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Transport of Ascorbic and Dehydroascorbic Acids across Protoplast and Vacuole Membranes Isolated from Barley (*Hordeum vulgare* L. cv Gerbel) Leaves[1]

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Protoplasts, vacuoles, and chloroplasts were isolated from leaves of 8-d-old barley (*Hordeum vulgare* L. cv Gerbel) seedlings. Transport of ascorbate and dehydroascorbate into protoplasts and vacuoles was investigated. Contents of ascorbic acid, glutathione, and α-tocopherol and ascorbate peroxidase activity and glutathione reductase activity were analyzed in protoplasts, vacuoles, and chloroplasts. Uptake of ascorbate and dehydroascorbate by protoplasts showed saturation kinetics (K_m = 90 μM reduced ascorbic acid, 20 μM dyhydroascorbic acid). Effects of various membrane transport inhibitors suggested that transport was carrier mediated and driven by a proton electrochemical gradient. Translocation of ascorbate and dehydroascorbate into vacuoles did not show saturation kinetics. Neither was it influenced by effectors or by ATP but only by Mg^{2+}, suggesting that translocation did not occur by carrier. Ascorbic acid was predominantly localized in the cytosol. Contents in the chloroplasts and vacuoles were low. The results are consistent with the view that ascorbate is synthesized in the cytosol and released to chloroplasts, apoplast, and vacuole following a concentration gradient. Translocation from the apoplast into the cytosol is against a steep gradient and appears to control the concentration of ascorbic acid in the apoplast. In its function as an antioxidant, ascorbate in the apoplast may be oxidized to dehydroascorbate, which can be efficiently transported back into the cytosol for regeneration to ascorbate.

AA is an important antioxidant defense substance in plant cells (Foyer and Halliwell, 1976). It protects plant cells against damage by oxygen free radicals, which may be produced as a result of a disturbance of electron transfer processes or via autoxidation. In chloroplasts, electrons may be diverted from the electron transport chain when its efficiency is overtaxed due to high light intensity. Electron transfer processes in mitochondria may be hindered as a result of damage to, or interaction of xenobiotic compounds with, the electron transport chain (Winston, 1990). The apoplastic compartment becomes a site of production of oxygen free radicals if the air pollutant ozone interacts with the cellular surface (Penel and Castillo, 1991). Thus, the cellular site where oxygen free radicals are produced and where antioxidant defense compounds such as AA are needed may vary depending on the specific environmental conditions.

AA as an antioxidant is oxidized to DHA. Oxygen free radicals may be scavenged directly by AA or may lead to the production of H_2O_2, which can be detoxified by AA in the presence of ascorbate peroxidase (Castillo and Greppin, 1986). An additional involvement of AA as an antioxidant was proposed for the reduction of oxidized tocopherol (Wefer and Sies, 1988). Tocopherol is associated with membrane lipids and is oxidized as it protects lipids against peroxidation (Suarna and Southwell-Keely, 1991).

The protective effect of AA against oxygen free radicals depends on its subcellular distribution and on the presence of compounds that are related to its antioxidant function (ascorbate peroxidase, tocopherol). The subcellular distribution of AA depends on the site of AA synthesis, on its intracellular translocation, and on the site where it can be regenerated from DHA. AA appears to be synthesized in the cytosol (Loewus, 1980) and then translocated into the chloroplasts, vacuole, and apoplast. Translocation of AA into chloroplasts has been studied in spinach and was suggested to occur by facilitated diffusion (Anderson et al., 1983a; Beck et al., 1983). Regeneration of AA from DHA occurs in the cytosol (enzymatic) and chloroplasts (nonenzymatic), with glutathione as a reductant. The same compartments contain glutathione reductase for the regeneration of reduced glutathione (Foyer and Halliwell, 1976; Halliwell, 1984). Regeneration of AA from DHA does not seem to occur in the vacuole or in the apoplast. This suggests that apoplastic and vacuolar DHA cannot be reduced to AA unless it is translocated to the cytosol. However, no data are available on the transport of AA and DHA across the plasmalemma and the tonoplast. This study investigates the characteristics of these transport processes and relates them to the subcellular distribution of ascorbic acid (AA and DHA), of ascorbate peroxidase and tocopherol as antioxidant defense compounds associated with AA, and of glutathione and glutathione reductase as compounds associated with the regeneration of AA from DHA.

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Abbreviations: AA, reduced ascorbic acid; DHA, oxidized ascorbic acid (= mono-plus didehydroascorbic acid); DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonate; NEM, *N*-ethylmaleimide; pCMBS, *p*-chlormercuribenzenesulfonic acid; V_{max}, maximal rate of uptake.

MATERIALS AND METHODS

Plants

Barley (*Hordeum vulgare* L. cv Gerbel) seeds were soaked in tap water for 24 h and sown in potting medium of roughly equal amounts of quartz sand, peat, and garden soil. No additional nutrients were applied. Seedlings were grown in a growth chamber for 8 d. The light period was 12 h, and the PPFD was 100 μmol quanta m^{-2} s^{-1} or as indicated. Day/night temperatures were 22°C/18°C. RH was 75%, and water was supplied.

Preparation of Protoplasts, Chloroplasts, and Vacuoles

Protoplasts were prepared according to Kaiser et al. (1982). For the isolation of organelles, purified protoplasts were suspended in 0.4 M sorbitol, 30 mM potassium gluconate, 2 mM EDTA, 10% (v/v) Percoll, and 25 mM Hepes-imidazole (pH 7.5). The suspension was forced through a needle of 0.6 or 1.0 mm diameter to release chloroplasts or vacuoles, respectively. Vacuoles were purified by flotation according to Martinoia et al. (1986). Chloroplasts were purified on a density gradient consisting of 3 mL of 0.4 M sorbitol, 30 mM potassium gluconate, 100% Percoll, 20 mM Hepes-imidazole (pH 7.2), and 10 mL of 0.4 M sorbitol, 30 mM potassium gluconate, 35% (v/v) Percoll, 20 mM Hepes-imidazole (pH 7.2). Five milliliters of the suspension of broken protoplasts were layered onto this gradient. Chloroplasts were collected from the lower interphase after 5 min of centrifugation at 700*g*.

Uptake of [^{14}C]AA and [^{14}C]DHA by Protoplasts

[^{14}C]AA was purchased from DuPont (Regensdorf, Switzerland). [^{14}C]DHA was prepared from [^{14}C]AA in the presence of iodine immediately before use. ^3H_2O was purchased from Amersham. Uptake of [^{14}C]AA and [^{14}C]DHA by protoplasts was followed in 0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM Mes-imidazole (pH 6.0), 44 Bq μL^{-1} [^{14}C]AA/DHA, and 89 Bq μL^{-1} ^3H_2O. Suspensions were stirred in tubes in a water bath at 20°C. After 1 and 5 min, aliquots of 100 μL were transferred into 400-μL centrifuge tubes containing 50 μL of 0.5 M sorbitol, 1 mM $CaCl_2$, 20% (v/v) Percoll, 10 mM Mes-imidazole (pH 6.0) and 200 μL of silicon oil AR 200 (Fluka). Uptake was stopped by centrifugation in a Beckman Microfuge E. Tubes were frozen at −18°C. The lower interphase contained the protoplasts and was cut out for determination of ^{14}C and ^{3}H in a β counter. ^{14}C and ^{3}H in the uptake medium were determined for reference. ^3H_2O equilibrates rapidly between the medium and the protoplastic space and was used to quantify the number of protoplasts (10^7 protoplasts corresponded to 200 μL; Martinoia et al., 1986). The rate of uptake was calculated using a linear regression between ^{14}C uptake and time. The response of uptake rate to the substrate concentration was calculated in a weighted nonlinear regression analysis (Wilkinson, 1961).

Uptake of [^{14}C]AA and [^{14}C]DHA by Vacuoles

Uptake of [^{14}C]AA and [^{14}C]DHA by vacuoles was investigated following the method described by Martinoia et al. (1986). Thirty microliters of vacuole suspension were mixed with 70 μL of 0.4 M sorbitol, 30 mM potassium gluconate, 30% (v/v) Percoll, 20 mM Hepes-imidazole (pH 7.2), 0.2% (w/v) BSA, 200 Bq μL^{-1} [^{14}C]AA/DHA and 150 Bq μL^{-1} ^3H_2O in a 400-μL centrifuge tube. The mixture was overlayered with 200 μL of silicon oil and 60 μL of water. Uptake was terminated between 1 and 30 min by centrifugation in a Beckman Microfuge E. ^{14}C and ^{3}H, which were contained in the water phase, were determined in a β counter. ^{14}C and ^{3}H in the uptake medium were determined as a reference. ^{3}H was used to quantify the number of vacuoles (10^7 vacuoles corresponded to 162 μL; Martinoia et al., 1986). Uptake rate was calculated as described for protoplasts.

Analytical Methods

Protoplast, chloroplast, and vacuole fractions for analysis of enzyme activities were frozen and stored at −70°C for not more than 15 d. Samples for the analysis of ascorbic acid and glutathione were stabilized in 3% (w/v) *m*-phosphoric acid and stored for less than 2 weeks at −70°C. Samples for the determination of α-tocopherol were stabilized in 90% (v/v) methanol and stored for less than 2 weeks at −20°C.

Ascorbic acid (AA + DHA) was determined fluorimetrically after oxidation of AA to DHA and condensation of DHA with 1,2-phenylenediamine to quinoxaline (Brubacher and Vuilleumier, 1974). Glutathione (oxidized and reduced) was determined in an enzymatic cycling assay based on glutathione reductase (Griffith, 1980). α-Tocopherol was determined in the laboratories of F. Hoffmann-LaRoche AG (Basel, Switzerland) using HPLC and fluorimetric detection. Chl was determined spectrophotometrically (Bruinsma, 1963).

Ascorbate peroxidase activity was measured spectrophotometrically following the oxidation of ascorbate in the presence of H_2O_2 (Gerbling et al., 1984). Glutathione reductase activity was determined spectrophotometrically following oxidation of NADPH in the presence of oxidized glutathione (Foyer et al., 1989). α-Mannosidase activity was assayed by measuring the liberation of *p*-nitrophenol from *p*-nitrophenol-α-mannopyranoside (Martinoia et al., 1981). Glyceraldehyde-3-P dehydrogenase activity was determined from the oxidation of NADPH (Kaiser et al., 1982). Glc-6-P dehydrogenase activity was measured following the reduction of NADP (Kuby and Noltmann, 1966).

Nonaqueous Compartmental Analysis

The method of Gerhardt and Heldt (1984) was used. Leaf samples were frozen in liquid nitrogen, homogenized to a fine powder, and freeze-dried. The freeze-dried powder was homogenized in a mixture of *n*-heptane and carbon tetrachloride by ultrasonication and separated on a density gradient as described. Four gradients were prepared in two experiments. The gradients were fractionated into five fractions. The leaf material was separated from the media by centrifugation and evaporation. Ascorbic acid and three marker enzymes (glyceraldehyde-3-P dehydrogenase for chloroplasts, Glc-6-P dehydrogenase for cytosol, and α-mannosidase for vacuoles) were analyzed in each fraction.

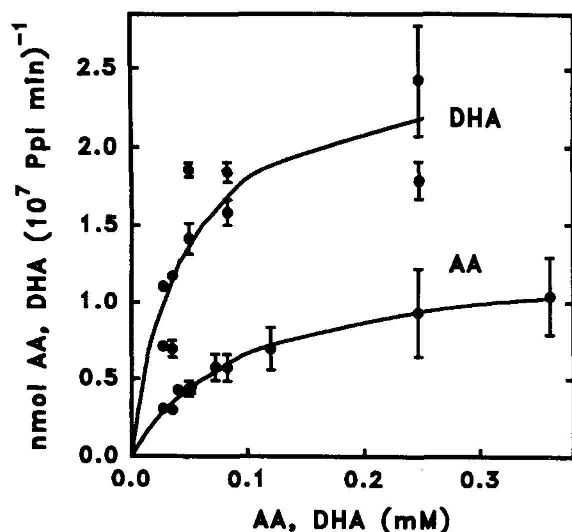

Figure 1. Uptake of AA and DHA by protoplasts (Ppl) of barley leaves as affected by the concentration of the substrates. Slopes of the regression of the uptake-time response are means of three replications. SE values are indicated.

Calculations

In the aqueous compartmental analysis, proportions of ascorbic acid, glutathione, and ascorbate peroxidase and glutathione reductase activity in chloroplasts and vacuoles were calculated by relating the contents of these compounds to the contents of the activities of the marker enzymes glyceraldehyde-3-P dehydrogenase (for chloroplasts) and α-mannosidase (for vacuoles). Concentrations of ascorbic acid and glutathione in the cytosol, chloroplasts, and vacuoles were calculated assuming volumes of 15, 25, and 162 μL/10^7 protoplasts, respectively (Martinoia et al., 1986).

In the nonaqueous analysis, the data of the two gradients were combined in each of the two experiments, and means were calculated. The significance of an association of ascorbic acid with a marker enzyme was calculated in a multiple linear regression analysis ($n = 10$) with the three marker enzymes as independent variables and ascorbic acid as the dependent variable. The significance of an increase in variance after omission of one of the marker enzymes in the analysis was calculated. This gave an indication as to whether the respective marker enzyme was significantly associated with ascorbic acid.

RESULTS AND DISCUSSION

Uptake of AA and DHA by Protoplasts

Uptake of AA and DHA by protoplasts followed saturation kinetics (Fig. 1), suggesting that both transport processes were carrier mediated. DHA was taken up more readily than AA. Double-reciprocal plots show that V_{max} was higher for DHA than for AA (Figs. 2 and 3). The K_m was lower for DHA than for AA (20 and 90 μM, respectively), indicating that the affinity of the carrier was higher for DHA than for AA. AA uptake was inhibited by DHA and vice versa (Figs. 2 and 3). In both cases, V_{max} was clearly affected; the effects on K_m were not significant, suggesting that the two substrates did not compete for the same binding site. Uptake of AA and DHA by protoplasts was examined in the presence of various membrane transport inhibitors (Table I). NEM and pCMBS decreased the uptake of both substrates, suggesting that protein-bound sulfhydryl groups were involved in the transport processes. The anion channel inhibitor DIDS inhibited the uptake of AA but not of DHA. This is consistent with the view that AA is taken up as an anion but DHA is electroneutral. The ionophores valinomycin and gramicidin inhibited the uptake of both substrates, indicating that the transport was driven by a proton electrochemical gradient.

Carrier-mediated transport of AA and DHA has been shown for cells of various animal tissues. In brush border cells of guinea pig ileum, AA was taken up more readily than DHA (Bianchi et al., 1986), which is in contrast to our findings with plant protoplasts. The K_m values of AA and DHA were between 0.1 and 0.4 mM in these animal cells and tended to be higher than K_m values of plant protoplasts in this study.

Uptake of AA and DHA by Vacuoles

The rates of uptake of AA and DHA by vacuoles were very slow at low substrate concentrations but increased linearly as substrate concentrations were increased (Fig. 4). DHA was taken up more readily than AA. Uptake of both substrates was not significantly inhibited by the membrane transport inhibitors DIDS, NEM, gramicidin, and valinomycin (Table II). DHA did not affect AA uptake and vice versa. Adding MgATP (3 mM) resulted in an almost 2-fold increase of the uptake rates (Table III). However, this effect was most likely due to the provision of Mg^{2+} ions, which alone had a similar

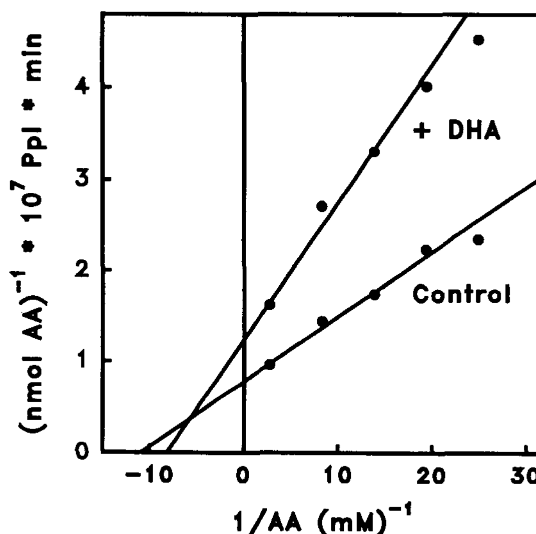

Figure 2. Double-reciprocal plot of the uptake of AA by protoplasts (Ppl) of barley at various substrate combinations. The effect of 0.5 mM DHA is shown. Enzyme kinetic constants ± SE were calculated for the control: V_{max} = 1.29 ± 0.061 nmol (10^7 protoplasts min)$^{-1}$; K_m = 0.09 ± 0.010 mM; r^2 = 0.971. Kinetic constants for the uptake in the presence of DHA: V_{max} = 0.81 ± 0.056 nmol (10^7 protoplasts min)$^{-1}$; K_m = 0.12 ± 0.018 mM; r^2 = 0.982.

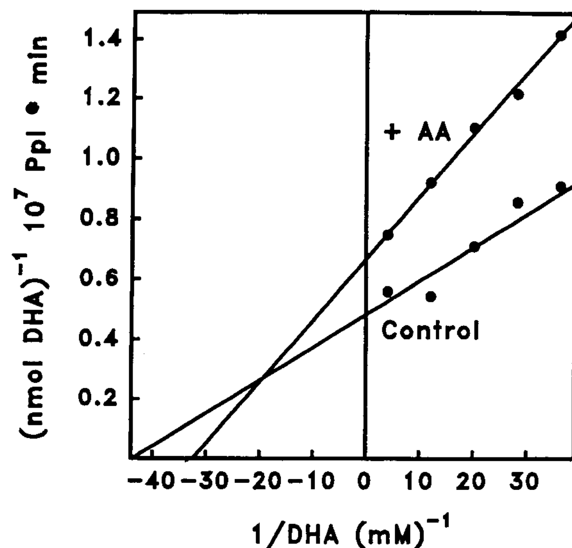

Figure 3. Double-reciprocal plot of the uptake of DHA by protoplasts (Ppl) of barley at various substrate concentrations. The effect of 0.5 mM AA is shown. Enzyme kinetic constants ± SE were calculated for the control: V_{max} = 2.07 ± 0.199 nmol (10^7 protoplasts min)$^{-1}$; K_m = 0.02 ± 0.008 mM; r^2 = 0.921. Kinetic constants for the uptake in the presence of DHA: V_{max} = 1.50 ± 0.024 nmol (10^7 protoplasts min)$^{-1}$; K_m = 0.03 ± 0.002 mM; r^2 = 0.995.

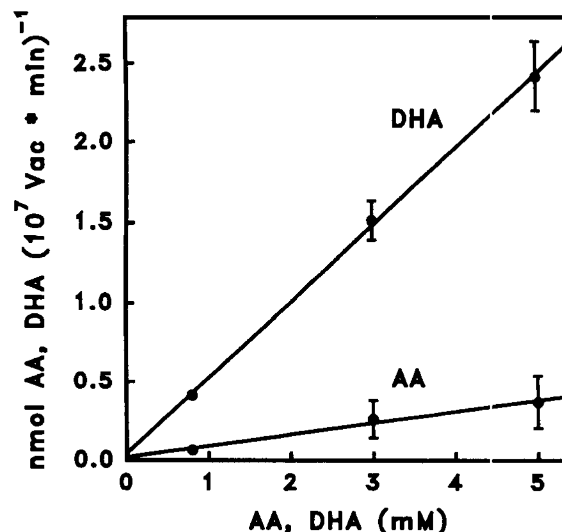

Figure 4. Uptake of AA and DHA be vacuoles (Vac) of barley as affected by the concentrations of the substrates. SE values of the slopes of the regressions of the uptake-time responses (n = 12) are indicated. The r^2 of regression of uptake rates were 0.980 for AA and 0.999 for DHA.

effect on the uptake rates of AA and DHA. MgGTP had the same effect as MgATP. Bafilomycin, a specific inhibitor of the tonoplast H^+-ATPase (Bowman et al., 1988), in combination with MgATP did not inhibit transport rates.

These data do not give any indications for a carrier-mediated translocation of AA and DHA into the vacuole. Neither carriers with sulfhydryl groups nor anion channels nor a proton electrochemical gradient appear to be involved in the translocation of AA and DHA. MgATP as an energy supply for the buildup of a proton electrochemical gradient across the tonoplast membrane is not essential for the translocation of AA and DHA. Only Mg^{2+} ions at concentrations between 3 and 6 mM had a beneficial effect on the uptake rates of AA and DHA. As a divalent cation, Mg^{2+} may affect membrane properties by bridging phosphate and carboxylate groups of phospholipids.

Assuming uptake by diffusion, the higher rate for DHA compared with AA appears to be due to the electroneutral character of DHA and the anionic character of AA at physiological pH (Hirst, 1933). In contrast to AA, the electroneutral DHA is expected to be soluble in membrane lipids and to diffuse readily across membranes (Martin, 1961) as shown in our data. However, even DHA was not taken up rapidly by vacuoles. Rose (1987) showed that the oil-water distribution of DHA was similar to that of mannitol, which is known for its slow diffusion across membranes.

Table I. *Uptake of AA and DHA by protoplasts as affected by various effectors*

Concentrations of AA and DHNA were 0.5 mM. SE values of the slopes of the regressions of the uptake-time responses are shown. The numbers of determinations are indicated in parentheses.

	AA	DHA
	nmol (10^7 protoplasts min)$^{-1}$	
Control[a]	0.88 ± 0.119 (6)	1.18 ± 0.060 (6)
NEM (0.50 mM)	0.21 ± 0.031 (6)	0.56 ± 0.142 (6)
pCMBS (0.50 mM)	0.29 ± 0.056 (6)	0.73 ± 0.109 (5)
DIDS (0.25 mM)	0.33 ± 0.012 (6)	1.03 ± 0.196 (6)
Valinomycin[b] (1.0 μM)	0.14 ± 0.060 (5)	0.66 ± 0.090 (6)
Gramicidin[b] (50 μM)	0.51 ± 0.085 (4)	0.53 ± 0.011 (4)

[a] Did not contain ethanol. [b] Solubilized in 100% ethanol. Ethanol content in the final incubation media was 0.5% (v/v).

Table II. *Uptake of AA and DHA by vacuoles as affected by various effectors*

Concentrations of AA and DHA were 0.8 mM. SE values of the slopes of the regressions of the uptake-time responses are shown. The numbers of determinations are indicated in parentheses.

	AA	DHA
	nmol (10^7 vacuoles min)$^{-1}$	
Control[a]	0.06 ± 0.023 (18)	0.41 ± 0.033 (18)
DIDS (0.25 mM)	0.08 ± 0.028 (18)	0.45 ± 0.037 (12)
NEM (0.5 mM)	0.06 ± 0.036 (12)	0.43 ± 0.047 (11)
Gramicidin[b] (5 μM)	0.07 ± 0.034 (12)	0.38 ± 0.035 (11)
Valinomycin[b] (1 μM)	0.05 ± 0.037 (12)	0.40 ± 0.043 (12)
AA (0.8 mM)		0.39 ± 0.046 (10)
DHA (0.8 mM)	0.06 ± 0.037 (11)	

[a] Did not contain ethanol. [b] Solubilized in 100% ethanol. Ethanol contents in final incubation media were 0.5% (v/v).

The Content of Ascorbic Acid (AA + DHA) in the Subcellular Compartments

The content of ascorbic acid in isolated barley protoplasts was affected considerably by the PPFD at which the plants had been grown (Table IV). The content was much higher when PPFD was 500 compared to 100 μmol quanta m^{-2} s^{-1} and compared well with 235 μg mg^{-1} Chl, which was found in protoplasts of spinach that had been grown in the glasshouse (Foyer et al., 1983). The main proportion of ascorbic acid was localized in the cytosol, whereas the proportions in the chloroplasts and in the vacuole were low. Calculated concentrations showed high levels in the cytosol, lower levels in the chloroplasts, and lowest levels in the vacuole (Table V). The proportions and concentrations in the chloroplasts and vacuole increased as the PPFD level increased during growth. This is especially true for the chloroplasts, the concentration in which was 5 times higher when they were exposed to a PPFD of 500 compared to 100 μmol quanta m^{-2} s^{-1}.

Considering the possibility of an underestimation of the proportion of ascorbic acid in the chloroplasts due to a loss from chloroplasts during aqueous isolation, the subcellular distribution of ascorbic acid in plants grown at 100 μmol quanta m^{-2} s^{-1} PPFD was reexamined using a nonaqueous technique. Ascorbic acid appeared to be associated exclusively with the cytoplasmic compartment. An analysis of the percent distribution of ascorbic acid and of the cytosolic marker enzyme (Glc-6-P dehydrogenase) between the five fractions of the density gradient showed a highly significant relationship between the two parameters ($P \leq 0.001$) with a regression coefficient (±SE) of 1.0 ± 0.11 (n = 10). No significant association was found between ascorbic acid and the chloroplastic and vacuolar marker enzymes (glyceraldehyde-3-P dehydrogenase and α-mannosidase). This confirmed the conclusion, following the aqueous compartmental analysis, that proportions in chloroplasts and in the vacuole were low.

The proportion of ascorbic acid in the chloroplast was much lower in barley than in spinach, as has been found by other authors (Franke and Heber, 1964; Gerhardt, 1964; Foyer et al., 1983). Proportions of 30 to 40% (aqueous analysis; Foyer et al., 1983) and 35 to 50% (nonaqueous analysis; Franke and Heber, 1964; Gerhardt, 1964) were found in chloroplasts of spinach that had been grown in the glasshouse. Ascorbic acid concentrations were 15 to 25 mM or even 46 mM. Leaf orientation strongly influences the interception of light energy; leaves normal to the light path receive the most energy. This may explain the observed differences in the relative amounts of AA found in the chloroplasts of barley, whose leaves are oriented vertically, and spinach, the leaves of which are more horizontal. Considering the effect of PPFD on the proportion of ascorbic acid in chloroplasts, it is suggested that the difference between barley and spinach could be due primarily to a difference in leaf orientation and hence light exposure of chloroplasts. However, genetic factors may also be involved in this difference.

No data on vacuolar ascorbic acid contents are available for leaves of species other than barley. Data for vacuoles of root cells of horseradish show a very high proportion (close to 100%) of ascorbic acid in the vacuole (Grob and Matile, 1980). Ascorbic acid in horseradish roots has a special function. It is used as a cofactor and activator for the enzyme myrosinase, which is responsible for the rapid conversion of glucosinolates into mustard oils (Grob and Matile, 1980).

The proportion of ascorbic acid in the apoplastic compartment has not been investigated in this study. However, significant contents of ascorbic acid in the apoplast have been found for sedum (Castillo and Greppin, 1988) and spinach (Takahama and Oniki, 1992; Luwe et al., 1993). Ascorbic

Table III. *Uptake of AA and DHA by vacuoles as affected by Mg^{2+}, nucleotides, and bafilomycin*

Concentrations of AA and DHA were 0.8 mM. SE values of the slopes of the regressions of the uptake-time responses are shown. The numbers of determinations are indicated in parentheses.

		AA	DHA
		nmol (10^7 Vac min)$^{-1}$	
Control		0.26 ± 0.041 (47)	0.58 ± 0.046 (45)
MgATP	(3 mM)	0.52 ± 0.028 (38)	0.95 ± 0.038 (37)
$MgSO_4$	(3 mM)	0.44 ± 0.052 (13)	0.77 ± 0.064 (14)
	(6 mM)	0.60 ± 0.071 (8)	0.84 ± 0.097 (6)
MgGTP	(3 mM)	0.64 ± 0.039 (7)	0.7 ± 0.075 (7)
MgATP	(3 mM)		
+ Bafilomycin	(1 μM)	0.64 ± 0.043 (15)	0.86 ± 0.050 (16)

Table IV. *Content in leaf protoplasts and subcellular distribution of ascorbic acid, glutathione, and α-tocopherol*

Barley plants were grown at a PPFD of 100 (L) or 500 μmol quanta m^{-2} s^{-1} (H). SE values are shown. Numbers of determinations are indicated in parentheses.

	Total in Protoplasts	Proportion in	
		Chloroplasts	Vacuoles
	μg (mg Chl)$^{-1}$	%	%
AA + DHA			
L	120.0 ± 10.3 (6)	8 ± 1.4	15 ± 1.0
H	280.0 ± 38.7 (3)	16 ± 1.5	26 ± 6.0
Glutathione			
L	60.0 ± 12.0 (6)	20 ± 5.9	5 ± 0.4
H	92.0 ± 34.0 (3)	20 ± 3.2	6 ± 0.8
α-Tocopherol			
L	8.0 ± 1.2 (2)	57 ± 1.0	18 ± 0.6
H	615.9 ± 1.4 (2)	48 ± 4.5	18 ± 0.8

Table V. *Concentration of ascorbic acid and glutathione in the subcellular compartments*

Values were calculated from data presented in Table IV. Barley plants were grown at a PPFD of 100 (L) or 500 μmol quanta m^{-2} s^{-1} (H).

		Cytosol	Chloroplasts	Vacuoles
			mM	
AA + DHA	L	35	2	0.6
	H	61	10	2.6
Glutathione	L	9.8	1.6	0.06
	H	14.9	2.4	0.11

acid concentrations in the apoplast were 1 mM in sedum and 0.1 to 0.5 mM in spinach.

Glutathione and Glutathione Reductase

The content of glutathione and glutathione reductase activity were relatively high in the cytosol and in the chloroplasts, whereas both were barely detectable in the vacuoles. This suggests that the regeneration of AA from DHA was confined to the former compartments (Tables IV and VI). Regeneration of AA from DHA in the apoplastic space appeared to be unlikely but cannot be excluded (Heath, 1988).

Ascorbate Peroxidase and α-Tocopherol

The subcellular distribution of ascorbate peroxidase corresponded with the distribution of ascorbic acid, showing highest activity in the cytosol (Table VI). A significant proportion was found in the vacuole. The proportion in the chloroplasts was low, possibly due to the low PPFD at which the plants had been grown. The presence of ascorbate peroxidase in the chloroplasts and apoplast has been shown by other authors (Anderson et al., 1983b; Castillo and Greppin, 1988). The consistency of the subcellular distribution of ascorbic acid and ascorbate peroxidase suggests that ascorbic acid can be important for the detoxification of H_2O_2. In contrast, the subcellular distribution of α-tocopherol was inconsistent with that of ascorbic acid. About 50% of the tocopherol was contained in the chloroplasts (Table IV), whereas the ascorbic acid content was low in this compartment. This suggests that the main proportion of ascorbic acid was not involved in the regeneration of oxidized α-tocopherol. The distributions of ascorbic acid and α-tocopherol at higher PPFD appear to be more consistent, since the proportion of ascorbic acid in the chloroplasts is higher under these conditions.

CONCLUSIONS

There is agreement between the concentrations of ascorbic acid in the subcellular compartments and the characteristics of the translocation of AA and DHA across membranes. The concentration of ascorbic acid is highest in the cytosol. The transport from the cytosol into the chloroplasts, vacuole, and apoplast follows a relatively steep gradient. The transport of ascorbic acid into the vacuole does not appear to be carrier mediated. It is slow at low substrate concentrations but increases linearly with increasing concentrations. At the relatively high concentration of AA in the cytosol a remarkable rate of translocation of ascorbic acid into the vacuole appears to be enabled. The question as to whether or not vacuolar AA and DHA are transported back to the cytosol has not been answered convincingly in our study.

The transport of ascorbic acid from the cytosol to the apoplast has not yet been investigated. However, non-carrier-mediated diffusion would appear to be sufficiently fast to establish a concentration in the apoplast that is comparable to that in the vacuole. The gradient between cytosol and chloroplasts is less steep than that among the cytosol and the vacuole and the apoplast. The translocation of ascorbic acid into chloroplasts has been investigated for spinach (Anderson et al., 1983a; Beck et al., 1983). It takes place by facilitated diffusion with a low affinity for ascorbic acid. The K_m (20 mM) of the transport of ascorbic acid into the chloroplasts compares with the concentration in the cytosol found for barley.

The translocation of ascorbic acid from the apoplast to the cytosol across the plasmalemma is against a steep gradient. Accordingly, the transport must be actively driven either through co-transport with another molecule moving down its electrochemical potential gradient or through the action of a transporter that uses the energy of ATP or PPi hydrolysis. The affinity of this transport process for AA is high. The K_m (0.1 mM) lies in the range of the AA concentration in the apoplast of spinach (Takahama and Oniki, 1992; Luwe et al., 1993). The characteristics of this transport process appear to be important to maintain the concentration of ascorbic acid in the apoplast at an appropriately low level. The concentration of DHA in the apoplast (and in the other subcellular compartments) is much lower than that of AA (less than 10% of AA + DHA) but may increase in the presence of ozone (Castillo and Greppin, 1988). Accordingly, the K_m of the transport is lower for DHA than for AA. DHA is more readily transported into the cytosol than is AA, suggesting that DHA that is produced from AA in the apoplast can be efficiently transported back into the cytosol for regeneration to AA.

Table VI. *Subcellular distribution of the activities of ascorbate peroxidase and glutathione reductase in protoplasts of barley leaves*

Plants were grown at a PPFD of 100 μmol quanta m^{-2} s^{-1}.

	Cytosol	Chloroplasts	Vacuoles
		%	
Ascorbate peroxidase	89	2	9
Glutathione reductase	46	53	1

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