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Amine Transport in *Riccia fluitans*[1]

CYTOPLASMIC AND VACUOLAR pH RECORDED BY A pH-SENSITIVE MICROELECTRODE

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ABSTRACT

The cytoplasmic and vacuolar pH and changes thereof in the presence of ammonia (NH_4Cl) and methylamine (CH_3NH_3Cl) have been measured in rhizoid cells of *Riccia fluitans* by means of a pH-sensitive microelectrode.

On addition of 1 micromolar NH_4Cl, the cytoplasmic pH of 7.2 to 7.4 drops by 0.1 to 0.2 pH units, but shifts to pH 7.8 in the presence of 50 micromolar NH_4Cl or 500 micromolar CH_3NH_3Cl. The pH of the vacuole increases drastically from 4.5 to 5.7 with these latter concentrations. Since a $NH_4^+/CH_3NH_3^+$ uniporter has been demonstrated in the plasmalemma of *R. fluitans* previously (Felle 1983 Biochim Biophys Acta 602:181–195), the concentration-dependent shifts of cytoplasmic pH are interpreted as results of two processes: first, acidification through deprotonation of the actively transported NH_4^+; and second, alkalinization through protonation of NH_3 which is taken up to a significant extent from high external concentrations. Furthermore, it is concluded that the determination of intracellular pH by means of methylamine distribution is not a reliable method for eucaryotic systems.

Reports on amine transport across membranes can be followed back to the last century (8). Though unspecific diffusion of NH_3 has been favored by some authors (8), evidence for carrier-mediated NH_4^+ transport has been accumulating in the last decade (8). In these studies, the nonmetabolized methylamine has been used as a model substrate for NH_3/NH_4^+. But methylamine is also used for estimating the ΔpH across biological membranes, especially if these are not accessible to standard electrophysiological techniques. This method is based on the proven concept that the unprotonated and thus uncharged amine base is able to permeate a lipid membrane far better than the protonated and positively charged species (8). Since the pK of methylamine (10.65) is far from any known intracellular pH, the unspecific diffusional equilibration of CH_3NH_2 across a given membrane builds up a $CH_3NH_3^+$ gradient which, according to the Hasselbalch-Henderson equation, reflects the pH gradient in question. However, a carrier-mediated transport of the protonated form would make the estimation of ΔpH by this method unreliable. Vice versa, unspecific transmembrane diffusion of the unprotonated amine must be considered in studies of carrier-mediated amine transport. Analysis becomes even more complicated by the intracellular compartmentation into cytoplasm and vacuole; clearly, carrier-mediated transport and unspecific diffusion of amines are operating in series across plasmalemma and tonoplast.

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In early reports it has been stated that addition of ammonium salts to the external medium cause a rapid alkalinization of the cell interior (8). Reliable information on such changes in the intracellular pH is essential for analysis of proton-dependent transport processes including amine transport.

Obviously, the intracellular pH has to be monitored continuously and independently in both cytoplasm and vacuole. We feel that only pH-sensitive microelectrodes can provide this information. Sanders *et al.* (10) adapted the Thomas electrode (recessed tip glass-glass electrode [11]) for penetration of rigid cell walls like those of *Neurospora*. The response time of $t_{1/2} \approx 8$ s was sufficient to record pH changes occurring in the presence of CN^- or weak acids. Since in *Riccia* the membrane depolarization in the presence of NH_4Cl or CH_3NH_3Cl is very fast, *i.e.* greater than 50 mv/s (3), we had to turn to the faster ion-exchanger filled glass electrode (1). By means of this electrode, the effects of amine transport and dissociation on intracellular pH have been measured in rhizoid cells of *Riccia fluitans*; in this aquatic liverwort, the existence of a high-affinity $NH_4^+/CH_3NH_3^+$ uniporter has been demonstrated previously (3).

The results are discussed with respect to amine transport and, second, to the established methylamine technique of pH determination.

MATERIALS AND METHODS

Culture Conditions. Green thalli of the acquatic liverwort *Riccia fluitans* were cultured under semisterile conditions in deep Petri dishes similar to the method developed by Hüsemann and Barz for suspension cultures of *Chenopodium rubrum* (7). The growth medium contained 1% of the medium of Murashige and Skoog (9), and 99% of a medium which contained 0.1 mM KCl, 0.1 mM $CaCl_2$, and 2 mM sodium phosphate buffer; external pH was 6.5. One to two h before measurement, the plants were transferred into the test medium, which contained 2 mM Tris-buffer, 1 mM KCl, 1 mM NaCl, and 1 mM $CaCl_2$; external pH was adjusted to 8.0 with 1 N HCl. All other conditions were identical to those described previously (5).

Electrical Experiments. The measurement of membrane potential was carried out by means of standard electrophysiological technique (3). The bilateral open Plexiglas chamber permitted the controlled approach of two oppositely directed microelectrodes, one of which monitored the voltage difference across the membrane, the other was the pH-sensitive microelectrode. Since the pH electrode always detects the sum of membrane potential plus the e.m.f. corresponding to the pH difference between the electrodes, a high impedance (10^{15} ohm) differential amplifier (W. P. Instruments, New Haven, CT, model FD 223) recorded and immediately subtracted the two signals. These three traces were recorded separately on a pen chart (Kontron, W&W 314).

Fabrication of the pH-Sensitive Microelectrode. Glass capillaries with solid filament (Hilgenberg) were acid-cleaned overnight in 15% chrome-sulfuric acid, rinsed six to eight times in

distilled H_2O, and then dried at 120°C. The clean capillaries were then pulled on a vertical electrode puller (Getra, Weilheim) to make electrodes with tips of roughly 0.4 μm diameter. The electrodes were dipped with the blunt end into a mixture of 0.2% dimethyl-dichloro-silane/benzene and baked at 180°C for 30 min to provide a water-repellent interior surface of the capillary. For further stabilization of the tips, 0.1% polyvinylchloride dissolved in tetrahydrofuran was sucked into the tip. By means of a second glass capillary, the proton exchanger resin (Fluka, Buchs, No. 82500) was backfilled into the tip, free of air bubbles. The remainder of the capillary was filled with 3 M KCl. The thus prepared pH-electrodes were stored until use in a sealed damp chamber with the tips dipped into 0.5% chrome-sulfuric acid. Typically, the electrodes had a resistance of 5 to $8 \cdot 10^{10}$ ohms, and displayed a slope of 56 to 58 mv per pH unit between pH 4 and pH 9, as shown in Figure 1.

Tracer Experiments. Uptake of [^{14}C]methylamine was measured on green *Riccia* thalli, as described before (3).

RESULTS AND DISCUSSION

The Uniporter of $NH_4^+/CH_3NH_3^+$ at the Plasmalemma of *Riccia fluitans*. Figures 2 and 3 illustrate some of the more noticeable phenomena of amine transport at the plasmalemma of *R. fluitans*. Very low external concentrations of NH_4Cl and CH_3NH_3Cl depolarize the plasmalemma rapidly and reversibly with half-maximal depolarization at about 2 μM NH_4Cl and about 20 μM CH_3NH_3Cl, respectively. Spontaneous repolarization, as usually observed with cotransport systems (*e.g.* amino acids [6]), only occurs in the presence of low amine concentrations (Fig. 2). Both uptake of [^{14}C]methylamine and the electrical net current triggered by the addition of methylamine are concentration dependent and follow similar saturation kinetics (Fig. 3). The apparent half-maximal saturation compares well with the half-maximal depolarization derived from Figure 2.

NH_3/H^+ Cotransport? With respect to the current cotransport hypothesis it could be suspected that the previously proposed $NH_4^+/CH_3NH_3^+$ uniport might actually be a proton-driven NH_3/CH_3NH_2 cotransport. Apart from the fact that this mechanism would require an extremely high affinity of the carrier for NH_3 or CH_3NH_2 of only a few nmol, Figure 4 clearly disproves this idea of a H^+/amine-cotransport: the pH-dependence of [^{14}C] methylamine uptake displays a typical pH-scan along the dissociation curve of methylamine. Between pH 5 and 7.7 [^{14}C] methylamine uptake is essentially constant and very likely represents mainly the uptake of the protonated base. This is followed by a much steeper increase of uptake between pH 7.7 and 10.3, where presumably the high permeability of the membrane to the unprotonated base increasingly dominates the uptake of methylamine. The indicated course of the protonmotive force ($\Delta\tilde{\mu}_H^+/F$) clearly is different and rules out a proton cotransport mechanism for amine transport. Also, at pH 10, depolarization of the plasmalemma in the presence of NH_4Cl (not shown) is reduced according to the low NH_4^+ concentration at this pH compared with lower pH (pK = 9.25).

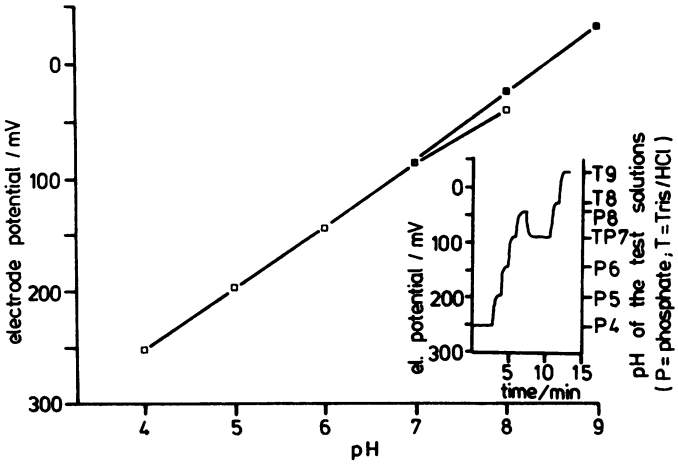

FIG. 1. Calibration curve of a typical ion exchanger pH microelectrode in sodium-phosphate buffer (□——□) and Tris/HCl buffer (■——■); other ions: 1 mM KCl, 1 mM $CaCl_2$, 10 mM NaCl. Reference electrode: 3 M KCl. Inset: Original trace of electrode response to external pH.

Repolarization. During amine accumulation the cell membrane, after initial depolarization, could repolarize for different reasons: first, the hyperpolarizing proton pump (4) may increase its activity upon depolarization and second, because of substrate accumulation within the cytoplasm and subsequent reduction of the driving force for the amine influx. Figure 2 shows that repolarization only occurs at low amine concentrations, whereas at higher concentrations the plasmalemma remains depolarized. An inhibition of oxidative or photosynthetic electron transport by the amine concentrations used in this study is not taken into consideration, since a slight increase in cellular ATP (not shown) rather than a decrease has been detected. This may point to an inhibition of an ATP consumer; for instance, the electrogenic proton pump (4).

Intracellular pH. *(a) Cytoplasmic pH.* The cytoplasmic pH of *R. fluitans* recorded by a pH-sensitive microelectrode was 7.2 to 7.4 (Fig. 5). Addition of 1 μM NH_4Cl or 50 μM CH_3NH_3Cl caused a transient acidification of the cytoplasm. Upon step-wise increase of the external amine concentration to 500 μM CH_3NH_3Cl and 50 μM NH_4Cl, respectively, an immediate alkalinization of the cytoplasm of 0.3 to 0.5 pH units is observed. As Figure 6 demonstrates, this alkalinization also occurs if only the high amine concentrations are added. Removal of the amine from the perfusion fluid rapidly restores the original pH_{cyt} because of amine efflux from the cell.

(b) Vacuolar pH. The recorded vacuolar pH of rhizoid cell is 4.5 to 4.6, confirming an earlier estimate on thallus cells of this plant (2). Addition of 10 and 100 μM methylamine caused a fast and drastic alkalinization of the vacuole (Fig. 7) which clearly exceeds that observed with the cytoplasm. While in the presence of 10 μM methylamine no significant pH change could be detected in the cytoplasm, the vacuolar pH shifted from 4.5 to 4.7, whereas 100 μM methylamine caused shifts of 1.2 pH units. How can these pH changes be explained?

Addition of the amine sets up a concentration gradient of the unprotonated base from outside to the cytoplasm causing a net influx of that molecule into both cytoplasm and vacuole. According to the dissociation curve of the amines (pK is 9.25 for ammonia and 10.65 for methylamine), the amine base will take up protons from the cytoplasm to form NH_4^+ or $CH_3NH_3^+$, respectively. This diffusional flux of the amine base is obviously sufficiently high so as to shift the cytoplasmic pH from 7.3 to 7.8 despite the buffer capacity, provided a reasonably high amine concentration is given. At an external pH of 8, addition of 500 μM methylamine and 20 μM ammonia will establish equal concentrations of the unprotonated base, *i.e.* $1.12 \cdot 10^{-6}$ M NH_3 and $1.06 \cdot 10^{-6}$ M CH_3NH_2. Figure 6 indeed demonstrates comparable changes of the cytoplasmic pH of 0.4 to 0.5 units. However, Figure 5 also shows that small amine concentrations do not result in alkalinization, but slightly acidify the cytoplasm.

Why did the vacuolar pH change so much more than the cytoplasmic pH (Fig. 7)? The cell sap obtained from *Riccia* thalli has a pH of roughly 6. Considering the volume ratio of the cytoplasm to vacuole of approximately 1 to 10, the pH of the cell sap should effectively indicate the vacuolar pH. In fact, the

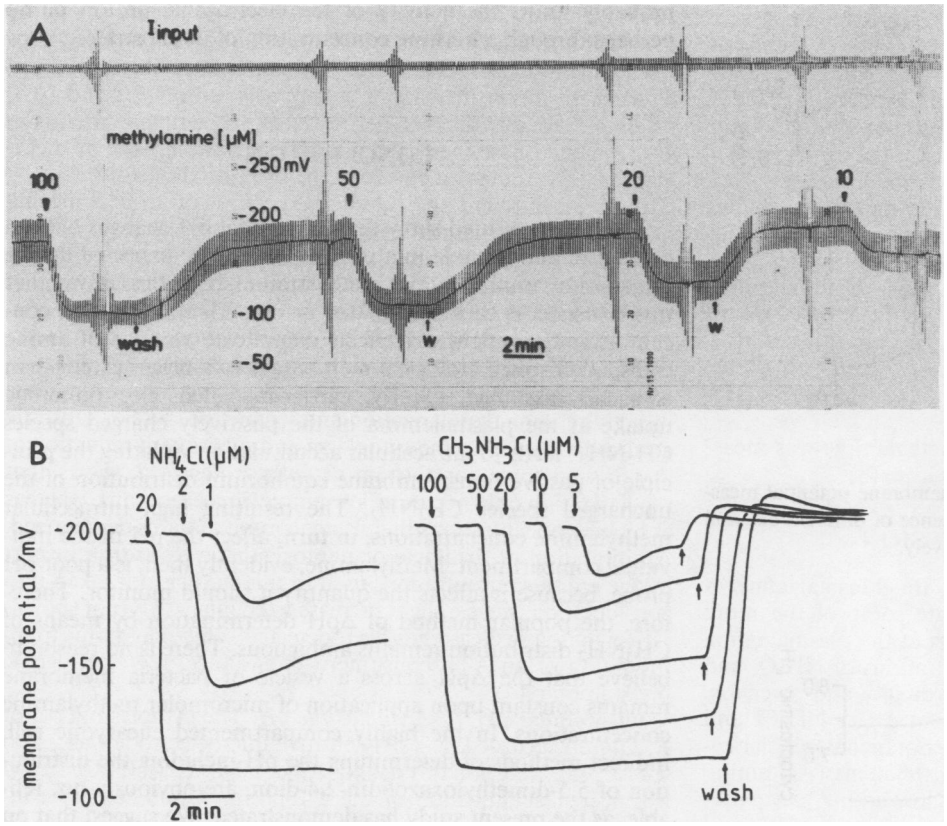

FIG. 2. A, Original record of membrane potential and membrane resistance of *R. fluitans* showing the effect of varying external methylamine concentrations. The upper trace gives the input current pattern superimposed upon the membrane potential (lower trace). B, Depolarization and spontaneous repolarization in the presence of different concentrations of NH_4Cl and CH_3NH_3Cl.

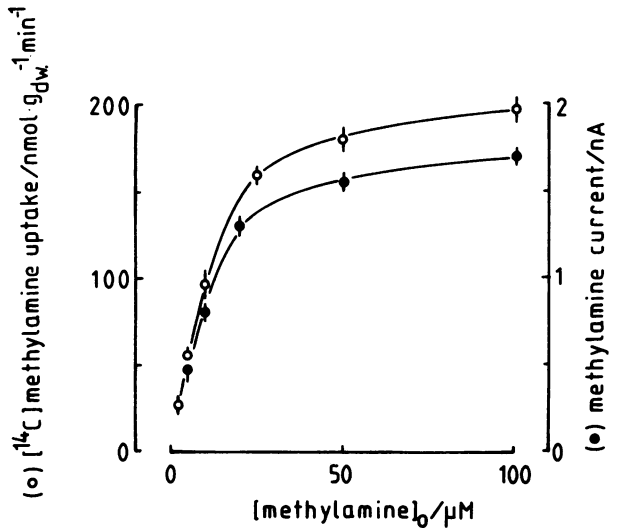

FIG. 3. Comparison of half-maximal saturation of (a) [^{14}C]methylamine uptake (1 min) into green thalli, and (b) methylamine-induced electrical current measured on rhizoid cells of *R. fluitans*.

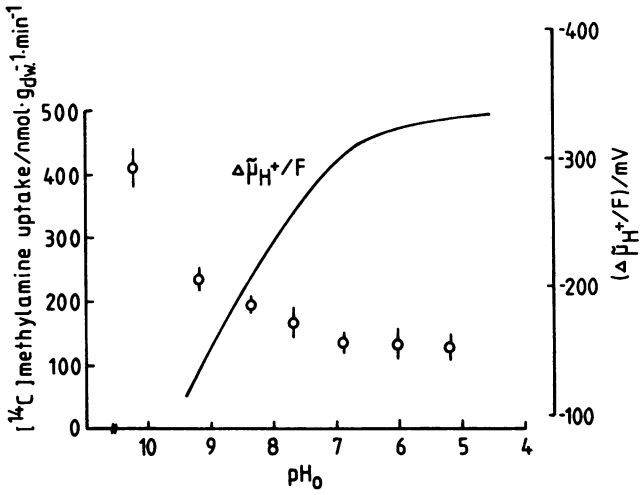

FIG. 4. [^{14}C]methylamine uptake into green thalli of *R. fluitans* as function of external pH from 5 to 10. Buffer: Mes/Tris 5 mM. The protonmotive force ($\Delta\tilde{\mu}_{H^+}/F$) indicates that [^{14}C]methylamine uptake is not a function of ΔpH.

vacuolar pH is still more acid, probably because the vacuole is not as well buffered as the cytoplasm. Since the diffusional distance from the outside medium to the vacuole is short (1–2 μm), no significant lag exists between amine addition and vacuolar signals (Fig. 7). Finally, active electrophoretic uptake of NH_4^+ causing initial acidification of the vacuole, does not exist at the tonoplast according to the pH trace of Figure 7. Thus, diffusional transport of NH_3 across the tonoplast and subsequent protonation explains the observed strong alkalinization of the vacuole. This observed marked change of the vacuolar pH upon addition of methylamine is plausible because of the high methylamine accumulation in this very acid compartment.

Does the Proton Pump React to Changes in Cytoplasmic pH? Protons are the substrate for an electrogenic pump at the plasmalemma of *R. fluitans* (4). It has been shown in *Riccia* and in *Sinapis* root cells (H. Felle, unpublished data) that acidification of the cytoplasm stimulates the pump. Thus, alkalinization could decrease pump activity. This decrease could well lead to depolarization of the plasmalemma independently of the depolarization caused by the carrier-mediated NH_4^+ or $CH_3NH_3^+$ transport. But while this latter transport slows down with time, leading to ultimate repolarization, the persisting cytoplasmic alkalinization

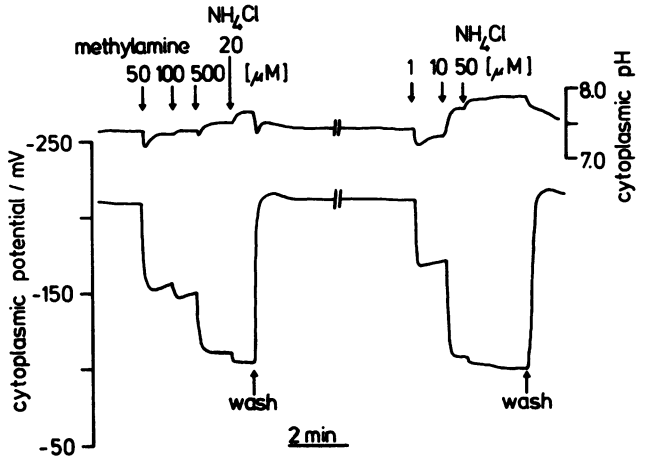

FIG. 5. Changes in cytoplasmic pH and membrane potential measured in rhizoid cells of *R. fluitans* in the presence of different external NH_4Cl and CH_3NH_3Cl concentration, respectively.

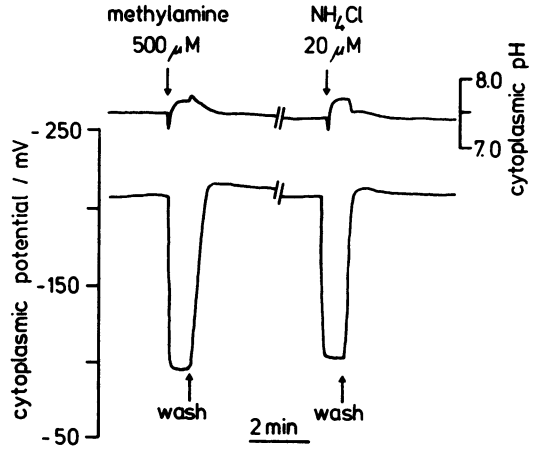

FIG. 6. Cytoplasmic alkalinization and change in membrane potential measured in rhizoid cells of *R. fluitans* in the presence of 20 μM NH_4Cl and 500 μM CH_3NH_3Cl, respectively.

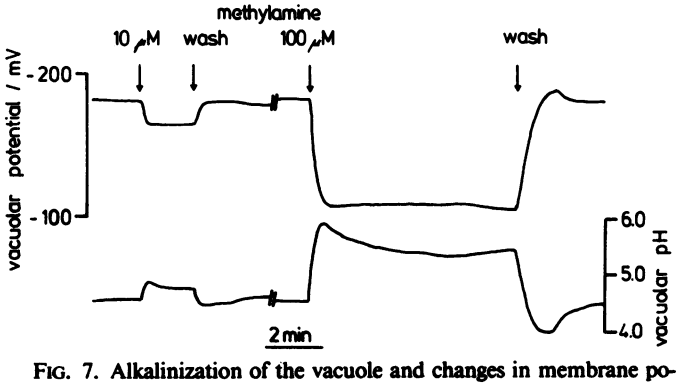

FIG. 7. Alkalinization of the vacuole and changes in membrane potential measured in rhizoid cells of *R. fluitans* in the presence of 10 and 100 μM CH_3NH_3Cl.

probably limits the activity of the electrogenic proton pump perhaps through a limiting concentration of its substrate.

CONCLUSIONS

The data presented show that substantial pH changes of both cytoplasm and vacuole in a plant cell are to be expected during transport of ammonia (and methylamine), regardless of whether this transport is carrier-mediated or not. These time- and concentration-dependent pH effects complicate analysis of amine transport through biological membranes to a bigger extent than is usually assumed. Clearly, carrier-mediated, electrophoretic uptake at the plasmalemma of the positively charged species $CH_3NH_3^+$ leads to intracellular accumulation violating the principle of passive transmembrane equilibrium distribution of the uncharged species CH_3NH_2. The resulting high intracellular methylamine concentrations, in turn, affect the pH of the individual compartment. Methylamine, evidently then, is a poor pH probe, because it affects the quantity it should monitor. Therefore, the popular method of ΔpH determination by means of CH_3NH_2 distribution remains ambiguous. There is no reason to believe that the ΔpH across a vesicle or bacteria membrane remains constant upon application of micromolar methylamine concentrations. In the highly compartmented eucaryotic cell, indirect methods of determining the pH including the distribution of 5,5-dimethyloxazolidin-2,4-dion, are obviously not reliable, as the present study has demonstrated. We suggest that on eucaryotic cells a reliable pH measurement can only be accomplished by use of pH-sensitive microelectrodes.

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