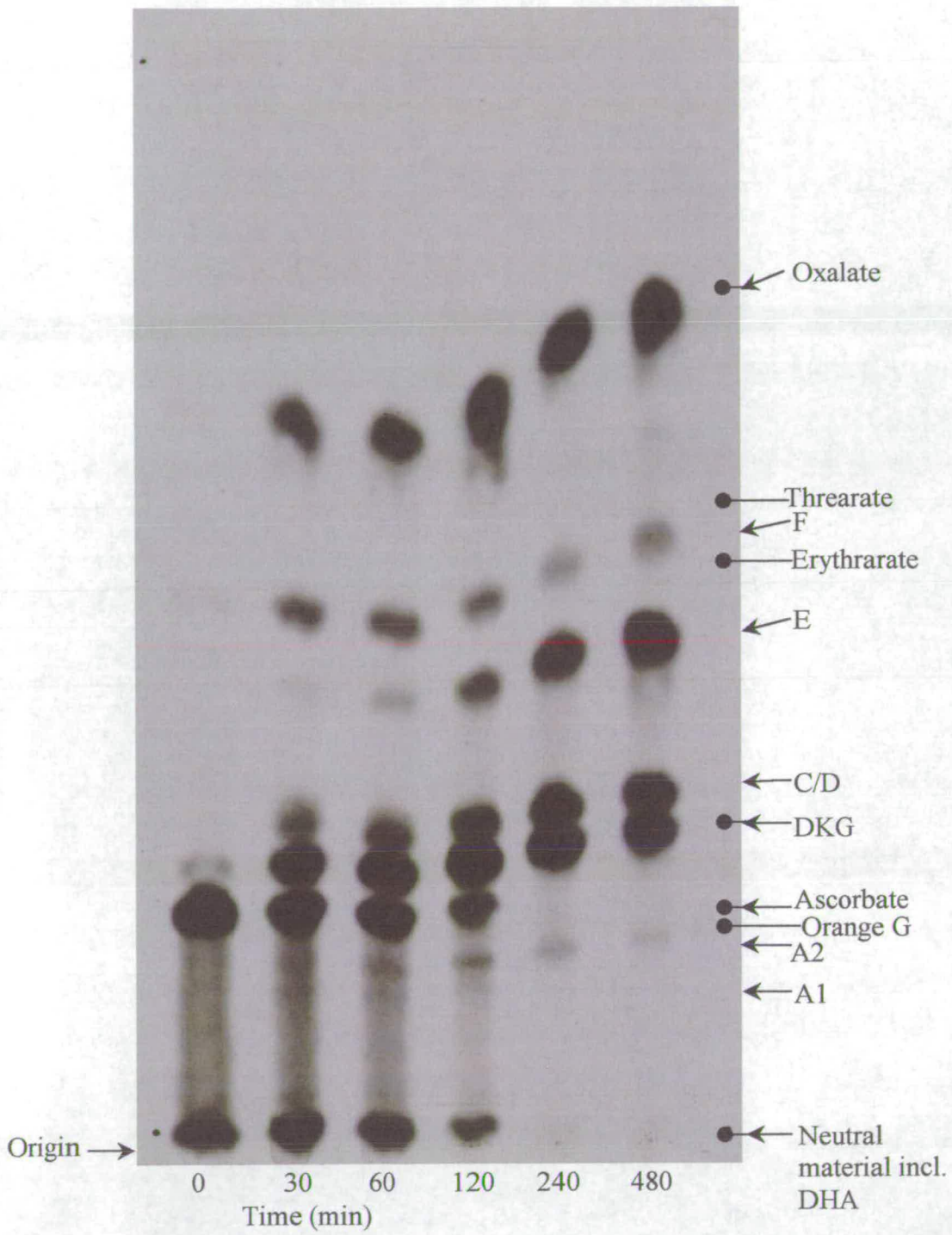
Fig. 32

C. Breakdown of [1-^{14}C]ascorbate in spent culture medium from 5-d-old rose culture. Analysis as described in Figure 32 A.
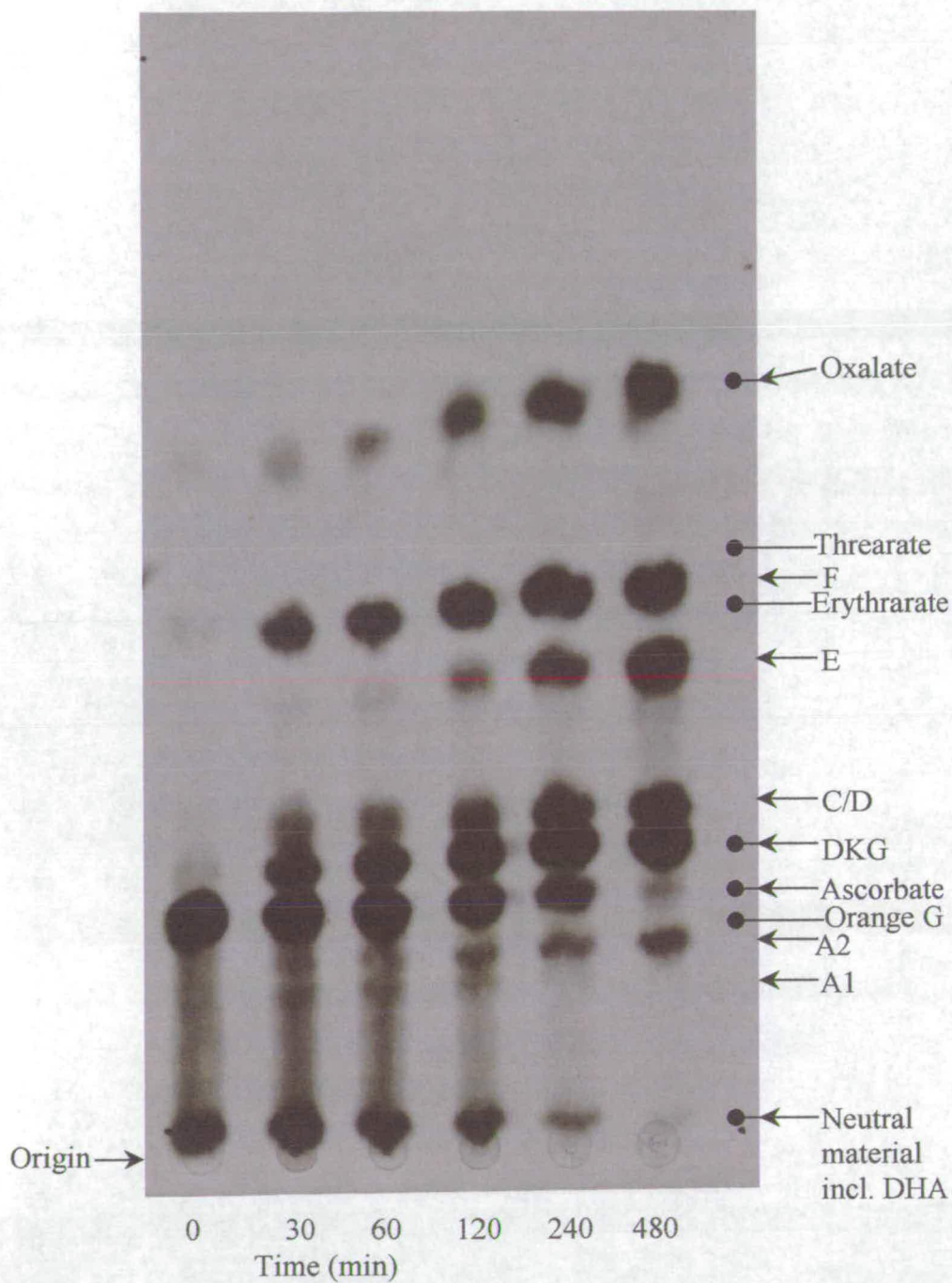
Fig. 32

D. Breakdown of [1-^{14}C]ascorbate in boiled spent medium from 5-d-old rose culture. Soluble products analysed as described in Fig. 32 A.

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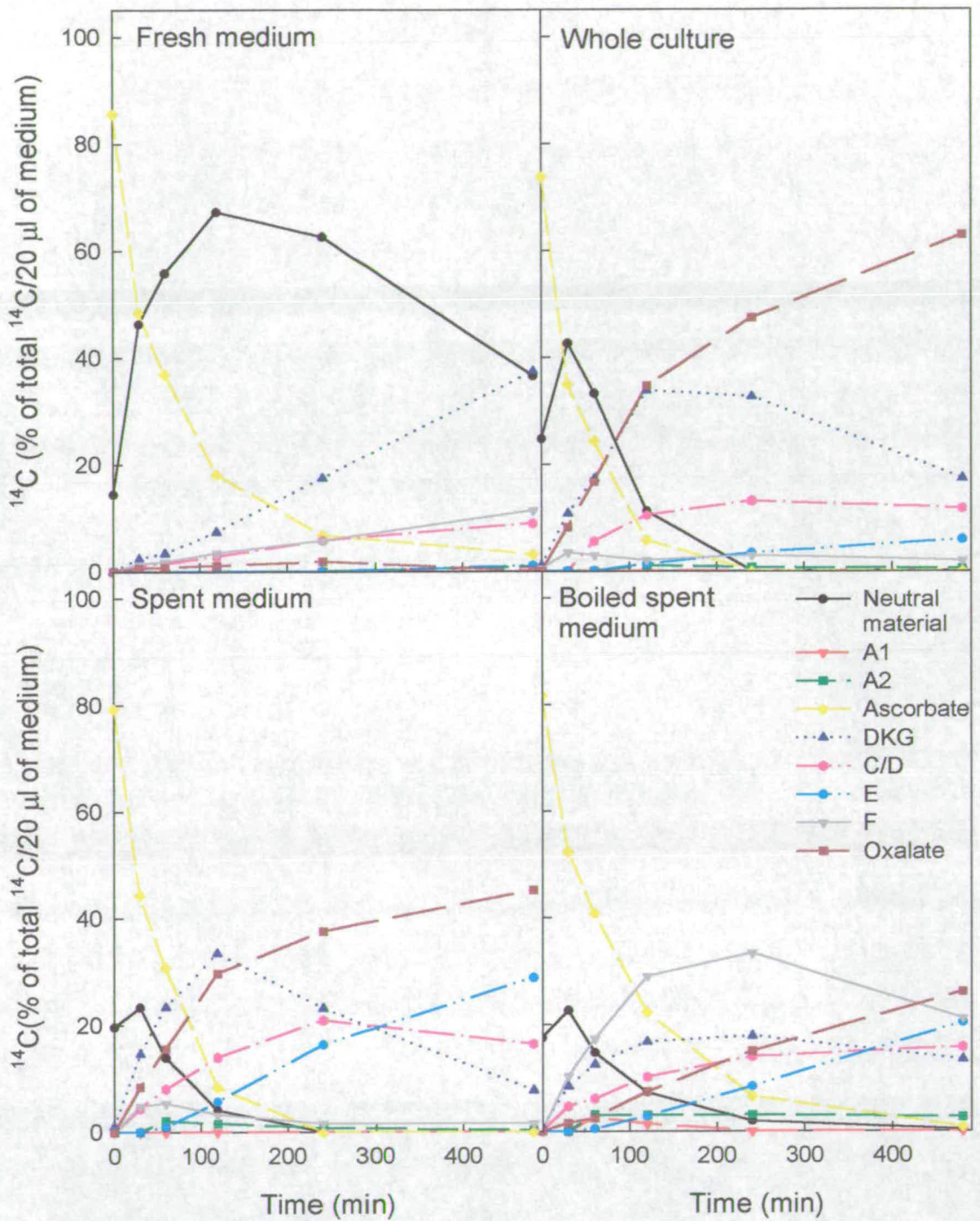
Figure 32

E. Fate of [1-^{14}C]ascorbate in rose culture and culture medium was quantitatively analysed by scintillation counting the areas of electrophoretograms corresponding to metabolites detected by autoradiography. Immobile material that remained on the origin was not included as dehydroascorbate.

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The consensus of the results of all three experiments on the feeding [1-^{14}C]ascorbate to rose cultures and culture medium has been summarised in Table 4

[1-^{14}C]Ascorbate and dehydroascorbate were both removed from the medium of whole rose culture, fresh and spent culture medium. Metabolism of [1-^{14}C]ascorbate was fastest in whole culture and spent medium and although the metabolism of dehydroascorbate was slower than that of [1-^{14}C]ascorbate it followed a similar pattern.

Radiolabelled compounds A1 and A2 were difficult to detect because of [^{14}C]DHA streaks. A1 and A2 were detected after 2 hours in a small but consistent amount.

[^{14}C]Diketogulonate accumulated in the medium of whole rose culture, fresh medium, unboiled spent medium and boiled spent medium. Its accumulation reflected the removal of [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate.

Radiolabelled compounds C and D were only partially resolved. Radiolabelled compounds C and/or D were detected early in the time course, after 1 hour, and increased gradually by a small amount over the rest of the time course.

Radiolabelled compound E was formed in a small amount in fresh medium and whole culture. It accumulated in much larger amounts in spent medium and boiled spent medium. This could indicate that radiolabelled compound E is produced non-enzymically from a precursor which may be subject to uptake by cells. Alternatively, radiolabelled compound E, produced in the apoplast, may be taken up by cells or adhere to cell surfaces in whole rose culture. Components of fresh rose culture could prevent the formation of compound E, or promote its breakdown, thus preventing its accumulation fresh medium. The uptake of such

components of fresh medium by rose cells during a 5-d growth period could allow radiolabelled compound E to accumulate.

Radiolabelled compound F accumulated most in boiled spent medium but not as much in whole culture or unboiled spent medium. The formation of radiolabelled compound F is likely to be non-enzymic since it forms in boiled spent medium. Formation of compound F could be promoted by a heat-stable compound secreted by rose cells during a 5-d growth period. More compound F accumulates in fresh medium than whole culture or spent medium, indicating that it is more likely to be a heat-stable component of fresh medium that is involved in the formation of this compound. Uptake of radiolabelled compound F or adhesion to cell surfaces is unlikely since it does not accumulate when in spent medium when cells are absent. The accumulation of radiolabelled compound F in fresh and boiled medium indicates that it is usually broken down by the action of an apoplastic enzyme.

$[^{14}C]$Oxalate accumulated in whole culture and spent medium more than in fresh medium or boiled spent medium. The formation of $[^{14}C]$oxalate occurs non-enzymically in fresh and boiled medium. The presence of apoplastic enzymes in whole culture and spent medium promotes the formation of larger amounts of $[^{14}C]$oxalate. The extent of the accumulationof $[^{14}C]$oxalate suggests that it may be an end product of $[1-^{14}C]$ascorbate metabolism. Since $[^{14}C]$oxalate accumulates when radiolabelled compound F declines, it was suggested that radiolabelled compound F may be broken down to $[^{14}C]$oxalate.

Table 4 Summary of all three experiments examining the fate of $[1\text{-}^{14}C]$ascorbate in rose culture and culture medium

Compound	Fresh medium	Whole culture	Spent medium	Boiled spent medium
DHA	OOOOO	OOO	OOO	OOO . .
A1	??? . . .		?? .	??? .
A2	??? . . .	??? . . .	??? . .	??? . . .
Ascorbate	OOOoo	Ooo	Ooo	OOOo .
DKG	. . oO	. ooO	. oOo	. oOO
C/D	. O	. . OO	. . OO	. OOO
E	. .	. .	. oO	. O
F	. . OO	. . .	. .	.ooOOO
Oxalate	. .	.oooO	. ooO	. . O

Each symbol represents the relative amount of each radioactive compound present at a time point (O>o> >.). ? represents a compound that may be obscured by another compound or by streaking. A blank indicates that no radiolabelled compound was detected.

3.5 Fate of radiolabelled metabolites of ascorbate in rose culture medium

The experiments described in this section aimed to determine whether the compounds formed from [1-^{14}C]ascorbate in rose culture medium were produced directly from ascorbate or whether they were derived indirectly *via* other compounds. The fate of [^{14}C]dehydroascorbate was examined in a series of three experiments differing only in the method used to prepare [^{14}C]dehydroascorbate. The methods used to prepare [^{14}C]dehydroascorbate were adapted to try to increase the amount of dehydroascorbate present and minimise the amounts of ascorbate and diketogulonate. The results showed that the main fate of dehydroascorbate was conversion to diketogulonate. Two different methods were used to prepare [^{14}C]diketogulonate but neither method produced pure diketogulonate. [^{14}C]Diketogulonate was stable in rose culture medium as was oxalate, one of the contaminants present. Compound F, the other contaminant present was degraded in rose culture medium. These findings were confirmed by the application of radiolabelled compound F and [^{14}C]oxalate to rose culture medium. Overall, the results were used to piece together a pathway for the metabolism and/or degradation of ascorbate.

3.5.1 Fate of dehydroascorbate—experiment I

The oxidation of ascorbate to dehydroascorbate makes it difficult to differentiate metabolites formed directly from ascorbate from those formed from dehydroascorbate.

Unbuffered L-[1-^{14}C]ascorbate (14 Ci/mol) was treated with an ascorbate oxidase-impregnated spatula for 5 minutes at room temperature to form [^{14}C]dehydroascorbate. [^{14}C]Dehydroascorbate (1 µCi in 10 µl) was added to rose

culture and culture medium (133 μl). Samples of medium were removed to storage on liquid nitrogen at intervals over 8 hours. Filtered cells were retained for analysis by scintillation counting.

[^{14}C]Ascorbate was effectively converted to [^{14}C]dehydroascorbate (Fig. 33 A–D time-0 tracks) but other acidic compounds, including [^{14}C]diketogulonate were also formed. An acidic radiolabelled compound with mobility similar to the metabolite of [1-^{14}C]ascorbate designated A2 was present. This compound is referred to as A2i. When quantified by scintillation counting the amount of acidic contaminants present at time 0 was minimal (Fig. 33 E).

In fresh medium at pH 6.0, [^{14}C]dehydroascorbate was rapidly hydrolysed to [^{14}C]diketogulonate (Fig. 33 A, E). Other radiolabelled compounds including radiolabelled C/D, E, F and oxalate were also formed (Fig. 33 A).

[^{14}C]Dehydroascorbate was removed from the culture medium of whole culture over a period of 8 hours at a rate similar to breakdown of [^{14}C]dehydroascorbate in fresh medium. [^{14}C]Diketogulonate increased slightly during the first 60 minutes but then remained constant for the rest of the time course (Fig. 33 B, E). The formation of [^{14}C]diketogulonate in culture medium of whole culture followed a pattern similar to its formation in fresh medium (Fig. 33 A, B, E). The amount of radioactivity associated with filtered cells was increased during the first 30 minutes but then gradually declined over the following 7.5 hours (Fig. 33 E). Little or no extracellular [^{14}C]ascorbate was regenerated.

The removal of [^{14}C]dehydroascorbate from spent medium (Fig. 33 C) appeared to proceed at a similar rate to removal from whole culture (Fig. 33 B) and fresh medium (Fig. 33 A). When the radioactivity present was quantified by scintillation counting (Fig. 33 E) the removal of [^{14}C]dehydroascorbate was much

slower than from whole cultures or fresh medium (Fig. 33. E). The amount of [^{14}C]diketogulonate present increased steadily over the 8-hour period in spent medium (Fig. 33 C, E). The final amount formed was comparable to the amount formed in whole culture and fresh medium (Fig. 33 E). No contaminants other than [^{14}C]diketogulonate were detected in the stock [^{14}C]dehydroascorbate fed to spent medium (Fig. 33. C). The same stock was fed to fresh medium, spent medium and boiled spent medium. Despite its appearance of being underexposed, the autoradiogram presented in Fig. 33 C was exposed for the same time and under the same conditions as the other autoradiograms.

In boiled spent medium [^{14}C]dehydroascorbate underwent degradation to form [^{14}C]diketogulonate and other radiolabelled compounds including C/D, E, F and oxalate (Fig. 33 D, E), indicating that hydrolysis of dehydroascorbate occurs non-enzymically.
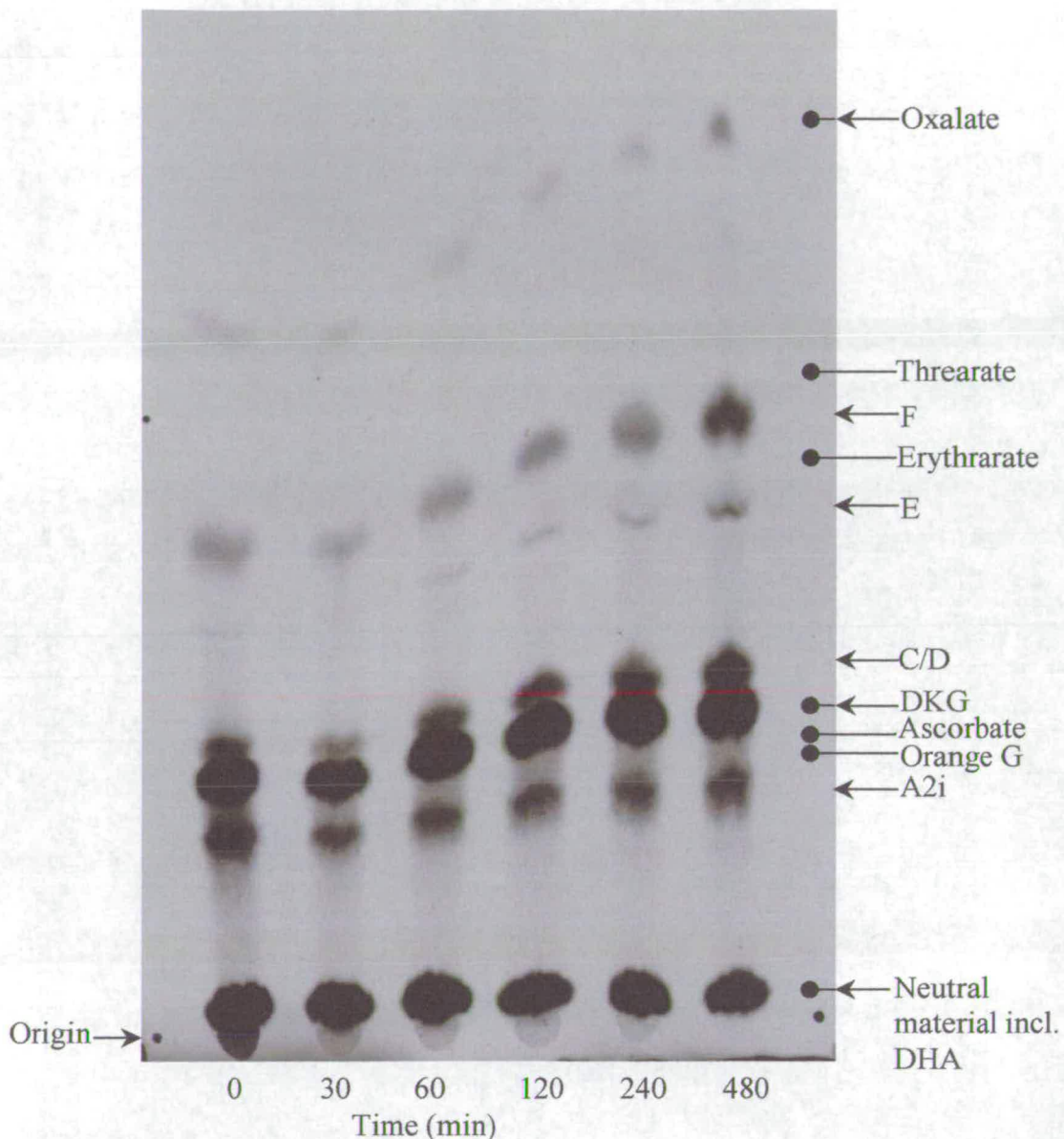
Fig. 33. Fate of ^{14}C labelled dehydroascorbate in rose cultures and culture medium over a period of 8 hours.

A. Breakdown of $[^{14}C]$dehydroascorbate in fresh culture medium. Samples of culture medium were analysed by electrophoresis run in pH 6.5 buffer, 3 kV for 30 minutes followed by autoradiography.

● ———— represents the approximate mobility of external standards
←———— represents radioactive metabolites of DHA
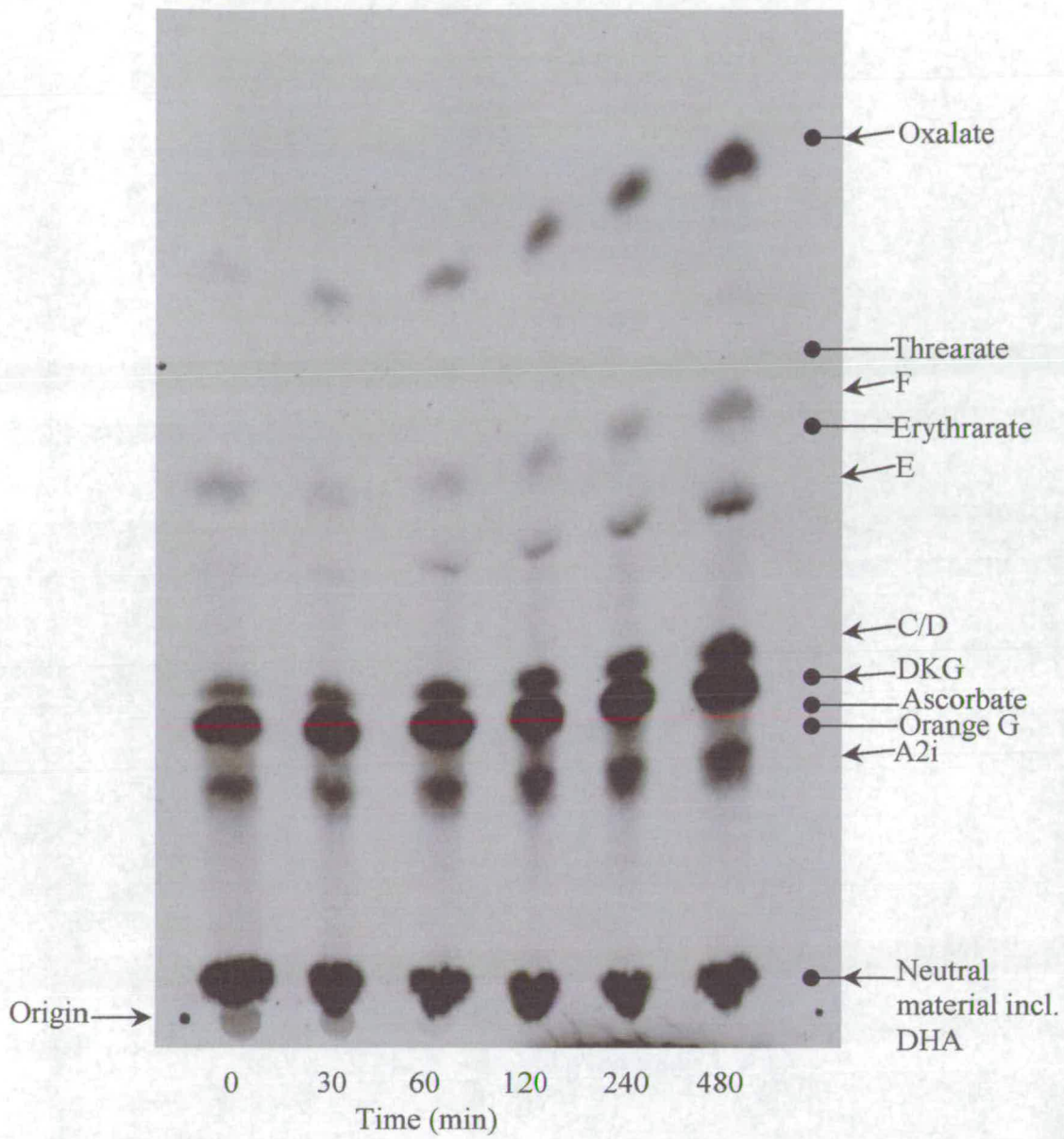
Fig. 33

B. Metabolism of [1-^{14}C]DHA in whole, 5-d-old rose cell culture. Soluble extracellular products were analysed as described in Fig 33 A.

169
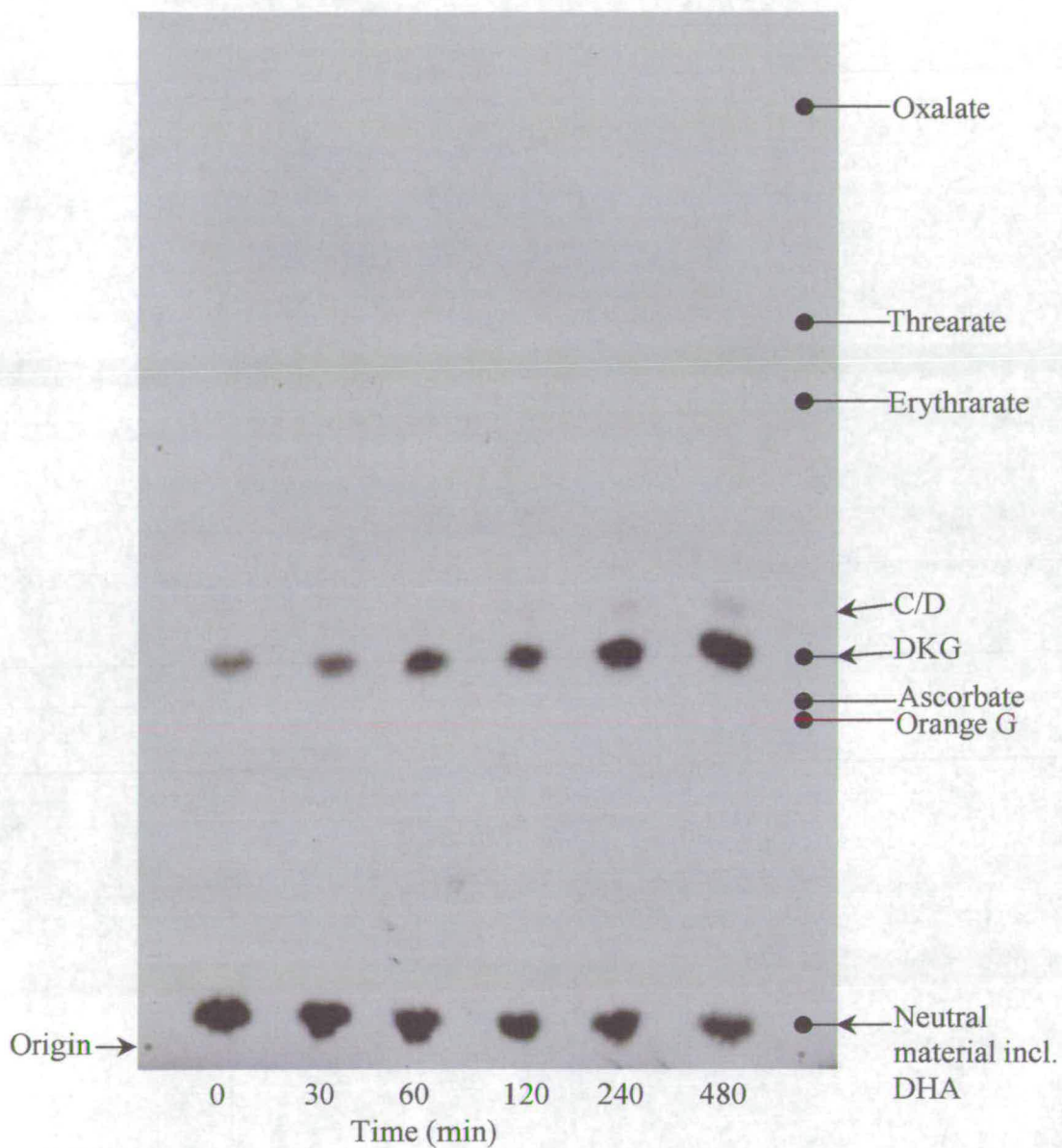
Fig. 33

C. Breakdown of [^{14}C]DHA in spent culture medium obtained by removing cells from 5-d-old rose cell culture. Soluble products were analysed as described in Fig 33 A.
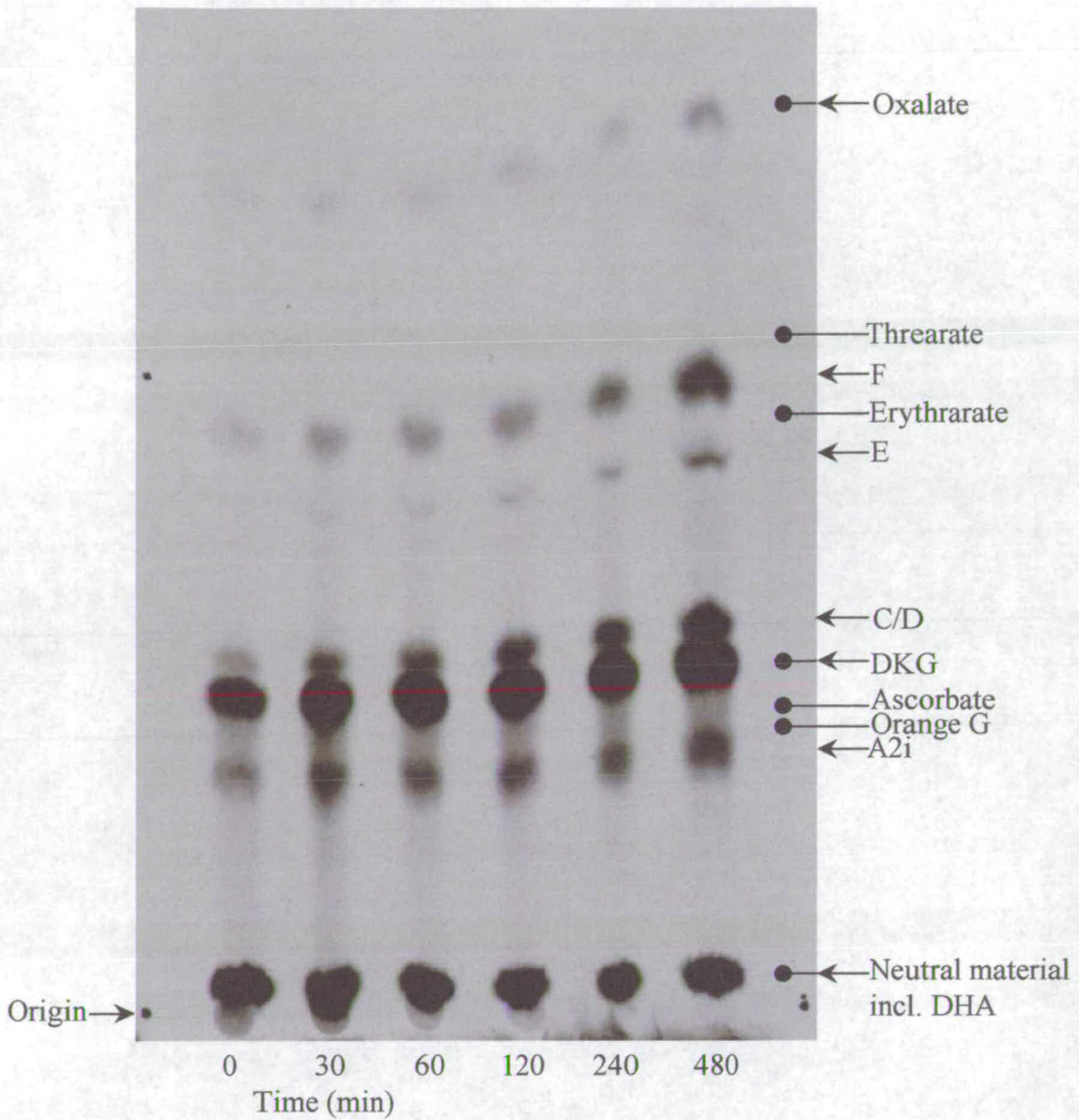
Fig. 33

D. Fate of [^{14}C]DHA in boiled spent medium. Spent medium obtained as described in Fig. 33 C was boiled 5 minutes then cooled to room temperature. Soluble products were analysed as described in Fig. 33 A.

171
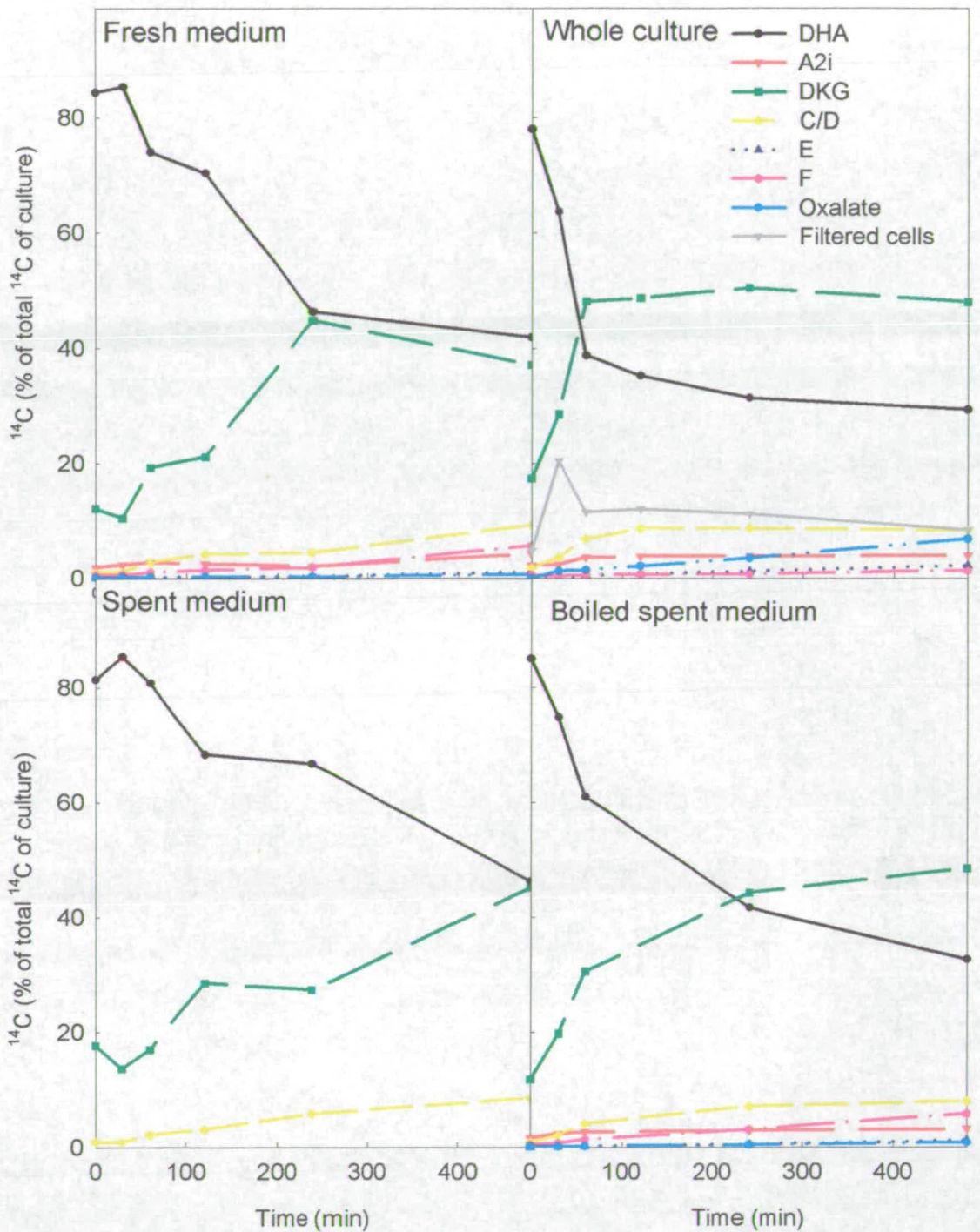
Figure 33

E. Fate of [^{14}C]DHA in rose culture and culture medium was quantitatively analysed by scintillation counting the areas of electrophoretograms corresponding to metabolites detected by autoradiography. Material which remained on the origin was not included with dehydroascorbate. Radioactivity associated with cells was analysed by scintillation counting of cells removed from a 20-μl sample of whole culture by filtration.

3.5.2 Fate of dehydroascorbate —experiment II

[1-^{14}C]Ascorbate was treated with ascorbate oxidase as described in 33. Exposure to ascorbate oxidase was prolonged from 5 minutes as described in section 3.5.1 to 20 minutes to try and increase the removal of ascorbate and increase the amount of dehydroascorbate formed.

Conversion of [1-^{14}C]ascorbate to [^{14}C]dehydroascorbate was complete but other acidic compounds—including [^{14}C]diketogulonate and radiolabelled A2i— were also present (Fig. 34 A–E, 0-minute time point).

The amount of [^{14}C]dehydroascorbate in fresh medium decreased over the 8-hour time course but it was not completely removed (Fig. 34 A, E). [^{14}C]Dehydroascorbate was hydrolysed non-enzymically to form increasing amounts of [^{14}C]diketogulonate. Small but increasing amounts of the almost indistinguishable radiolabelled compounds C and/or D (Fig. 34 A, E) were formed. Radiolabelled compounds E, F and [^{14}C]oxalate were detected (Fig. 34. A, E).

[^{14}C]Dehydroascorbate present in whole culture decreased during the first 4 hours and then remained constant for the rest of the 8-hour time course (Fig. 34. B, E). [^{14}C]Diketogulonate increased slightly over the 8-hour time course (Fig. 34.B, E). The increase in the amount of [^{14}C]diketogulonate was similar to the increase observed in fresh medium (Fig. 34 A, E). The decrease in [^{14}C]dehydroascorbate from 30 to 240 minutes was reflected by an increase in radioactivity associated with filtered cells (Fig. 34. E). This decreased during the following 4 hours, coinciding with a more rapid increase in [^{14}C]diketogulonate (Fig. 34. B, E). Radiolabelled compound E, detected by autoradiography, appeared to accumulate slightly over the 8-hour period (Fig. 34 B). Radiolabelled compound F present in whole culture was detected by autoradiography in a small but consistent quantity throughout the

experiment (Fig. 34 B). Slightly greater amounts of [^{14}C]oxalate were formed and accumulated in whole culture (Fig. 34 B, E) compared to fresh medium (Fig. 34 A, E). Little or no extracellular [^{14}C]ascorbate was regenerated.

[^{14}C]Dehydroascorbate fed to spent culture medium was rapidly but incompletely removed (Fig. 34 C, E) in the first 240 minutes. After this time there was little decrease in [^{14}C]dehydroascorbate. The rate of removal of [^{14}C]dehydroascorbate from spent medium (Fig. 34 C, E) was comparable to the rate at which it was removed from whole culture (Fig. 34 B, E). This suggests that dehydroascorbate is removed from culture medium by the action of apoplastic enzymes and that while some uptake may occur it is not the major metabolic fate of dehydroascorbate. The amount of [^{14}C]diketogulonate present in spent medium increased steadily throughout the 8-hour time course (Fig. 34 C, E) as did radiolabelled compounds C and/or D (Fig. 34 C, E). Radiolabelled compounds E and F were present in spent medium and radiolabelled compound E appeared to increase in amount (Fig. 34 C). The amount of [^{14}C]oxalate present in spent medium increased steadily over the 8-hour time course (Fig. 34 C, E).

The removal of [^{14}C]dehydroascorbate from boiled spent medium (Fig. 34 D, E) was slower than its removal from spent medium (Fig. 34 C, E). This supports the suggestion that dehydroascorbate is removed by the action of one or more apoplastic enzymes. However, the rate of [^{14}C]diketogulonate formation was only slightly slower in boiled spent medium (Fig. 34 D, E) than in spent medium (Fig. 34 C, E), indicating that [^{14}C]diketogulonate is also degraded non-enzymically. Radiolabelled compounds C and/or D accumulated slightly in boiled spent medium (Fig. 34 D, E) as previously observed in fresh medium (Fig. 34 A, E), whole culture (Fig. 34 B, E) and spent medium (Fig. 34 D, E). This suggests that radiolabelled

compounds C and/or D are formed by the non-enzymic degradation of [^{14}C]dehydroascorbate. The accumulation of radiolabelled compound E in boiled spent medium (Fig. 34 D, E) followed the same pattern as previously described in whole culture (Fig. 34 B) and spent medium (Fig. 34 C). Radiolabelled compound F accumulated considerably more in boiled spent medium (Fig. 34 D, E) than in spent medium (Fig. 34 C, E) or whole culture (Fig. 34 B, E) again leading to the suggestion that compound F is broken down by an apoplastic enzyme. [^{14}C]Oxalate did not accumulate as much in boiled spent medium (Fig. 34 D, E) as in whole culture or spent medium. Compound F is suggested to be enzymically broken down to form oxalate (Chapter 3.4, 3.5).
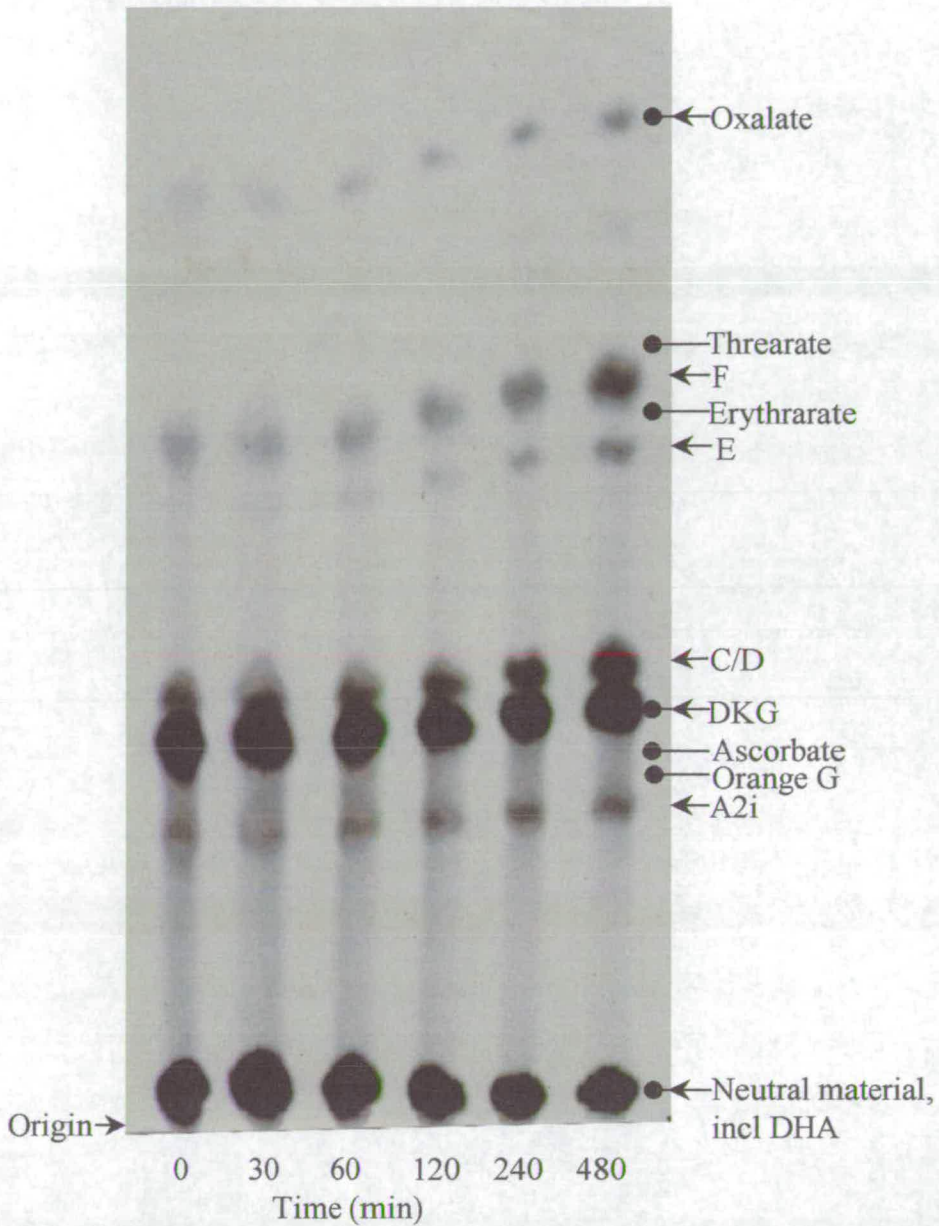
Figure 34. Fate of [^{14}C]dehydroascorbate fed to rose culture and culture medium—experiment II

A. Non-enzymic breakdown of [^{14}C]deydroascorbate in fresh rose culture medium. Samples of medium were subjected to electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes and then autoradiographed.

●—— represents the approximate mobility of external standards
←—— indicates radiolabelled metabolites of DHA

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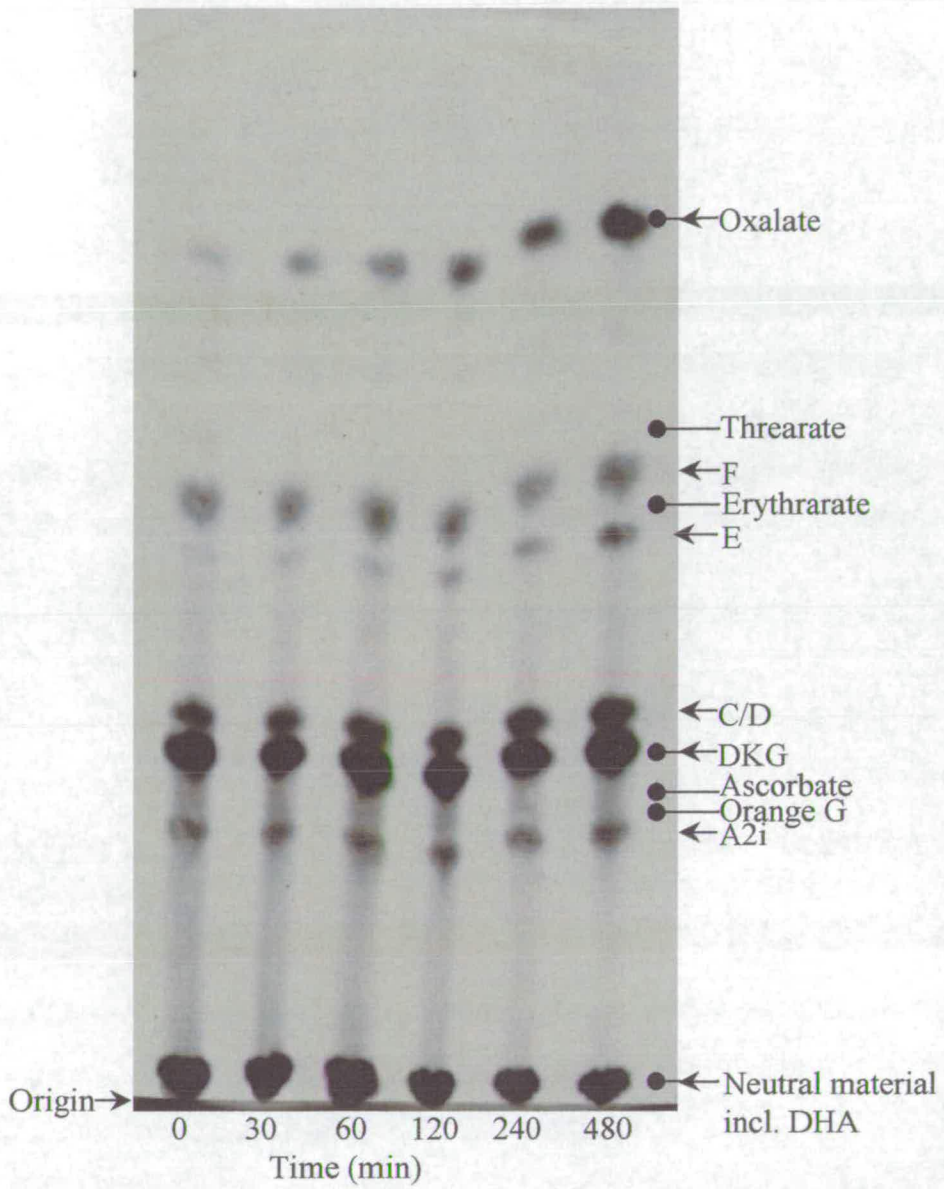
Figure. 34

B. Metabolism of [^{14}C]dehydroascorbate in whole, 5-d-old rose cell culture. Soluble, extracellular products were analysed as described in Fig. 34. A.
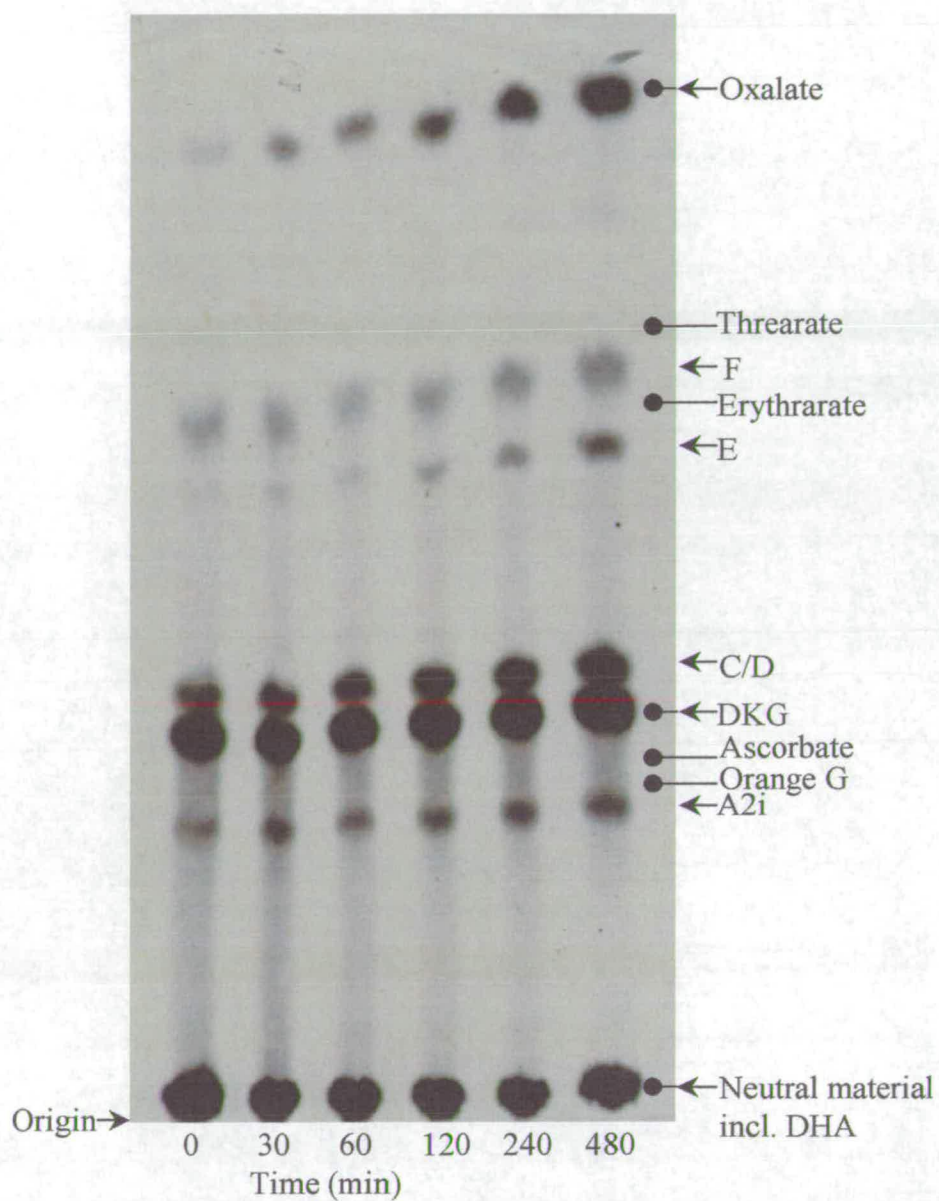
Figure 34.

C. Breakdown of [^{14}C]dehydroascorbate in spent medium obtained from 5-d-old rose cell culture. Soluble, extracellular products were analysed as described in Fig. 34 A.
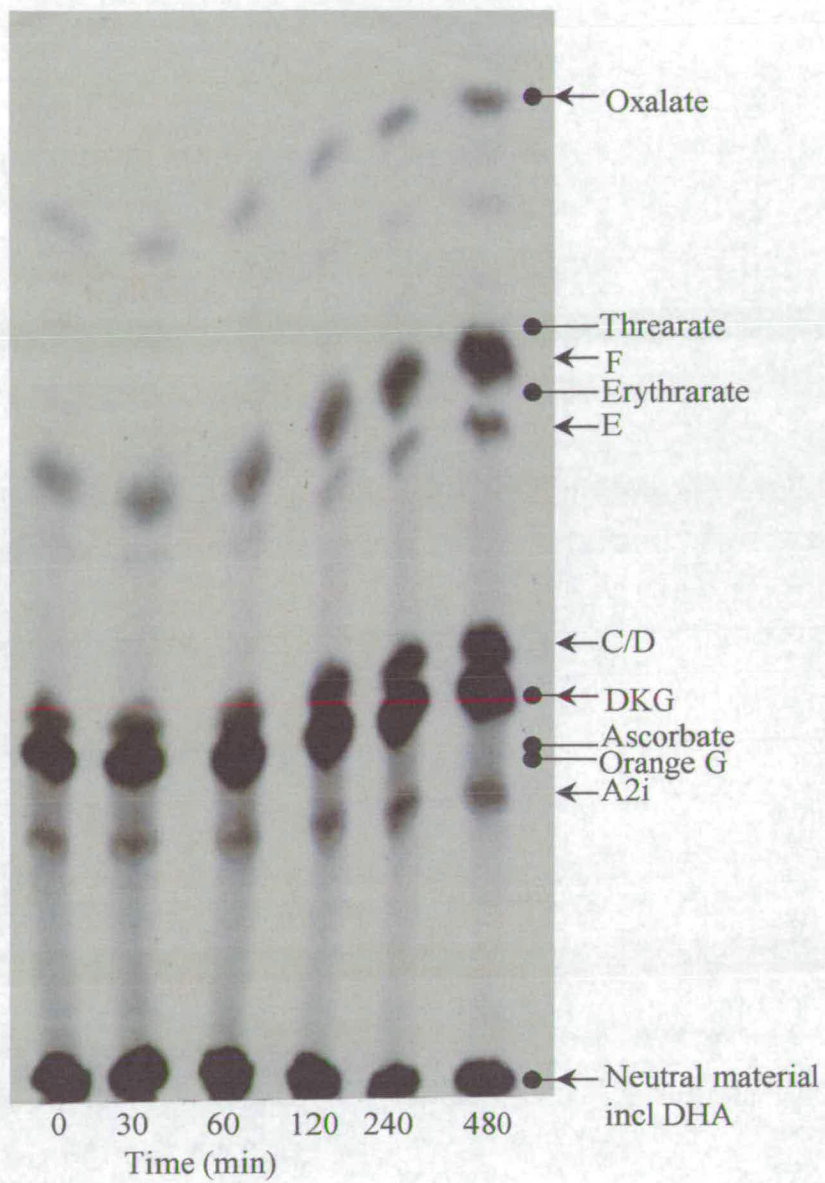
Figure 34.

D. Fate of [^{14}C]dehydroascorbate added to boiled spent medium obtained from 5-d-old rose cell cultures. Analysis of soluble products as described in Fig. 34. A.
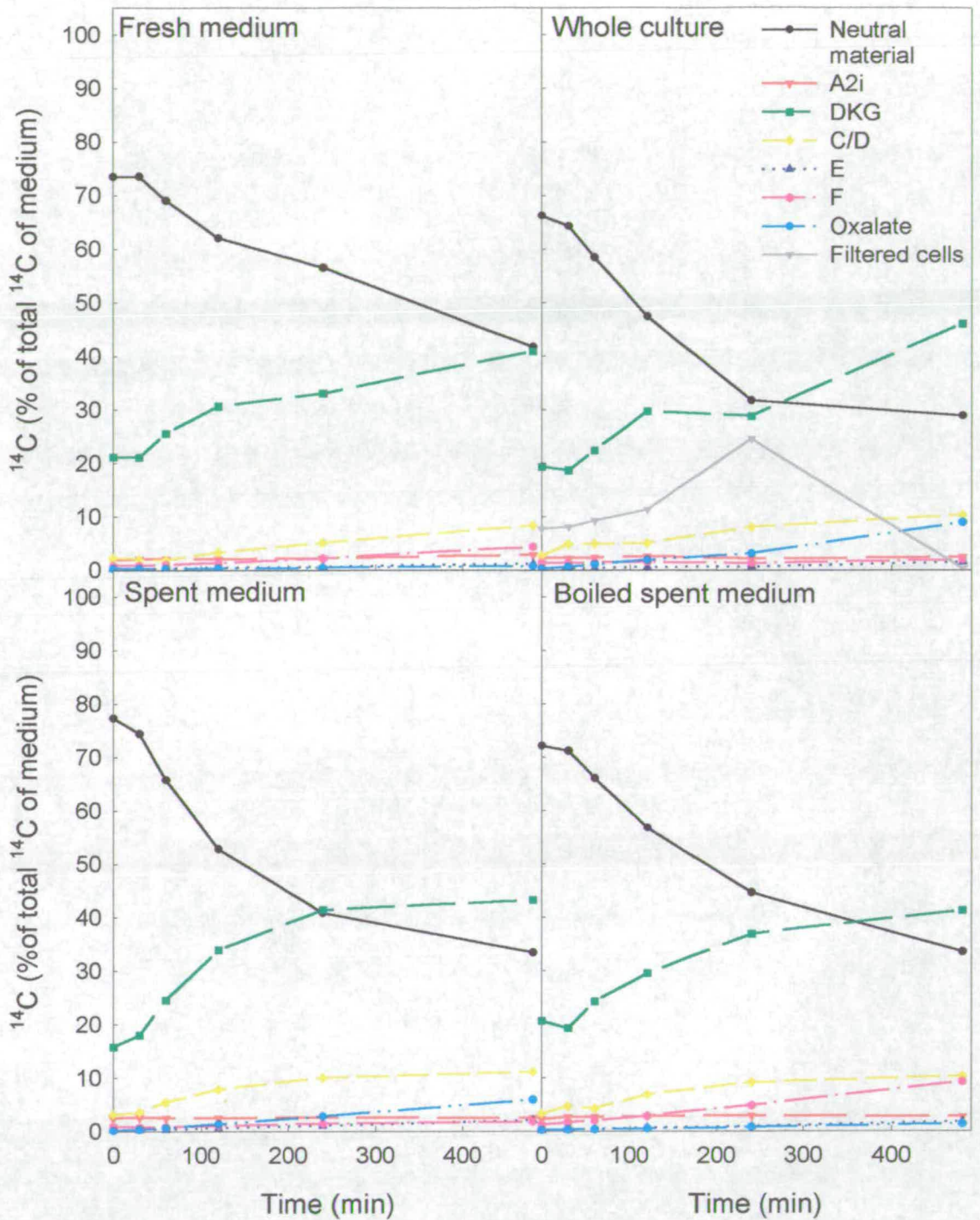
Figure 34

E. Fate of [^{14}C]dehydroascorbate in 5-d-old rose culture and culture medium analysed by scintillation counting sections of electrophoretograms corresponding to metabolites. Material present on the origin was not included with dehydroascorbate. Radioactivity associated with cells was analysed by scintillation counting of cells extracted by filtration from a 20-μl sample of whole culture.

3.5.3. Fate of [^{14}C]dehydroascorbate—experiment III

[1-^{14}C]Ascorbate (14 Ci/mol) was treated with an ascorbate oxidase-impregnated spatula for 20 minutes to oxidise ascorbate to dehydroascorbate. This also resulted in the formation of considerable amounts of diketogulonate (Fig. 33 A–E and Fig. 34 A–E). A Dowex 1 (MES□) anion-exchange resin (20 μl) was added to the prepared solution of [^{14}C]dehydroascorbate (100 μl) and gently shaken for 5 minutes in an attempt to remove [^{14}C]diketogulonate. The ion-exchange resin was sedimented by centrifugation and the supernatant (10 μl) immediately added to rose culture or culture medium (133 μl). [^{14}C]Diketogulonate was not completely removed (Fig. 35 A–D time-0 tracks) or was immediately replenished after removal of the Dowex. The amount of [^{14}C]diketogulonate present was less (Fig. 35 E) than in previous preparations of [^{14}C]dehydroascorbate (Fig. 33 A–E, Fig. 34 A–E). The negatively charged radiolabelled compound A2i was also present. This compound was present in a consistently small quantity in rose culture and culture medium (Fig. 35 A–E).

The amount of [^{14}C]dehydroascorbate present in fresh medium decreased steadily during the first 240 minutes (Fig. 35 A, E) while the amount of [^{14}C]diketogulonate increased (Fig. 35 A, E). Over the last 4 hours of the time course the amount of [^{14}C]dehydroascorbate increased slightly but the amount of [^{14}C]diketogulonate decreased (Fig. 35 A, E). Radiolabelled compound F increased during the first 4 hours (Fig. 35 A, E) but then decreased during the last half of the time course. These results suggest that conversion to diketogulonate is the main fate of dehydroascorbate in fresh medium.

The amount of [^{14}C]dehydroascorbate present in the medium of a whole culture decreased rapidly during the first 30 minutes (Fig. 35 B, E) and continued to decrease slightly and gradually over the rest of the time course (Fig. 35. B, E). The amount of radioactivity associated with filtered cells increased rapidly during the first 30 minutes. It then decreased from 30 to 120 minutes and increased steadily over the next 6 hours (Fig. 35. B, E). The amount of [^{14}C]diketogulonate present increased during the first 2 hours and then gradually and slightly decreased over the next 6 hours (Fig. 35 B, E).

In spent medium, the amount of [^{14}C]dehydroascorbate present decreased during the first 2 hours then remained constant for the rest of the time course (Fig. 35 C, E). [^{14}C]Diketogulonate mirrored the change in [^{14}C]dehydroascorbate, increasing in amount during the first 2 hours and only decreasing very slightly during the following 6 hours (Fig, 35 C, E). Radiolabelled compounds C and/or D remained constant throughout the time course while radiolabelled compound E increased very slightly (Fig 35 C, E). The amount of radiolabelled compound F remained reasonably constant over the 8-hour period (Fig. 35 C, E).

[^{14}C]Dehydroascorbate decreased in the first 2 hours after addition to boiled spent medium then continued to decrease slightly over the next 6 hours (Fig. 35 D, E). The amount of [^{14}C]diketogulonate increased slightly during the first 2 hours then remained reasonably constant before decreasing slightly during the final 4 hours of the time course (Fig 35 D, E). This mimicked the pattern of [^{14}C]dehydroascorbate metabolism observed in spent medium (Fig. 35 C, E). Radiolabelled compounds C and/or D were present in a larger quantity than previously observed but did not change in amount during the 8-hour period (Fig 35 D, E). The amount of radiolabelled compound E increased slightly over the 8-hour

time course while radiolabelled compound F increased considerably in boiled spent medium (Fig. 35 D, E). This is consistent with the suggestion that compound F is broken down by enzymes present in spent medium.

The amounts of radiolabelled compounds C (and/or D), E and F that accumulated when [1-^{14}C]dehydroascorbate was fed to cultures and culture medium (Fig. 33–35) were much less than when [1-^{14}C]ascorbate was fed (Ch. 3.4, Fig. 30–32). I propose that dehydroascorbate is not the major precursor for these compounds and suggest that they are usually derived directly from ascorbate. Radiolabelled compounds C (and/or D), E and F may have been formed in the present experiments as a result of incomplete removal of ascorbate or, formation of ascorbate from dehydroascorbate. These results indicate the conversion of dehydroascorbate to diketogulonate is limited by a non-enzymic, heat-stable component of spent culture medium from 5-d-old rose cell suspension cultures.

The data presented in figures 33 E, 34 E and 35 E were combined and presented as an average (Fig. 36) for the three experiments.

The results confirm that the main fate of [^{14}C]dehydroascorbate was conversion to [^{14}C]diketogulonate although the removal of [^{14}C]dehydroascorbate was incomplete. [^{14}C]Dehydroascorbate was removed faster during the first 4 hours from the medium of whole cultures than from fresh medium, spent medium or boiled spent medium. This was coupled with a slight increase in the amount of radioactivity asscoiated with filtered cells suggesting that some uptake of [^{14}C]dehydroascorbate may occur.

Other radiolabelled metabolites including compounds C (and/or D), E, F and oxalate were also formed in small amounts. The formation of radiolabelled compounds C (and/or D) was greater in whole culture, spent medium and boiled

spent medium than in fresh medium. This suggests that the formation of radiolabelled compound C (and/or D) is promoted by a heat stable component of the apoplast.

Radiolabelled compound F accumulated considerably more in boiled spent medium than in fresh medium, whole culture or spent medium. It is suggested that radiolabelled compound F is broken down by the action of an apoplastic enzyme.
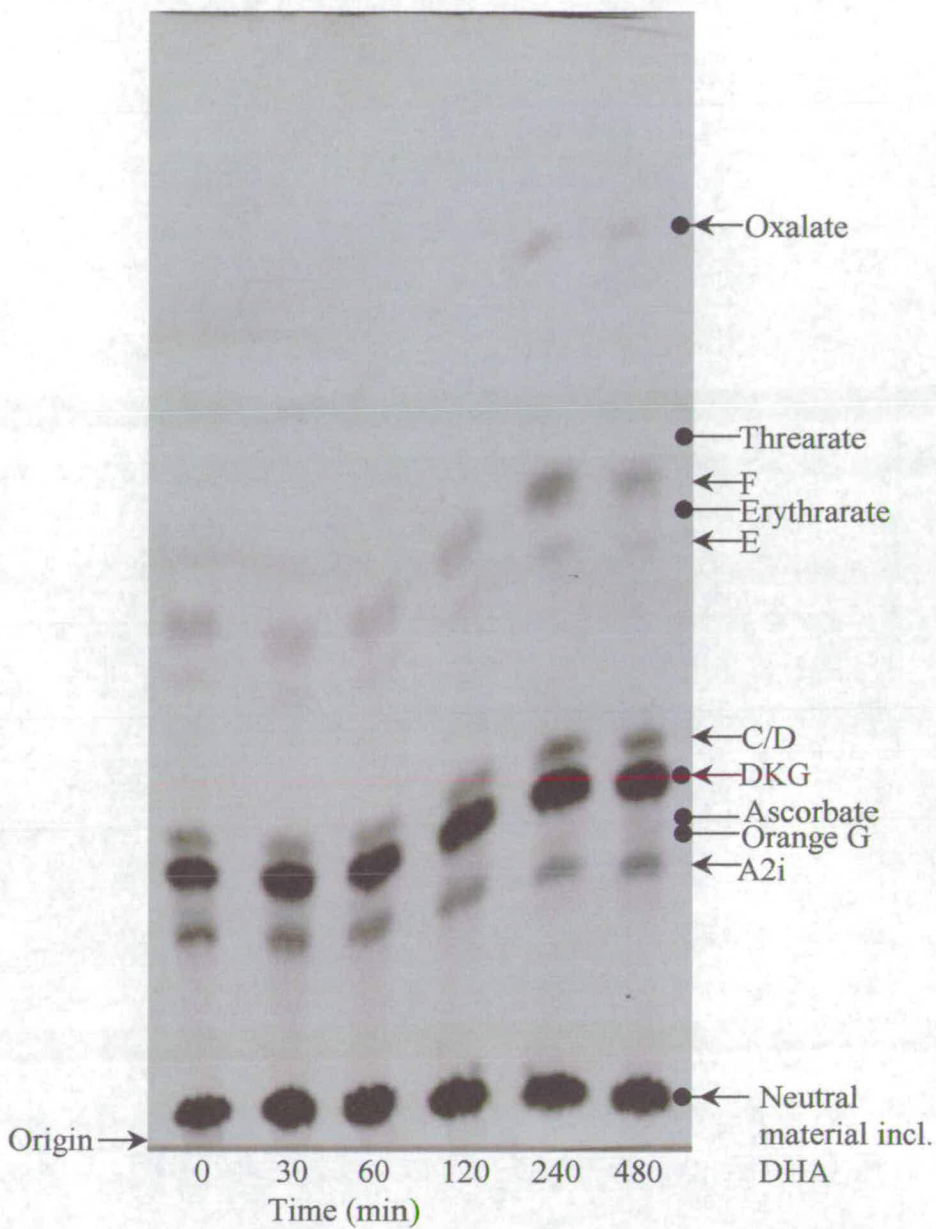
Figure 35. Fate of [^{14}C]dehydroascorbate—experiment III

A. Non-enzymic breakdown of [^{14}C]dehydroascorbate in fresh medium. Soluble products were analysed by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes followed by autoradiography.

●— represents the approximate mobility of external standards
←— indicates metabolites of [^{14}C]DHA
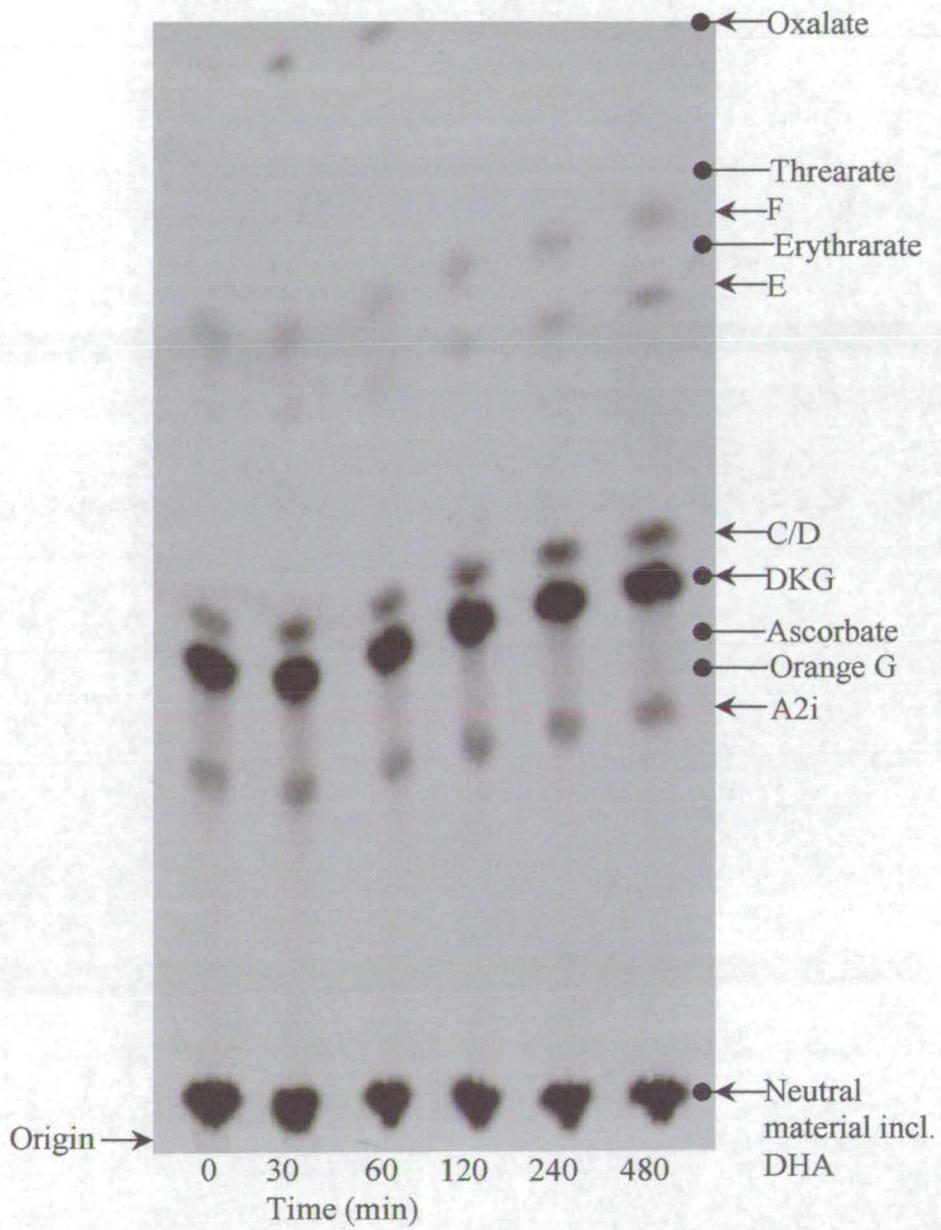
Figure 35

B. Metabolism of [^{14}C]dehydroascorbate in whole, 5-d-old rose culture. Soluble extracellular products analysed as described in Fig. 35. A.
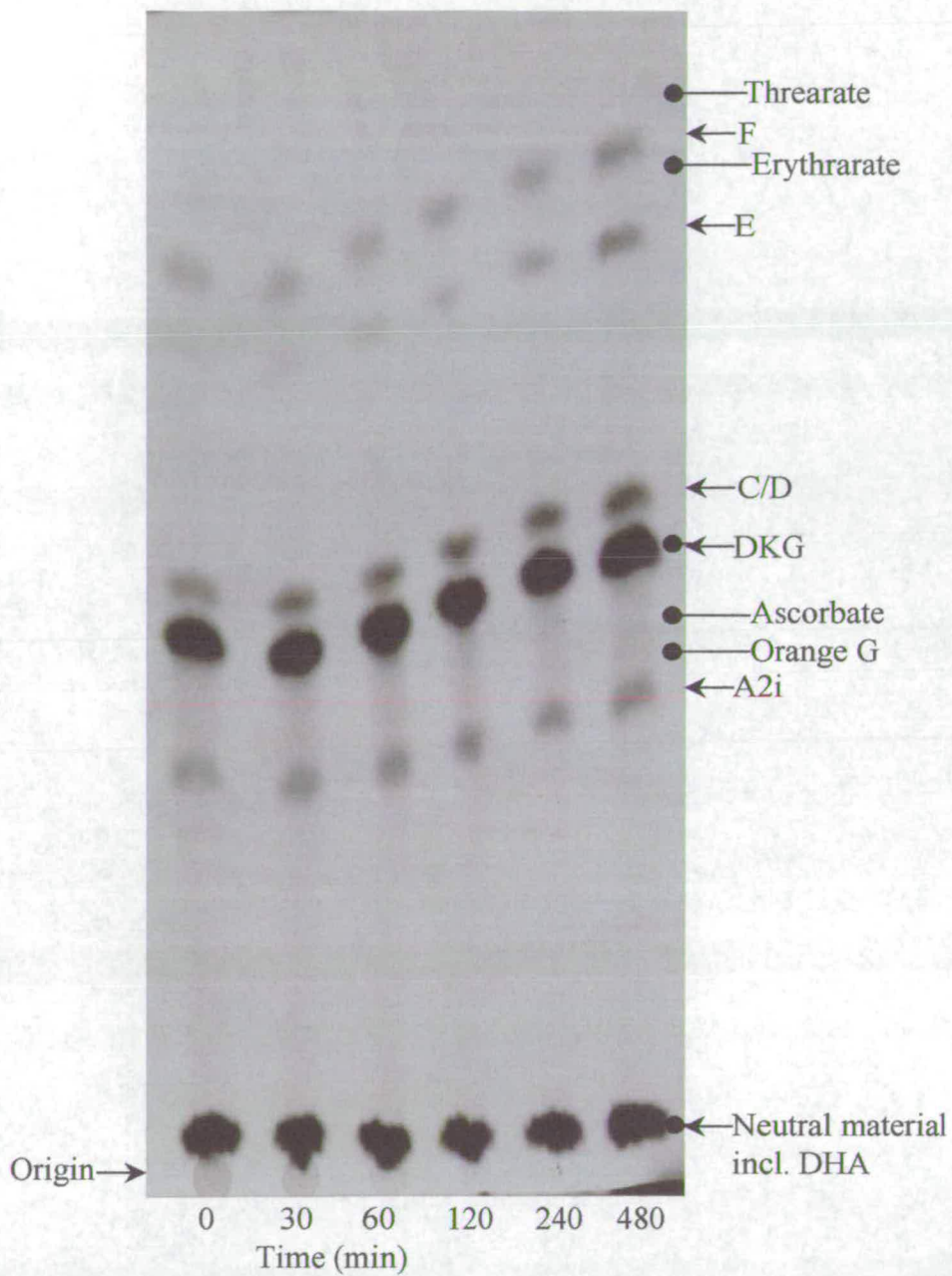
Figure 35

C. Breakdown of [^{14}C]dehydroascorbate in spent medium from 5-d-old rose culture medium. Soluble products analysed by electrophoresis and autoradioagraphy as described in Fig. 35 A.
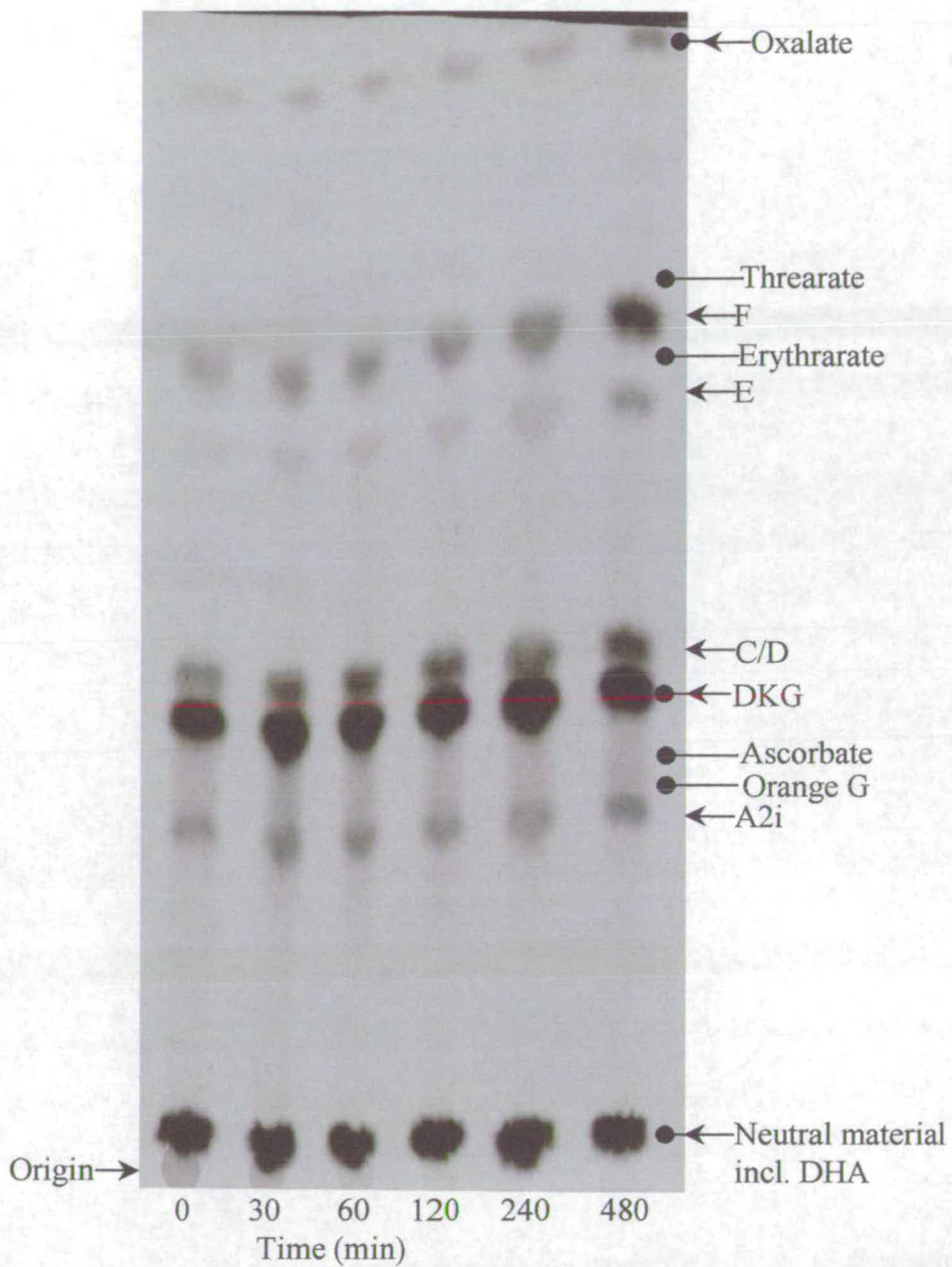
Fig. 35

D. Non-enzymic breakdown of [^{14}C]dehydroascorbate fed to boiled spent medium obtained from 5-d-old rose culture. Soluble products were analysed as described in Fig. 35 A.
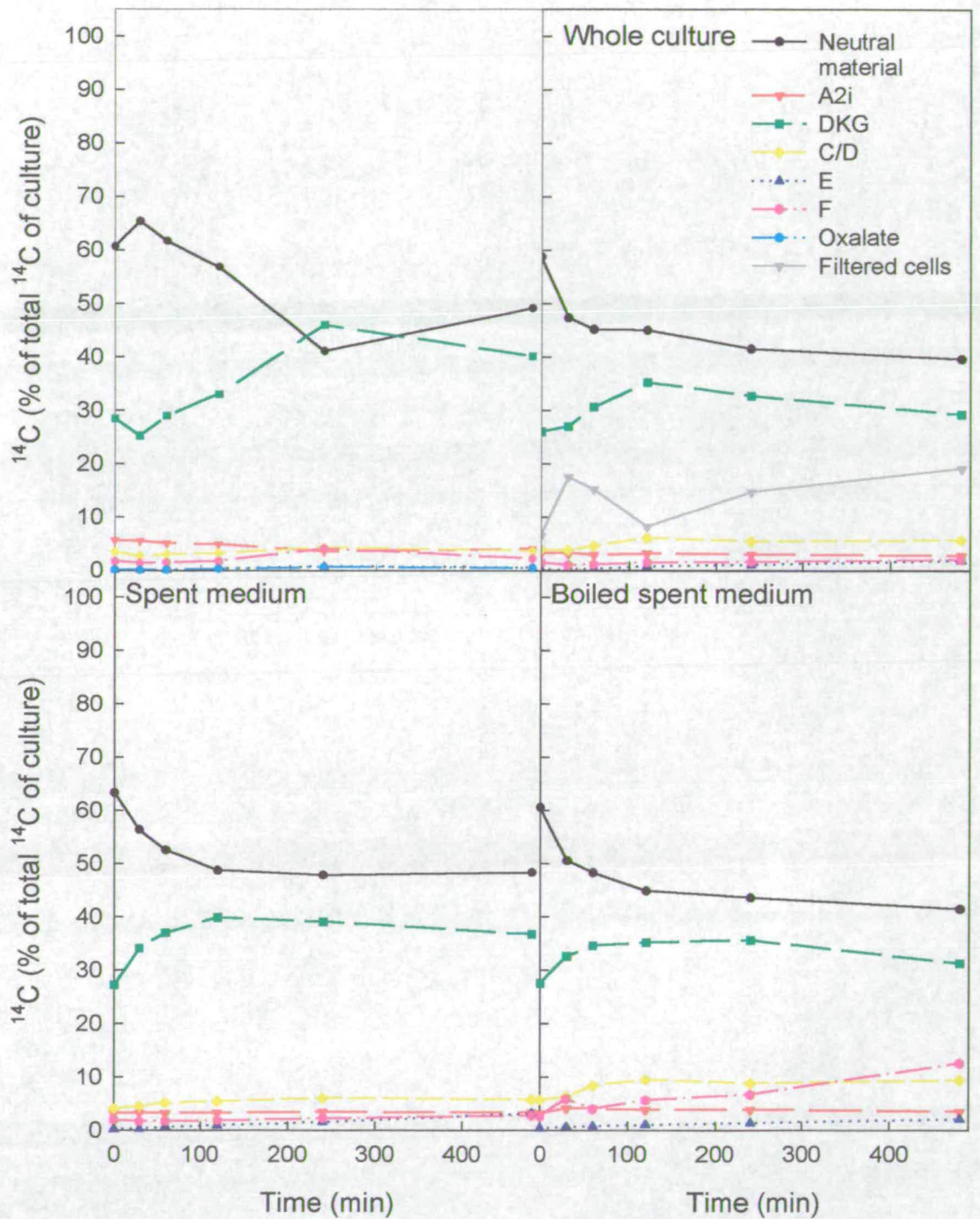
Fig. 35

E. Fate of $[^{14}C]$dehydroascorbate in 5-d-old rose culture and culture medium analysed by scintillation counting sections of electrophoretograms corresponding to metabolites. Material present on the origin was not included with dehydroascorbate. Radioactivity asscoiated with cells was analysed by scintillation counting of cells extracted by filtration from a 20-μl sample of whole culture.
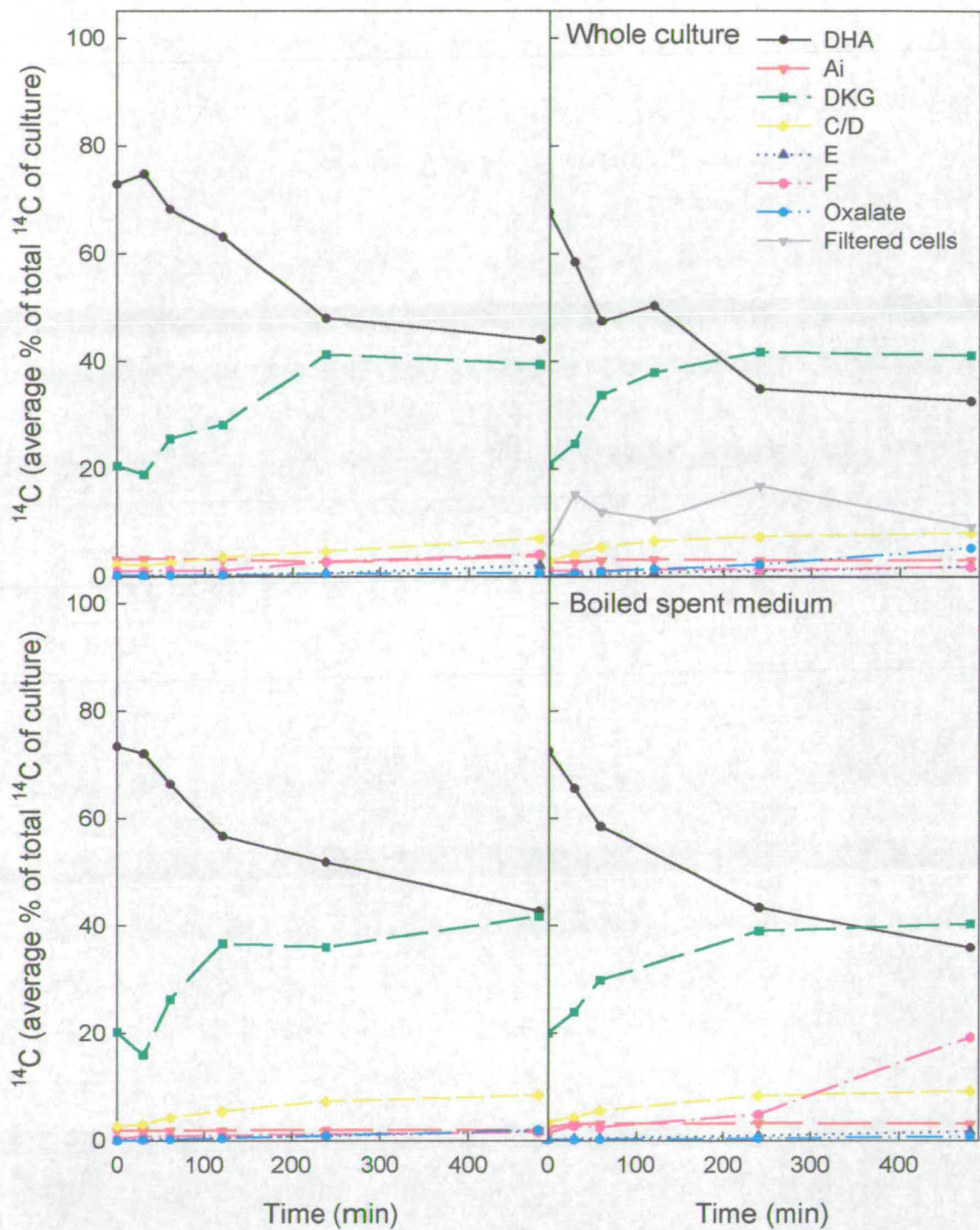
Figure 36. Fate of [14C]dehydroascorbate in rose culture and culture medium presented as an average of data presented in figures 33 E, 34 E and 35 E.

3.5.4 Metabolism of [^{14}C]diketogulonate—experiment I

[^{14}C]Diketogulonate formed by feeding rose culture and culture medium [1-^{14}C]ascorbate (Chapter 3.4.3) was purified from electrophoretograms, dried, then redissolved in water (60 μl). [^{14}C]Diketogulonate (0.002 μCi in10 μl) was added to rose culture and culture medium (133 μl). Samples of culture medium (20 μl) were removed to storage on liquid nitrogen at intervals over the 8-hour time course. Samples were subjected to electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes then detected by autoradiography.

Despite its apparent stability in culture medium (Chapter 3.5.1–3) [^{14}C]diketogulonate could not be satisfactorily purified from electrophoretograms. Only a small proportion of 'purified [^{14}C]diketogulonate' was actually [^{14}C]diketogulonate. 'Purified [^{14}C]diketogulonate' consisted mostly of radiolabelled compound F and [^{14}C]oxalate (Fig. 37 A–E, 0-minute time point). The amount of [^{14}C]diketogulonate, radiolabelled compound F and [^{14}C]oxalate remained constant in fresh medium throughout the 8-hour time course (Fig. 37 A, E).

The small amount of [^{14}C]diketogulonate added to whole culture remained constant over the 8-hour time course (Fig 37 B, E). Radiolabelled compound F decreased rapidly in the first 2 hours and was present in a consistently low quantity for the remainder of the time course(Fig. 37 B, E). The amount of [^{14}C]oxalate present in whole culture increased during the first 4 hours(Fig 37 E)then remained constant for the rest of the time course. Some fluctuation in the amount of radioactivity associated with filtered cells was observed but there was no overall change (Fig. 37 B, E).

In spent culture medium the amount of $[^{14}C]$diketogulonate present remained constant throughout the time course (Fig. 37 C, E). A rapid decrease in radiolabelled compound F was observed in the first 4 hours (Fig. 37 E) and was followed by a more gradual decline over the remainder of the time course (Fig. 37 C). This suggests that the removal of compound F from the apoplast of whole culture is due to extracellular breakdown rather than uptake by cells. After a rapid increase during the first 2 hours the amount of $[^{14}C]$oxalate did not change during the remainder of the time course (Fig. 37 C, E).

The amount of $[^{14}C]$diketogulonate in boiled spent medium remained constant over the 8-hour time course (Fig. 37 D, E). The amounts of radiolabelled compound F and $[^{14}C]$oxalate also remained constant throughout the time course (Fig. 37 D, E).

Radiolabelled compound E was detected in fresh medium (Fig. 37 A, E) and was also present in a small quantity in whole culture, spent medium and boiled spent medium (Fig. 37. B–E). Radiolabelled compound E did not appear to be metabolised in rose culture or culture medium (Fig. 37 A–E).

$[^{14}C]$Diketogulonate is not metabolised by rose culture or culture medium although it does break down forming radiolabelled compound F and $[^{14}C]$oxalate when stored and/or eluted from electrophoretograms. Radiolabelled compound F, present as a contaminant of $[^{14}C]$diketogulonate, is converted to $[^{14}C]$oxalate by the action of an apoplastic enzyme.
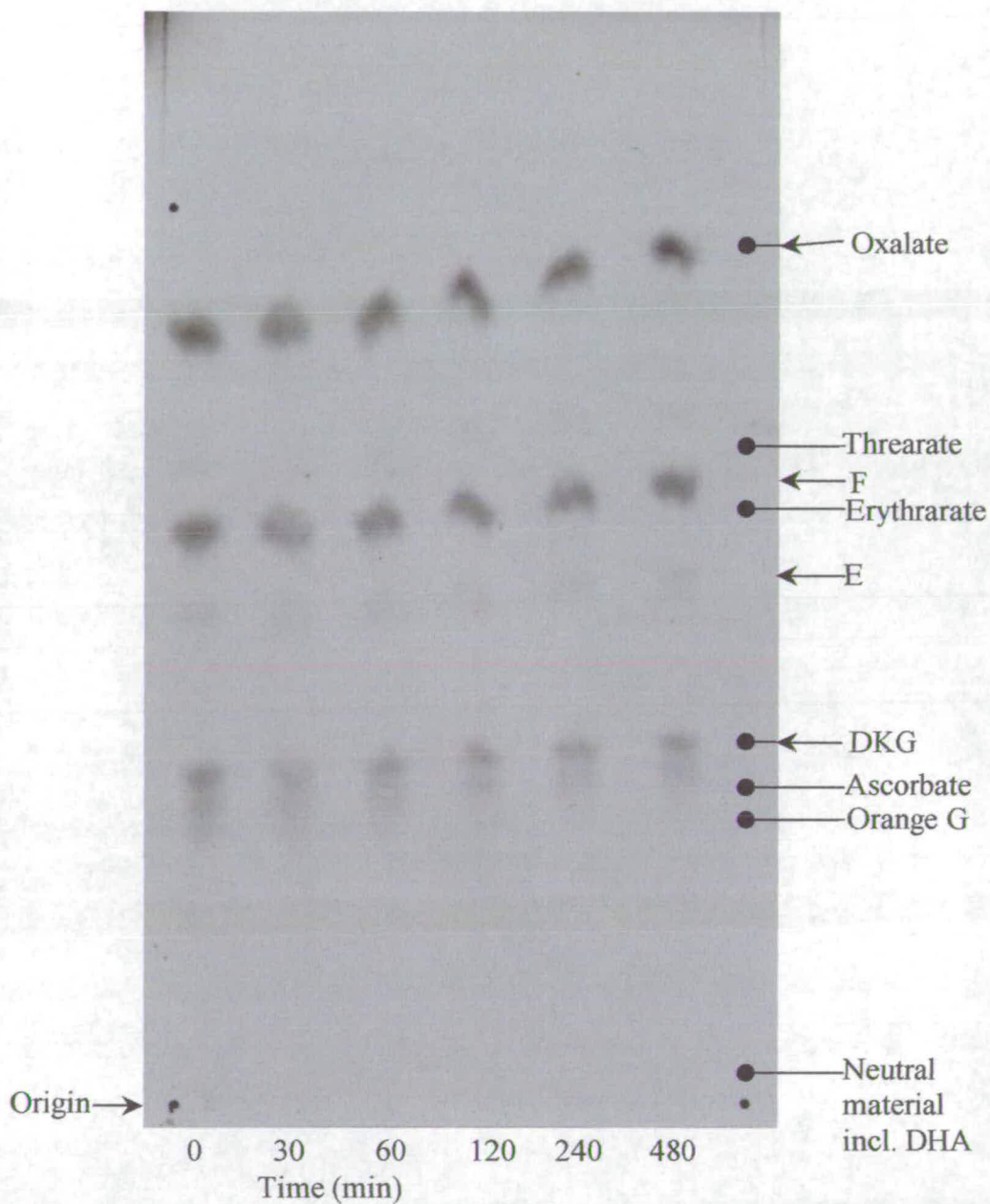
Figure 37. Metabolism of [^{14}C]DKG

A. Breakdown of [^{14}C]diketogulonate in fresh rose medium. Soluble products were analysed by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes followed by autoradiography.

●——— represents the approximate mobility of external standards
←——— indicates radiolabelled metabolites

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Figure 37

B. Metabolism of [^{14}C]diketogulonate in whole, 5-d-old rose culture. Soluble, extracellular products were analysed as described in Fig. 37 A.

Figure 37

C. Breakdown of [^{14}C]diketogulonate in spent medium obtained from 5-d-old rose culture. Soluble products were analysed by electrophoresis and autoradiography as described in Fig. 37 A
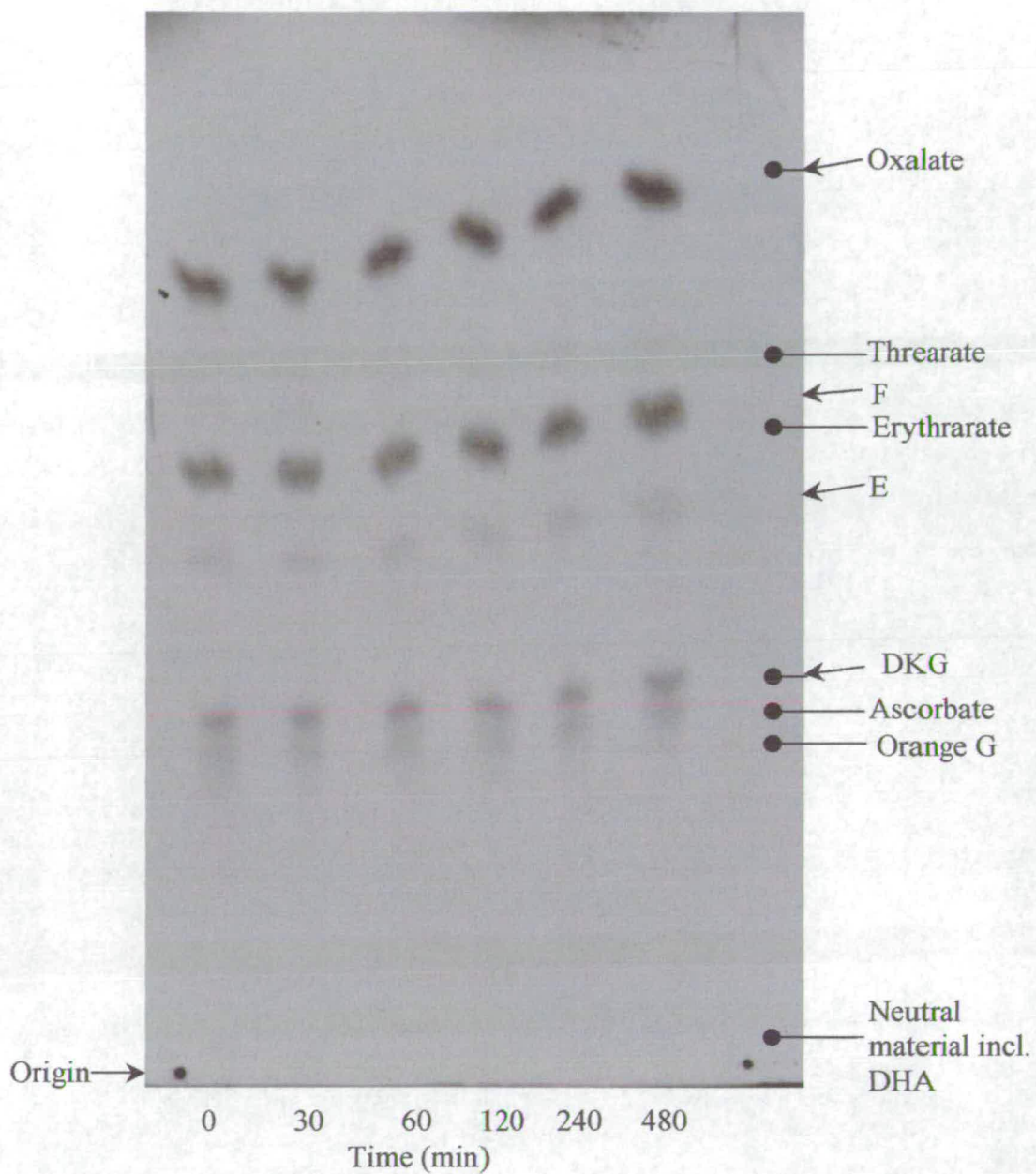
Figure 37

D. Non-enzymic breakdown of [^{14}C]diketogulonate in boiled spent medium obtained form 5-d-old rose culture. Soluble products analysed as described in Fig. 37 A
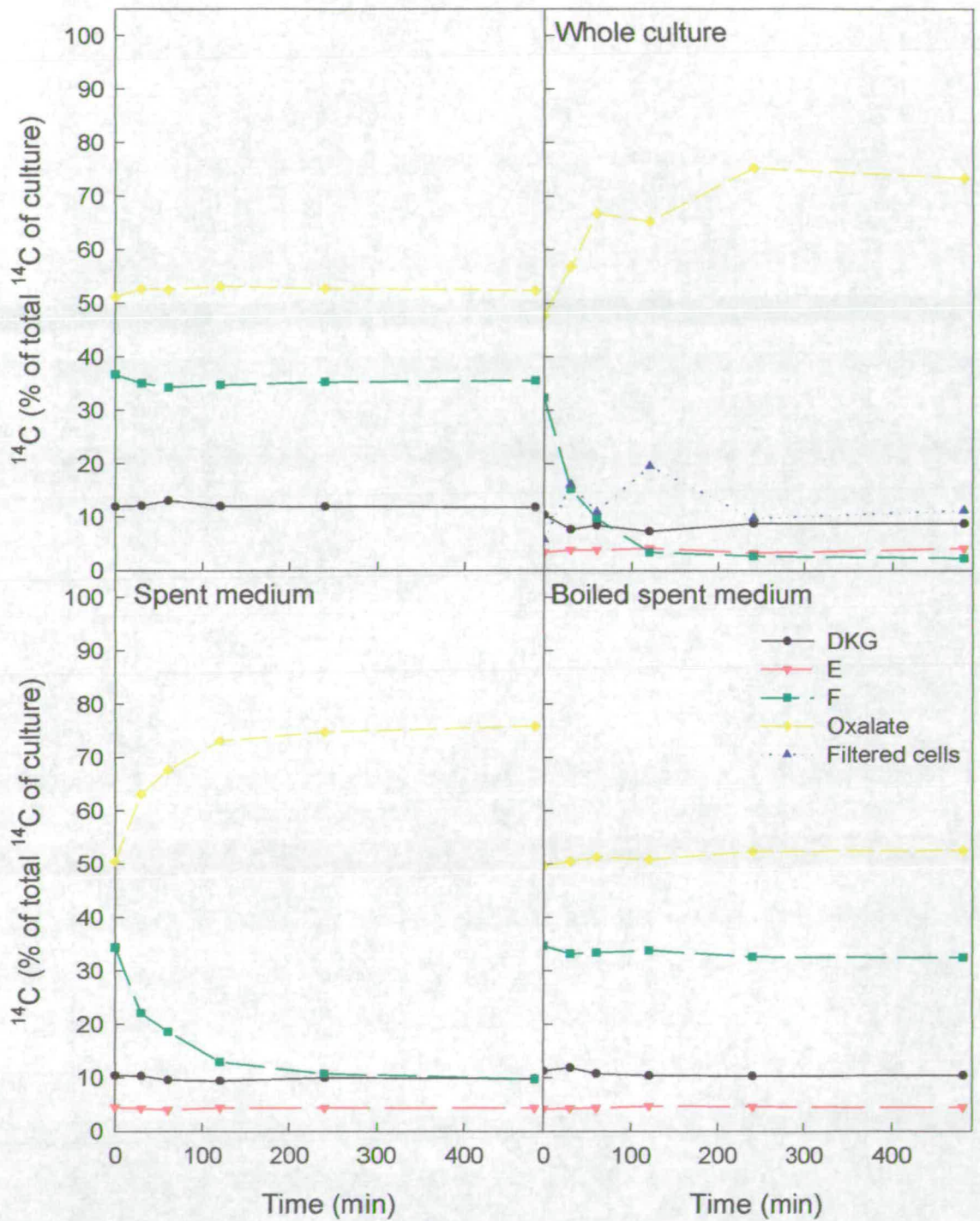
Figure 37

E. Fate of ^{14}C labelled diketogulonate in 5-d-old rose culture and culture medium analysed by scintillation counting areas of electrophoretograms corresponding to metabolites. Radioactivity associated with cells was analysed by scintillation counting of cells extracted from a 20-µl sample of whole culture by filtration.

3.5.5 Fate of [^{14}C]diketogulonate—experiment II

[1-^{14}C]Ascorbate (13 Ci/mol) stored at –20°C for 2 years had broken down forming [^{14}C]diketogulonate and trace amounts of other radiolabelled compounds including [^{14}C]oxalate and compounds with similar mobility to compounds C/D and F. [^{14}C]Diketogulonate (5 µCi) was purified by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes and located by autoradiography. It was eluted from the electrophoretogram in water (50 µl). [^{14}C]Diketogulonate (1 µCi) was added to rose culture and culture medium (133 µl).

Purified [^{14}C]diketogulonate contained trace amounts of radiolabelled compounds including C/D, F and oxalate (Fig. 38 A–D). These were formed in purification after electrophoresis, during drying and/or elution.

[^{14}C]Diketogulonate and radiolabelled compounds C and/or D were stable in 5-d-old rose culture and culture medium (Fig. 38 A–E). Radiolabelled compound F was stable in fresh medium and boiled spent medium (Fig. 38 A, D, E) but was almost completely removed from whole culture and spent medium (Fig. 38 B, C, E). The decrease in the amount of radiolabelled compound F present in whole culture and spent medium was reflected by an increase in the amount of [^{14}C]oxalate present (Fig. 38 B, C, E). This shows that radiolabelled compound F is converted to [^{14}C]oxalate by the action of an apoplastic enzyme.
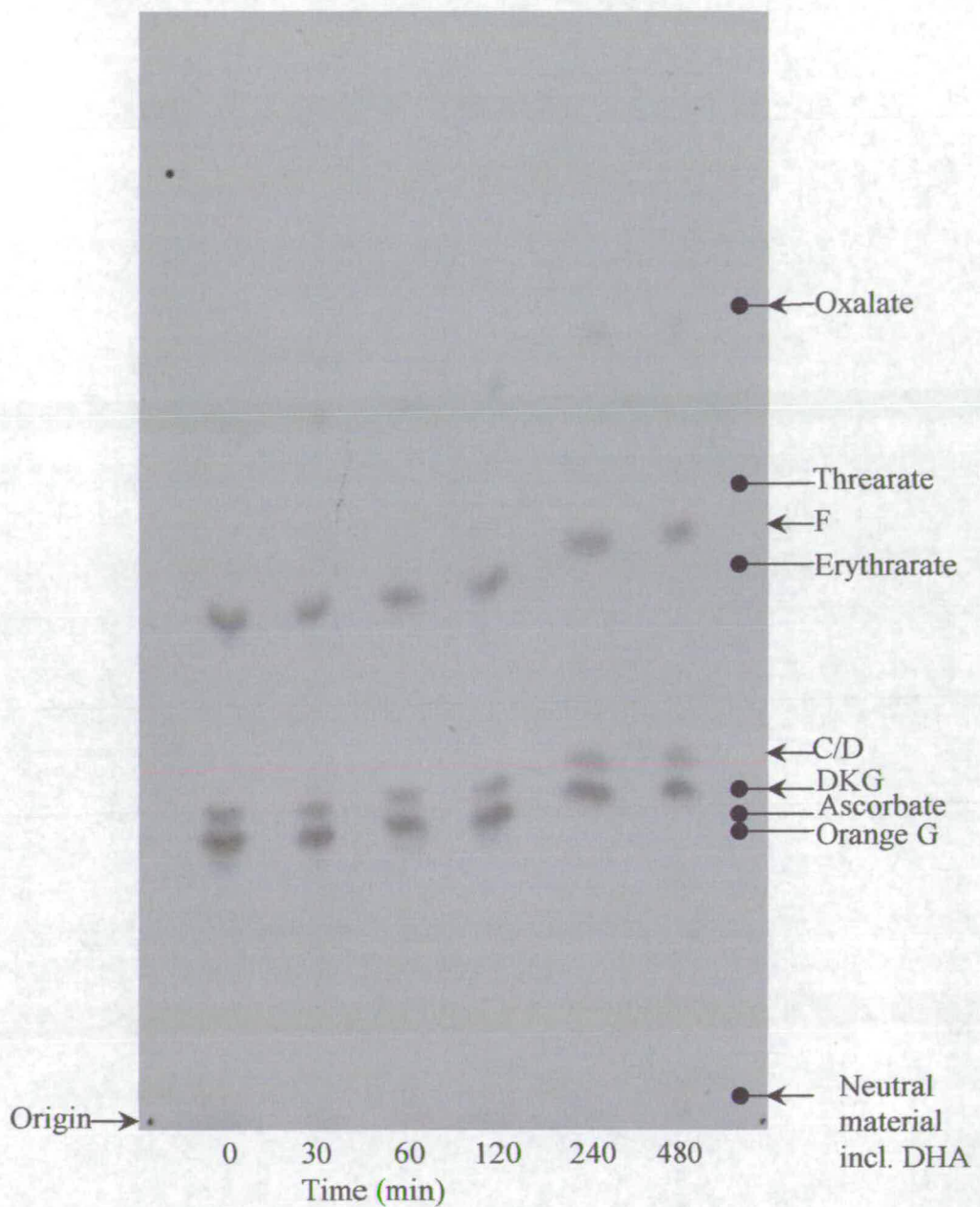
Fig. 38. Fate of [^{14}C]DKG in rose culture and culture medium—experiment II

A. Breakdown of [^{14}C]DKG in fresh medium. Samples of medium were analysed by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes. Autoradiography was used to detect radiolabelled compounds.

● —— represents the approximate mobility of external standards
← —— indicates radiolabelled compounds derived from [^{14}C]DKG

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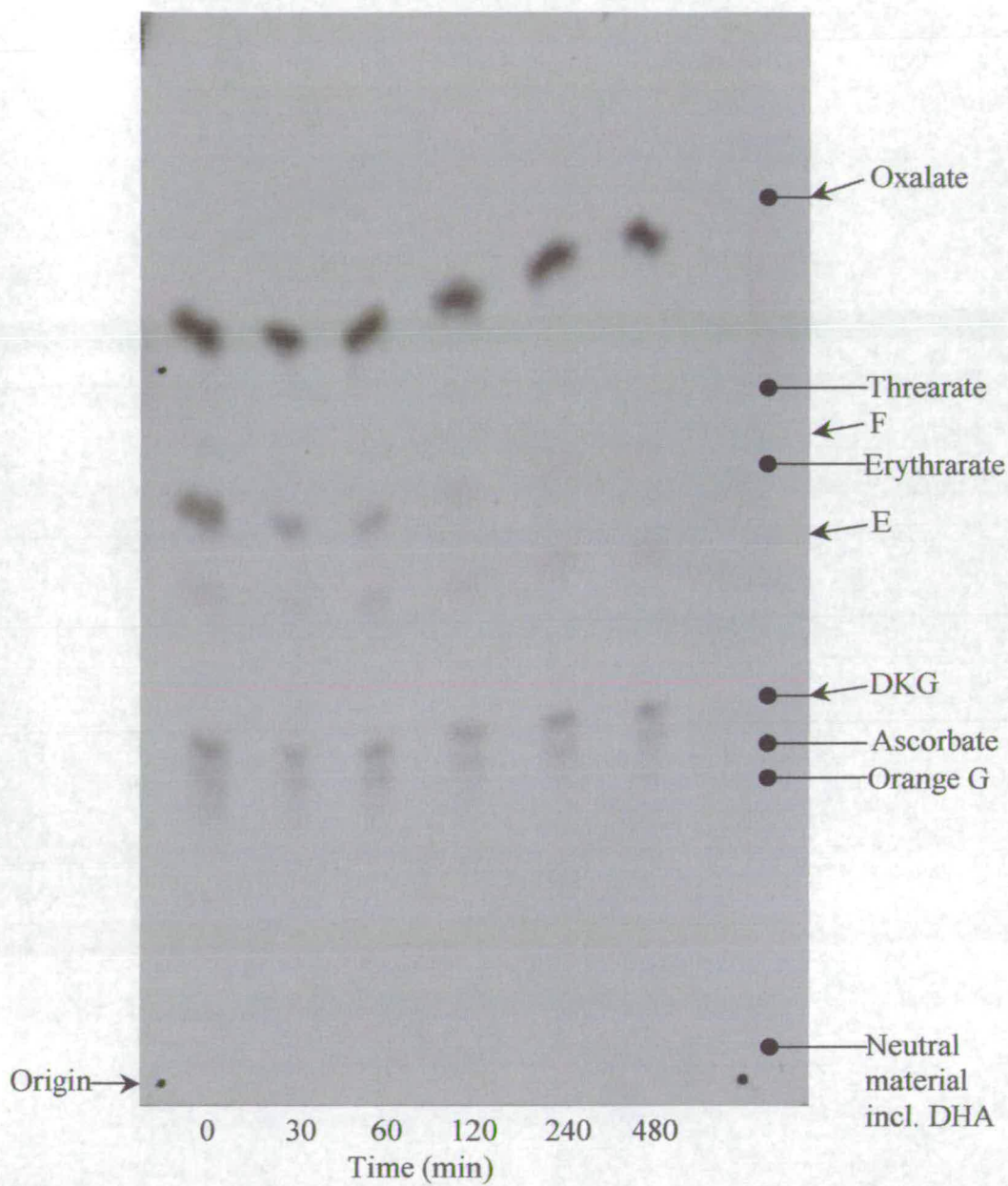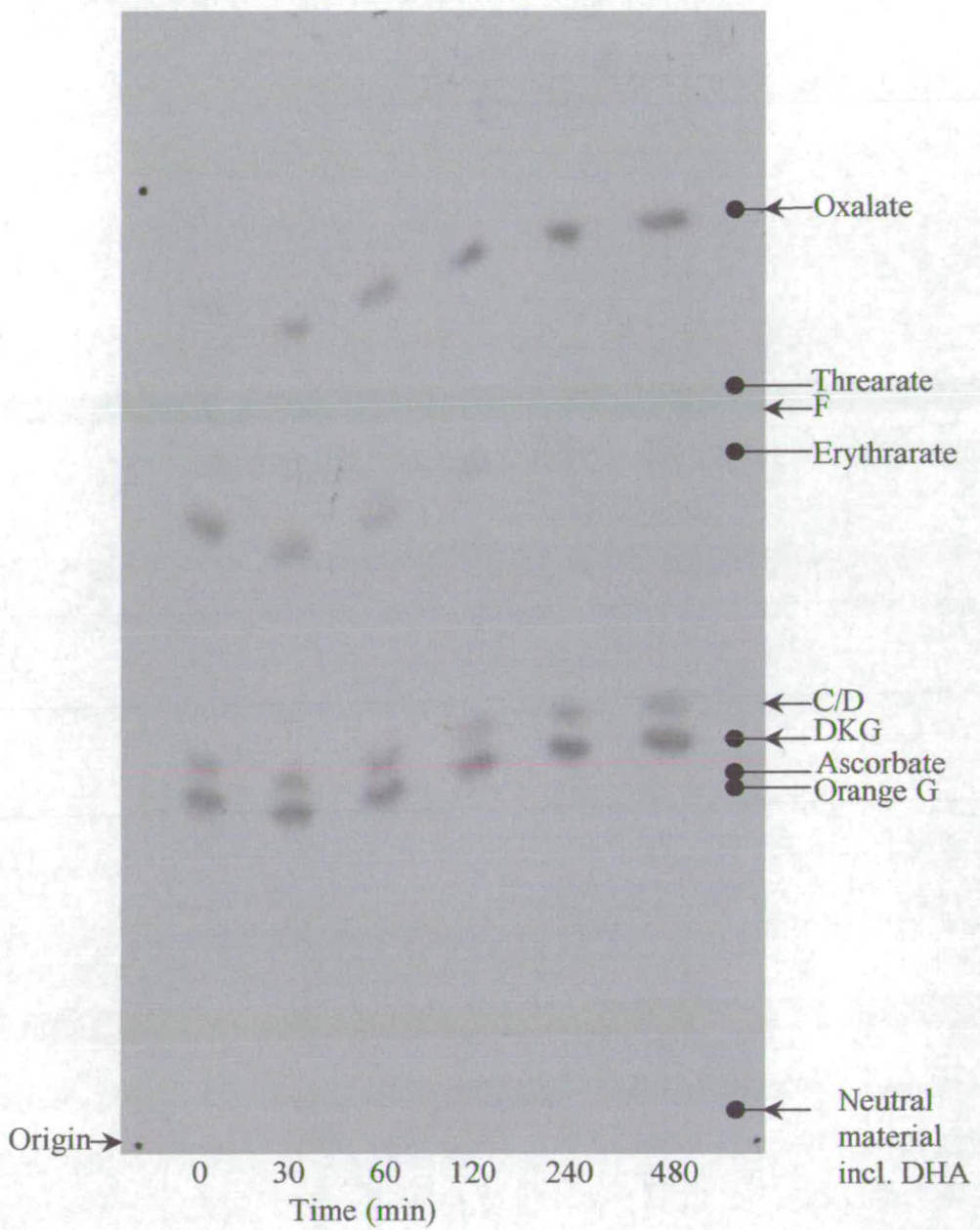
Figure 38

B. Metabolism of [^{14}C]DKG in whole culture. Soluble, extracellular products were analysed as described in Fig. 38 A.
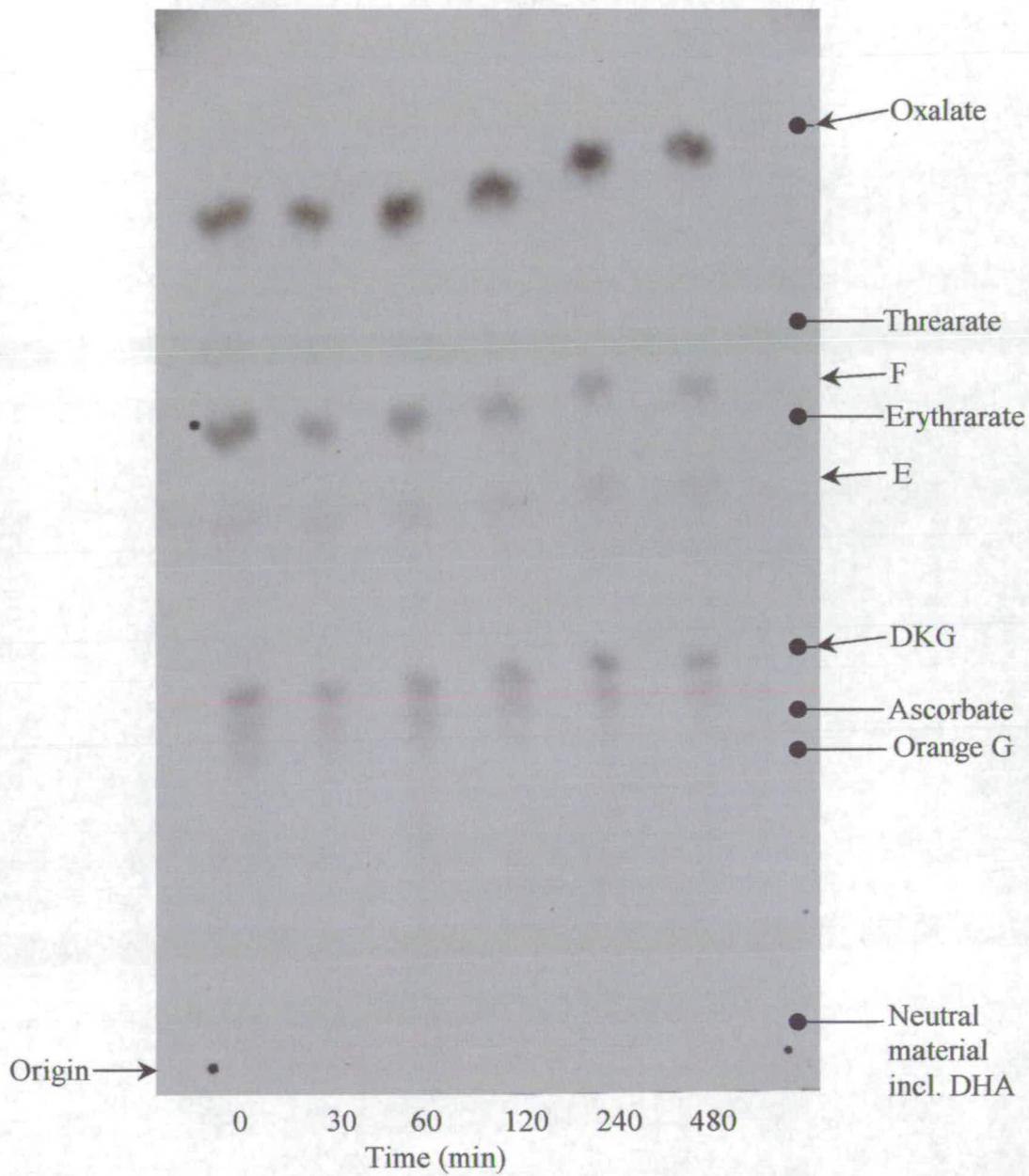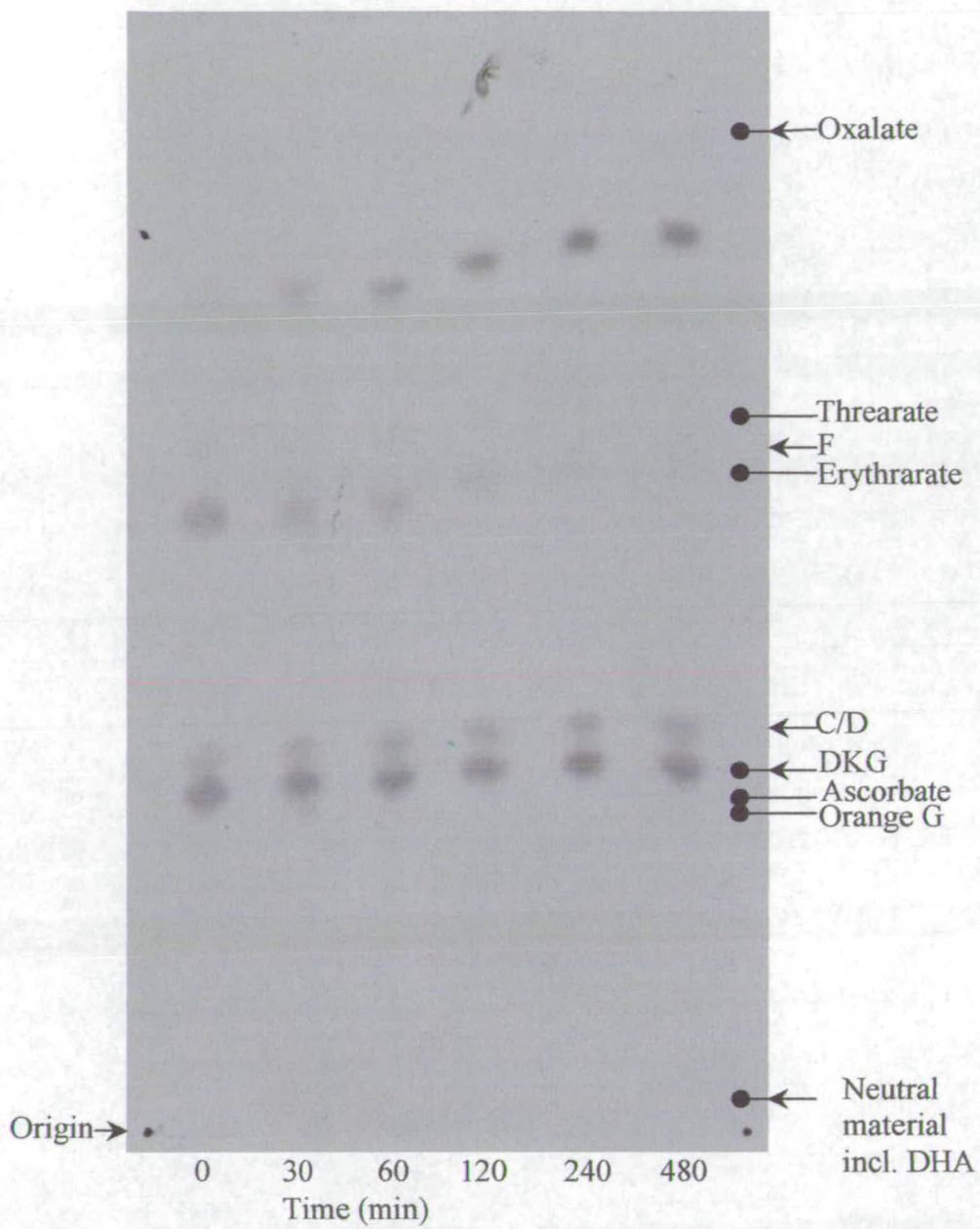
Figure 38

C. Fate of $[^{14}C]$DKG in spent culture medium. Samples of culture medium were analysed as described in Fig. 38 A.
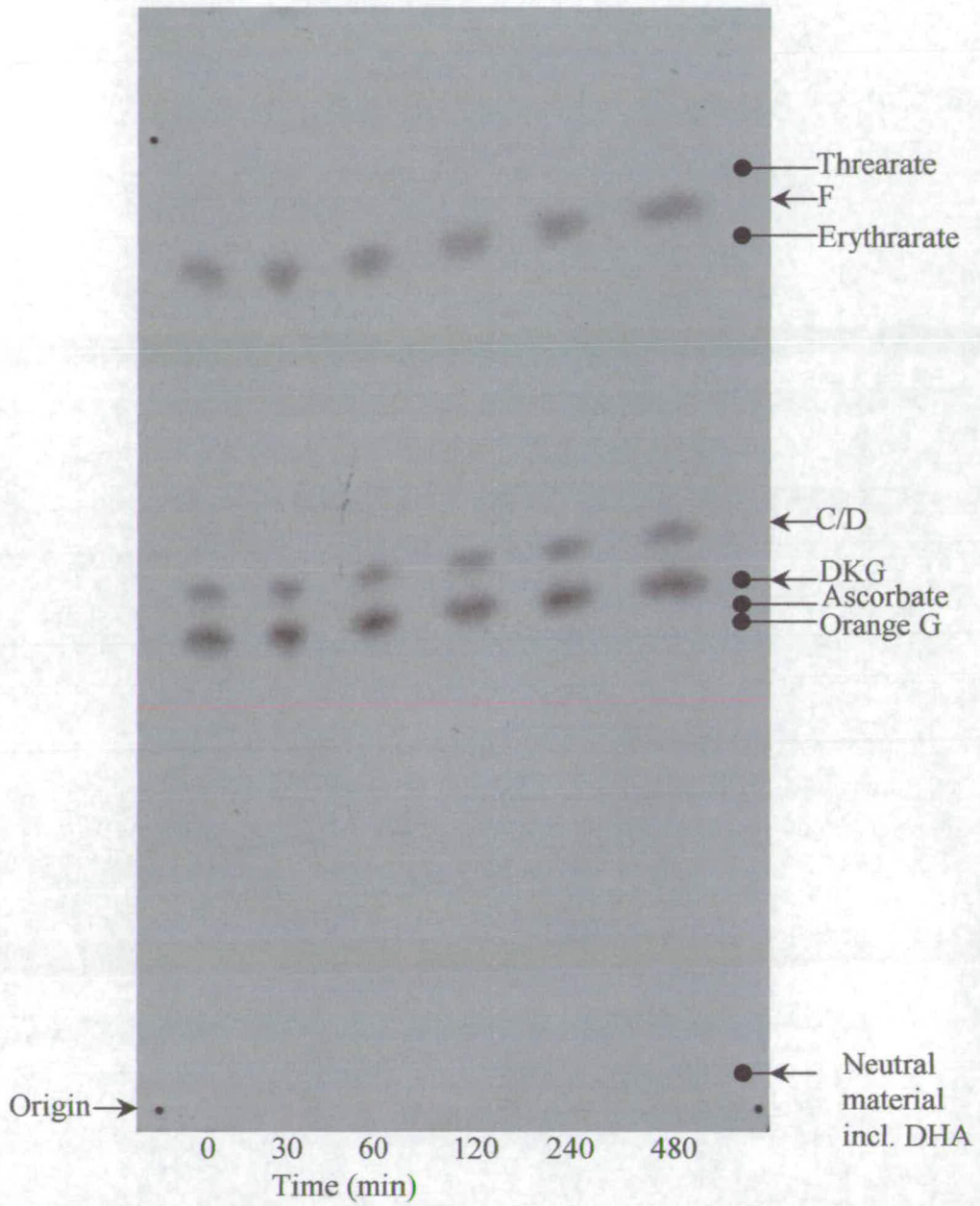
Figure 38

D. Breakdown of [^{14}C]DKG in boiled spent medium. Samples of boiled spent medium were analysed as described in Fig. 38 A.
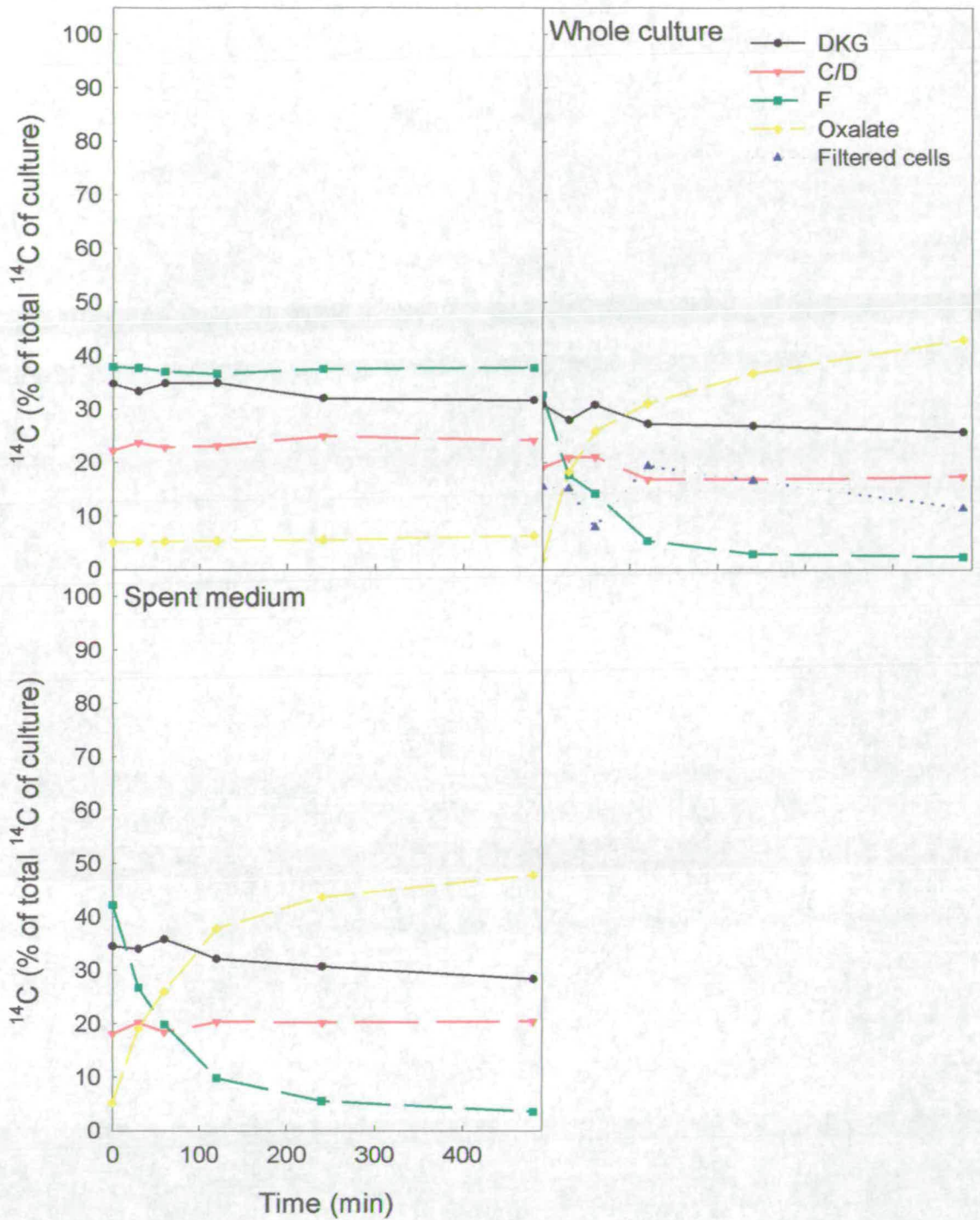
Figure 38

E. Fate of [14C]diketogulonate in rose culture and culture medium. The areas of electrophoretograms corresponding to metabolites identified by autoradiography in Fig. 38 A–D were cut out and the radioactivity present was quantified by scintillation counting. Radioactivity associated with cells was analysed by scintillation counting of cells obtained by filtration of a 20-μl sample of whole culture. Results could not be expressed as a % of total in boiled spent medium since data for [14C]oxalate were not available.

3.5.6. Fate of radiolabelled compound F

Radiolabelled compound F, formed by metabolism of [1-^{14}C]ascorbate in rose culture and culture medium, was eluted from the electrophoretograms described in Chapter 3.4.3, dried and redissolved in water (60 µl). Radiolabelled compound F (0.0023 µCi in 10 µl) was added to rose culture and culture medium (133 µl). Samples of culture and culture medium (20 µl) were removed at intervals for analysis by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes followed by autoradiography and scintillation counting.

The sample of ^{14}C-labelled compound F fed to rose cultures was reasonably pure and although some [^{14}C]oxalate was present (Fig. 39 A–D 0-minute time point) the proportion was negligible (Fig. 39 A–E).

Radiolabelled compound F fed to fresh medium remained constant over the 8-hour period (Fig. 39 A, E). A small amount of [^{14}C]oxalate was present throughout the time course (Fig. 39 A, E).

Radiolabelled compound F fed to whole, 5-d-old rose culture was steadily and almost completely removed from culture medium over the 8-hour time course (Fig. 39 B, E). Radiolabelled compound F was converted to [^{14}C]oxalate, which steadily increased over the time course (Fig. 39 B, E). There was no change in the amount of radioactivity associated with cells extracted from whole culture by filtration (Fig. 39 E). These results imply that compound F, present in the apoplast, is converted to oxalate with no net uptake of radioactivity by cells.

Radiolabelled compound F added to spent medium was almost completely removed during the 8-hour time course (Fig. 39 C, E). Removal of radiolabelled compound F was concurrent with an increase in the amount of [^{14}C]oxalate present

(Fig. 39 C, E). This shows that compound F is converted to oxalate by the action of an enzyme present in the apoplast.

Radiolabelled compound F, fed to boiled spent medium, was not converted to [^{14}C]oxalate and was stable throughout the 8-hour time course (Fig. 39 D, E). When apoplastic enzymes were denatured no conversion of compound F to oxalate occurred (Fig. 39 D, E).
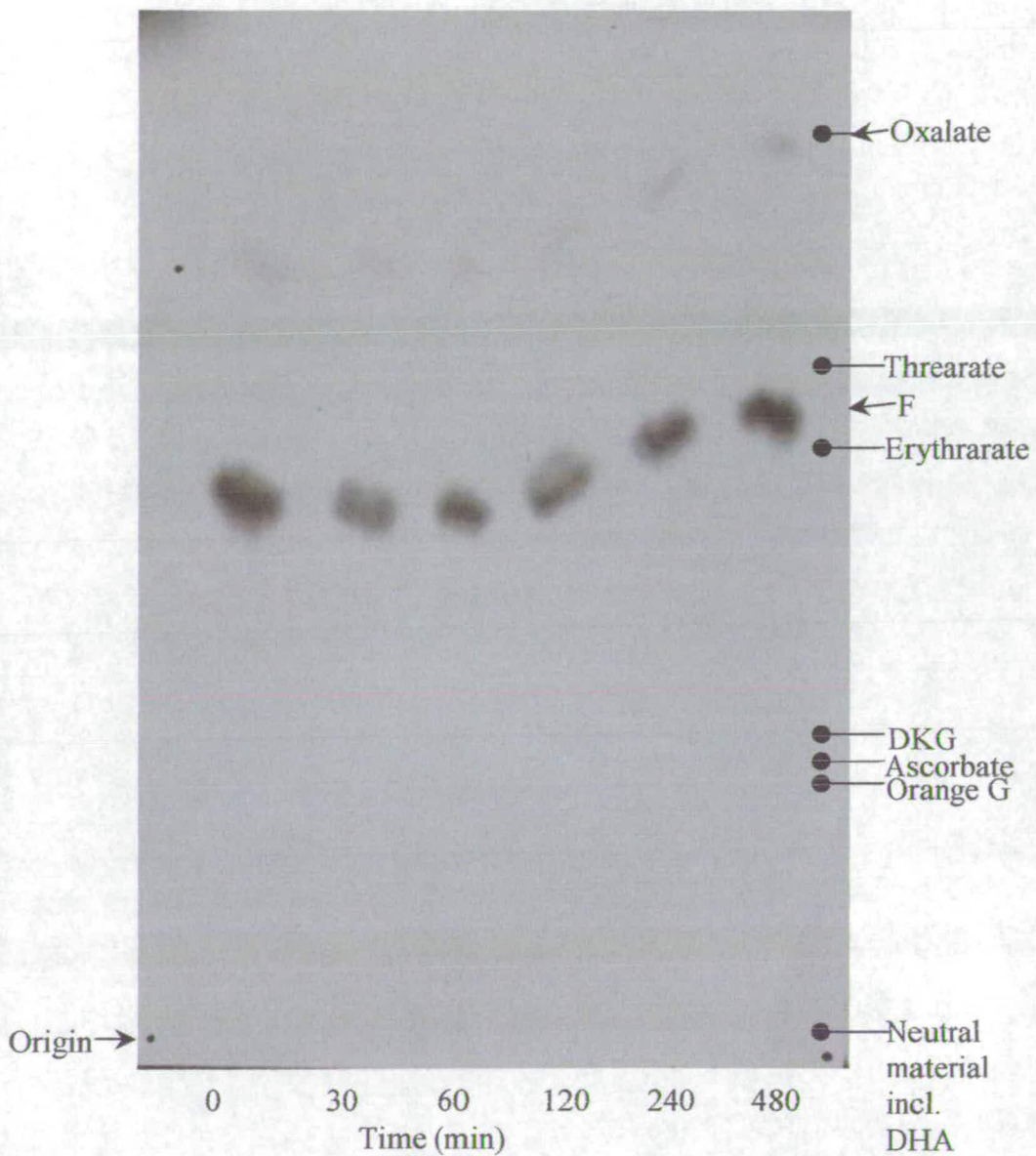
Figure 39. Fate of ^{14}C-labelled compound F fed to rose culture and culture medium

A. Non-enzymic breakdown of radiolabelled compound F added to fresh medium. Soluble products were analysed by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes followed by autoradiography.

●——— represents the approximate mobility of external standards
←——— indicates radiolabelled metabolites

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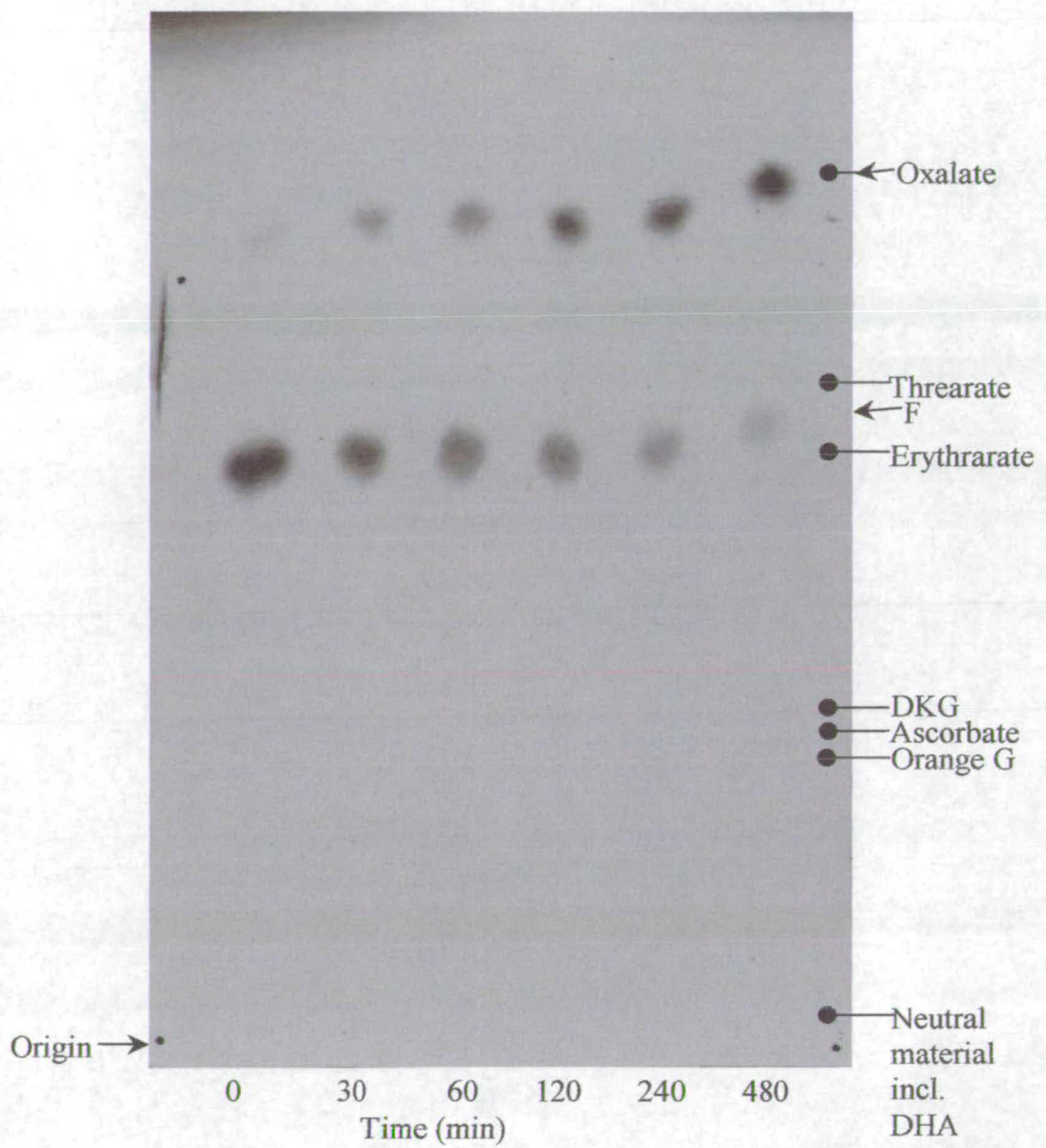
Figure 39

B. Metabolism of ^{14}C-labelled compound F in whole, 5-d-old rose culture. Soluble, extracellular products were analysed by electrophoresis and autoradiography as described in Fig. 39 A.
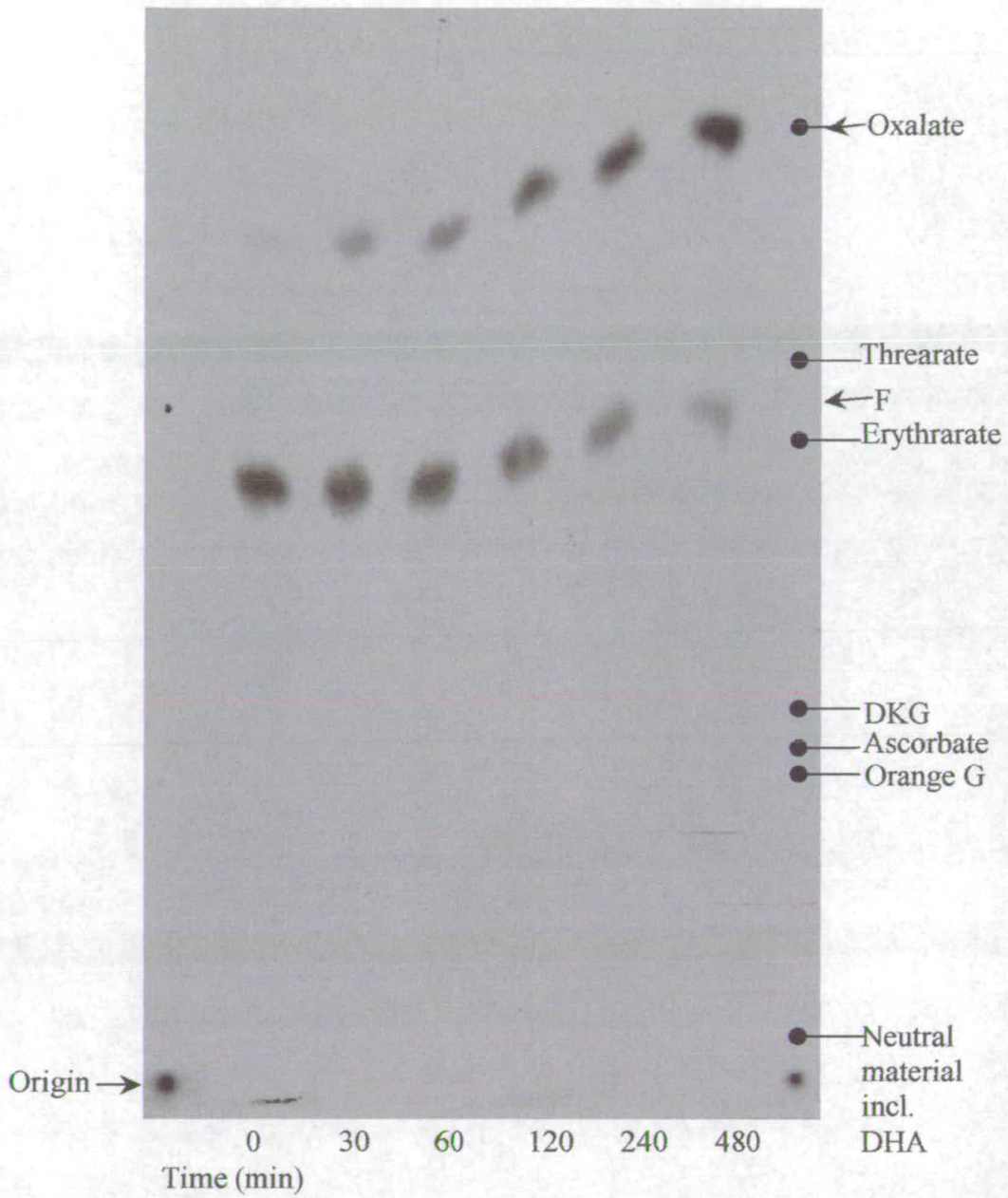
Figure 39

C. Breakdown of ^{14}C-labelled compound F in spent medium obtained from 5-d-old rose culture. Soluble products were analysed as described in Fig. 39 A.
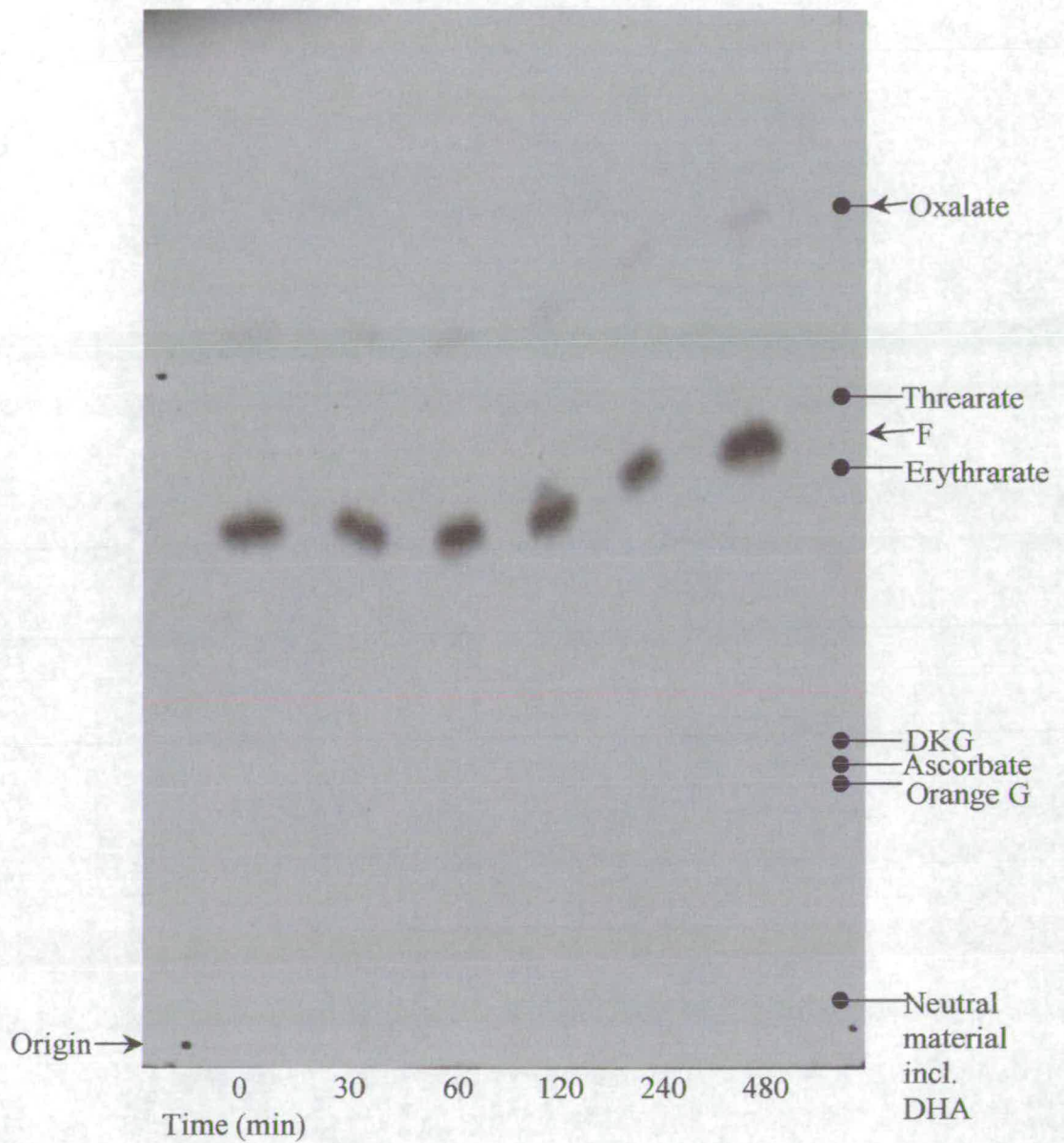
Figure 39

D. Non-enzymic breakdown of ^{14}C-labelled compound F in boiled spent medium obtained from 5-d-old rose culture. Soluble products were analysed by electrophoresis and autoradiography as described in Fig. 39. A
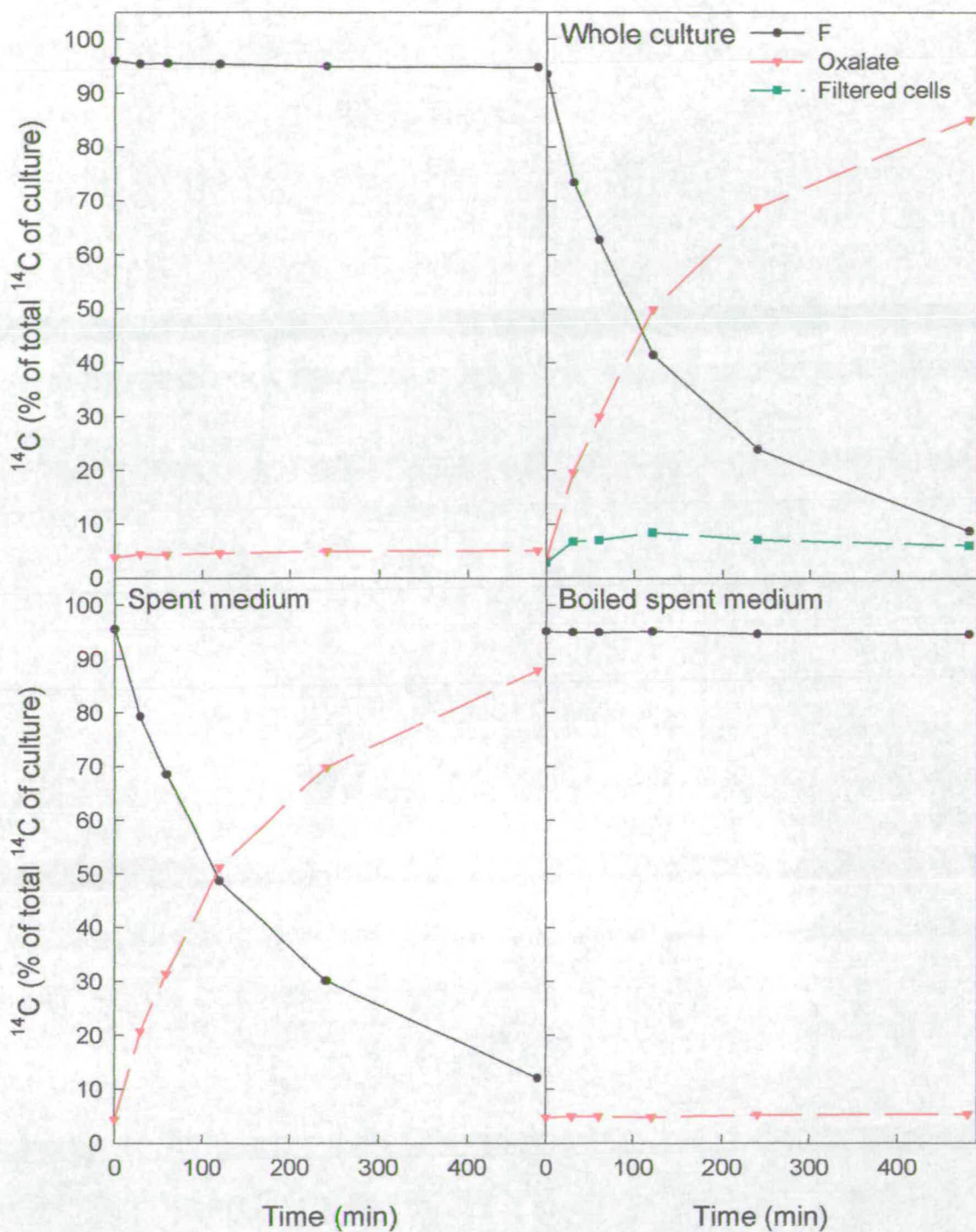
Figure 39

E. Fate of ^{14}C labelled compound F added to rose culture and culture medium. Radiolabelled compounds detected by autoradiography (Fig. 39. A–D) were excised from electrophoretograms and radioactivity present was quantified by scintillation counting. Radioactivity associated with cells was analysed by scintillation counting of cells extracted by filtration of a 20-µl sample of whole culture.

3.5.7. Fate of [^{14}C]oxalate

[^{14}C]Oxalate (10 Ci/mol) was dissolved to a concentration of 0.5 mM in rose cultures and culture medium (143 µl). Samples of culture medium were removed at intervals and subjected to electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes followed by autoradiography.

The [^{14}C]oxalate was contaminated (Fig. 40 A–D 0-minute time point) but the contaminants appeared not to influence the fate of [^{14}C]oxalate over the 8-hour time course. A darker patch in the ^{14}C streak was observed in the [^{14}C]oxalate fed to culture and culture medium. This had similar mobility to radiolabelled compound F but was not metabolised in culture or culture medium during the 8-hour time course (Fig. 40 A–D).

The amount of [^{14}C]oxalate present in fresh medium did not change during the 8-hour experimental period (Fig. 40 A, E). Overall, oxalate was not removed from whole culture (Fig. 40 B, E). The amount of [^{14}C]oxalate in spent medium and boiled spent medium did not show any appreciable change during the 8-hour period (Fig. 40 C–E). [^{14}C]Oxalate is stable in rose culture and culture medium and is not converted to $^{14}CO_2$ or other radiolabelled volatile products.

The accumulation of oxalate in culture medium as a result of feeding [1-^{14}C]ascorbate and [^{14}C]dehydroascorbate suggested that it may be an end product of ascorbate metabolism. The stability of [^{14}C]oxalate in rose culture and culture medium indicates that oxalate does not undergo further metabolism, confirming the suggestion that it is an end product.
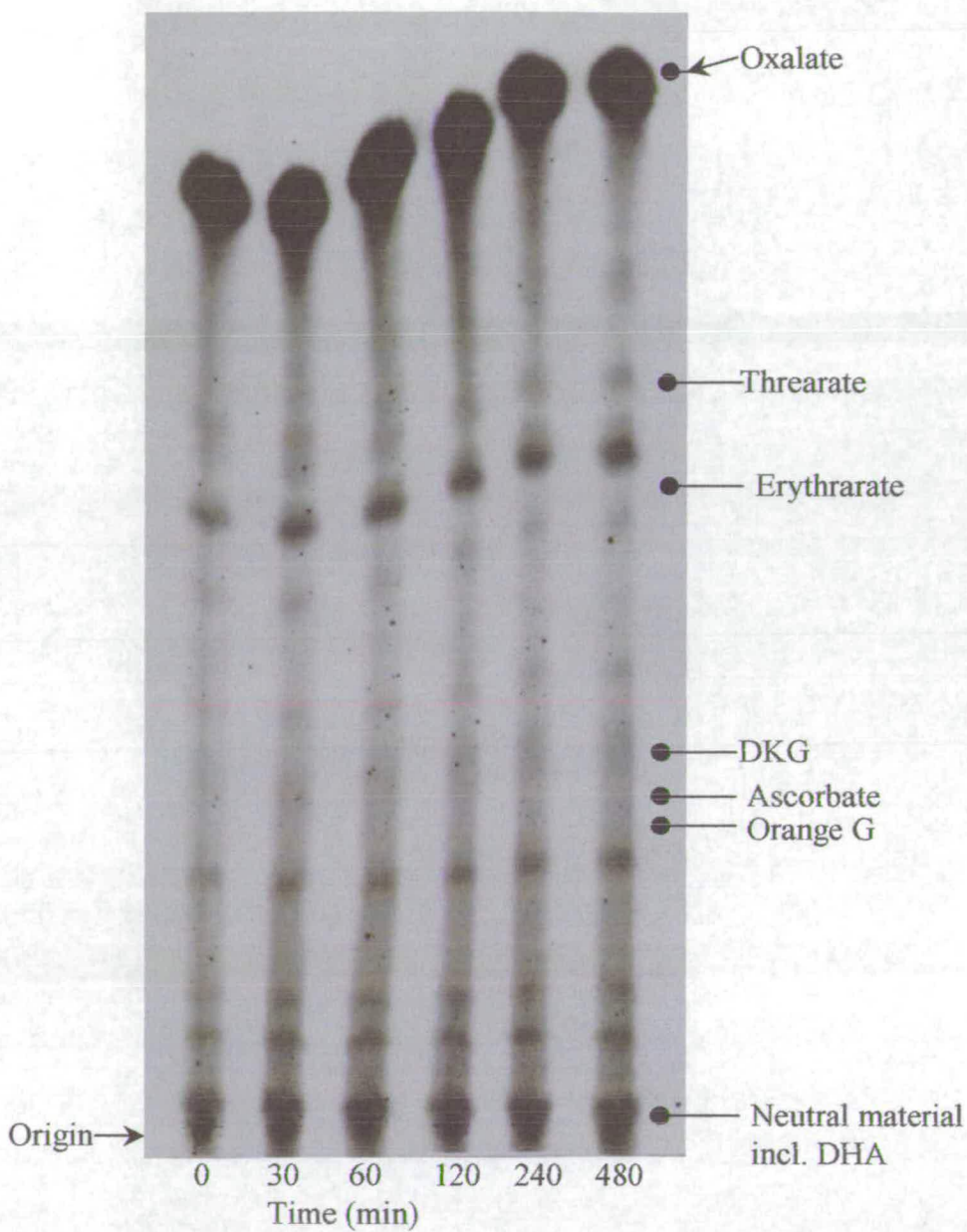
Figure 40. Fate of [^{14}C]oxalate in rose culture and culture medium.

A. Breakdown of [^{14}C]oxalate in fresh medium. Soluble products were analysed by electrophoresis in pH 6.5 buffer at 3 kV for 30 minutes followed by autoradiography.

●——— represents the approximate mobility of external standards
←——— indicates the mobility of [^{14}C]oxalate
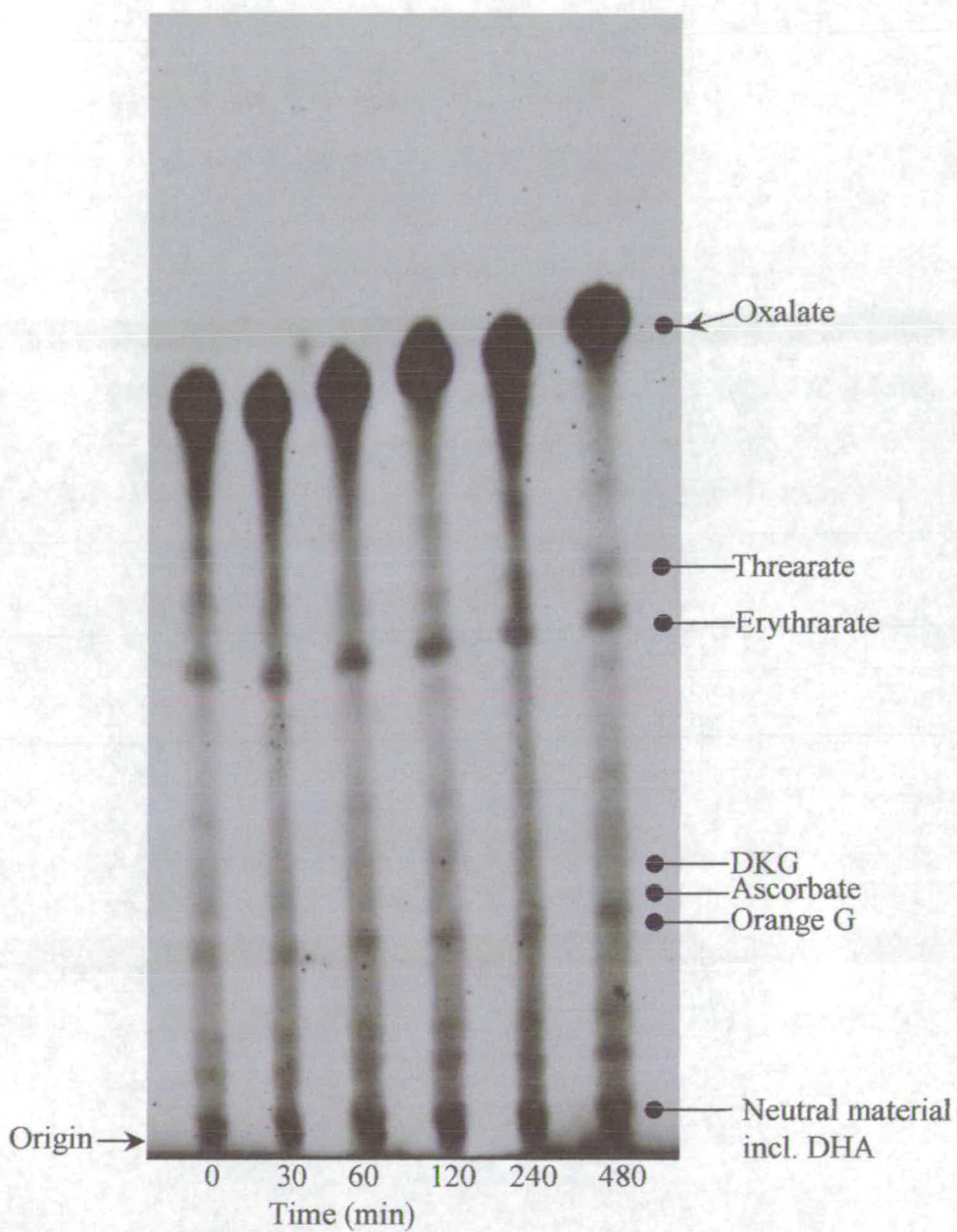
Figure 40

B. Metabolism of [^{14}C]oxalate in whole, 5-d-old rose culture. Soluble, extracellular products were analysed as described in Fig. 40 A.
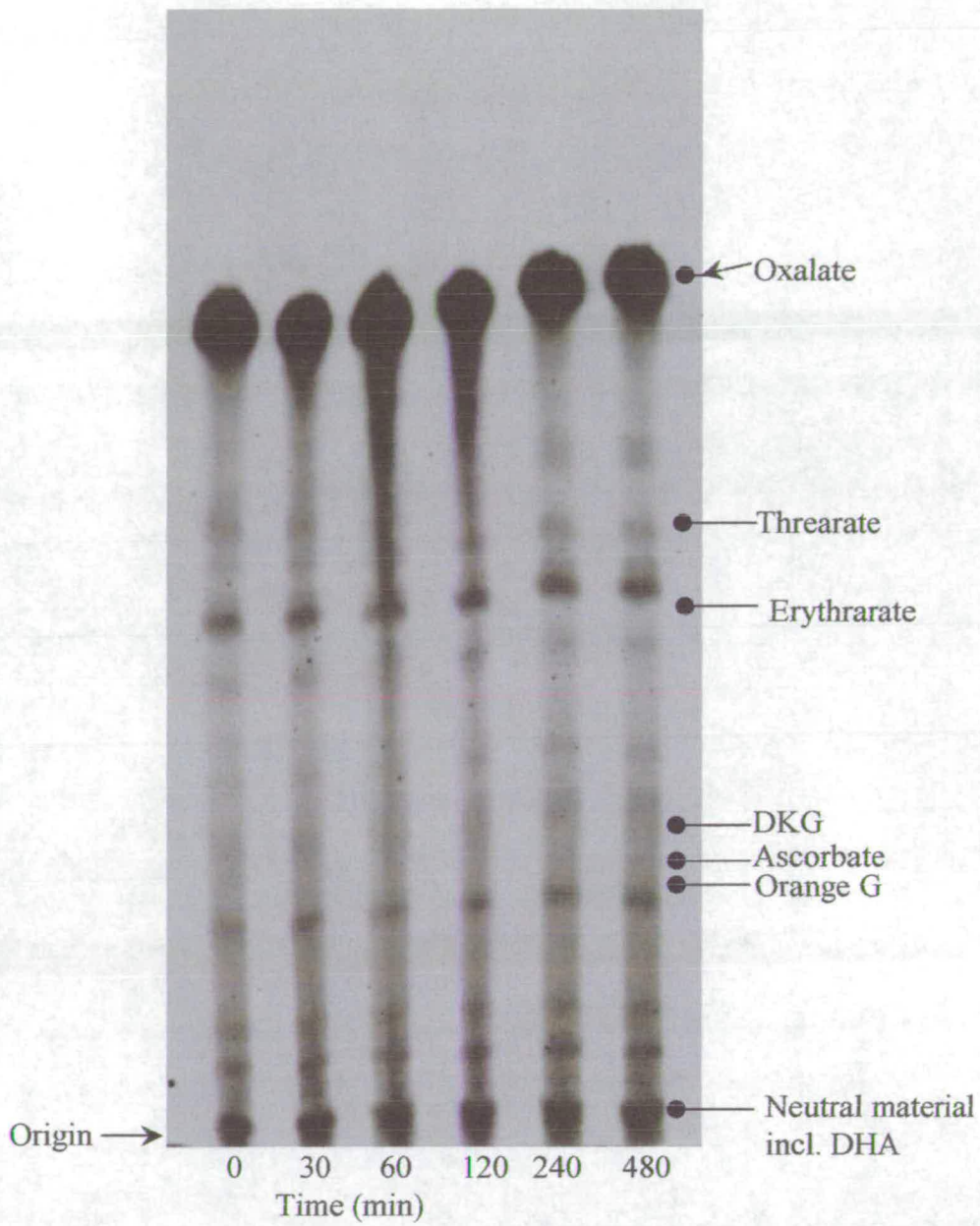
Figure 40

C. Fate of [^{14}C]oxalate in spent culture medium obtained from 5-d-old rose culture. Soluble products were analysed by electrophoresis and autoradiography as described in Fig. 40 A.
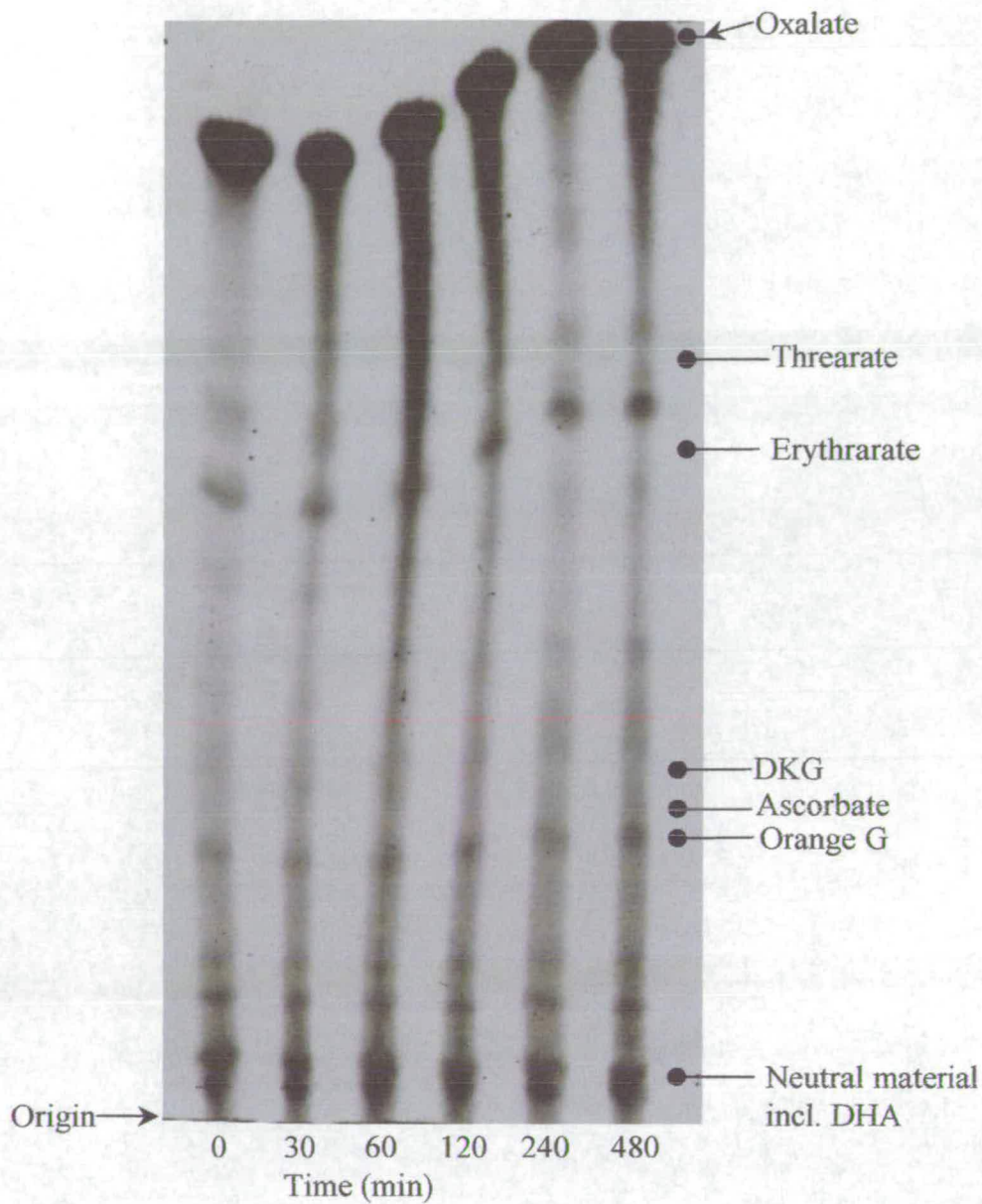
Figure 40

D. Fate of [^{14}C]oxalate in boiled spent medium obtained from 5-d-old rose culture. Soluble products were analysed as described in Fig 40 A.

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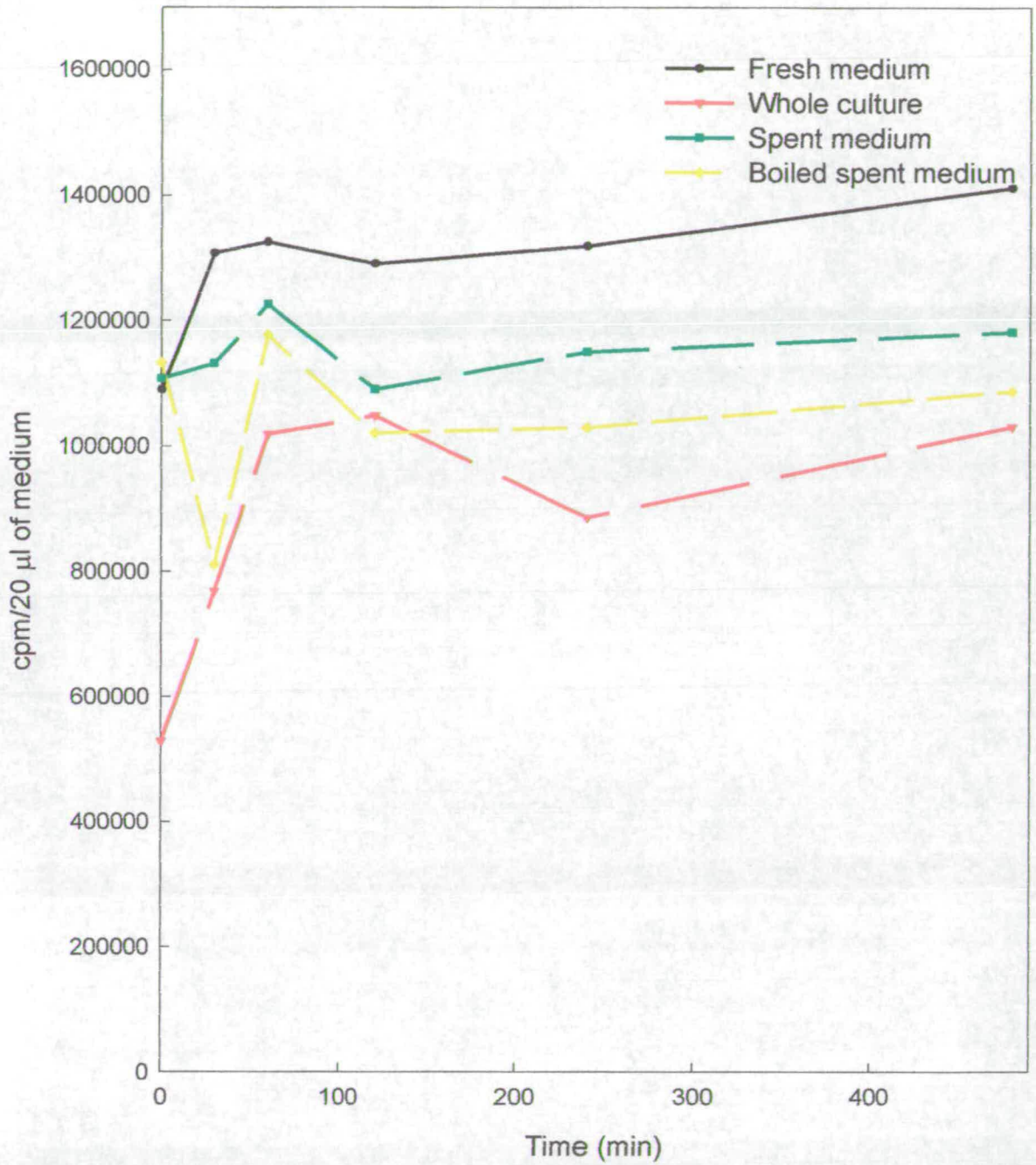
Figure 40

E. Fate of [^{14}C]oxalate in rose culture and culture medium. The area of the electrophoretogram corresponding to [^{14}C]oxalate was analysed by scintillation counting.

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4. Discussion

My work has shown that ascorbate is extensively degraded in the medium of 5-d-old rose cell suspension cultures via a range of compounds to oxalate during an 8-hour period. Degradation and/or metabolism occurs in the apoplast although some uptake of degradates and/or metabolites occurs. Major metabolites derived from [1-^{14}C]ascorbate include compounds C, D, E, F and oxalate. Efforts were made to identify these compounds and their characteristics in order to piece together a pathway for the metabolism of apoplastic ascorbate.

Previous research into intraprotoplasmic ascorbate metabolism showed that ascorbate is converted to L-tartrate in the Vitaceae with L-tartrate derived from carbons 1 to 4 of ascorbate. In the Geraniaceae, oxalate is derived from carbons 1 and 2 and L-threonate from carbons 3 to 6 of ascorbate. L-Tartrate is formed by oxidation of threonate. Neither of these pathways appear to be representative of the predominant pathway for ascorbate metabolism in plants in general.

My results show that oxalate is derived from carbons 1 and 2 of ascorbate. [1-^{14}C]Ascorbate was fed to cultures and therefore any radiolabelled metabolites detected must contain the carbon 1 of ascorbate. Non-radiolabelled compounds were not examined and it is not possible to speculate that ascorbate metabolism in rose follows the same pathway as in the Geraniaceae with threonate derived from carbons 3 to 6 of ascorbate. Administration of universally labelled ascorbate or ascorbate labelled in positions other that carbon 1 would provide more information on other metabolites of ascorbate.

Compound F, a major metabolite of ascorbate, has not yet been identified but many likely candidates have been eliminated. The high electrophoretic mobility of F at pH 2.0 is indicative of a compound with low pK values. The observed

mobility of F at pH 6.5 was similar to the observed mobility of L-threarate (L-tartrate) and erythrarate (meso-tartrate). L-Tartrate (L-threarate), a product of presumably intraprotoplasmic C4–C5 cleavage of ascorbate in the Vitaceae (Williams et al. 1979), was considered a likely product of apoplastic ascorbate metabolism. Threarate and erythrarate both possess two acid groups and have a molecular weight of 150. The observed mobility of F could indicate that it has two acid groups and a molecular weight of approximately 150. However, although F had similar mobility at pH 6.5, it did not exactly co-migrate with either threarate or erythrarate. At pH 2.0, F was highly mobile while threarate and erythrarate were almost immobile.

Threonate was also considered as a potential of the cleavage of the C4–C5 bond of ascorbate. Threonate has only one negative charge and a M_r of 136. At pH 6.5 threonate is mobile ($m_{OG} = 1.54$) but not as mobile as F ($m_{OG} = 2.34$).

The results indicate that F is not threonate, threarate or erythrarate and that apoplastic ascorbate metabolism in rose does not follow a similar pathway to the presumably intraprotoplasmic metabolism of ascorbate in the Vitaceae.

Dihydroxyfumarate ($M_r = 148$) and oxaloacetate ($M_r = 132$) could be formed by oxidative cleavage of the C4–C5 bond of ascorbate. Both of these compounds possess two acid groups and have similar charge:M_r ratio to the estimated charge:M_r ratio of F. Dihydroxyfumarate and oxaloacetate were both mobile at pH 2.0 but neither migrated as far as F. Fumarate, malate and succinate were also considered potential products of ascorbate metabolism. Malate could be formed after hydrolysis of ascorbate between C4 and C5 but the formation of succinate would require a reduction reaction. Oxidation and hydrolysis reactions are more likely than

reduction reactions to occur in the apoplast because O_2 and H_2O are more abundant than reductants such as NADH and NADPH.

Intraprotoplasmic ascorbate metabolism in the Geraniaceae involves the cleavage of the C2–C3 bond of ascorbate and formation of oxalate from C1 and C2 of ascorbate and threonate from C3 to C6 (Williams et al. 1979). L-Threonate is then oxidised to L-tartrate. In *Rumex*, threonate is converted to CO_2.

In rose, oxalate is formed from carbons 1 and 2 of ascorbate and accumulates as a major product of apoplastic ascorbate metabolism. It does not undergo further metabolism to CO_2 or uptake during an 8-hour period. Oxalate is formed non-enzymically in boiled spent medium although to a lesser extent than in unboiled medium or whole culture.

Glycollate and glyoxylate were suggested as possible products of apoplastic ascorbate metabolism. They could be formed from carbons 1 and 2 of ascorbate by hydrolysis and oxidation and could undergo further oxidation to oxalate. Glycollate and glyoxylate are mobile at pH 6.5 with a similar charge to area ratio (calculated as charge/$M_r^{2/3}$) as the estimated charge to area ratio for F. For this reason glycollate and glyoxylate were considered as candidates for F. However, neither glycollate nor glyoxylate migrated as far as F during electrophoresis at pH 6.5. These results indicate that direct C2–C3 cleavage of ascorbate in apoplastic ascorbate metabolism in rose is unlikely and formation of oxalate from C1 and C2 of ascorbate may occur via an intermediate compound. It was not possible to detect if tartrate or threonate were formed from carbons 3–6 of ascorbate since the ascorbate administered to rose cultures was only radiolabelled in carbon 1.

The identity of F remains a mystery although several likely compounds that could correspond to F have been ruled out. Examination of the identity of F has

indicated that apoplastic ascorbate metabolism may be very different from intraprotoplasmic ascorbate metabolism. Further analysis of F by NMR may help to identify this metabolite of apoplastic ascorbate.

Compound F is converted to oxalate by cold, dilute alkali and in culture medium by the action of an azide-inhibited enzyme. The effect of alkali on compound F indicates the presence of an alkali-labile bond such as an ester bond. The formation of oxalate as a result of alkaline treatment would suggest that F is an oxalyl ester.

The formation of oxalate from F was inhibited by azide. Azide inhibits enzymes that require copper as part of their catalytic mechanism and decarboxylase enzymes. The inhibitory effect of azide on the enzymic formation of oxalate from F would suggest that either an oxidase or a decarboxylase was involved. If the conversion of F to oxalate involved an oxidation reaction it would have been inhibited by a reducing agent such as dithiothreitol. However, dithiothreitol did not inhibit conversion of F to oxalate and therefore it is assumed that formation of oxalate does not involve oxidation. F is susceptible to hydrolysis resulting in the formation of oxalate. This occurs enzymically in spent medium indicating that an apoplastic enzyme is involved. The conversion of F to oxalate does not involve oxidation as demonstrated by the effects of azide and dithiothreitol although the enzyme inhibited by azide could be a decarboxylase.

The radiolabelled metabolites of apoplastic ascorbate C and D had similar observed mobilities at pH 6.5 after 30 minutes. C and D were eluted from 30-minute-electrophoretograms and resolved by electrophoresis at pH 6.5 for 45 minutes. After 45 minutes other compounds of interest including oxalate, F, threarate and erythrarate had migrated off the paper. Since the resolution of C and D

showed C to be predominant, with only a small amount of D present, a mixture of C and D was used for further analysis. The mobilities of C and D at pH 6.5 were similar to those of diketogulonate, 2-ketogluconate and threonate although neither C nor D exactly co-migrated with any of these compounds. Diketogulonate and 2-ketogluconate are 6-carbon compounds and each possesses one negative charge. Threonate also possesses one negative charge but it contains four carbons making its charge/$M_r^{2/3}$ ratio less than that of diketogulonate or 2-ketogluconate. For this reason threonate is more mobile at pH 6.5. The mobility of C and D could indicate a six-carbon compound that has one negative charge. C and D were mobile during electrophoresis at pH 2.0 indicating that C and D have low pKs and suggesting that they may possess two negative charges.

Compound E was more mobile ($m_{OG} = 2.06$) than C ($m_{OG} = 1.53$) at pH 6.5. The mobility of E was suggestive of a compound with six carbons and two negative charges or with four carbons and one negative charge. Samples of E eluted from pH 6.5 electrophoretograms were found to contain almost equivalent amounts of C. It is unlikely that contamination occurred when the radiolabelled areas were excised from the whole electrophoretogram because E and C are clearly resolved. Any C present in samples of E must be formed from E during aqueous elution and/or during storage and further analysis of E.

The nature of the conversion of E to C was examined using cold, dilute, aqueous NaOH. Treatment with NaOH resulted in the removal of C an increase in the amount of E present. C was completely converted to E in the presence of NaOH. These results indicate that C is hydrolysed to E and that C contains an easily hydrolysed bond such as a lactone bond.

A sample of E, purified form pH 6.5 electrophoretograms, easily forms C without the addition of any other compounds. In reverse, this would indicate that the hydrolysis of C has only one product, E, formed by the hydrolysis of an intramolecular ester bond in C. C is thought to be a lactone form of E, a suggestion that is supported by comparison of the mobility of C with E at pH 6.5.

The mobility of C is suggestive of a compound with six carbons and one negative charge while the mobility of E suggested a either a 6-carbon compound with two negative charges or a four-carbon compound with one negative charge. Lactonisation of a six-carbon compound with two negative charges would result in the formation of another six-carbon compound but with only one free negatively charged group. Hence E probably has six carbons and two negative charges, one of which is involved in lactonisation to form C, which would have only one free negatively charged group.

E does not accumulate as much in whole cultures as in spent culture medium, suggesting that some uptake of E occurs. C does not appear to be subject to uptake by cells. Lactonisation of E to form C may prevent uptake of this compound by cells and constitute a mechanism for the control of the uptake of E.

Compounds C and E were difficult to analyse owing to their instability. Oxalate and F were detected in samples of what began as C (and/or D). The formation of F and oxalate in samples of C (and/or D) suggests that these compounds precede F and oxalate in a metabolic pathway leading from ascorbate to oxalate. Purified F does not yield C (and/or D) so while C (and/or D) may form F, the reaction does not appear to be reversible.

Diketogulonate is formed from apoplastic ascorbate in rose cultures. Its formation by hydrolysis of dehydroascorbate occurs non-enzymically in fresh

medium and in boiled spent medium and was not promoted or inhibited in whole culture or spent medium. The formation of diketogulonate in the apoplast is therefore believed to be non-enzymic. [^{14}C]Diketogulonate fed to rose cultures is not metabolised, its formation appears to be irreversible and it does not undergo further metabolism. Diketogulonate appears to be an end-product of apoplastic ascorbate metabolism.

The fate of dehydroascorbate in rose culture and culture medium was examined. Treatment of ascorbate with ascorbate oxidase resulted in the formation of dehydroascorbate and other acidic compounds including diketogulonate. An attempt was made to remove acidic compounds but was unsuccessful. Metabolism of dehydroascorbate in rose culture and culture medium is limited, its main fate being hydrolysis to diketogulonate. Apoplastic ascorbate is metabolised to oxalate in rose and dehydroascorbate is metabolised to diketogulonate. Diketogulonate does not undergo further metabolism. Loewus et al. (1975) also found that diketogulonate was not converted to oxalate, providing support to my suggestion that diketogulonate produced from ascorbate is not further metabolised. Contrary to my results, Loewus et al. (1975) found dehydroascorbate to be equivalent to ascorbate as a precursor of oxalate. I propose that in the apoplast of rose, ascorbate is the main precursor for oxalate and that dehydroascorbate is not involved in oxalate biosynthesis.

From the results of this work I have suggested a pathway for the apoplastic metabolism of ascorbate and biosynthesis of oxalate (Fig. 41) in 5-d-old rose culture medium. In the putative pathway represented, the involvement of enzymes and other reactants and products is shown based on conclusions drawn from the results of my research and deductions as to the identity of some metabolites.
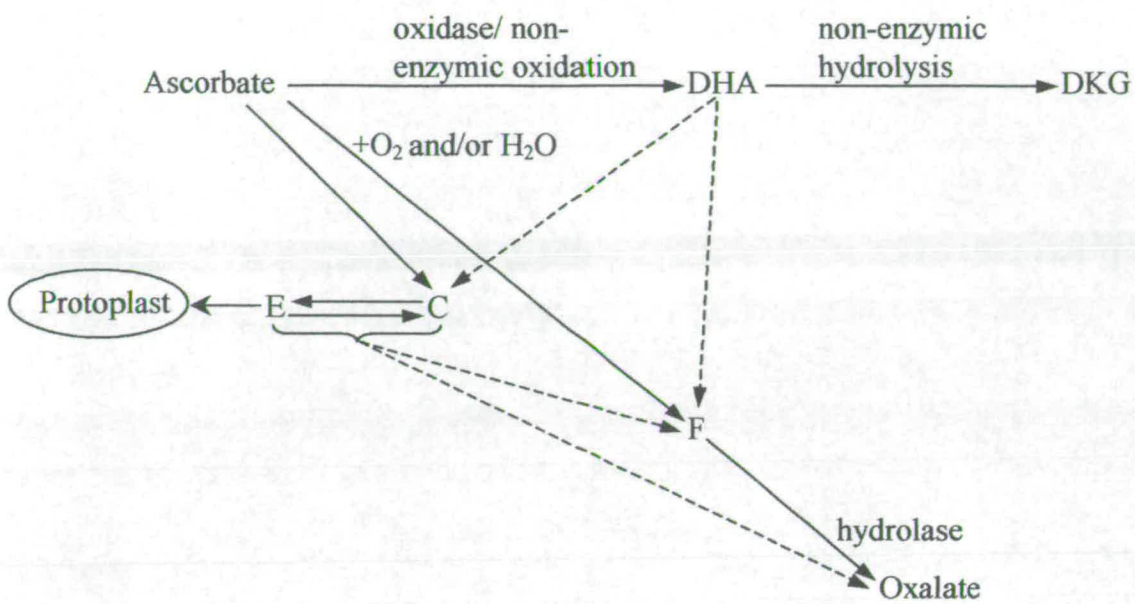
Figure 41. Metabolism of exogenous apoplastic ascorbate

⟶ represents confirmed reactions
---⟶ represents speculative reactions

Ascorbate is extensively metabolised in culture medium, which is representative of the apoplast. Apoplastic ascorbate is broken down by the components of rose culture medium and is metabolised both enzymically— by hydrolase and possibly decarboxylase enzymes as suggested by the effect of azide— and non-enzymically as demonstrated in spent and boiled spent medium. Apoplastic ascorbate does not appear to be taken up by cells in suspension culture and most metabolites of apoplastic ascorbate appear to remain in the culture medium. I have not investigated the presence of $[1-^{14}C]$ascorbate in the symplast of cultured cells or other fates of apoplastic ascorbate such as uptake by cells followed by rapid secretion of symplastic metabolites into the apoplast. Exogenous apoplastic ascorbate may be taken up by cells and metabolised within the protoplast. Apoplastic ascorbate could adhere to cell surfaces—the cell wall and plasma membrane—and thus be undetectable in the apoplast. However, negligible radiolabel was associated with cells from whole culture suggesting that apoplastic ascorbate does not adhere to cell surfaces.

It is difficult to deduce the significance of apoplastic ascorbate metabolism without knowing the identity of the metabolites involved. The pathway I have postulated may function to remove excess apoplastic ascorbate. Ascorbate is not usually transported into protoplasts. It is regenerated from either monodehydroascorbate radicals or dehydroascorbate by electron transfer from the cytosol. Dehydroascorbate is reported to be taken up by protoplasts (Horemans et al. 1997, Rautenkrantz 1994), although no dehydroascorbate uptake was observed in this study. Exchange of apoplastic dehydroascorbate with cytosolic ascorbate maintains the redox state of ascorbate in the apoplast (Horemans et al. 1998). There are potential disadvantages of too high levels of apoplastic ascorbate. Ascorbate has

been shown to mediate the generation of hydroxyl radicals and thereby promote scission of cell wall polysaccharides (Fry 1998, Fry et al. 2002). Excess apoplastic ascorbate could compromise the integrity of the cell wall and since it does not appear to be removed by uptake, an alternative mechanism for the removal of excess ascorbate would be required. Thus the pathway that I have identified could serve as a mechanism for the removal of ascorbate.

Some of the products of apoplastic ascorbate metabolism— C, E, F— may merely be intermediates in the removal of ascorbate from the apoplast. Alternatively, these metabolites could be intermediates in a biosynthetic pathway that results in the formation of oxalate. Apoplastic oxalate could be oxidised by the enzyme oxalate oxidase to produce CO_2 and H_2O_2. Oxalate oxidase has been shown to be cell-wall-bound in wheat, barley and maize (Vuletić and Šukalović 2000). The H_2O_2 produced by oxalate oxidase catalysed oxalate oxidation could be involved in the oxidative cross-linking of cell wall polysaccharides which would strengthen the cell wall, or it could facilitate cell wall loosening as a result of polysaccharide scission through its role in hydroxyl radical generation. Thus the biosynthesis of oxalate could be part of a mechanism which regulates cell wall expansion. The oxalate produced from ascorbate in rose cell suspension cultures is stable and does not appeared to be removed by the action of oxalate oxidase.

The apoplastic pathway leading from ascorbate to oxalate may utilise and/or produce H_2O_2 in formation of intermediate metabolites. D-Erythroascorbate— a fungal five-carbon analogue of ascorbate— is cleaved between carbons 2 and 3 in the presence of H_2O_2 to form oxalate (Loewus 1999). It is possible that a similar process occurs during the metabolism of apoplastic ascorbate in plants. This may proceed via one or more intermediates to produce F which would then be cleaved to

form oxalate. F is postulated to be a four-carbon compound with 2 negative charges similar to L-threarate (and erythrarate) or dihydroxyfumarate. If cleaved in the presence of H_2O_2, L-threarate (and erythrarate) and dihydroxyfumarate could produce oxalate and either glycollate or glyoxylate respectively. This would also result in the conversion H_2O_2 to water. My results have not identified F as L-threarate, erythrarate or dihydroxyfumarate and also suggest that conversion of F to oxalate is the result of hydrolysis without oxidation. If formation of oxalate and glycollate or glyoxylate from L-threarate (and erythrarate) or dihydroxyfumarate did occur in the apoplast it could provide a mechanism for the removal of H_2O_2 and thereby limit hydroxyl radical generation and cell wall polysaccharide scission. The oxalate formed in the apoplast is stable rose cell suspension cultures and does not appear to be removed by the action of oxalate oxidase. This indicates that oxalate oxidase, if present either cell wall bound or free in the apoplast, is not active within the apoplast of rose cell cultures.

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6. Appendix

Consumption of Exogenous Ascorbate and Measurement of Symplastic and Apoplastic Ascorbate in Suspension-Cultured Plant Cells

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Introduction Ascorbate is synthesised in all members of the plant kingdom. It has a multitude of roles in photosynthesis, defence against oxidative stress, cell division, cell wall metabolism and cell expansion. Despite the importance of ascorbate in plants its biosynthetic pathway has only recently been elucidated[1]. Ascorbate is unstable and is oxidised to dehydroascorbate which is subsequently hydrolysed to diketogulonate. These may be metabolised to oxalic, threonic and/or tartaric acid. In this way ascorbate may be a precursor for such organic acids. The aim of this project is to examine the metabolic fate of ascorbate in the apoplast of suspension-cultured rose, spinach and maize cells. Recent work has examined uptake of exogenous ascorbate by suspension cultures and attempted to measure symplastic and apoplastic ascorbate.

Methods Removal of exogenous 1mM ascorbate from the medium by cell cultures was examined in suspension cultures. At intervals over an 8-hour incubation period at room temperature, samples of culture media (0.5 ml) were removed and added to metaphosphoric acid (0.5 ml, 10%) to stabilise ascorbate. Ascorbate was assayed by titration with 2,6-dichlorophenolindophenol (DCPIP).
Symplastic concentration of endogenous ascorbate was measured after cultures were filtered to remove spent media and cells resuspended in metaphosphoric acid (5%). This was filtered and assayed for ascorbate by timed titration with DCPIP.
Apoplastic ascorbate was examined in spent culture media. Samples of culture media were filtered to remove cells and stabilised in metaphosphoric acid (5% final concentration). Ascorbate was measured by titration with DCPIP.

Results Exogenous ascorbate in maize cultures was removed faster in old and young cultures but slowly in cultures of intermediate age. In rose cultures, removal was faster in cultures of intermediate age and slower in old and young cells. The trend in spinach cultures was less obvious.
Symplastic endogenous ascorbate concentration ranged from a minimum of 0.05 mmol kg^{-1} fresh weight in 0-h rose cultures to a maximum of 1.06 mmol kg^{-1} fresh weight in 5-d cultures. Ascorbate ranged from 0.19 to 0.61 mmol kg^{-1} fresh weight in spinach and 0.47 to 0.84 mmol kg^{-1} fresh weight in maize.
Ascorbate may be present in the apoplast at less than 10 μM but has not been detected at higher concentrations. Titration with DCPIP is sensitive to detect ascorbate down to a concentration of 10 μM.

References
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Apoplastic Ascorbate Metabolism in Rose Cell Suspension Cultures

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Introduction

Ascorbate is present in cell walls and is thought to be involved in cell wall metabolism and expansion. Its role in formation of hydrogen peroxide may lead to oxidative scission of cell wall polysaccharides[1]. Ascorbate is a precursor for oxalate and other organic acids— possibly tartrate[2].

The metabolism of [1-^{14}C]ascorbate has been examined in 5-d-old rose cultures, the identity of extracellular metabolites investigated and a putative metabolic pathway presented.

Methods

Experimental rose cultures were prepared from rose cell suspension culture (CSC) buffered with MES (Na$^+$, 5 mM, pH 7.0). L-[1-^{14}C]Ascorbate (1 μCi, 14 Ci/mol) was added to 133 μl fresh medium, whole culture, spent medium or boiled spent medium to a final concentration of 0.5 mM. At intervals over an 8-hour period, samples of 20 μl were removed to storage on liquid nitrogen. Samples were analysed by high-voltage electrophoresis on Whatman 3MM paper, in pH 6.5 buffer, at 3 kV for 30 minutes. Electrophoretograms were autoradiographed. [^{14}C]Labelled metabolites were quantified by scintillation counting. After washing to remove scintillation fluid, metabolites were eluted from the paper. Internal markers were used to identify some ^{14}C-metabolites. The different metabolites were fed back to rose cultures to monitor their subsequent fate following the procedure described above.

Results

Ascorbate was metabolised both enzymically and non-enzymically in the medium of 5-d-old rose cultures. Dehydroascorbate, diketogulonate and oxalate were among the metabolites formed. Oxalate was not readily taken up by cells. Other metabolites included unknown compounds E, thought to be an oxalyl ester of a C$_4$ acid, and F, which may be glycollate. Compound E was produced enzymically and removed from medium of whole cultures. Compound F did not accumulate in culture medium and was enzymically oxidised to oxalate.

Conclusion

Apoplastic ascorbate metabolism proceeds enzymically and non-enzymically in 5-d-old rose CSC. Work is proceeding to identify ^{14}C-labelled metabolites and the nature of their formation.

1. Fry S. C., (1998) Biochemical Journal 332: 507-515
2. Loewus F. A., (1999) Phtyochemistry 52: 193-210

ASCORBATE METABOLISM IN ROSE CELL SUSPENSION CULTURES

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Introduction Ascorbate is involved in cell wall metabolism and expansion. It is reversibly oxidised to dehydroascorbate (DHA) which can undergo irreversible hydrolysis to diketogulonate (DKG). Ascorbate is a precursor for tartaric and oxalic acid. In grapes, carbon 1 of ascorbate becomes a terminal carbon of tartaric acid while in geranium, carbon 6 of ascorbate is incorporated into tartaric acid and carbons 1 and 2 into oxalic acid [1].

Ascorbate has been shown to mediate the formation of hydroxyl radicals and subsequent scission of polysaccharides *in vitro* [2]. Ascorbate-induced production of hydroxyl radicals in the cell wall could have a useful role in loosening the cell wall.

Exogenous ascorbate supplied to cell suspension culture (CSC) may be taken up and metabolised in the cells or, it may be metabolised in the culture medium and the products taken up by cells. Ascorbate metabolism and/or uptake by rose CSC has been examined and an attempt to identify apoplastic metabolites made.

Materials and Methods [^{14}C]Ascorbate (0.1 µCi) mixed with unlabelled ascorbate (1mM) was supplied to rose cultures. Ascorbate concentration was measured by titration with DCPIP (0.1% w/v). Uptake of ^{14}C was measured by scintillation counting.

Identification of metabolic products was attempted by feeding cultures (160 µl) with [^{14}C]ascorbate (1 mM, 2.72 µCi). Culture medium and a formic acid extract of cell contents (20 µl) were subjected to high voltage electrophoresis. Electrophoretograms were autoradiographed for 60 hours.

Ascorbate concentration in culture medium decreased to 0 over 8 hours. Removal was fastest in 3-d and 5-d cultures and slowest in 0-d, 9-d and 14-d cultures. ^{14}C was removed by all rose cultures. Metabolite concentration was estimated by subtracting ascorbate concentration from the total (calculated as cpm/specific radioactivity). A continuous increase in metabolic products indicated ascorbate metabolism in culture medium and accumulation of products (0 d and 1 d). A subsequent decrease indicated uptake was proceeding, e.g. in 3-d, 5-d and 7-d cultures. A low concentration of metabolic products throughout the time course (9-d, 14-d) indicated ascorbate was taken up by cells or metabolites instantly removed from culture medium.

Autoradiography confirmed the removal of ascorbate from culture medium and indicated the formation of some unknown compounds.

Conclusion The results show that ascorbate is metabolised apoplastically by rose CSC. The nature of metabolites remains unknown but work is continuing to reveal their identity.

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