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Amine Ion Porter in *Chara australis:* Effects of Alkyl Substitution and External pH

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Summary. The rate of transport of amine ions into *Chara australis* internodes is studied by measuring changes in membrane current when amine solutions are presented to voltage-clamped cells. The dependence of this rate on ion concentration is investigated for a series of alkyl-amine ions: methyl-, ethyl-, isopropyl-, dimethyl-, trimethyl- and tetramethylammonium. A Michaelis-Menten relationship is displayed by all except tri- and tetramethylammonium, where currents are irregular and difficult to reproduce. Evidence suggests that the different ions cross the plasmalemma via a common uniport. K_M values for this porter increase as the amine ion becomes more highly substituted. The V_m values are similar for all amines and lie within the range 10 to 100 mA m^{-2} (for cell potential at −200 mV). The changes in K_M indicate that hydrogen bonding may be involved in the binding interaction. V_m varies with external pH in a way which suggests that an ionizable group on the transport protein with pK_a ~5.8 directly affects the transport rate. K_M is independent of external pH over the range 4.5 to 10.5.

Key Words amine · porter · *Chara australis*

Introduction

The classic view of amine transport into plant cells was that only passive diffusion of the neutral species was likely to occur (*see*, e.g., Cooper & Osterhout, 1930). This view originated in experiments in animal cells (e.g., Chambers, 1922).

Studies of the effects of ammonium and of external pH on methylammonium transport suggest the existence of specific cation porters in a variety of cell types: bacteria (Stevenson & Silver, 1977; Bellion & Wayland, 1982), marine diatoms (Wheeler, 1980; Wheeler & Hellebust, 1981), fungi (Hackette et al., 1970; Roon et al., 1975; Cook & Anthony, 1978), algae (Smith, Raven & Jayasuriya, 1978; Wheeler, 1979), Charophytes (Smith, Walker & Raven, 1977; Smith & Walker, 1978; MacFarlane & Smith, 1982) and terrestrial angiosperms (Smith, 1980*b*; Raven & Farquhar, 1981).

Direct observations of changes in membrane electric potential difference (PD) or current, attributed to electrogenic amine cation transport, have been reported for *Neurospora* (Slayman & Walker, *see* Slayman, 1977), *Hydrodictyon* (Smith et al., 1978) *Chara, Nitella* (Smith & Walker, 1978) and *Riccia* (Bertl, Felle & Bentrup, 1984). Electrogenic transport can be monitored by measuring membrane current changes in voltage-clamped cells. Changes in membrane current on presentation of amine to voltage-clamped *Chara* cells were measured by Walker, Smith and Beilby (1979*b*) and were shown to be equal to the amine flux over the pH range 6–9 by Walker, Beilby and Smith (1979*a*). This identified the major transport process as an (electrogenic) amine ion uniport. There was significant entry of uncharged free base at pH greater than 9.

The study quoted found that V_m values for ammonium and methylammonium transport were similar and ranged from 10 to 100 mA m^{-2} in different cells. K_M values were ~3 μM for ammonium and ~250 μM for methylammonium. It was earlier shown that these two amines enter *Chara* by the same porter, since the saturated current is the same for either amine separately as for both together (Smith & Walker, 1978).

This paper reports studies of the transport of a number of substituted amines into voltage-clamped *Chara* cells. We investigated (1) the effect of alkyl substitution of the substrate on the transport parameters and (2) the effect of external pH on methylammonium transport.

Materials and Methods

Axial internodal cells of *Chara australis* R. Br of length 30–70 mm were harvested from plants grown in outdoor tanks and maintained for 1–2 days in a standard experimental medium (SW) containing (in mM): NaCl, 1.0; $CaSO_4$, 0.05; KCl, 0.2; zwitterionic buffer, 5; NaOH was required to adjust pH.

Cells were then mounted into a three compartment experimental chamber. A glass microelectrode was inserted into the cell vacuole in the central compartment (8 mm wide) for measurement of the PD across the plasmalemma and tonoplast in series. This PD was clamped to −200 mV: clamp current was supplied via external Ag-AgCl electrodes in the two outer chambers and recorded after passing through the cell and across both membranes via an external Ag-AgCl electrode in the central compartment. The rest of the electrical apparatus was as described by Walker et al. (1979*a*). The central compartment was continually irrigated with SW. Changes in clamp current were observed during brief introductions of amine into the flowing solutions.

The amines used, with their abbreviations and pK_a values (*cf.* Sober & Harte, 1968; Weast & Selby, 1968) were:

ammonium	—	9.25
methylammonium	MA	10.66
ethylammonium	EA	10.81
isopropylammonium	IPA	10.71
dimethylammonium	DMA	10.73
trimethylammonium	T3MA	9.81
tetramethylammonium	T4MA	—

These were obtained from Sigma Chemical Co. and were added to the SW as chlorides. In the case of DMA, T3MA and T4MA, the range of solutions required was made up with constant ionic strength, using NaCl (or occasionally LiCl) as supplement. This precaution was not thought necessary for MA solutions, since MA was used at low concentrations; it was inadvertently ommitted when EA and IPA solutions were prepared. (Variations in chloride ion concentrations during EA and IPA experiments are not considered significant, as chloride currents are very small (less than 10 mA m^{-2}, even when enhanced by the presence of ammonium or MA (Smith, 1970, 1980*a*) and saturate by 100 μM (Beilby & Walker, 1981).) All solutions were prepared using glass-distilled water that had been passed through a column of Dowex-50 cation exchange resin, in the acid form. This reduced the exposure of cells to ammonium (otherwise present in the water), which significantly depresses amine uptake (Walker et al., 1979*a*).

Different concentrations of the various amine solutions were applied to cells in varying orders and 2 or 3 times each. To obtain values for the transport parameters, K_M and V_M, current/concentration data were fitted to the Michaelis-Menten equation (Michaelis & Menten, 1913) using a Fortran program calling subroutines for least squares fitting from the NAG library.

Experiments were performed at pH 7.5 except for MA, where data were collected at pHs 4.5–10.5. The buffers used, each at or near its pK_a, were MES, PIPES, HEPES, TAPS, CHES and CAPS.[1]

Cytoplasmic streaming rates were measured before and after the exposure of each cell to amine solutions. These were compared using the Wilcoxon Signed-Rank Test. Consistency of streaming rates was taken to indicate consistency of cell condition, given that streaming rate depends on the cytoplasmic ATP concentration (Williamson, 1975; Reid & Walker, 1983.)

[1] *Abbreviations:* MES, 2-(N-morpholino)ethanesulfonic acid; PIPES, piperazone-N,N′-bis(2-ethanesulfonic acid); HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; TAPS, ((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino-1-propanesulfonic acid; CHES, 2-(N-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

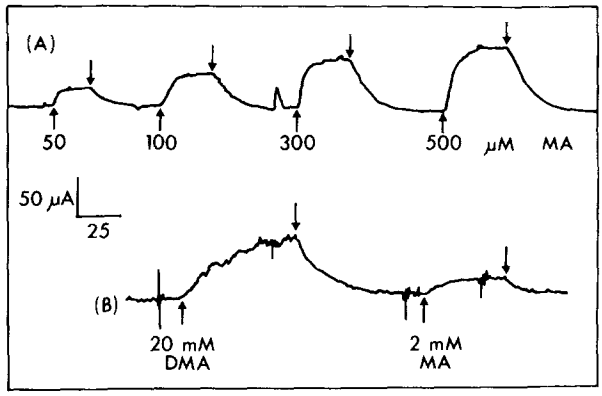

Fig. 1. Time-course of membrane current, showing effect of introducing amine into the fast-flowing medium, and of then removing it (unmarked arrows). (*A*) Methylammonium, *MA*. (*B*) Dimethylammonium, *DMA*, and *MA* in the same cell

Results

Effect of Amines on Rate of Cytoplasmic Streaming

Cytoplasmic streaming was significantly slower after exposure of cells to DMA and significantly faster after exposure of cells to a combination of MA and EA ($\alpha = 0.05$). These amines may have affected cell condition, or these two cases may merely reflect random fluctuations of streaming rate. (The Wilcoxon Test was applied 19 times.) It is noted that less than 25% of a cell's surface was ever exposed to amine.

Effect of Amine Solutions on Membrane Current

Rapid, reproducible changes in membrane current were observed when cells were briefly exposed to MA, EA and IPA solutions: these were in the depolarizing direction (Fig. 1*A*). Current changes were roughly exponential with half times about 2–3 sec for solutions flowing at 25–30 ml min^{-1}. In most instances, the current remained constant after 5–15 sec and then during the amine exposure (20–50 sec) before returning to the original value when the amine was washed out (SW irrigation; Fig. 1*A*).

During exposure of cells to DMA, T3MA and T4MA, the current did not change to a steady value but instead continued to increase with time (Fig. 1*B*). This was most obvious at high amine concentrations. Such current data were discarded if they could not be easily resolved by eye into linear and exponential components. T3MA- and T4MA-associated currents were seldom reproducible and so

Table 1.

Amine ion	n (cells)	K_M (μM)	V_m (mA m^{-2})
MA	8	0.31 ± 0.09	85 ± 12
EA	6	14 ± 2	54 ± 5
IPA	5	59 ± 11	89 ± 13
DMA (Na)	7	29 ± 54	80 ± 130
DMA (Li)	6	34 ± 35	29 ± 25

Table 2.

	0 mM Li	20 mM Li
K_M (μM)	420 ± 80	520 ± 110
V_m (mA m^{-2})	100 ± 8	67 ± 5

transport parameters for these amines could not be derived.

Effect of Alkyl-Substitution on Transport Parameters

Current/concentration data yielded the values shown in Table 1 for the transport parameters, K_M and V_m. There seemed to be a reduction in the V_m for DMA transport where LiCl rather than NaCl was used to maintain the ionic balance throughout an experiment. This was investigated: MA currents were observed in the presence of 0 and 20 mM lithium in $n = 4$ cells. V_m(MA) was significantly lower under the high lithium conditions shown in Table 2.

Mutual Saturation?

Several cells ($n > 4$) were exposed to MA, then to MA plus EA/IPA/DMA/T3MA/T4MA to test whether the different amine ions enter cells via the same porter.

The saturated current was the same for MA as for MA plus EA, IPA (Fig. 2*A*) but was reduced by ~20% for MA plus DMA (Fig. 2*B*). T3MA and T4MA results were inconclusive, again because of poor reproducibility.

Effect of External pH on Methylammonium Transport

The effects of external pH (pH_o) on the parameters for MA transport are summarized in Table 3. At pH_o 4.5, MA currents, even at high concentrations, were extremely small and could not be reliably resolved from the current baseline. When MA was

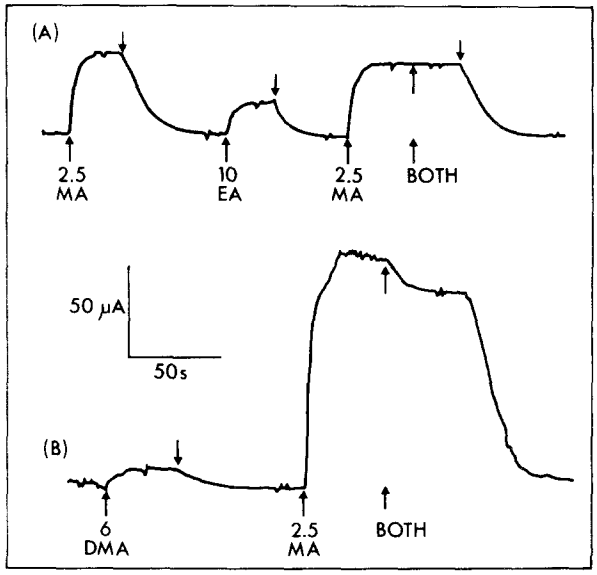

Fig. 2. (*A*) Time-course of membrane current, showing effect of introducing methylammonium, *MA*, and ethylammonium, *EA*, into the fast-flowing medium separately and then together (concentrations in mM). Unmarked arrows indicate amine removal. (*B*) As for *A*, but replacing EA with dimethylammonium, *DMA*

Table 3.

pH_o	$\frac{[CH_3NH_3^+]}{[CH_3NH_2]}$	n	K_M ($\leq\mu$M)	V_m (mA m^{-2})
4.5	$1.44\ 10^6$	—	—	—
5.5	$1.44\ 10^5$	9	270 ± 120	26 ± 5
6.5	$1.44\ 10^4$	6	280 ± 110	64 ± 15
7.5	$1.44\ 10^3$	6	310 ± 90	85 ± 12
8.5	144	6	200 ± 90	54 ± 11
9.5	14.4	6	180 ± 70	55 ± 10
10.5	1.44	—	—	—

presented to cells at pH_o 10.5, the membrane current continued to increase slowly with time instead of reaching a steady value (Fig. 3, *cf.* the irregular DMA-associated rise shown in Fig. 1*B*).

The apparent dependence of V_m on pH_o was tested by measuring changes in membrane current during exposure of the same cell ($n = 3$) to 1.5 mM MA at pH_o 4.5 to 10.5 (avoiding intercellular variations in V_m). Results are summarized in Fig. 4.

Discussion

Maximum Rates

Values of V_m for MA, EA, IPA and DMA (Na) transport lie within the range 10–100 mA m^{-2},

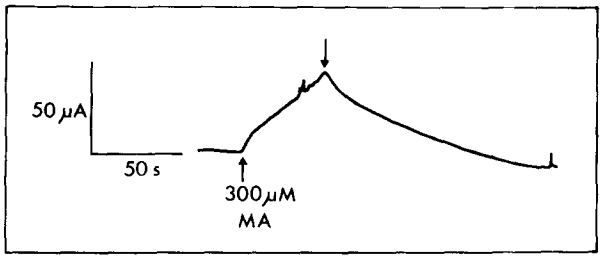

Fig. 3. Time-course of membrane current, showing effect of introducing and removing (unmarked arrow) methylammonium, *MA*, into the fast-flowing medium at pH_o 10.5

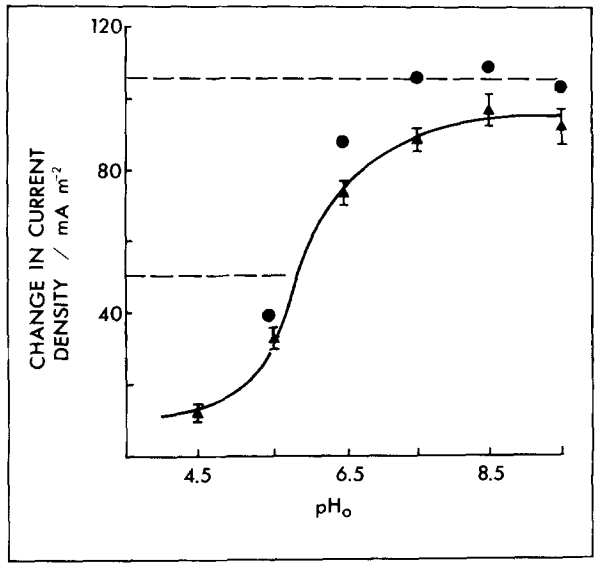

Fig. 4. Effect of external pH (pH_o) (i) on the change in membrane current density produced by 1.5 mM methylammonium (triangles, mean of 3 cells); (ii) on V_m values as calculated from the Michaelis-Menten equation (circles)

which is similar to that given by Walker et al. (1979*a*,*b*) for different cells.

The MA transport rate was reduced by ~33% in the presence of 20 mM lithium; the DMA transport rate also appeared to be reduced (Tables 1 and 2). K_m values and cytoplasmic streaming rates remained fairly constant, however. Such effects of monovalent cations on amine transport have been reported in various systems. Roon et al. (1975), for example, observed a ~24% reduction in the uptake of ^{14}C-labeled methylamine by *Saccharomyces cerevisiae* in the presence of 10 mM lithium.

V_m values were not derived for T3MA and T4MA due to the unusual current time-courses observed when these ions were presented to cells. Highly substituted amines have been found to affect membrane permeability and channel conductances in a variety of plant (and animal) cell types. Coleman and Findlay (Findlay & Coleman, 1983; Coleman & Findlay, 1985) report blocking of potassium channels in *Hydrodictyon africanum* and *Chara inflata* by tetraethylammonium.

A Common Uniport?

That ammonium and MA share a common uniport was suggested by Smith and Walker (1978). In the present study, currents observed when cells were exposed to a combination of MA and EA or IPA resembled currents observed when MA alone was presented to the same cells at concentrations high enough to virtually saturate the previously described porter (*see* Fig. 2*A*). This result may suggest competition between the two substrates (i.e., between MA and EA, or MA and IPA) for common binding sites on the transporting protein, since one or both component currents must be significantly lower than if the amines had been applied separately. (The relative contributions of MA and EA or IPA currents to the net current cannot be gauged from this experiment.) The similarity of V_m values for transport of these different amines is consistent with a common uniport hypothesis, although it remains possible that there are different but interdependent amine ion porters operating in the membrane.

Currents observed in the presence of MA plus DMA were reduced to ~80% of the saturated rate in a given cell (Fig. 2*B*). Again, this may imply competition for common binding sites, but with DMA (or the MA/DMA combination) slowing the transport rate, e.g. because of steric hindrance. Complete current/concentration data were obtained for both MA and DMA(Na) transport in one cell. V_m(DMA) was lower than V_m(MA) in this cell:

V_m(DMA): 25 ± 13 mA m^{-2}
V_m(MA): 58 ± 9 mA m^{-2}.

K_M Values

The K_M(MA) derived here (310 ± 90 μM) is slightly higher than that given by Walker et al. (1979*a*): ~200 μM at −200 mV. If the available K_M values are placed in order from lowest to highest (*viz.* Table 1):

ammonium > MA > EA > DMA (>) IPA (Walker et al., 1979*a*)

then the sequence may be compared with the K_I sequence given by Wheeler (1979) for alkylamine

transport in the giant kelp, *Macrocystis pyrifera:*

ammonium > MA > EA,
propylamine (PA) > DMA.

PA is the unbranched conformational isomer of IPA. It would seem plausible that the stereochemical difference between PA and IPA may explain the discrepancy between these two sequences.

The transport of different alkylamines by a common uniport depends on their being able to interact with the common binding site. Successful binding will depend on factors such as: (1) an ion striking this site at a favorable orientation. The probability of this happening will decrease as the ion becomes more highly substituted; (2) the degree of steric hindrance encountered by an ion as it approaches the binding site (which will be especially important if it is located well into the membrane, as postulated by Walker et al. (1979*a*)); (3) energy constraints. Where a binding reaction is in equilibrium such that the binding constant far exceeds the dissociation constant, K_M may be taken as a measure of binding (*see* Briggs & Haldane, 1925). Differences in the K_M values for the transport of two amines, *A* and *B*, may then be used to calculate differences in their binding energies, ΔBE, where $\Delta BE = RT \ln (K_M(A)/K_M(B))$ if the V_m values for their transport are the same. Where *A* and *B* have different V_m values (e.g., where *A* or *B* is DMA), then the relationship between K_M and binding is more tenuous; where the binding reaction is not in equilibrium, K_M will include the rate constant as well as the equilibrium constant.

Changes in binding energy values calculated in this way suggest, for example, that the replacement of one N-bound H atom by a methyl group (*A* = ammonium, *B* = MA) reduces the BE by 11.3 kJ mol^{-1}, while the replacement of two such atoms by two methyl groups (*A* = ammonium, *B* = DMA) reduces the binding energy by 22.4 kJ mol^{-1}. Such incremental changes in BE on mono- and di-substitution of the substrate may (subject to the qualifications noted above) suggest the involvement of hydrogen bonds in the binding interaction. Hydrogen bond energies have been estimated at between 11 and 30 kJ mol^{-1} in various cases (*see* Fersht, 1977). Pullman (1983) has proposed that binding of ammonium to the carrier protein, nonactin, involves four hydrogen bonds (to four tetrahydrofuran oxyens).

pH and Methylammonium Transport

Smith and Walker (1978) found that the influx of ^{14}C-methylamine into *C. corallina* internodes is strongly dependent on external pH (pH_o). However, pH_o is known to affect the plasmamembrane PD in Charophyte cells (*see* Walker, 1982). Walker et al. (1979*a*) maintained this PD constant throughout a preliminary investigation of the effect of pH_o on ammonium and MA currents. The present study resolves the effects of pH_o on K_M and V_m values, using MA as substrate.

There is no significant variation in K_M over the pH_o range tested, whereas V_m is strongly pH_o dependent (Table 3, Fig. 4). These results are consistent with the earlier findings referred to above. In particular, Fig. 4 suggests that the transport rate is affected by one or more acid-base residues on the transporting protein with $pK_a \sim 5.8$. This value is comparable with that proposed by Walker et al. (1979*a*): 4.5–5.5, based on their preliminary experiments and theoretical uniport model.

At pH_o 10.5, K_M and V_m values could not be derived because of the continual increase in membrane current on presentation of amine to cells (Fig. 3). At this pH_o, acid and base forms of MA are present in similar concentrations in the experimental solution (*see* Table 3). The observed current increase may therefore be a response to a significant increase in cytoplasmic pH (pH_c) resulting from greater influx of base (methylamine) by passive diffusion across the plasmamembrane at this pH_o. (The permeabilities of ammonia, methylamine and ethylamine in *C. australis* have recently been measured (Ritchie, 1987).) Amines have been shown to affect pH_c in *C. australis* by Smith (1980*a*, 1986): short-term (5 min) exposure to 4 μM ammonium or long-term (>2 hr) exposure to 200 μM MA cause pH_c to increase by less than one pH unit (Smith, 1986). Smith (1980*a*) suggests that such effects may be due to a temporary increase in the operation of the plasmamembrane H^+ pump. Smith (1986) comments that the rate of change of pH_c will depend (in part) on the rate of uptake of the amine concerned at a given pH_o (hence on pH_o) and on that amine's acid-base properties. Similar changes in pH_c in the presence of ammonium and MA have been reported in other cell types (e.g., in rhizoid cells of *Riccia fluitans* (Bertl et al., 1984). Effects on pH_c (and on vacuolar pH) must therefore be considered when interpreting any amine transport data.

At pH_o's higher than 10.5 the *C. australis* membrane becomes highly permeable to protons (or OH^-), with cells hyperpolarizing by 59 mV per pH unit (Bisson & Walker, 1980). Experiments were not performed above pH_o 10.5, therefore.

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