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SOME PROPERTIES OF A NEW ELECTROGENIC TRANSPORT SYSTEM: THE AMMONIUM (METHYLAMMONIUM) CARRIER FROM *CLOSTRIDIUM PASTEURIANUM*

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Summary

Clostridium pasteurianum is able to build up about 100-fold gradients of methylammonium across the cell membrane. Methylammonium enters the cell by means of a carrier as shown by the energy requirement, saturation kinetics and a pH profile with a narrow maximum between pH 6.2 and 6.8. The methylammonium transport (apparent K_m = 150 μM, V = 100 μmol/min per g dry weight) is competitively inhibited by ammonium (apparent K_i = 9 μM). The low K_i value and the observation that methylammonium cannot serve as a carbon or nitrogen source for *Cl. pasteurianum* strongly indicate that ammonium rather than methylammonium is the natural substrate. Uncouplers and inhibitors of energy metabolism or of the membrane-bound ATPase inhibit transport. *Cl. pasteurianum* maintains a membrane potential (interior negative) in the range 80—130 mV. This membrane potential was identified as the energy source: the same agents that block transport also decrease the membrane potential, and artificial generation of a membrane potential (by addition of valinomycin to K^+-loaded cells) induces concentrative uptake of methylammonium. Thus NH_4^+ (or $CH_3NH_3^+$) must be the transported species. Digestion of the cell wall by lysozyme does not abolish the transport activity.

Introduction

In the last decade, the discovery and some properties of ammonium transport systems in eukaryotes have been reported [1—9]. For most studies, the

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ammonium analog [^{14}C]methylammonium was used as a substrate, and the energy-dependent formation of large concentration gradients across the cell membrane was observed. Since methylammonium uptake was competitively inhibited by small amounts of ammonium salts, and for other reasons, it is generally accepted that the natural substrate for the carrier is ammonium rather than methylammonium.

Only few reports have appeared which indicate that ammonium transport systems should also exist in prokaryotes [10—14]. Concentrative uptake of methylammonium and its partial inhibition by uncouplers has been demonstrated in *Escherichia coli* [13] and the obligate anaerobe, N_2-fixing *Clostridium pasteurianum* [14].

In this paper, we present investigations which extend our previous studies [14], and demonstrates that in *Cl. pasteurianum*:

(1) ammonium transport is mediated by a specific carrier,

(2) a membrane potential (interior negative) is required as energy source, and therefore

(3) NH_4^+ (or $CH_3NH_3^+$) is the transported species.

Materials and Methods

Growth of organisms. Cl. pasteurianum W5 (a gift from Professor R.H. Burris, University of Wisconsin, Madison) was grown under N_2-fixing conditions as described before [15] at pH 6.4 and at temperatures between 22 and 30°C. Growth was followed spectrophotometrically at 660 nm. Extinctions were adjusted to 0.1—0.3 by dilution.

Analytical procedures. Dry weight determinations were carried out with washed cells. Centrifuged pellets were dried to constant weight over silica gel at room temperature. Intracellular volumes were estimated by assuming the cell-bound water to be 4-times the bacterial dry weight (upper limit in Fig. 1 of Ref. 16). Protein concentrations were determined by the microbiuret method [17] after sonication of the cells. A linear relationship exists between bacterial density, protein content and dry weight: 1 A_{660} unit corresponds to 0.48 mg/ml protein or 0.69 mg/ml dry weight.

Assays of CH_3NH_2 transport. Routinely, 0.5 ml samples of the culture were withdrawn quickly, incubated for the times indicated with $^{14}CH_3NH_2$ (18 μM, except for Fig. 3), and rapidly centrifuged for 5 s in an Eppendorf centrifuge (Netheler and Hinz, Hamburg, F.R.G.). After separation from the supernatant and removing the adhering liquid by aspiration, the pellets were suspended in 0.5 ml of 1 M $HClO_4$ for 10 min. The total separation time was 15 s. Both the supernatant and the extract were assayed for radioactivity by liquid scintillation counting.

Measurements of membrane potentials. The distribution of the lipophilic cation, triphenylmethylphosphonium ($TPMP^+$), between the intra- and extracellular space was used as a probe for measuring membrane potentials $\Delta\psi$ [18]. Samples were incubated with 14 μM [^{14}C]$TPMP^+$ and 1 μM $NaB(C_6H_5)_4$ until uptake was complete (in less than 3 min). Then the cells were separated from the supernatant and extracted as described above. The concentration gradients were used for the calculation of $\Delta\psi$ according to the Nernst equation.

Analysis of intracellular radioactive compounds. In order to estimate the extent to which [^{14}C]methylammonium was metabolized after uptake, the extracts prepared with 1 M $HClO_4$ were neutralized with an equal amount of $KHCO_3$, centrifuged, and the supernatant was analyzed by thin-layer chromatography on poly(ethylene imine cellulose) with an isopropanol/water mixture (70 : 30, v/v). After drying, the plates were subjected to autoradiography.

Preparation of [methyl-^{14}C]TPMP$^+$ iodide. A break-seal tube containing 2.2 mg $^{14}CH_3I$ (0.25 mCi, New England Nuclear, Boston, MA) was cooled to −78°C, and 7 mg triphenylphosphine in 1 ml of toluene were added. The mixture was gradually warmed to room temperature while white crystals of $TPMP^+I^-$ formed at the walls. When radioactivity could no longer be detected in the supernatant (after about 60 h), the toluene was carefully removed, and the crystals were dissolved in 1 ml water to produce a 15 mM stock solution of [^{14}C]TPMP$^+$. Elemental analysis of unlabeled TPMP$^+$ prepared the same way showed that the compound was essentially pure (more than 95%).

Materials. [^{14}C]Methylammonium chloride (specific activity 5 Ci/μmol) was purchased from Rohstoff-Einfuhr (Düsseldorf, F.R.G.), iodoacetate, *p*-hydroxymercuribenzoate and 5,5′-dithiobis(2-nitrobenzoate) from Serva (Heidelberg, F.R.G.), *N*,*N*′-dicyclohexylcarbodiimide (DCCD) from Merck-Schucchardt (Hohenbrunn, F.R.G.), *N*-ethylmaleimide from Ega (Steinheim, F.R.G.), and valinomycin and lysozyme from Sigma (Munich, F.R.G.).

Results

Uptake kinetics. Our preliminary results [14] had shown that at 30°C equilibration of methylammonium across the membrane was achieved in less than 30 s, and that therefore kinetic measurements could not be carried out with our previous methods. By employing a faster centrifuge and by lowering the temperature to 24°C, however, it was possible to observe linear uptake kinetics up to 20 s and the establishment of approx. 100-fold gradients after 1 min (Fig. 1). Both the rate and the extent of methylammonium uptake, however, varied for unknown reasons from one day to another. The largest gradients found were about 120-fold, the lowest about 20-fold.

Effect of pH. The pH dependence of methylammonium transport (Fig. 2) shows a relatively narrow optimum between pH 6.2 and 6.8, which was found repeatedly in the temperature range between 22 and 30°C.

Inhibitors. Inhibition of methylammonium uptake by various agents and the effect of the primary assimilation products of N_2 fixation are summarized in Table I. While glutamate and glutamine had little inhibitory effect (the small inhibition by glutamine is probably due to traces of ammonium), ammonium acetate was a strong, and as will be discussed below, competitive inhibitor. Of the SH reagents, only iodoacetate and *N*-ethylmaleimide were strongly inhibitory. Both *p*-hydroxymercuribenzoate and 5,5′-dithiobis(2-nitrobenzoate) were ineffective at concentrations that are sufficient to block many enzymes [19]. Inhibitors of energy metabolism, like azide or DCCD, are very effective. Azide inhibits the membrane-bound H^+-transporting ATPase in

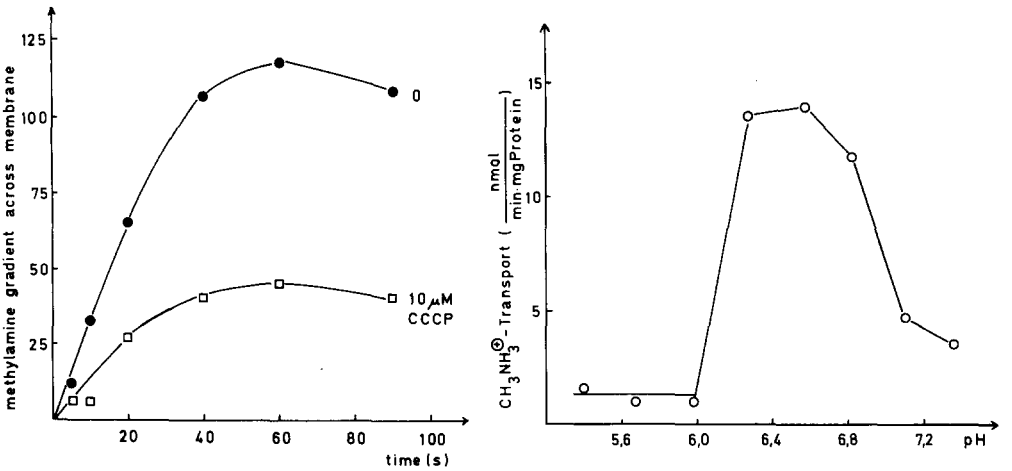

Fig. 1. Kinetics of methylammonium uptake in the absence (•) and presence (□) of 10 μM CCCP. Samples of growing cells (t = 24°C, pH 6.4, A_{660} = 1.8) were incubated for 1 min with CCCP before CH_3NH_3Cl was added.

Fig. 2. pH profile of methylammonium uptake. The pH of the continuous culture (t = 24°C) was maintained for at least 30 min at the desired value before initial uptake rates were measured, then the pH was changed to a new value. A_{660} remained constant at 2.0 in the whole pH range.

TABLE I

THE EFFECT OF VARIOUS COMPOUNDS ON METHYLAMMONIUM TRANSPORT AND MEMBRANE POTENTIAL ($\Delta\psi$) IN *Cl. pasteurianum*

Samples of the culture were incubated for 6 min under argon at 25°C, pH 6.4, with the indicated compounds. Methylammonium uptake and $\Delta\psi$ were measured in different experiments. DCCD and CCCP were added as solutions in ethanol (final ethanol concentration 0.5%); ethanol alone has no effect. All other compounds were added as aqueous solutions.

Compound	Final concentration (mM)	Residual uptake rate (%)	$\Delta\psi$ (mV)
No addition	—	100	95
Nitrogenous metabolites			
Ammonium acetate	1	2	95
Glutamate	1	100	—
Glutamine	1	77	—
Inhibitors			
p-Hydroxymercuribenzoate	0.25	100	—
5,5'-Dithiobis(2-nitrobenzoate)	5	95	—
Iodoacetate	5	7	62
N-Ethylmaleimide	5	18	70
Azide	5	10	58
DCCD	0.01	15	59
	0.1	7	—
Uncouplers			
2,4-Dinitrophenol	0.2	69	—
CCCP	0.005	85	71
	0.01	35	67
	0.05	2	56

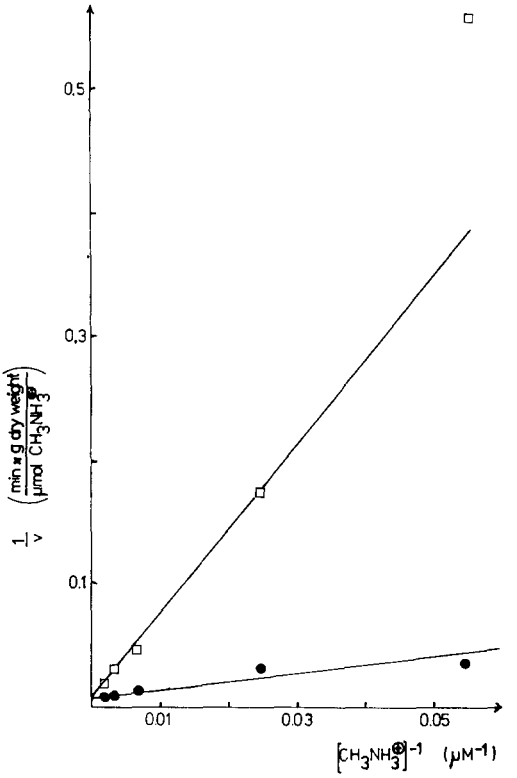

Fig. 3. Lineweaver-Burk plot of methylammonium uptake in the absence (•) and presence (□) of 50 μM CH_3COONH_4. CH_3NH_3Cl and CH_3COONH_4 were added simultaneously to otherwise untreated culture samples (t = 24°C, pH 6.4, A_{660} = 1.9).

E. coli [20], while DCCD inhibits prokaryotic ATPases in general, inter alia, the *Cl. pasteurianum* enzyme [21]. Uncouplers have little effect in concentrations (5 μM), at which they dissipate the pH gradient across clostridial membranes [16] whereas higher concentrations proved inhibitory.

Competitive inhibition by ammonium. Competitive inhibition of methylammonium uptake by ammonium has been repeatedly regarded as evidence that the natural substrate of this transport system is ammonium rather than methylammonium [1,4,6,8]. Analyzing our data by a Lineweaver-Burk plot (Fig. 3), competitive inhibition of methylammonium uptake by ammonium was observed. The apparent K_m value for methylammonium is about 150 μM, and thus in the same range as that found for *E. coli* [13], while the K_i value for ammonium varied at about 9 ± 4 μM. If we assume that this K_i is identical to the K_m for ammonium uptake, it reflects a far higher affinity of the transport system for ammonium than for methylammonium, suggesting the former is the natural substrate.

Metabolization of methylammonium. The evolution of a special methylammonium transport system would only make sense if this compound could be utilized as a nitrogen or carbon source. In our previous paper [14], we reported that after prolonged incubation, a significant fraction of the radio-

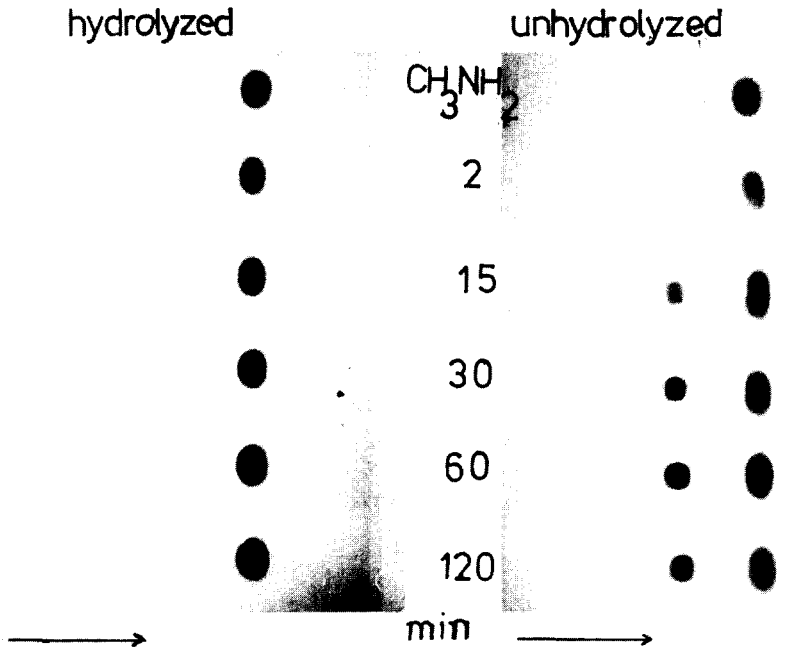

Fig. 4. Thin-layer chromatography of *Cl. pasteurianum* extracts after incubation for various lengths of time with $^{14}CH_3NH_3Cl$. Samples were extracted with 1 M $HClO_4$ and either neutralized immediately (upper part) or, after hydrolysis for 2 h at 90°C (lower part), chromatographed on poly(ethylene imine cellulose)-coated glass plates and analyzed by autoradiography. Reference: $^{14}CH_3NH_3Cl$ (left spots).

activity (30% after 30 min) was extractable in forms other than the methylammonium from the cells. These results were confirmed by thin-layer chromatography of cells pre-incubated with [^{14}C]methylammonium chloride for various lengths of time and detection of radioactive metabolites by autoradiography. As can be seen in Fig. 4 (upper part), almost no metabolization occurs during the first 2 min, whereas about 30% of the methylammonium is converted to a compound with a lower R_f value after 30 min of incubation or more. From a comparison of R_f values of unlabeled compounds the newly formed metabolite might be γ-*N*-methylglutamine. When the extracts were hydrolyzed (prior to neutralization) for 2 h at 90°C, only one radioactive spot with the R_f value of methylammonium appeared on the chromatogram (Fig. 4, lower part). Since the main route of ammonium metabolization in *Cl. pasteurianum* starts with the formation of glutamine [15], these results indicate that this organism is able to incorporate slowly methylammonium into the amide group of glutamine, but then no further metabolization occurs, and this compound cannot serve as a carbon or nitrogen source.

Energy source. One of the central problems concerning active transport is its connection with the energy metabolism of the cell. The best conceptual framework available is the chemiosmotic theory [22—25]. In relation to the energy source, one generally distinguishes between primary and secondary active transport systems [24,25]: the first ones convert chemical energy (stored in chemical systems out of equilibrium) into electrical and osmotic

energy (stored in membrane potentials and concentration gradients), whereas the latter transfer energy from one electro-osmotic gradient into another. In *Cl. pasteurianum* probably the most important primary system for the generation of an energy source for the secondary systems is the membrane-bound H^+-transporting ATPase, which builds up a pH gradient [16]. By using the equilibration of the lipophilic $TPMP^+$ as a probe, we could demonstrate that actively metabolizing cells of this organism maintain a $\Delta\psi$ (interior negative) in the range of 80—130 mV, the existence of which has been previously inferred from transport studies by Booth and Morris [26].

The survey of inhibitors of methylammonium transport (Table I) already hints at the nature of the energy source. Blockage of glycolytic enzymes by *N*-ethylmaleimide [27] and iodoacetate [28], and energy (carbon) starvation abolish this transport, indicating a requirement for an energy-generating metabolism. Blockage of the membrane-bound ATPase also shuts down transport, demonstrating the requirement of either a pH gradient or $\Delta\psi$. Finally, uncouplers in concentrations sufficient to cause the complete break-dwon

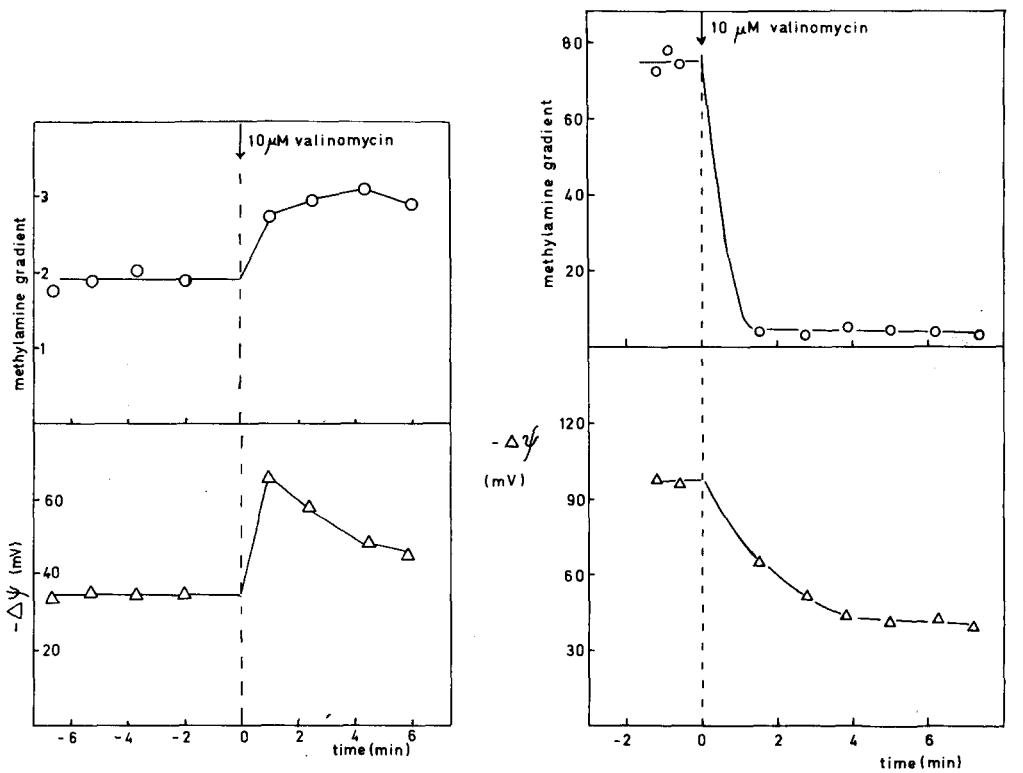

Fig. 5. Uptake of methylammonium in response to an artificially induced membrane potential. Cells from the continuous culture (extracellular $[K^+]$ = 30 mM) were suspended in K^+-free sodium phosphate buffer (50 mM, pH 6.7, t = 24°C, A_{660} = 3.4, containing 5 mM iodoacetate and 1 mM DCCD) for 10 min, and then equilibrated with $^{14}CH_3NH_3Cl$ or $TPMP^+$ for 3 min before measurements were started. At the indicated time, 10 μM valinomycin was added. ○, methylammonium gradient; △, membrane potential.

Fig. 6. Break-down of $\Delta\psi$ (△) and release of accumulated methylammonium (○) after addition of 10 μM valinomycin (arrow) to a culture sample containing 0.2 M extracellular KCl (pH 6.4, t = 24°C, A_{660} = 1.9).

of the pH gradient (5 μM, see Ref. 16) only slightly affect the methylammonium transport. These results suggest that $\Delta\psi$ is the energy source. This assumption is supported by the findings that the same compounds (with the exception of ammonium) that inhibit methylammonium uptake also decrease $\Delta\psi$ (Table I).

For a more direct proof of this type of coupling we artificially induced a $\Delta\psi$ and could so drive methylammonium uptake. Induction of an artificial $\Delta\psi$ (interior negative) can be generally achieved by suspending K^+-loaded cells in K^+-free buffer, inhibiting the ATPase and energy metabolism, and adding the K^+-specific ionophore valinomycin, thus inducing electrogenic efflux of K^+ [24,25]. Fig. 5 demonstrates that in *Cl. pasteurianum* this treatment leads to the creation of $\Delta\psi$, and that this artificially induced $\Delta\psi$ can drive concentrative uptake of methylammonium. On the other hand, if valinomyin was added to an uninhibited culture with a high extracellular K^+ concentration (0.2 M), electrogenic K^+ flux was directed inward, causing break-down of $\Delta\psi$ and release of pre-accumulated methylammonium (Fig. 6). The induction of $\Delta\psi$ and methylammonium uptake was not inhibited by azide, iodoacetate, *N*-ethylmaleimide, and, surprisingly, not even by 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) which at these concentrations completely abolishes methylammonium uptake and strongly decreases $\Delta\psi$ in cells where $\Delta\psi$ is generated by the ATPase (Table I). Uncouplers have been reported to inhibit valinomycin-induced transport in *E. coli* [29], *Staphylococcus aureus* [30] and *Streptococcus faecalis* [31] by electrogenic H^+ influx. Our deviating results are at present unexplainable.

Valinomycin is an ionophore with a high specificity for K^+ and NH_4^+, but cannot transport $CH_3NH_3^+$ through mitochondrial membranes [32]. But even if valinomycin were able to facilitate methylammonium flow through clostridial membranes, only the rate but not the extent of accumulation should increase.

The bacterial wall is not involved. The function in solute transport of cell walls and outer membranes from gram-negative bacteria has been studied intensively in recent years (see Ref. 33), but little is known in this respect about Gram-positive organisms.

When *Cl. pasteurianum* cells were incubated anaerobically in 50 mM potassium phosphate, pH 6.6, with 20% sucrose and 0.3 mg/ml lysozyme at 30°C, protoplast formation was complete in about 1 h. During this time, the methylammonium transport rate remained unchanged (results not shown), indicating no function of the cell wall in this activity.

Discussion

NH_3 has long been regarded as highly diffusible through most, if not all biological membranes (see Ref. 32). N_2-fixing bacteria, however, are able to maintain 20—100-fold ammonium gradients across the cell membrane [11,12, 14] without major leakage of NH_3, the first product of N_2 fixation to the outside (except under certain conditions). Such a leakage should be facilitated by a more alkaline interior with respect to the outside. This pH gradient has been demonstrated in several bacteria, including *Cl. pasteurianum* [16]. N_2-

fixing bacteria are even able to absorb ammonium against this gradient [11, 12,14]. This process must be energy dependent and can be expected to be mediated by a carrier. Such ammonium carriers have been recently discovered in lower eukaryotes and in prokaryotes [1—9,13,14]. Extending our previous report [14], we describe here some properties of the ammonium (methylammonium) carrier from *Cl. pasteurianum*. Apart from the ability of gradient formation, the existence of a carrier is further substantiated by the demonstration of a distinct pH profile, energy requirement and saturation kinetics. Such characteristics are not expected for simple diffusion. Although most studies have been carried out with the ammonium analog, [^{14}C]methylammonium, it is highly probable that the natural substrate for the translocase is ammonium rather than methylammonium: (1) Methylammonium uptake is competitively inhibited by ammonium (Fig. 3). The K_i value for ammonium (9 μM) is much lower than the K_m value for methylammonium (150 μM), indicating a far higher affinity for ammonium. (2) 2 h after uptake, methylammonium is not further metabolized except to an easily hydrolyzable compound, probably *N*-methylglutamine. Subsequent incorporation into the α-amino group of glutamate is not found.

Our studies provide strong evidence that the energy source for the ammonium translocation is $\Delta\psi$, and that therefore NH_4^+ ($CH_3NH_3^+$) is the transported species:

(1) Selective inhibition of the ATPase by small amounts of DCCD inhibits transport (Table I). This strongly indicates involvement of the proton-motive force, since this inhibition should not affect primary transport systems (see Ref. 34).

(2) The reversibility of accumulation (Fig. 6) also indicates secondary energy coupling, since uptake driven by primary coupling is usually unidirectional (see Ref. 34).

(3) Break-down of the pH gradient does not totally abolish methylammonium transport.

(4) Artificially induced $\Delta\psi$ can drive uphill transport.

(5) Decrease of $\Delta\psi$ by specific inhibitors decreases the methylammonium gradient.

(6) The observed membrane potentials (80—130 mV) are large enough to produce the observed gradients (20—120-fold).

The residual $\Delta\psi$ of 40—60 mV after total inhibition of the ATPase (Figs. 5 and 6 and Table I) might be due to either nonspecific adsorption of $TPMP^+$ (see Ref. 25) or to a Donnan potential, as has been discussed for *S. aureus* [30]. The V value for methylammonium transport (about 100 μmol/min per g dry weight) indicates a higher capacity of the *Cl. pasteurianum* than of the *Penicillium chrysogenum* [1], *Aspergillus nidulans* [3] or *Saccharomyces cerevisiae* [4] systems (10, 11 and 17 μmol/min per g dry weight, respectively). From all bacterial cation transport systems listed in a recent review [35], only the K^+TrA and the Kdp systems for K^+ in *E. coli*, and the citrate-inducible Mg^{2+}-transport system in *Bacillus subtilis* have higher V values. The high V and the low K_m (assumed to be equal to the K_i of 9 μM) values stress the importance of this carrier. As has been pointed out by Stevensen and Silver [13], a specific NH_4^+ carrier should be of selective advantage under nitrogen-

limited growth, since under physiological conditions the equilibrium between NH_4^+ and NH_3 is far on the side of NH_4^+. For N_2-fixing organisms, NH_4^+ transport is far less energy consuming than N_2 assimilation, and thus nitrogenase synthesis is derepressed only, when all traces of NH_4^+ have been scavanged from the environment.

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