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Transport and Subcellular Localization of Polyamines in Carrot Protoplasts and Vacuoles[1]

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ABSTRACT

Putrescine and spermidine uptake in carrot (*Daucus carota* L., cv "Tip top") protoplasts and isolated vacuoles was studied. Protoplasts and vacuoles accumulated polyamines very quickly, with maximum absorption within 1 to 2 minutes. The insertion of a washing layer containing 100 millimolar unlabeled putrescine or spermidine did not change this pattern, but strongly reduced the uptake of putrescine and spermidine in protoplasts and in vacuoles. The dependence of spermidine uptake on the external concentration was linear up to the highest concentrations tested in protoplasts, while that in vacuoles showed saturation kinetics below 1 millimolar (K_m = 61.8 micromolar) and a linear component from 1 to 50 millimolar. Spermidine uptake in protoplasts increased linearly between pH 5.5 and 7.0, while there was a distinct optimum at pH 7.0 for vacuoles. Preincubation of protoplasts with 1 millimolar Ca^{2+} affected only surface binding but not transport into the cells. Nonpermeant polycations such as La^{3+} and polylysine inhibited spermidine uptake into protoplasts. Compartmentation studies showed that putrescine and spermidine were partly vacuolar in location and that exogenously applied spermidine could be recovered inside the cells. The characteristics of the protoplast and vacuolar uptake system induce us to put forward the hypothesis of a passive influx of polyamines through the plasmalemma and of the presence of a carrier-mediated transport system localized in the tonoplast.

Polyamines are ubiquitous in higher plants. They may act as growth regulators, membrane stabilizers, senescence retardants, stress metabolites, and so forth (for reviews, see Refs. 5 and 18). For many of these functions, transport of polyamines has to be postulated. Recently, evidence has increased for both long and short distance transport of polyamines in higher plants (2, 3, 7, 14, 15).

Using petals of *Saintpaulia* (3, 14) and cultured carrot cells (15), the uptake of polyamines into cells could be demonstrated. In the case of carrot cells, the uptake of putrescine and spermidine was remarkably rapid, reaching a maximum within only 1 min (15). The concentration dependence displayed a biphasic behavior for both polyamines tested. Two saturable components have been observed for putrescine uptake with K_m values of about 40 μM and 30 mM for low (less than 5 mM) and high (up to 100 mM) external putrescine concentration, respectively. Spermidine uptake was composed of a saturable component with a K_m value of about 30 μM for low and a linear component for high external spermidine concentrations. Meaningful interpretations of these kinetics data on polyamine uptake were difficult since cell fractionation studies revealed that most of the spermidine taken up by the cells (about 70%) had been adsorbed to the cell wall, whereas most of the putrescine taken up (70%) was recovered in the cytoplasm (15).

From this differential subcellular distribution of polyamines, it is obvious that two overlapping processes have to be studied separately in order to gain the desired information on the uptake of polyamines: (a) the interactions of the positively charged polyamines with the negatively charged cell wall components and (b) the uptake of polyamines into the cell as a transport process across the plasmalemma (and then the tonoplast). The present study deals mainly with the second point.

The availability of protoplasts and vacuoles had the added benefit of also allowing a study of the subcellular compartmentation of accumulated polyamines with the vacuole as the focal point. The vacuole as a temporary storage site of polyamines would explain the often observed discrepancy between the high intracellular polyamine concentrations of up to 1 mM and the low physiological concentrations needed for a proper functioning of the polyamines (10–100 μM) (4). Unfortunately, the study of the vacuolar compartmentation of polyamines has proved to be difficult, most probably due to leakage of polyamines out of the vacuoles during the isolation procedure.

MATERIALS AND METHODS

Plant Material. Mature taproots of carrot, *Daucus carota* L. cv "Tip Top," were a gift from VLG, Ins, Switzerland. They were stored unwashed in the cold room (5°C) for up to 3 months.

Isolation of Protoplasts and Vacuoles. Protoplasts and vacuoles from carrot phloem parenchyma were prepared essentially as described by Keller (11). Briefly, the chopped tissue was incubated with 2% (w/v) cellulase Y-C (Sheishin Pharm. Co.) and 0.1% (w/v) pectolyase Y-23 (Seishin Pharm. Co.) in 0.7 M glycinebetaine and 25 mM Mes-Tris (pH 5.5) for 3 h at 24°C. The released protoplasts were purified by filtering through cheesecloth and washing in the above medium minus enzymes by sedimentation (twice at 25*g* for 5 min). The purified protoplasts were lysed in a 10-fold volume of 0.4 M glycinebetaine, 50 mM Tricine-NaOH (pH 8), 0.1% BSA, and 1 mM Na_2-EDTA for 1 min. The vacuoles were purified by filtering through cheesecloth and washing with a medium containing 0.6 mM glycinebetaine, 25 mM Hepes-AMPD[2] (pH 7.6), and 0.1% BSA by sedimentation twice at 25*g* for 5 min.

Protoplasts were counted in a Burker counting chamber. The pellet contained 10^7 protoplasts/ml.

Polyamine Uptake. Incubation was performed in 400 μl microtubes that contained (from bottom to top) 240 μl of silicone oil (Wacker AR 200 for protoplasts; Wacker AR 20:AR 200 = 1:1.5 [v/v], for vacuoles) and 125 μl of protoplast or vacuole medium supplemented with 3.7 kBq [^{14}C]polyamine (final po-

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[2] Abbreviation: AMPD, 2-amino-2-methylpropanediol.

lyamine concentration as indicated in the text). When protoplasts and vacuoles had to be washed with a cold polyamine solution, 50 μl of 100 mM putrescine or spermidine in protoplasts or vacuoles medium were inserted accordingly between two silicone oil layers. Uptake was initiated by adding 25 μl of the protoplast or vacuole pellet (prepared as described above) to the corresponding medium. After a 10 s to 5 min incubation, with gentle manual shaking at room temperature (24°C), the uptake was terminated by centrifuging the microtubes in a Beckman Microfuge E at 14,250*g* for 20 s. The microtubes were then immediately snapfrozen in liquid N_2. Their tips containing the protoplasts or vacuoles were excised with a razor blade and placed into 3 ml tubes containing 0.5 ml of distilled water. After sonication for 1 min, 250 μl aliquots were used for the determination of radioactivity in 4 ml of Beckman Ready-Solv MP with a Beckman LS 1800 scintillation spectrometer. Further aliquots were used for the determination of protein and fructose.

Protein content of the pellets served as a measure of the number of protoplasts and fructose content as a measure of the number of vacuoles. As it has been previously noted that the procedure for vacuole preparation gave an artifactual 25% rise in the number of vacuoles (11), direct counting of the vacuoles was avoided and their number determined by a more correct indirect method. The number of vacuoles was calculated assuming (a) that *in vivo* one protoplast contains one vacuole, (b) that the total fructose content (in free fructose and sucrose) of protoplasts is located in the vacuole, and (c) that the vacuolar fraction is virtually free from protoplasts. The validity of these assumptions is discussed by Keller (11).

Polyamine Analysis. Polyamines were extracted, separated, and detected by the method of direct dansylation as described by Smith and Best (19) using precoated silica gel 60 TLC plates with a concentrating zone (Merck). The solvent was chloroform:triethylamine (5:1, v/v). Fluorescent spots were scraped off the plates and extracted with 2 ml of acetone. Fluorescence was measured with a Jasco FP 550 fluorescence spectrometer (excitation at 360 nm, emission at 505 nm). Quantification was performed by comparison with dansylated standards chromatographed under the same conditions, and the identity of the spots was confirmed by co-chromatography.

Determination of Fructose and Protein. Total fructose was measured colorimetrically with a modified cysteine and carbazole reaction according to Nakamura (13). Protein was measured by the method of Bradford (6) with bovine serum albumine as a standard.

Enzyme Assay. NAD-malate dehydrogenase and α-mannosidase were assayed as described by Keller (11).

RESULTS

Silicone Oil Centrifugation. The study of polyamine transport across membranes proved to be a difficult task because multiple charged cations polyamines have a strong tendency to bind to negatively charged sites of the membranes such as phospholipids (1, 16). In order to assess the extent of surface polyamine binding, isolated protoplasts and vacuoles were incubated with ^{14}C-labeled spermidine (6.6 μM) and putrescine (5.5 μM) for 5 min and then were separated from the incubation medium by rapid centrifugation through a layer of silicone oil with or without the insertion of a washing layer of excess unlabeled polyamine. Table I clearly shows that substantial binding of the polyamine to the plasmalemma and tonoplast does indeed occur. The insertion of an osmotically adjusted, aqueous washing layer of unlabeled polyamine proved to effectively remove the surface binding by an exchange process which seems to be very rapid and saturable at about 50 to 100 mM (Table I). The extent of the surface binding ranged from 50% (spermidine to the tonoplasts) to 91.5% (spermidine to the plasmalemma). It is therefore imperative that special attention be given to a careful distinction between surface binding and transport. Further evidence for binding of polyamines to membranes was obtained by incubation of protoplasts with [^{14}C]spermidine (6.6 μM) for 5 min in the presence of the impermeant polycations polylysine (mol wt = 3300) or La^{3+} (as $La[NO_3]_3$). Polylysine at 125 μM and La^{3+} at 1 mM showed the same effect as a washing layer of 100 mM unlabeled spermidine as they removed 91.9 and 89.5%, respectively, of the radioactivity associated with the protoplasts compared to the control (data not shown).

Table I. *Effect of the Purification Procedures Used in the Studies of Polyamine Transport into Protoplasts and Vacuoles of Carrot Taproot Cells*

Data are the mean ± SD of three different experiments.

^{14}C-Polyamine Supplied to	Concentration of Unlabeled Polyamine in Washing Layer	Radioactivity Recovered in Pellet
		dpm
Protoplasts		
Putrescine (5.6 μM)	No washing layer	14800 ± 3700 (100%)
	100 mM	2380 ± 550 (16%)
Spermidine (6.6 μM)	No washing layer	62100 ± 8100 (100%)
	10 mM	16800 ± 3200 (27%)
	50 mM	5600 ± 800 (9.0%)
	100 mM	5270 ± 690 (8.5%)
Vacuoles		
Putrescine (5.6 μM)	No washing layer	4830 ± 970 (100%)
	100 mM	1400 ± 290 (29%)
Spermidine (6.6 μM)	No washing layer	90000 ± 4700 (100%)
	100 mM	45000 ± 3500 (50%)

The possibility of a carryover of incubation medium during the silicone oil centrifugation was tested by incubation of protoplasts with [^{14}C]hydroxymethylinulin (3.7 kBq in 2 μl; specific activity: 492 MBq/mmol), which may be considered as a fairly inert and impermeant compound in carrot protoplasts. Centrifugation of the protoplasts through silicone oil removed at least 99.6% of the radioactivity present in the incubation medium.

Time Course of Spermidine Uptake and Binding. Spermidine uptake from a dilute polyamine solution (6.6 μM) into carrot taproot protoplasts and vacuoles was very rapid. As shown in Figure 1 the uptake of spermidine had reached a maximum after 1 to 2 min. Spermidine surface binding, as calculated from the difference between isolation with or without a washing layer of 100 mM unlabeled spermidine, was also very rapid. It had reached a maximum after 30 sec in the case of vacuoles (tonoplast) and 2 min in the case of protoplasts (plasmalemma).

No further binding nor uptake could be observed for incubation times of up to 20 min (data not shown). It was calculated that after 5 min the washed protoplasts and vacuoles had taken up 3.3 and 20% of the externally supplied spermidine, respectively.

Concentration Dependence of Spermidine Uptake. Spermidine uptake into protoplasts showed a linear dependence on the external concentration (6.6 μM to 100 mM) (Fig. 2). Uptake into isolated vacuoles, however, showed a biphasic concentration dependence. A saturable component below 1 mM (K_m = 61.8 μM) was followed by a linear component up to the highest concentration tested (50 mM).

In experiments carried out with high concentrations of external polyamines, the insertion of the washing layer was less effective in decreasing polyamine uptake: the spermidine taken up by washed protoplasts was 47, 64, and 100% of that taken up by unwashed protoplasts in the presence of an external spermidine concentration of 1, 5, and 10 mM, respectively.

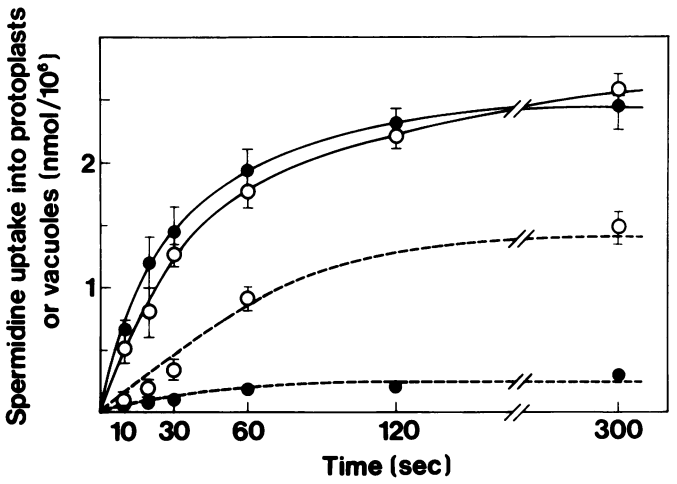

FIG. 1. Time course for spermidine uptake into carrot protoplasts and vacuoles. 3.7 kBq [^{14}C]spermidine (final concentration: 6.6 μM) were added to the incubation medium (error bars not given when smaller than symbols). Each point represented the mean ± SD of three different experiments. (●), Protoplasts; (○), vacuoles; (——), no washing layer; (- - -), washing layer with incubation medium plus 100 mM unlabeled spermidine.

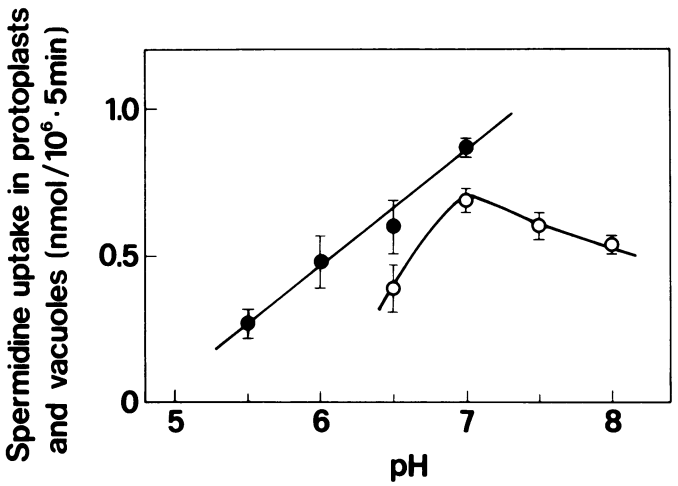

FIG. 3. Effect of pH on spermidine uptake into carrot protoplasts and vacuoles. 3.7 kBq [^{14}C]spermidine (final concentration: 6.6 μM) were added to the protoplast and vacuole medium containing the indicated buffer. The points are the mean ± SD of three different experiments. (●), Protoplasts (25 mM Mes-Tris); (○), vacuoles (25 mM Hepes-AMPD).

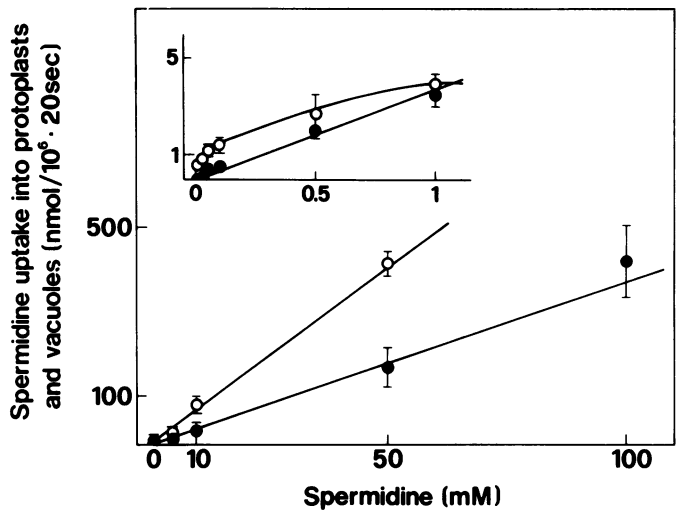

FIG. 2. Spermidine uptake into carrot protoplasts and vacuoles over a concentration range of 6.6 μM to 1 mM (system I) and of 1 to 100 mM (system II). 3.7 kBq [^{14}C]spermidine and unlabeled spermidine at various concentrations were added to the incubation medium for 20 s. Each point represents the mean ± SD of three different experiments. (●), Protoplasts; (○), vacuoles.

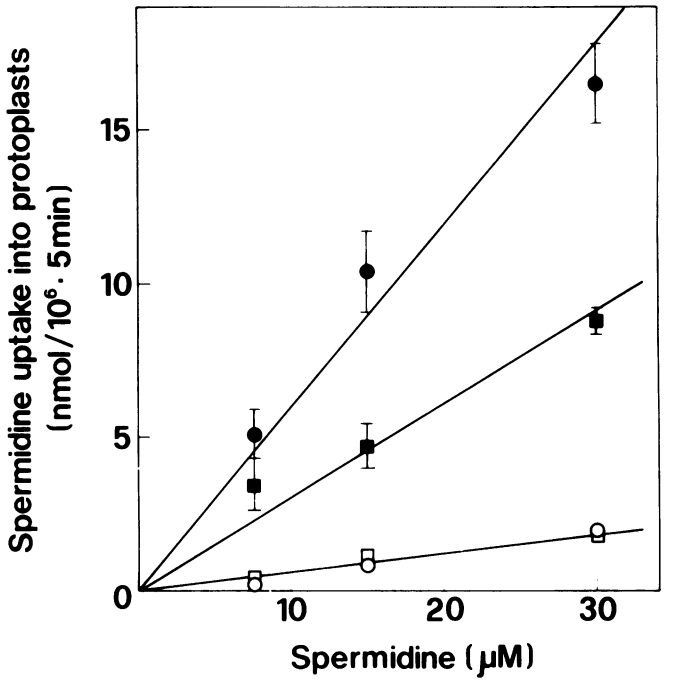

FIG. 4. Effect of preincubation of protoplasts with 1 mM Ca^{2+} plus or minus a washing layer with 100 mM unlabeled spermidine. Square symbols (■, □) represent the protoplasts preincubated with 1 mM Ca^{2+}, pelleted to eliminate excess calcium and then incubated with [^{14}C]spermidine (3.7, 8.5, and 16.6 kBq for the three different concentrations) with (□) or without (■) washing. Circles (○, ●) represent the controls incubated with [^{14}C]spermidine and then washed (○) or not (●). Each point represents the mean ± SD of two or three different experiments; error bars not given when smaller than symbols.

pH Dependence of Spermidine Uptake. Spermidine uptake from a 6.6 μM solution into protoplasts showed a linearly increasing pH dependence between pH 5.5 and 7.0 (Fig. 3). Uptake into isolated vacuoles, however, showed a distinct optimum at pH 7.0. This study was performed only with the useful range of the buffering capacity of the two buffers employed in the transport studies.

Effect of Calcium on Spermidine Uptake into Protoplasts. To understand the interactions between calcium and spermidine in regard to surface binding and uptake, protoplasts were preincubated with 1 mM Ca^{2+} for 5 min and then incubated with [^{14}C]spermidine at different concentrations (6.6, 15, and 30 μM) for 5 min. Figure 4 shows that spermidine uptake into protoplasts was unaffected by the preincubation with 1 mM Ca^{2+} (results after washing with 100 mM unlabeled spermidine). Surface binding of spermidine, however, was reduced considerably when the protoplasts were preincubated with Ca^{2+} (results without washing).

Subcellular Compartmentation of Polyamines. The content of

endogenous polyamines in cells of mature carrot taproot phloem parenchyma was shown to be 3.57 nmol putrescine/10^6 cells and 10.67 nmol spermidine/10^6 cells (Table II). Only traces of spermine but no other polyamines could be detected by the direct dansylation TLC method used. The putrescine content of the protoplasts was similar to that of the cells. The spermidine content of the protoplasts, however, was only one-third that of the cells, indicating that two-thirds of the cellular spermidine is located in the cell wall. A similar proportion of apoplastic spermidine was observed in cultured carrot cells (15).

By comparing the endogenous polyamine content of protoplasts with that of vacuoles isolated from them, it appeared that 42% and 28% of the intracellular putrescine and spermidine, respectively, was vacuolar in location (Table II). However, a preferential leakage of polyamines out of the vacuole during the isolation procedure cannot be ruled out. When vacuoles were preloaded with [^{14}C]spermidine (6.6 μM for 5 min), a rapid efflux was observed in the presence of 100 μM external spermidine (efflux of 58 and 69% of the accumulated spermidine in 30 and 40 s, respectively) or in the absence (64% in 40 s) (data not shown). Neither $La(NO_3)_3$ at 1 mM nor the polycation DEAE-dextran at 0.01 mg/ml was able to abolish the efflux of polyamines out of the vacuoles. To further elucidate the role of the vacuole as a possible storage site for polyamines, protoplasts were preloaded with spermidine by incubating them in the presence of 100 mM spermidine for 5 min and then isolating vacuoles without washing the protoplasts with unlabeled spermidine but only with the usual incubation medium. The results are shown in Table II. Preloading of protoplasts with spermidine did not alter the content and the vacuolar compartmentation of endogenous putrescine. However, the total spermidine content increased by a factor of almost 20 as compared to the original protoplasts. The vacuolar compartmentation of spermidine after preloading changed to about 8% of that present in protoplasts. In absolute terms, the spermidine content of the vacuoles increased by a factor of 6 after preloading as compared to the endogenous content, suggesting a transport of excess spermidine into the vacuole. A different approach was chosen when the compartmentation analysis was performed by incubating the protoplasts with 6.6 μM [^{14}C]spermidine, washing them through oil plus 100 mM unlabeled spermidine, and then isolating the vacuoles. The distribution of the [^{14}C]spermidine taken up between the vacuole and the cytoplasm was then calculated on the basis of the distribution of the marker enzymes for cytoplasm (NAD-malate dehydrogenase) and vacuoles (α-mannosidase) according to Keller (11). Up to 27% of the [^{14}C]spermidine was localized in the vacuole. However, care must be taken in the interpretation of this result as a redistribution due to surface binding and/or leakage of spermidine out of the vacuole cannot be ruled out.

An interesting side-effect of the preloading treatment of protoplasts with 100 mM spermidine was observed. It caused the protoplasts to be more stable, an effect which manifested itself in an incomplete lysis. Therefore, the impurity of the vacuoles was higher (12%) as compared to the controls (1%).

DISCUSSION

Protoplasts and vacuoles isolated from the phloem parenchyma of mature carrot taproots transport polyamines as rapidly as cultured carrot cells (15). In contrast to cowpea protoplasts, which begin to take up exogenous spermidine only after a lag period of about 20 h (9), there was no delay prior to uptake in carrot protoplasts and vacuoles. In the case of carrot cells, the apparently rapid uptake may be partially due to binding of polyamines to negative charges of the cell wall (15). This kind of binding was also shown in mung-bean hypocotyl cells (8). Although these complications inherent to the cell walls were eliminated in the present study by using protoplasts, the problem of possible interactions of the polyamines with the negatively charged phospholipids of the plasmalemma still remained. In fact it was demonstrated that polyamines bind to membrane phospholipids. Many papers suggest a role of polyamines in the stabilization of plant protoplasts *per se* (1) in addition to an inhibition of protease (10) and ribonuclease activity that delays senescence (17). Also, the decrease of betacyanin efflux from discs of beet root storage tissue (12) could probably be due to a stabilization of cell membranes. The recent work of Roberts *et al.* (16) using fluorescent

Table II. *Endogenous Polyamine Content of Carrot Taproot Cells, Protoplasts, and Vacuoles and Subcellular Localization of Exogenously Applied Spermidine (100 mM cold or 6.6 μM labeled Spermidine for 5 min) to Protoplasts*

The purity of the vacuoles isolated from the protoplasts was assessed by using NAD-malatedehydrogenase as a marker for extravacuolar contamination. The vacuoles were contaminated by 1 and 12% in the experiments concerning the endogenous and exogenous spermidine, respectively. When the protoplasts were preloaded with 100 mM spermidine, the concentration of glycinebetaine in the incubation medium was reduced from 0.7 to 0.3 M. Data are the mean ± SD of two or three different determinations.

	Putrescine	Spermidine
	nmol/10^6 cells or protoplasts or vacuoles	
Endogenous content in		
Cells	3.57 ± 0.54	10.70 ± 1.40
Protoplasts	3.23 ± 0.15 (100%)	3.74 ± 0.38 (100%)
Vacuoles	1.36 ± 0.23 (42%)	1.06 ± 0.16 (28%)
Exogenous content in protoplasts preloaded with 100 mM spermidine		
Protoplasts	2.98 ± 0.38 (100%)	73.70 ± 2.87 (100%)
Vacuoles	1.28 ± 0.33 (43%)	6.04 ± 0.10 (8%)
Exogenous content in protoplasts preloaded with 6.6 μM spermidine		
Protoplasts	ND[a]	0.41 ± 0.06 (100%)
Vacuoles	ND	0.11 ± 0.01 (27%)

[a] Not determined.

lipid-soluble probes, suggests that polyamines at physiological concentrations reduce membrane fluidity. The interactions between polyamines and membranes are further supported by our present findings that the washing of protoplasts and vacuoles with an unlabeled 100 mM polyamine solution reduced the amount of polyamine associated with them substantially at least at the lower external concentrations of external spermidine. This effect was greater with protoplasts than with vacuoles, reflecting a difference in the chemical composition of the plasmalemma and tonoplast and/or in the capacity for polyamine uptake. The decrease of spermidine association with protoplasts in the presence of permeant (Ca^{2+}) and nonpermeant (La^{3+} and polylysine) cations also points to a competition for the same anionic sites on the cell surface. In addition, Ca^{2+} seems not to be necessary for spermidine transport in protoplasts.

Apart from the surface binding, our results also demonstrate that polyamine transport does occur across the plasmalemma and tonoplast. This is supported, first, by the sixfold increase in endogenous spermidine content in vacuoles after administration of high (100 mM) concentrations of unlabeled spermidine to the protoplasts and, second, by the recovery of 27% of the radioactivity in the vacuoles with respect to the protoplasts after the addition of a low (6.6 μM) concentration of the labeled compound (Table II). This transport at the cellular level could explain the net translocation of polyamines from apple leaves to fruitlets and vice versa reported previously (2).

The differences between polyamine transport in protoplasts and vacuoles led us to the hypothesis that polyamines enter the cell by diffusion across the plasmalemma and are then transported further out of the cytosol into organelles, particularly into vacuoles, by a carrier-mediated process.

The observation in favor of a polyamine uptake into protoplasts by diffusion is the linearity of both the concentration- and pH-dependent uptake. The latter datum is in agreement with the general observation that basic substances usually permeate the membrane at pH values in which they are less protonated, although in our experiments we did not reach the pH value at which spermidine is unprotonated. The observations supporting the hypothesis of a carrier-mediated polyamine uptake into vacuoles are (a) the saturation kinetics observed in the concentration dependence study at the lowest concentration tested and (b) the narrow pH optimum observed for spermidine uptake in these organelles. Therefore, the tonoplast could be considered a site controlling the steps of polyamine uptake into cells. Further investigations on the driving forces responsible for polyamine transport into vacuoles are difficult because the organelles become easily unstable in the presence of many reagents. Thus, it is still unclear whether spermidine transport in carrot vacuoles occurs via facilitated diffusion or active transport.

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