Transport Properties of the Tomato Fruit Tonoplast[1]

II. CITRATE TRANSPORT

NANCY OLESKI[2], PEIMAN MAHDAVI, AND ALAN B. BENNETT*
Mann Laboratory, Department of Vegetable Crops, University of California, Davis, California 95616

ABSTRACT

Citrate transport across the membrane of tomato fruit tonoplast vesicles was investigated. In the tonoplast vesicles, [^{14}C]methylamine uptake was stimulated 10-fold by MgATP and strongly inhibited by NO_3^-. Under identical experimental conditions, [^{14}C]citrate uptake was inhibited by 5 millimolar free Mg^{2+}, and this inhibition was reversed in the presence of ATP, presumably by ATP chelation of free Mg^{2+}. No evidence was obtained in support of energy-linked ATP stimulation of citrate uptake. Citrate uptake showed saturation kinetics, and was inhibited by 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid and by other organic acids. The pH-dependence of uptake suggested that citrate^{3-} was the transported species. Our results indicate that citrate transport across the tomato fruit tonoplast occurs by facilitated diffusion of citrate^{3-}. The carrier shares some features in common with anion channels in that it is relatively nonspecific for organic acids and is inhibitable by 4,4'-diisothyocyano-2,2'-stilbenedisulfonic acid.

One of the major organic acids present in tomato fruit tissue is citric acid (6, 14). This acid accumulates to concentrations of 6 to 30 mmol per kg fresh weight in the whole fruit, and of 10 to 34 mmol per kg fresh weight in the pericarp tissue (6). It is likely that this high level of citric acid is largely confined to and indeed concentrated in the vacuole, and therefore must be transported across the tonoplast. The goal of the present study was to elucidate the mechanism(s) by which citric acid is transported into the vacuole of developing tomato fruit.

Although information on accumulation of organic acids in plant cell vacuoles is scarce, a few studies have contributed valuable insight into mechanisms that are operative in some plants. Lüttge and Ball (7) studied malic acid accumulation in the vacuole of the CAM plant *Kalanchoë daigremontiana* and concluded that malic acid in the malate^{2-} form moved passively into the vacuole across the tonoplast and became protonated to the H-malate^{-1} form. The H$^+$ available to protonate malate^{2-} anions is presumably pumped into the vacuole by an H$^+$-ATPase. Buser-Suter *et al.* (5) evaluated malic acid uptake into isolated vacuoles of *Bryophyllum daigremontianum* and suggested that a carrier protein transported malic acid across the tonoplast. In addition, they found the uptake rate of malic acid to be unaffected by ATP and various electrochemical gradient uncouplers. In contrast, Marin *et al.* (9) found citrate uptake in *Hevea* tonoplast vesicles to be stimulated by MgATP, implicating the involvement of the tonoplast H$^+$-ATPase in citric acid accumu-

lation. Marin and Chrestin (8) proposed the existence of a citrate/H$^+$ antiport in the tonoplast of *Hevea*. In their model H$_2$-citrate$^-$ was transported into the vacuole in exchange for H$^+$ and was sequestered in the nonpermeant form of H$_2$-citrate$^-$ or Mg-citrate$^-$. Uptake of malate into barley leaf vacuoles has also been shown to be stimulated by MgATP (10).

Based on these studies, it appeared that any of several mechanisms could be operative in citrate transport across the tomato tonoplast, including simple diffusion, facilitated diffusion, or carrier-mediated active transport, possibly by an antiport mechanism. It also appeared that the functional mechanism could be either dependent upon, or independent of the electrochemical gradient for H$^+$ established by the tonoplast H$^+$-ATPase.

MATERIALS AND METHODS

Plant Material. Tomato fruit (*Lycopersicon esculentum* cv UC82 were harvested from greenhouse or field grown plants (depending on availability) at the mature green stage (approximately 40 d after pollination).

Membrane Preparation. Tomato fruit tonoplast membranes were prepared as previously described (11) except that a 16%/30% discontinuous sucrose gradient interface was collected to increase yield of tonoplast vesicles.

Protein Determination. Protein was determined by the method of Schaffner and Weismann (13).

Citrate Uptake Assays. The typical reaction mix for these assays consisted of 50 mM Tris/Mes (pH 7.0), 50 mM KCl, and 3.6 μM [^{14}C]citrate (New England Nuclear; specific activity = 56.9 mCi/mmol) diluted to 33 μM with unlabeled citrate in a final volume of 110 μl. The reaction was started by the addition of 15 μl membrane vesicles (20–25 μg protein) to each tube at 1 min intervals. Citrate uptake was linear with time to 50 min, after which the rate of citrate uptake began to decrease. Determination of initial rates of citrate uptake were, therefore, measured using 30 min incubations. At the end of the incubation period the reaction was diluted with 5 ml of resuspension buffer (250 mM sucrose, 1 mM DTT, 5 mM Tris/Mes [pH7]) and rapidly filtered on millipore 0.45 μm HA filters. The filters were subsequently placed in 5 ml liquid scintillation fluid (Liquiscint, National Diagnostics) and counted.

Methylamine Uptake Assays. [^{14}C]Methylamine (New England Nuclear; 49.0 mCi/mmol) uptake assays were done in an identical manner to the citrate uptake assays. The concentration of methylamine in each assay was 1.15 μM.

H$^+$ Transport Assays. Formation of interior-acid pH gradients was monitored by fluorescence quenching of acridine orange (3). The reaction mix consisted of 5 mM Tris/Mes (pH 7.0), 125 mM sucrose, 5 mM MgSO$_4$, 25 mM KCl, 3 μM acridine orange, 25 mM K$_2$SO$_4$ or 50 mM KNO$_3$, and sodium citrate at 100 μM or 50 mM. The reaction was started by the addition of 10 μl of 0.5 M ATP.

[2] Present address: Plant Biology Laboratory, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92138-9216.

Free Mg^{2+} Calculations. Free Mg^{2+} was calculated as described by Bennett *et al.* (2).

RESULTS

Initial experiments in this study were designed to establish whether the tonoplast H^+-ATPase was active under the conditions used for citrate uptake assays. Therefore, [^{14}C]methylamine uptake was evaluated and compared to [^{14}C]citrate uptake, as shown in Table 1. MgATP greatly stimulated uptake of [^{14}C] methylamine, and NO_3^- inhibited uptake in the presence of MgATP. Mg^{2+} alone or Mg^{2+} in combination with NO_3^- were not effective in stimulating [^{14}C]methylamine uptake. These data indicated that the NO_3^- sensitive H^+-ATPase on the tonoplast was functional under the conditions used to evaluate citrate uptake.

Citrate uptake was also measured in the absence and presence of MgATP (Table I). Citrate uptake was progressively inhibited by 0.5 and 5 mM $MgSO_4$. The inhibition by 5 mM $MgSO_4$ was reversed by the addition of 5 mM ATP leading to an apparent stimulation of citrate uptake in the presence of ATP. The inclusion of 5 mM $MgSO_4$ and 5 mM ATP results in a concentration of free Mg^{2+} of 0.5 mM. When rates of citrate uptake are compared in the absence and presence of ATP when free Mg^{2+} is maintained at 0.5 mM it is apparent that citrate uptake is not stimulated by ATP. The inhibition of citrate uptake by free Mg^{2+} may be accounted for by formation of impermeant Mg-citrate$^-$. ATP chelation of Mg^{2+} liberates the permeant form of citrate, leading to the apparent stimulation of uptake by ATP. Interestingly, citrate uptake is inhibited by NO_3^- (Table I). This inhibition by NO_3^-, however, is not related to the known action of NO_3^- on the tonoplast H^+-ATPase (12) since NO_3^- inhibits citrate uptake to the same extent in the absence or presence of ATP, when free Mg^{2+} is maintained constant at 0.5 mM.

To investigate further the possibility of a relation between tonoplast H^+-ATPase activity and citrate uptake, various inhibitors and ionophores were evaluated in the absence and presence of MgATP (Table II). In the presence of KCl methylamine uptake was stimulated by ATP and inhibited to varying degrees by DCCD[3], CCCP, nigericin, and DIDS. In contrast citrate uptake was not stimulated by ATP, and DCCD, and CCCP and nigericin did not inhibit citrate uptake. The anion channel blocker DIDS, however, strongly inhibited citrate uptake. In the presence of K_2SO_4 ATP-dependent methylamine uptake was reduced relative

Table I. *Uptake of [^{14}C]Citrate or [^{14}C]Methylamine across Tonoplast Vesicles in Presence of Mg^{2+}, MgATP, and NO_3^-*

Assays were done in the presence of 50 mM Tris/Mes (pH 7.0) and 50 mM KCl at 28°C for 30 min, using 22 μg protein per assay. The concentration of KNO_3 was 50 mM. In order to hold K^+ constant, those assays without 50 mM KNO_3 contained 25 mM K_2SO_4.

Treatment	Uptake Rate			
	[^{14}C]Citric acid		[^{14}C]Methylamine	
	nmol/mg protein·h	% of control	nmol/mg protein·h	% of control
H_2O (control)	2.72	100	0.18	100
0.5 mM Mg^{2+}	2.46	90	0.28	155
5.0 mM Mg^{2+}	1.64	60	0.18	100
5.0 mM MgATP	2.54	94	2.10	1168
5.0 mM MgATP + NO_3^-	1.36	50	0.46	256
0.5 mM Mg^{2+} + NO_3^-	1.64	60	0.18	100

[3] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenyhydrazone, DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid.

Table II. *Effect of H^+-ATPase Inhibitors and Ionophores on Uptake of Citrate and Methylamine in Presence of KCl or K_2SO_4*

Assays were done in the presence of 50 mM Tris/Mes pH 7.0 and 50 mM KCl or 25 mM K_2SO_4 at 28°C for 30 min, using 15 μg protein per assay. In order to hold free Mg^{2+} constant, 0.5 mM $MgSO_4$ was added to the −ATP assays, while 5.0 mM $MgSO_4$ was added to the +ATP assays. Data for methylamine uptake is the difference in uptake measured in the presence and absence of MgATP.

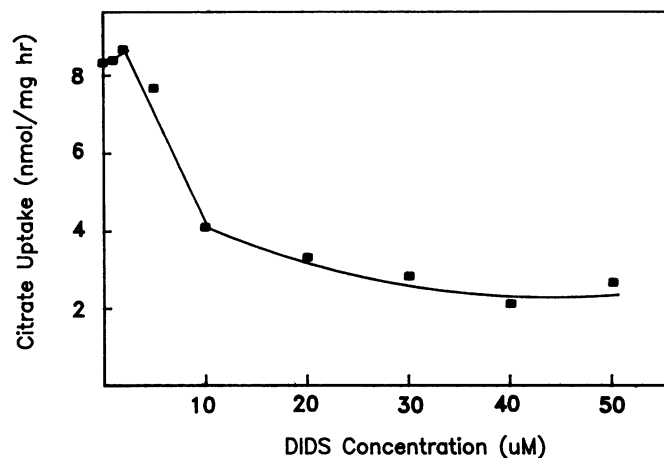| Treatment	Uptake Rate			
	Citrate		Methylamine	
			ATP-stimulated uptake	
	−ATP	+ATP		
	nmol/mg protein·h		nmol/mg protein·h	% of KCl control
+KCl	9.06	8.14	2.39	100
+ DCCD (50 μM)	9.29	8.23	1.82	76
+ CCCP (2 μM)	8.87	9.53	1.57	66
+ Nigericin (2 μM)	10.85	9.6	0	0
+ DIDS (25 μM)	1.07	1.27	0.41	17
+K_2SO_4	5.30	5.69	1.90	79
+DCCD (50 μm)	5.79	6.24	1.57	66
+CCCP (2 μM)	9.98	7.53	0.50	21
+Nigericin (2 μM)	6.67	6.87	0	0
+DIDS (25 μM)	0.32	0.51	0.16	7

FIG. 1. Concentration dependence of DIDS inhibition of citrate uptake in tomato fruit tonoplast vesicles. Assays were carried out as described in "Materials and Methods" with the indicated concentration of DIDS and 15 μg membrane protein.

to uptake in the presence of KCl. This is consistent with previous observations that Cl^- stimulates ΔpH formation by relieving formation of membrane potential (1). Citrate uptake in the presence of K_2SO_4 was somewhat reduced relative to uptake in the presence of KCl and was not significantly stimulated by ATP, indicating that citrate uptake is not driven by H^+-ATPase generated membrane potential. Citrate uptake was not inhibited by DCCD, CCCP, or nigericin (Table II) and in other experiments citrate uptake was similarly uneffected by valinomycin or gramicidin (data not shown). As in the presence of KCl, however, DIDS effectively inhibited citrate uptake in the presence of K_2SO_4. The inhibition of citrate uptake by DIDS was further evaluated over a range of DIDS concentrations and uptake was strongly inhibited at concentrations above 10 μM (Fig. 1).

Although the lack of inhibition of citrate uptake by ionophores suggested the H^+ gradients did not play a role in citrate transport, this was further tested by examining the effect of citrate on ATP-
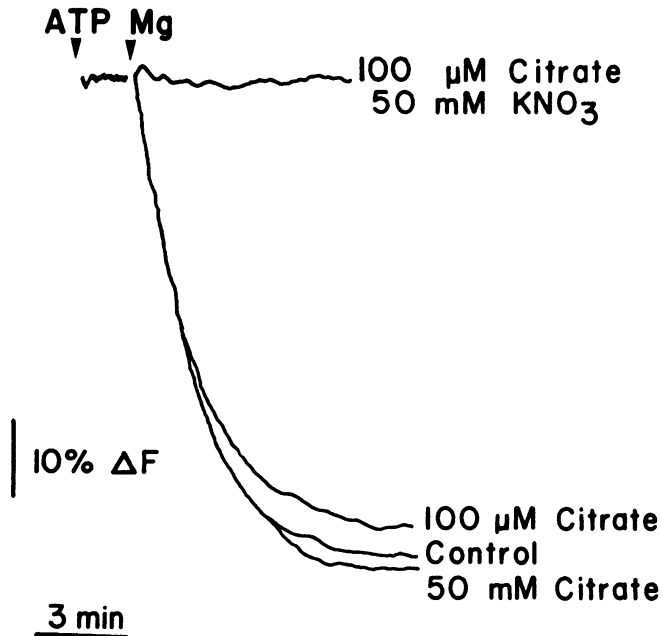
FIG. 2. The effect of citrate on ATP-dependent quenching of acridine orange fluorescence by tomato fruit tonoplast vesicles. Assays were carried out at 28°C with 15 μg membrane protein as described in "Materials and Methods."
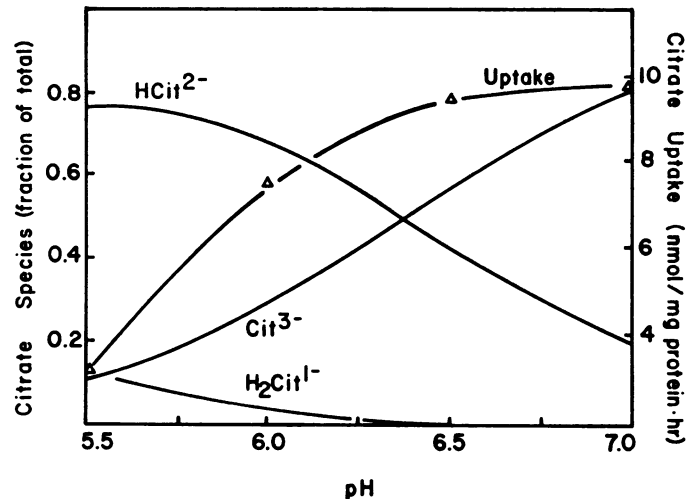

FIG. 3. pH dependence of citrate uptake in tomato fruit tonoplast vesicles. Assays were carried out as described in "Materials and Methods" except that pH was varied by varying the ratio of Tris/Mes. The levels of citrate ionic species were calculated using published values of PK_{a1} = 3.1, pK_{a2} = 4.8, and PK_{a3} = 6.4.

dependent pH gradient formation in the tonoplast vesicles (Fig. 2). As demonstrated using [^{14}C]methylamine as an indicator of pH gradient formation (Table I), quenching of acridine orange fluorescence also indicated that the ATP-dependent H$^+$ pump in tomato tonoplast vesicles is active (Fig. 2). H$^+$ transport was completely inhibited by NO$_3^-$, but unaffected by either 100 μM or 50 mM citrate. If H$^+$/citrate antiport or symport mechanisms were operative in these vesicles, the presence of citrate in the H$^+$ transport assay would be expected to alter the steady-state level of fluorescence quench. The lack of any effect of citrate on fluorescence quenching suggests that its transport is not directly coupled to H$^+$ fluxes.

Although citrate uptake did not appear to be either ATP or H$^+$ gradient-dependent, inhibition by DIDS suggested that a proteinaceous anion carrier may be involved in citrate transport. To characterize citrate transport further, the pH- and concentration-dependence of citrate uptake were examined. The pH dependence of citrate uptake indicated the citrate uptake was favored at pH above pH 5.5 (Fig. 3). When compared to ionic species of citrate (H$_2$-citrate$^-$, H-citrate^{2-} or citrate^{3-}) present over this pH range (Fig. 3), the uptake rate approximately paralleled the level of citrate $^{3-}$, suggesting that this species was the most permeant ionic form of citrate. Alternatively, the pH dependence may reflect the pH optimum of a carrier protein.

Uptake of citrate across tonoplast vesicles was saturable (Fig. 4, upper panel). However, when the data were transformed and graphed in a Hanes-Woolf plot, citrate uptake appeared to consist of two saturating components (Fig. 4, lower panel). Citrate uptake at low concentration (Fig. 4, lower panel inset) could be resolved as a saturating system with a K_m of 125 μM, while uptake at high concentration (Fig. 4, lower panel) indicated a second saturating system with a K_m of 1.5 mM. Because most of our experiments were performed at 33 μM citrate our results may reflect only the high affinity (i.e. K_m = 125 μM) uptake system.

The effect of other organic acids on citrate uptake was also examined (Table III). Citrate uptake was inhibited by all of the organic acids tested. The relative effectiveness of the organic acids in inhibiting citrate uptake was citrate > malate = succinate
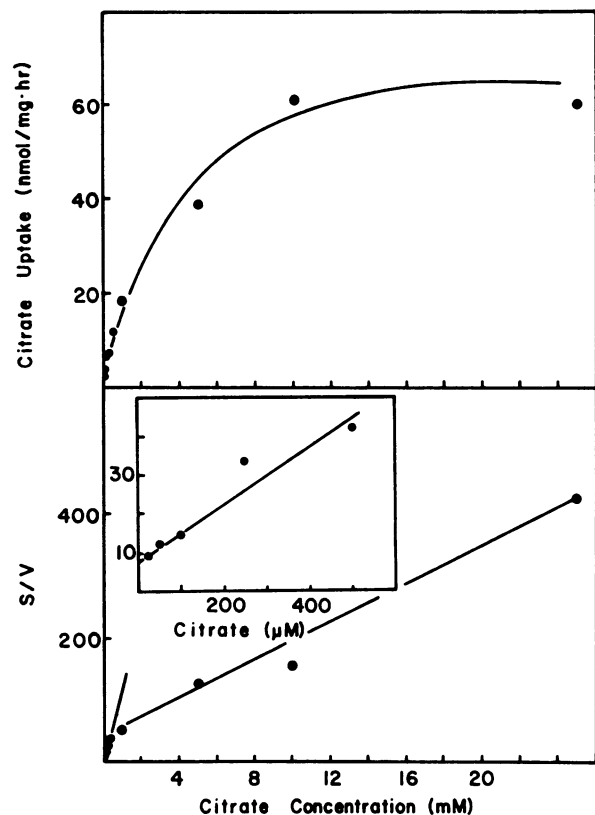

FIG. 4. Upper panel: concentration dependence of citrate uptake in tomato fruit tonoplast vesicles. Citrate concentration was varied between 25 μm and 25 mM and uptake measured as described in "Materials and Methods." Lower panel: Hanes-Woolf plot of data in upper panel. In this transformation two linear segments were apparent, indicating two saturating uptake mechanisms. The linear region at low concentration is expanded in the inset panel.

> isocitrate > fumarate ≫ oxaloacetate. The ability of other organic acids to inhibit citrate uptake suggests that the mechanism responsible for citrate uptake is saturable and relatively nonspecific for organic acids. Inorganic anions such as Cl$^-$ and

Table III. *Effect of Various Organic Acids on [^{14}C]Citrate Uptake across Tonoplast Vesicles*

All acids were present in the sodium salt form at 1 mM. Assays were done in the presence of 50 mM Tris/Mes pH 7 and 50 mM KCl at 28°C for 30 min, using 33 μg protein per assay.

Organic Acid	Rate of Citrate Uptake	
	nmol/mg·hr	*% of control*
Control	3.10	100%
Citric	0.48	16%
Malic	3.10	36%
Oxaloacetic	1.30	84%
Succinic	1.16	37%
Fumaric	1.46	47%
Isocitric	1.28	41%

SO_4^{2-}, however, did not effect citrate uptake (data not shown) indicating that organic and inorganic anions do not compete for common transport mechanisms.

DISCUSSION

The H^+-ATPase in tomato fruit tonoplast vesicles is active in citrate transport assays, as evidenced by ATP-dependent methylamine uptake. However, the activity of the H^+-ATPase does not affect the rate of citrate uptake, since citrate uptake is the same in the presence or absence of ATP, provided that free Mg^{2+} is held constant. Free Mg^{2+} inhibits citrate uptake, possibly by formation of impermeant Mg-citrate$^-$. Under certain experimental conditions ATP can lead to an apparent stimulation of uptake by a reversal of Mg^{2+} inhibition. The lack of inhibition of citrate uptake by DCCD or protonophores further indicates that in tomato fruit tonoplast vesicles the rate of citrate uptake is not dependent on activity of the H^+-ATPase.

Our results, especially with regard to ATP-dependence of citrate uptake, contrast with those of Marin and Chrestin (8) and Martinoia *et al.* (10) who show a stimulation of citrate uptake in *Hevea* tonoplast vesicles and of malate uptake in barley leaf vacuoles, respectively. Marin and Chrestin (8) suggest that H-citrate^{2-} is transported by antiport with H^+, thereby accounting for ATP-stimulation of uptake. While our results do not support this interpretation, ATP-stimulation of citrate uptake may alternatively be explained by facilitated uptake of citrate^{3-} followed by trapping of impermeant H-citrate^{2-} as suggested for malate accumulation in CAM mesophyll cells (9). In this case, ATP-dependent citrate uptake could result from the role of the H^+-ATPase in decreasing the pH of the vesicle lumen. While this should result in an increase in the final accumulation of citrate, this mechanism would not necessarily stimulate the rate of citrate uptake. Because we only measured initial rates of citrate uptake, our results failing to detect ATP-stimulation of the rate of citrate uptake could be consistent with this latter mechanism of organic acid accumulation in the vacuole.

Overall, our results indicate that citrate accumulation in the tomato fruit vacuole is not directly energy-dependent. Accumulation of citrate in the vacuole may occur by the formation of impermeant species including H-citrate^{2-} or Mg-citrate$^-$. In addition, accumulation of citrate may occur in response to membrane potential across the tonoplast. If we assume that citrate^{3-} is the most permeant form of citrate (via the proposed organic anion carrier) then a 15-300-fold accumulation of citrate between the vacuole and cytoplasm could be accounted for by a membrane potential of 30 to 50 mV across the tonoplast. At concentrations of 20 to 30 mM citrate in tomato fruit tissue (estimate from Davie and Hobson [6]), even conservative estimates of the tonoplast membrane potential would result in a cytoplasmic citrate concentration of less than 5 mM.

Citrate transport across the tomato tonoplast appears to operate by facilitated diffusion. This probably involves an anion carrier or channel, since DIDS effectively inhibits citrate uptake at low concentration (10 μM). Uptake of citrate through this carrier/channel is saturable and inhibition of citrate uptake by other organic acids suggest that the organic anion carrier/channel is not highly specific for citrate. Rather, it may be a common mechanism to facilitate the transport of a number of organic anions into the tomato fruit vacuole.

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