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Short- and Long-Term Effects of Dehydroascorbate in *Lupinus albus* and *Allium cepa* Roots

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Administration of 1 mM dehydroascorbate (DHA) results in a rapid and large increase in cellular ascorbate (AA) content in both *Lupinus albus* L. and *Allium cepa* L. root tips. Uptake of DHA from the medium occurs at a high rate within 10–12 h of incubation, and is slowed down thereafter. In the first few h, DHA reduction to AA is apparently correlated to GSH depletion and slightly higher DHA reductase activity. DHA incubation also seems to induce new GSH synthesis. Longer DHA incubation (24 h) affects root growth by inhibiting cell proliferation. At this stage, an apparently generalised oxidation of SH-containing proteins is observed in DHA-treated roots. Treatment with 1 mM L-galactono-γ-lactone, the last precursor of AA biosynthesis, results in an increase in AA content similar to that obtained with DHA, but stimulates growth and affects the redox state of SH-containing proteins in the opposite way. A possible multi-step mechanism of DHA reduction/removal is suggested and the hypothesis that DHA inhibits cell cycle progression by affecting the redox state of SH-containing proteins is discussed.

Key words: *Allium cepa* L. — Ascorbate — Cell cycle progression — Dehydroascorbate — *Lupinus albus* L. — Root growth.

Abbreviations: AA, ascorbate; BSO, L-buthionine-S,R-sulfoximine; DHA, dehydroascorbate; GL, L-galactono-γ-lactone; mBBr, monobromobimane.

Introduction

Dehydroascorbate (DHA) is formed in cell metabolism as a consequence of ascorbate (AA) utilisation (Arrigoni 1994), via spontaneous disproportionation of ascorbate free radical (Bielski 1981). Early reports identified a tight correlation between the AA/DHA ratio and the growth of auxin-stimulated isolated pea internodes (Marrè and Laudi 1955, Marrè and Arrigoni 1957, Marrè et al. 1957). The authors showed that the AA/DHA ratio was high in actively growing tissues, and lower when the growth rate decreased. It was suggested that DHA could be responsible for growth inhibition, since it inhibited the activity of pyridine nucleotide dependent-dehydrogenases measured in cell-free extracts (Marrè et al. 1955). Recently, it has been shown that DHA at low concentrations inhibits the activity of several enzymes in vitro, including malate dehydrogenase, fructose 1,6-bisphosphatase (Morell et al. 1997) and hexokinase (Fiorani et al. 2000). Moreover, root growth inhibition has been observed in response to DHA administration in vivo (Cordoba-Pedregosa et al. 1996), whereas an increase in AA content stimulates growth (Cordoba-Pedregosa et al. 1996, Arrigoni et al. 1997).

It has been reported that DHA is accumulated in the apoplast as a consequence of oxidative stress (Castillo and Greppin 1988, Plöchl et al. 2000), and Horemans and colleagues (Horemans et al. 1997, Horemans et al. 1998) suggested that apoplastic DHA is efficiently transported inside the cells. Recent data show that DHA transport results in an increased intracellular AA content in cultured cells (de Pinto et al. 1999, Potters et al. 2000), although the timing and mechanism of such AA accumulation has not been studied in detail. These observations induced us to study the effects of DHA administration to intact roots, in order to investigate the mechanism of both DHA transport/reduction and DHA-induced growth inhibition. Data presented here show that (i) DHA is not accumulated in the root, since it is transported inside the cells and rapidly reduced to AA by means of enzymic and non-enzymic mechanisms; (ii) although AA content is markedly increased in DHA-treated roots, the rate of cell division is slowed down, resulting in root growth inhibition; and (iii) cell cycle inhibition by DHA could be correlated with the oxidation of several thiol-containing proteins.

Results

DHA treatment resulted in a large rise in AA content in root tips of both *Lupinus albus* (Fig. 1) and *Allium cepa* (Fig. 2). AA content markedly increased in the first few h of incubation and reached a steady state after 12 h. At 48 h, incubation of lupin with 1 mM DHA raised intracellular AA content even slightly more than 1 mM GL, the immediate AA precursor in its biosynthetic pathway, the latter inducing a linear increase in AA content (Fig. 1).

DHA content in the cells was substantially unchanged in

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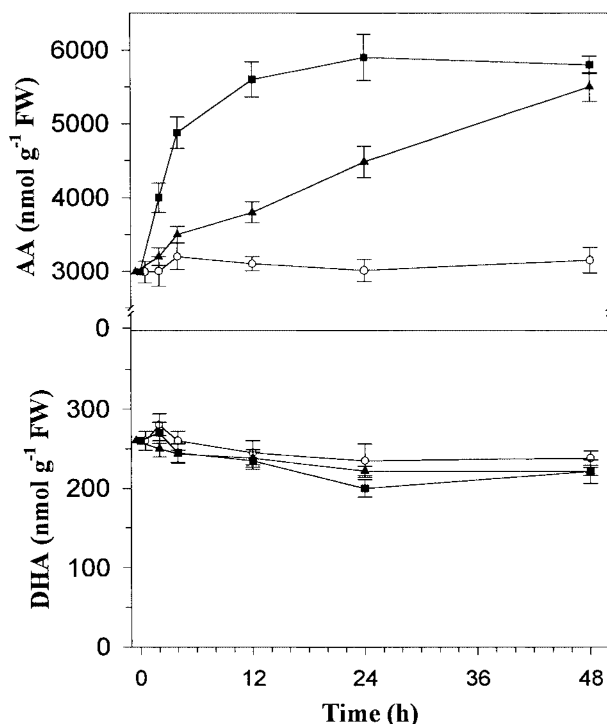

Fig. 1 AA and DHA contents in seedlings of *L. albus* treated with DHA or GL. Root tips (3 mm) were excised from control plants (open circles), and from plants treated with 1 mM DHA (closed squares) or 1 mM GL (closed triangles). Means of four experiments±SE.

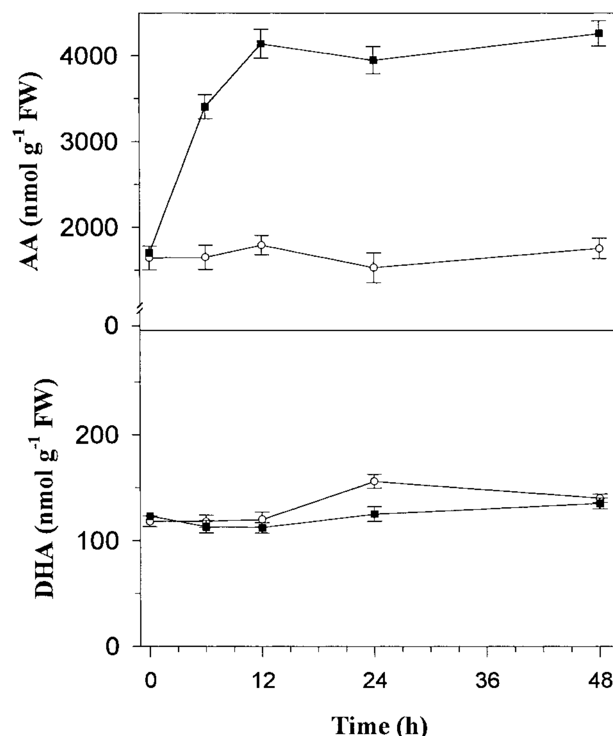

Fig. 2 AA and DHA contents in *A. cepa* roots treated with DHA. Roots were grown either in water (open circles) or in the presence of 1 mM DHA (closed squares). Root tips (3 mm) were excised and analysed. Means of four experiments±SE.

both lupin and onion (Fig. 1, 2). AA peroxidase activity increased in both DHA- and GL-treated samples and was positively correlated with AA (Table 1).

AA accumulation in DHA-treated roots induced us to investigate DHA uptake by measuring the decrease in DHA content in the incubation medium either in the presence or the absence of growing lupin roots (Fig. 3). The decrease in DHA content due to spontaneous decay of DHA in the solution maintained at pH 5.8 was linear, whereas, in the presence of plants, the rate of DHA decrease was higher during the first 10 h and qualitatively consistent with the observed intracellular AA increase. Thereafter, DHA content in the medium decreased at the same rate as spontaneous decay. This behaviour was not due to DHA depletion in the incubation medium. In fact, when plants incubated for 12 h in DHA were transferred to fresh DHA solution, the rate of DHA disappearance in the medium

Table 1 AA peroxidase activity in *L. albus* and *A. cepa* root meristem

Treatment	AA peroxidase (nmol AA oxidised min^{-1} (mg protein^{-1}))	
	L. albus	*A. cepa*
Control	78±6	88±9
1 mM DHA	141±12	150±16
1 mM GL	114±10	132±27

Samples were analysed after 24 h of incubation. Mean of three experiments ± SE.

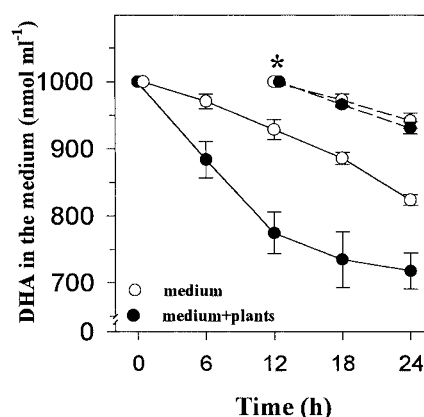

Fig. 3 DHA content in the growth medium. DHA was measured in a 200 ml beaker either in the absence of plants (spontaneous decay, open circles) or in the presence of 20 *L. albus* seedlings (spontaneous decay + root uptake, closed circles). In parallel experiments, plants were transferred to a new medium after 12 h of incubation (asterisk) and the rate of DHA disappearance measured over 12 more h (dashed line). Means of three experiments±SE.

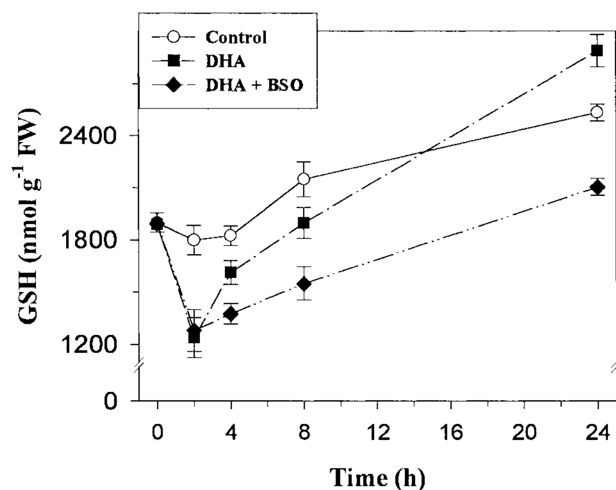

Fig. 4 Effect of different treatments on GSH content in *L. albus* roots. Root tips (3 mm) were excised from control plants (open circles), and from plants treated with 1 mM DHA (closed squares) or 1 mM DHA + 100 μM BSO (closed diamonds). Means of three experiments±SE.

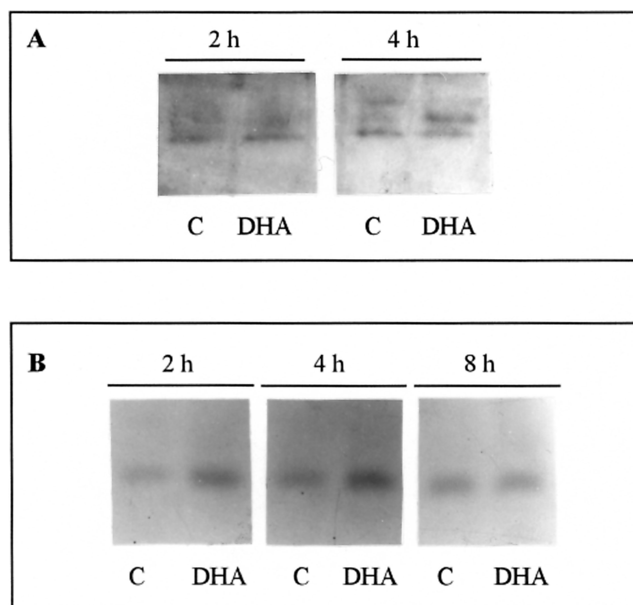

Fig. 5 Effect of DHA on enzyme activities detected by native-PAGE and activity staining. Panel A: GSSG reductase activity in control (C) and DHA-treated samples after 2 and 4 h of treatment. Panel B: DHA reductase activity in controls (C) and DHA-treated samples after 2, 4 and 8 h of incubation.

was the same irrespective of the presence of plants (Fig. 3), suggesting that DHA was not taken up anymore.

DHA can be reduced by enzymatic and non-enzymatic mechanisms, both mainly (but not exclusively) depending on GSH availability (Meister 1992). In order to evaluate GSH involvement in the observed DHA reduction, we measured GSH content in DHA-treated samples.

In lupin roots, GSH content decreased by approximately 35% within 2 h of DHA incubation (Fig. 4). Thereafter, GSH content increased and was 15% higher than that in controls at 24 h. Administration of the GSH biosynthesis inhibitor BSO showed that the increased GSH derived from de novo synthesis. This was also confirmed by long-term measurements in onion roots (Table 2). The content of GSSG was 109±12 nmol (g FW)$^{-1}$ (mean value of three experiments±SE) at the beginning of DHA incubation, increased to 333±27 after 2 h (GSH/GSSG ratio 5.3), then decreased to values similar to controls (between 90 and 120 nmol (g FW)$^{-1}$) at longer time points (not shown).

In lupin root, glutathione reductase activity was apparently not affected by DHA treatment as evidenced by native-PAGE and activity staining after 4 h of DHA incubation, with an apparent change in isoform pattern (Fig. 5A). The activity of

Table 2 GSH content in *A. cepa* in response to different treatments

Treatment	GSH (μmol (g FW)$^{-1}$)
Control 8 h	1.24±0.03
1 mM DHA 8 h	1.18±0.04
Control 24 h	1.55±0.02 [a]
1 mM DHA 24 h	1.74±0.039 [b]
1 mM DHA + 100 μM BSO 24 h	1.08±0.026 [c]

Mean of three experiments ± SE. Statistically significant differences in the Student's *t* test between comparable treatments (P<0.05) are indicated by different letters. BSO, L-buthionine-(S,R)-sulfoximine.

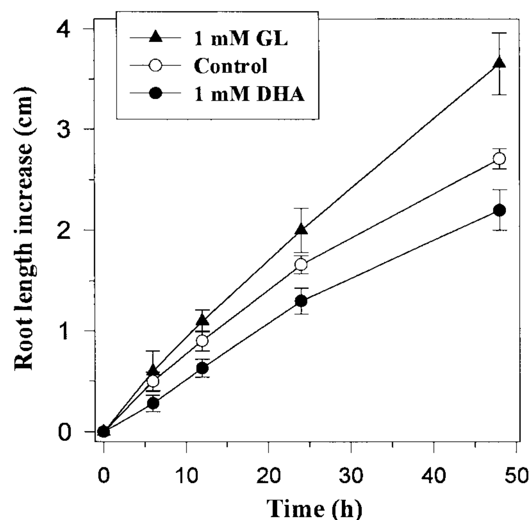

Fig. 6 Effect of DHA and GL on root growth of *L. albus*. After 72 h of germination, seedlings with roots of similar length (1.5 cm) were incubated in the different media. Increase in root length was measured at different time points. Means±SE of five experiments are given.

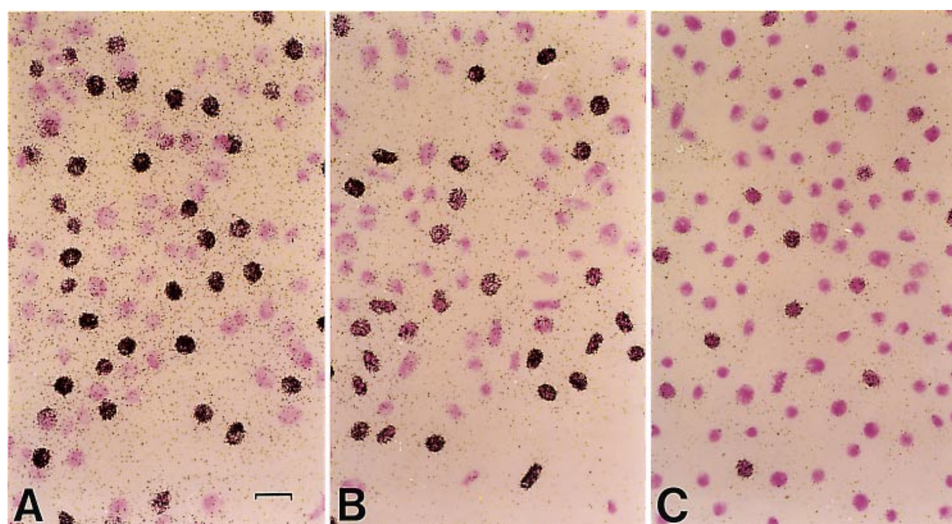

Fig. 7 Effect of DHA on thymidine incorporation in the meristematic nuclei of onion roots. Roots incubated either in distilled water (control) or in 1 mM DHA for different times were exposed to [methyl-^{3}H]thymidine for 1 h; excised tips (1 mm) were prepared as Feulgen squashes and subsequently autoradiographed. The drastic drop in the number of labelled nuclei is evident as well as the high level of chromatin compactness following DHA treatment. A, control 8 h; B, treatment with 1 mM DHA for 12 h; C, treatment with 1 mM DHA for 24 h. Controls at 12 and 24 h did not significantly differ from control at 8 h (see Table 3), and have been omitted. Scale bar: 25 µm.

GSH-dependent DHA reductase markedly increased between 2 and 4 h of DHA incubation, as compared to controls (Fig. 5B). Controls themselves showed a slight increase in DHA reductase activity between 2 and 4 h of incubation in distilled water.

It is well known that a positive correlation exists between AA content, cell division and expansion (see Introduction). However, although DHA treatment induced a large increase in AA content, root growth was inhibited by 1 mM DHA, whereas 1 mM GL treatment induced both an increase in AA content and root growth stimulation in *L. albus* (Fig. 6). Similar results have previously been reported by Cordoba-Pedregosa et al. (1996) in *A. cepa* roots.

DHA-induced root growth inhibition was mainly due to a decrease in the rate of cell proliferation. The mitotic index in onion root meristem dropped from 9.18±0.5 to 4.38±0.3 after 24 h of incubation in 1 mM DHA. A similar decrease in the mitotic index could be observed in lupin root meristem (not shown). The fraction of rapidly cycling cells in onion roots was investigated by pulse with tritiated thymidine. Thymidine incorporation was progressively slowed down by DHA treatment up to 24 h (Table 3; Fig. 7) and virtually blocked at 48 h (not shown), whereas it appeared to be constant in controls (Table 3).

Table 3 Labelling index with [methyl-^{3}H]thymidine of excised tips (1 mm) of onion roots incubated in distilled water (control) or in 1 mM DHA for indicated times

Treatment	Time		
	8 h	12 h	24 h
Control	29±2	24±3	23±3
1 mM DHA	30±3	23±4	9±3

For each treatment, five slides were scored. Values ± SE are given.

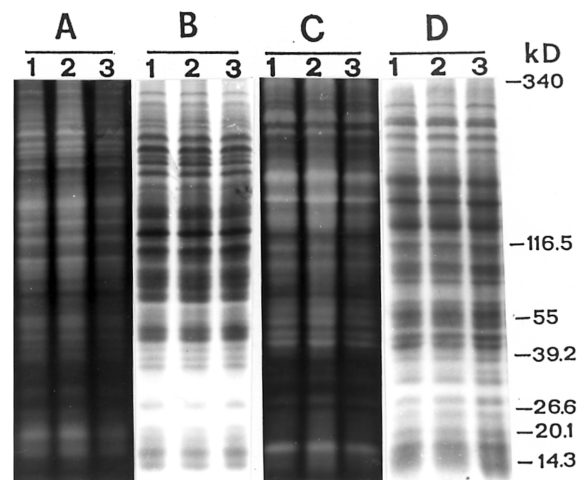

Fig. 8 Analysis of the thiol/disulfide state of proteins with mBBr. Roots of *L. albus* (A, B) and *A. cepa* (C, D) were analysed after 24 h of treatment. Proteins extracted from root tips (3 mm) were subjected to SDS-PAGE. Panels A and C: fluorescence emission of controls (lanes 1), and of samples treated with 1 mM GL (lanes 2) or 1 mM DHA (lanes 3). Panels B and D: the same gels stained with Coomassie brilliant blue R-250 (*A. cepa*: 150 µg protein per lane; *L. albus*: 80 µg protein per lane). Molecular masses of marker proteins are indicated on the right in kD.

It has been demonstrated that several different proteins containing thiol groups are capable of reducing DHA to AA (see Discussion). In order to assess the contribution of protein thiols to DHA reduction, we measured the thiol/disulfide status of proteins extracted after 24 h treatment with DHA or GL using monobromobimane (mBBr) derivatization of SH groups. As shown in Fig. 8, at 24 h GL treatment slightly enhanced fluorescence, which is indicative of the presence of thiol groups, whereas DHA treatment shifted the redox balance towards the oxidised (disulphide) status. The percentage increase or decrease in the average fluorescence intensity in comparison to controls, as measured using UTHSCA Image Tool software, was +16% and +18%, after GL treatment, and –41% and –30% after DHA treatment in lupin and onion, respectively. It is noteworthy that the effect of DHA on thiols was not evident when proteins were extracted after 12 h of incubation (not shown).

Discussion

Assessing the fate of DHA in plant cells is of great importance for several reasons: (i) in the extracellular matrix of plants, DHA is generated by the AA and AA peroxidase system, acting as a first barrier to oxidative stress conditions caused by pollutants (Castillo and Greppin 1988, Plöchl et al. 2000) and pathogens (Vanacker et al. 1998); (ii) in both plant and animal cells, DHA is generated as a consequence of AA utilisation by dioxygenases, a large class of enzymes involved in many important metabolic reactions (Arrigoni and De Tullio 2000); and (iii) DHA inhibits several enzyme activities in vitro and growth in vivo (see Introduction).

Data reported here show that DHA is not accumulated in the cells even in the presence of 1 mM DHA in the growth medium. AA accumulated largely in DHA-treated roots, indicating that DHA rapidly enters the root, where it is concomitantly reduced to AA. These results support data on the presence of a preferential DHA transport mechanism located at the plasma membrane (Horemans et al. 1997, Horemans et al. 1998). Our data suggest that in the short term the massive amount of DHA entering the cells is mainly reduced by available GSH. Moreover, DHA treatment apparently induces de novo synthesis of GSH but no significant change in glutathione reductase activity. At this stage (0–4 h), increased DHA reductase activity also seems to contribute to DHA reduction. After 8–10 h of incubation, DHA uptake is virtually blocked, intracellular AA content is more or less stable, GSH content increases and DHA reductase activity is apparently lower. However, from this time-point on, other symptoms of DHA incubation can be observed. Root growth is inhibited, the rate of thymidine incorporation is progressively slowed down and a general oxidation of protein thiols occurs. These data induced us to discriminate between "short-term" and "long-term" responses to DHA administration.

Metabolic responses to DHA in intact plants are apparently different and more complex than those reported for cell suspensions (de Pinto et al. 1999, Potters et al. 2000). Our data show a rapid and transient decrease in GSH content. This is extremely important, since GSH is essential for cell cycle progression (Vernoux et al. 2000). De novo synthesis of GSH occurs at high rates in DHA-treated roots, leading to rapid recovery. Indeed, it is known that stress conditions can stimulate GSH synthesis in plants (May and Leaver 1993). Moreover, stimulation of the pentose phosphate pathway and increased GSH content in animal cells treated with DHA has been observed (Puskas et al. 2000). The increase in the GSH pool in cells is of great relevance because it could affect DHA reduction to AA in at least two different ways: by favouring GSH-mediated non-catalytic reduction of DHA to AA (Meister 1992); and by increasing the enzymic activity of DHA reductase as a consequence of the increased availability of its physiological electron donor GSH (Kato et al. 1997, Shimaoka et al. 2000).

An additional contribution to DHA reduction could be ascribed to thiol-containing proteins and especially those containing the more or less classic dicysteinyl motif -C-X-X-C- peculiar to thioredoxins (Follmann and Häberlein 1996), protein disulfide isomerase and many other proteins (De Tullio et al. 1998). Our data on the protein thiol/disulphide status, showing that DHA treatment induces a shift towards the more oxidised form, seem to support this view. DHA treatment results in a general effect on the redox state of several different proteins. Furthermore, it has long been known that DHA, beside oxidising thiols, can form addition compounds as a consequence of the interaction of its carbonyl groups with amino acid residues (Drake et al. 1942). Recent results showed that DHA irreversibly inhibits human type I hexokinase, and the amino acid analysis of the DHA-modified enzyme reveals a decrease in the number of cysteine residues (Fiorani et al. 2000).

It is conceivable that protein-thiol oxidation does not quantitatively contribute to the observed rate of DHA reduction to AA. Nevertheless, we believe that this process could have an important qualitative role. A recent report (Nardai et al. 2001) stressed the close relationship existing between DHA reduction and the oxidation of protein thiols in the endoplasmic reticulum, where DHA reduction mediated by protein thiols and/or protein disulfide isomerase activity could have an important role in protein folding. In addition, our data suggest that cell cycle inhibition resulting from DHA treatment cannot be fully explained only by the observed transient ("short term") decrease in GSH content and that the effect of DHA on the redox state of thiol-containing proteins should also be considered to understand the inhibiting effect of DHA on cell cycle progression and consequently on growth. In fact, it has been reported that non-proliferating cells blocked in their cell cycle because of GSH shortage can re-enter the cell cycle when GSH becomes available (Vernoux et al. 2000), whereas our data show a progressive "long-term" effect of DHA incubation on cell cycle and root growth. Interestingly, the effect of DHA on

protein thiols could not be observed after short-time incubation, but proceeded in parallel with a decrease in thymidine incorporation and root growth inhibition.

Our data also raise an important question concerning the fate of AA formed by DHA reduction and indicate that this AA pool is not completely equivalent to AA formed by biosynthesis via GL. We hypothesise that AA *improperly* formed outside its specific cellular context could be less available for AA-requiring reactions than *properly* generated AA. On the other hand, AA formed by DHA reduction can also have a positive role: AA peroxidase activity is stimulated by both DHA and GL treatments, suggesting that increased AA availability, irrespective of the way in which it is obtained, is sufficient to upregulate this enzyme. Of course these hypotheses need further investigation.

We believe that our data showing a long-term effect of DHA on the redox state of some thiol-containing proteins could be a key to understanding the mechanism of DHA action on cell metabolism and to realise that DHA is, in both plants and animals, a compound with peculiar features and specific reactivity (Jung and Wells 1998, Deutsch 2000), rather than just the *B-side* of AA.

Materials and Methods

Plant material and growth conditions

Seeds of *L. albus* L., imbibed for 2 h, were sown in moist vermiculite in a dark room at 22°C with 54% humidity. After 3 d, seedlings with roots of similar length were suspended in beakers containing distilled water, 1 mM DHA or 1 mM L-galactono-γ-lactone (GL), the last AA precursor in its biosynthetic pathway (Wheeler et al. 1998). Bulbs of *A. cepa* L. were locally purchased and their basal plates immersed in water. When roots had reached a length of 2–3 cm, the bulbs were transferred to beakers for the different treatments. All the solutions were aerated by continuous bubbling at a rate of 10–15 ml air min^{-1} and their pH was set at 5.8 with 1 M HCl or 1 M KOH. Incubation media were renewed every 24 h. Root length was measured using a flexible plastic ruler, and root tips (3 mm) were cut with a razor blade for biochemical analysis.

Determination of AA, DHA, GSH and GSSG

Root tips (3 mm) of onion and lupin were ground in chilled mortar with pestle in ice-cold 5% (w/v) metaphosphoric acid. The homogenate was centrifuged for 15 min at 25,000×*g* and the supernatant used for determinations. AA and DHA were measured according to Zhang and Kirkham (1996).

GSH and GSSG were measured using 5-5′-dithiobis(2-nitrobenzoic) acid (Ellman's reagent) following the procedure described by Anderson (1985). Where indicated, the GSH biosynthesis inhibitor L-buthionine-(S,R)-sulfoximine (BSO) was added (Griffith and Meister 1979).

Enzyme assays

Root tips (3 mm) of onion and lupin were homogenised using a mortar and pestle at 4°C with an ice-cold medium containing 0.3 M mannitol, 1 mM EDTA, 50 mM Tris-HCl, pH 7.8 and 0.05% (w/v) cysteine. The homogenate was centrifuged for 20 min at 25,000×*g* and the supernatant was collected and used to determine the enzyme activities. Spectrophotometric assay of AA peroxidase (EC 1.11.1.11) activity in the supernatant was carried out according to Arrigoni et al. (1997).

Proteins were determined as described by Bradford (1976), using bovine serum albumin as a standard.

Electrophoretic assay

Native-PAGE of proteins with DHA reductase activity was performed using a 14×24 cm slab gel with a stacking gel containing 4.3% acrylamide, a running gel containing 7.3% acrylamide and a running buffer composed of 4 mM Tris-HCl, pH 8.3 and 38 mM glycine. After the run, gels were incubated in 0.1 M sodium phosphate buffer (pH 6.2) containing 4 mM GSH and 2 mM DHA, for 15 min. The gels were then washed in distilled water and stained in the dark with a solution of 0.125 M HCl containing 0.1% potassium ferricyanide and 0.1% ferrichloride (w/v) for 20 min. DHA-reducing proteins were observed as dark blue bands on a light blue background.

Electrophoresis of GSSG reductase was performed as above. After the run, gels were stained according to Ye et al. (1997).

Protein thiol labelling with monobromobimane

Root tips (3 mm) of onion and lupin (approximately 0.3 g) were homogenised in liquid nitrogen with a mortar and pestle. Protein SH groups were labelled with mBBr according to published procedures (Wong et al. 1995, Gobin et al. 1997). Immediately after the disappearance of the last trace of liquid N_2, 1 ml of a 2 mM solution of mBBr (previously dissolved in acetonitrile) in 0.1 M Tris-HCl buffer (pH 7.9) was added and the sample further homogenated for 1 min. To stop the reaction and derivatise excess mBBr, 25 μl of a solution of 10% SDS and 25 μl of 100 mM 2-mercaptoethanol were added to 200 μl of mBBr-labeled sample. SDS-PAGE was carried out on 10% acrylamide gels with a 3% stacking gel. After the electrophoretic run, the proteins were fixed with 12% (w/v) trichloroacetic acid for 1 h and then in a methanol : acetic acid : water (4 : 1 : 5 v/v) solution for 8–10 h to remove excess mBBr. The fluorescence of protein-bound mBBr was visualised by placing the gel on a light box fitted with an UV light source (365 nm). The resulting fluorescence emission is indicative of the relative thiol/disulphide state of proteins analysed. After fluorescence photography, total proteins were stained with Coomassie as a loading control and re-photographed. The intensity of fluorescent bands was analysed utilising the UTHSCA Image Tool software.

Cytological procedures

Onion roots were grown as above, then treated with 1 mM DHA for 7, 11 or 23 h and media renewed every 12 h. At the end of each treatment, roots were exposed for 1 h to [methyl-^{3}H]thymidine at a concentration of 9.25×10^7 Bq $litre^{-1}$; specific activity 92.5×10^7 Bq $mmol^{-1}$ in 1 mM DHA. Control roots were also supplied with a solution of [methyl-^{3}H]thymidine at the same concentration for 1 h. Roots were then sampled and fixed in 3 : 1 ethanol : acetic acid (v/v). The root meristems were Feulgen-stained after hydrolysis in 1 M HCl at 60°C for 7 min and prepared as permanent autoradiographs using Ilford L4 emulsion. Exposure time was 20 d. On the autoradiographed slides all the nuclei were scored for the presence or absence of overlying silver grains. For the labelling index (percentage of the ratio between labelled nuclei number and total nuclei number) five slides per treatment time per sampling were analysed. The mitotic index was determined according to Liso et al. (1984). The data represent the mean value of at least three bulbs. For each treatment, five squash preparations were made and for each slide, 1,000 cells were scored, giving a total of 5,000 cells scored.

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