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Oxidation of dehydroascorbic acid and 2,3-diketogulonate under plant apoplastic conditions

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ABSTRACT

The rate of L-ascorbate catabolism in plants often correlates positively with the rate of cell expansion. The reason for this correlation is difficult to explore because of our incomplete knowledge of ascorbate catabolism pathways. These involve enzymic and/or non-enzymic oxidation to dehydroascorbic acid (DHA), which may then be hydrolysed to 2,3-diketogulonate (DKG). Both DHA and DKG were susceptible to further oxidation under conditions of pH and H_2O_2 concentration comparable with the plant apoplast. The kinetics of their oxidation and the identity of some of the products have been investigated here. DHA, whether added in pure form or generated *in situ* by ascorbate oxidation, was oxidised non-enzymically to yield, almost simultaneously, a monoanion (cyclic-oxalyl-threonate; cOxT) and a dianion (oxalyl-threonate; OxT). The monoanion was resistant to periodate oxidation, showing that it was not oxalic threonic anhydride. The OxT population was shown to be an interconverting mixture of 3-OxT and 4-OxT, differing in pK_a. The 3-OxT appeared to be formed earlier than 4-OxT, but the latter predominated at equilibrium. DKG was oxidised by H_2O_2 to two partially characterised products, one of which was itself further oxidised by H_2O_2 to yield threonate. The possible occurrence of these reactions in the apoplast *in vivo* and the biological roles of vitamin C catabolites are discussed.

1. Introduction

L-Ascorbate is a major solute undergoing turnover in all metabolically active plant tissues (Loewus, 1999; Smirnoff et al., 2001). It has several vital roles in plant protoplasm, especially the regeneration of the reduced forms of glutathione and $NADP^+$, e.g. in the highly oxidising milieu of the photosynthesising chloroplast (Banhegyi and Loewus, 2004; Foyer and Noctor, 2009; Foyer and Shigeoka, 2011; Mano et al., 2004; Noctor, 2006; Noctor and Foyer, 1998). It is also a co-factor involved in the formation of hydroxyproline residues, e.g. during extensin synthesis (Tiainen et al., 2005), and is a precursor for several other plant metabolites (Debolt et al., 2007). It is essential in animals, and must be consumed as 'vitamin C' from mainly plant-based sources by those animals that have lost (e.g. primates) or diminished (Cui et al., 2011) the ability to synthesise ascorbate endogenously. Ascorbate is also synthesised by the protist *Euglena*, and is presumed to serve anti-oxidative protective roles in this organism too (Ishikawa and Shigeoka, 2008).

Ascorbate is the major redox buffer of the plant apoplast (aqueous solution that permeates the cell wall) (Parsons and Fry, 2010; Pignocchi et al., 2006), where it acts as an anti-oxidant, protecting for example against tropospheric ozone (Feng et al., 2010; Polle et al., 1995; Sanmartin et al., 2003; Takahama and Oniki, 1992). Curiously, apoplastic ascorbate may also act as a pro-oxidant by non-enzymically reducing O_2 and Cu^{2+} to H_2O_2 and Cu^+ respectively, thus enabling a Fenton reaction to occur, locally generating apoplastic hydroxyl radicals (Fry, 1998; Kärkönen and Fry, 2006). These, the most highly reactive and shortest-lived of the reactive oxygen species, may cause non-enzymic scission of neighbouring wall-bound polysaccharides (Fry, 1998; Miller and Fry, 2001; Schweikert et al., 2000), thereby contributing a non-enzymic mechanism of wall-loosening during the softening of ripening fruits (Dumville and Fry, 2003; Fry et al., 2001) and the extension of growing cells (Müller et al., 2009; Schopfer et al., 2002). For these and numerous other reasons, the turnover of apoplastic ascorbate and dehydroascorbic 'acid' (DHA; not strictly an acid because it does not ionise) is biologically interesting, and has been explored in the present paper.

Abbreviations: cOxT, cyclic oxalyl threonate; cOxTL, cyclic oxalyl threonolactone; DHA, dehydroascorbic acid; DKG, 2,3-diketogulonate; HVPE, high-voltage paper electrophoresis; *m*OG, electrophoretic mobility relative to that of orange G (m_{OG} = 1.0) and glucose (m_{OG} = 0.0), corrected for electro-endo-osmosis; OG, orange G; OxA, oxalate; OxT, oxalyl threonate; ThrO, threonate.

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Although high ascorbate concentrations are characteristic of rapidly growing tissues, it is intriguing that such tissues also tend to possess high activities of ascorbate oxidase (Lin and Varner, 1991), a cell-wall-localised enzyme that initiates ascorbate catabolism. This may indicate a role for dehydroascorbic acid and/or its further metabolites in the mechanism of cell expansion. Further exploration of this matter is hampered by our lack of detailed knowledge of the ascorbate turnover pathways.

Simpson and Ortwerth (2000) have described non-oxidative routes of ascorbate degradation. However, its major fate *in vivo* is oxidation, first generating DHA. Intraprotoplasmic DHA can be reduced back to ascorbate in the ascorbate–glutathione cycle (Foyer and Noctor, 2011). However, DHA can also be irreversibly degraded *in vivo* (Deutsch, 2000) to products from which ascorbate can no longer be restored. This degradation be initiated either by hydrolysis or by oxidation.

Hydrolysis of DHA irreversibly yields 2,3-diketogulonate (DKG) in a reaction that is highly pH-dependent (Cioffi et al., 2000; Kagawa et al., 1960; Tolbert and Ward, 1982), bicarbonate-promoted (Koshiishi et al., 1998) and possibly catalysed by a plant enzyme (Tewari and Krishnan, 1960). In the absence of H_2O_2, DKG is relatively stable both *in vivo* and *in vitro*, but slowly generates a range of products, including those reported in Green and Fry (2005) as compounds '**C**' and '**E**' and subsequently proposed to be 2-carboxy-L-xylonolactone plus 2-carboxy-L-lyxonolactone (**C**) and 2-carboxy-L-*threo*-pentonate (**E**) (Parsons et al., 2011).

Major oxidative end-products of irreversible DHA degradation, both *in vitro* and in most plant species *in vivo*, are ThrO plus OxA, which are formed by oxidative cleavage of the C-2–C-3 bond (Deutsch, 1998a; Isbell and Frush, 1979). A series of intermediates between DHA and these end-products was reported (Green and Fry, 2005), and it was proposed that the first-formed product of DHA oxidation was cyclic oxalyl L-threonate (cOxT), whose two ester bonds were successively hydrolysed to produce first oxalyl L-threonate (OxT) and finally OxA + ThrO (Green and Fry, 2005). Subsequently, it was found that cOxT, OxT and OxA + ThrO could all be formed, essentially simultaneously, from DHA non-enzymically in the presence of H_2O_2 *in vitro* (Parsons et al., 2011). Only in the presence of apoplastic plant esterases or during very lengthy non-enzymic incubations did appreciable hydrolysis of cOxT → OxT → OxA + ThrO occur. A more accurate pathway was suggested (Parsons et al., 2011) in which DHA was first oxidised to a highly reactive intermediate, proposed to be cyclic-2,3-*O*-oxalyl-L-threonolactone (cOxTL), which was then quickly hydrolysed via any of three routes to the more stable intermediates reported earlier (Fig. 1) (Green and Fry, 2005).

However, important uncertainties still surrounded the identity of some of the intermediary metabolites of DHA oxidation. It was not excluded, for example, that the compound proposed (Green and Fry, 2005) to be cOxT might be oxalic threonic anhydride (Fig. 1), which would resemble cOxT in being a C_6 compound with one ionisable carboxy group and thus have a similar electropho-

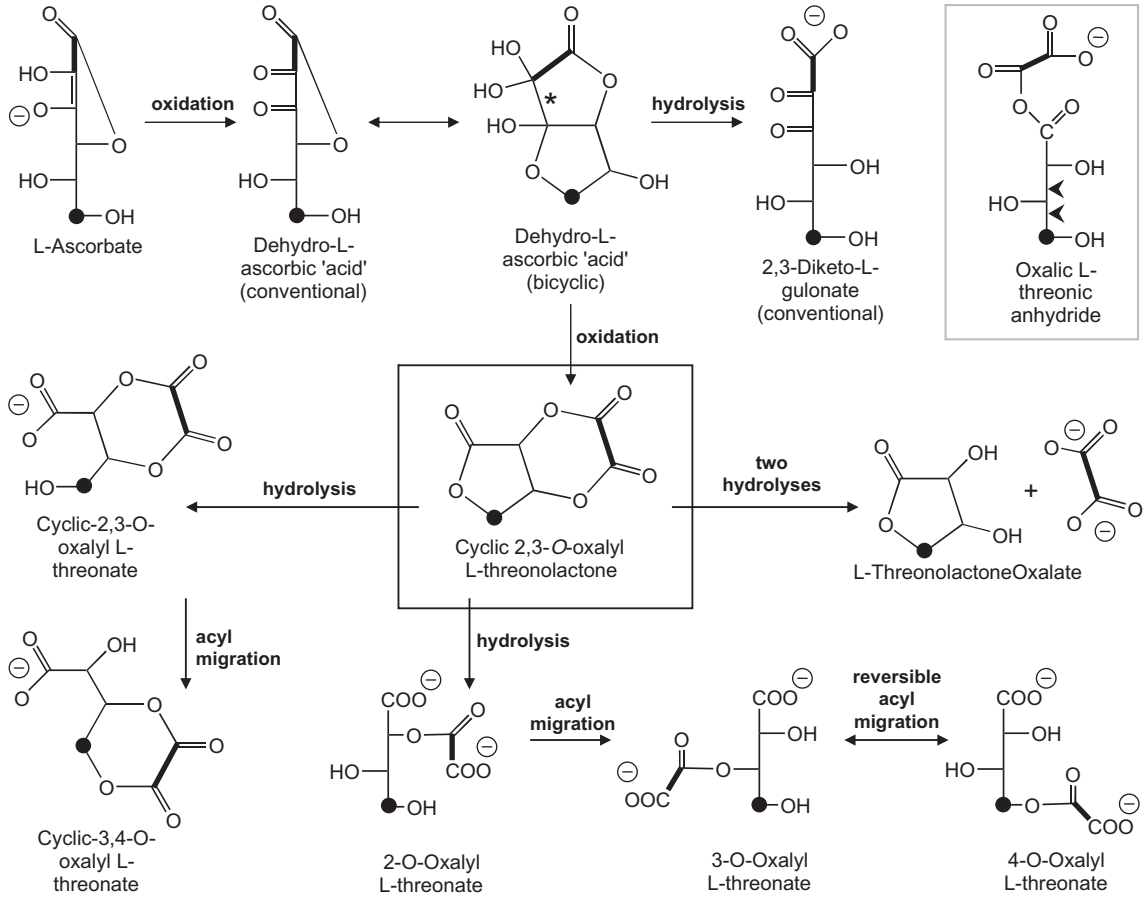

Fig. 1. Proposed structures and interconversion of ascorbate metabolites Ascorbate (top left) is oxidised non-enzymically or by ascorbate oxidase to yield dehydroascorbic acid, which is shown both in its conventional structure and as the more realistic bicyclic structure adopted in aq. soln (Kerber, 2008). DHA is further oxidised by H_2O_2 non-enzymically (and possibly enzymically *in vivo*), in a reaction that cleaves the bond marked '∗'. The product is a highly reactive intermediate proposed (Parsons et al., 2011) to be cyclic-2,3-*O*-oxalyl-L-threonolactone (cOxTL; centre structure), which is subject to any of the three hydrolytic reactions shown, selected stochastically. If not rapidly oxidised, DHA can be hydrolysed to diketogulonate (DKG), which is shown only in its conventional structure, although this too is likely to be hydrated at the 'di-keto' group in aq. soln. Oxalic threonic anhydride is a hypothetical product, not found; ⮜ shows where periodate would cleave this structure. The C–C bond originating from C-1 and C-2 of ascorbate is in bold; the carbon derived from C-6 of ascorbate is indicated by ●.

retic mobility. We have now tested this possibility. Furthermore, it had been assumed that all OxA and ThrO was produced via the oxidation of DHA, whereas an alternative would be that some of the OxA and/or its esters are formed by oxidation of DKG (Kagawa, 1962). We have therefore now directly investigated the oxidation products of DKG. Another possibility not yet explored is that the cOxT or OxT occur as multiple positional isomers, potentially interconvertible for acyl migration. Acyl migration of cOxT had been proposed on theoretical grounds (Green and Fry, 2005) but without experimental evidence. We have now obtained evidence for positional isomers of OxT. Overall in this work *in-vitro* branched pathways of DHA degradation, including both intermediates and end-products, have been compiled which can be used as comparative models for the *in-vivo* fate of these compounds.

Products were analysed by high-voltage paper electrophoresis (HVPE) and HPLC. HVPE has proven particularly valuable in kinetic analyses of low-M_r, unstable organic acids such as ascorbate metabolites. A great advantage of this technique is that short-lived intermediary compounds may be visualised and, in the case of radiolabelled metabolites, accurately quantified even if their identity is uncertain.

2. Results

2.1. Non-enzymic oxidation of ascorbate by H_2O_2: Transient formation of DHA

At pH 6.0, in the absence of deliberately added metal ions, H_2O_2 rapidly oxidised [1-^{14}C]ascorbate (Fig. 2a). Initially, while the [^{14}C]ascorbate concentration was rapidly decreasing, neutral [^{14}C]DHA increased slightly and then persisted for the first hour (Fig. 2a and b). The dose of H_2O_2 supplied was theoretically sufficient for both the complete oxidation of ascorbate to DHA and the complete oxidation of the DHA to products with the oxidation-state of OxA + ThrO (including their esters). Therefore, it was expected that DHA would also diminish, once it was no longer being replenished from ascorbate. In agreement with this, between 60 and 480 min, the [^{14}C]DHA concentration decreased (Fig. 2a and b).

2.2. Non-enzymic oxidation of dehydroascorbic acid by H_2O_2: Absence of oxalic threonic anhydride

Major ^{14}C-labelled downstream products were those previously suggested (Green and Fry, 2005) on the basis of HVPE at pH 6.5 to be cOxT, OxT and free OxA + ThrO (Green and Fry, 2005; Parsons et al., 2011). Very little [^{14}C]DKG was produced under the oxidising conditions used in the present experiments because most of the DHA was oxidised before it could be hydrolysed to DKG.

One of the products of DHA oxidation was a compound previously suggested to be cOxT ('compound **D**' of (Green and Fry, 2005)). On HVPE at pH 3.5, a single spot could be attributed to cOxT (Fig. 2a). Its proposed identity had been based on its electrophoretic mobility at pH 6.5 being approximately as expected (on the basis of Offord's rules (Fry, 2011; Offord, 1966)) for a C_6 compound with one negative charge, and on its hydrolysis to [^{14}C]OxA + ThrO. Of the three conceivable structures of cOxT (2,3-cOxT, 3,4-cOxT and 2,4-cOxT) the two shown in Fig. 1 are the more plausible ones because they lack a 7-membered ring. However, a theoretical alternative structure with the same predicted characteristics is [^{14}C]oxalic threonic anhydride (Fig. 1). As a test of this possibility, a sample of the compound was eluted from an electrophoretogram (similar to that shown in Fig. 2a) and treated with $NaIO_4$, which cleaves the C–C bonds of vicinal diols. $NaIO_4$ is expected to have no effect on any of the possible isomers of cOxT because they are not vicinal diols, but would attack the anhydride's two vicinal diol bonds (dashed lines in Fig. 1). The predicted radioactive product ([^{14}C]oxalic glyoxylic anhydride) would be smaller but still possess a single charge and would therefore migrate faster than the starting material on HVPE at pH 6.5 (Fry, 2011). We observed that, under conditions shown previously to oxidise 4-OxT (Green and Fry, 2005), $NaIO_4$ had no effect on the compound (Fig. 3), which is thus supported as being cOxT.

2.3. Non-enzymic oxidation of dehydroascorbic acid by H_2O_2: Two isomers of oxalyl threonate

Using high-voltage paper HVPE at pH 6.5, Green and Fry (2005) had detected 4-OxT as a major ascorbate oxidation product, whereas 2-OxT and 3-OxT were not reported. Evidence for the identity of 4-OxT included its double negative charge and the oxidation of its vicinal diol group by periodate to a less electrophoretically mobile radioactive product (oxalyl glycollaldehyde). However, Fig. 2b of Green and Fry (2005) shows a small proportion of the radioactivity remaining electrophoretically unaltered during periodate treatment – behaviour expected of 3-OxT because this compound does not contain a vicinal diol group (Fig. 1).

Therefore, to explore further the range of oxalyl esters formed from DHA, we eluted a sample of the putative OxT from a pH 6.5 electrophoretogram similar to that shown in Fig. 1b of Green and Fry (2005) and re-electrophoresed it under different conditions. It was resolved into at least two spots by HVPE at pH 3.5, as was a sample of the ester that had been stored frozen from the 2005 work (data not shown). Thus at least two of the three possible isomers of OxT were present. The time-course for production of these isomers in the presence of H_2O_2 suggested that the faster-migrating one was the first-formed; however, both were present at all time-points analysed (Fig. 2a).

To test whether these isomers were readily interconvertible by acyl migration, we used 2-dimensional HVPE. A sample of the 480-min reaction products shown in Fig. 2a was run by HVPE at pH 3.5; then the lane of resolved products was re-electrophoresed under identical conditions but at right-angles (Fig. 4). As expected, most of the radioactive spots were found along a diagonal, indicating that they had the same mobility during both runs, but in the OxT region there were four spots arranged in a square – behaviour typical of a pair of gradually interconverting isomers (Fry, 1979). In both dimensions, the slower-migrating isomer predominated somewhat. It is assumed to be the previously reported 4-OxT, which owing to the wider separation of the two free carboxy groups would indeed be expected to have a higher pK_a than the 2- or 3-*O*-isomer and thus to migrate slower at pH 3.5 [compare the first pK_a values of oxalic, malonic and succinic acids (HOOC–COOH, HOOC–CH_2–COOH and HOOC–CH_2–CH_2–COOH), which are 1.23, 2.83 and 4.19 respectively; http://research.chem.psu.edu/brpgroup/pKa_compilation.pdf]. It is not known whether the faster-migrating OxT spot in Fig. 2a is 2-OxT, 3-OxT, or a mixture of both; however, 3-OxT is likely to be more stable because it has the two negatively charged carboxy groups further apart than in 2-OxT.

We conclude that in the presence of H_2O_2, DHA is oxidised to at least two rapidly interconverting OxT isomers and at least one isomer of cOxT.

We recently showed that cOxT, OxT and OxA are produced essentially simultaneously, and in a ratio of ~6:1:1, when DHA is oxidised with H_2O_2 (Parsons et al., 2011). It was proposed that this is due to initial oxidation of DHA to a highly unstable intermediate, cOxTL, which may hydrolyse via any of three routes, selected stochastically, to generate the 6:1:1 ratio noted. A time-course of DHA oxidation analysed by HVPE at pH 3.5 to resolve the OxT isomers (Fig. 2a and b) broadly resembled that recorded at pH 6.5 (Parsons

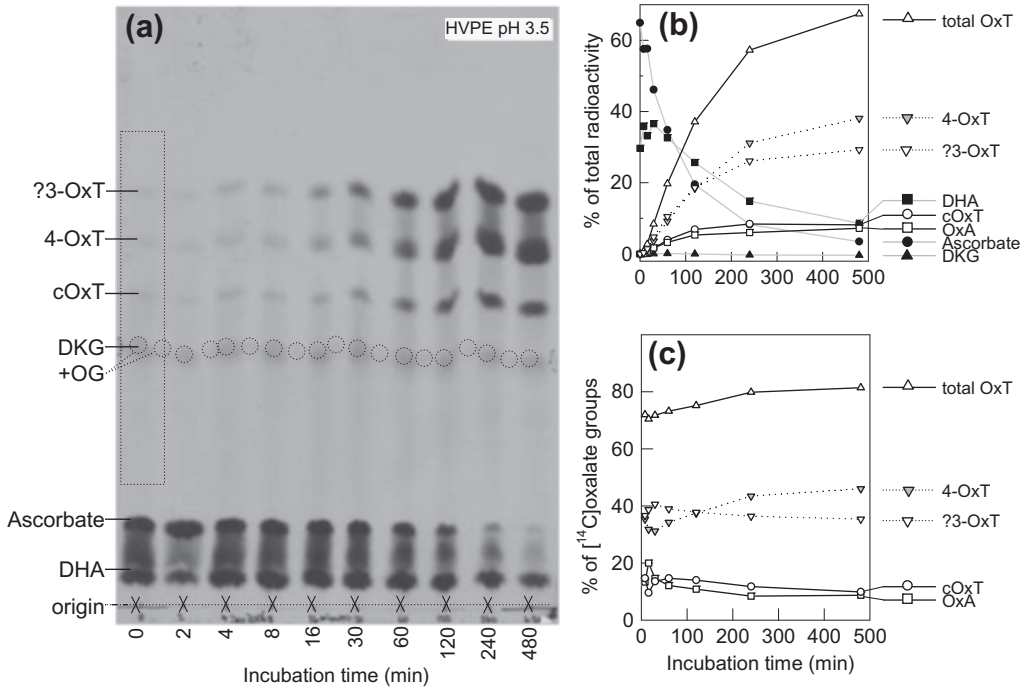

Fig. 2. Kinetics of formation of oxalyl threonate isomers during the *in-vitro* oxidation of [^{14}C]ascorbate under strongly oxidising conditions L-[1-^{14}C]Ascorbic acid (10 mM; 3.2 kBq/μmol) was incubated at pH 6.0 with 2 M equivalents of H_2O_2. At intervals, aliquots (5 μl) were analysed by HVPE in pH 3.5 buffer. (a) An autoradiogram of the electrophoretogram. The marker OG spots are faintly visible (dotted circles). (b) Radioactive zones of the same electrophoretogram were assayed for ^{14}C scintillation counting. The ^{14}C of each metabolite is plotted as a fraction of the total ^{14}C detected at that time point. Except in the cases of ascorbate and DHA, the trace of ^{14}C present at time 0 (*dotted box in a*) has been subtracted from the percentage. The tail of ^{14}C observed between ascorbate and DHA, representing DHA formed from ascorbate *during* electrophoresis, have been taken as [^{14}C]ascorbate. Values for [^{14}C]OxA were obtained from a similar electrophoretogram run at pH 6.5. (c) Relative yields of the various oxalate products.

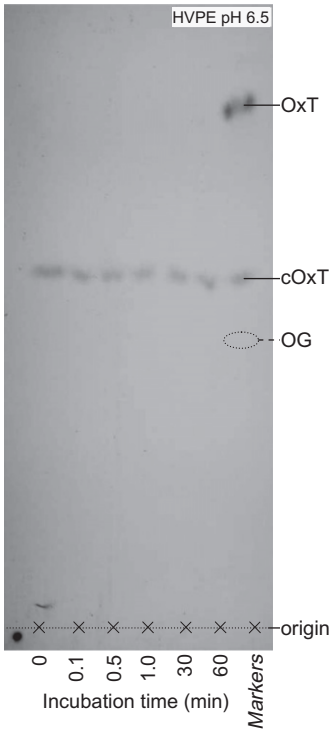

Fig. 3. Identity of the singly charged product formed from ascorbate in the presence of H_2O_2 Distinguishing cyclic oxalyl threonate from oxalic threonic anhydride. A tracer concentration of the monoanionic compound tentatively identified as [^{14}C]cOxT was incubated with $NaIO_4$ for the times indicated and then subjected to HVPE at pH 6.5; an autoradiogram is shown. OG = Orange G (coloured marker).

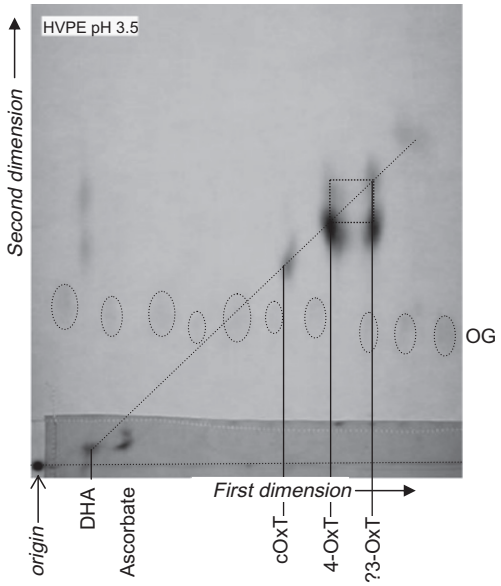

Fig. 4. Interconversion of oxalyl threonate isomers A portion of the 480-min products shown in Fig. 2a was analysed by 2-dimensional HVPE (pH 3.5 in both dimensions). Additional spots of OG were pipetted onto the lane of separated products after the 1st dimension run. Dotted circles, final positions of orange G spots.

et al., 2011), supporting the simultaneous production of cOxT and OxT during oxidation by H_2O_2. [OxA was difficult to detect on HVPE at pH 3.5, probably because it was pptd by traces of Ca^{2+} present in the paper.] The ratios suggested that the faster-migrating 3(?)-OxT

was formed slightly earlier than 4-OxT (Fig. 2d) – as expected since the oxalyl group originates from the C-1 end of DHA (Fig. 1).

We conclude that, under non-enzymic conditions, H_2O_2 oxidises DHA to give, essentially simultaneously, cOxT, OxT and free OxA, with the OxT somewhat more slowly setting up an equilibrium mixture of at least two positional isomers.

2.4. Fate of non-radioactive DHA in presence of H_2O_2

Although ascorbate and DHA gave negligible DKG accumulation under strongly oxidising conditions, it might be suggested that DKG was formed transiently and then quickly broken down to different products. It was also possible that DHA might give products lacking the original C-1 atom and thus undetectable radiochemically. To test more critically the individual contributions of DHA and DKG to the formation of end-products under strongly oxidising conditions, we examined the fate of highly purified, non-radioactive DHA and, separately, DKG.

We confirmed that when pure DHA was treated with excess H_2O_2, very little DKG accumulated but OxT (both isomers) and cOxT appeared rapidly and almost simultaneously (Fig. 5). At the pH used during oxidation in this experiment (pH 6.0), the cyclic ester was gradually hydrolysed to the simple esters, and some free ThrO was also formed, indicating that the simple esters were also gradually hydrolysed. No novel products were detected (comparable to compounds **H** and **J** mentioned below).

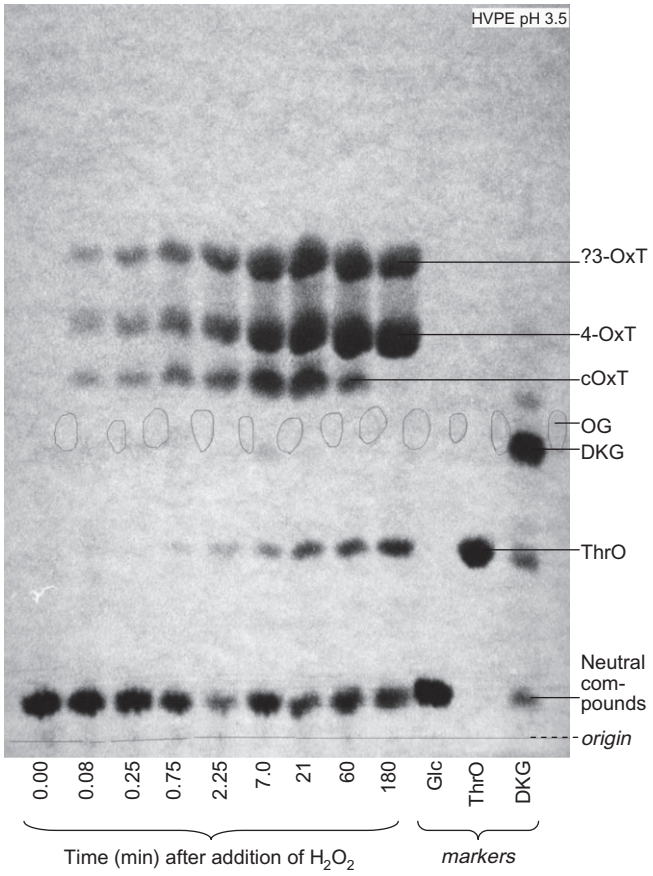

Fig. 5. Oxidation of purified dehydroascorbic acid under strongly oxidising conditions by H_2O_2 DHA (20 mM) was incubated at pH 6.0 with 2 mol eq H_2O_2. After the indicated times, 20-µl aliquots were subjected to HVPE at pH 3.5 and stained with $AgNO_3$. OG (pencilled) was loaded between each sample; it washes out of the paper during $AgNO_3$ staining.

2.5. Fate of non-radioactive DKG in presence of H_2O_2

We confirmed that purified DKG was relatively long-lived in weakly oxidising conditions (in the presence of dissolved O_2 but with no obvious mechanism for endogenous H_2O_2 production) (Fig. 6a). However, when incubated under strongly oxidising conditions (with excess H_2O_2) and analysed by HVPE at pH 2.0, DKG ($m_{OG} \approx 0.23$) was gradually converted to free ThrO ($m_{OG} \approx 0.03$) plus two unknowns: a transient 'compound **H**' ($m_{OG} = 0.12$) and a stable 'compound **J**' ($m_{OG} = 0.73$) (Fig. 6b).

Compound **H** co-migrated with DKG on HVPE at pH 3.5 and with ThrO at pH 6.5 respectively; however, HVPE at pH 2.0 resolved them. The timing of the appearance/disappearance of compound **H** (Fig. 6b) suggested that it turned over to form free ThrO. We confirmed that **H**, when eluted from an unstained electrophoretogram, was indeed further oxidised by H_2O_2 to ThrO but not to compound **J** (Fig. 6c). Compound **H** is unidentified but was shown not to be 2-carboxy-L-pentonolactone (compound **C**; mention this in Intro) or 2-carboxy-L-pentonate (**E**) (Fig. 6a and b), or xylonate (data not shown).

The compound **J** spot approximately co-electrophoresed with OxT at pH 2.0, but the majority of the material in this spot differed from authentic OxT in susceptibility to alkaline hydrolysis (Fig. 7). This was revealed by HPLC, which was preferred over HVPE in this case because free OxA does not stain well with $AgNO_3$. Authentic OxT gave a sharp peak on HPLC (retention time 10.0 min) and was, as expected, completely hydrolysed by a 2-h treatment in 0.1 M NaOH (in which OxT has a half-life of $\sim$ 1 min; data not shown) (Fig. 7a); in contrast, eluted compound **J** gave a broader HPLC peak (retention time $\sim$10.15 min) and was only partially hydrolysed to OxA, leaving a sharper peak of residual alkali-resistant material with a retention time of 10.25 min (Fig. 7b). Thus, the 'compound **J**' spot was probably accompanied by a trace of OxT, but was mainly a distinct, alkali-resistant, unidentified substance. Like **H**, **J** was shown not to be xylonate, 2-carboxy-L-pentonate or its lactone. Compound **J** gave no spot in the 2-carboxy-L-pentonate region on HVPE at pH 6.5 or 2.0 (data not shown); and although **J** ran close to 2-carboxy-L-pentonolactone at pH 2.0, it failed to convert to 2-carboxy-L-pentonate on treatment with NaOH (data not shown) and was thereby distinguished from 2-carboxy-L-pentonolactone.

In conclusion, purified DKG did not give appreciable yields of 2-carboxy-L-pentonate or its lactone under strongly oxidising conditions, being degraded instead to compounds **H** and **J**. Since **H** and **J** were not formed during ascorbate or DHA degradation, we conclude that DKG is not even a transient intermediate in the degradation of DHA under strongly oxidising conditions.

3. Discussion

3.1. Range of ascorbate degradation pathways and products

There have been several previous studies of the *in-vitro* breakdown of ascorbate and DHA (Deutsch, 1998a,b, 2000; Isbell and Frush, 1979), which are relevant to our understanding of the metabolic fate and roles of apoplastic ascorbate. However, the broad metabolic landscape of ascorbate turnover had remained rather confusing. Recently, a forked pathway of ascorbate degradation was proposed that included several novel compounds and enzyme activities (Green and Fry, 2005; Parsons et al., 2011). Both branches of the forked pathway are initiated by ascorbate → DHA oxidation. The two principal branches differ in the subsequent fate of the DHA: oxidation *versus* hydrolysis. These reactions, not recorded in detail in previous studies, were discovered by HVPE, an excellent method for resolving organic anions. We have now further applied

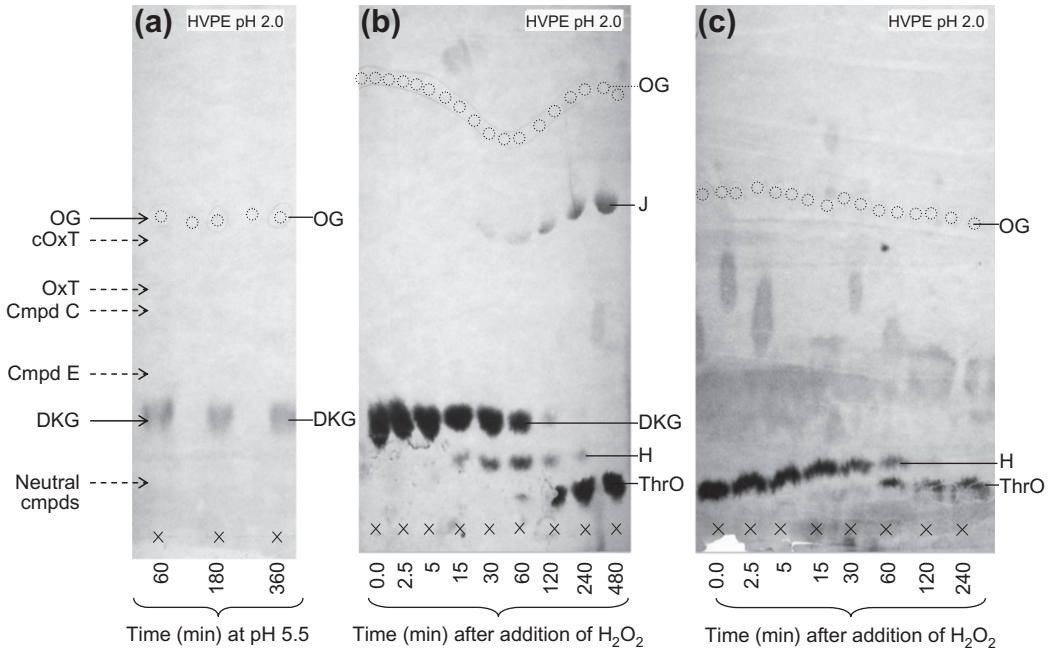

Fig. 6. Oxidation of purified diketogulonate under strongly oxidising conditions by H_2O_2 DKG was incubated with or without excess H_2O_2. After the indicated times, 20-μl aliquots were subjected to HVPE at pH 2.0 and stained with $AgNO_3$. × = origin. Dotted circles indicate the positions of OG added within and between each radioactive sample. (a) Control: non-radioactive DKG (25 mM) was incubated in MES buffer, pH 5.5. The approximate positions expected of some potential products, relative to DKG and OG, are indicated at left (dashed lines). (b) Non-radioactive DKG (60 mM) was incubated in 1 M H_2O_2 (pH ≈ 4.5). Samples were treated with catalase, frozen and then electrophoresed. (c) Compound H was eluted from an electrophoretogram similar to that shown in (b), diluted to ~10 mM and treated with 0.5 M H_2O_2 at pH 4.5. Cmpd C, 2-carboxy-L-*threo*-pentonolactones; Cmpd E, 2-carboxy-L-*threo*-pentonate; H, J, new unknowns.

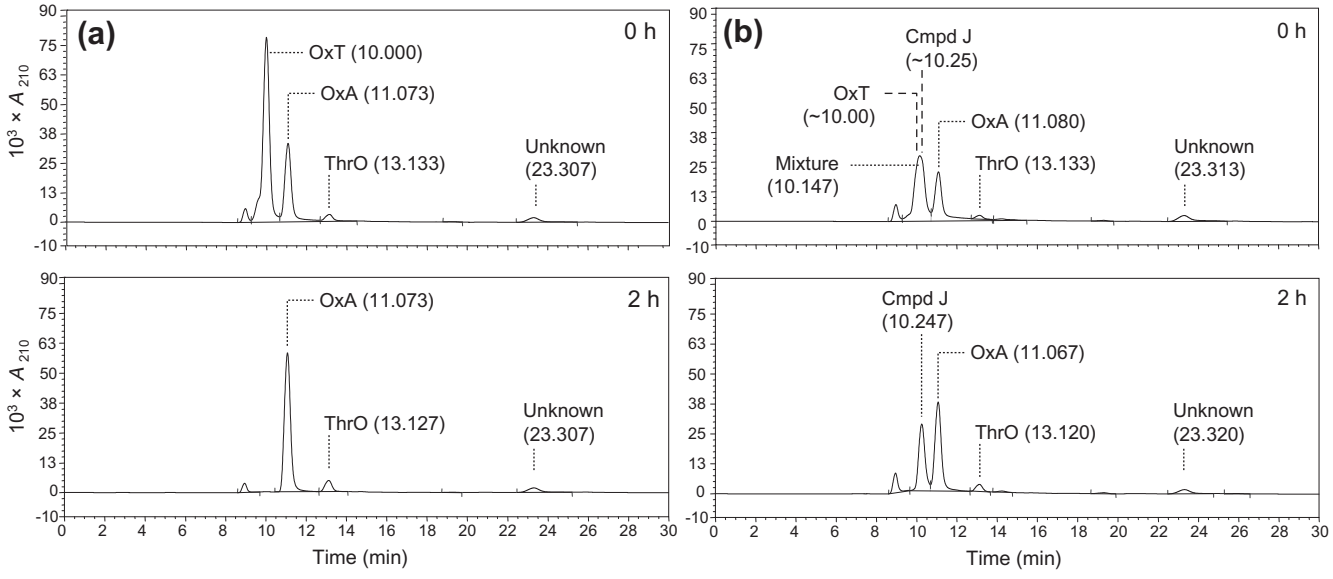

Fig. 7. Comparison of the alkaline hydrolysis of oxalyl threonate and compound J(a) OxT and (b) a mixture of OxT plus compound J (a product of DKG with similar electrophoretic mobility to that of OxT; see Fig. 6b) was treated with 0.1 M NaOH for 0 or 2 h. For '0 h', acetic acid was added immediately before the NaOH; total final Na^+ and acetate were equal in both samples. Products were analysed by HPLC on a Rezex ROA organic acid anion exclusion column. Peaks are labelled with their proposed identity and retention time (minutes).

this technique, using different HVPE buffers that alter the metabolites' ionisation, to monitor the breakdown of ascorbate and DHA under various conditions mimicking the plant apoplast in various respects. By applying HVPE to both ^{14}C-labelled and non-radioactive substrates and intermediates, we have attempted to give a balanced idea of what may occur *in vivo*. Building this model of ascorbate breakdown *in vitro* will help to identify the repertoire of biologically relevant products and reaction sequences.

3.2. The DHA oxidation branch

Both branches of apoplastic ascorbate degradation begin with its oxidation to DHA. This step was very rapid under strongly oxidising conditions in the presence of added H_2O_2.

Under these conditions, DHA was oxidised further. Recent data suggest that H_2O_2 oxidises DHA to a reactive intermediate, cOxTL, which generates almost simultaneously three alternative end-

products – OxT, cOxT and free OxA in a ratio of ~6:1:1 (Parsons et al., 2011). According to the scheme proposed, early-formed free OxA would be accompanied by threonolactone (not distinguishable electrophoretically from DHA), which would itself then delactonise to free ThrO. This interpretation may account for the slight lag observed in free ThrO formation (Fig. 5).

In previous work, we have treated OxT as a single compound. However, it was shown for the first time in the present work to comprise a mixture of at least two rapidly and non-enzymically interconverting isomers: 4-OxT and a second pool concluded to be 3-OxT (possibly also with some 2-OxT). The HVPE results gave some indication that the latter pool appeared prior to the 4-OxT, as would be expected if it arose from cOxTL (Fig. 1).

3.3. The DHA hydrolysis branch

When oxidation is minimised, buffered DHA solns undergo appreciable hydrolysis to DKG. We therefore also explored the fate of the DKG. In previous studies of DKG metabolism in various systems (Deutsch, 1998b; Kagawa, 1962), the products were found to be highly dependent on reaction conditions. However, the compounds produced in the present work were different from those, such as L-xylonate or L-lyxonate, previously reported in mammalian systems (Deutsch, 1998b) or *in vitro* (Kagawa, 1962).

The fate of DKG depended on the oxidative environment. In the presence of dissolved O_2, but no added metal ions, DKG was stable for long periods (Fig. 6a). In the presence of living plant cells, 2-carboxy-L-*erythro*-pentonate and its monolactones were formed (Parsons et al., 2011). Finally, under strongly oxidising conditions (with added H_2O_2), we show here that DKG was oxidised to a stable compound (**J**) plus an unstable one (**H**) which itself was finally further oxidised to free ThrO.

It is not clear whether compounds **H** and **J** would form during ascorbate/DHA metabolism *in vivo*. This is because (a) appreciable concentrations of their precursor, DKG, are formed only when DHA oxidation is minimised and thus an appreciable proportion of the DHA has time to be hydrolysed, whereas (b) conversion of DKG to **H** and **J** requires a highly oxidising medium. These two conditions might be mutually exclusive, unless the apoplastic DKG could either be stored until the apoplast became more oxidising or else be transported into a more oxidising cellular compartment.

Although we have not fully identified compounds **H** and **J**, we nevertheless note some of their interesting properties:

3.3.1. The nature of compound *H*

ThrO has been reported as a product of DKG decarboxylation in the presence of H_2O_2. Various C_5 decarboxylation products of DKG have been found *in vivo* in mammalian systems, such as xylonate and lyxonate (Kagawa et al., 1960). Deutsch (1998a) found an intermediate during the formation of ThrO from DKG with H_2O_2 *in vitro*, which had the correct *m*/*z* to be 2-keto-L-xylonate and had similar precursor–product properties to our compound **H**. The structure of 2-keto-L-xylonate is indeed the simplest predictable product of the oxidative decarboxylation of DKG. The intermediary nature of this compound and the similarity of the reaction conditions and response to H_2O_2 compared to those used by Deutsch (1998a) suggest that compound **H** may be the same intermediate compound. Further work is required, however, because 'DKG' can theoretically adopt at least six hemiketal ring forms (α- and β-2,5-furanose; α- and β-2,6-pyranose; and α- and β-3,6-furanose), and of these it is likely that the 3,6-furanose forms are the most susceptible to oxidative decarboxylation because only they possess a free 2-oxo-carboxylate [R–(C=O)–COO^-] group. The initial product of the action of H_2O_2 on 'DKG' may thus be a 2,5-furanose hemiketal form of 2-ketoxylonate, which would not be oxidatively decarboxylated until its ring had opened during gradual mutarotation.

3.3.2. The nature of compound *J*

Future work, for example involving HPLC purification, MS and NMR, will be necessary for identification of compound **J.** Its immediate precursor is also still uncertain. **J**, a stable product of DKG, had an unusually high electrophoretic mobility at pH 2.0 (Fig. 6), comparable to that of OxT and much higher than that of threarate (a simple C_4 dicarboxylate). It therefore had at least one highly acidic anionic group. Small amounts of **J** had started to be formed by 30 min (Fig. 6b), at which time there was no detectable free ThrO, suggesting that ThrO was not the precursor of **J**. However, curiously, the majority of **J** was formed after 240 min, by which time ThrO was the only potential substrate still available, most of the DKG and **H** having been consumed. This apparent discrepancy remains unexplained.

4. Conclusions

This work has further defined the range of metabolites and branching pathways stemming from vitamin C under oxidising and slightly acidic conditions resembling those of the plant apoplast. It remains to be seen what proportion of a plant cell's endogenous ascorbate is secreted into the apoplast and subjected to these pathways, and which of the steps may be enzymically catalysed or non-enzymic *in vivo*. Apoplastic ascorbate and DHA may be sacrificially oxidised in the presence of oxidising pollutants such as ozone and reactive oxygen species generated by ultraviolet or other radiation, thus protecting the protoplasm (Sanmartin et al., 2003). It is also possible that some of the specific ascorbate metabolites described here serve signalling roles, triggering other defence responses.

5. Experimental

5.1. Chemicals

Ascorbic acid, DHA, H_2O_2 and catalase were from Sigma Chemical Co. Solid L-[1-^{14}C]ascorbic acid (0.52 or 0.407 MBq/μmol) from GE Healthcare was dissolved in water, aliquotted and stored at −80 °C until required.

Commercial DHA was dissolved at 2 M in dimethylformamide and stored for up to 3 months at −80 °C. To remove anionic contaminants, immediately before each experiment, we diluted a small aliquot into 0.5 ml water and immediately passed the soln through a 0.5-ml bed-volume column of Dowex-1 × 4 100–200 mesh (acetate$^-$ form), followed by 2 ml of 2 mM pyridine-acetate buffer (pH 4.0, the pH at which DHA is most stable). The eluate was quickly used in oxidation/hydrolysis experiments.

For DKG preparation, solid ascorbic acid was dissolved at 0.12 M in 0.36 M potassium iodate (Kagawa, 1962). After 5 min, 1 M KOH was added gradually until the soln became colourless. DKG was precipitated by addition of eight volumes of cold ethanol (−20 °C) and filtered, and the crystals were rinsed in ethanol, dried, and stored solid at −80 °C.

5.2. Oxidation of ascorbate and its metabolites with H_2O_2

All incubations were performed at 20 °C in open tubes (thus with dissolved O_2). For ascorbate/DHA oxidation experiments with added H_2O_2, the reaction mixture (500 μl) contained 10–20 mM substrate [ascorbic acid (including 16 kBq L-[1-^{14}C]ascorbate) or purified DHA], 80 mM pyridinium acetate buffer, pH 6.0 (i.e., 80 mM pyridine, pH adjusted to 6.0 with acetic acid), and 2 mol eq

H_2O_2 (added last). For other oxidation experiments, self-buffered DKG (~60 mM) or compound **H** (~10 mM) in water (pH ≈ 4.5) were treated with H_2O_2 (to 1.0 M and 0.5 M H_2O_2 respectively). With all reaction mixtures containing exogenous H_2O_2, 20-µl aliquots were taken at intervals, treated with 1–4 U catalase for ~10 s, frozen in liquid N_2 and subjected to HVPE.

5.3. Alkaline hydrolysis of compound *J*

Additional tracer-level aliquots of ^{14}C-labelled compound **C**, and non-radioactive OxT or compound **J** were alkali-hydrolysed in 0.1 M NaOH at 20 °C for 2 h and then adjusted to pH 4.7 with acetic acid. Controls were treated with the same amounts of acetic acid and NaOH but in reverse order so that the sample was never at an alkaline pH. Radioactive and non-radioactive products were analysed by HVPE and HPLC respectively.

5.4. Periodate oxidation of cOxT

[^{14}C]cOxT (tracer concentration), buffered in 250 mM formate (NH_4^+, pH 3.7), was oxidized with $NaIO_4$ (10 mM). Reactions were stopped by addition of ethane-1,2-diol to 1% (v/v). Products were analysed by HVPE.

5.5. High-voltage paper electrophoresis (HVPE)

Samples were dried on Whatman No. 1 paper and electrophoresed in a volatile buffer at pH 6.5 (acetic acid–pyridine–water, 1:33:300 by volume, containing 5 mM EDTA), pH 3.5 (pyridine–acetic acid–water, 1:10:189 by volume) or pH 2.0 (formic acid–acetic acid–water, 1:35:355 by volume), usually at 3.0 kV for 40 min (Fry, 2011). The papers were cooled to 20–25 °C with toluene (pH 6.5) or white spirit (pH 3.5 and pH 2.0) during the run. Orange G (OG) was used as an internal reference marker and/or was loaded between samples. Non-radioactive compounds were stained with $AgNO_3$ (Fry, 2000). ^{14}C-Labelled compounds were detected by autoradiography and quantified by scintillation counting. Samples required for further analysis were isolated by elution from electrophoretograms in water.

5.6. Analysis of compounds by HPLC

Organic acids (40 µl injection volume) were separated on a Dionex HPLC system (Dionex, Sunnyvale, CA, USA) with a 300 × 7.8-mm Rezex ROA organic acid anion exclusion column (Phenomenex Torrance, CA, USA). Samples were eluted isocratically in 47 mM H_2SO_4 at 0.5 ml/min. Compounds were detected by UV-absorbance at 210 nm. Catalase was removed from samples prior to analysis by HPLC with Amicon ultra 10 k centrifugal devices (Millipore corporation) used according to manufacturer's instructions.

5.7. Detection of radioactivity

Radioactive compounds on paper electrophoretograms were detected by autoradiography on Kodak BioMax MR-1 film. In some cases, the radioactive areas of the paper were cut out and ^{14}C was quantified by scintillation counting in 'OptiScint HighSafe'.

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