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MONOVALENT CATION/PROTON ANTIPORTERS IN BACILLUS ALCALOPHILUS

City University of New York PH.D. 1980

MONOVALENT CATION/PROTON ANTIPORTERS IN *BACILLUS ALCALOPHILUS*

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York.

1980

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/28/90
date

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4/28/90
date

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The City University of New York

ABSTRACT

MONOVALENT CATION/PROTON ANTIPORTERS IN

BACILLUS ALCALOPHILUS

by

Kenneth G. Mandel

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Bacillus alcalophilus is an obligately alkalophilic bacterium. It grows only from pH 8.5 to 11.5, with optimal growth at pH 10.5. Growth at extremely alkaline pH presents a special bioenergetic dilemma with respect to generation of transmembrane proton gradients. Indeed, at its optimal pH for growth, B. alcalophilus maintains a cytoplasmic pH considerably more acidic than the external pH. However, in accord with the chemiosmotic theory, bacteria have been found to extrude protons, thus establishing a transmembrane proton gradient, outside acid and positive with respect to the intracellular milieu. The present work describes the role of monovalent cation/proton antiporters in allowing B. alcalophilus to maintain an acidified cytoplasm when suspended at extremely alkaline external pHs. Isolated membrane vesicles were extensively utilized in these studies.

Upon energization with ascorbate/TMPD, right-side-out Na^+-loaded membrane vesicles of B. alcalophilus generated a transmembrane pH gradient (ΔpH, inside acid) over a range of external pH from 8 to 10.5; above pH 11, the ΔpH was zero. A transmembrane electrical potential ($\Delta\psi$, inside negative),

increasing from -125 to -135 mV from pH 8 to 11.5 was also observed. Over the same pH range, the intravesicular and external pHs of K^+-loaded vesicles were equal ($\Delta pH = 0$); only a $\Delta\psi$, inside negative, was generated. Vesicles prepared without Na^+ or K^+ generated a small ΔpH and $\Delta\psi$, interior alkaline and negative. Incubating K^+-loaded vesicles with Na^+ resulted in acidification of the intravesicular space upon energization; a half maximal effect was observed with 0.7 mM Na^+.

A non-alkalophilic mutant strain, designated as B. alcalophilus KM23, was isolated. This strain grew between pH 5.5 and 9.0, but not above pH 9.0, and could not acidify its cytoplasm at highly alkaline pH. Upon energization, right-side-out K^+-loaded membrane vesicles of KM23 did not generate a ΔpH, and in contrast to the wild type, incubation with Na^+ did not cause energy-dependent acidification of the intravesicular space. In the absence of K^+, vesicles of KM23 generated a ΔpH, interior alkaline. Furthermore, while starved cells of wild type B. alcalophilus exhibited a $\Delta\psi$-dependent efflux of passively loaded Na^+, cells of the mutant strain failed to do so. Everted K^+-loaded vesicles of B. alcalophilus, energized with NADH catalyzed Na^+-dependent alkalinization of the intravesicular space, which correlated with uptake of $^{22}Na^+$. The proton extrusion and $^{22}Na^+$ uptake were dependent upon a $\Delta\psi$, and were half maximal at a sodium concentration of about 0.7 mM. Vesicles of the non-alkalophilic mutant lacked these activities.

Everted vesicles of B. alcalophilus made in the absence of K^+ and Na^+, acidified their interior upon energization, and addition of K^+ dissipated the pH gradient slightly. K^+-loaded vesicles treated with SCN^-, to dissipate the membrane potential, still failed to exhibit a ΔpH, indicating that the K^+/H^+ exchange was electroneutral. The observed bioenergetic patterns were compatible with the presence of: an electroneutral K^+/H^+ antiporter which exchanged protons extruded by respiration with internal K^+, thus dissipating a ΔpH in both strains; and an electrogenic Na^+/H^+ antiporter which acidified the intravesicular space of the wild type relative to the external milieu. The non-alkalophilic mutant was defective in the Na^+/H^+ antiporter function and could not acidify its cytoplasm, nor grow at highly alkaline pH.

Finally, Na^+-AIB symport was also defective in cells and membrane vesicles of KM23. The defect is probably not due only to the impaired sodium circulation across the membrane of the mutant; the results indicate a possibility that the defect is in a "common" sodium translocating subunit, shared by the Na^+/H^+ antiporter and the Na^+-symporter in B. alcalophilus.

ACKNOWLEDGEMENTS

To Dr. Terry A. Krulwich, my advisor, for her thoughtful and helpful supervision and guidance throughout the course of my graduate career.

To Dr. Arthur Guffanti for his invaluable assistance and friendship.

To. Dr. David Calhoun who most generously allowed my use of his French pressure cell.

To Nick for his five years of fiendship, and at times commiseration.

To Robert Bornstein and Svetlana Belkina for their excellent assistance on some of the experiments

To Haruko for her friendship, love, and understanding.

To my parents and my brother for their unquestioning support in all my endeavors.

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AIB	α-aminoisobutyric acid
Ammediol	2-amino-2-methyl-1,3-propandiol
CCCP	carbonyl-m-chlorophenylhydrazone
DDA^+	dibenzyldimethylammonium
DCCD	N,N'-dicyclohexylcarbodiimide
FCCP	carbonylcyanide-p-trifluromethoxyphenyl-hydrazone
$K\text{-}CO_3$	potassium bicarbonate/potassium carbonate buffer
$Na\text{-}CO_3$	sodium bicarbonate/sodium carbonate buffer
NADH	nicotinamide adenine dinucleotide
PCB^-	phenyldicarbaundecaborane
PMS	phenazinemethosulphate
SCN^-	thiocyanate
TMG	methylthio-β-D-galactoside
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
$TPMP^+$	triphenylmethylphosphonium
TPP^+	tetraphenylphosphonium
Tris	Tris(hydroxymethyl)aminomethane
$\Delta\bar{\mu}_{H^+}$	electrochemical gradient of protons
$\Delta\psi$	membrane potential
ΔpH	proton gradient

INTRODUCTION

Alkalophilic bacteria present special bioenergetic problems with respect to the generation of transmembrane concentration gradients of protons. Specifically, at the highly alkaline, optimal pHs for their growth, alkalophilic organisms, such as Bacillus alcalophilus (Guffanti et al., 1978) and Bacillus firmus RAB (Guffanti et al., 1980), maintain a cytoplasmic pH that is considerably lower than the external pH. Yet, in accord with the chemiosmotic hypothesis (Mitchell, 1961, 1963, 1966, 1973), bacteria have been found to extrude protons (reviewed by Harold, 1977a). In conventional bacteria this proton extrusion, from respiration or ATP hydrolysis, establishes an electrochemical gradient of protons, outside acid and positive.

At the highly alkaline pHs at which alkalophilic bacteria can grow, the proton gradient maintained across the plasma membrane could only be maintained if the cytoplasmic pH were 10 or above. Clearly, no such gradient, and even a "reversed" proton gradient would be expected; a reversed progradient could be compensated for the purposes of energetic functions, by an increased membrane potential.

How do the alkalophiles retain a conventionally oriented transmembrane electrical potential (the $\Delta\psi$ component of the electrochemical gradient) while reversing the usual chemical gradient of protons (the ΔpH)? In non-alkalophilic bacteria a similar but less extreme problem is apparent. At the alkaline end of their pH ranges for growth, non-alkalo-

philic bacteria maintain a $\Delta\psi$ while exhibiting cytoplasmic pHs no higher than the external pH (Padan et al., 1976; Guffanti et al., 1979b; Mandel and Krulwich, 1979; Zilberstein et al., 1979; Guffanti et al., 1980). The activity of monovalent cation/proton antiporters at alkaline pH could account for these observations. Such antiporters have been suggested (Padan et al., 1976; Skulachev, 1978; Schuldiner and Fishkes, 1978; Brey et al., 1980) to function in the regulation of cytoplasmic pH in Escherichia coli, but unequivocal evidence has not been presented. Since the physiological problem is exaggerated in the alkalophiles, these bacteria have offered a system in which the role of antiporters can readily be tested.

Recently, Guffanti et al. (1980) reported the isolation of a non-alkalophilic mutant strain of B. firmus RAB. The parent strain grew only above pH 8.0, with optimal growth at pH 10.5. In contrast, the non-alkalophilic mutant grew over a range of pH from pH 5.5 to 9.0. These physiological data correlated with the loss in whole cells of the non-alkalophilic mutant, of an energy-dependent Na^+ efflux activity. Thus a Na^+/H^+ antiporter was implicated in the acidification of the cytoplasm relative to the external milieu of the alkalophiles. This acidification apparently facilitates growth at high pH, but precludes growth at lower pH. In view of the above findings, an extensive study of monovalent cation/proton antiporters in B. alcalophilus and in a newly isolated non-alkalophilic mutant derivative of this species was under-

taken. This work, largely employing isolated membrane vesicles, further documents the presence of these antiporters and supports the proposed role of the Na^+/H^+ antiporter in the regulation of cytoplasmic pH in alkalophilic bacteria.

LITERATURE REVIEW

The impermeability of the bacterial membrane to protons and other cations may be part of the mechanistic basis of the energization of many cellular processes. According to Mitchell's chemiosmotic hypothesis (Mitchell, 1961, 1963, 1966, 1973; Greville, 1969; Harold, 1972), oxidation of respiratory substrates by the cytochrome chain, or hydrolysis of ATP by the membrane bound F_oF_1-ATPase, catalyzes a net, electrogenic proton extrusion from the cell. This proton extrusion establishes a transmembrane proton gradient, ΔpH, exterior acid, and a transmembrane electrical potential, $\Delta\psi$, exterior positive. Together, the proton gradient and the membrane potential comprise the protonmotive force ($\Delta\bar{\mu}_{H^+}$). The total protonmotive force, in terms of millivolts, is calculated from the sum of the two gradients:

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - 2.3RT/F(\Delta pH)$$

The constant term, 2.3RT/F, reduces to 58.8 mV at 25°C.

It has been demonstrated that respiring bacteria do indeed translocate protons to the external milieu (Lawford and Haddock, 1973; Brookman et al., 1975; Jones et al., 1975; Harold, 1972, 1977a; Cox and Haddock, 1978). Proton translocation can also be energized by ATP hydrolysis by the F_oF_1 ATPase (Asghar et al., 1973; Altendorf et al., 1974; Rosen and Adler, 1975; Lancaster and Hinkle, 1977; Singh and Bragg, 1977). The role of both the respiratory chain and the membrane bound ATPase in generation of the protonmotive force has been the subject of extensive and thorough reviews, and

shall not be further discussed in this thesis (reviewed by; Harold, 1972, 1977a,b; Haddock and Jones, 1977; Skulachev, 1977; Kagawa, 1978).

The protonmotive force has been postulated to drive several energy-dependent functions in bacterial cells. Evidence that ATP synthesis is energized by a protonmotive force has been presented from experiments with both cells and membrane vesicles (Hertzberg and Hinkle, 1974; Wilson et al., 1976; Tsuchiya and Rosen, 1976a; Tsuchiya, 1976, 1977; Maloney, 1977, 1978). Many respiration-coupled transport systems also appear to be energized by the electrochemical potential or by a ΔpH. Rottenberg has presented a model to account for the energetic requirements of proton(cation)-substrate symport (Rottenberg, 1976), and general reviews of bacterial transport have been recently published (Simoni and Postma, 1975; Kaback, 1976; Kaback et al., 1977; Rosen and Kashket, 1978). There are also indications that the protonmotive force can energize motility (Larsen et al., 1974; Manson et al., 1977; DeJong and van der Drift, 1978).

It is of interest that in membranes of Halobacterium halobium there is a special system capable of generating the protonmotive force. Membranes of this organism contain a protein, bacteriorhodopsin, which, upon absorption of a photon of light energy, translocates protons to the external environment. Thus bacteriorhodopsin functions as a light-driven primary proton pump. This electrogenic proton pump generates a $\Delta\bar{\mu}_{H}^{+}$, acid and positive outside, independently of

respiration or ATP hydrolysis. The bioenergetics of this system have been described in considerable detail (Lanyi, 1978a; Stoeckenius, 1978).

This review will concentrate on the magnitude and orientation of the protonmotive forces generated by a variety of prokaryotes. Studies utilizing both whole cells and membrane vesicles are discussed. The evidence and postulated roles for the involvement of cation/proton antiporters in generation of the observed electrochemical gradients will be elaborated.

Protonmotive forces in bacterial cells and membrane vesicles.

The protonmotive forces generated across plasma membranes of cells and in membrane vesicles have been determined for several bacterial species. Limitations of intracellular and intravesicular size largely precludes the use of electrodes. Therefore the measurement of the ΔpH and $\Delta\psi$ are determined by indirect methods. The ΔpH is usually measured from the distribution of permeant weak acids (such as DMO, benzoate, acetate, butyrate) and weak bases (methylamine, ethylamine) across the membrane (Maloney et al., 1975; Rottenberg, 1975, 1979; Guffanti et al., 1978; Ramos et al., 1979). The weak acids diffuse across the cell membrane as the neutral, undissociated, form, and become trapped by dissociation if the intracellular or intravesicular pH is more alkaline than the external milieu. A weak base diffuses across the membrane as the dissociated form, a neutral molecule; it becomes trapped upon protonation which occurs when

the intravesicular space is more acidic than the external environment (Waddell and Butler,1959; Rottenberg et al., 1972; Schuldiner et al., 1972). The fluorescent dyes 9-aminoacridine and quinacrine have been utilized to qualitatively monitor generation of a ΔpH (Schuldiner et al., 1972; Rosen and Adler, 1975). These dyes however, were not reliable for quantitative determination of the proton gradient, possibly due to their interactions with the membrane surface. (Fiolet et al., 1974; Reenstra et al., 1980).

The $\Delta\psi$ is commonly determined from the accumulation of diffusible lipophilic cations ($TPMP^+$, DDA^+, TPP^+) which were originally developed by Liberman and his colleagues (Liberman and Topaly, 1968; Liberman and Skulachev, 1970; Liberman et al., 1970), or from the lipophilic anion, SCN^- (for a review on the use of lipophilic ions see: Rottenberg, 1975, 1979). A second method for determining the $\Delta\psi$ is from measuring the potassium concentration ratio across the cell membrane in cells which have been made permeable to K^+. The potassium-specific ionophore valinomycin is usually employed to make the membranes highly permeable to potassium (Maloney et al., 1975). It is also possible to measure the membrane potential from distribution of $^{86}Rb^+$ in valinomycin-treated cells and membrane vesicles (reviewed by Rottenberg, 1979). Finally, a variety of fluorescent dyes have been used as indicators of the membrane potential; both the theory and results of their utilization have been reviewed (Waggoner, 1979).

In Streptococcus lactis, the steady-state levels of

TMG accumulation has been utilized as an independent measure of the $\Delta\bar{\mu}_{H^+}$ (Kashket and Wilson, 1973, 1974; Kashket et al., 1980) supporting the use of other indirect probes for determination of the ΔpH and $\Delta\psi$.

In all but the fluorescence assays, the cells and vesicles can be separated from their reaction buffers by filtration or centrifugation methods and the internal concentrations of the probes determined from the levels of accumulation. Alternatively, the flow dialysis technique (Ramos et al., 1976, 1979) allows continuous measurement of the external concentration of the probe while the cells or vesicles remain at essentially steady-state conditions. In addition, the flow dialysis technique does not subject the experimental system to the stresses imposed by filtration or centrifugation which may cause leakage of the probes out of the cells. Protonmotive forces in bacterial cells which grow at a neutral range of pH -

The protonmotive force was first quantitated for glycolyzing cells of Streptococcus faecalis (Harold et al.,1970a; Harold and Papineau, 1972a). S. faecalis cells were dependent upon hydrolysis of ATP by the F_oF_1 ATPase for generation of the protonmotive force. The ΔpH, interior alkaline, measured by uptake of DMO in a filtration assay, decreased from -71 mV at an external pH of 6.0 to -35 mV at pH 7.5. The pH gradient was abolished by treatment with proton conductors, nigericin, or DCCD, an ATPase inhibitor (Harold et al., 1970a).

Uptake of the lipophilic cations DDA^+ and $TPMP^+$ by glycolyzing cells indicated that a $\Delta\psi$ of -150 to -200 mV, exterior positive was generated (Harold and Papineau, 1972a).

Energized cells of S. lactis, suspended between pH 5.0 and 8.0 also generated a $\Delta\bar{\mu}_{H^+}$ (Kashket and Wilson, 1974; Barker and Kashket, 1977; Kashket and Barker, 1977; Kashket et al., 1980). At pH 5.0, fermenting cells of S. lactis maintained a ΔpH of 1 unit, interior alkaline; the ΔpH was measured from accumulation of benzoate by a centrifugation assay. As the external pH increased to pH 8.0, the ΔpH was diminished, and the $\Delta\psi$, measured from accumulation of TPP^+, increased. Thus, between pH 5.0 and 7.0, the total protonmotive force remained fairly constant at -150 to -160 mV (Kashket et al., 1980). Furthermore, addition of K^+ ($\geq$2.5 mM) decreased the $\Delta\psi$ and increased the ΔpH. At pH 6.0, adding K^+ decreased the $\Delta\psi$ from -92 mV to -55 mV, while the ΔpH was increased from -47 to -75 mV. Adding Na^+ had no effect on the ΔpH and very little effect on the $\Delta\psi$ (Kashket et al., 1980)

Grinius and his colleagues (Griniuviene et al., 1974 1975) measured a membrane potential of -140 mV in energized cells of Escherichia coli, suspended at pH 7.0, from the uptake of DDA^+ or $TPMP^+$. Catalytic amounts of a lipid-soluble anion, PCB^-, were also included to allow uptake of the cations in these experiments. Collins and Hamilton (1976) estimated that E. coli cells, suspended at pH 6.5, maintained a $\Delta\bar{\mu}_{H^+}$ of approximately -211 $\pm$ 15 mV. They estimated the $\Delta\psi$

from K^+-distribution across the membrane of valinomycin treated cells; the pH gradient was estimated by measuring changes in the pH of the medium upon lysis of cells. They also obtained similar values for the protonmotive force in Staphylococcus aureus cells using identical methods.

In studies by Padan et al. (1976), the ΔpH of E. coli was estimated by DMO or methylamine uptake in a centrifugation assay using EDTA-treated cells; the $\Delta\psi$ was estimated from the distribution of $^{86}Rb^+$ across the plasma membrane of EDTA/valinomycin-treated cells. Uptake of Rb^+ was also measured in a centrifugation assay. At pH 6.0, the ΔpH was 2 units, interior alkaline, which decreased to 0.6 to 0.75 unit at pH 7.0. At pH 7.65, the ΔpH was abolished, and when the external pH was raised above pH 7.65, the pH gradient appeared to slightly reverse, becoming 0.5 unit, interior acid, at pH 9.0. Anaerobiosis, or treatment of the cells with cyanide or FCCP abolished the ΔpH. The $\Delta\psi$ was maintained at -74 to -88 mV between pH 6.0 and 8.0, when extracellular K^+ was at low levels (1 mM). At higher levels of K^+ (150 mM) the membrane potential was decreased and the pH gradient increased slightly in EDTA/valinomycin-treated cells. Thus, Padan et al. (1976) concluded that respiring cells of E. coli maintain a relatively constant internal pH (7.63 to 7.75) over a range of external pH from 6.0 to 7.9. They proposed that an electroneutral Na^+/H^+ antiporter, originally described by West and Mitchell (1974), might be involved in regulating the internal pH. The magnitude of the $\Delta\bar{\mu}_{H^+}$ decreased as the ex-

ternal pH was raised, since the $\Delta\psi$ remained essentially constant.

In a more recent series of experiments (Zilberstein et al., 1979), the protonmotive force was again measured in EDTA-treated cells of E. coli. The ΔpH was determined as described by Padan et al. (1976), while the $\Delta\psi$ was measured from the accumulation of TPP^+ by a flow dialysis assay. Measurement of the ΔpH gave virtually identical results to those reported by Padan et al. (1976); as the pH was increased from pH 6.0 to 8.0, the ΔpH decreased from 1.8 units to -0.2 units. The magnitude of the $\Delta\psi$ however, increased from -90 mV at pH 6.0 to -135 mV at pH 7.0, and -150 mV at pH 8.0. Thus the total protonmotive force, rather than decreasing (Padan et al., 1976), remained essentially constant as the external pH was raised (Zilberstein et al., 1979). Determination of the internal pH in cells of E. coli by ^{31}P-nuclear magnetic resonance methods (Ogawa et al., 1978) indicated that cells maintained an internal pH of pH 7.5 $\pm$ 0.1, which was in very close agreement with results obtained by indirect methods (Collins and Hamilton, 1976; Padan et al., 1976; Zilberstein et al., 1979).

Cells of Halobacterium halobium suspended at pH 6.0 and energized by illumination, generated a $\Delta\psi$, interior negative of -120 mV, and a ΔpH, interior alkaline, of 1.1 to 1.2 units. The gradients were measured from the uptake of $TPMP^+$ and DMO respectively, using centrifugation assays (Michel and Oesterhelt, 1976). As the external pH was in-

creased to pH 8.0, the ΔpH decreased to zero, essentially as observed with E. coli (Padan et al., 1976; Zilberstein et al., 1979). Adding DCCD to H. halobium, to block the proton channel of the ATPase, increased the ΔpH and the Δψ, so that the $\Delta\bar{\mu}_{H}+$ was increased by -100 mV (Michel and Oesterhelt, 1976). Bakker et al. (1976) measured a protonmotive force of -140 mV generated by energized cells of H. halobium suspended at pH 6.6; the ΔpH was 0.6 to 0.8 units, interior alkaline (somewhat less than that observed at pH 6.0), and the Δψ was -100 mV. Bakker et al. (1976) have also observed that raising the external pH above pH 7.5, dissipated the ΔpH.

Protonmotive forces have been determined in several other bacterial species which also grow optimally in the neutral ranges of pH. Arthrobacter pyridinolis, grown on D-gluconate, generated a ΔpH (measured from butyrate uptake by flow dialysis) which decreased from -77 mV at pH 5.5 to zero at pH 7.5 and did not reverse at higher pHs; the membrane potential of -87 mV, calculated from $TPMP^{+}$ distribution was relatively constant over this range of pH (Mandel and Krulwich, 1979). A protonmotive force of -90 mV at neutral pH has been estimated for Bacillus subtilis based on the transmembrane potassium gradient and an estimation of the internal pH (Shioi et al., 1978). Decker and Lang (1978) have measured a $\Delta\bar{\mu}_{H}+$ of -118 mV in Bacillus megaterium cells, grown on fructose plus malate, and suspended at pH 7.4. The internal pH of 8.0 and the Δψ, exterior positive, of -80 mV, were determined from DMO and $TPMP^{+}$ uptake respectively, using

filtration assays. Cells of Clostridium pasteurianum, grown on glucose, maintained an internal pH of 0.8 to 0.4 units more alkaline than the external medium, at external pHs between 5.1 and 7.0. The ΔpH, determined from the distribution of DMO, acetate, or methylamine was abolished by treating the cells with CCCP or DCCD, or by starving the cells (Riebeling et al., 1975). Micrococcus lysodeikticus cells, energized with ascorbate plus PMS exhibited a relatively constant $\Delta\bar{\mu}_{H^+}$ of -193 to -223 mV over the pH range of 5.5 to 8.5 (Friedberg and Kaback, 1980). The ΔpH, measured from uptake of weak acids by a flow dialysis assay, decreased from 1.4 units, interior alkaline, at pH 5.5 to zero at pH 7.8 and above. Over this same pH range, the $\Delta\psi$, interior negative, measured from uptake of TPP^+ by flow dialysis, or filtration, increased from -110 to -211 mV. Thus, the internal pH of M. lysodeikticus cells remained relatively constant at pH 7.1 to 7.8 over the pH range examined (Friedberg and Kaback, 1980). A small protonmotive force has been measured in glucose-grown, energized cells of Paracoccus denitrificans (Deutsch and Kula, 1978). Between pH 6.3 and 7.7, the $\Delta\psi$ was maintained at -40 mV (determined from the distribution of $TPMP^+$ and SCN^-). The ΔpH, exterior acid, which was 0.9 unit at pH 6.3, decreased to zero at pH 7.7 (Deutsch and Kula, 1978). In Staphylococcus epidermidis (Horan et al., 1978), a facultative aerobe, the $\Delta\psi$, measured from uptake of ^{137}Cs by valinomycin treated cells, corresponded to measurements of the membrane potential by $TPMP^+$ accumulation. The ΔpH, measured from DMO

distribution by flow dialysis, decreased from 1.1 to 1.5 units interior alkaline at pH 5.0 to zero at pH 6.0; the $\Delta\psi$ reciprocally increased from -40 mV at pH 5.0 to -90 mV at pH 6.0, thus, the $\Delta\bar{\mu}_{H}+$ was relatively constant over the pH range.

A general bioenergetic scheme emerges from these studies in intact cells which grow optimally at a relatively neutral pH range. At the lower end of the pH range, pH 5.0 to 6.0, the ΔpH is maximal, as high as 2 pH units interior alkaline, and the $\Delta\psi$ is often minimal. As the external pH is raised, the ΔpH decreases dramatically, and is dissipated at external pHs between pH 7.5 and 8.0. The membrane potential however, can either increase, thus maintaining a relatively constant $\Delta\bar{\mu}_{H}+$ over a range of external pH, or the potential remains constant, so that the protonmotive force decreases as the environment becomes more alkaline (Bakker et al., 1976; Horan et al., 1978; Mandel and Krulwich, 1979; Zilberstein et al., 1979; Friedberg and Kaback, 1980). The relative magnitudes of maximal ΔpH, $\Delta\psi$, and the $\Delta\bar{\mu}_{H}+$ values generated by the different organisms varies greatly. These differences may be due, in part, to differences in growth substrates, relative anaerobiosis, presence of various ions in the assay buffers, or, as indicated by the comparison of the results obtained by Padan et al. (1976) and Zilberstein et al. (1979), the techniques utilized to measure the components of the $\Delta\bar{\mu}_{H}+$ are of paramount importance. For example, it is very possible that the centrifugation assay used by Padan et al. (1976) allowed leakage of the probe and

decreased their values for the $\Delta\psi$.

Protonmotive forces in cells of acidophilic bacteria -

Bacillus acidocaldarius grows from pH 2 to 6 at 45 to 70 ^{o}C. The pH optimum is 3 to 4 at 60 to 65 ^{o}C (Garland and Brock, 1971). Oshima et al. (1977) measured the intracellular pH in cells suspended at pH 2.6 from the uptake of DMO in a centrifugation assay. They estimated the internal pH to be between 6.0 and 6.3; however, addition of uncouplers to the cells did not dissipate the ΔpH. Krulwich et al. (1978) measured the protonmotive force generated by L-malate-grown cells of B. acidocaldarius. At pH 2.0, the ΔpH, measured from distribution of (^{14}C)-aspirin in a flow dialysis assay, was 4 units interior alkaline; at pH 4.5, the ΔpH diminished to 1.8 units. At both pHs the $\Delta\psi$, measured from accumulation of the lipid soluble anion SCN^-, was +34 mV, interior positive. Treatment of the cells, to abolish the membrane potential, also collapsed the proton gradient, and in contrast to the findings of Oshima et al. (1977), uncouplers did indeed abolish the ΔpH. Studies with Thermoplasma acidophila (Hsung and Haug, 1975, 1977; Searcy, 1976) which grows optimally at pH 1 to 2, indicated that cells maintained a large ΔpH, interior alkaline. Cells grown on an undefined medium, generated a ΔpH of 3.8 units when suspended at pH 1.7. Cells of T. acidophila grown on glucose and suspended at pH 2.0, maintained their cytoplasm at pH 6.4 to 6.8. Hsung and Haug (1977) measured a $\Delta\psi$, from uptake of SCN^-, of 109 to 125 mV, interior positive; therefore the total

protonmotive force in these cells was -170 mV. Cells of Thiobacillus ferrooxidans (Cox et al., 1979), maintained their internal pH at pH 6.5 over an external pH range of 1 to 8, as measured from uptake of acetate or methylamine by a filtration assay. At pH 2.0, a small $\Delta\psi$, interior positive, was measured in these cells from uptake of SCN^-.

It was quite apparent that acidophilic bacteria generate much larger ΔpHs than organisms which grow at more neutral pH ranges. The ΔpH comprises the entire electrochemical gradient. The existence of a "reversed" $\Delta\psi$, interior positive, may be necessary compensation for the large pH gradient, lowering the $\Delta\bar{\mu}_{H^+}$ to levels within the capacitance of the membrane (Krulwich et al., 1978).

Protonmotive forces of alkaline-tolerant and alkalophilic bacteria -

Bacillus alcalophilus grew on L-malate between pH 8.5 and 11.5 with optimal growth at pH 10.5 to 11.0. The $\Delta\psi$, interior negative, measured from $TPMP^+$ distribution by a filtration assay, increased from -84 mV at pH 9.0 to -152 mV at pH 11.5. The ΔpH, measured from methylamine distribution by flow dialysis, was 0 mV at pH 9.0 (or below), 0.6 units, interior acid, at pH 10.0, and 1.4 units, interior acid, at pH 11.0. The organism maintained its cytoplasmic pH at or below 9.5, thus generating a "reverse" ΔpH (Guffanti et al., 1978). The membrane potential was always larger and of opposite polarity than the ΔpH, so that a small protonmotive force was maintained; the $\Delta\bar{\mu}_{H^+}$ decreased from -80 mV at pH

10.0 to -15 mV at pH 11.5. Uptake of AIB by L-malate-grown cells of B. alcalophilus also occured optimally at pH 10.5 with a Km of 9.3 μM. AIB transport was Na^+-dependent, and required the $\Delta\psi$; dissipation of the membrane potential abolished AIB uptake (Guffanti et al., 1978).

Lactose-grown cells of B. alcalophilus grew optimally at pH 9.0 to 9.5. At pH 8.5, the internal pH was pH 7.8, 0.7 pH units interior acid; the $\Delta\psi$ at this pH was -135 mV. Thus the total protonmotive force at pH 8.5 was only -93 mV Optimal uptake of β-galactosides also occured at pH 8.5 to 9.0. When the external pH was raised to pH 10, a pH compatible with growth on lactose, the internal pH was maintained at pH 8.5, and the $\Delta\psi$ was increased to -180 mV. The total protonmotive force, -100 mV, was essentially equal to that observed at pH 8.5 (Guffanti et al., 1979a). The techniques for measuring the ΔpH and $\Delta\psi$ in lactose-grown cells were the same as those described for the L-malate-grown cells.

A comparitive study has been performed on the energetics of Bacillus firmus RAB, an alkalophilic bacterium, and Bacillus firmus ATCC, an alkaline-tolerant strain of the same species (Guffanti et al., 1980). Cells of B. firmus ATCC grew between pH 6.5 to 8.5, while cells of B. firmus RAB grew between pH 8.0 and 11.0. Over a range of pH from pH 6.5 to 8.0 B. firmus ATCC maintained its cytoplasm at pH 8.0; above pH 8.0, the ΔpH was zero, and no reversal of the ΔpH occured. The ΔpH was monitored from accumulation of DMO or methylamine by flow dialysis. The $\Delta\psi$,interior negative, measured

from $TPMP^+$ uptake, increased from -77 mV at pH 6.5 to -137 mV at pH 9.0. Thus, the $\Delta\bar{\mu}_{H}+$ generated by the alkaline-tolerant strain, B. firmus ATCC, decreased very slightly as the external pH increased from pH 6.5 to 9.0. The alkalophilic strain, B. firmus RAB, maintained a ΔpH, interior acid, at external pHs above pH 9.5, so that at pH 11.0, the internal pH was only 9.5. Between pH 8.0 and 9.5, no ΔpH was generated. B. firmus RAB thus resembled B. alcalophilus since neither organism could maintain a conventionally oriented proton gradient. The $\Delta\psi$, interior negative generated by B. firmus RAB increased from -89 mV at pH 8.0 to -145 mV at pH 11.0, and the $\Delta\bar{\mu}_{H}+$ was therefore between -89 mV at pH 8.0 and -65 mV at pH 11.0.

Protonmotive force patterns have also been described for a second alkaline-tolerant bacterium, Bacillus circulans (Guffanti et al., 1979b). B. circulans grew on lactose at pHs up to, but not above pH 9.0. At pH 6.6 a ΔpH of -71 mV, measured from DMO distribution in a flow dialysis assay, was generated by energized cells; the internal pH therefore was pH 7.4. A $\Delta\psi$, interior negative, of -83 mV was also generated at pH 6.6. At pH 9.0, no ΔpH was maintained, and energized cells did not accumulate DMO or methylamine, but did accumulate $TPMP^+$, indicating that a $\Delta\psi$ of -138 mV was produced. At both pH 6.6 and 9.0 the $\Delta\bar{\mu}_{H}+$ was approximately the same (Guffanti et al., 1979b), and the overall energetic pattern resembled that observed with B. firmus ATCC (Guffanti et al., 1980).

Two other alkalophilic organisms have been reported in the literature. One, an alkalophilic bacillus isolated from indigo balls (Ohta et al., 1975) grew optimally at pH 10 to 11, and was estimated to maintain its internal pH in the neutral range, based on the pH-optima for a number of cytoplasmic enzyme activities. The F_1-ATPase has been purified from this bacterium (Koyama et al., 1980) and did not appear to differ significantly from ATPases from other nonalkalophiles. Souza and Deal (1977) have isolated an alkalophile which when grown at pH 10.0, maintained an internal pH between pH 7.4 and 7.7. They determined the internal pH by lysing the cells and measuring the change in the pH of the suspension.

The bioenergetic studies of the alkalophiles provides interesting insights into the energetic adaptations for growth at extreme environments. Alkalophilic bacteria never appear to generate a conventionally oriented ΔpH, and have adapted to withstand cytoplasmic pHs up to pH 9.5, higher than the intracellular pHs reported for any other bacterial species. At external pHs above pH 9.5, these organisms acidify their cytoplasm, maintaining an intracellular pH no higher than 9.5. The inability of these organisms to generate a conventional ΔpH may actually limit them to an alkaline environment. It may be possible that alkalophiles obligately acidify their cytoplasm, and that intracellular buffering abilities prevent formation of a "reversed" ΔpH at pH 8 to 9.5. Therefore at more neutral pH it is very probable that these

organisms would still acidify their cytoplasm and lower the intracellular pH even further. In contrast to the alkalophiles, alkaline-tolerant organisms do not "reverse" the proton gradient. Rather, these organisms can withstand raising their cytoplasmic pH to pH 9.0, but not above. At lower pHs however, they generate a normally oriented ΔpH (Guffanti et al., 1979b, 1980). Therefore, the major adaptation allowing an organism to become obligately alkalophilic appears to be the ability to maintain its cytoplasmic pH at a more acidic pH than the external milieu.

Protonmotive forces in bacterial membrane vesicles -

The electrochemical proton gradient generated in energized membrane vesicles of Escherichia coli has been extensively studied. The membrane potential has been measured from the uptake of lipophilic cations by filtration, flow dialysis, or by direct and indirect electrode measurements (Hirata et al., 1973; Altendorf, et al., 1975; Schuldiner and Kaback, 1975; Ramos et al., 1976; Boonstra and Konings, 1977; Porter et al., 1979; Singh and Bragg, 1979b; Reenstra et al., 1980); the ΔpH has been measured from the accumulation of weak acids by flow dialysis (Ramos et al., 1976; Ramos and Kaback, 1977; Boonstra and Konings, 1977; Reenstra et al., 1980). At pH 5.5, right-side-out membrane vesicles of E. coli, energized with D-lactate or ascorbate/PMS, generated a large ΔpH, interior alkaline, of 1.8 to 2.0 pH units. As the external pH was increased, the magnitude of the pH

gradient decreased, becoming zero at pH 7.5 and above (Ramos et al., 1976; Ramos and Kaback, 1977). The internal pH of the vesicles was therefore maintained at pH 7.5, and the decrease in the ΔpH with increasing external pH was almost identical to that observed in whole cells (Padan et al., 1976; Zilberstein et al., 1979). In vesicles however, the $\Delta\psi$ remained relatively constant at -70 to -100 mV over this pH range (Hirata et al., 1973; Ramos et al., 1976; Ramos and Kaback, 1977), while in whole cells, using identical techniques, the $\Delta\psi$ increased dramatically as the external pH was raised (Zilberstein et al., 1979). Similar protonmotive force patterns were observed in right-side-out vesicles from Salmonella typhimurium (Tokuda and Kaback, 1977) and Micrococcus lysodeikticus (Friedberg and Kaback, 1980), where the ΔpH decreased from a maximal value at pH 5.5 to zero at pH 7.5 to 8.0, while the $\Delta\psi$ remained essentially constant over the pH range examined. Cells of Micrococcus lysodeikticus resembled those of E. coli, by increasing the $\Delta\psi$ and diminishing the ΔpH as the external pH became more alkaline, so that the $\Delta\bar{\mu}_{H}+$ remained essentially constant between pH 5.5 and 8.0 (Friedberg and Kaback, 1980). Boonstra and Konings (1977; Konings and Boonstra, 1977) have demonstrated that vesicles prepared from anaerobically grown E. coli generated a protonmotive force from oxidation of formate. Their results were virtually identical to those from vesicles prepared from aerobically grown cells.

Protonmotive forces have also been measured in everted

vesicles from E. coli energized by oxidation of D-lactate, succinate, NADH, and ascorbate/PMS, or by ATP hydrolysis (Singh and Bragg, 1976, 1979a,b; Reenstra et al., 1980). Singh and Bragg (1976) have measured a ΔpH of 3.3 to 3.7 units, interior acid, in everted vesicles suspended at pH 7.5, from the quenching of fluorescence of 9-aminoacridine. They have also monitored the generation of a $\Delta\psi$, interior positive, using a SCN^- sensitive electrode to measure the SCN^- distribution across the membrane. The $\Delta\psi$ was determined to be 100 to 150 mV; however, treating potassium-loaded vesicles with valinomycin only decreased the potential, measured by this technique, by approximately 30% (Singh and Bragg, 1979a,b). Reenstra et al. (1980) have measured the $\Delta\bar{\mu}_{H^+}$ in everted vesicles from E. coli using the flow dialysis technique to monitor both the ΔpH and the $\Delta\psi$. At pH 5.5 to 8.0, the $\Delta\psi$ was of similar magnitude, but opposite orientation, to the membrane potential measured in right-side-out vesicles; the $\Delta\psi$ was approximately 70 to 80 mV, interior positive. A ΔpH, interior acid, was about 80 mV when the intravesicular pH was 5.5 and decreased to 0 mV when the intravesicular pH reached 7.5 or 8.0. Thus, the ΔpH generated by everted vesicles was somewhat smaller at the lower pH than that observed with right-side-out vesicles. With this discrepency considered, the $\Delta\bar{\mu}_{H^+}$ in both right-side-out and everted E. coli vesicles was quite similar when measured by the flow dialysis technique (Reenstra et al., 1980). It was also demonstrated (Reenstra et al., 1980) that determina-

tion of the ΔpH by distribution of methylamine, ethylamine or N-methylmorphine, in a flow dialysis assay, gave identical values over a wide range of protein concentrations. However, the use of 9-aminoacridine, in either a fluorescent assay or by flow dialysis, did not give results consistent with the other three probes, and the values differed widely as the protein concentration was varied.

Protonmotive forces have also been measured in energized membrane vesicles of Azotobacter vinelandii (Bhattacharyya et al., 1977), Halobacterium halobium (Renthal and Lanyi, 1976; Lanyi, 1978b), Paracoccus denitrificans (Kell et al., 1978), and Rhodopseudomonas sphaeroides (Michels and Konings, 1978). L-Malate energized vesicles of A. vinelandii, loaded with K^+, generated a $\Delta\psi$ of -75 to -80 mV, while Na^+-loaded vesicles generated a $\Delta\psi$ of -104 mV. The K^+-loaded vesicles translocated more protons than the Na^+-loaded vesicles, generating a larger ΔpH and indicating the possibility of a Na^+/H^+ antiporter functioning in these membranes (Bhattacharyya and Barnes, 1978). The $\Delta\bar{\mu}_{H^+}$ generated by Na^+-loaded vesicles of H. halobium increased from -145 mV to -229 mV when the external pH was increased from pH 5.0 to 6.8 (Renthal and Lanyi, 1976; Lanyi, 1978b). Potassium-loaded vesicles at pH 6.8, generated a ΔpH, interior alkaline, of 2 units, and a small $\Delta\psi$ (-34 mV); the $\Delta\bar{\mu}_{H^+}$ in K^+-loaded vesicles was -150 mV (Renthal and Lanyi, 1976). Michels and Konings (1978) have measured a $\Delta\bar{\mu}_{H^+}$ of -110 mV in illuminated membrane vesicles from R. sphaeroides suspended at pH

7.0. The energetic pattern in these vesicles was also quite similar to that observed in E. coli vesicles at pH 7.0; the ΔpH was about 0.6 units, interior alkaline, and the $\Delta\psi$ was -70 mV. Everted cytoplasmic membrane vesicles from P. denitrificans (Kell et al., 1978) generated a $\Delta\psi$ of 145 mV, interior positive, upon oxidation of NADH. Adding valinomycin to dissipate the $\Delta\psi$ allowed generation of a ΔpH, 1.5 units, interior acid. The $\Delta\bar{\mu}_{H^+}$ was not determined in these experiments.

Role of cation/proton antiporters in bacterial membranes.

Bacterial cells tend to extrude sodium and accumulate potassium (reviewed by Harold and Altendorf, 1974). It has been proposed that the cells can use the potassium and sodium gradients maintained across the plasma membrane to interconvert the $\Delta\psi$ and ΔpH generated by the initial H^+-translocation; such interconversion would result in a buffering of the intracellular pH of the organism (Skulachev, 1978). Two modes of transmembrane ion transport are proposed to catalyze these interconversions. A coupled, carrier-mediated cation-proton antiport can interconvert the ion and pH gradients. Diffusion of cations down the electrochemical potential could be coupled to the $\Delta\psi$ and would not be directly coupled to proton extrusion (Skulachev, 1978; Lanyi, 1979).

Sodium/proton antiports have been described for several organisms and their possible roles discussed. Harold and Papineau (1972b) characterized a Na^+/H^+ antiporter activity

in cells of S. faecalis and isolated a mutant which appeared defective in Na^+-extrusion. Na^+-loaded cells of the wild type maintained a smaller ΔpH than K^+-loaded cells, or cells of the mutant strain. Addition of monensin, which catalyzes an electroneutral exchange of Na^+ with H^+, restored the ability of the mutant cells to regulate their ΔpH by sodium extrusion. Similar findings were reported in cells from Anacystis nidulans, a cyanobacterium; H^+-extrusion by the membrane ATPase energized secondary Na^+-efflux, partially dissipating the ΔpH (Paschinger, 1977).

A Na^+/H^+ antiporter was first described in cells of E. coli by West and Mitchell (1974). Upon pulsing anaerobic cells with oxygen, protons were extruded. These protons reequilibrated more rapidly in the presence of Na^+ than in its absence. In addition, pulsing the cells with Na^+ caused proton efflux. Since SCN^- was present in these experiments to dissipate the $\Delta\psi$, West and Mitchell proposed that the Na^+/H^+ antiporter in E. coli was electroneutral. Similar respiratory pulse experiments performed with anaerobic cells of Alteromonas haloplanktis, a marine pseudomonad, led to the proposal of an electroneutral $Na^+(Li^+)/H^+$ antiporter in this organism (Niven and MacLeod, 1978). An electroneutral Na^+/H^+ antiport activity was also observed in anaerobic cells of E. coli where, following respiratory or energy pulses, ion movements were monitored with a Na^+-sensitive electrode (Tsuchiya and Takeda, 1979a). Interestingly, Tsuchiya and Takeda (1979a) also observed that there was a higher Na^+/H^+

antiport activity when the cells were induced for melibiose transport. Melibiose transport was catalyzed by a Na^+-symport mechanism (Stock and Roseman, 1971; Tsuchiya et al., 1977; Lopilato et al., 1978). The sodium/proton antiporter has also been studied in both right-side-out and everted E. coli membrane vesicles (Brey et al., 1978; Schuldiner and Fishkes, 1978; Beck and Rosen, 1979; Tsuchiya and Takeda, 1979b; Reenstra et al., 1980). Efflux of passively loaded $^{22}Na^+$ from energized right-side-out vesicles appeared to be electroneutral at pH 6.6 while at pH 7.5, efflux appeared electrogenic. Na^+-efflux was concomitant with acidification of the intravesicular space of the right-side-out vesicles (Schuldiner and Fishkes, 1978). Right-side-out vesicles from Salmonella typhimurium also exhibited energy-dependent efflux of preloaded Na^+ (Tokuda and Kaback, 1977). Rosen and his colleagues have shown that adding Na^+ or Li^+ to energized everted vesicles loaded with choline chloride and Tris buffer, partially dissipated a ΔpH, interior acid. They observed this effect by monitoring reversal of ΔpH-dependent quenching of quinacrine dye fluorescence (Brey et al, 1978; Beck and Rosen, 1979). They also reported that in the presence of SCN^-, which dissipated the membrane potential, interior positive, the velocity of the Na^+/H^+ exchange was reduced and thus proposed that the antiporter functioned electrogenically (Beck and Rosen, 1979). Similar results and interpretations were reported by Tsuchiya and Takeda (1979b) in everted membrane vesicles of E. coli.

More recent data by Reenstra et al. (1980), have indicated that the Na^+/H^+ exchange in everted E. coli vesicles was driven by either the $\Delta\psi$ or the ΔpH, and functioned equally well over a pH range from 5.7 to 8.0. They also noted that inclusion of high levels of chloride dissipated the $\Delta\psi$. This may make the reports of an electrogenic antiporter by Brey et al., Beck and Rosen, and Tsuchiya and Takeda somewhat tentative since high levels of chloride were present in all their buffer systems.

Several roles for the Na^+/H^+ antiporter in E. coli have been proposed, but have not been conclusively demonstrated. It is quite probable that this antiporter can convert the proton gradient to an electrochemical sodium gradient to energize the sodium-dependent transport functions (Tokuda and Kaback, 1977; Schuldiner and Fishkes, 1978; Beck and Rosen, 1979). The Na^+/H^+ antiporter could also have some role in buffering the internal pH of E. coli, simply by its ability to translocate protons across the cell membrane (Schuldiner and Fishkes, 1978; Skulachev, 1978; Beck and Rosen, 1979). Finally, it has been proposed that this exchange functions as the regulator of the internal pH in E. coli cells and membrane vesicles, acting to dissipate the ΔpH at alkaline pH (Padan et al., 1976; Ramos and Kaback, 1977a; Schuldiner and Fishkes, 1978). However, as shown by Reenstra et al. (1980), it is unlikely that the Na^+/H^+ antiporter alone can regulate the internal pH in E. coli, since it is equally active over a wide range of pH,

and does not effect the magnitude of either the ΔpH or the $\Delta\psi$.

Bhattacharyya and Barnes (1978) have described an electroneutral Na^+/H^+ antiporter in right-side-out and everted membrane vesicles from A. vinelandii based on studies of sodium efflux and uptake respectively. Its energetic function, if any, is unresolved.

An electrogenic Na^+/H^+ antiporter has been characterized in membrane vesicles from H. halobium (Lanyi et al., 1976; Lanyi and MacDonald, 1976; Lanyi, 1977; Eisenbach et al., 1977). The proton/solute stoichiometry was determined to be greater than 1. Presumably, the primary function of this antiporter is in generating a large $\Delta\bar{\mu}_{Na}+$ with the Na^+-concentration gradient oriented out to in. The electrochemical sodium gradient energizes most transport systems in this halophilic organism. In addition, by virtue of transmembrane proton movement, this antiporter can convert a portion of the pH gradient to the membrane potential, buffering the internal pH of the organism (reviewed by Lanyi, 1979). A light-energized sodium-pump has recently been characterized (Lindley and MacDonald, 1979; Lanyi and Weber, 1980) in a "red mutant" of H. halobium which lacks bacteriorhodopsin (Matsuno-Yagi and Mikohata, 1977). The light-induced Na^+-pump is spectrally distinguishible from bacteriorhodopsin, and extrudes Na^+, generating both a sodium gradient and a $\Delta\psi$. Protons can move passively, down the resulting electrochemical gradient. Unlike the Na^+/H^+ antiporter, the sodium

pump is insensitive to valinomycin or FCCP treatment. The primary sodium pump actually represents a system for sodium extrusion and is not believed to be involved in generating the $\Delta\bar{\mu}_{Na}^{+}$ (Lanyi and Weber, 1980).

Recently, a K^+/H^+ antiporter has been characterized in everted membrane vesicles from E. coli (Brey et al., 1978, 1980; Rosen et al., 1980). It was electroneutral, had an alkaline pH optimum of pH 7.8 to 8.2, and was relatively non-specific for monovalent cations, responding to K^+, Na^+, Li^+, Rb^+, and Tl^+. Rosen and his colleagues (Brey et al, 1980; Rosen et al., 1980) have proposed that the K^+/H^+ antiporter regulates the internal pH in E. coli based on the following evidence: 1) it was electroneutral and thus could dissipate the ΔpH without dissipating the $\Delta\psi$; 2) it was optimally active at alkaline pH, where the ΔpH in cells and vesicles was dissipated (Padan et al., 1976; Ramos et al., 1976; Ramos and Kaback, 1977; Zilberstein et al., 1979); and 3) a mutant lacking the K^+/H^+ antiport activity was isolated which could not grow at as high a pH as the wild type strain (Rosen et al., 1980). Kashket and Barker (1977) have also proposed the existence of a K^+/H^+ antiporter in S. lactis. Addition of potassium to fermenting cells was coupled to proton efflux which increased the ΔpH and decreased the $\Delta\psi$.

Finally, calcium/proton antiporters have been identified in A. vinelandii (Bhattacharyya and Barnes, 1976; Barnes et al., 1978) and E. coli (Tsuchiya and Rosen, 1975, 1976b; Brey et al., 1978; Brey and Rosen, 1979; Tsuchiya

and Takeda, 1979b). Calcium uptake by everted vesicles of A. vinelandii occured in response to the ΔpH, interior acid, in the presence or absence of valinomycin, when the vesicles were energized with ATP (Bhattacharyya and Barnes, 1976) or respiratory substrates (Barnes et al., 1978). Abolition of the membrane potential did not inhibit calcium uptake and thus the exchange was considered to be electroneutral. Calcium accumulation by everted vesicles from E. coli could be energized by resipratory substrates and by ATP (Tsuchiya and Rosen, 1975), or by an artificially imposed pH gradient, interior acid (Tsuchiya and Rosen, 1976b). Brey and Rosen (1979) and Tsuchiya and Takeda (1979b) have proposed that the antiporter was electrogenic based on studies using fluorescence methods to measure changes in the ΔpH and $\Delta\psi$. However, these vesicles contained large concentrations of chloride ion and quite possibly did not generate significant membrane potentials (Reenstra et al., 1980). The role of the calcium/proton antiporter in both E. coli and A. vinelandii, is most likely to regulate the intracellular levels of calcium.

Cation/proton antiporters exchanging sodium, potassium and calcium for protons have thus been described in several bacterial species. They can function in extrusion of potentially cytotoxic ions such as sodium and calcium. The Na^+/H^+ antiporter has been shown to generate an electrochemical sodium gradient, $\Delta\bar{\mu}_{Na^+}$, which energizes sodium-dependent transport systems in both E. coli and H. halobium. This

antiporter could also be involved in buffering the intracellular pH. However, as shown by Reenstra et al. (1980), the Na^+/H^+ antiporter in E. coli cannot be responsible for dissipating the proton gradient at alkaline pH. By contrast, Rosen and his colleagues (Beck and Rosen, 1979; Rosen et al., 1980), have some evidence that the K^+/H^+ antiporter in E. coli may be involved in such a function. These studies have not, however, correlated measurements of the ΔpH and $\Delta\psi$ with assays of the putative antiporters.

MATERIALS AND METHODS

Organism and growth conditions -

Bacillus alcalophilus was obtained from The American Type Culture Collection (ATCC No., 27647). The basal growth medium (Guffanti et al., 1978) consisted of 25 mM sodium carbonate buffer at pH 10.5, 0.1% (w/v) $(NH_4)_2SO_4$, 1 mM K_2HPO_4, and 0.1 mM $MgSO_4$. This medium was supplemented with 0.1% (w/v) yeast extract (Difco) and 1% (v/v) trace salts solution (Hegeman, 1966) added from separate sterile solutions. Sodium L-malate, added to 50 mM, was the carbon source. In some experiments, 25 mM lactose was added as the carbon source.

The non-alkalophilic mutant strain, B. alcalophilus KM23, derived from B. alcalophilus, was grown on a medium referred to as PT6.8 (Guffanti et al., 1979b; Krulwich et al., 1979). PT6.8 consisted of 25 mM potassium phosphate buffer at pH 6.8, 25 mM Tris-HCl at pH 6.8, 0.1% (w/v) $(NH_4)_2SO_4$, and 0.1 mM $MgSO_4$. This medium was also supplemented by the addition of 0.1% yeast extract and 1% (v/v) trace salts solution (Hegeman, 1966) from separate sterile solutions; 50 mM potassium L-malate was added as the carbon source.

Both strains were grown at 30 ^{0}C, with shaking at 200 rpm on a New Brunswick G25 rotatory shaker, Growth was monitored turbidometrically with a Klett-Summerson colorimeter (No. 42 filter); for all experiments cells were grown to the late logarithmic stage of growth.

Mutagenesis and isolation of non-alkalophilic mutant strains-

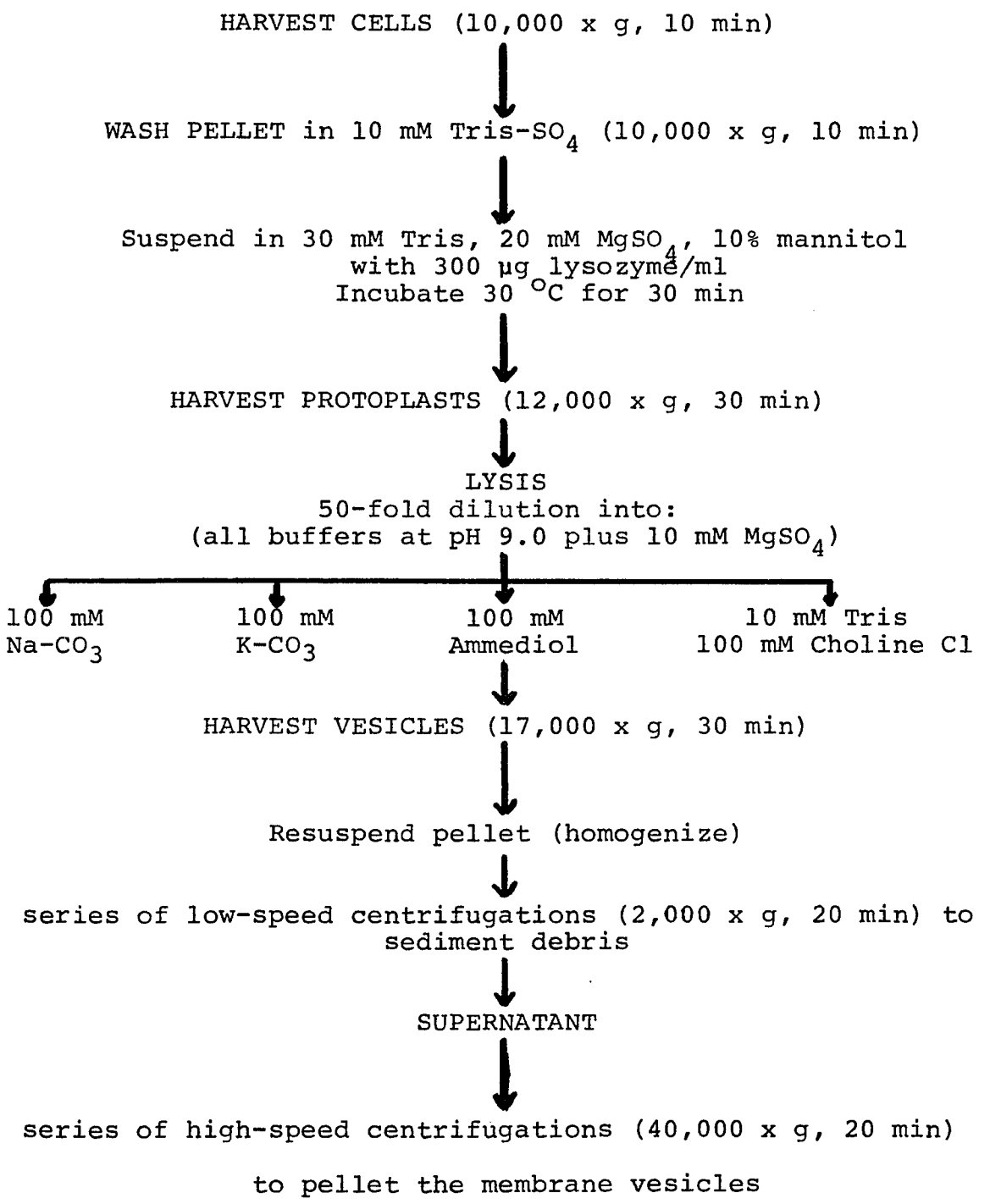
B. alcalophilus was grown to the late logarithmic stage on potassium carbonate medium at pH 9.0 with 50 mM potassium L-malate as the carbon source. The medium consisted of 25 mM K-CO_3 buffer, pH 9.0, 0.1% (w/v) $(NH_4)_2SO_4$, 1 mM K_2HPO_4, 0.1 mM $MgSO_4$; trace salts solution and yeast extract were added as described above. The cells were sterilely harvested (10,000 x g, 10 min), resuspended and washed with the K-CO_3 pH 9.0 medium. The cells were then resuspended in 10 ml of the same medium and mutagenized by adding 1% (v/v) ethyl methansulfonate as previously described (Wolfson and Krulwich, 1972). Following the mutagenesis, the cells were harvested, washed three times with K-CO_3 pH 9.0 medium, and finally resuspended in 10 ml of the complete K-CO_3 pH 9.0 medium (50 mM potassium L-malate present as carbon source). The cells were grown out for one doubling time in the liquid medium, then plated directly onto potassium L-malate containing PT6.8 plates (1.5% purified agar, Difco). Wild type B. alcalophilus formed no colonies on such plates. However, following mutagenesis, colonies were readily obtained; over twenty colonies were randomly selected and examined. One of the strains, designated B. alcalophilus KM23 was further characterized and utilized in this study. Several of the other strains exhibited properties identical to those of B. alcalophilus KM23.

Preparation of right-side-out membrane vesicles -

Right-side out membrane vesicles were prepared from L-malate-grown cells of B. alcalophilus and B. alcalophilus KM23, and from lactose-grown cells of B. alcalophilus, using a modification of a method described by Kaback (1971). A general scheme is presented in Figure 1. Cells were harvested (10,000 x g, 10 min), washed once by resuspension in 10 mM Tris-SO_4 pH 8.0, and collected (10,000 x g, 10 min). Protoplasts were prepared in 30 mM Tris, 20 mM $MgSO_4$, 10% (w/v) mannitol at pH 8.0 in the presence of 300 µg lysozyme/ml, with shaking at 30 ^{o}C for 30 min. After harvesting the protoplasts (12,000 x g, 30 min), vesicles were prepared by lysis in various buffers in the presence of DNase and RNase at 10 µg/ml. Sodium-loaded (Na^+-loaded) vesicles were prepared by lysing protoplasts in 100 mM sodium carbonate buffer at pH 9.0. Potassium-loaded (K^+-loaded) vesicles were formed from lysis in 100 mM potassium carbonate buffer, pH 9.0. Vesicles were also made in the absence of both Na^+ and K^+ by lysing protoplasts in 100 mM ammediol (ammediol-loaded) or in 10 mM Tris, 100 mM choline chloride (choline-Tris-loaded) at pH 9.0. $MgSO_4$, 10 mM, was included in all lysis buffers, and in buffers used in the washing procedures. Vesicles were harvested (17,000 x g, 30 min). The pellet was resuspended in ice cold buffer, and subjected to a series of low speed centrifugations (2,000 x g, 20 min) followed by high speed centrifugations (40,000 x g, 20 min) which sedimented debris and pelleted the vesicles respectively. Right-side-out ves-

FIGURE 1

Scheme for preparing right-side-out membrane vesicles



icles could be frozen in liquid nitrogen and stored at -80 ^{o}C for several weeks without loss in activity. The internal volume of the vesicles, determined by the method of Stock et al. (1977), was found to be 1.1 µl/mg vesicle protein. (^{14}C)-Inulin was utilized as a marker for the extravesicular space.

Preparation of everted membrane vesicles -

Everted vesicles were prepared from cells using a modification of the procedure of Kobayashi et al. (1978). Protoplasts were prepared as described above, in 30 mM Tris, 20 mM $MgSO_4$, 10% mannitol at pH 8.0. The protoplasts were then lysed in 100 mM potassium carbonate buffer pH 8.7 (K^+-loaded), or in 10 mM Tris, 100 mM choline chloride pH 8.7 (choline-Tris-loaded) with DNase present (10 µg/ml). $MgSO_4$, 10 mM, was present in both buffers during lysis and subsequent washings. The membranes were collected by centrifugation (17,000 x g, 30 min), resuspended in the lysis buffer, omitting the DNase, by gentle homogenization, and recentrifuged (2,000 x g, 20 min) to remove debris and unlysed cells. The supernatant was removed, centrifuged (40,000 x g, 20 min) to pellet the membranes, and the membranes were resuspended to a final volume of 80 ml. This suspension was passed once through an American Instrument Co., French pressure cell at 8,000 pounds/in^2. The membranes were then centrifuged at 20,000 x g (20 min) to remove debris, and the remaining supernatant was centrifuged at 100,000 x g (1 h) to collect the membranes. The everted vesicles were resuspended, washed once (100,000 x g,

1 h) and assayed immediately following resuspension. The internal volume, determined by the method of Stock et al. (1977), was 0.6 µl/mg protein.

Determination of the protonmotive force in membrane vesicles - 1. Measurement of the ΔpH in right-side-out membrane vesicles

The ΔpH was measured at 25 °C from the distribution of a diffusible weak acid, (^{14}C)-5,5-dimethyl-2,4-oxazolidine dione (DMO), or weak base, (^{14}C)-methylamine, using a flow dialysis assay (Colowick and Womack, 1969; Ramos et al., 1976, 1979). The weak acid diffuses across the cell membrane in its neutral, undissociated, form,and becomes trapped by dissociation if the intracellular or intravesicular space is more alkaline than the external milieu. A weak base diffuses across the membrane as the dissociated form, a neutral molecule; it becomes trapped upon protonation which occurs when the internal space is more acidic than the external environment (Waddel and Butler, 1959; Rottenberg et al., 1972; Schuldiner et al., 1972). The assay utilizing the membrane vesicles from the two strains of B. alcalophilus was essentially the same as that described for whole cells of B. alcalophilus (Guffanti et al., 1978). Vesicles were suspended in various buffers at approximately 10 mg protein/ml, and added to the upper chamber of the flow dialysis apparatus (final volume was 0.8 ml). The specific suspension buffers are indicated in the description of individual figures and tables. Buffer was pumped through the lower chamber at approximately 6 ml/min with a Buchler Polystaltic Pump. The two

chambers were separated by a Spectropor/dialysis membrane (6,000 to 8,000 molecular weight cutoff, Fisher Scientific). Both chambers were stirred with magnetic stirring bars and the vesicles in the upper chamber were oxygenated by passing a stream of water-saturated oxygen over the surface. The weak acid, DMO, or the weak base, methylamine, were added to final concentrations of 55 µM and 25 µM respectively to initiate the assay. Vesicles were energized by addition of 20 mM ascorbate (neutralized with the appropriate base) plus 2 mM TMPD. Fractions of 1.7 ml were collected; 1 ml aliquots were removed from each fraction, dissolved in 10 ml of Liquiscint (National Diagnostics) and assayed for radioactivity by liquid scintillation spectrometry.

The internal and external concentrations of the probes used in determining the ΔpH were calculated as described by Ramos et al. (1979). The internal pH was calculated from the distribution of the acid or the base using the equations derived by Waddell and Butler (1959). These equations, for weak acid and weak base distribution, were respectively:

weak acid distribution:

$$pH_{in} = pK_{acid} + \log\{\frac{(acid)_{in}}{(acid)_{out}} (10^{pH_{ext} - pK_{acid}} + 1) - 1\}$$

weak base distribution:

$$pH_{in} = pK_{base} - \log\{\frac{(base)_{in}}{(base)_{out}} (10^{pK_{base} - pH_{ext}} + 1) - 1\}$$

2. Validation of measurements of ΔpH in vesicles

The approximate pH used in most of the experiments, pH 9.0, was relatively far from the pK's of both DMO and methylamine; the pK of DMO was 6.3, and the pK for methylamine was 10.6. The Waddell and Butler equation contains a term which accounted for the percentage dissociation of the probes. Under the conditions employed, at an external pH of 9.0, a ΔpH of 0.3 units could be measured quantitatively with either probe, and smaller gradients qualitatively demonstrated. The methods were tested in a series of experiments described below. A suspension of unenergized K^+-loaded vesicles in 100 mM potassium carbonate buffer containing 10 mM $MgSO_4$ at pH 9.0, were subjected to a known, sudden shift in the external pH by addition of acid or base. The magnitude of the imposed pH gradient calculated from the distribution of the weak acid or base, using an intravesicular volume of 1.1 μl/mg protein, was within 10% of the expected value of the actual pH shift. The pH gradient was slowly diminished over several minutes. These experiments also verified that the determination of the intravesicular volume was accurate. Further evidence that the methods employed to determine the ΔpH were indeed valid included: a) the ΔpH measured was the same at several concentrations of the probe; b) vesicles treated with 10 μM gramicidin or 100 μM CCCP did not accumulate DMO or methylamine upon energization; c) observations of methylamine uptake in right-side-out vesicles always correlated with observations of DMO uptake by everted vesicles

(and vice versa).

A representative flow dialysis run of methylamine uptake by Na^+-loaded right-side-out vesicles is presented in Figure 2. (^{14}C)-Methylamine (25 μM) was added to initiate the assay. The vesicles were energized by addition of 20 mM sodium ascorbate plus 2 mM TMPD and accumulated methylamine. This was indicated by a decrease in radioactivity appearing in the dialysate fractions. Addition of 10 μM gramicidin dissipated the $\Delta\bar{\mu}_{H^+}$ (Harold, 1972; Guffanti et al., 1978) and caused release of accumulated methylamine. The accumulation represented a ΔpH of 1.5 units, interior acid.

3. The ΔpH in everted membrane vesicles

The ΔpH generated by energized, everted membrane vesicles was measured by flow dialysis, essentially as described for right-side-out vesicles. Everted vesicles were suspended at approximately 20 mg protein/ml and were energized by addition of NADH (appropriate salt). The assay buffers are described for the individual figures in the "Results".

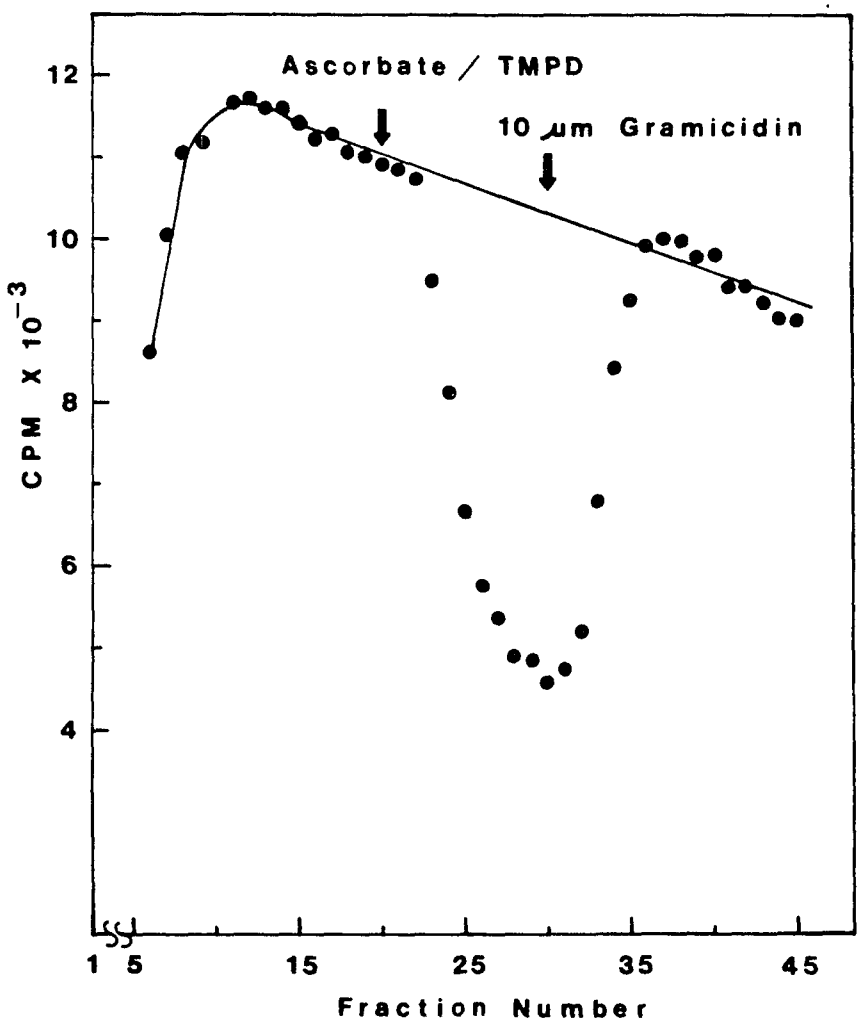
4. Determination of the membrane potential, Δψ, in right-side-out membrane vesicles

The Δψ generated by right-side-out membrane vesicles upon energization with ascorbate/TMPD was measured from the distribution of 25 μM (^3H)-$TPMP^+$ (100 μCi/ml by a filtration assay (Schuldiner and Kaback, 1975). A typical assay contained membrane vesicles suspended in 100 mM potassium carbonate buffer, 100 mM sodium carbonate buffer, or 100 mM ammediol buffer at the indicated pHs. The assay mixtures also

FIGURE 2

Methylamine uptake by right-side-out membrane vesicles of Bacillus alcalophilus

Na^+-loaded membrane vesicles were suspended in 100 mM sodium carbonate buffer, 10 mM $MgSO_4$, pH 9.0, and added to the upper chamber of the flow dialysis apparatus. The same buffer was pumped through the lower chamber at 6 ml/min. The assay was initiated by adding 25 µM (^{14}C)-methylamine at fraction # 1; 20 mM ascrobate plus 2 mM TMPD, and 10 µM gramicidin were added when indicated.


Ascorbate / TMPD
10 µm Gramicidin
CPM X 10^{-3}
12
10
8
6
4
1
5
15
25
35
45
Fraction Number

contained 10 mM $MgSO_4$, 25 μM (^3H)-$TPMP^+$, 20 mM ascorbate, and 2 mM TMPD. Uptake of $TPMP^+$ was initiated by the addition of membrane vesicles; the suspensions were oxygenated with water-saturated oxygen. Vesicles treated with 10 μM gramicidin were used as controls for the nonspecific binding of the isotope to membranes and filters. At various times, 0.1 ml aliquots, containing 100 μg of membrane protein, were filtered (Millipore type EH, 0.5 μ pore size) and washed with 2 ml of 100 mM LiCl. The filters were dried, dissolved in 10 ml of Betafluor (National Diagnostics) and counted for radioactivity as described above.

$TPMP^+$, a lipophilic cation, distributed across the membrane in response to the membrane potential, and steady-state levels of accumulation could be used to calculate that potential using the Nernst equation (Rottenberg, 1975, 1979):

$$\Delta\psi \text{ (in mV)} = -(RT/zF) \ln (TPMP^+)_{in}/(TPMP^+)_{out}$$

at room temperature the equation reduced to:

$$\Delta\psi = -58.8 \log (TPMP^+)_{in}/(TPMP^+)_{out}$$

5. Verification of the use of $TPMP^+$ as a probe for the $\Delta\psi$

To verify using $TPMP^+$ as an indicator of the membrane potential, right-side-out vesicles were swollen by decreasing the osmolarity of the buffer. Accumulation of $TPMP^+$ was greater in the swollen vesicles, but the calculated membrane potential was not significantly different from that in smaller vesicles.

6. Calculation of the protonmotive force

The total protonmotive force, $\Delta\bar{\mu}_H+$, was calculated from the following equation:

$$\Delta\bar{\mu}_H+ = \Delta\psi - 58.8\ \Delta pH$$

Determination of the protonmotive force in B. alcalophilus KM23 cells -

1. Measurement of the ΔpH

The ΔpH, interior alkaline, was determined from the distribution of 25 μM (^{14}C)-DMO in a flow dialysis assay, essentially as described in the preceeding sections. Initially, the upper chamber contained 25 mM potassium phosphate buffer at the indicated pH; the same buffer was pumped through the lower chamber. (^{14}C)-DMO was added to the upper chamber. After achieving equilibration, an aliquot of a thick suspension of cells, in the same buffer, was added to the upper chamber together with 10 mM L-malate (K^+-salt). The final cell suspension in the apparatus was 2 mg protein/ml in a total volume of 0.8 ml. Suspensions in the upper chamber were continuously gassed with water saturated oxygen. Gramicidin (10 μM) was added several fractions after the cells to dissipate the ΔpH. Fractions were collected and assayed as described for membrane vesicles. At every pH, controls were run for non-specific binding of DMO using heat-killed cells.

2. Measurement of the Δψ

The transmembrane potential was determined for cells of

B. alcalophilus KM23, suspended in 25 mM potassium phosphate buffer, at the indicated pHs, from the distribution of 10 µM (^{3}H)-TPMP$^+$ (100 µCi/ml) as described previously (Guffanti et al., 1978; Mandel and Krulwich, 1979).

Measurement of $^{22}Na^+$-uptake by everted membrane vesicles -

Uptake of $^{22}Na^+$ by everted vesicles was assayed by a flow dialysis assay as described for the determination of the ΔpH. Vesicles (0.8 ml) suspended (at 20 mg protein/ml) in 100 mM potassium carbonate buffer at pH 8.7, containing 10 mM mM $MgSO_4$, were added to the upper chamber. The vesicles were gassed with water-saturated oxygen. The same buffer was pumped through the lower chamber. After five minutes the experiment was initiated by adding $^{22}Na^+$ (250 µCi/ml) to the indicated concentrations. Fractions of 1.7 ml were collected and assayed for radioactivity as described for the ΔpH determinations. After 10 min (fraction # 30) NADH (K^+-salt) was added to 10 mM. In experiments in which vesicles were preincubated with 10 mM KSCN, the dialysate buffer also contained thiocyanate.

Fluorimetric studies with right-side-out membrane vesicles of B. alcalophilus and B. alcalophilus KM23 -

Energy-dependent generation of a ΔpH, interior acid, was monitored qualitatively in right-side-out membrane vesicles from the quenching of quinacrine dye fluorescence (Rosen and Adler, 1975; Brey et al., 1978). Fluorescence quenching was measured in a Perkin-Elmer model 650-10S Fluorescence Spectrophotometer coupled to a Perkin-Elmer mo-

del 023 chart recorder; the sample was excited at 425 nm and the emission recorded at 500 nm. The assay contained 200 µg of K^+-loaded or Na^+-loaded membrane vesicles protein, 100 mM potassium or sodium carbonate buffer at pH 9.0, 10 mM $MgSO_4$, and 1 µM quinacrine dye in a final volume of 1.0 ml. After recording an initial level of fluorescence, the vesicles were energized by adding 10 mM ascorbate plus 1 mM TMPD, and the change in the quenching of the dye was recorded. Correction was made for the non-specific quenching effects of ascorbate/TMPD on the quinacrine fluorescence by using heat-killed vesicles, or vesicles that had been pretreated with 10 µM gramicidin for 10 min prior to the assay.

Efflux of $^{22}Na^+$ from starved cells of B. alcalophilus and B. alcalophilus KM23 -

Sodium efflux from cells was measured essentially using methods developed by others (Tokuda and Kaback, 1977; Schuldiner and Fishkes, 1978) to study Na^+-efflux from membrane vesicles. Cells of B. alcalophilus were harvested (14,000 x g, 10 min), washed twice, and resuspended in 25 mM potassium carbonate buffer at pH 9.0 or 10.5 to their original density. The suspensions were incubated at 30 ^oC , with shaking for 7 to 24 h., depending on the experiment. Starved cells were then harvested (14,000 x g, 10 min) and resuspended in 25 mM potassium carbonate buffer, pH 9.0 or 10.5 at approximately 1 mg protein/ml. The suspensions were loaded with 1 mM $^{22}NaCl$ (6,000 cpm/nmol) for 1 h. at 25 ^oC; cells treated with 10 µM valinomycin were preincubated for 10 min prior to

addition of the energy source. KCl (20 mM) was added to un-energized cells instead of ascorbate/TMPD. At intervals, 0.2 ml aliquots were filtered and washed with 2 ml of 25 mM potassium carbonate buffer at the appropriate pH. Filtration and washing occured within 5 sec.

Sodium efflux from B. alcalophilus KM23 cells was compared to efflux from the wild type strain as described above except that: the buffer used was 25 mM potassium phosphate at pH 9.0; cells were starved for a total of 4 h.; and cells were energized by addition of 10 mM potassium L-malate.

Uptake of AIB by cells and right-side-out membrane vesicles -

Uptake of AIB was measured in cells of B. alcalophilus and B. alcalophilus KM23 as previously described (Guffanti et al., 1978; Krulwich et al., 1979). Cells were harvested, resuspended and washed twice with 25 mM potassium phosphate buffer at pH 9.0, and then resuspended to approximately 0.1 mg protein/ml. The cells were aerated by rapid mixing, KCl or NaCl were added to 10 mM, and uptake was initiated by adding 20 μM (^{14}C)-AIB. At various times, 1 ml aliquots were removed and filtered with washing.

Uptake of 20 μM (^{14}C)-AIB was also measured in right-side-out K^+-loaded membrane vesicles of B. alcalophilus and the non-alkalophilic mutant. The complete assay mixture contained 100 mM potassium carbonate buffer pH 9.0, 10 mM $MgSO_4$, 20 mM ascorbate (K^+-salt), 2 mM TMPD, 1 mM NaCl, and 20 μM (^{14}C)-AIB (100 μCi/ml). Vesicles were added to initiate the uptake. At various times, 0.1 ml aliquots were re-

removed and filtered and washed with 2 ml of 100 mM LiCl. The filters were dried and the radioactivity was counted as described above. For some assays sodium and/or the energy source were omitted from the suspensions.

Determination of protein concentration -

Protein concentrations were determined for all assays by the method of Lowry et al. (1951), using egg white lysozyme as the standard.

Reagents -

Ascorbic acid, ascorbate (sodium salt), ammediol, CCCP, choline chloride, choline base, DNase I, ethyl methansulfonate, gramicidin, lysozyme (egg white, EC 3.2.1.17), NADH (Tris and potassium salts), RNase, N,N, N', N'-tetramethyl-p-phenylenediamine, and valinomycin were all purchased from Sigma Chemical Co. (^{14}C)Methylamine hydrochloride (52.2 mCi/mmol), α-(1-^{14}C)-aminoisobutyric acid (9 mCi/mmol), (Carboxyl-^{14}C)inulin-carboxyl (2.1 mCi/g), (^{3}H)-H_2O (1 mCi/ml), (^{3}H) $TPMP^+$ (3.59 Ci/mmol), and ^{22}NaCl (4.6 mCi/ml, carrier free) were from New England Nuclear Corp. 5,5-Dimethyl (2-^{14}C) oxazolidine-2,4-dione (57 mCi/mmol) was purchased from Amersham Corporation. Purified agar and yeast extract were provided by Difco, and $TPMP^+$ was from ICN K+K Laboratories. All other reagents were obtained commercially at the highest purity available and were routinely provided by Fisher Scientific Co., or Sigma Chemical Co.

RESULTS

A. Measurement of components of the protonmotive force in membrane vesicles and cells -

1. The protonmotive force in right-side-out vesicles from L-malate grown cells of Bacillus alcalophilus

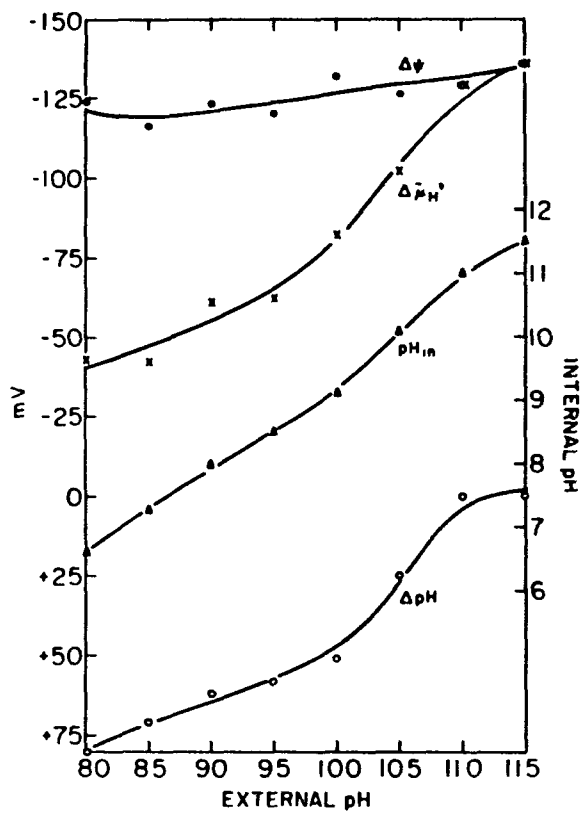
The protonmotive force was measured in right-side-out vesicles of B. alcalophilus isolated from cells grown on L-malate. Vesicles loaded with and resuspended in 100 mM sodium carbonate buffer at pH 9.0 (all containing 10 mM $MgSO_4$) were compared over a range of external pH. The vesicles were energized with ascorbate/TMPD, and the ΔpH was measured from distribution of either DMO or methylamine in a flow dialysis assay. The $\Delta\psi$ was measured from uptake of $TPMP^+$ by a filtration assay. All assays were as described in the "Materials and Methods".

Upon energization, Na^+-loaded vesicles, generated a ΔpH, interior acid, over a range of external pH from pH 8.0 to 10.5 (Figure 3, left panel; Table 1). Between pH 8.0 and 10.5 these vesicles accumulated methylamine and did not accumulate DMO. The ΔpH was zero at and above pH 11.0. The $\Delta\psi$ increased very slightly, from -120 to -136 mV, over the pH range studied; the total protonmotive force ($\Delta\bar{\mu}_{H^+}$) increased from -44 mV at pH 8.0 to -136 mV at pH 11.5, essentially following the shape of the ΔpH curve (Fig. 3, left panel). Vesicles loaded with and resuspended in potassium carbonate buffer

FIGURE 3

The effect of external pH on the internal pH, ΔpH, $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ generated by right-side-out vesicles isolated from L-malate-grown cells of B. alcalophilus

Vesicles were prepared and assayed as described in the "Materials and Methods". Left panel: Na^+-loaded vesicles suspended in 100 mM sodium carbonate buffer containing 10 mM $MgSO_4$ at the indicated pHs. Right panel: K^+-loaded vesicles suspended in 100 mM potassium carbonate buffer containing 10 mM $MgSO_4$ at the indicated pHs.


-150
-125
-100
-75
-50
-25
0
+25
+50
+75
mV
12
11
10
9
8
7
6
INTERNAL pH
80 85 90 95 100 105 110 115
EXTERNAL pH
Δψ
Δμ̃H+
pHin
ΔpH

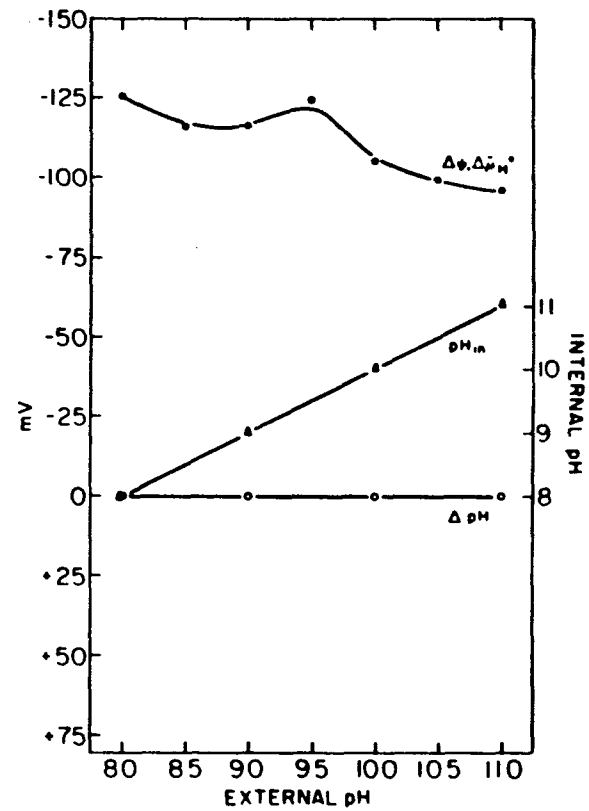

-150
-125
-100
-75
-50
-25
0
+25
+50
+75
mV
11
10
9
8
INTERNAL pH
80 85 90 95 100 105 110
EXTERNAL pH
Δψ.Δμ̃H+
pHin
Δ pH

TABLE 1

Protonmotive force generated by right-side-out membrane vesicles of B. alcalophilus loaded with sodium carbonate buffer

External pH	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)	Internal pH
8.0	-124	+80	- 44	6.6
8.5	-116	+71	- 45	7.4
9.0	-123	+62	- 61	8.0
9.5	-120	+58	- 62	8.5
10.0	-132	+51	- 81	9.2
10.5	-127	+25	-102	10.1
11.0	-129	0	-129	11.0
11.5	-136	0	-136	11.5

Right side out Na^+-loaded vesicles were prepared and the internal pH, ΔpH, $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ determined as described in the "Materials and Methods" and legend to Figure 3.

(K^+-loaded), exhibited a markedly different energetic pattern, As illustrated in Figure 3 (right panel), and Table 2, K^+-loaded vesicles did not generate a ΔpH over the entire pH range examined; these vesicles accumulated neither DMO nor methylamine upon energization. Since the ΔpH was zero, the internal and external pHs were the same, and the $\Delta\bar{\mu}_{H^+}$ therefore corresponded to the $\Delta\psi$. The membrane potential of K^+-loaded vesicles appeared to decrease slightly from -125 mV to -96 mV, as the external pH was increased from pH 8.0 to 11.0 (Fig. 3, right panel; Table 2). As shown in Figure 4, steady-state levels of $TPMP^+$ accumulation by K^+-loaded vesicles were achieved within 2 - 3 minutes of energization. These results, for vesicles suspended at pH 9.5, were typical of those observed at this and other pHs with K^+-, Na^+-, and ammediol-loaded right-side-out vesicles of B. alcalophilus. Addition of 10 µM gramicidin abolished the protonmotive force and prevented $TPMP^+$ uptake. Likewise, adding 10 µM valinomycin, a K^+-specific ionophore (Asghar et al., 1973) to K^+-loaded vesicles, dissipated the $\Delta\psi$ and prevented $TPMP^+$ accumulation.

Vesicles were isolated in the absence of both Na^+ and K^+, by loading them with 100 mM ammediol and 10 mM $MgSO_4$ at pH 9.0. Upon energization, ammediol-loaded vesicles generated both a $\Delta\psi$ and a ΔpH or conventional orientation, acid and positive outside (Table 3). Over a range of external pH from pH 8.0 to 9.5, ammediol-loaded vesicles generated a small ΔpH of -35 to -45 mV, alkalinizing the intravesicular

TABLE 2

Protonmotive force generated by right-side-out membrane vesicles of B. alcalophilus loaded with potassium carbonate buffer

External pH	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)	Internal pH
8.0	-125	0	-125	8.0
8.5	-116	n.d.*	-116	
9.0	-116	0	-116	9.0
9.5	-123	n.d.	-123	
10.0	-105	0	-105	10.0
10.5	- 99	n.d.	- 99	
11.0	- 96	0	- 96	11.0

*, not determined

Right-side-out K^+-loaded vesicles were prepared and the internal pH, ΔpH, $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ determined as described in the "Materials and Methods" and legend to Figure 3.

FIGURE 4

TPMP$^+$ uptake by K^+-loaded right-side-out membrane vesicles of B. alcalophilus

Uptake of 25 μM (^{3}H)-TPMP$^+$ was assayed as described in the "Materials and Methods". Vesicles were suspended in 100 mM potassium carbonate buffer containing 10 mM $MgSO_4$ at pH 9.5; 20 mM ascorbate plus 2 mM TMPD were added as the energy source. (●) Ascorbate/TMPD only; (Δ) vesicles treated with 10 μM gramicidin; (O) vesicles treated with 10 μM valinomycin.

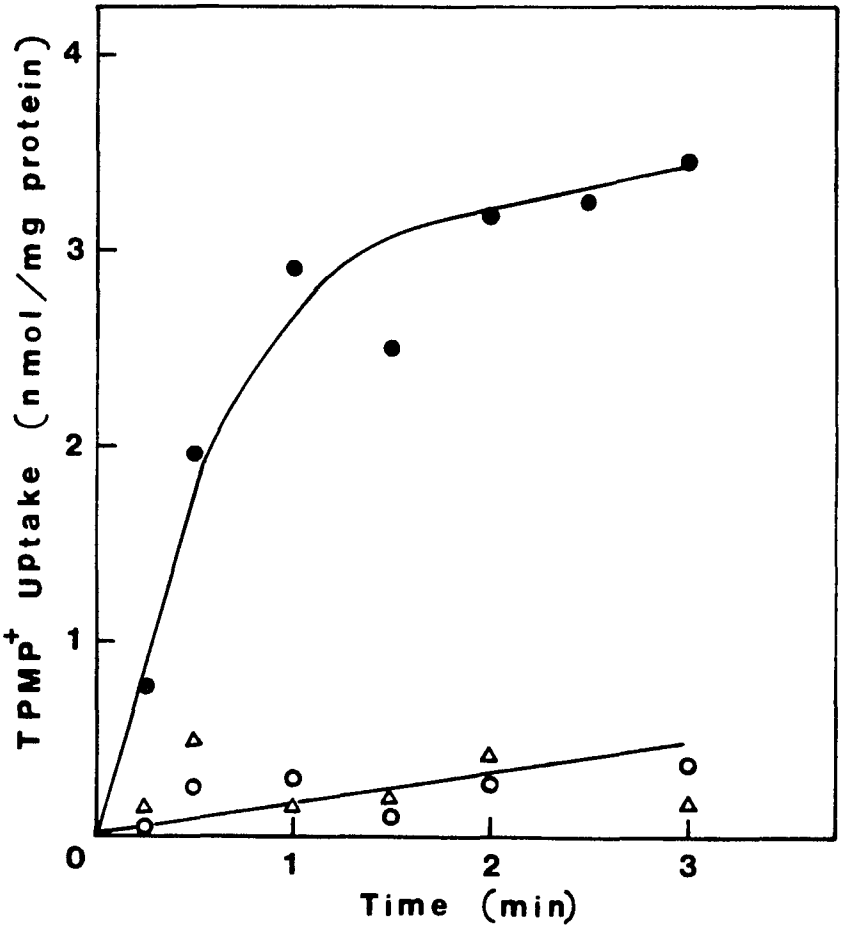

TPMP+ UPtake (nmol/mg protein)
4
3
2
1
0
1
2
3
Time (min)

TABLE 3

Protonmotive force generated by right-side-out membrane vesicles of B. alcalophilus loaded with ammediol buffer

External pH	$\Delta\psi$ (mv)	ΔpH (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)	Internal pH
8.0	- 64	- 44	-108	8.75
8.5	- 84	- 42	-126	9.21
9.0	- 84	- 41	-125	9.70
9.5	-100	- 35	-135	10.10
10.0	- 96	n.d.*	$\geq$-96	-

*, not determined

Right-side-out ammediol-loaded vesicles were prepared and the internal pH, ΔpH, $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ determined as described in the "Materials and Methods" The vesicles were suspended in 100 mM ammediol and 10 mM $MgSO_4$ at the indicated external pH at approximately 10 mg protein/ml.

space, and therefore accumulated DMO and not methylamine. A $\Delta\psi$ of -64 to -100 mV, interior negative, was also generated over this pH range. Similar results to those observed with ammediol-loaded vesicles were observed with B. alcalophilus vesicles loaded with 10 mM Tris, 100 mM choline chloride, and 10 mM $MgSO_4$ at pH 9.0 (data not shown).

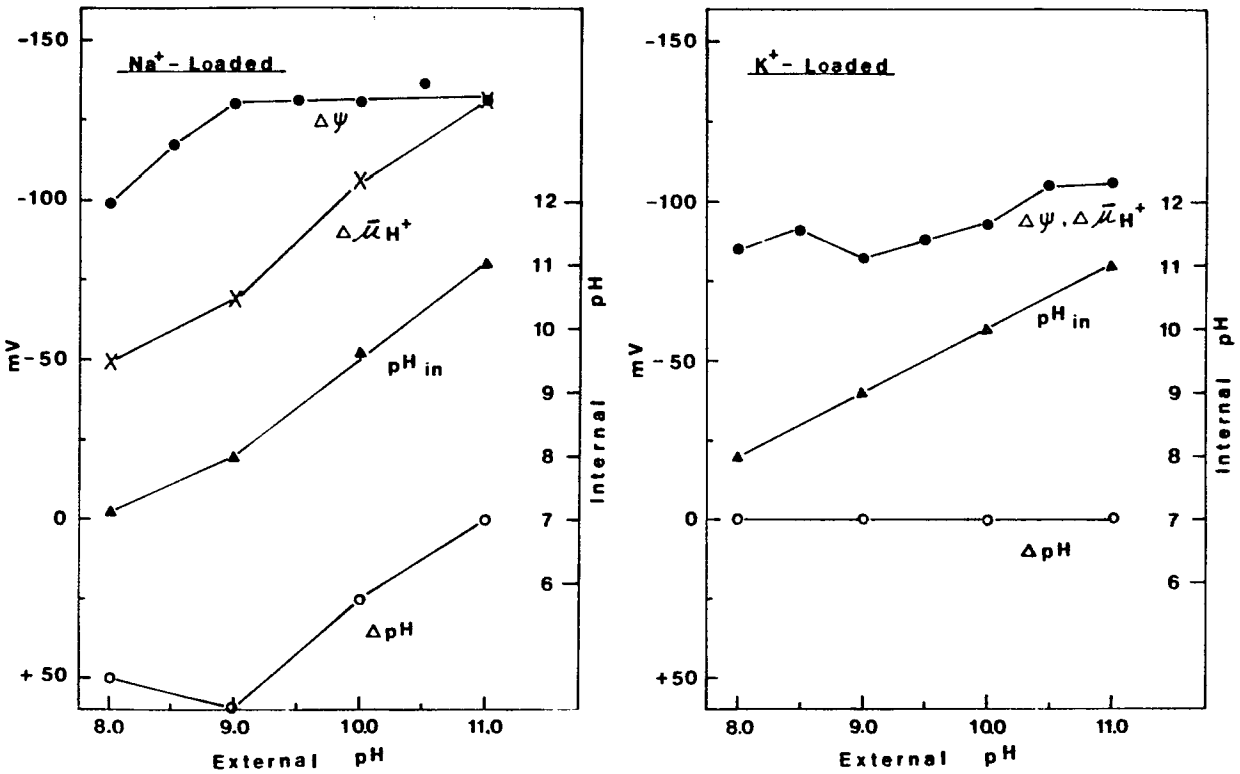
2. The protonmotive force in right-side-out vesicles from lactose-grown cells of B. alcalophilus

Na^+-loaded and K^+-loaded membrane vesicles were prepared from lactose-grown cells of B. alcalophilus in order to compare their protonmotive force patterns with those from the L-malate-grown cells. These vesicles were also energized by addition of ascorbate/TMPD. As shown in Figure 5 (left panel), Na^+-loaded membrane vesicles from lactose-grown cells, suspended in 100 mM sodium carbonate buffer containing 10 mM $MgSO_4$, at the indicated pHs exhibited an acidification of the intravesicular space. These vesicles accumulated methylamine but not DMO. The ΔpH, interior acid, was between +50 and +60 mV at pH 8.0 to 9.0, and then decreased to zero at pH 11. The $\Delta\psi$ increased from -100 mV at pH 8.0 to -130 mV at pH 9.0 and then remained constant. The $\Delta\bar{\mu}_{H^+}$ therefore increased from -50 mV at pH 8.0 to -130 mV at pH 11.0. K^+-loaded vesicles from lactose-grown cells, like those from L-malate-grown cells, did not generate a ΔpH, either conventional or "reversed". The $\Delta\psi$, which corresponded to the $\Delta\bar{\mu}_{H^+}$, increased slightly as the pH was increased from 8.0 to 11.0 (Fig. 5,

FIGURE 5

The effect of external pH on the internal pH, ΔpH, $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ generated by right-side-out vesicles isolated from lactose-grown cells of B. alcalophilus

Vesicles were prepared from lactose-grown cells and assayed as described in the "Materials and Methods". Left panel: Na^+-loaded vesicles suspended in 100 mM sodium carbonate buffer containing 10 mM $MgSO_4$ at the indicated pHs. Right panel: K^+-loaded vesicles suspended in 100 mM potassium carbonate buffer containing 10 mM $MgSO_4$ at the indicated pHs.


Na+ - Loaded
Δψ
Δμ̄H+
pH in
ΔpH
-150
-100
-50
0
+50
mV
12
11
10
9
8
7
6
Internal pH
8.0
9.0
10.0
11.0
External pH
K+ - Loaded
Δψ , Δμ̄H+
pH in
ΔpH
-150
-100
-50
0
+50
mV
12
11
10
9
8
7
6
Internal pH
8.0
9.0
10.0
11.0
External pH

right panel).

3. The protonmotive force in cells and right-side-out vesicles from B. alcalophilus KM23

B. alcalophilus KM23, the non-alkalophilic mutant strain derived from B. alcalophilus, was grown to the late logarithmic stage on PT6.8 medium with 50 mM potassium L-malate as the carbon source as described in the "Materials and Methods". The non-alkalophilic strain grew in a range of pH between 5.0 and 9.0, but not above pH 9.0; its pH optimum for growth was between pH 5.5 and 6.8 (Figure 6). By contrast, the wild type organism did not grow below pH 8.5 on L-malate, and grew at pHs up to and including pH 11.5, with optimal growth at pH 10.5 (Guffanti et al., 1978). Both B. alcalophilus and B. alcalophilus KM23 utilized the same carbon sources for growth when cultured on PT medium buffered at pH 9.0 (Table 4).

The components and magnitude of the protonmotive force generated by cells of B. alcalophilus KM23 were determined over a range of pH from pH 5.5 to pH 9.0 as described in the "Materials and Methods". The results of these studies are presented in Table 5. At pH 5.5, cells of B. alcalophilus KM23 generated a $\Delta\bar{\mu}_{H^+}$ of -136 mV which decreased to -92 mV at pH 9.0. The $\Delta\psi$, interior negative, measured from accumulation of $TPMP^+$, was -53 mV at pH 5.5 and increased to -92 mV at pH 8.0 to 9.0. B. alcalophilus KM23 also generated a ΔpH, interior alkaline, measured from the accumulation of

FIGURE 6

Growth of B. alcalophilus KM23 on L-malate as a function of the external pH

The initial growth rate was determined before changes in pH occurred. Growth was determined using the PT6.8 medium described in the "Materials and Methods", except that the pH was adjusted to the indicated values. Potassium L-malate added to 50 mM was the carbon source.

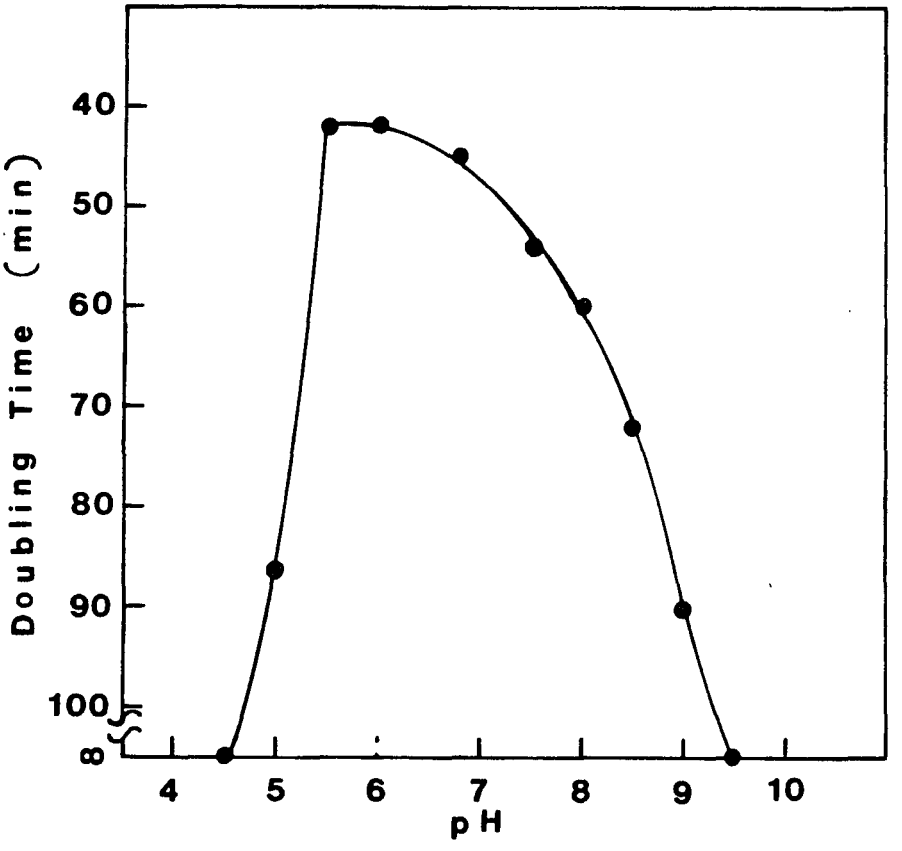

Doubling Time (min)
40
50
60
70
80
90
100
∞
4
5
6
7
8
9
10
pH

TABLE 4

Utilization of various carbon sources for growth by B. alcalophilus and B. alcalophilus KM23

Carbon source	B. alcalophilus	B. alcalophilus KM23
L-Malate	+	+
β-D-Fructose	+	+
α-Lactose	+	+
L-Glutamate	-	-
L-Alanine	+	+
L-Aspartate	+	+
D-Gluconate	+	+
D-Galactose	+	+
Succinate	+	+
L-Rhamnose	+	+

Cells of B. alcalophilus and B. alcalophilus KM23 were grown on PT9 medium (Guffanti et al., 1979b) supplemented with 0.1% (w/v) yeast extract, and 1% (v/v)trace salts solution as described in "Materials and Methods". Carbon sources were added to final concentrations of 50 mM, except the α-lactose, which was added to 25 mM.

TABLE 5

Protonmotive force generated by cells of B. alcalophilus KM23

External pH	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)	Internal pH
5.5	- 53	- 83	-136	6.9
7.0	- 93	- 30	-123	7.5
8.0	- 93	0	- 93	8.0
9.0	- 92	0	- 92	9.0

L-Malate-grown cells of B. alcalophilus KM23 were harvested and resuspended in 25 mM potassium phosphate buffer at the indicated pHs. The internal pH, ΔpH, $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ were determined as described in the "Materials and Methods".

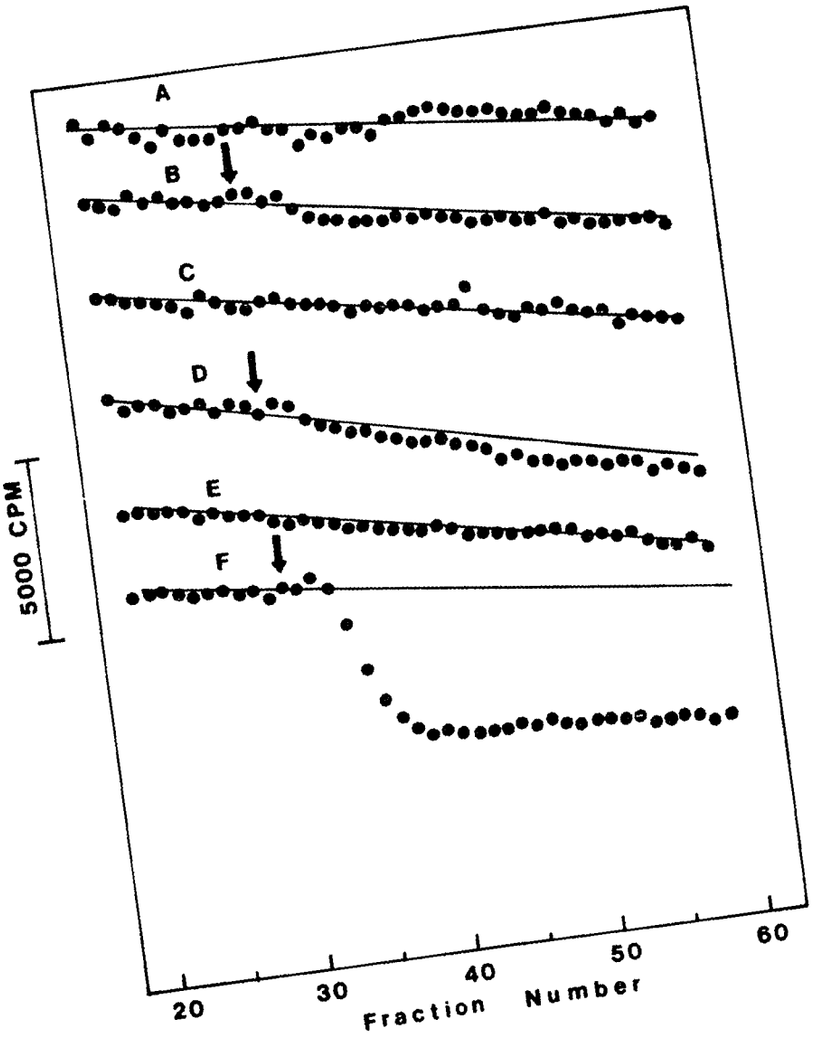
DMO. The ΔpH was -83 mV at pH 5.5 (1.4 units, interior alkaline) and decreased to 0 mV at pH 8.0 to 9.0. The non-alkalophilic strain could not "reverse" its ΔpH to acidify its cytoplasm. The $\Delta\bar{\mu}_{H}+$ of -90 mV generated by B. alcalophilus KM23 at pH 9.0 is approximately of the same magnitude as that generated by the wild type strain at pH 9.0 (Guffanti et al., 1978). At pH 9.0, the wild type strain maintained a protonmotive force of -87 mV, consisting of only a membrane potential.

It was also of interest to measure the protonmotive force generated by energized right-side-out membrane vesicles of B. alcalophilus KM23. Right-side-out vesicles were isolated from L-malate-grown cells as described in "Materials and Methods" and loaded with: a) 100 mM potassium carbonate buffer and 10 mM $MgSO_4$ at pH 9.0 ($K-CO_3$-loaded vesicles); b) 100 mM potassium phosphate buffer with 10 mM $MgSO_4$ at pH 6.0 ($K-PO_4$-loaded vesicles); and c) 10 mM Tris, 100 mM choline chloride, with 10 mM $MgSO_4$ at pH 9.0 (choline-Tris-loaded vesicles). Uptake of 55 μM DMO was measured by flow dialysis to monitor generation of a ΔpH, interior alkaline. Vesicles loaded and suspended in potassium carbonate buffer at pH 9.0 (Figure 7, A & B), or potassium phosphate buffer at pH 6.0 (Figure 7, C & D), failed to generate a ΔpH, interior alkaline, upon energization with ascorbate/TMPD. Similarly, unenergized choline-Tris-loaded vesicles failed to accumulate DMO (Figure 7E). However, upon energization, choline-Tris-loaded vesicles of B. alcalophilus KM23 accumulated DMO,

FIGURE 7

Generation of a ΔpH, interior alkaline, by right-side-out membrane vesicles of B. alcalophilus KM23 in the absence of potassium ions

Uptake of 55 μM (^{14}C)-DMO was measured by flow dialysis as described in "Materials and Methods" in vesicles loaded with and suspended in: 100 mM potassium carbonate buffer, pH 9.0 (A & B); 100 mM potassium phosphate buffer, pH 6.0 (C & D); and 10 mM Tris, 100 mM choline chloride, pH 9.0 (E & F). $MgSO_4$, 10 mM, was present in all buffers, and the same buffer was pumped through the lower chamber of the apparatus. At the arrows, 20 mM ascorbate (appropriate salt) and 2 mM TMPD were added to energize the vesicles.


A
B
C
D
E
F
5000 CPM
20
30
40
50
60
Fraction Number

generating a ΔpH, interior alkaline, of 1.3 units (-74 mV) (Figure 7F). Similar results were observed with choline-Tris vesicles from B. alcalophilus KM23 at pH 6.6 (data not shown). Thus, as observed with right-side-out vesicles from wild type B. alcalophilus, vesicles of B. alcalophilus KM23 generated a ΔpH, interior alkaline, when prepared in the absence of potassium, but not in its presence. The $K\text{-}CO_3$-loaded B. alcalophilus KM23 vesicles generated a membrane potential of -75 mV as determined from accumulation of $TPMP^+$.

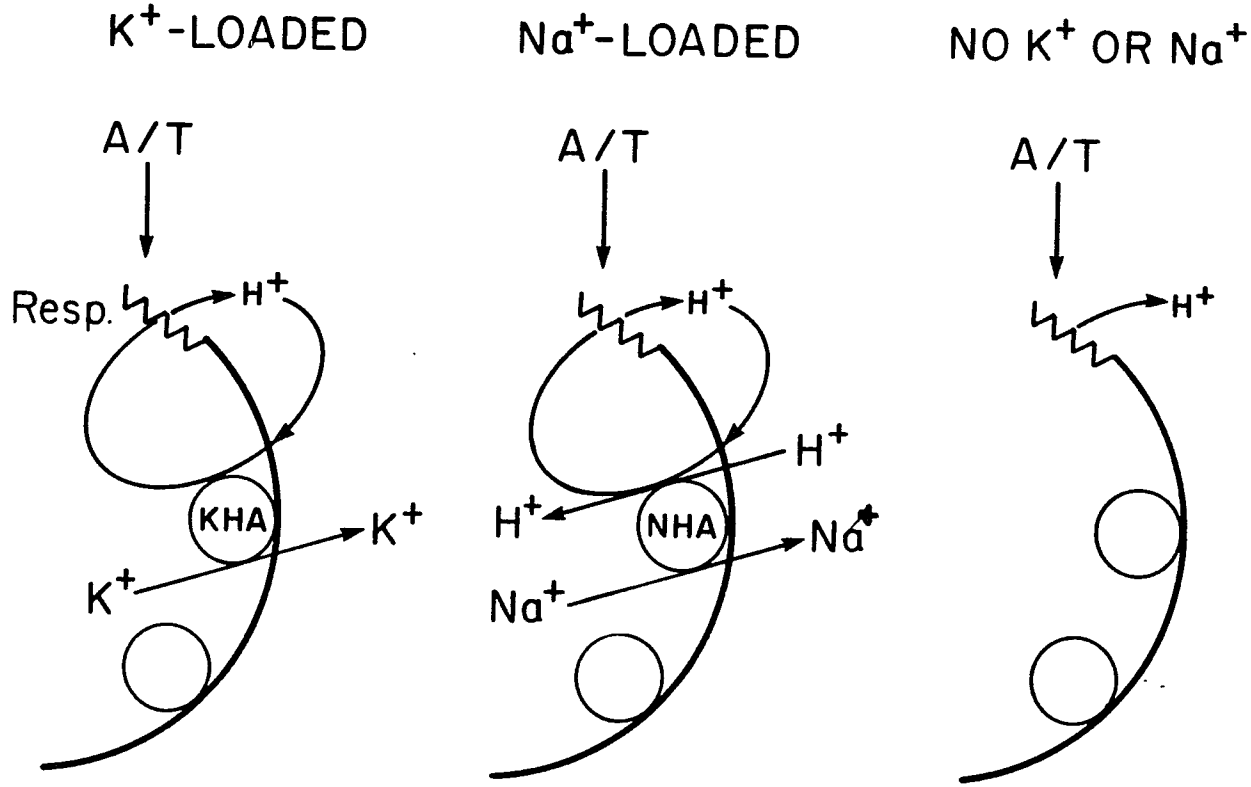
A model which could account for the energetic patterns observed with right-side-out membrane vesicles from wild type B. alcalophilus is presented in Figure 8. In the absence of both K^+ and Na^+, i.e., conditions achieved with ammediol-loaded or choline-Tris-loaded vesicles, protons were extruded by respiration upon addition of an energy source. The primary proton extrusion produced the observed protonmotive force pattern, exterior acid and positive. An electroneutral K^+/H^+ antiporter, active in the potassium-loaded vesicles, could exchange K^+ for extruded protons, dissipating the ΔpH and maintaining the $\Delta\psi$, outside positive. The bioenergetic pattern observed in Na^+-loaded vesicles could be accounted for by a Na^+/H^+ antiporter. In exchange for Na^+, this antiporter could inwardly translocate more protons than are effectively extruded by respiration. The antiporter would be energized, at least in part, by the membrane potential. Depending on the rates of Na^+/H^+ exchange and of proton extrusion from respiration, the $\Delta\psi$ generated could be of consid-

erable magnitude. The non-alkalophilic strain might retain the K^+/H^+ antiporter while lacking the Na^+/H^+ antiporter. The activities of both the Na^+/H^+ and K^+/H^+ antiporters in B. alcalophilus and the non-alkalophilic mutant were further documented in the following series of experiments.

FIGURE 8

A model illustrating possible antiporter involvement in generation of the observed protonmotive force patterns

A/T, ascorbate/ TMPD; KHA, K^+/H^+ antiporter; NHA, Na^+/H^+ antiporter; and the respiratory chain is the site of primary proton extrusion.


K+-LOADED
Na+-LOADED
NO K+ OR Na+
A/T
A/T
A/T
Resp.
H+
H+
H+
KHA
K+
K+
H+
H+
NHA
Na+
Na+

B. Monovalent cation/proton antiporters of Bacillus alcalophilus -

1. Sodium efflux from starved cells of B. alcalophilus and B. alcalophilus KM23

Energy-dependent efflux of Na^+ from starved cells of B. alcalophilus was examined. Cells were starved and passively loaded with 1 mM $^{22}NaCl$ as described in the "Materials and Methods". Addition of 20 mM potassium ascorbate plus 2 mM TMPD to starved, unenergized cells, elicited a rapid efflux of $^{22}Na^+$ from the cells at either pH 9.0 or 10.5 (Figure 9, A & B, closed circles). Cells treated with 10 µM valinomycin, 10 min prior to energization failed to exhibit efflux of $^{22}Na^+$. Parallel measurement of the membrane potential, demonstrated that cells incubated with ascorbate plus TMPD generated a $\Delta\psi$ of -120 to -130 mV; addition of 10 µM valinomycin reduced the $\Delta\psi$ to 0 to -10 mV (data not shown). Starved cells to which neither valinomycin nor the energy source was added failed to exhibit efflux of $^{22}Na^+$ as long as the endogenous $\Delta\psi$ was less than -95 mV. In experiments, in which short starvation times were used, and the $\Delta\psi$ of the cells was above -95 mV, $^{22}Na^+$-efflux was observed without further addition of an energy source. Cells of B. alcalophilus KM23 were starved for 4 hours as described in the "Materials and Methods". The cells were suspended in 25 mM potassium phosphate buffer at pH 9.0 and passively loaded with 1 mM $^{22}NaCl$. As shown in Figure 10, B. alcalophilus

FIGURE 9

Efflux of passively loaded $^{22}Na^{+}$ from starved cells of B. alcalophilus

Cells were starved for 16 h., and assayed for $^{22}Na^{+}$-efflux as described in the "Materials and Methods". Key to figure: (x) unenergized cells; (O) cells energized with 20 mM ascorbate plus 2 mM TMPD; (O) cells treated with 10 µM valinomycin prior to energization with ascorbate/TMPD.

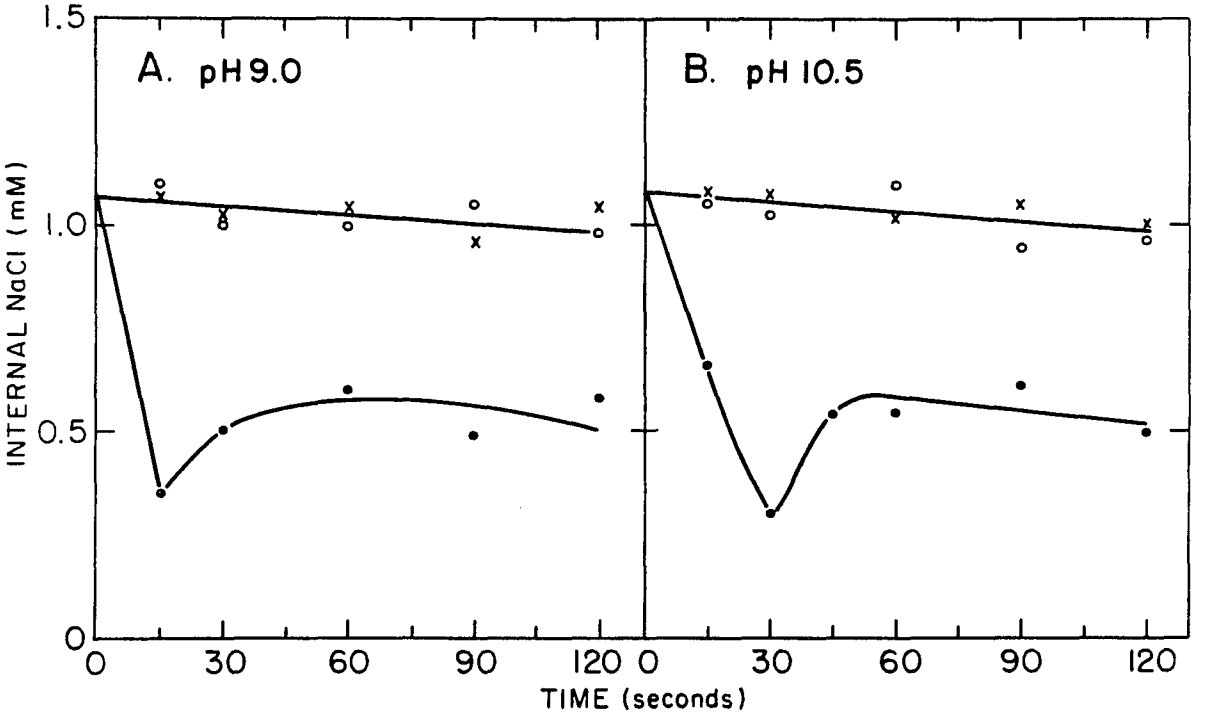
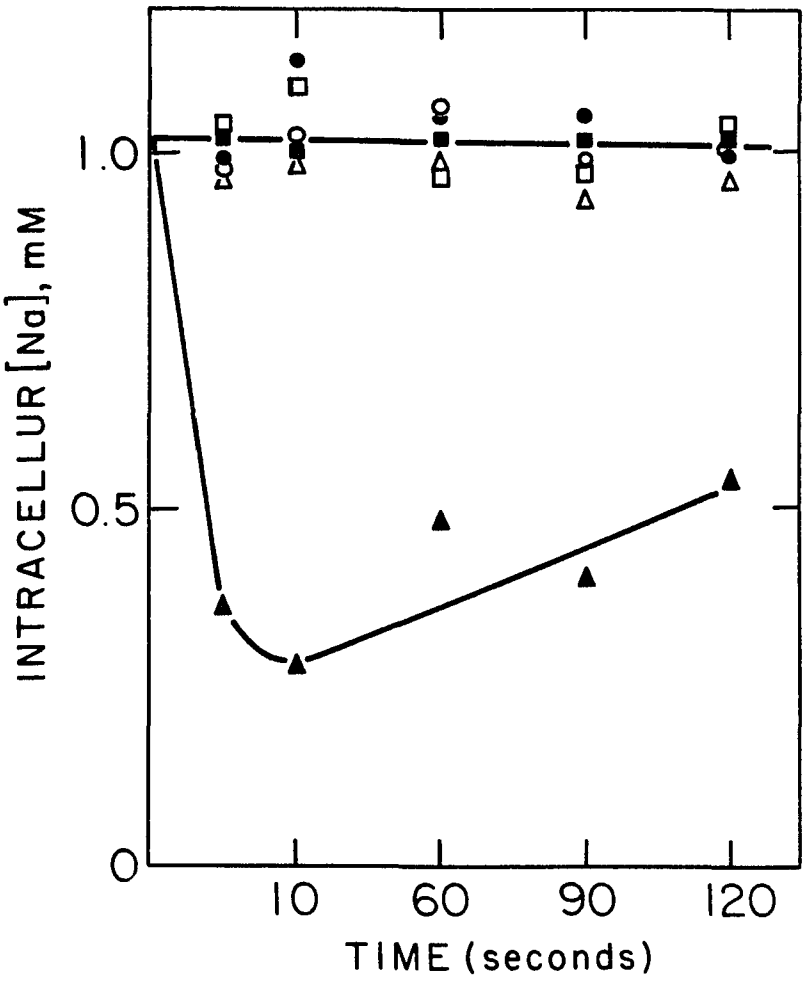

A. pH 9.0
B. pH 10.5
INTERNAL NaCl (mM)
1.5
1.0
0.5
0
0
30
60
90
120
TIME (seconds)

FIGURE 10

Efflux of passively loaded $^{22}Na^{+}$ from cells of B. alcalophilus and B. alcalophilus KM23

Cells of B.alcalophilus (closed symbols) and B. alcalophilus KM23 (open symbols) were starved, suspended in 25 mM potassium phosphate buffer at pH 9.0, and assayed for $^{22}Na^{+}$-efflux as described in the "Materials and Methods". After loading with sodium, the cells were warmed to 25°C and separated into aliquots. One aliquot of cells was heat-killed (●,○) by placing them in a boiling water bath for 5 min.; one aliquot was treated with 10 µM valinomycin for 10 min. prior to energization (■,□); the final aliquot was kept at 25°C for 10 min. (▲,△): The efflux of Na^{+} was initiated by the addition of 10 mM potassium-L-malate, pH 9.0.


INTRACELLUR [Na], mM
1.0
0.5
0
10
60
90
120
TIME (seconds)

KM23 cells failed to exhibit energy-dependent efflux of $^{22}Na^+$ while similarly treated cells of the wild type strain exhibited valinomycin sensitive efflux of sodium. In these experiments the cells were energized by addition of 10 mM L-malate; energization with ascorbate/TMPD did not cause sodium efflux from B. alcalophilus KM23 (data not shown).

2. Sodium-dependent acidification of the intravesicular space of right-side-out vesicles

Na^+-dependent acidification of the intravesicular space of right-side-out vesicles of B. alcalophilus was measured qualitatively by an assay in which the quenching of quinacrine dye fluorescence was used as an indicator of proton influx (Rosen and Adler, 1975). Vesicles of B. alcalophilus and B. alcalophilus KM23 were prepared in 100 mM potassium carbonate buffer and 10 mM $MgSO_4$ at pH 9.0. The vesicles were diluted 10-fold into either 100 mM potassium carbonate or sodium carbonate buffer at pH 9.0 containing 10 mM $MgSO_4$, incubated at 0°C for 10 min., allowing the ions to equilibrate across the membrane, and then warmed to 25°C. Quinacrine fluorescence was monitored as described under "Materials and Methods". In the presence of intravesicular Na^+, but not K^+, quinacrine fluorescence was markedly quenched in suspensions of energized vesicles (Table 6). The quenching was prevented by treating the vesicles with 10 µM gramicidin. Vesicles from B. alcalophilus KM23 failed to exhibit Na^+-dependent quenching of quinacrine fluorescence.

TABLE 6

Na^+-dependence of acidification of the interior of right-side-out membrane vesicles

Vesicles	Diluting and Assay Buffer	Gramicidin present	% Quenching of quinacrine fluorescence
Wild type	$K\text{-}CO_3$	-	10
	$Na\text{-}CO_3$	-	49
	$Na\text{-}CO_3$	+	0
KM23	$K\text{-}CO_3$	-	0
	$Na\text{-}CO_3$	-	0
	$Na\text{-}CO_3$	+	0

Quinacrine fluorescence was monitored as described in the "Materials and Methods".

Furthermore, addition of 20 mM or 50 mM Na-CO_3 simultaneously with the energy source, to B. alcalophilus vesicles suspended in potassium carbonate buffer, did not increase the relative quenching of the quinacrine dye (data not shown). Thus it appeared that sodium must be present within the intravesicular space to effect an energy-dependent acidification of the intravesicular space.

As shown in Figure 11, K^+-loaded right-side-out vesicles of B. alcalophilus (solid line) generated a ΔpH, interior acid, in the presence of concentrations of Na^+ as low as 1 mM; the magnitude of the ΔpH increased as the Na^+ concentration was raised. In the experiments shown, Na^+ was added as the carbonate salt at pH 9.0 two minutes prior to energization. Identical results were obtained with vesicles preincubated in the presence of Na^+ for 30 min. Linear regression analysis indicated that a half-maximal effect of sodium occured at 0.7 mM sodium. As shown by the dashed line, at the bottom of the figure, vesicles of B. alcalophilus KM23 did not acidify their intravesicular space upon addition of sodium.

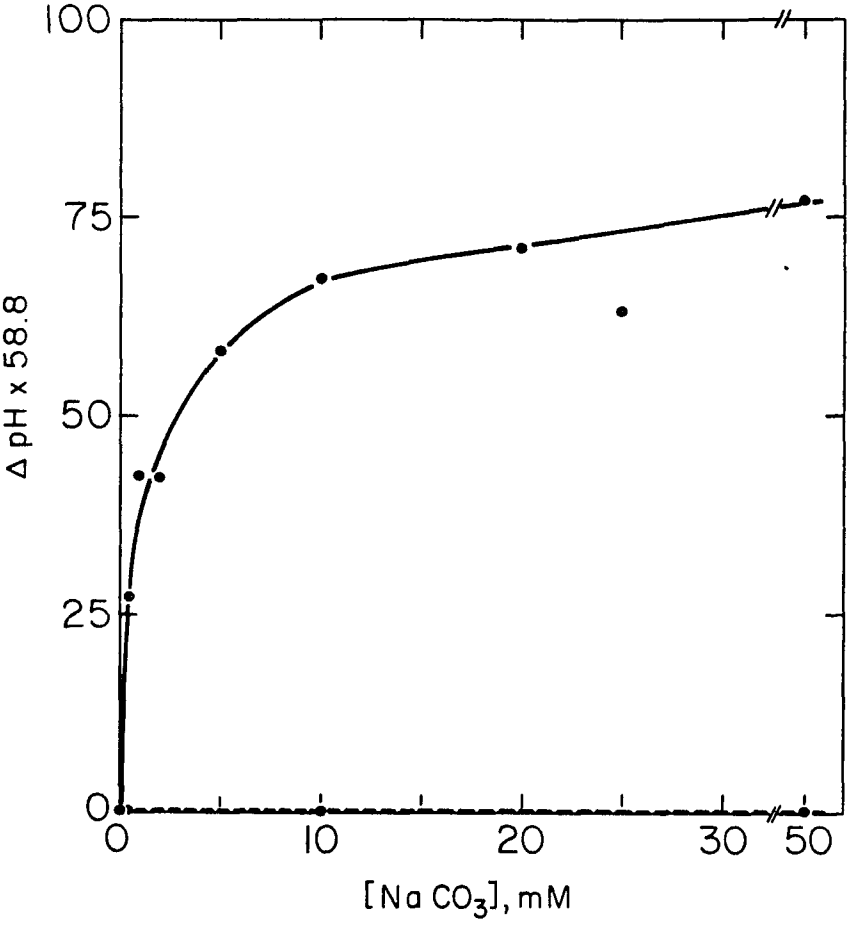
3. Sodium-dependent alkalinization of the intravesicular space in everted membrane vesicles

Everted membrane vesicles were prepared in potassium carbonate buffer at pH 8.7 from L-malate-grown cells of B. alcalophilus and B. alcalophilus KM23. The everted vesicles were energized from addition of 10 mM NADH (K^+-salt).

FIGURE 11

Acidification of the intravesicular space of right-side-out vesicles as a function of Na^+ concentration

K^+-loaded vesicles of wild type B. alcalophilus (solid line) and B. alcalophilus KM23 (dashed line) were suspended in 100 mM K-CO_3 buffer, 10 mM $MgSO_4$, pH 9.0, at 10 mg vesicle protein/ml. Na-CO_3, pH 9.0, was added to the indicated concentrations 2 min prior to energization with 20 mM K-ascorbate/ 2 mM TMPD. The ΔpH was determined from the distribution of 25 μM methylamine as described in "Materials and Methods". Each point represents the average of several determinations.


100
75
50
25
0
Δ pH x 58.8
0
10
20
30
50
[Na CO3], mM

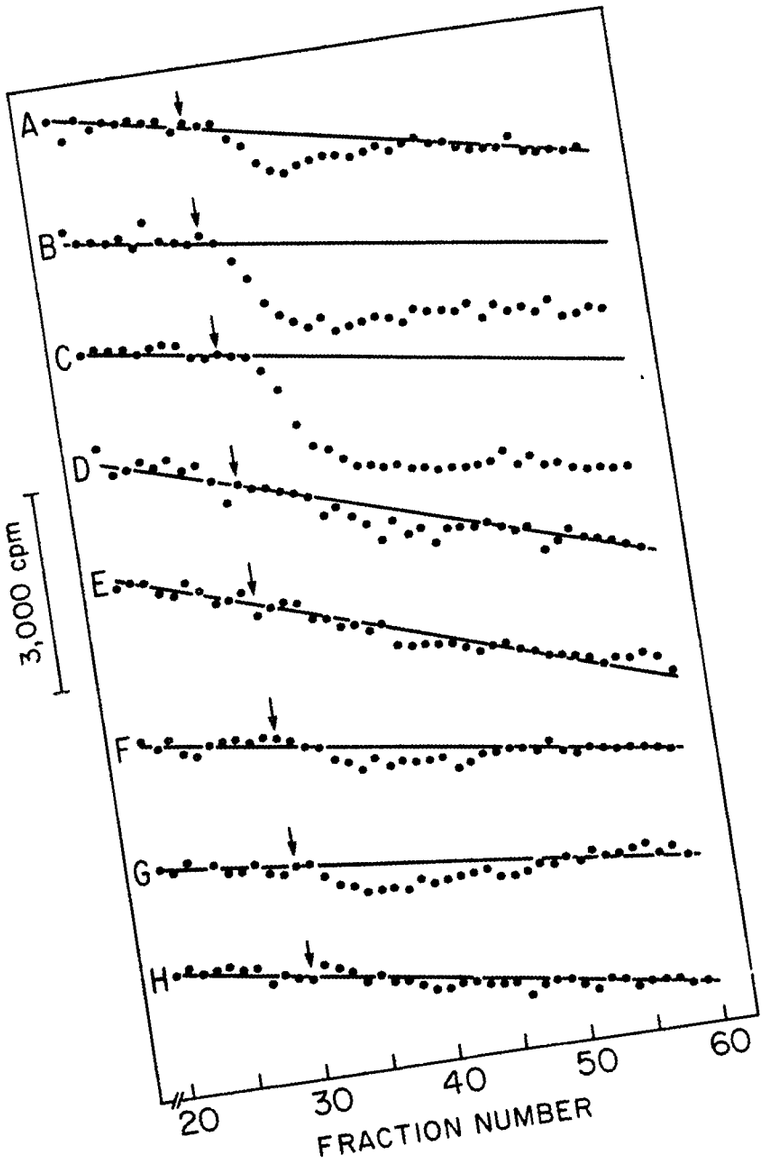
Alkalinization of the intravesicular space was measured from accumulation of the weak acid DMO, while acidification could be demonstrated by uptake of methylamine.

K^+-loaded everted vesicles of B. alcalophilus, suspended in potassium carbonate buffer, exhibited no methylamine uptake (data not shown) and little or no DMO uptake upon energization with NADH; the transient DMO uptake sometimes observed in the absence of exogenously added Na^+ (Figure 12, A) correlated with somewhat variable sodium contamination of the solutions. The levels of sodium contamination were in the range of 10 to 100 μM Na^+. Treatment of vesicles with 10 μM gramicidin prior to energization completely abolished any DMO uptake (data not shown). Addition of 10 mM Na^+ or Li^+ (carbonate salts) with the energy source, stimulated DMO uptake (Figure 12, B & C respectively). This observation was consistant with the Na^+-dependent uptake of methylamine observed in right-side-out vesicles (Figure 11). A ΔpH of -53 mV was calculated from the DMO accumulation by the everted vesicles, in the presence of 10 mM Na^+. Similar results were observed when 10 mM $NaNO_3$ or 5 mM Na_2SO_4 were added to energized everted vesicles of B. alcalophilus (data not shown). Treating the everted vesicles with either 10 mM KSCN (Figure 12, D & E) or 10 μM valinomycin (Figure 12, F & G) to abolish the membrane potential (Asghar et al., 1973; Krulwich et al., 1978), prevented Na^+-dependent alkalinization of the intravesicular space (Figure 12, E & G). DMO was not accumulated by energized everted vesicles of the

FIGURE 12

Sodium-dependent alkalinization of the intravesicular space of K^+-loaded everted membrane vesicles

K^+-loaded everted membrane vesicles from wild type B. alcalophilus (A-G) and B. alcalophilus KM23 (H) were suspended in 100 mM K-CO_3 buffer, 10 mM $MgSO_4$, pH 8.7 at approximately 20 mg protein/ml and uptake of 55 µM (^{14}C)-DMO (250 µCi/ml) was assayed as described in the "Materials and Methods". DMO was present from the beginning of the experiment. At the times indicated by the arrows, 10 mM NADH (K^+-salt) was added together with: (A), (D), and (F), no further additions; (B), (E), and (G), 10 mM Na-CO_3, pH 8.7; and (C), 10 mM $LiCO_3$. For the experiments shown in (D) and (E), vesicles were preincubated with 10 mM KSCN for 30 min; 10 mM KSCN was also included in the dialysate buffer. For the experiments shown in (F) and (G), vesicles were preincubated with 10 µM valinomycin. In the experiment shown in (H), 10 mM NADH and 10 mM Na-CO_3 were added to KM23 everted vesicles.


A
B
C
D
E
F
G
H
3,000 cpm
20
30
40
50
60
FRACTION NUMBER

non-alkalophilic mutant in the presence of 10 mM Na^+ (Figure 12, H); nor was methylamine uptake observed with these membrane vesicles (data not shown). These observations were consistent with the lack of both DMO uptake (Figure 7) and methylamine uptake (Figure 11) by right-side-out K^+-loaded membrane vesicles of B. alcalophilus KM23.

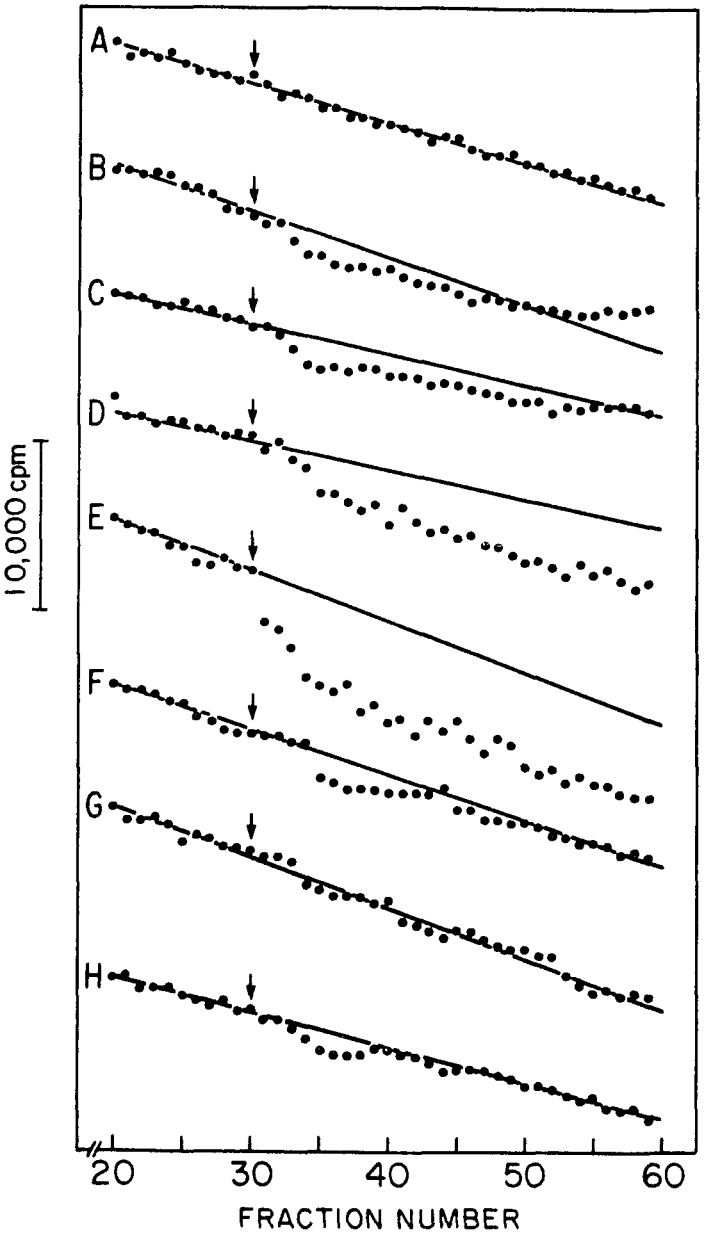
4. Sodium uptake by everted membrane vesicles

Na^+-uptake by potassium-loaded everted membrane vesicles from B. alcalophilus and B. alcalophilus KM23 correlated with the results of DMO uptake as described in the preceeding section. Sodium uptake was assayed as described in the "Materials and Methods". Unenergized vesicles did not accumulate 10 mM $^{22}Na\text{-}CO_3$ (Figure 13, A). Uptake of sodium was dependent upon energization and increased with increasing concentrations of sodium, up to 10 mM Na^+ (Figure 13, B-E). A $\Delta\mu_{Na}+$ of +70 mV, representing a 15-fold concentration gradient, interior > exterior, was generated by energized vesicles incubated with 10 mM Na^+. The treatment of everted vesicles with 10 mM KSCN or 10 µM valinomycin (Figure 13, F & G respectively) to dissipate the $\Delta\psi$, abolished energy-dependent uptake of 10 mM $^{22}Na\text{-}CO_3$. Everted vesicles of the non-alkalophilic mutant B. alcalophilus KM23, did not accumulate $^{22}Na^+$ upon energization (Figure 13, H).

FIGURE 13

Accumulation of $^{22}Na^{+}$ by everted membrane vesicles

K^{+}-loaded everted membrane vesicles from wild type B. alcalophilus (A-G) and B. alcalophilus KM23 (H) were suspended as described in the legend to Figure 12, and $^{22}Na^{+}$ uptake assayed as described in the "Materials and Methods". $^{22}Na\text{-}CO_3$ was present from the beginning of the experiment at the following concentrations: (A) and (E - H), 10 mM; (B), 0.5 mM; (C), 1.0 mM; and (D), 2.5 mM. In the experiments shown in (F) and (G) vesicles were treated with KSCN and valinomycin respectively as described in the legend to Figure 12. At the time indicated by the arrows the following additions were made: (A), none; (B) - (H), 10 mM NADH.


A
B
C
D
E
F
G
H
10,000 cpm
20
30
40
50
60
FRACTION NUMBER

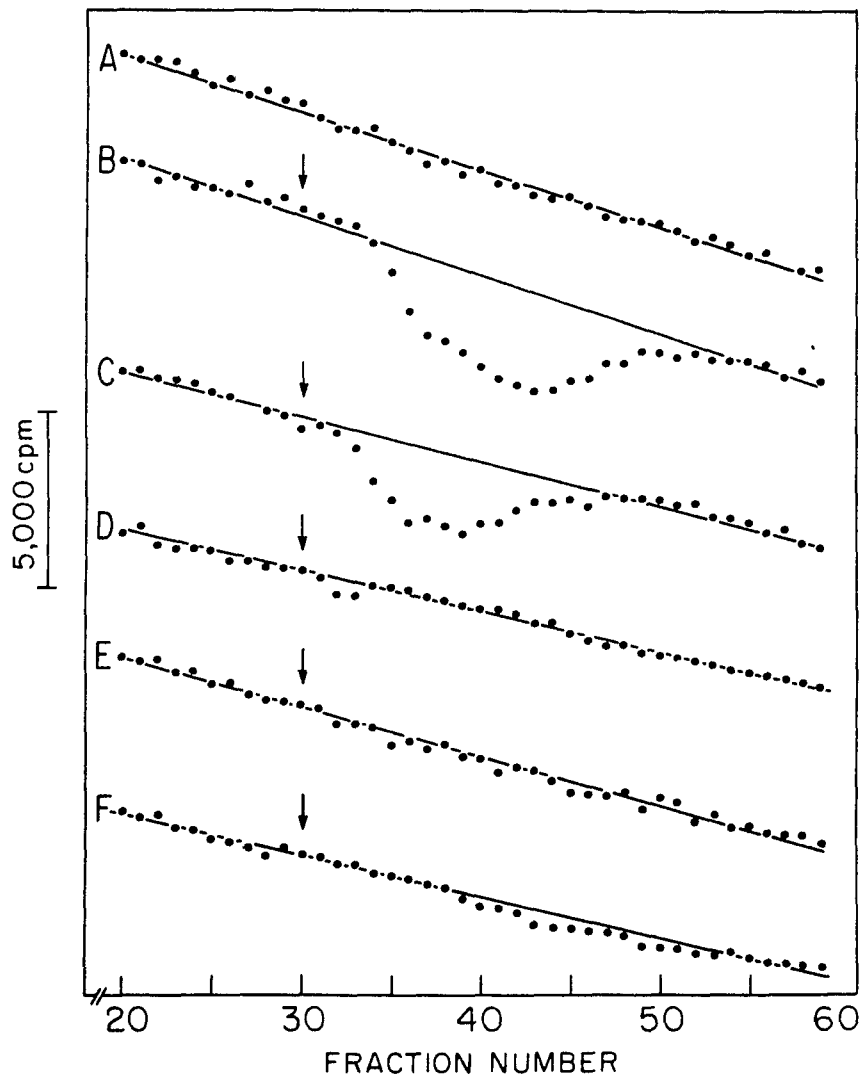
5. The potassium/proton antiporter in everted membrane vesicles of B. alcalophilus

The existence of a K^+/H^+ antiporter in the membrane of B. alcalophilus was first suggested by the observed differences between the energetic patterns in K^+-loaded and ammediol-loaded right-side-out vesicles (Figure 3, Table 2 & 3) Upon energization, K^+-loaded right side-out vesicles did not generate a ΔpH, while vesicles loaded with ammediol generated a small, conventionally oriented ΔpH. Similarly, vesicles of B. alcalophilus KM23, loaded with potassium failed to alkalinize the intravesicular space, while those loaded with choline and Tris did so (Figure 7). It proved difficult to study both the sodium and potassium/proton antiporters in the ammediol and choline-Tris buffers because of apparent inhibitory effects of buffer constituents. However, at least some K^+-dependent proton movements were demonstrable in the choline-Tris-loaded everted vesicles of B. alcalophilus. As shown in Figure 14, upon energization, B. alcalophilus everted membrane vesicles loaded with choline-Tris at pH 8.7, acidified their intravesicular space and accumulated methylamine (Figure 14, B). Addition of KCl simultaneously with energization, caused a consistantly reproducible reduction in the magnitude and duration of the methylamine uptake (Figure 14, C). Addition of KCl to unenergized vesicles had no effect upon methylamine uptake (Figure 14, D). K^+-loaded everted vesicles of B. alcalophilus have been shown to exhibit no DMO uptake (Figure 12, A), nor did they exhibit

FIGURE 14

Effect of potassium on the acidification of the intravesicular space of everted membrane vesicles of B. alcalophilus

Everted membrane vesicles were prepared from B. alcalophilus in either 10 mM Tris, 100 mM choline chloride, 10 mM $MgSO_4$, pH 8.7 (A - D) or in 100 mM K-CO_3, 10 mM $MgSO_4$, pH 8.7 (E & F). Acidification of the intravesicular space was monitored by the accumulation of methylamine as described in the "Materials and Methods". 25 µM (^{14}C)-Methylamine was present from the beginning of the experiment. The vesicles in experiment (F) were treated with 10 mM KSCN as described in the legend to Figure 12. At the times indicated by the arrows, the following additions were made: (B), (E), and (F), 10 mM NADH (Tris salt); (C), 50 mM KCl and 10 mM NADH (Tris salt); (D), 50 mM KCl.


A
B
C
D
E
F
5,000 cpm
20
30
40
50
60
FRACTION NUMBER

energy-dependent uptake of methylamine (Figure 14, E). The treatment of K^+-loaded vesicles with 10 mM KSCN did not effect the pattern of methylamine uptake and thus the potassium-dependent abolition of the ΔpH did not appear to require a $\Delta\psi$. Potassium-loaded everted vesicles from B. alcalophilus KM23 also failed to accumulate both DMO and methylamine upon energization (data not shown).

6. Uptake of α-aminoisobutyric acid (AIB) by cells and right-side-out membrane vesicles of B. alcalophilus and B. alcalophilus KM23

Uptake of AIB was assayed in cells of B. alcalophilus and the non-alkalophilic mutant, B. alcalophilus KM23, as described in the "Materials and Methods". It was previously shown that AIB uptake by B. alcalophilus occurred in symport with Na^+, and was energized by the $\Delta\psi$ (Guffanti et al., 1978). As shown in Figure 15, cells of B. alcalophilus suspended in 25 mM potassium phosphate buffer at pH 9.0, accumulated AIB in the presence of 10 mM NaCl but not in the presence of 10 mM KCl. Under identical conditions, cells of B. alcalophilus KM23 failed to accumulate AIB in either the presence or absence of Na^+.

Identical results were observed using K^+-loaded right-side-out vesicles of both the wild type and the non-alkalophilic mutant strains. The results are presented in Figure 16. Just as observed with cells, AIB uptake by B. alcalophilus membrane vesicles was sodium dependent and monensin,

which catalyzed an electroneutral exchange of Na^+ for H^+, inhibited only slightly. Vesicles from B. alcalophilus KM23 did not exhibit AIB uptake. The addition of monensin did not stimulate AIB uptake by the mutant strain. It is therefore possible that the defect in Na^+-AIB transport in the KM23 strain was not due to a defect in the circulation of sodium across the membrane, but rather to a defect in a sodium subunit that is part of this symport system as well as part of the antiporter.

FIGURE 15

The uptake of AIB by cells of B. alcalophilus and B. alcalophilus KM23

AIB uptake was assayed as described in the "Materials and Methods". Key to figure: B. alcalophilus, circles; B. alcalophilus KM23, squares. Cells treated with 10 mM NaCl are represented by the open symbols; cells treated with 10 mM KCl, the solid symbols.

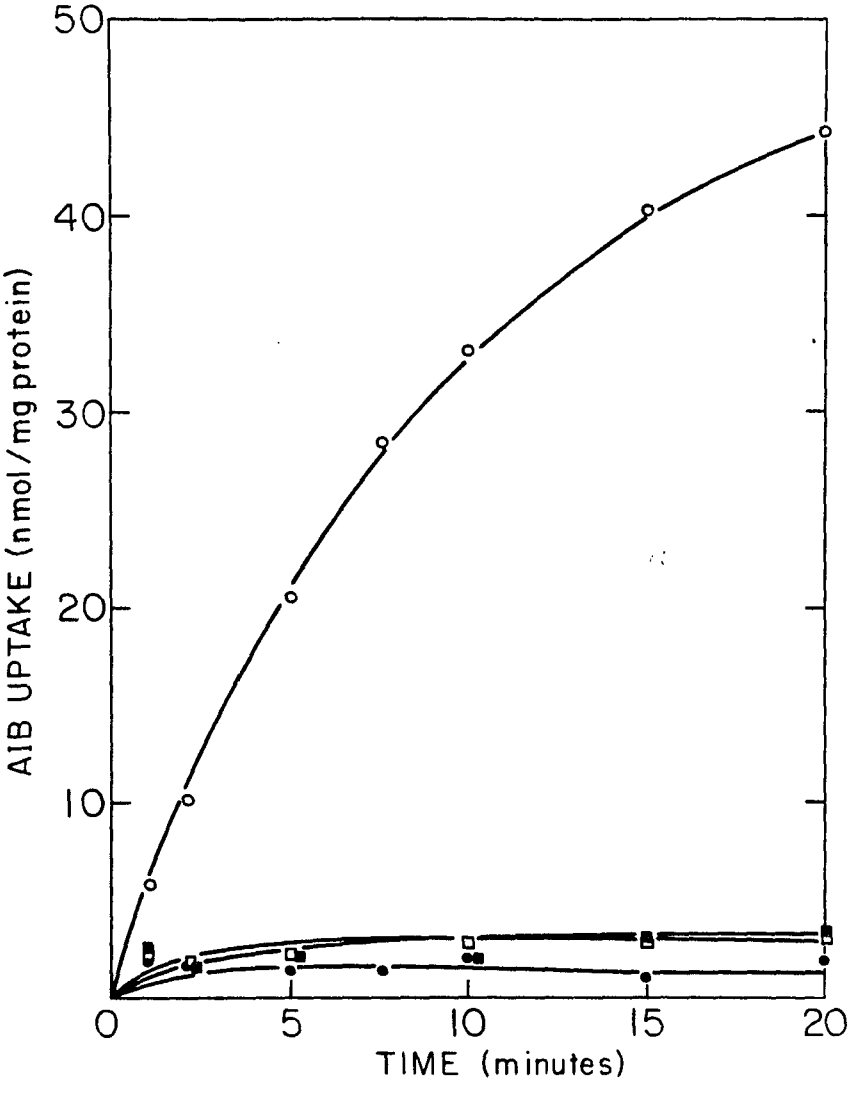
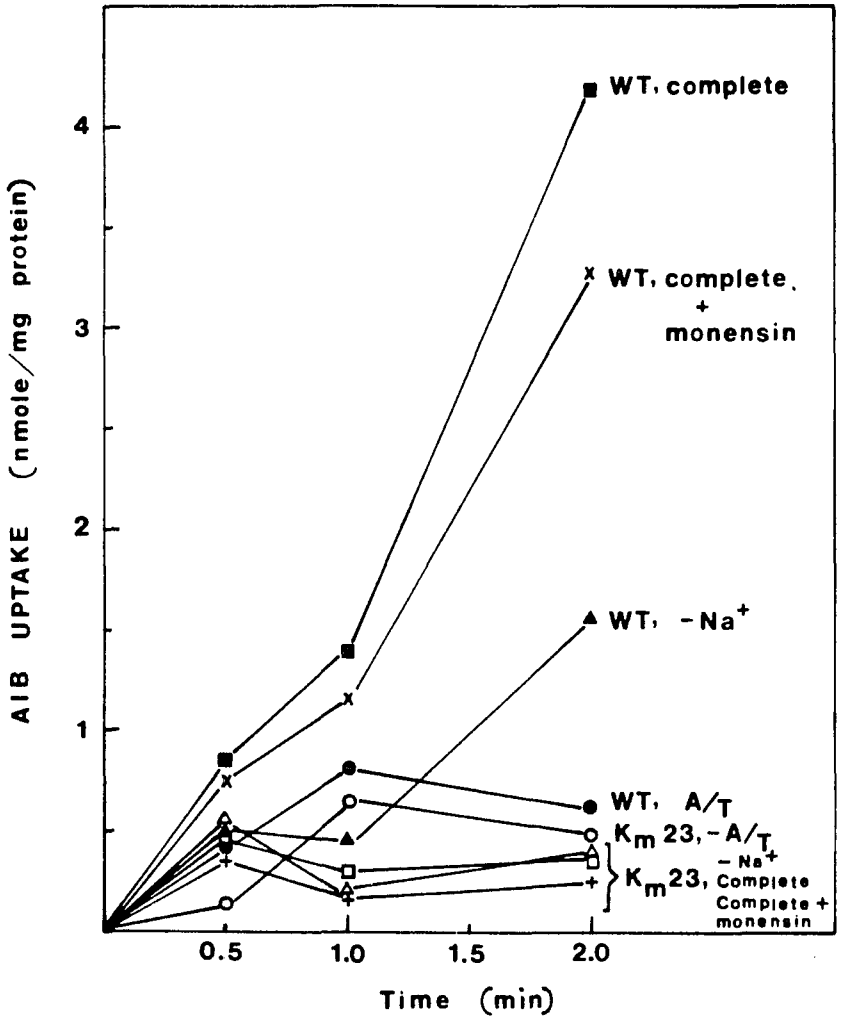

50
40
30
20
10
AIB UPTAKE (nmol/mg protein)
0
5
10
15
20
TIME (minutes)

FIGURE 16

AIB uptake by K^+-loaded right-side-out membrane vesicles of B. alcalophilus and B. alcalophilus KM23

K^+-loaded membrane vesicles were suspended in a buffer at pH 9.0 containing 100 mM K-CO_3 buffer, 10 mM $MgSO_4$, 20 µM (^{14}C)-AIB, and 1 mM Na-CO_3 unless otherwise indicated. Uptake was initiated by addition of 20 mM K-ascorbate plus 2 mM TMPD (A/T) as the energy source. At various times, samples containing 200 µg vesicle protein were removed, filtered and washed with 100 mM LiCl. Where indicated monensin was added to 2 µg/ml. Key to figure: WT, wild type strain; KM23, non-alkalophilic mutant.


AIB UPTAKE (nmole/mg protein)
4
3
2
1
0.5
1.0
1.5
2.0
Time (min)
WT, complete
WT, complete, + monensin
WT, -Na+
WT, A/T
Km 23, -A/T
Km 23, -Na+ Complete
Complete + monensin

DISCUSSION

Monovalent cation/proton antiporters have been characterized in several bacterial species (Harold and Papineau, 1972b; West and Mitchell, 1974; Lanyi and MacDonald, 1976; Brey et al., 1978; Niven and MacLeod, 1978; Schuldiner and Fishkes, 1978; Beck and Rosen, 1979; Tsuchiya and Takeda, 1979b; Brey et al., 1980; Reenstra et al., 1980). Their existence may well prove to be ubiquitous among prokaryotic organisms. One of the functions attributed to the Na^+/H^+ antiporter is the extrusion of potentially cytotoxic Na^+ ions from the cell (Beck and Rosen, 1979). A Ca^{2+}/H^+ antiporter, described in membranes of A. vinelandii (Bhattacharyya and Barnes, 1978) and E. coli (Brey and Rosen, 1979), has been proposed to catalyze a similar extrusion of calcium. The Na^+/H^+ antiporter has also been shown to generate an electrochemical sodium gradient, $\Delta\bar{\mu}_{Na^+}$, utilized for the energization of sodium-dependent transport systems in H. halobium (Lanyi et al., 1976; Lanyi and MacDonald, 1976; Lanyi, 1979). The involvement of the sodium/proton antiporter in generating a sodium-motive force has also been proposed from studies with E. coli (Schuldiner and Fishkes, 1978; Beck and Rosen, 1979) and S. typhimurium (Tokuda and Kaback, 1977) membrane vesicles. Finally, monovalent cation/proton antiporters have been postulated to have a role in regulation of the cytoplasmic pH (West and Mitchell, 1974; Padan et al., 1976; Tokuda and Kaback, 1977; Schuldiner and Fishkes, 1978;

Skulachev, 1978; Brey et al., 1980), but the evidence presented has not been conclusive. Recently, from studies with everted membrane vesicles of E. coli, Reenstra et al. (1980) have demonstrated that the Na^+/H^+ antiporter did not appear to be capable of regulating the magnitude of the ΔpH. They showed that antiporter activity was energized by the ΔpH or the $\Delta\psi$, and that sodium-proton exchange did not dissipate the ΔpH or the $\Delta\psi$. Furthermore, Rosen and his colleagues (Brey et al., 1980; Rosen et al., 1980), have preliminary evidence that a K^+/H^+ antiporter in E. coli everted membrane vesicles may be involved in the regulation of the intravesicular pH.

The results of the study presented here however, clearly support a specific role for the Na^+/H^+ antiporter of B. alcalophilus in acidifying the cytoplasm of this alkalophilic organism. This antiporter translocates protons in exchange for intracellular sodium, establishing chemical gradients for these two ions across the cell membrane. The Na^+/H^+ exchange would thus account for the observed lower pH of the cytoplasm relative to the external milieu (Guffanti et al., 1980).

The experiments in which energy-dependent efflux of passively loaded sodium from starved whole cells of B. alcalophilus was monitored indicate the presence of a $\Delta\psi$-dependent efflux. Efflux was completely inhibited by addition of valinomycin, which dissipated the $\Delta\psi$ under conditions employed. Similar observations of $\Delta\psi$-dependent sodium efflux, using

energized right-side-out membrane vesicles, have been made with E.coli at pH 7.5 (Schuldiner and Fishkes, 1978), and H. halobium (Lanyi et al., 1976; Eisenbach et al., 1977). Those observations were interpreted as supportative of Na^+/H^+ antiporters in these organisms. In B. alcalophilus the $\Delta\psi$ may be required not only to establish chemical gradients, but also because of some gating effect. Sodium efflux was observed only when the membrane potential, generated by energization of the starved whole cells, exceeded -95 mV. Cells starved for short periods of time, which did not lower the membrane potential below -95 mV exhibited Na^+-efflux in the absence of an exogenous energy source. A similar gating effect of the Na^+/H^+ antiporter has been reported in membrane vesicles from H. halobium (Lanyi and Silverman, 1979); the critical potential in that species was -130 to -155 mV.

B. alcalophilus KM23, a non-alkalophilic mutant derived from wild type B. alcalophilus, could no longer grow at highly alkaline pH and had gained the ability to grow at pHs below 8.0. The bioenergetic pattern exhibited by cells of B. alcalophilus KM23 (Table 5) resembled that of the alkaline-tolerant organisms B. circulans (Guffanti et al., 1979b) and B. firmus ATCC (Guffanti et al., 1980); these organisms could also grow at pHs up to, but not above pH 9.0. As the external pH was increased to pH 8.0 to 9.0, the ΔpH generated by B. alcalophilus KM23 and the alkaline-tolerant species, was dissipated, but did not "reverse", and there was some compensatory elevation of the $\Delta\psi$. Concomitant with the loss of

the ability to acidify its cytoplasm, and consequently grow at pHs greater than pH 9.0, was a loss of energy-dependent sodium efflux from starved cells of B. alcalophilus KM23 (Figure 10). Thus, the studies with whole cells of the wild type and the mutant strain indicated a role for a Na^+/H^+ antiporter in facilitating growth at high pH and precluding growth at lower pHs by acidifying the cytoplasm.

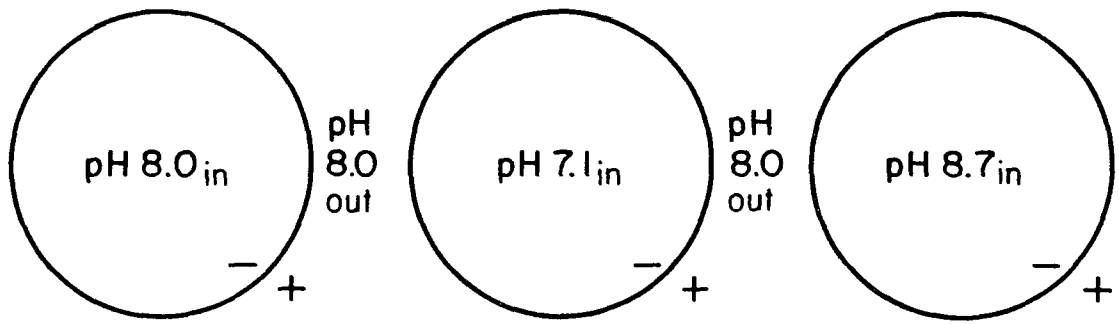
Isolated membrane vesicles proved to be a fruitful system for more detailed characterization of antiporters vis-a-vis protonmotive force patterns. Upon energization, Na^+-loaded right-side-out membrane vesicles of B. alcalophilus, suspended between pH 8.0 and 10.5, acidified their intravesicular space. Treating the vesicles with gramicidin or CCCP, abolished this acidification. A $\Delta\psi$ of -120 to -135 mV was generated over this pH range; therefore the $\Delta\bar{\mu}_{H^+}$ actually increased as the external pH was increased (Figure 3). This protonmotive force pattern was the opposite of that observed in membrane vesicles frcm the more neutral pH-range bacteria E. coli (Ramos et al., 1976; Ramos and Kaback, 1977), S. typhimurium (Tokuda and Kaback, 1977) and M. lysodeikticus (Friedberg and Kaback, 1980). Interestingly, K^+-loaded vesicles from B. alcalophilus did not generate any ΔpH upon energization; only a $\Delta\psi$ was measured in K^+-loaded vesicles (Figure 3), whereas vesicles prepared in the absence of both Na^+ and K^+, and loaded with ammediol, generated a small, conventional ΔpH and a small $\Delta\psi$ (Table 3).The bioenergetic patterns observed with the membrane vesicles of B. alcalophilus

loaded with Na^+, K^+, or ammediol are summarized in Figure 17.

It is of interest that a ΔpH, inside acid was observed in B. alcalophilus vesicles at external pHs from pH 8.0 up to pH 10.5 only. Whole cells exhibited a ΔpH at pHs from 10.0 to 11.5; at pH 9.0 and 9.5, the ΔpH was zero (Guffanti et al., 1978). The differences between whole cells and vesicles suggested a role for cytoplasmic constituents in buffering the protons taken up by the antiporter and/or affecting the activity of the antiporter. It is likely that a limited ability of the cytoplasm to buffer the inwardly translocated protons below pH 8.0 is the basis for the obligately alkalophilic nature of B. alcalophilus; i.e., below pH 8.0, the antiporter may lower the cytoplasmic pH below the minimum for viability. This hypothesis is substantiated by the properties of the non-alkalophilic mutant strain, KM23, which gains the ability to grow from pH 5.5 to 9.0, while exhibiting loss of antiporter activity and ability to grow at pHs above pH 9.0 (Krulwich et al., 1979).

Acidification of the intravesicular space of right-side-out B. alcalophilus vesicles required sodium to be present within the intravesicular space. Addition of sodium, simultaneously with energization did not cause acidification of the intravesicular space of K^+-loaded vesicles. However, upon loading these vesicles with sodium, acidification of the vesicle interior was found to be dependent upon the sodium concentration, with a half-maximal effect occuring with 0.7 mM Na^+. In comparison, Km's for Na^+ of 3 mM and 44 mM have

PROTONMOTIVE FORCE IN
ISOLATED MEMBRANE VESICLES FROM *BACILLUS ALCALOPHILUS* *



K^+-LOADED	Na^+-LOADED	AMMEDIOL-OR CHOLINE-TRIS-LOADED
Δ pH = 0	Δ pH reversed	Δ pH conventional
$\Delta\Psi = -85$ mV	$\Delta\Psi = -100$ mV	$\Delta\Psi = -64$ mV

*Energized with ascorbate / TMPD at external pH of 8.0

FIGURE 17

been determined for the Na^+/H^+ antiporters in E. coli (Beck and Rosen, 1979) and A. vinelandii (Bhattacharyya and Barnes, 1978) respectively. Right-side-out membrane vesicles from the non-alkalophilic mutant, KM23, did not exhibit sodium-dependent acidification of the intravesicular space.

Studies with everted membrane vesicles supported the conclusion that the Na^+/H^+ antiporter in B. alcalophilus was $\Delta\psi$-dependent. Upon energization, everted vesicles exhibited sodium-dependent alkalinization of their intravesicular space that correlated with accumulation of $^{22}Na^+$. Valinomycin or thiocyanate treatment inhibited both of these effects and the non-alkalophilic strain, B. alcalophilus KM23, did not exhibit these activities.

The dependence of the Na^+/H^+ antiporter on the membrane potential could reflect an electrogenic translocation of > 1 H^+/Na^+ as has been described for the Na^+/H^+ antiporter in H. halobium (Lanyi and Silverman, 1979). Neither whole cells nor right-side-out vesicles of B. alcalophilus required a sodium gradient for acidification of the cell or vesicle interior, but only required that Na^+ be present. Therefore, the Na^+/H^+ antiporter probably catalyzes an electrogenic exchange, energized by the $\Delta\psi$. An electrogenic Na^+/H^+ antiporter would be expected to convert the membrane potential, at least in part, to a $\Delta\mu_{Na^+}$ and ΔpH, interior acid, so that the expected $\Delta\psi$ would be lowered. However, the $\Delta\psi$ in Na^+-loaded vesicles was as high or higher than that observed in K^+-loaded membrane vesicles. It was conceivable

that the activity of the Na^+/H^+ antiporter, in generating the "reverse" ΔpH, facilitated the generation of a much higher $\Delta\psi$ than is produced in the absence of Na^+, perhaps by stimulating respiration.

An electroneutral K^+/H^+ antiporter was consistent with the absence of a ΔpH in both right-side-out and everted K^+-loaded vesicles from B. alcalophilus and B. alcalophilus KM23, and in thiocyanate-treated K^+-loaded everted vesicles of the wild type strain. Since this activity is apparently present in membranes of B. alcalophilus KM23, it must not be involved in facilitating growth at high pHs. In the absence of both Na^+ and K^+, right-side-out vesicles of both the wild type and the non-alkalophilic mutant, generated a ΔpH, interior alkaline. Everted vesicles prepared in the absence of K^+ and Na^+ generated a ΔpH, interior acid, which appeared to be reduced by addition of K^+. Corroborative studies on K^+ (or Rb) movements will be required to properly document this activity. The physiological role of the K^+/H^+ antiporter remains unclear. It might serve as a secondary system for proton reentry, or as one of several processes for regulating the intracellular potassium levels. It is however, possible that this antiporter could be involved in the regulation of the cytoplasmic pH in a lower pH range. An electroneutral K^+/H^+ antiporter in E. coli has been characterized and suggested to regulate the cytoplasmic pH in that organism (Brey et al., 1980) at its upper limits of pH for growth. A mutant strain of E. coli, unable to grow at as high a pH as the wild

type, and apparently defective in the K^+/H^+ antiporter has also been reported (Rosen et al., 1980). As noted in the "Literature Review" however, parallel studies of the antiporter activity and the protonmotive force have not been conducted on this mutant.

Finally, it was observed that the non-alkalophilic mutant strain, B. alcalophilus KM23, which lacked the Na^+/H^+ antiporter, was also defective in Na^+-AIB symport at pH 9.0. At this pH, wild type cells and vesicles accumulated AIB, while the non-alkalophilic mutant failed to do so. Harold et al. (1970b) have isolated a Na^+/H^+ antiport mutant of S. faecalis. Harold and Papineau (1972b) reported that addition of monensin, which catalyzes electroneutral Na^+/H^+ exchange across a membrane, to this mutant strain of S. faecalis restored the ability of the strain to cycle protons in exchange for intracellular Na^+. Energetically, addition of monensin to the mutant restored the wild type energetic pattern. Addition of monensin to membrane vesicles of B. alcalophilus KM23 did not stimulate AIB uptake. This suggested that the defect in the non-alkalophilic strain was not merely caused by an inability to cycle protons across the membrane through the antiporter. Rather, the lack of stimulation of AIB uptake by monensin, together with the pleiotropic defect in Na^+-coupled translocations in B. alcalophilus KM23, suggested that the genetic defect might be in a common "sodium-subunit" shared by both the Na^+/H^+ antiporter and Na^+-AIB symporter.

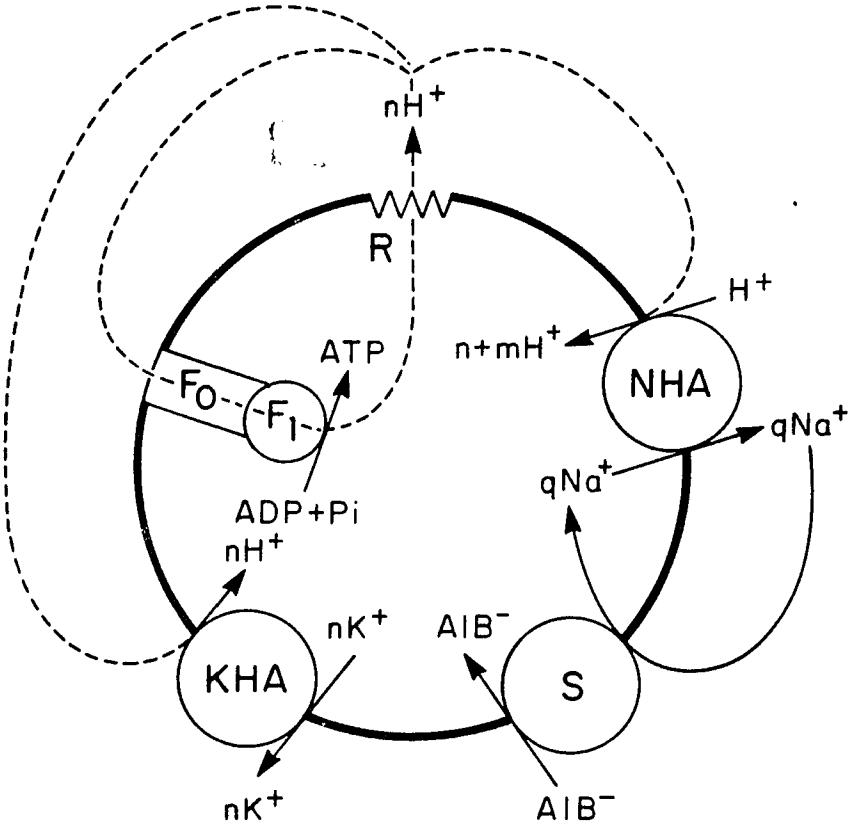
Lieberman, Hong, and their colleagues, have characterized a series of E. coli mutants, designated as ecf mutations. These strains are pleiotropically defective in coupling the protonmotive force to active transport. They have proposed that the ECF-protein is a common proton-translocating-subunit shared among the various proton-symport systems in the E. coli membrane (Lieberman and Hong, 1974; Lieberman et al., 1977; Hong, 1977).

A summary model of ion and proton movements in B. alcalophilus membrane vesicles is presented in Figure 18. Protons extruded by respiration (R) can be channeled through the F_oF_1-ATPase, and in the presence of ADP and phosphate, synthesize ATP (Guffanti and Krulwich, 1980). The K^+/H^+ antiporter (KHA) is shown to catalyze electroneutral, 1:1 exchange. Protons are returned to the intravesicular space by the Na^+/H^+ antiporter (NHA), acidifying the interior. Though a stoichiometry for this process is unknown, it can be proposed that for nH^+ extruded by respiration, $(n+m)H^+$ are inwardly translocated by the antiporter in exchange for qNa^+. Since the Na^+/H^+ is electrogenic, (n+m) is probably greater than q. If n and q are assumed to be 2, and m = 1, then this proposed model acidifies the intravesicular space and allows generation of a membrane potential, outside positive. Sodium can be recycled into the vesicles or cells by symport with some substrate (i.e., AIB), and as discussed above it is possible that the Na^+/H^+ antiporter and the Na^+-coupled symporter share a common subunit for sodium translocation.

FIGURE 18

A summary of ion and proton movements in Bacillus alcalophilus

The sodium/proton antiporter is represented by NHA and the potassium/proton antiporter by KHA. The symporter for AIB and Na^+ is represented by S. R designates the respiratory chain, and F_oF_1 is the membrane-bound ATPase.


nH+
R
H+
n+mH+
NHA
qNa+
qNa+
ATP
Fo
F1
ADP+Pi
nH+
nK+
AIB−
KHA
S
nK+
AIB−

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