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Amine Uniport at the Plasmalemma of Charophyte Cells
II. Ratio of Matter to Charge Transported and Permeability of Free Base

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Summary. We have previously reported that inward positive current flows across the plasmalemma of voltage-clamped *Chara* cells when ammonia or methylamine is added to the medium. This is attributed to (inward) uniport of the cation. We have measured the stoichiometric ratio of the quantity of methylamine transported to the quantity of positive charge transported. We find 0.9 mol/faraday from pH 5.7 to 8.5, as expected if the cation flux is much larger than that of free base. The ratio increases progressively above pH 9 as the concentration of free base becomes comparable with that of cation: the fluxes fit those predicted if neutral methylamine has a permeability of 1.8×10^{-5} m/sec. This is comparable with the permeability of the methylammonium ion, 6×10^{-6} m/sec, at low concentration and -200 mV, as previously reported. Low concentrations of NH_4^+ are found to inhibit entry of $CH_3NH_3^+$ when membrane PD is constant. Half maximum inhibition is found at $\sim 20\,\mu M$ NH_4^+, in agreement with the apparent K_M for NH_4^+ binding to its uniporter site. This suggests that NH_4^+ and $CH_3NH_3^+$ enter by the same uniporter, competing for binding to its binding site.

It was believed for many years that weakly basic compounds entered cells in the form of the free base, the cationic form being impermeant (e.g., Chambers, 1922; Zarlengo & Abrams, 1963). It is becoming increasingly clear, however, that many cells have selective, saturable transport systems for NH_4^+ which may also transport $CH_3NH_3^+$. This seems to be the case for fungi (Hackette *et al.*, 1970; Slayman & Walker, *unpublished*, in Slayman, 1977), for algae (Smith, Raven & Jayasuriya, 1978; Wheeler, 1979), and for charophyte plants (Smith, Walker & Raven, 1977; Smith & Walker, 1978; Walker, Beilby & Smith, 1979, referred to below as *I*).

In previous publications, cited above, we showed that the rate of

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entry of ^{14}C-labeled methylamine was broadly independent of pH between 7 and 9, in which range the concentration of CH_3NH_2 was low and varied by $100\times$. We also showed that there was a large inward positive current through the voltage-clamped charophyte cell membrane when NH_4^+ or $CH_3NH_3^+$ was added to the bathing medium. These observations both indicated that methylamine enters as $CH_3NH_3^+$; the electrical observations also indicated that ammonia enters as NH_4^+. There appeared to be competition between these two amines for entry, since ammonia inhibited the entry of labeled methylamine and since the electric currents produced by the two amines were not additive but approached the same saturation value as produced by either amine alone (Smith & Walker, 1978).

We have postulated that the *Chara* membrane contains a passive, selective transport system (uniporter) for NH_4^+, which carries $CH_3NH_3^+$ as well, and that this is the dominant mode of entry of each substrate at normal pH. The free base is, of course, also expected to enter at a rate determined by its concentration and its permeability coefficient. The influx of cation is not proportional to concentration, but follows Michaelis-Menten kinetics with voltage-dependent parameters. The low-concentration permeability coefficients for NH_4^+ and $CH_3NH_3^+$, at -200 mV, are 3×10^{-4} m/sec and 6×10^{-6} m/sec respectively (*1*). The permeability coefficients of NH_3 and CH_3NH_2 in *Chara* have not previously been measured.

The present work is an investigation of the ratio of matter to charge entering the cell during uptake of methylamine. The primary goal is to establish whether the mechanism at normal pH is cation uniport (as contrasted with, e.g., uniport of the free base, or coupled cotransport of H^+ and amine cation). The present work also gives a value for the permeability coefficient of CH_3NH_2. We also investigate the effect of NH_4^+ on the influx of $CH_3NH_3^+$ while the membrane PD is held constant, with the aim of showing that the effect is not caused only by depolarization.

We have in the past commented on the need to have amine moved from cytoplasm to vacuole: the present work allows an estimate to be made of the permeability of the tonoplast to amine.

Materials and Methods

Chara corallina Klein ex. Will em. R.D. Wood (= *C. australis* R. Br) was grown as previously described (*1*). Plants from several different cultures were used; direct comparisons (e.g., that of Fig. 6) are made only between plants of the same culture.

Stock solutions of amines were prepared as salts of MES acid (*I*), and were added as required to buffered solution (SW) which contained: NaCl, 1 mM; K_2SO_4, 0.1 mM; $CaSO_4$, 0.5 mM; zwitterionic buffer, 5 mM; NaOH, about 2.5 mM, to give the required pH value. This solution was made up with deionized distilled water (*I*). ^{14}C-methylamine was obtained from the Radiochemical Centre, Amersham.

The electrical apparatus has been described (*I*); it was used to obtain time-courses of membrane current before, during, and after exposure of the cell to amine solution. As before, the PD between medium and vacuole was clamped. The results do not differ significantly from those which would be obtained by clamping the PD between medium and cytoplasm (*I*).

Short internodal cells (<25 mm in length) were cut from the plants 1–3 days before the experiment, and were kept in SW made from deionized water. The selected cell was mounted in a plastic chamber and two microelectrodes were inserted into its vacuole near the center. SW at the desired pH flowed past the cell for about 15 min, until the measured PD was steady; the flow rate was about 10 mm/sec.

For the measurement of the stoichiometric ratio, the cell was first exposed briefly, as a check, to unlabeled methylamine solution, which was then washed away. It was then exposed to ^{14}C-methylamine solution, which was allowed to flow until the current had ceased to change. The flow was then stopped until, 200–300 sec later, the labeled methylamine was washed out, the wash continuing for 300 sec in flowing SW. A typical record of membrane current (at pH 7.5) is shown in Fig. 1. There is some decline in the current after flow stops, as the concentration of methylamine in the unstirred region near the cell membrane falls: the current increases again when the medium is stirred (at *S* and when flow resumes). For the present purpose this is not important.

The total electric charge entering the cell during transport of ^{14}C-methylamine was found from the area between the curve and the interpolated base-line (shown dashed in Fig. 1). Usually the current returned to nearly its initial value, as in Fig. 1; at high pH it did not do so (Fig. 2), and it was then assumed that the base-line had changed linearly with time. This will result in only a second-order error.

At the end of the wash period the electrodes were withdrawn, and the cell was removed to a wax block. After its turgor had been reduced, the nodes were cut off and discarded and the rest of the cell was transferred to a vial of scintillation mix for counting. Since some pigments were leached from the cell, and a variable amount of

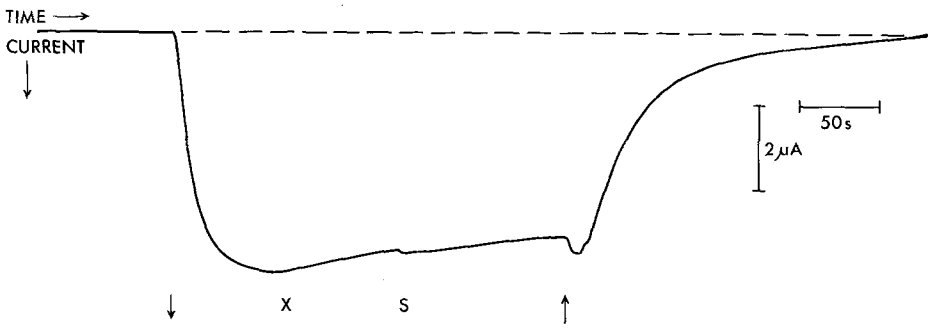

Fig. 1. Time-course of membrane current in a voltage-clamped cell of *C. corallina*. At ↓, the flowing medium (SW) was changed to SW + 1 mM total methylamine (labeled with ^{14}C); at *X*, flow of the bathing medium ceased; at *S*, a sample was taken for specific-activity determination; at ↑, the methylamine was washed away by a flow of SW. pH, 7.5

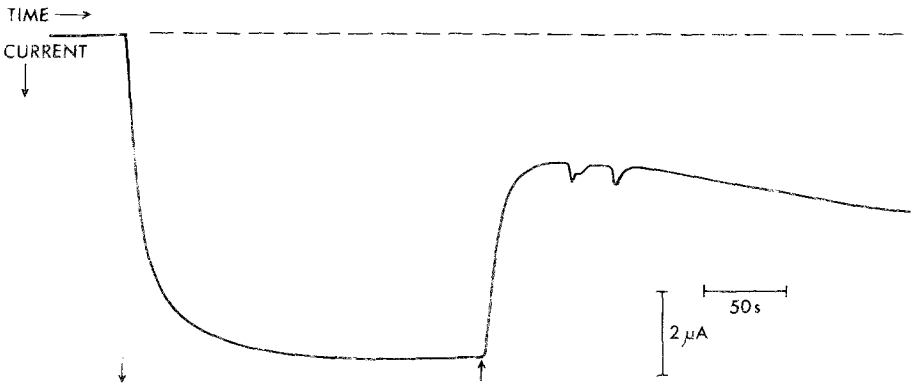

Fig. 2. As in Fig. 1. pH, 10.5

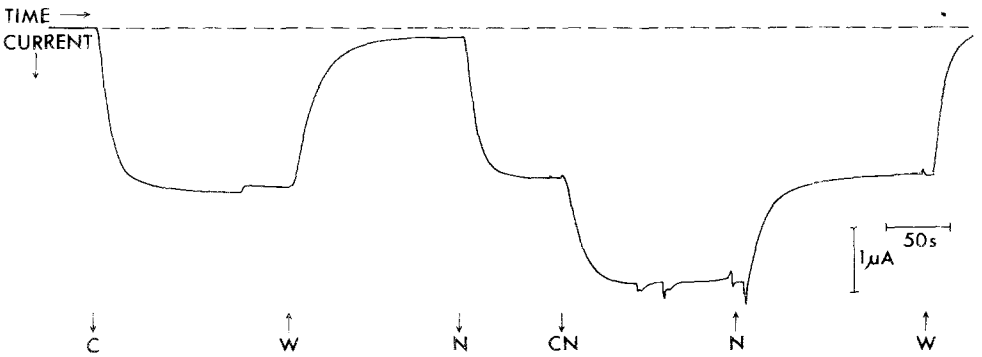

Fig. 3. Time-course of membrane current in a voltage-clamped cell of *C. corallina* during an inhibition experiment. At ↓*C*, unlabeled methylamine at 100 μM; at ↑*W*, washed away by SW; at ↓*N*, ammonia at 20 μM; at ↓*CN*, both amines, the methylamine labeled with ^{14}C; at ↑*N*, the labeled methylamine removed; at ↑*W*, the ammonia washed away

quenching might have resulted, internal standards were used. They showed that cell and aliquot samples counted at the same efficiency.

In the experiments on competition, the cell was exposed to unlabeled methylamine for 100–200 sec; then in turn to ammonia, ammonia plus ^{14}C-methylamine, ammonia, and SW. A typical current record is shown in Fig. 3. In these experiments the solution flowed continuously.

Experiments on the intracellular distribution of labeled methylamine were performed as above, a sample of sap, about 1–5 μl in volume, being taken up in a "Microcap" and counted separately, as described by Findlay, Hope and Walker, 1971, except that the cell wall was not discarded. From the radioactivity of this sample and that of the remainder of the cell, the fractions of label associated with the vacuole and cytoplasm could be calculated. We allowed for the loss of 6% of the cell volume when the nodes were

excised; and for the overestimate of the cell diameter caused by measuring it to the outside of the cell wall, taken to be 10 μm thick.

Calculated values of the ratio were obtained by iterative numerical solution of the differential equations for fluxes through 2 membranes in series (3-compartment model), run on a desk calculator (H.P. 9810 A). We assumed a cytoplasm 7 μm thick, a cytoplasmic pH given by

$$pH_c = 6.28 + 0.22\, pH_o$$

(*see* Smith & Walker, 1976), a vacuole of infinite volume, and influx and wash periods of 250 and 300 sec, respectively.

Results

At pH 7.5, with the cells voltage-clamped at −200 mV, the ratio of the quantities of methylamine and electric charge was found to be:

Methylamine Concentration/mM	Ratio/mol faraday^{-1}
0.1	0.89 ± 0.05 (5)
1.0	1.08 ± 0.06 (5)

In this tabulation, as elsewhere, we give the mean, the SEM, and the number of observations.

At a constant total methylamine concentration of 1 mM, this ratio changed with pH of the medium in the way shown in Fig. 4. Between pH 5.7 and 8.5, the ratio is apparently independent of pH, and in this range the mean value is 0.88. This value is close to 1.0, but significantly less. It appears that in this pH range methylamine enters the cell essentially as $CH_3NH_3^+$, which should ideally result in a ratio of 1.0. The slightly lower value observed is discussed later; it implies that about 10% more charge enters the cell than is accounted for by the quantity of $^{14}CH_3NH_3^+$ found in the cell at the end of the experiment. The curves on Fig. 4 are also discussed later.

The explanation of the rise in stoichiometric ratio above pH 9 is established by the plot of influx against pH in Fig. 5, where influx of electric charge is plotted as well as influx of labeled methylamine. It is clear that the influx of charge is approximately independent of pH up to 10.5, the highest value used, while the influx of methylamine rises rapidly above pH 9.

If this rapid rise in influx of label were due to the permeation of CH_3NH_2, whose concentration becomes appreciable in this pH range, one would expect the additional influx to be directly proportional to

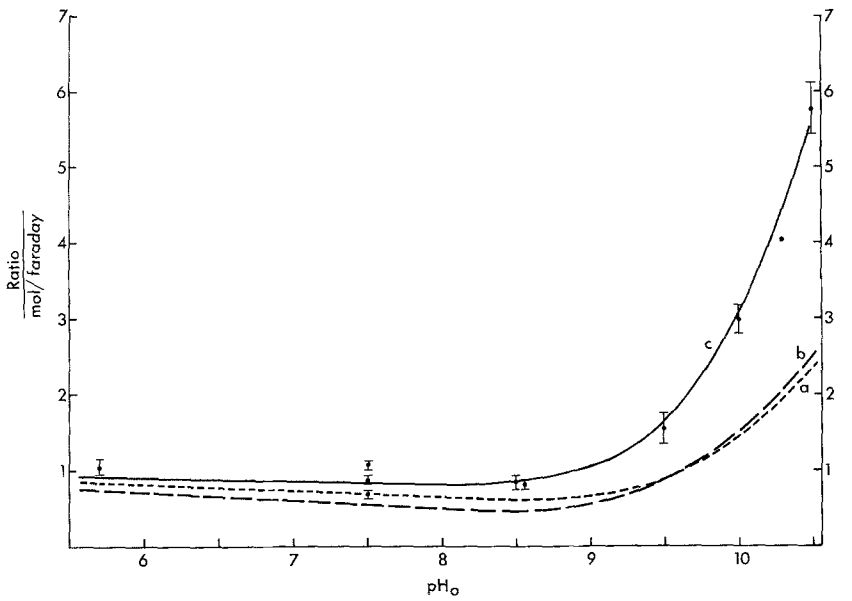

Fig. 4. Stoichiometric ratio of moles of methylamine transported to faradays of charge transported into voltage-clamped cell of *C. corallina*, as a function of pH of medium. Bars show SEM where this is larger than symbol. Curves are based on 3-compartment model (*see Materials and Methods*) with the following values for the permeabilities:

Curve	Tonoplast		Plasmalemma
	$P_{CH_3NH_2}$/m sec^{-1}	$P_{CH_3NH_3^+}$/m sec^{-1}	$P_{CH_3NH_2}$/m sec^{-1}
a	0	3×10^{-8}	8×10^{-6}
b	0	3×10^{-8}	1.2×10^{-5}
c	5×10^{-7}	10^{-7}	1.2×10^{-5}

concentration of this species. Figure 6 shows that this expectation is confirmed: here for each individual cell the difference between the influx of label and the influx of charge is plotted against the concentration of CH_3NH_2 as calculated from the Henderson-Haselbalch Equation. The slope of the linear regression is 8.1×10^{-6} m/sec, which is therefore an initial estimate of the permeability coefficient of CH_3NH_2.

During the influx period of about 250 sec a relatively large quantity of methylamine enters the cell. If much of the amine is retained in the cytoplasm, which is typically about 6% of the cell volume (Bostrom, 1976), it would be a serious question whether much of the label which entered the cell during the experiment might not leave it during the uptake and wash periods. We therefore determined, for a batch of 6 cells, the distribution of label between cytoplasm and vacuole, by removing a

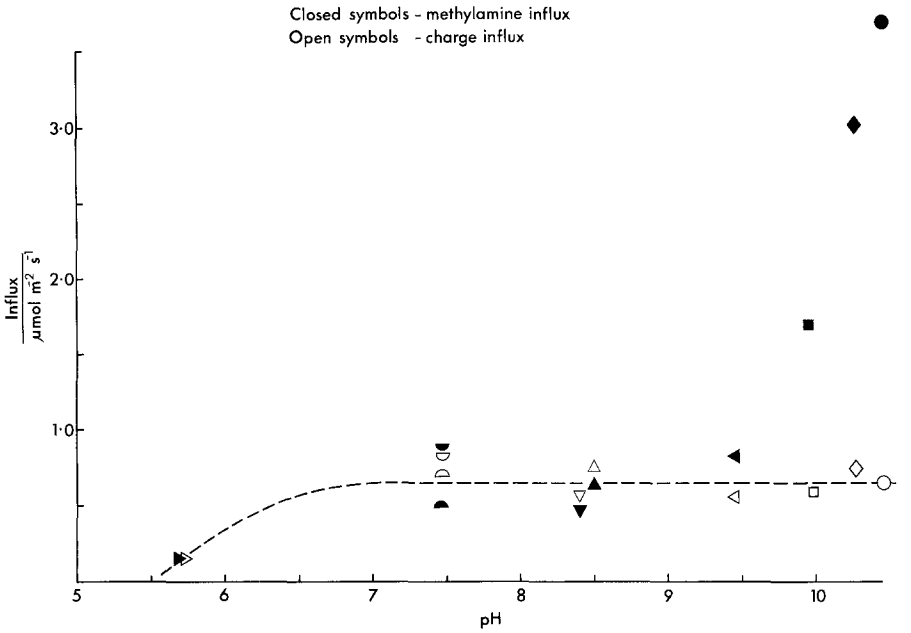

Fig. 5. Fluxes of matter (solid symbols) and electric charge (open symbols) as function of pH_o. Symbols of the same shape refer to the same batch of cells

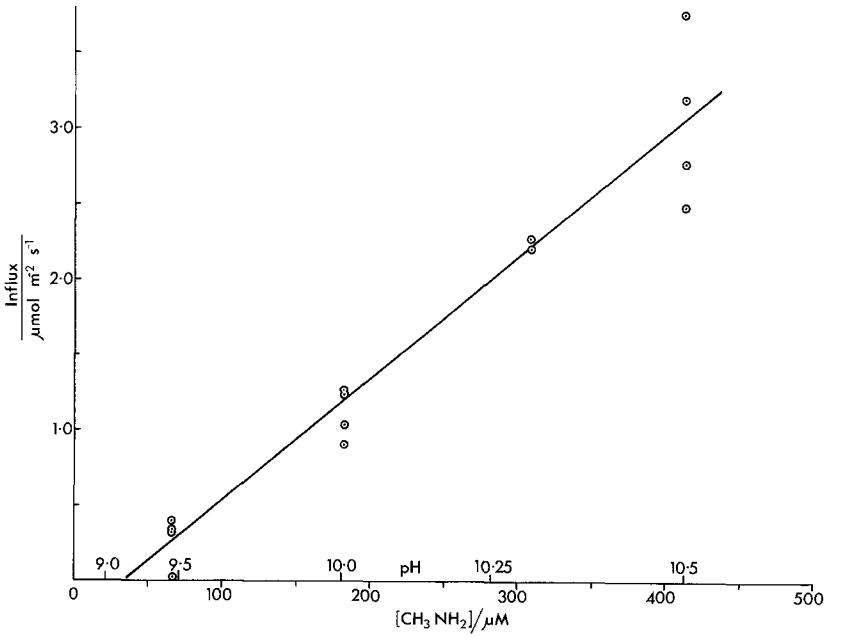

Fig. 6. Difference between flux of methylamine and flux of electric charge as a function of (calculated) concentration of free methylamine base in the medium. Line fitted by linear regression; slope 8.1×10^{-6} m sec^{-1}

sample of the vacuolar sap and counting it separately. The results, after influx and wash periods of standard length, were as follows:

Fraction in:	
Cytoplasm	Vacuole
<0	1.26 ± 0.03 (6)

Since the fraction in the vacuole cannot exceed 1.00, there is clearly a consistent experimental error in this measurement. However, it does make it reasonable to conclude that most of the ^{14}C-label is found in the vacuole even after so short an exposure.

The Inhibition of $CH_3NH_3^+$ Entry by NH_4^+

In a number of experiments at pH 7.5 the inhibition of ^{14}C-methylamine entry by NH_4^+ was determined. In each experiment the initial current flow caused by unlabeled methylamine at 100 μM was used as a measure of the control rate; the subsequently measured influx of ^{14}C-methylamine in the presence of ammonia gave the inhibited rate. The results of 10 such experiments are shown in Table 1, together with the results of the 5 experiments already quoted, in which 100 μM methylamine was used. The concentration of NH_4^+ giving half-maximum inhibition appears from these results to be about 20 μM.

Table 1. Inhibition of $CH_3NH_3^+$ [a] entry by NH_4^+

Concentration of NH_4^+ (μM)	Entry of $CH_3NH_3^+$ in 120 sec in absence of NH_4^+ (by electric method) (mC)	Entry of $CH_3NH_3^+$ in 120 sec in presence of NH_4^+ (by ^{14}C tracer method) (mC)	Inhibition of $CH_3NH_3^+$ entry by NH_4^+, as fraction	Inhibition of NH_4^+ entry by $CH_3NH_3^+$, as fraction
∅	0.26 ± 0.03 [b]	0.22 ± 0.02	0.89 ± 0.05	–
20	0.30 ± 0.05 [b]	0.17 ± 0.03	0.57 ± 0.05	1.05 ± 0.06
100	0.27 ± 0.01 [b]	0.016 ± 0.001	0.057 ± 0.003	0.98 ± 0.01

[a] Concentration of $CH_3NH_3^+$: 100 μM throughout.

[b] Number of observations in each row: 5.

Table 2. Change in resting potential after exposure to amine at high pH

Cell	ψ_i (mV)	ψ_f (mV)	Quantity of uncharged amine entered (mmol m^{-2})	pH_o
11.09.6	−170	−140	0.58	10.0
11.09.7	−170	−130	0.50	10.0
11.10.1	−170	−150	0.76	10.5
11.10.2	−170	−150	0.89	10.5
11.10.3	−150	−140	0.86	10.5
11.10.4	−160	−130	0.83	10.5

The Inhibition of NH_4^+ Entry by $CH_3NH_3^+$

If the measured $^{14}CH_3NH_3^+$ influx is converted to a current density, it is possible to find by subtraction the NH_4^+ current in the presence of methylamine in such experiments. Such a measurement of the current density of NH_4^+ entry allows us to calculate the inhibition of NH_4^+ by methylamine (Table 1, last column). At the concentrations used, there was found no significant inhibition of NH_4^+ entry by methylamine.

Shift of Membrane Potential Difference

After exposure of a cell to amine at high pH, a considerable current was required to maintain the same membrane PD as before the exposure (Fig. 2). When the clamp was released, the membrane PD was found to be more positive by about 25 mV, after the entry of some 0.74 mmol m^{-2} of uncharged amine (Table 2).

Discussion

Ion Uniport

The results presented here, especially in the form of Fig. 5, indicate clearly that below pH 9 nearly equal quantities of methylamine and of positive electric charge enter the cell during a brief presentation of amine. Above pH 9, the results, especially as in Fig. 6, are equally persuasive that there is also in this range an entry of CH_3NH_2, un-

accompanied by charge, with an apparent diffusion coefficient of 8×10^{-6} m/sec, in parallel with the mechanism that is present at lower pH.

The evidence previously available has already convinced us (Smith & Walker, 1978; Walker *et al.*, 1979) that the mechanism at low pH is the entry of the methylammonium ion, and this is strengthened by the present finding that essentially one positive electronic charge enters with every methylamine molecule. We conclude that this mechanism operates over the whole range of pH values studied, 5.5 to 10.5, with some reduction of rate at the lower end of the range, as was also found previously (*I*). We have already postulated that the ion enters through a specific, passive uniporter, which selects NH_4^+ over $CH_3NH_3^+$ by some $50\times$, and which is clearly not at all sensitive to Na^+ or K^+ at the levels used (1–2 mM and 0.2 mM, respectively).

We had already concluded that NH_4^+ inhibited the entry of $CH_3NH_3^+$ (Smith & Walker, 1978) in *Chara* cells without voltage-clamp. The mechanism of this inhibition might have been competition for the binding site of the uniporter or the depolarization of the membrane by NH_4^+ with a consequent reduction in V_m and increase in K_M for $CH_3NH_3^+$ (*see I*). The present experiments show directly that NH_4^+ inhibits the entry of labeled methylamine even when the membrane PD is constant. The inverse phenomenon, the inhibition of NH_4^+ entry by $CH_3NH_3^+$, was not observed, but given the relative K_M values, it would not be expected at the concentrations we used. This experiment would ideally require the use of labeled NH_4^+, and we have not yet attempted it. The inhibition observed does strengthen the argument for a common mechanism in the entry of $CH_3NH_3^+$ and NH_4^+. Although the data are not capable of giving an exact K_i for the effect of NH_4^+ on $CH_3NH_3^+$, the $K_{\frac{1}{2}}$ appears from Table 1 to be of the order of 20 μM. This value is not corrected for the presence of unstirred layers of solution outside the cell membrane, and is in reasonable agreement with values of K_M for NH_4^+ entry when these are not corrected for unstirred layer effect. The observed $K_{\frac{1}{2}}$ is consistent with the suggested mechanism, therefore, though this point cannot be critically examined.

Diffusive Influx of Free Base

The estimate of permeability coefficient, 8×10^{-6} m/sec is an apparent value, not corrected for unstirred layer effect. The experiments at

high pH were carried out with a flow-rate which would yield an unstirred layer about 50 μm thick. Such a layer would itself have a permeability ($P = D/\delta$) of some 35×10^{-6} m/sec, so that the true permeability of the cell membrane would be about 10.5×10^{-6} m/sec if the apparent net permeability is 8.1×10^{-6} m/sec. This estimate will be refined in a later section.

Efflux of Free Base: Error in the Stoichiometric Ratio

An estimate of the apparent permeability coefficient of the free base allows us to estimate the maximum error in the present measurements which would result from the efflux of labeled methylamine during the influx and wash periods. If the efflux occurs via the ion uniporter, it will not affect the measurement of the stoichiometric ratio; in any case, the uniport reaction is so far from equilibrium that efflux by that route will be small compared with the influx. However, labeled molecules may enter via the uniporter and then, having dissociated in the cytoplasm, return to the medium as free base. In this case the quantity of electric charge found to enter will exceed the quantity of label found in the cell at the end of the influx and wash periods. The worst case can be simply estimated: if all the methylamine remains in the cytoplasm (impermeable tonoplast), the maximum concentration achieved during an experiment is typically:

$$\frac{\phi \cdot t}{d} = \frac{7 \times 10^{-7} \times 250}{13 \times 10^{-6}} = 13 \text{ mM}.$$

At any external pH_o, we can predict an approximate cytoplasmic pH_c from:

$$pH_c = 6.28 + 0.22\, pH_o,$$

(Smith & Walker, 1976). At say $pH_o = 8$, the maximum equilibrium concentration of CH_3NH_2 in the cytoplasm is thus:

$$C_c \times (1 + 10^{pK - pH})^{-1} = 13 \times 2.45 \times 10^{-3} = 33 \text{ μM}$$

and the maximum efflux is 0.27 μmol m^{-2} sec^{-1}, which is not much less than the measured influx. It is clear that in such a situation—with a tonoplast impermeable to amine—the present experimental protocol would give quite erroneous results. The correctness of the present results must therefore depend on the permeation of the amine through the tonoplast.

It is clear that in fact the amine entering the cell does cross the tonoplast relatively rapidly (Smith *et al.*, 1977). The present experiments on the distribution of amine between cytoplasm and vacuole were designed to produce an approximate value for the tonoplast permeability so that the maximum error resulting from efflux could be estimated. Their finding, that at pH 7.5 the vacuole contains 1.25 of the content of the whole cell, represents of course a physical impossibility; thus there is unfortunately no way to set a precise lower limit for the true fraction of label in the vacuole. If it is taken as >0.9, which is reasonable, this would correspond to a permeability of the tonoplast to total amine (largely cation) of $>3\times10^{-8}$ m/sec. This would allow us to predict the ratio of matter to charge that should be found at various pH values, using the same numerical solution techniques. The results of these calculations are displayed in Fig. 4, curve *a*. This line may be taken as an indication of the worst case. Since we cannot measure the permeability of the tonoplast directly, we estimate it as in the next section.

Final Estimation of Parameters

In computing the curves of Fig. 4 for the three-compartment model, the chief unknowns are the three permeabilities: of the tonoplast, to CH_3NH_2 and to $CH_3NH_3^+$, and of the plasmalemma, to CH_3NH_2. The curves shown were computed using the following values:

Curve	Tonoplast		Plasmalemma
	$P_{CH_3NH_2}$/m sec^{-1}	$P_{CH_3NH_3^+}$/m sec^{-1}	net $P_{CH_3NH_2}$/m sec^{-1}
a	0	3×10^{-8}	8×10^{-6}
b	0	3×10^{-8}	1.2×10^{-5}
c	5×10^{-7}	1×10^{-7}	1.2×10^{-5}

Of these, curve *b* illustrates that a moderate increase in net plasmalemma permeability will not help much in fitting the actual data, while curve *c* shows a reasonably good fit, in which the plasmalemma permeability is largely determined by the values at high pH, while the tonoplast permeability is determined by the results at low pH. The distribution of the tonoplast permeability between the ionized and unionized forms is more arbitrary, but is reasonable. If the tonoplast were permeable only to CH_3NH_2, the result at low pH would be less well fitted. If it were permeable only to $CH_3NH_3^+$, the curve would be little altered.

We conclude that the values for curve *c* are the best values for the present experiments; thus the net permeability of plasmalemma and unstirred layers to CH_3NH_2 is about 1.2×10^{-5} m/sec and the true permeability of the plasmalemma is estimated to be about 1.8×10^{-5} m/sec. This is a rather higher value than an earlier estimate, 1.5×10^{-6} m/sec (Smith & Walker, 1978). It is, however, quite a reasonable value: with a molecular weight of 31 daltons it presumably crosses the plasmalemma by the polar "route" (Wright & Diamond, 1969).

Changes of Resting Potential

We draw attention to the results of Table 2, which suggests that the value of the resting PD is changed by the flux of amine into the cytoplasm. Explanation of this phenomenon is postponed to another publication.

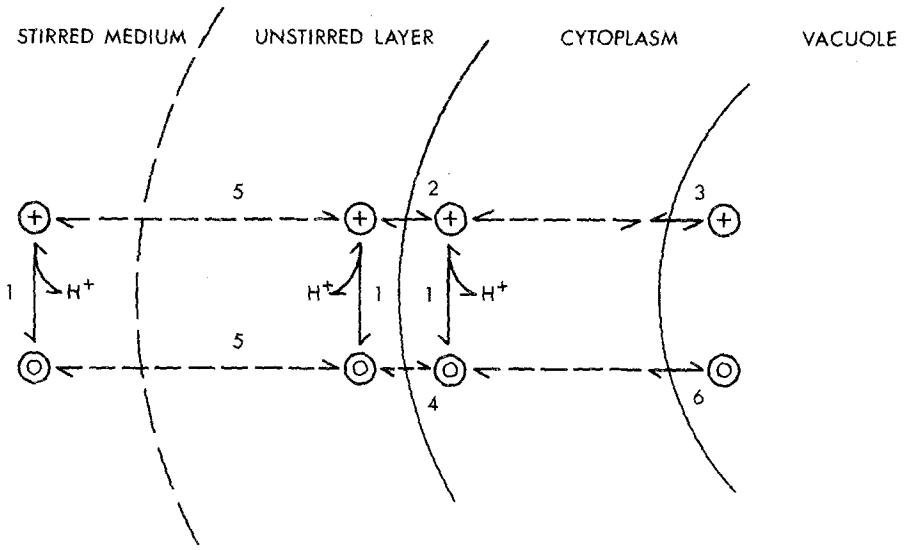

Fig. 7. Schema for entry of methylamine into *Chara*, based on this paper and previous work (*1*). Symbols: ⊕, cation; ⊙, free base; H^+, hydrogen ion. Reaction characteristics: (1) protonation reaction: $pK_a = 10.65$; (2) selective, passive, uniport: $V_m = 1.5 \exp(-(\psi + 200\text{ mV})/175\text{ mV})$ μmol m^{-2} sec^{-1}. $K_M = 200 \exp(\psi + 200\text{ mV})/75\text{ mV})$ μM; (3) presumed selective *uniport*: $V_m/K_M = 10^{-7}$ m/sec; (4) diffusive entry: $P = 1.8 \times 10^{-5}$ m/sec; (5) diffusion in aqueous solution: $D = 1.7 \times 10^{-9}$ m^2/sec (derived from the value for NH_4^+ by Stokes's Law); and (6) presumed diffusive entry

Conclusions

We conclude that methylamine enters *Chara* by two parallel mechanisms. The free base has a permeability of about 1.8×10^{-5} m/sec. The ion enters by selective, passive uniport (*I*). Over the pH range 5.5 to 9.5, ion uniport is the dominant mechanism: the crossover point is approximately 9.6–9.7. The permeability of the tonoplast to $CH_3NH_3^+$ is on the order of 10^{-7} m/sec (in estimating this we have taken the PD across the tonoplast to be zero, which is adequate for an approximate calculation). This is summarized in Fig. 7.

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