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by

Richard J. Lewis

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1982

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Chairman of Examining Committee

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Abstract

THE RESPIRATORY CHAIN AND BIOENERGETICS IN ALKALOPHILIC BACTERIA

by

Richard J. Lewis

Adviser: Professor Terry Ann Krulwich

Bacillus firmus RAB and *Bacillus alcalophilus* grow optimally at pH 10.5-11.0. Certain bioenergetic problems that are also found in other organisms are thus exaggerated in these obligate alkalophiles. Maintaining an acidic cytoplasmic pH relative to the external *milieu* is accomplished through electrogenic Na^+/H^+ antiport whose operation is one of the peculiar energy demands of alkalophiles. In addition, the energy requirement for ATP synthesis is somewhat greater due to a higher $\Delta G°$ at elevated pH's.

Difference spectroscopy revealed extraordinary levels of a-, b- and c-type cytochromes in alkalophile membranes (>5 nmol heme/mg protein). Non-alkalophilic mutants derived from the alkalophiles had greatly reduced quantities of membrane cytochromes (<2 nmol heme/mg protein). Redox potentiometry revealed an abundance of different cytochrome species in the alkalophiles. Cytochromes from *B. alcalophilus* vesicles prepared at pH 9.0 (typical cytoplasmic pH of whole cells) had the following midpoint potentials: cytochromes a and a_3, +240 mV; cytochrome b's, +20, -120, -240 and -320 mV; cytochrome c, +70 mV. One a-type cytochrome and at least one b-type cytochrome exhibited pH-dependent midpoint potentials. Alkalophile vesicles contained an unusually low potential Rieske FeS (EPR signal $g = 1.90$) protein. A cytochrome a, Em_7 + 100 mV, one cytochrome b, Em_7 -120 mV and a cytochrome c Em_7 +140 mV were identified in the non-alkalophilic mutant. No Rieske protein was distinguished. Analogous results were obtained in *B. firmus* RAB and its non-alkalophilic mutant. Both alkalophiles have high molar growth yields (Ymal $\sim$40 g dry weight/mole malate consumed) and conventional oxygen utilization rates. The above data raised the possibility that special properties of the alkalophile respiratory chain make them particularly efficient in energy transduction; this possibility was supported by H^+/O determinations at alkaline but not neutral pH. A very rapid initial phase of respiration-linked proton ejection by cells of *B. firmus* RAB at pH 9.0 had a stoichiometry of at least 9. No such fast phase was seen at pH 7.0 or in the non-alkalophilic mutant, whose H^+/O stoichiometry was approximately 4. Furthermore, growth yields for non-alkalophilic derivatives of both species were much lower (Ymal $\sim$20) than those of the wild type parents. The results indicated that the function of respiratory chains of the mutants are severely compromised. This is also true of the wild type respiratory chain operating at neutral pH and may be related to the inability of alkalophiles to grow at pH 7.0.

FOREWORD

Parts of this thesis have appeared in the following publications:

Lewis, R.J., Belkina, S. and Krulwich, T.A. (1980) Alkalophiles have much higher cytochrome contents than conventional bacteria and their own non-alkalophilic mutant derivatives. Biochem. Biophys. Res. Commun. 95: 857-863

Lewis, R.J., Knaff, D.B. and Krulwich, T.A. (1980) The electron transport chain of *Bacillus alcalophilus*. Fed. Proc. 39: 2147

Lewis, R.J., Prince, R.C., Dutton, P.L., Knaff, D.B. and Krulwich, T.A. (1981) The respiratory chain of *Bacillus alcalophilus* and its non-alkalophilic mutant derivative. J. Biol. Chem. 256: 10543-10549

Lewis, R.J., Kaback, E. and Krulwich, T.A. (1981) The role of Na^+/H^+ antiport and the consequences of its mutational loss in alkalophiles. Abstracts of the VIIth International Biophysics Congress and IIIrd Pan-American Biochemistry Congress. p. 142.

Krulwich, T.A. and Lewis, R.J. (1981) Microbial adaptations to stress: some lessons to be learned from aerobes in *Trends in the Biology of Fermentations for Fuels and Chemicals* (Hollaender, A., Rabson, R.; Rogers, Pietro and Wolfe, eds.) pp. 493-497, Plenum, New York

Lewis, R.J., Kaback, E. and Krulwich, T.A. (1982) Pleiotropic properties of mutations to non-alkalophily in *Bacillus alcalophilus*. J. Gen. Microbiol. 128: 427-430

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AQ-1,5-disulfonic acid	anthraquinone-1,5-disulfonic acid
AQ-2-sulfonic acid	anthraquinone-2-sulfonic acid
AIB	α-aminoisobutyric acid
BV	benzyl viologen
CCCP	carbonyl-*m*-chlorophenylhydrazone
DAD	2,3,5,6-tetramethylphenylenediamino (diaminodurene)
E_h	The oxidation-reduction potential referred to the standard hydrogen electrode
Em_x	the midpoint potential at pH_x
EMS	ethyl methane sulfonate
EPR	electron paramagnetic resonance
HOQNO	2-heptyl-4-hydroxyquinoline
MOPS	3-(N-morpholino)propane sulfonic acid
MV	methyl viologen
NADH	nicatinamide adenine dinucleotide
PES	N-ethyl phenazonium ethosulfate
PMS	N-methyl phenazonium methosulfate
TMPD	N,N,N',N'-tetramethyl-*p*-phenylenediamine
TRIS	Tris(hydroxymethyl) aminomethane
1,4 BQ	1,4-benzoquinone
1,2 NQ	1,2-naphthaquinone
1,4 NQ	1,4-naphthaquinone
1,2 NQ-4 S	1,2-naphthaquinone-4 sulfonate
2(OH)-1,4 NQ	2-hydroxy-1,4-naphthaquinone

2,5$(OH)_2$-1,4 BQ	2,5-dihydroxy-1,4-benzoquinone
$\Delta\tilde{\mu}_{H^+}$	the electrical gradient of protons, protonmotive force
$\Delta\psi$	transmembrane electrical potential
ΔpH	transmembrane proton gradient

INTRODUCTION

In meeting the requirements for life in their extreme environment, alkalophilic bacteria have evolved adaptative properties which make them particularly interesting organisms for the study of various bioenergetic processes. *Bacillus alcalophilus* and *Bacillus firmus* RAB are two obligately alkalophilic bacteria that are the subjects of this study. Both species actively grow over the pH range of 8.5 to 11.5 with optimal growth occurring at pH 10.5 (Guffanti *et al.*, 1978, 1980). Earlier work with these bacteria demonstrated that even at external pH values as high as 11.5, these organisms maintain a cytoplasmic pH no greater than 9.5 (Guffanti *et al.*, 1978, 1980). Nevertheless, as predicted in Mitchell's chemiosmotic hypothesis (Mitchell, 1961, 1963) bacteria extrude protons by ATP hydrolysis and/or electron transport through their respiratory chains (reviewed by Harold, 1977). This establishes an electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$), outside acidic and electrically positive with respect to the inside. ATP synthesis via proton return through an F_1F_0-ATPase, and transmembrane proton/solute symport, can both be driven by the $\Delta\tilde{\mu}_{H^+}$ (West and Mitchell, 1972; Harold, 1977; Konings and Boonstra, 1977). Thus, in conventional bacteria, a predominantly proton-based energetic currency is found.

Obligate alkalophiles, growing in a proton poor environment (i.e., $[H^+]<10^{-10}M$) and possessing comparatively low $\Delta\tilde{\mu}_{H^+}$, utilize solute symport systems that generally couple to sodium ions rather than protons (Guffanti *et al.*, 1978, 1981). Investigations with membrane vesicles, however, indicated that, like other bacteria (Harold, 1977), *B. alcalophilus* and *B. firmus* RAB extrude protons during respiration (Mandel *et* al., 1980; Guffanti et al., 1981; Krulwich et al., 1982). The internal pH is apparently maintained below 9.5 by a secondary exchange involving an electrogenic Na^+/H^+ antiporter (Mandel et al., 1980; Guffanti et al., 1980). This antiporter results in net acidification of the intravesicular space (or cytoplasm) and in the generation of a transmembrane Na^+ gradient ($Na^+_{out} > Na^+_{in}$). The Na^+ gradient is then part of the driving force for solute uptake (Fig. 1). Na^+/H^+ antiport, a vital physiological function for pH homeostasis in these alkalophiles, is one special energy-consuming process peculiar to these organisms. In addition, a second energy cost for alkalophiles is associated with the synthesis of ATP via oxidative phosphorylation and results from the fact that the free energy of hydrolysis for ATP is considerably higher at the typical cytoplasmic pH of 9.0 than at neutral pH (Rosing and Slater, 1977).

In view of the unusual energy demands just outlined, it was particularly intriguing to note that isolated membranes prepared from B. alcalophilus and B. firmus RAB are rather intensely red in color (see Fig. 2) somewhat resembling the color of mitochondria. It thus seemed possible that part of the specific adaptive machinery developed for alkalophily might involve high levels of cytochromes and/or other special properties of the respiratory chain. Characterization of the respiratory chain of two obligately alkalophilic bacteria is the focus of this work. The studies have generally been conducted in comparison with non-alkalophilic mutant strains of the two obligate alkalophiles. The non-alkalophilic mutants have lost Na^+/H^+ antiport activity and hence have lost the ability to grow at extremely alkaline pH (Krulwich et al., 1979; Guffanti et al., 1980).

FIGURE 1

Model showing proton and cation-translating processes in alkalophilic bacteria (Krulwich, 1982)

Primary proton pumping is shown to occur by the respiratory chain. A Na^+/H^+ antiporter catalyzes net proton uptake in exchange for Na^+. Na^+/solute symport systems complete the Na^+ cycle and are suggested to contain a Na^+-translocating subunit in common with that of the antiporter. ATP is synthesized by proton backflow through the membrane F_1F_0-ATPase.

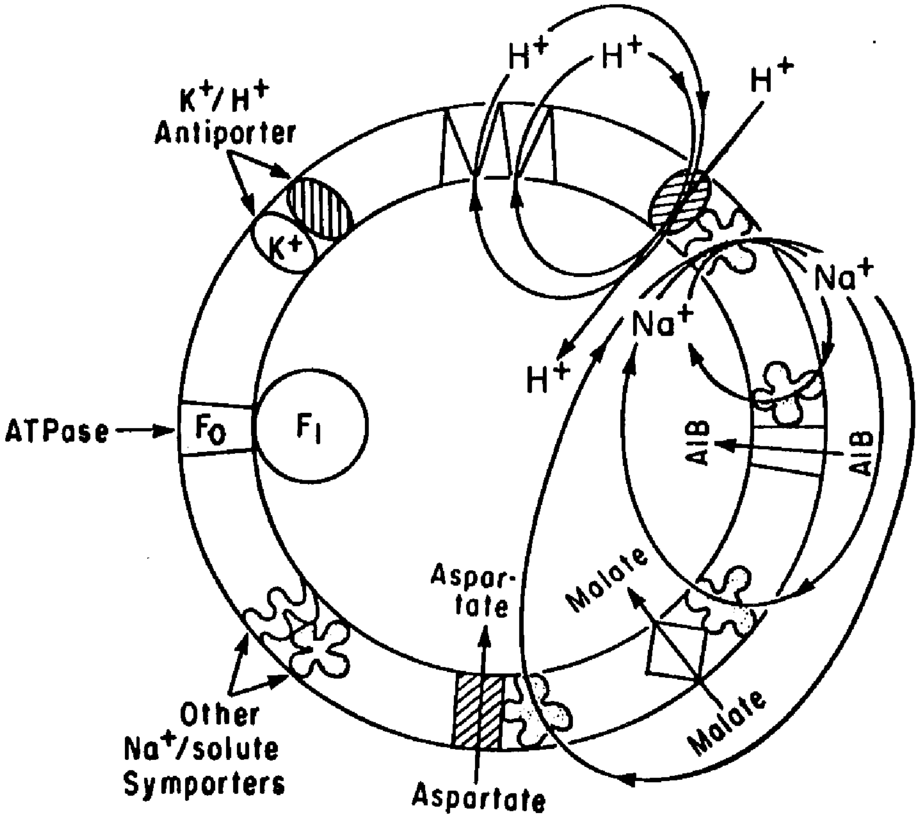
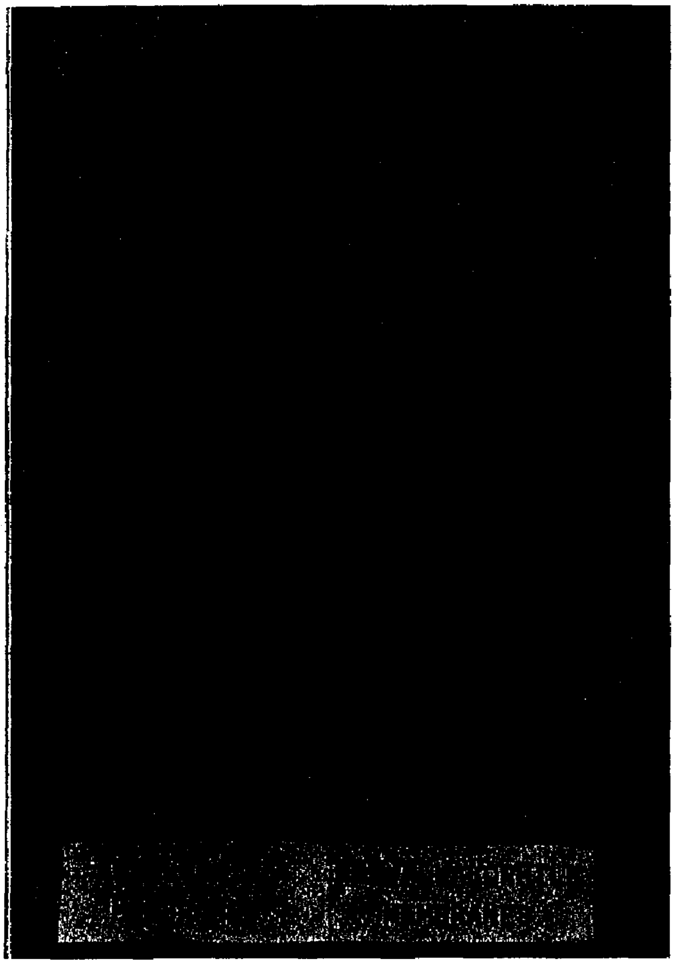

K+/H+ Antiporter
H+
H+
H+
K+
Na+
Na+
H+
ATPase
Fo
F1
AIB
AIB
Aspar-tate
Malate
Other Na+/solute Symporters
Aspartate
Malate

FIGURE 2

Membranes from an alkalophile and its non-alkalophilic mutant

Vesicles were prepared from *B. firmus* RAB and its non-alkalophilic derivative RABN as described in "Materials and Methods". Both membrane suspensions were adjusted to 10 mg protein/ml. The *B. firmus* RAB membranes on the left appear red due to their very high cytochrome content. In comparison membranes from the non-alkalophile (RABN) have a pale, creamy color.



LITERATURE REVIEW

The phenomenon of oxidative phosphorylation, i.e., the process by which the energy derived from the oxidation of substrates is conserved in the form of phosphate bond energy, was first observed in the 1930's. Engelhardt (1930, 1931), studying respiration in bird erythrocytes, observed inorganic phosphate incorporation with the oxidation of methylene blue (while anaerobic metabolism was suppressed with NaF) and recognized the relationship between oxidation and phosphorylation. The real significance of oxidative phosphorylation was not appreciated, however, until ATP formation by cell free kidney preparations was shown to be respiration-dependent and independent of glycolytic phosphorylation (Kalckar, 1937, 1939). By carefully measuring oxygen consumption in Warburg vessels and inorganic phosphate and acid labile phosphate to determine phosphate esterification, Belitzer and Tsibakova (1939), working with minced muscle, and later, Ochoa (1943), studying cell free cat heart and pigeon brain preparations, concluded that the transport of one pair of hydrogen electrons from substrate to oxygen results in at least 2-3 high energy phosphate bonds. The stoichiometry and a measure of the efficiency of this process could be expressed in a P:O ratio (i.e., the ratio of molecules of inorganic phosphate esterified per atom of oxygen consumed).

As early as 1925, Keilin had identified cytochromes as cellular enzymes crucial for respiration (Keilin, 1925). This conclusion was based on his observations that: 1. cytochromes were present in cells of all aerobic organisms studied; 2. there was an apparently direct relationship between the respiratory activity of a tissue or cell suspension and its cytochrome content; 3. cytochromes were observed to undergo reversible oxidations and reductions in living cells of yeast and insect wing muscles; and 4. these oxidations and reductions were inhibited by known respiratory inhibitors such as cyanide. With his use of these inhibitors, especially ethyl urethane which prevents electron transfer from cytochrome b to cytochrome c, Keilin is often credited with the first observation that the sequence of electron transfer between cytochromes is from b → c → a.

Redox potentials are frequently used to describe enzymatic oxidation-reduction equilibria. For the reaction

$$A_{\text{reduced}} = A_{\text{oxidized}} + ne^-$$

where n is the number of electrons (e^-) stripped from the reduced component as it is oxidized, the standard relationship used is

$$E_h = E_m + \frac{RT}{nF} \ln \frac{[A_{ox}]}{[A_{red}]}$$

a form of the Nernst equation. R, T and F are the gas constant, the absolute temperature, and the Faraday constant; E_h is the measured potential relative to the standard hydrogen half cell, and E_m, the midpoint potential, is the redox potential of the system when the concentrations of A_{ox} and A_{red} are equal.

Early determinations of the midpoint potentials for the ferrocytochrome c $\rightleftarrows$ ferricytochrome c system at physiological pH were found to be +260 mV for crude yeast extracts (Coolidge, 1932) and between +253 and +270 mV for ox heart preparations (Ball, 1938; Wurmser and Filitti-Wurmser, 1938; Stotz et al., 1938). Midpoint potentials for ox heart cytochrome b and cytochrome oxidase (cytochrome aa_3) were measured at approximately 0.0 mV and +290 mV respectively (Ball, 1938). The average high energy phosphate bond ($\sim$P) contains approximately 12 Kcal of energy, which is equivalent to a 250 mV potential difference, and the average potential between oxygen and a pair of substrate hydrogen electrons is approximately 1200 mV (Lipmann, 1946). With these determinations Lipmann (1946) was able to calculate as a theoretical upper limit a value above 4 for P/O ratios (i.e., $\frac{1200 \text{ mV}}{250 \text{ mV}}$). Since only 3 inorganic phosphates were found to be esterified experimentally, Lipmann drew a scheme of electron transport through the respiratory chain with components grouped according to redox potential, indicating 3 redox potential spans of approximately 250 mV each wherein some mechanism for energy conservation was assumed to operate (Lipmann, 1946).

Oxidative phosphorylation was located in the mitochondria organelle of eukaryotic cells (Kennedy and Lehninger, 1949) soon after an isolation procedure for intact mitochondria was developed (Hogeboom _et_ _al_., 1948). While the precise identification of the individual sites of phosphorylation within the respiratory chain is still in progress, partial resolution was obtained rapidly. Oxidation of ascorbate via cytochrome c in liver mitochondria resulted in ^{32}Pi incorporation (Friedkin and Lehninger, 1949), with a P/O ratio of about 1 (Judah, 1951), thus establishing a site of oxidative phosphorylation between cytochrome c and oxygen, generally referred to as Site 3. With NADH as a hydrogen donor and ferricyanide as acceptor, phosphorylation was demonstrated in mitochondria, establishing Site 1 as lying between the dehydrogenase and the lowest potential cytochrome, cytochrome b (Copenhauer and Lardy, 1952). Further oxidation by the cytochromes of the respiratory chain was prevented by addition of the inhibitor, antimycin A. One phosphorylation was found to be coupled to the oxidation of succinate with added cytochrome c as hydrogen acceptor, establishing Site 2 (Slater, 1955).

Electron transport can proceed through the respiratory chain without the esterification of inorganic phosphate or even its presence (Slater, 1949; Bonner, 1954). Carefully prepared mitochondria, however, were found to be absolutely dependent on the presence of both ADP and Pi for respiration (Lardy and Wellman, 1953). Lardy proposed that the concentration of ADP might actually control the rate of respiration (Lardy and Wellman, 1953). Chance and Williams, in a series of papers, proved Lardy correct and introduced the following definitions and conventions (Chance and Williams, 1955 a-d, 1956). The state of respiration of mitochondria in the presence of substrate, inorganic phosphate and ADP is called the active state (state 3). When ADP is exhausted, respiration ceases and tightly coupled mitochondria are in a controlled state (state 4). This is known as respiratory control. A respiratory or acceptor control index, defined as

$$\frac{\text{the rate of respiration with ADP present}}{\text{the rate of respiration without ADP}} = \frac{\text{respiration state 3}}{\text{respiration state 4}}$$

serves as a measure of the coupling of oxidation with phosphorylation. Tightly coupled mitochondria have control indicies > 40. The addition of uncoupling reagents, such as 2, 4-dinitrophenol, causes respiration rates to increase while inhibiting phosphorylation. Furthermore, P/O ratios could be readily determined by causing a shift from state 4 to state 3 by the addition of a small known amount of ADP, while monitoring the oxygen consumption until the ADP is consumed and respiration reverts back to state 4.

Direct Measurements of Oxidative Phosphorylation in Bacteria

Bacterial respiratory chains share many similarities with those found in eukaryote mitochondria; the similarities are greatest with respiratory chains from the aerobes, *Paracoccus denitrificans* and *Alcaligenes eutrophus*. In these species respiratory chain redox components are much like those of mitochondria and display similar sensitivities to rotenone, antimycin A, and cyanide (Asano, *et al.*, 1967; Scholes and Smith, 1968; Ishaque and Aleem, 1970; Shipp, 1972; Drozd and Jones, 1974; Lawford *et al.*, 1976). In fact, the theory has been advanced that the inner mitochondrial membrane of eukaryote mitochondria may have evolved from the plasma membrane of an early bacterial species related to *P. denitrificans* (John and Whatley, 1975). Most bacterial respiratory chains, however, are significantly different from mitochondria. There is an enormous diversity in the cytochrome species (especially different terminal cytochrome oxidases) which are present in bacterial respiratory chains (see, for reviews, Gel 'man *et al.*, 1967; Bartsch, 1968; Smith, 1968; Kamen and Horio, 1970; Horio and Kamen, 1970; Lemberg and Barret, 1973; Meyer and Jones, 1973a; Jurtshuk *et al.*, 1975; Gel'man *et al.*, 1975). These frequently result in multiple or branched pathways for electron transport (White and Sinclair, 1971; Haddock and Jones, 1977).

The procedure for measuring oxidative phosphorylation by monitoring oxygen consumption in state 4 → 3 → 4 transitions (Chance and Williams, 1956), unfortunately cannot be applied to intact bacteria due to their lack of a membrane ADP translocase (Garland and Haddock, 1977).

Some of the earliest and most extensive studies of bacterial respiration were conducted with Azotobacter vinelandii extracts because of their high respiratory rate and cytochrome levels (Castor and Chance, 1959; Jones and Redfearn, 1966). Determinations of P/O ratios by monitoring oxygen uptake and phosphate esterification, however, were very low for this organism. They were much less than 1 even though different substrates and many different types of cell free extracts and membrane preparations were employed in these measurements (Hyndman et al., 1953; Tissieres and Slater, 1955; Rose and Ochoa, 1956; Tissieres et al., 1957; Cota-Robles et al., 1958; Temperli and Wilson, 1958; Hovenkamp, 1959; Schils et al., 1960; Temperli and Wilson, 1960; Brodie, 1963). Determinations of P/O ratios in most other bacteria were equally disappointing (Harold, 1972), consistently falling well below 1 and exhibiting no respiratory control, e.g., Aerobacter aerogenes and Proteus vulgaris (Nossal et al., 1956), Alcaligenes faecalis (Pinchot, 1957 a-c), Micrococcus lysodeikticus (Ishikawa and Lehninger, 1962), Escherichia coli (Kashket and Brodie, 1963), Streptococcus faecalis (Gallin and Van Denmark, 1964), Nitrosomonas europaea (Ramaiah and Nicholas, 1964), Pseudomonas saccharophilia (Ishaque et al., 1971, 1973).

Some cell free extracts and membrane preparations, however, exhibited higher P/O ratios. Downey (1964) found a P/O ratio of 1.85 for Bacillus subtilis using succinate as substrate. Values close to 2 were also observed in Mycobacterium phlei (Brodie, 1959), and Thiobacillus novellus (Cole and Aleem, 1970). A value of 2.8 was observed in Nitrobacter winogradskyi (Kiesow, 1964) and N. agilis (Aleem, 1968) employing NADH as the source of reducing equivalents. A total lack of respiratory control, together with the generally observed low P/O ratios, led some investigators to believe that oxidative phosphorylation was rather inefficient in bacteria compared to mitochondria (Gunsalus and Shuster, 1961). Others maintained, however, that the observed low P/O ratios reflected damage to the membranes and bacterial respiratory chains incurred as a result of the methods of preparation. This view was supported by the fact that to obtain any phosphorylation at all in some bacterial preparations, solubilized extracts, containing unknown coupling factors, frequently had to be restored to the membranes. Moreover, some higher values and indications of respiratory control have been reported in bacteria. Membranes from Paracoccus denitrificans had relatively high phosphorylation activity (Imai et al., 1967). Although P/O ratios of only 1.5 for NADH and 0.5 for succinate were observed, addition of ADP and/or phosphate resulted in an increase in respiratory rate, and classical uncouplers of oxidative phosphorylation, such as 2, 4-dinitrophenol, caused a 3-fold stimulation in oxygen consumption while completely inhibiting phosphorylation (John and Whatley, 1970; John and Hamilton, 1970, 1971; John and Whatley, 1975). Respiratory control has been reported in preparations of A. vinelandii (Eilermann et al., 1970; Jones et al., 1971 a, b) and N. winogradskyi (Cobley and Chappel, 1974), although the P/O ratio obtained from these preparations were very low. More recently, Burstein et al. (1979) reported respiratory control in E. coli under limiting respiratory substrate conditions or when the substrate is not a carbon source. Tsuchiya and Rosen (1980) have confirmed these results demonstrating an increase in respiratory rate upon addition of uncoupler. As the calculated P/O ratios are still low, and the respiratory rate was very low, the question of true respiratory control in bacteria remains unsettled.

Other methods for assaying oxidative phosphorylation in bacteria were clearly needed. Hempfling (1970), using intact cells of *E. coli* and rapid stopped flow techniques, measured esterification of inorganic phosphate and correlated it with oxidation of endogenous NADH as measured spectrally. P/O ratios between 2.5 and 3.0 were obtained and he concluded that oxidative phosphorylation in *E. coli* was just as efficient as in mitochondria. Using this technique, a P/O ratio of 3.0 was calculated for resting whole cells of *A. vinelandii* (Baak and Postma, 1971). Van der Beek and Strouthamer (1973), however, showed that changes in NADH did not accurately represent the amount of oxygen consumed and that, therefore, Hempfling's method could result in severe overestimates in P/O ratios. By measuring changes in ATP, ADP and AMP levels in anaerobic resting cells of different intact bacteria after the addition of a small known amount of oxygen, the following P/O ratios were calculated: *Proteus mirabilis*, 0.6-1.0, *Aerobacter aerogenes*, 0.3, *Pseudomonas aeruginosa*, 0.5 and *A. vinelandii*, 0.5 (Van der Beek and Stouthamer, 1973). They concluded that P/O ratios for intact "resting" bacterial cells are not as high as mitochondrial values.

Indirect Measurements of Oxidative Phosphorylation in Bacteria

Use of growth yields as an indirect method for estimating the efficiency of oxidative phosphorylation in growing bacteria was sparked by the study of Bauchop and Elsden (1960). These investigators showed that under anaerobiosis, several organisms, whose fermentation pathways are well characterized, have a molar growth yield of ATP (Y_{ATP}) equal to 10.5 grams dry weight of cells per mole of ATP generated with glucose as the energy source. Using this concept, a determination of $Y_{ATP} = 10.2$ for Aerobacter aerogenes grown anaerobically on glucose was made (Hadjipetrou et al., 1964; Hadjipetrou and Stouthamer, 1965). By simultaneously measuring the molar growth yield for glucose (Y_{glu} = grams dry cell weight per mole glucose consumed) and the total oxygen consumption, they first calculated the growth yield per gram-atom oxygen (Y_O = grams dry cell weight per gram-atom O consumed). Having obtained this value, they simply divided Y_O by Y_{ATP}, to obtain the P/O ratio of 3. The concept of Y_{ATP} as a biological constant for all bacteria was challenged early by investigators studying the rumen bacteria Bacteroides amylophilus and Selenomonas ruminatium (Hobson and Summers, 1967) who found that Y_{ATP} for these species appeared dependent on growth rate and could attain values as high as 20. Similarly a value for Y_{ATP} of 17 was found in certain strains of Streptococcus (Moustafa and Colins, 1968). Since more conventional values for these same strains were found by Forrest and Walker (1971) and since the metabolic pathways were not completely worked out for these species, these exceptionally high Y_{ATP} values were classified as "anomalous" growth yields. Many investigators accepted a "normal" value of 10.5 for Y_{ATP} as an upper limit for most cells growing on a substrate which allowed full energetic coupling (Stouthamer, 1969; Payne, 1970; Forrest and Walker, 1971). Using the "normal" Y_{ATP} value of 10.5 and measuring molar growth yields to obtain Y_O, P/O ratios close to 2.0 were calculated for E. coli and Pseudomonas fluorescens, and close to 3.0 for A. aerogenes, Acetomonas oxydans, and Aerobacter cloacae (Stouthamer, 1969). A P/O ratio of 2.0 was also obtained for Proteus mirabilis (Stouthamer and Bettenhaussen, 1972).

After more careful evaluation, Stouthamer and Bettenhaussen (1973) concluded that the method for the determination of P/O ratios and the efficiency of oxidative phosphorylation by measuring molar growth yields could not be used so easily. Greatly different values for Y_{ATP} were obtained in bacteria with well characterized metabolic pathways (de Vries *et al.*, 1970; McGill and Dawes, 1971) forcing abandonment of the concept that Y_{ATP} was a biological constant. Their studies, furthermore, showed that growth rates greatly affected Y_{ATP} values. Since growth rates are appreciably slower in anaerobic than aerobic cultures, even for the same strain, Y_{ATP} values obtained under anaerobic conditions could not be used to make P/O ratio determinations without great overestimation (Stouthamer and Bettenhaussen, 1973).

Growth Yields to Measure Efficiency of Bacterial Oxidative Phosphorylation

Studies of bacteria growing in continuous culture chemostats revealed an apparent linear relationship between the specific rate of oxygen consumption and the growth rate of the culture. Plots of these two growth parameters, however, failed to extrapolate back to zero. To explain these findings, Pirt (1965) concluded that there were growth independent "maintenance" functions which required a constant small amount of the energy released from substrate catabolism. Stouthamer and Bettenhaussen (1973) recognized the importance of taking maintenance energy requirements into account when trying to estimate ATP production.

A value of 14.0 for Y_{ATP}^{Max}, the molar growth yield for ATP corrected for the energy of maintenance, was obtained for an anaerobic culture of Aerobacter aerogenes (Stouthamer and Bettenhaussen, 1975). In an attempt to compensate for the problem of growth rate dependent Y_{ATP} values, both anaerobic and aerobic cultures were grown at approximately the same growth rates in continuous culture chemostats. Using the Y_{ATP}^{Max} value obtained from the anaerobic culture, a P/O ratio of only 1.3 was determined for this organism compared with the previous determination of close to 3 (Hadjipetrou and Stouthamer, 1965). Stouthamer and Bettenhaussen (1975) concluded that the P/O ratio in bacteria was not as efficient as oxidative phosphorylation in mitochondria.

The greatest difficulties and errors in determining the efficiencies of oxidative phosphorylation from growth yield studies have been, and remain, in evaluating Y_{ATP}^{Max}. Highly intricate mathematical models were developed to determine this value for cultures growing at different growth rates (de Kwaadsteniet et al., 1976). Paracoccus denitrificans is an obligate aerobe and, therefore, anaerobic Y_{ATP} values cannot be measured for it. With this model it became possible for van Verseveld and Stouthamer (1976) to calculate P/O ratios for P. denitrificans equal to 1.7. They concluded that there are 2 sites of energy coupling when cells are grown under gluconate-limiting conditions. Y_{ATP} values in themselves have been reported as a measure of the efficiency in which bacteria conserve energy. Theoretical Y_{ATP}^{Max} values have been calculated for a number of bacteria growing on various substrates (Gunsalus and Shuster, 1961; Forrest and Walker, 1971; Stouthamer, 1973; Harder and van Dijken, 1976). To obtain theoretical Y_{ATP}^{Max} values, the elemental and macromolecular composition of the cells is determined and the ATP requirement for the synthesis of this cell material is calculated. Theoretical Y_{ATP}^{Max} values were found to be fairly close for growth with preformed monomers and for growth on glucose and inorganic salts for a number of bacteria. Special corrections have to be performed when cells that make large quantities of storage compounds are studied to use these Y_{ATP}^{Max} values as a measure of efficiency (see, for review, Stouthamer, 1977, 1978).

Chemiosmotic Hypothesis

According to the chemiosmotic hypothesis (Mitchell, 1961, 1963, 1966, 1973; Greville, 1969; Harold, 1972), the energy from the oxidation of substrates via the respiratory chain is conserved in the form of a protonmotive force ($\Delta\tilde{\mu}_{H^+}$). The protonmotive force is generated by the extrusion of protons out of the mitochondrion or bacterial cell during electron transport. The protonmotive force is comprised of a transmembrane electrical potential ($\Delta\psi$), outside positive, and a transmembrane proton gradient (ΔpH), outside acid. The total protonmotive force, in millivolts, is calculated from the sum of these gradients ($\Delta\tilde{\mu}_{H^+} = \Delta\psi - 58.8\Delta pH$ at 25° C). The protonmotive force is postulated to drive several energy-dependent processes. Experiments have shown that ATP synthesis (Hertzberg and Hinkle, 1974; Wilson *et al.*, 1976; Maloney, 1977, 1978), reversed electron flow (Klingenberg and Schollmeyer, 1961; Chance and Hollunger, 1961; Hinkle *et al.*, 1967), a transhydrogenase (Singh and Bragg, 1979), solute transport systems (Simoni and Postma, 1975; Kaback, 1976; Kaback *et al.*, 1977; Rosen and Kashket, 1978), and even bacterial motility (Larsen et al., 1974; Manson et al., 1977; De Jong and van der Drift, 1978), can all be energized by a protonmotive force.

The first direct measurements of proton efflux by mitochondria were detected by Mitchell and Moyle (1965), as a drop in pH outside respiring mitochondria. To determine the H^+/O ratio quantitatively, i.e., the number of protons translocated across the mitochondrial membrane per oxygen atom consumed, they developed the oxygen pulse technique (Mitchell and Moyle, 1967). In these experiments an anaerobic mitochondrial suspension is given a calibrated small pulse of dissolved oxygen while monitoring the pH of the suspension with a pH electrode. In these early experiments, H^+/O ratios close to 6 were obtained leading to the conclusion that the $H^+/2e^-$ ratio was 2 for each of the 3 sites of energy conservation within the mitochondrial respiratory chain. This conclusion was supported on theoretical grounds by the fact that most simple protolytic oxidation-reduction reactions also have a stoichiometry of one proton released per electron transferred. Further support came from ATP pulse experiments which measured outward translocation of 2 gram ions H^+ per mole ATP hydrolyzed by oligomycin sensitive ATPase in anaerobic whole mitochondria (Mitchell and Moyle, 1968; Brand and Lehninger, 1977) and the inward translocation of 2 H^+ by submitochondrial particles (Moyle and Mitchell, 1973; Thayer and Hinkle, 1973). This linked the stoichiometry of 2 protons pumped per site with the 2 protons required to drive ATP synthesis by the reversible mitochondrial ATPase.

An $H^+/2e^-$ ratio of two was inconsistent, however, with earlier findings of respiration-coupled transport in mitochondria. Studies showed that 2.0 Ca^{2+} ions were accumulated per site (Rossi and Lehninger, 1963, 1964; Chance, 1965; Rossi et al., 1967; Rossi and Azzone, 1969; Azzone and Massari, 1971, 1973). Mitchell argued that calcium ions were translocated in obligatory symport with phosphate as Ca_2^{4+} - HPO_4^{2-}, resulting in only 1 net (+) charge per calcium ion (Moyle and Mitchell, 1977). Using both hydrogen and calcium sensitive electrodes simultaneously, Reynefarje and Lehninger (1977) established that the uptake of calcium was indeed through operation of a bivalent electrophoretic uniport process. For these experiments, both phosphate limited assays and N-ethylmaleimide, known to block phosphate transport, were employed with the result that 2.0 Ca^{2+}/site are accumulated with 4 (+) charges. Also in conflict with a $H^+/2e^-$ ratio equal to 2.0 were studies of potassium transport in mitochondria. With valinomycin present to allow potassium free permeability as a charge compensating ion, $K^+/2e^-$ per site ratios significantly greater than 2.0 were obtained (Cockrell et al., 1966; Massari and Azzone, 1970).

Thermodynamic arguments against Mitchell's proposed stoichiometry were first advanced by Rottenberg (1970). According to chemiosmotic principles, the electrochemical gradient of protons generated across the membrane by electron transport is directly balanced against the phosphorylation potential, ΔG_p, a measure of the energy required to synthesize 1 ATP molecule. This relationship can be expressed as

$$\underset{\text{(electron transport)}}{n \times \Delta\tilde{\mu}_{H^+}} = \underset{\text{(ATP synthesis)}}{1 \times \Delta G_p}$$

where n is the number of H^+ ions ejected per pair of electrons per ATP-generating site (i.e., H^+/site). Theoretically at least, n can be any number, but if the H^+/site ratio is low, then the thermodynamic driving force must be high, and vice versa. Rottenberg (1970) argued that an

H^+/site ratio of only 2 would not provide enough energy to drive ATP synthesis against the highest phosphate potentials that isolated mitochondria can attain. Using valinomycin and varying concentrations of potassium, he partially collapsed the protonmotive force, yet still was able to demonstrate ATP synthesis. Investigators have taken Rottenberg's lead, and based on these thermodynamic considerations and measured ΔG_p and $\Delta\tilde{\mu}_{H^+}$ values, have postulated that at least 3 H^+ must be ejected per site (Padan and Rottenberg, 1973; Rottenberg, 1975; Nicholls, 1974). Studies on adenine nucleotide transport across the inner mitochondrial membrane showed that ATP^{4-}/ADP^{3-} exchange required energy (La Nove and Mueller, 1977). It was argued that if 1 H^+ per site was used by the electrogenic adenine nucleotide translocase to effect this ATP^{4-}/ADP^{3-} exchange, then a total H^+/site ratio of only 2.0 would certainly leave insufficient energy for ATP synthesis (Klingenberg and Rottenberg, 1977).

Lehninger and his colleagues have provided some of the strongest evidence for higher H^+/O (and $H^+/2e^-$ per site) ratios by improving the techniques for directly measuring the elements that comprise this ratio. Brand _et_ _al_. (1976 a,b) tried to determine the H^+ ejection stoichiometry by measuring the number of isotopically labeled proton-carrying weak acid anions, such as acetate and 3-hydroxybutyrate, accumulated along with the 2.0 Ca^{2+} ions entering the mitochondria per site. Their results indicated the need for 4 protons per site to compensate for this Ca^{2+} accumulation. Using the oxygen pulse technique of Mitchell and Moyle (1967), Lehninger also observed an H^+/site ratio equal to 2. Close examination of the results, however, revealed a large backdecay of protons attributed to the established H^+ gradient. Accounting for backdecay resulted in H^+/site ratios closer to 3 (Reynefarje et al., 1976 a). Another method for determining H^+/site ratios employed steady state measurements of the rates of H^+ ejection and oxygen consumption under conditions where interfering ion movements were presumably eliminated or controlled for (Brand et al., 1976 c). Values for H^+/O up to 12.0 were obtained consistent with an H^+/site value of 4.0. Lehninger found that higher values for H^+/O could be obtained even by the Mitchell and Moyle (1967) oxygen pulse technique if phosphate was very carefully washed out of the assay buffer or if mersalyl or N-ethylmaleimide were added to retard the proton leak accompanying the very rapid phosphate transport (Brand et al., 1976, b, d). H^+/O ratios consistent with an H^+/site value of 3.0 were obtained. Using an improved method whereby both H^+ ejection and oxygen consumption could be measured directly by sensitive electrodes after a reductant pulse (Brand et al., 1976 b), Lehninger obtained H^+/O ratios of 12.0 for NADH and 8.0 for succinate. These findings are consistent with H^+/site values of 4.0 and have been confirmed independently by Azzone (Pozzan et al., 1979).

Although early determinations showed that hydrolysis of ATP resulted in an H^+/ATP ratio of 2.0, Lehninger now believes that in fact 3.0 protons may be required for ATP synthesis (Brand and Lehninger, 1977; Lehninger et al., 1977; Alexandre et al., 1978). This view is based on thermodynamic considerations presented earlier and a new method for measuring both the steady-state rates of H^+ ejection and ATP hydrolysis (Alexandre et al., 1978). According to their current thinking (Alexandre et al., 1978), 4 protons are translocated across the membrane at each site. Three are used to drive ATP synthesis. The fourth H^+ is recycled via an electroneutral H^+-H_2PO_4 symport process which drives the electrogenic adenosine translocase.

Questions still remain, however, regarding the procedures for measuring H^+/O ratios and the interpretation of results obtained. Recent studies have employed respiratory inhibitors together with various electron donors and artificial electron acceptors to measure individual proton translocating sites. The question of proton pumping by the cytochrome oxidase segment of the respiratory chain (site 3) is especially controversial. According to the Mitchell theory of redox loops, no protons are pumped by this segment (Moyle and Mitchell, 1978). Papa (1976) also concluded that site 3 does not pump protons. By direct measurement, however, site 3 was shown to translocate 2 H^+ (Wikström and Saari, 1977; Sigel and Carafoli, 1978). The question of cytochrome oxidase as a proton translocase has been reviewed extensively by Wikström and Krab (1979, 1980). In a series of studies employing a new steady state method of investigating H^+/site and charge/site ratios, Brand _et al_. (1978) have concluded that there is unequal charge separation by the various coupling spans of the respiratory chain. This finding has led them to the conclusion that H^+/site ratios may not be equal and consequently more ATP may be synthesized per 2 e^- at some "sites" than at others resulting in non-integral P/O ratios as well.

H^+/O in Bacteria

Although the details of the chemiosmotic theory, including exact stoichiometries of proton translocation throughout the mitochondrial respiratory chain, are still highly controversial, measurements of proton pumping by bacteria can be used to compare efficiencies of bacterial respiration and to locate sites of energy conservation. The earliest experiments employing the oxygen pulse technique of Mitchell and Moyle (1967) failed to show any proton extrusion in *E. coli* K 12, *Klebsiella aerogenes*, *Pseudomonas fluorescens*, *Mycobacterium tuberculosis* BCG, *M. phlei*, or *Streptococcus lacti* but did yield an H^+/O value of approximately 4 for *Staphylococcus albus* and values between 6 and 8 for *P. denitrificans*, *Bacillus subtilis* and *B. megaterium* when gramicidin or valinomycin plus CCCP were present (Wimpeny, 1970). Reeves (1971), however, was able to detect proton extrusion after providing an oxygen pulse to anaerobic cells of *E. coli* when thiocyanate was present as a charge compensating ion.

H^+/O values of about 4 were obtained for *E. coli* cells grown under a variety of conditions when endogenous substrates were oxidized (Garland *et al.*, 1975; Jones *et al.*, 1975; Farmer and Jones, 1976). When a substrate was provided, slightly lower H^+/O values were obtained. Malate oxidation produced H^+/O values between 3.1 and 3.6 (Jones *et al.*, 1975), and formate oxidation resulted in 3.63 for H^+/O (Garland *et al.*, 1975). Garland noticed that the H^+ leak was very fast with formate, implicating a solute symport system in the lowering of H^+/O values with this substrate. In contrast, values close to 2.0 were found when succinate, lactate or glycerol were the substrates being oxidized (Poole and Haddock, 1975; Jones *et al.*, 1975). Supported by the earlier observation that 2.0 protons were extruded by the *E. coli* ATPase (West and Mitchell, 1974), the general conclusion reached was that the *E. coli* respiratory chain possessed 2 equivalent energy conservation sites, each responsible for the translocation of 2.0 protons (Lawford and Haddock, 1973; Brice _et al._, 1974; Jones _et al._, 1975). One site in the NADH dehydrogenase region of the respiratory chain, corresponding to site 1 of the mitochondrial chain, and a second site somewhere between oxygen and the entry points for the reducing equivalents from succinate, lactate and glycerol were proposed (Lawford and Haddock, 1973). A common entry point for the electrons derived from succinate, lactate and glycerol at the level of ubiquinone was further suggested. Verification of this scheme was provided when Haddock _et al._ (1974) demonstrated $H^+/2e^-$ ratios between 1 and 2 after providing pulses of ubiquinone -1 to anaerobic cell suspensions thereby confirming a site between NADH and ubiquinone. The relative ease in obtaining respiratory mutants of _E. coli_ (Gibson and Cox, 1973; Cox and Gibson, 1974) has no doubt been a major consideration in studying bioenergetics in this organism.

Concerned with findings in mitochondria (Brand and Lehninger, 1977) which showed that H^+/site ratios greater than 2.0 can be obtained when phosphate transport systems are controlled with N-ethylmaleimide, Cox and Haddock (1978) investigated the possibility of phosphate lowering the H^+/site ratios found for _E. coli_. They found that the same H^+/ site ratio of 2.0 was obtained in both wild type _E coli_ and in an organic phosphate auxotrophic mutant which had no functional inorganic phosphate transport system. Using the same oxygen pulse technique of Mitchell and Moyle (1967), Meyer and Jones (1973b), however, found a maximum H^+/O ratio of 6.0 for _E. coli_ cell suspensions taken from cultures at their highest growth rate, and values of 1.8 from cultures growing more slowly. This finding raised the possibility of a third site in *E. coli* grown under certain conditions. A more recent study, however, has shown that the H^+/O ratio in *E. coli* remained at approximately 3.7 regardless of growth rate or terminal oxidase (Rice and Hempfling, 1978).

Another bacterium which has received a considerable amount of attention is *Paracoccus denitrificans*. Scholes and Mitchell (1970 a) found that *P. denitrificans* membranes have a very low conductance for protons compared to other biological membranes. Relatively simple corrections could be employed to account for proton back-flux after an oxygen pulse. When they measured proton ejection in this species, they found an H^+/O ratio of 8 for oxidation of endogenous substrates (Scholes and Mitchell, 1970 b). Maintaining that, as in mitochondria, only 2 protons are translocated per site, Mitchell concluded that *P. denitrificans* had the same 3 sites of energy conservation as are typically found in mitochondria, and in addition, a transhydrogenase which contributed the remaining 2 protons observed under his non-physiological conditions. Further evidence for this scheme was obtained when Lawford *et al*. (1976) demonstrated H^+/O ratios of about 2.0 for the oxidation of ascorbate via TMPD, and between 3 and 4 for oxidation of glycerol or succinate. H^+/O values with malate as substrate as high as 8 were obtained with cells harvested at an early logarithmic stage of growth. Cells harvested at the late logarithmic stage, however, had H^+/O values only around 5, and H^+/O values of only 4 could be obtained from cells taken from stationary growth stages. This suggested that proton translocation by both NADH dehydrogenase and the transhydrogenase could be regulated by growth conditions.

Meijer *et al*. (1977) studied proton pumping by *P. denitrificans* cells grown under sulfate-limiting conditions in an attempt to functionally abolish site 1, which is known to require numerous iron-sulfur proteins. By first measuring molar growth yields and oxidative phosphorylation in membrane vesicles, they concluded that P. denitrificans cells grown under succinate-limiting conditions had 2 sites of energy conservation, whereas cells grown under sulfate-limiting conditions only had a single energy conservation site. This is in direct contrast to the findings of others that P. denitrificans has 3 active energy conserving sites when grown heterotrophically (Edwards et al., 1977; Jones et al., 1977; Lawford, 1977). Meijer et al. (1977) obtained H^+/O values similar to those obtained by Lawford et al. (1976) for the succinate-limited cells. H^+/O values of only 4, however, were obtained for the sulfate-limited grown cells, whether endogenous substrates or succinate were oxidized. They concluded that the H^+/site ratio was not 2.0 but rather between 3 and 4 (Meijer et al., 1977).

Support for this higher stoichiometry was obtained by measuring proton translocation in methanol grown cells (van Verseveld and Stouthamer, 1978 a). Methanol oxidation to formaldehyde was shown to involve electron transport to oxygen via a specially induced cytochrome c and an a-type cytochrome, implying just 1 site of energy conservation. H^+/O values of approximately 3.5 were obtained for these cells when methanol was the substrate, thus confirming the H^+/site ratios of between 3 and 4 found in heterotrophically grown P. denitrificans (Meijer et al., 1977). These methanol grown cells were shown to have 3 active sites of conservation both from molar growth yield studies (van Verseveld and Stouthamer, 1978 b) and measurements of H^+/O using endogenous substrates and succinate. H^+/O values of 6 were obtained for succinate oxidation and approximately 7 for endogenous substrates. Although endogenous substrates yielded variable results when grown on methanol, van Verseveld concluded that H^+/site had to be greater than 3. Lawford also has now confirmed H^+/site ratios closer to 3 than to 2 in P. denitrificans grown under both sulfate or carbon-limiting conditions. H^+/O values of 8.55 for endogenous substrates were lowered to 5.85 when piericidin, an inhibitor of the NADH dehydrogenase, was added (Lawford, 1978), and values of 5.3 and 5.2 were observed with oxidation of succinate and added ubiquinone to spheroplasts of P. denitrificans (Lawford, 1979). Additional support for an H^+/site ratio of 3 in P. denitrificans has resulted from measurements of respiration-driven proton translocation by spheroplasts prepared from cells grown autotrophically with H_2 as the energy source (Porte and Vignais, 1980). H^+/O ratios above 10 were obtained with endogenous substrates. This value decreased to 7.2 ± 1.3 upon the addition of rotenone, and a further reduction to 4.2 ± 0.6 occurred upon adding antimycin A. With H_2 as the energy substrate, H^+/O values of 6.8 ± 0.9 were only slightly effected by rotenone (6.2 ± 0.6) but dropped to 4.2 when antimycin A was included. H^+/O values of approximately 7 were obtained with succinate as the substrate.

Just as the uncertain stoichiometry of proton translocation by cytochrome oxidase in mitochondria has become a focus of attention, a recent study has cast similar doubt on the stoichiometry at site 3 in P. denitrificans (van Verseveld et al., 1981). $H^+/2e^-$ ratios were measured using endogenous substrates, added methanol, or added ascorbate/TMPD as electron donors, and ferricyanide and oxygen as the terminal electron acceptors. Ferricyanide prevents further oxidation of substrate via cytochrome oxidase and oxygen. In all cases, a difference of only 2.0 ejected protons was found between the results using ferricyanide, and oxygen. This suggests that, analogous to Wikström's findings in mitochondria (Wikström and Krab, 1979), the H^+/site ratio at site 3 is only 2 for P. denitrificans. This conclusion further implies that unequal stoichiometries may exist for each of the active sites of energy conservation in P. denitrificans (van Verseveld et al., 1981).

As stated earlier, more than just one type of terminal cytochrome oxidase is found in bacterial respiratory systems. Determinations of molar growth yields, efficiencies of oxidative phosphorylation, and proton translocation stoichiometries have all been complicated by the possibility and existence of electron transport through alternate branches of bacterial respiratory chains. At least 4 different cytochrome oxidases have been identified in bacteria: cytochromes aa_3, a_1, d(= a_2) and o (Keilin, 1966; Castor and Chance, 1955, 1959; Chance, 1953). In an attempt to determine the effect that terminal oxidases have on the efficiency of energy conservation in bacteria, a study was conducted on different bacterial species known to have only 1 type of terminal oxidase (at least under certain growth conditions) (Meyer and Jones, 1973b). Bacillus subtilis, and Microbacterium thermosphactum possess only cytochrome aa_3 as the terminal cytochrome oxidase of their respiratory chains. E. coli, Xanthomonas hyacynthi, Acinetobacter lwoffi, and Kurthia zopfii possess only cytochrome o as a cytochrome oxidase. Acetobacter T71 has cytochrome oxidase a_1, as its major oxidase and cytochromes a_1 and d could be induced by growing E. coli under oxygen limited conditions (see Pudek and Bragg, 1974; Ashcroft and Haddock, 1975). Molar growth yields were used to obtain the efficiency of oxidative phosphorylation. Those strains with either cytochrome aa_3 or cytochrome o had calculated P/O ratios of 3. On the other hand, Acetobacter T71 had a P/O ratio of about 1 and E. coli, specifically grown to induce cytochromes a_1 and d, had greatly reduced P/O ratios (a value of 1.4 was obtained, however, cytochrome o was still present) (Meyer and Jones, 1973b). Overall, these authors argued that proton pumping stoichiometries correlated with P/O determinations except in X. hyacynthi, A. lwoffi and M. thermosphactum where the H^+/O values obtained were higher than the number predicted from P/O measurements assuming $H^+/P = 2$. They explained the elevated H^+/O values as being due to a site o transhydrogenase which does not drive ATP synthesis.

Meyer and Jones (1973b) were careful to point out that the greater observed efficiency (higher P/O and H^+/O) found in these species with cytochromes aa_3 or o did not demand that the energy conservation at the terminal oxidase was the major factor resulting in these efficiencies. Another possibility is that the redox potentials of these cytochromes are sufficiently high to allow for more sites of energy conservation between NADH and the oxidase. In contrast, cytochrome oxidase a_1 and d may have lower midpoint potentials which, in effect, could result in a shorter respiratory chain. Meyer and Jones (1973b) have further speculated that a terminal oxidase such as a_1, which has a very high affinity for oxygen, may in fact allow a bacterial respiratory chain to carry out oxidative phosphorylation, even if at low efficiency, at very low oxygen tensions (Meyer and Jones, 1973b).

From proton translocation studies conducted on a variety of bacteria, certain correlations between respiratory chain composition and H^+/O ratios have become evident. Investigators have consistently reported the highest H^+/O values, often above 8, for *P. denitrificans* (Scholes and Mitchell, 1970 b; Jones *et al.*, 1975; Lawford *et al.*, 1976; van der Beek, 1976; Lawford, 1978; Stouthamer, 1980), *Hydrogenomonas eutropha* H16 (Beatrice and Chappel, 1974), *Pseudomonas-ovalis* Chester (Jones *et al.*, 1975), and *Staphylococcus aureus* (Jeacocke *et al.*, 1972). In the above 4 bacterial species, both an energy dependent pyridine nucleotide transhydrogenase and a high potential membrane bound cytochrome c are found in the respiratory chains. H^+/O values closer to 6 have been observed in *Pseudomonas aeruginosa* (van der Beek, 1976), *Micrococcus lysodeikticus* (Jones *et al.*, 1975), and *Acinetobacter lwoffi* (Jones *et al.*, 1975). In the first 2 species a high potential cytochrome c is present but no transhydrogenase. In the third, a transhydrogenase exists but cytochrome c is absent. H^+/O ratios very close to 4 are generally obtained in *E. coli* (Jones *et al.*, 1975; Lawford and Haddock, 1973; Haddock *et al.*, 1974; Rice and Hempfling, 1978), *Klebsiella pneumoniae* (Jones *et al.*, 1975), *Klebsiella aerogenes* (van der Beek, 1976), *Proteus mirabilis* (van der Beek, 1976), *B. megaterium* (Jones *et al.*, 1975), *B. licheniformis* (Jones *et al.*, 1975), *B. subtilis* (Jones *et al.*, 1975) and *Nitrosomonas europaea* (Drozd, 1976). None of these 8 bacteria has a cytochrome c or transhydrogenase.

Jones *et al.* (1977) have emphasized that H^+/O ratio determinations are made *in vitro* and, therefore, can reflect only the potential number of energy conserving sites. Thus, H^+/O ratios may not correlate perfectly with oxidative phosphorylation efficiencies obtained by measuring molar growth yields because these reflect the actual number of energy conserving sites in operation during growth. The transhydrogenase, for example, has been implicated in a site 0 redox loop which apparently can result in proton extrusion _in vitro_ (Scholes and Mitchell, 1970b; Jones _et al._, 1975). Examination of molar growth yields fails to reveal any increased ATP production by this segment. Physiologically this may be due to the fact that the total amount of NADPH produced by the cell is very small compared to its NADH levels and therefore any NADPH oxidation occurring at site 0 is negligible. Furthermore, the protonmotive force set up by the rest of the respiratory chain would probably drive the transhydrogenase in the opposite direction, providing NADPH needed for biosynthesis (Bragg _et al._, 1972).

Though the _in vivo_ direction of the transhydrogenase reaction and its role in proton ejection requires further clarification, the presence of a high potential cytochrome c has been clearly implicated with a site of energy conservation. Bacteria lacking cytochrome c generally have only sites 1 and 2, whereas, those possessing this respiratory chain component generally have 3 complete energy conservation sites (Jones _et al._, 1975; Harold, 1977; Haddock and Jones, 1977; Stouthamer, 1978). Willison and Haddock (1981), using molar growth yield and proton ejection measurements, compared wild type _P. denitrificans_ possessing cytochrome c with cytochrome c-deficient mutants. The parent strain was found to have an energy coupling site which was lost in the mutant, thus providing additional support for the requirement of cytochrome c for a 3rd site.

Jones _et al._ (1975) reported a wide divergence in H^+/O ratios which are apparently dependent on the respiratory chain make up and correlated with efficiencies determined by molar growth yield measurements. Species exhibiting the lowest efficiencies and H^+/O ratios usually occupy niches in highly nutritive environments, e.g., facultative anaerobes and certain aerobic Bacilli. Jones makes the observation that these fortunate bacteria can afford to be inefficient and waste potential energy. On the other hand, species demonstrating high efficiency and H^+/O ratios are those bacteria usually found under conditions where nutrients are severely limited in quantity and nature (e.g., Ps. ovalis Chester and H. eutropha). These of desperate necessity have developed the more highly efficient mechanisms for energy conservation.

MATERIALS AND METHODS

Organisms and Growth Conditions

Bacillus alcalophilus was obtained from The American Type Culture Collection (ATCC NO 27647). *Bacillus firmus* RAB was isolated from garden soil by others in this laboratory (Guffanti *et al.*, 1980). Both alkalophilic strains were routinely grown in sodium carbonate buffer, pH 10.5, consisting of 25 mM Na_2CO_3, 1 mM K_2PO_4, 0.1 mM $MgSO_4$ and 0.1%(w/v) $(NH_4)_2\ SO_4$ (Guffanti *et al.*, 1978). This basal growth medium was supplemented with 0.1%(w/v) yeast extract (Difco) and 1%(v/v) of a trace salts solution (Hegeman, 1966) added from separate sterile solutions. The carbon and energy source was 50 mM Na-L-malate which was also added from a separate sterile solution. For some experiments the basal growth medium was adjusted to pH 9.0 and experiments with 25 mM lactose as the carbon source were also conducted.

The non-alkalophilic strains derived from *B. alcalophilus* (Krulwich *et al.*, 1979) were designated KM23 and other KM strains, and those from *B. firmus* RAB were RAB strains. The non-alkalophilic mutants were grown at neutral pH. The basal growth medium for these strains, PT6.8, consisted of 25 mM $KHPO_4$, 25 mM Tris-HCl, 0.1 mM $MgSO_4$ and 0.1%(w/v) $(NH_4)_2$ SO_4 at pH 6.8. Added to this medium was 0.1%(w/v) yeast extract (Difco) and 1%(v/v) trace salts solution (Hegeman, 1966) from separate sterile solutions. For some studies the basal growth medium was adjusted to pH 8.5. The carbon and energy source was 50 mM K^+-L-malate.

All the above strains were grown and maintained in batch culture (250 ml erlenmeyer flasks containing 50 ml media) at 30°C with shaking at 200 rpm on a New Bruswick G25 rotary shaker. For experiments requiring greater amounts of cell material, 8 l batch cultures were grown at 30°C with forced aeration. For growth in complete darkness, growth vessels were wrapped with light-proof foils. For growth in "low iron" medium, cells were grown for four passages in media from which iron was omitted from the trace salts. Growth was monitored turbidometrically with a Klett-Summerson colorimeter (No. 42 filter) and cultures were grown to the late logarithmic stage of growth.

Isolation of Non-Alkalophilic Strains

The wild type alkalophilic strains were grown at pH 10.5 on Na^+-L-malate to late logarithmic stage. Using aseptic techniques, cells were harvested by centrifugation at 10,000 x g for 5 min., resuspended, washed and resuspended in 10 ml of a buffer, referred to as PT9.0, consisting of 25 mM K_2PO_4, 25 mM Tris-HCl, 0.1 mM $MgSO_4$ and 0.1% $(NH_4)_2SO_4$, pH 9.0. After addition of the mutagen ethyl methansulfonate to 1%(v/v) these cells were incubated at 30°C with shaking for 1 hour as described previously (Wolfson and Krulwich, 1972). The cells were then washed and resuspended in PT9.0 to which was added 50 mM K^+-L-malate, 1%(v/v) trace salts and 0.1% yeast extract. The cells were allowed to grow out for 4 hours in this medium. Mutagenized cells were then plated directly onto PT 6.8 plates (1-5% purified agar, Difco) containing K^+-L-malate. The wild type alkalophiles do not grow on these plates. Several colonies of non-alkalophilic mutants were observed after 2-3 days incubation at 30°C. Rates of spontaneous mutation to non-alkalophily and frequencies of reversion to alkalophily were also determined by plating alkalophiles at high concentration onto PT6.8 plates and similarly plating KM23 onto Na^+-CO_3 plates at pH 10.5. Revertants were designated KMR strains. Spontaneous mutation and reversion frequencies of 1 in 10^9 were obtained.

Measurement of Molar Growth Yields

Molar growth yields were determined according to the procedures outlined by Stouthamer (1969). Sidearm flasks containing 25 ml of the basal growth media described above (sodium carbonate pH 10.5 buffer for alkalophiles and PT6.8 for non-alkalophilic mutants) were prepared with 1%(v/v) trace salts, 0.04% yeast extract and different known amounts of L-malate in the range from 2.5 mM to 20 mM. The sidearm flasks were inoculated with cells that had been harvested in the mid-logarithmic stage of growth and washed with the appropriate basal growth medium. The sidearm cultures were incubated with shaking at 30°C. Growth was followed with a Klett-Summerson colorimeter until 2 turbidity readings taken an hour apart were the same. Cultures were then quantitatively harvested by centrifugation at 30,000 x g for 10 min. These cells were then washed 3 times with doubly distilled water placed in preweighed metal containers and dry weights were measured after drying in an oven at 105°C for at least 3 days. Molar growth yields on malate were expressed as Ymal = biomass dry weight/moles malate consumed.

Preparation of Right-Side-Out Membrane Vesicles

Right-side out membrane vesicles were prepared from L-malate grown cells of both alkalophilic species as well as from several non-alkalophilic mutant and revertant strains essentially by the lysozyme method of Kaback (1971). Cells grown to late logarithmic stage were harvested either by centrifugation (10,000 x g, 5 min.) or, in the case of large preparations, by tangential filtration employing the Millipore Pellicon system. Cells were generally washed once with 10 mM Tris-SO_4 pH 8.0 and again pelleted by centrifugation (10,000 x g, 5 min.). Cells were resuspended with gentle homogenization in 30 mM Tris-SO_4, 20 mM $MgSO_4$, 10%(w/v) mannitol, pH 8.0, with 30 μg lysozyme/ml and incubated with shaking at 37°C for 20-30 min. Protoplast formation was checked by inspection with oil immersion phase contrast microscopy. Protoplasts were harvested by centrifugation (25,000 x g, 20 min.) and vesicles obtained by osmotic lysis in various buffers in the presence of DNase. For studies conducted at pH 9.0, protoplasts were lysed and subsequently washed in 100 mM potassium carbonate buffer, pH 9.0, containing 10 mM $MgSO_4$. A pH of 9.0 was selected because it is a typical cytoplasmic pH for the wild type strains. For studies performed at pH 7.0, protoplasts were lysed and washed in either 100 mM potassium phosphate buffer containing 10 mM $MgSO_4$ pH 7.0, or for some redox titrations, 100 mM KCl, 20 mM MOPS, 5 mM $MgSO_4$ pH 7.0. Vesicles were harvested (17,000 x g, 30 min.) and the pellet was resuspended in ice cold buffer. Washing was conducted by a series of low speed centrifugations (2,000 x g, 15 min.) and relatively high speed centrifugations (40,000 x g, 20 min.) which succeeded in sedimenting debris and pelleting the vesicles, respectively. Right-side-out vesicles were frozen in liquid N_2 and stored at -80°C.

Preparation of Everted Membrane Vesicles

Everted membrane vesicles were prepared from cells essentially using the procedure of Kobayashi et al. (1978). Protoplasts were prepared and

FIGURE 3

Protocol for preparing membrane vesicles

HARVEST CELLS (Tangential flow filtration and centrifugation 10,000 x g, 5 min)

WASH CELLS in 10 mM Tris-SO_4, pH 8.0 (10,000 x g, 5 min)

Suspend in 30 mM Tris-SO_4, 20 mM $MgSO_4$, 10% mannitol, pH 8.0 with 300 g lysozyme/ml

Incubate at 37°C for 20-30 min

HARVEST PROTOPLASTS (25,000 x g, 20 min)

Lysis 50-fold dilution into specified buffers - DNase

100 mM $KHCO_3$	100mM $KHPO_4$	100 mM KCl
10 mM $MgSO_4$	10 mM $MgSO_4$	20 mM MOPS
pH 9.0	pH 7.0	5 mM $MgSO_4$
		pH 7.0

HARVEST VESICLES (17,000 x g, 20 min)

Resuspend pellet (gentle manual homogenization)

for right-side-out vesicles
low-speed centrifugation
(2,000 x g, 10 min) to
pellet any remaining whole
cells or protoplasts

SUPERNATANT
high-speed centrifugations
(40,000 x g, 15 min) to
pellet and wash membranes

for everted vesicles
EVERT VESICLES by passing
suspension through a French
Press at 8,000 lbs/in^2
low-speed centrifugation
(12,000 x g, 10 min) to
sediment any remaining
right-side-out vesicles

high-speed centrifugations
(100,000 x g, 50 min) to
pellet and wash membranes

lysed as detailed above resulting in right-side-out vesicles. A suspension of vesicles (approximately 10 ml/l cell culture) was passed through an American Instrument Co. French pressure cell at 8,000 pounds/in^2. Any right-side-out vesicles and cell debris were removed by centrifugation at 20,000 x g (20 min.) and the everted vesicles were collected and washed once by ultracentrifugation at 100,000 x g (50 min.).

Measurement of α-Aminoisobutyric Acid Uptake

Uptake of α-aminoisobutyric acid (AIB) by right-side-out membrane vesicles was determined by a filtration assay using oxygenated reaction mixtures containing 1 mg vesicle protein per ml, 100 mM K_2CO_3, 10 mM $MgSO_4$ and, when present, 10 mM Na_2CO_3, pH 9.0. Membranes were energized by a 1.0 min. preincubation at 30° with 20 mM ascorbate and 2.0 mM TMPD. The uptake reaction was initiated by the addition of 40 μM[^{14}C-]AIB. Aliquots were removed at specified times, filtered through 0.45 μm pore size HA Millipore filters and washed rapidly with 5.0 ml of the K_2CO_3 buffer. Filters were dried and the trapped radioactivity counted by liquid scintillation. Unenergized membranes failed to accumulate AIB and served as a control.

Measurement of Transmembrane pH Gradients

The production of a transmembrane pH gradient, inside acid, by right-side-out membrane vesicles was assayed by measuring the uptake of the weak base [^{14}C-]methylamine by flow dialysis (Colowick and Womack, 1969) in an apparatus modified according to the specifications of Ramos *et al*. (1979). The weak base employed could freely diffuse across the membrane in the neutral, dissociated form. If, however, the vesicle internal space was more acidic than the external milieu, the base became protonated and trapped inside (Waddel and Butler, 1959; Rottenberg et al., 1972; Schuldiner et al., 1972). The assays were performed essentially as described by Guffanti et al. (1978). Membrane vesicles at approximately 10 mg protein/ml were added to the upper chamber of the flow dialysis apparatus (final volume 0.8 ml). The buffers used are specified in the figure legends in the results section. The two chambers of the flow dialysis apparatus were separated by Spectrapor/dialysis tubing (6000 to 8000 molecular weight cutoff, Fisher Scientific). Mixing was accomplished with teflon coated magnetic stirring bars in each chamber. Buffer was pumped through the lower chamber at 6 ml/min. and 2 ml fractions were collected and assayed for radioactivity by liquid scintillation spectroscopy. $[^{14}C-]$ methylamine was added to a final concentration of 25 μM to start the assay. Membranes were energized by addition of 20 mM ascorbate plus 2 mM TMPD and a water saturated stream of oxygen was passed over the vesicle suspension.

Measurement of Oxygen Consumption

Respiratory rates of whole cells were assayed by oxygen uptake, using a Yellow Springs Instrument Model 53 Clark-type oxygen monitor. Cells in the late logarithmic phase were harvested, and resuspended in 25 mM potassium phosphate buffers at pH 6.8, or 25 mM potassium carbonate buffer at either pH 8.5 or 10.5. The cell protein concentrations were in a range from 0.03 to 0.1 mg cell protein/ml. Experiments were conducted at cell protein concentrations within that range so that the rate of oxygen uptake increased linearly with increasing protein concentration.

Samples (3.0 ml) were allowed to become air saturated in the monitor chamber and were equilibrated to 30°C for 3 min. The rate of oxygen uptake was determined both before and after the addition of 3.3 mM L-malate. The L-malate concentration used allowed the maximal rate of oxygen uptake. The respiratory rates were unaffected by the addition of 10 μM gramicidin.

Oxygen uptake by membrane vesicles was also examined. Everted membrane vesicles of B. alcalophilus and KM23 were freshly prepared in 100 mM potassium carbonate, 10 mM $MgSO_4$, pH 9.0 and 100 mM potassium phosphate, 10 mM $MgSO_4$, pH 7.0, respectively. The endogenous rates of oxygen uptake were determined as above for whole cells, and then 5 mM NADH was added to measure maximal rates of oxygen consumption.

Analysis of Respiratory Chain Components

1. Difference Spectroscopy

Dithionite-reduced minus air-oxidized difference spectra of membrane vesicles prepared from each strain were recorded at room temperature with a Perkin-Elmer 557 Dual Beam spectrophotometer to identify and quantitate membrane cytochrome content. Beer's Law,

$$\Delta A = \Delta \varepsilon b c$$

where ΔA = difference in absorbance between the reduced and oxidized vesicle sample

Δε = the difference millimolar extinction coefficient

b = the cell path length (routinely 1.0 cm)

c = the concentration of the cytochrome species

was used to calculate the cytochrome levels in these membranes. The following difference millimolar extinction coefficients and wavelength pairs were employed in these calculations: cytochrome $(a\text{-}a_3)$ $\Delta A_{605-630}$,

$\Delta\varepsilon = 20.5$/heme (Van Gelder, 1966); cytochrome b, $\Delta A_{560-575}$, $\Delta\varepsilon = 17.5$ (Deeb and Hager, 1964), cytochrome c $\Delta A_{551-538}$, $\Delta\varepsilon = 17.3$ (Jones and Redfearn, 1966). Spectra of samples at 77°K were obtained using the Perkin-Elmer liquid N_2 cryogenic accessory. Best results were obtained when samples were made approximately 5 mg protein/ml and 66% glycerol. Carbon monoxide difference spectra were obtained by first storing a dithionite-reduced spectrum in the microprocessor and then gently bubbling CO through the vesicle suspension for approximately 5 min. before recording a CO-reduced minus reduced difference spectrum.

Hemes could be extracted from the membrane vesicles of the two alkalophilic strains and pyridine hemochromagens were prepared according to the procedures described by Falk (1964). Frozen membrane vesicles (5-10 mg protein/ml) were thawed, acidified with 0.1 N HCl to pH 2.0, and homogenized with an equal volume of methyl ethyl ketone. The residue was reextracted with an additional volume of methyl ethyl ketone. The pooled extracts were evaporated to near dryness under a stream of N_2 and immediately dissolved in 1 ml of pyridine. An equal volume of 0.2 N KOH was added and spectroscopy performed on the resulting hemochromagens. Great care was exercised to perform these procedures shielded from light and at 0° to minimize heme decomposition. The protein residue after these extractions still contained cytochrome c. Direct conversion of the protein bound heme to the pyridine hemochromogen was accomplished by homogenization of the protein extract with equal volumes of pyridine and 0.2 N KOH. Dithionite-reduced minus ferricyanide-oxidized difference spectra were recorded for these compounds at room temperature using a Perkin-Elmer 557 Dual Beam spectrophotometer. The following millimolar extinction coefficients were used to calculate heme concentrations:

heme a, $\lambda = 587$, $\varepsilon = 24$ (Rieske, 1967) protoheme IX, $\lambda = 557$, $\Delta\varepsilon_{557-541} = 20.7$ (Falk, 1964); and heme c, $\lambda = 551$, $\Delta\varepsilon_{551-535} = 19.1$ (Rieske, 1967). As noted under "Results", the procedure gave very incomplete extraction of hemes from both non-alkalophilic strains relative to the alkalophilic strains.

2. Copper Determination

An analysis was performed on vesicles that had been dialysed for 24 h against 10 mM EDTA to remove adventitious Cu. After dialysis the vesicles were collected by centrifugation at 31,000 x g for 30 min. and resuspended in doubly distilled water. The suspension was made 10%(v/v) in 20% $HClO_4$, incubated for 30 min. and centrifuged at 31,000 x g for 30 min. Protein free supernatants were analyzed for Cu by Dr. David Knaff at Texas Tech University using a Perkin-Elmer Model 303 Atomic Absorbance Spectrometer calibrated against $CuCl_2$ standard solutions.

3. Quinone Determinations

Quinones were extracted from membrane vesicles using petroleum ether. Quinone identification and concentrations were determined as described by Kröger (1978). One ml of frozen membrane vesicles (2-5 mg protein/ml) were thawed and thoroughly mixed by vortexing with 5 ml of a mixture consisting of 60%(v/v) methanol and 40%(v/v) petroleum ether. To this was added 1 ml of acetone and this mixture was vortexed intermittently for 15 min. The petroleum ether layer was separated by centrifugation at 1500 x g for 5 min. and carefully removed with a Pasteur pipet. The residue was reextracted with 2 ml of petroleum ether and centrifuged as before. The petroleum ether extracts were combined, evaporated under a stream of N_2 and redissolved in 3.0 ml ethanol. Absolute ultraviolet spectra were recorded against ethanol blanks using a Perkin-Elmer 557 Dual Beam spectrophotometer. Samples were reduced by adding 5 µl of a 5 mg/ml $NaBH_4$ solution. Difference spectra were recorded as $NaBH_4$-reduced minus air oxidized reference and concentrations of ubiquinone calculated using the wavelength pair $\Delta A_{280-289}$ and the extinction coefficient $\Delta\varepsilon = 8.8\ mM^{-1}cm^{-1}$ (Kröger, 1978). Identical values were calculated using the absorbance at the peak at 275 nm and an extinction coefficient of 12.25 (Redfearn, 1967). To better identify the quinone species present in _B_. _alcalophilus_, an attempt was made to determine the length of the isoprenoid sidechain by reverse phase chromatography (Lester and Ramasarma, 1959). Whatman No. 1 filter paper was immersed in chloroform containing 5%(w/v) Dow-Corning Silicone Fluid No. 550 and allowed to dry at room temperature. Petroleum ether extracts were applied to the impregnated paper and descending paper chromatography was performed at room temperature using 7:3 (v/v) N-propanol:H_2O as the solvent. To detect the quinone compounds, the chromatogram was dipped into a 0.2%(w/v) aqueous solution of $KMnO_4$ for 30 sec. and immediately washed with hot tap water until all the unchanged $KMnO_4$ was removed. Ubiquinone species provided brown spots and preliminary identification was achieved by measuring RF values and comparing with standards and literature values (Lester and Ramasarma, 1959).

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.

4. Oxidation-Reduction Titration

Oxidation-reduction potentiometry was conducted using everted membrane vesicles from the alkalophiles *B. alcalophilus* and *B. firmus* RAB and their non-alkalophilic mutants KM23 and RABN respectively according to Dutton (1978). A Perkin-Elmer 557 Dual Beam spectrophotometer or a Johnson Foundation Scanning Dual Wavelength spectrophotometer interfaced with a Digital PDP 11/10 computer was used to follow cytochrome absorbance changes. The oxidation-reduction potential was measured using either a platinum-silver/silver chloride combination electrode (Metrohm EA 259) or by using a platinum electrode (Radiometer, Copenhagen, Type P101) and a saturated calomel electrode (Radiometer, Copenhagen, Type K401).

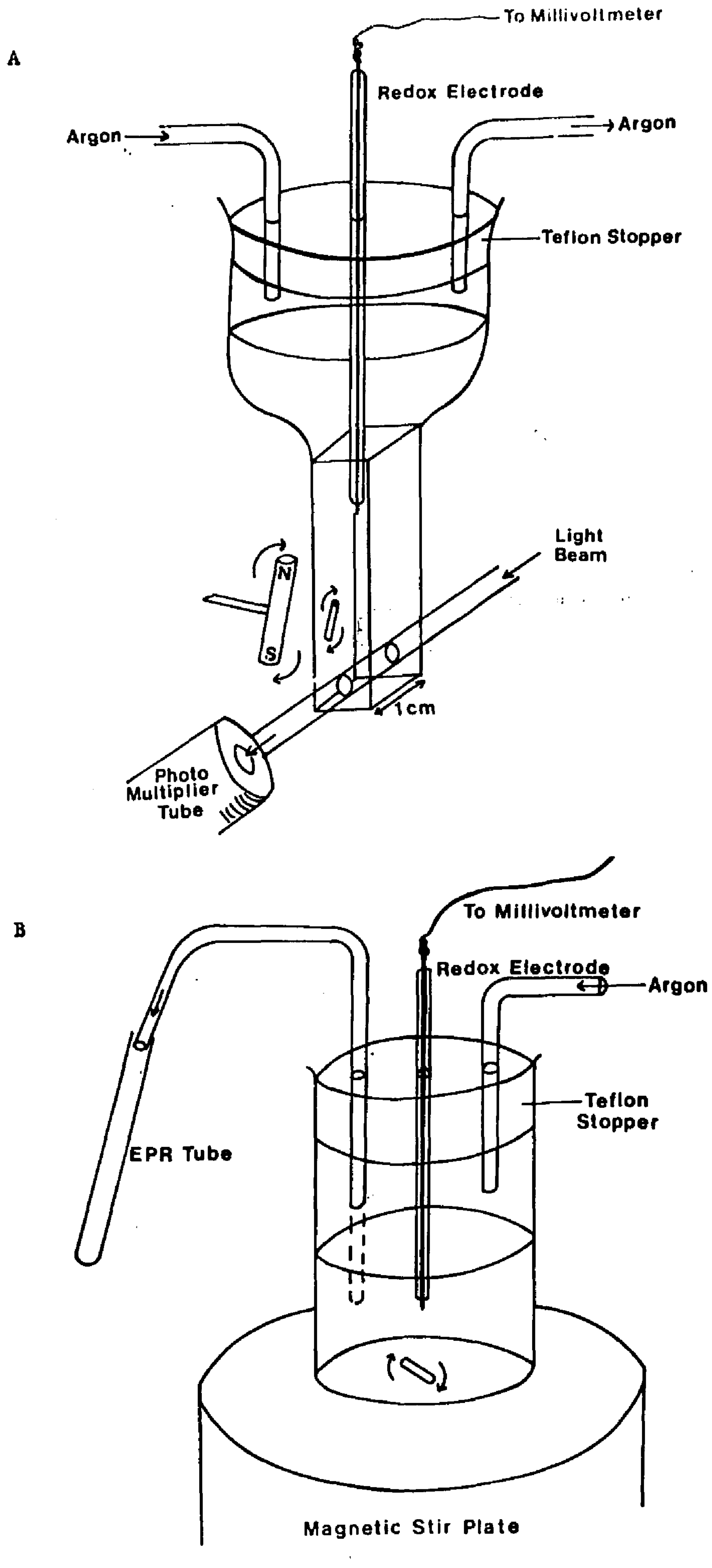
Complete anaerobiosis was established and maintained by passing a continuous stream of oxygen free argon (1 ppm O_2) over the sample. Continuous mixing was achieved with a teflon coated magnetic stirring bar suspended on the side of the sample cuvette and rotated by a magnetic stirrer. Figure 4a illustrates the anaerobic cuvette used for the simultaneous measurement of the redox potential and the state of reduction of cytochrome by optical spectroscopy. EPR was also employed to measure the state of reduction of respiratory chain components. Figure 4b shows the titration vessel used. When the sample was at the desired potential, the tube was lowered into the suspension. Pressure built up forcing the suspension through the tube and into an EPR tube which was immediately frozen in liquid N_2-cooled ligroin. A Varian E 109 spectrometer with an Air Products flowing helium cryostat with temperature control was used.

FIGURE 4

Reaction chambers for oxidation-reduction titrations

(A) For optical redox titrations a standard quartz cuvette with an expanded chamber was fitted with a teflon stopper. Mixing was provided by a teflon-coated magnetic flea held and rotated at one side by an external rotating bar magnet. A combination redox electrode was inserted through the stopper and potentials were measured with a millivoltmeter. Two additional tubes permitted passage of oxygen-free argon into the chamber above the reaction mixture to maintain anaerobiasis. The entire mechanism was placed into a Perkin-Elmer 557 dual beam spectrophotometer and the extent of cytochrome reduction was measured by changes in absorbance.

(B) For EPR redox titrations, a redox electrode was inserted through a teflon stopper into a closed chamber. Two tubes permitted passage of oxygen-free argon to maintain anaerobiasis. Stirring was accomplished through a teflon-coated magnetic flea and an external stir plate. The argon exit tube was lowered at appropriate potentials into the reaction mixture to remove a sample for EPR analysis.


A
To Millivoltmeter
Redox Electrode
Argon
Argon
Teflon Stopper
Light Beam
N
S
1cm
Photo Multiplier Tube
B
To Millivoltmeter
Redox Electrode
Argon
Teflon Stopper
EPR Tube
Magnetic Stir Plate

Preparation of Sintered Glass Oxygen Electrodes

Electrodes were built essentially as described by Davies and Grenell (1962). Very finely ground soft glass was made by crushing flint glass tubing, mixed with enough water to make a suspension of approximately 1 g glass per ml of water, and ground in a porcelain ball mill for 72 hours. Further grinding was achieved with an homogenizer fitted with a soft glass rod used as a pestle on the glass suspension in a flint glass test tube. The resulting glass was fined by centrifugation saving the supernatant from a 4 min. 4000 x g spin followed by a 4 min. 15,000 x g spin. The pelleted glass was resuspended in a minimal volume of distilled water using a Branson Sonifier (Model W185D) with a microtip. A drawn out Pasteur pipet was used to layer this glass suspension onto the tip of a platinum wire (28 or 30 gauge) inserted into a 3 mm diameter flint glass tube and made flush with one end. After the addition of each glass suspension microdrop, the electrode was baked in a furnace for 15 min. at 700°C. The procedure resulted in a number of electrodes with 90% response times of approximately 25 msec with movement artifacts of less than 15%. These specifications were determined with a special device designed by Davies (1962) which allowed the electrode tip to be exposed to different O_2 tensions very rapidly (estimated 4 msec). Electrode characteristics were measured with an oscilloscope.

Measurement of H^+/O Stoichiometries During Respiration

The methods used for the measurements of H^+/O stoichiometries during respiration are essentially those introduced by Mitchell and Moyle (1967) and further developed by Lehninger (personal communication; also

Reynefarje et al., 1982). Washed cells, resuspended in 120 mM KCl, 1.5 mM Tris-Cl at either pH 7.0 or pH 9.0, were added to an air saturated medium contained within a closed 2.0 ml thermostatically regulated plexiglass chamber using a Hamilton syringe inserted through a teflon port (see Fig. 5). After the cells had consumed all the oxygen in the reaction chamber, 1.0 μg of valinomycin was added as a concentrated ethanol solution. Rapid mixing was achieved with a teflon-coated magnetic flea driven at 2700 rpm by a magnetic stir plate located beneath the reaction chamber. Total mixing times of less than 200 msec was possible with this apparatus. Reactions were initiated by addition of small calibrated pulses of oxygen introduced by the rapid addition of air saturated medium of the proper temperature with a Hamilton microsyringe. Resulting acidification of the medium was detected with a Beckman combination pH electrode (Model No. 39505) connected to a Beckman Expandomatic pH meter (SelectIon 2000). The oxygen tension was simultaneously monitored with a fast responding oxygen electrode. These two electrodes shared the common reference electrode contained in the combination pH electrode. The small interference in the response of the pH electrode due to the current generated at the platinum surface of the oxygen probe was compensated for with an external bucking circuit. The currents generated by the oxygen electrode were in the nAmpere range and required construction of a highly sensitive low noise amplifier which also converted the signals to changes in potential. Great care had to be exercised to avoid ground loops and strict shielding precautions had to be observed. (See Appendix I for a schematic of amplifier circuit.) The amplified oxygen signal and that from the pH meter were fed into either a Soltec 330 stripchart recorder or into a Nicolet Digital Oscilloscope with pen output (Explorer III). Following each oxygen pulse, the extent of proton ejection was calibrated by adding a precisely known amount of HCl.

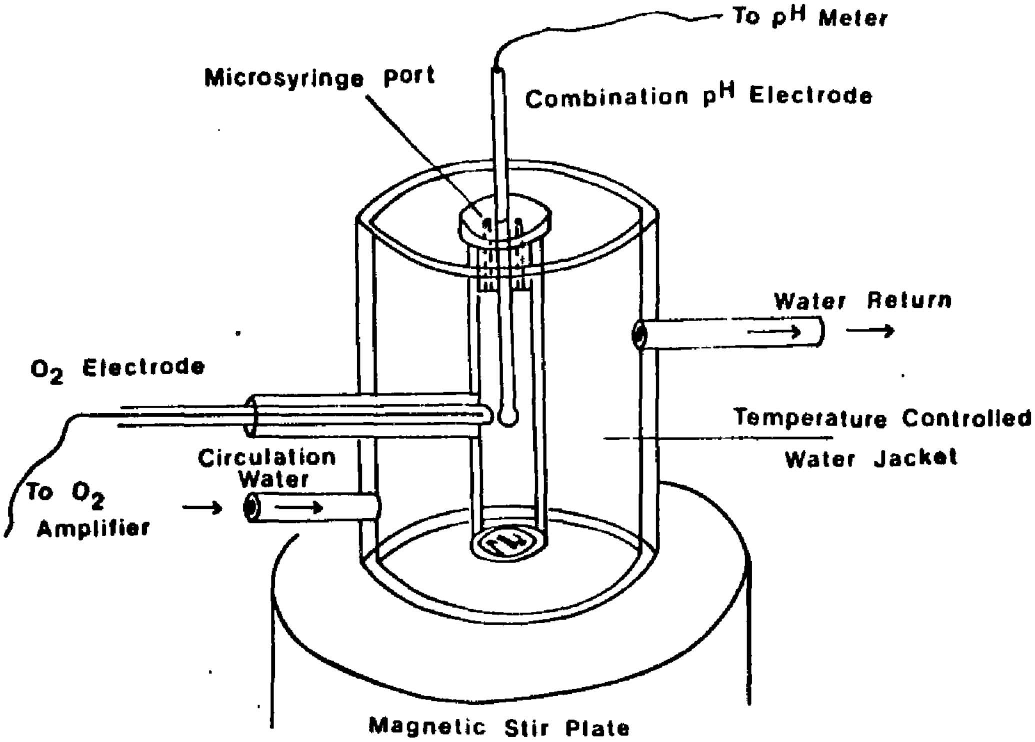
Reagents

Antimycin A, ascorbic acid, CCCP, cytochrome c (horse heart, type VI), DNase 1, EMS, gramicidin, HOQNO, lysozyme (egg white, EC 3.2.1.17), NADH (disodium salt), PMS, quinhydrone, TMPD, rotenone and valinomycin were all purchased from Sigma Chemical Co. The redox mediators: 2,5$(OH)_2$-1,4BQ, 1,4BQ, DAD, 1,2NQ-4S, 1,2NQ, 1,4NQ, 2(OH)-1,4NQ, AQ-1,5 disulfonic acid, AQ-2-sulfonic acid and MV were obtained from Aldrich Chemical Co. PES was purchased from ICN Pharmaceuticals, Inc. BV was purchased from BDH Chemicals, Ltd. [^{14}C-]methylamine hydrochloride(52.2mCi/mmol) and [1-^{14}C-]AIB(53mCi/mmol) were purchased from New England Nuclear Corp. Purified agar and yeast extract were purchased from Difco. All other reagents were reagent grade of the highest purity available and were routinely purchased from either Fisher Scientific Co. or Sigma Chemical Co.

FIGURE 5

Reaction vessel for measurement of H^+/O

A sealed plexiglass reaction chamber surrounded by a temperature-controlled water jacket had a membraneless oxygen electrode and a combination glass pH electrode (Beckman 39505) inserted through teflon ports. A teflon-coated magnetic flea achieved rapid stirring by means of an external stir plate (∿2700 rpm). Oxygen pulses and reagents were injected into the reaction chamber through use of Hamilton microsyringes. The oxygen electrode was connected to the input of a high gain, low noise amplifier.


To pH Meter
Microsyringe port
Combination pH Electrode
Water Return
O2 Electrode
Temperature Controlled
Water Jacket
Circulation Water
To O2 Amplifier
Magnetic Stir Plate

RESULTS

A. Characterization of the Respiratory Chain

1. Difference Spectroscopy

Difference spectra (dithionite-reduced minus air-oxidized) of membrane vesicles prepared from wild type B. alcalophilus indicated the presence of several cytochrome species. At toom temperature, α band absorption peaks were found at 600 and 550 nm (see Fig. 6A). These have beem attributed to a- and c- type cytochromes. A b- type cytochrome was indicated by a shoulder at approximately 560 nm. Additional absorption bands were found at 520 and 426 nm. When spectra were determined at 77° K, the sharpened individual absorption bands resolved additional peaks with a-, b- and c- type cytochromes clearly indicated by α bands at 602, 557 and 549 nm and Soret bands at 442, 426 and 418 nm (see Fig. 6B). Addition of a few grains of potassium ferricyanide to the reference cuvette generally did not increase the size of absorbance peaks but, nevertheless, was routinely performed to insure that the cytochromes in the reference were indeed in a fully oxidized state. The non-alkalophilic mutant strain, KM23, also contained a-, b- and c- type cytochromes as shown by its reduced minus oxidized difference spectrum (see Fig. 6C), although the spectra obtained were not as clear because of the greatly reduced concentrations of these cytochromes. Totally analogous spectra were obtained for the alkalophile, B. firmus RAB and its non-alkalophilic mutant derivative B. firmus RABN. Room temperature spectra of B. firmus RAB had α band peaks at 600 and 551 nm with a shoulder present at approximately 560 nm (Fig. 8d). Also present was a β band peak at 521 nm and a Soret

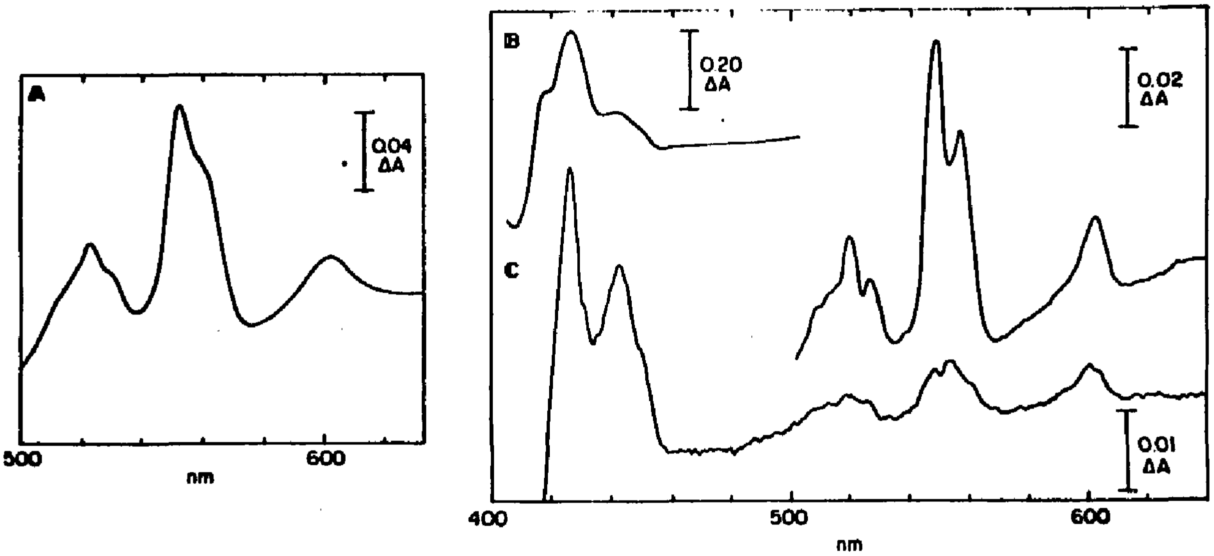
FIGURE 6

Difference spectra of B. alcalophilus and KM23

Dithionite-reduced minus air-oxidized samples were recorded as described under "Materials and Methods". (A) *B. alcalophilus* membrane vesicles (1.7 mg of protein/ml) recorded at room temperature; (B) *B. alcalophilus* vesicles (5.0 mg of protein/ml, 66% glycerol) recorded at 77°K; (C) KM23 vesicles (4.2 mg of protein/ml, 66% glycerol) recorded at 77°K.

B. alcalophilus

Dithionite minus air oxidized spectra



absorbance band at 434 nm (not shown). Dual beam spectroscopy performed at liquid N_2 temperatures resolved additional peaks with a-, b- and c-type cytochromes now clearly indicated by α bands at 602, 557 and 550 nm (see Fig. 7). Membrane bound cytochromes of B. alcalophilus were easily reduced by addition of NADH to everted vesicles. Indeed, by repeated rapid scanning (1200 nm/min) of vesicles after the addition of approximately 5 mM NADH, one could successively see cytochrome b reduction followed by reduction of the c and a cytochromes. Interestingly, addition of either malate or succinate produced minimal cytochrome reduction in these preparations and ascorbate/PMS or ascorbate/TMPD difference spectra were not distinguishable from those of NADH. On the other hand, addition of these same reductants to everted membrane vesicles prepared from B. firmus RAB, produced very different cytochrome spectra; several of these reductants displayed a pH dependence in their ability to reduce cytochromes. At pH 9.0, malate only reduced a small amount of cytochrome b (Fig. 8). At this pH cytochromes a, b and c were all reduced by NADH (Fig. 8). At pH 7.0, malate was now found to reduce most of the a- and c- type cytochromes as well as a little cytochrome b (Fig. 9). NADH, on the other hand, reduced only cytochromes a and c at pH 7.0 (Fig. 9). All difference spectra obtained from B. firmus RAB were made at pH 7.0. Malate failed to reduce appreciable amounts of any of the cytochromes of this strain but both ascorbate/PMS and NADH reduced most of the cytochrome a and c present as well as a little cytochrome b (Fig. 10).

Identification of CO-Binding Cytochromes

Carbon monoxide-difference spectra (dithionite plus CO, minus dithionite) of wild type B. alcalophilus membranes were conducted in order to

FIGURE 7

Difference spectrum of B. firmus RAB

Everted membrane vesicles of *B. firmus* RAB were prepared in 100 mM potassium carbonate buffer containing 10 mM $MgSO_4$, pH 9.0 to which was added glycerol (to 66%) for a final protein concentration of 6.43 mg/ml. Dithionite-reduced minus air-oxidized difference spectra at 77°K were obtained using the Perkin-Elmer liquid N_2 cryogenic accessory.

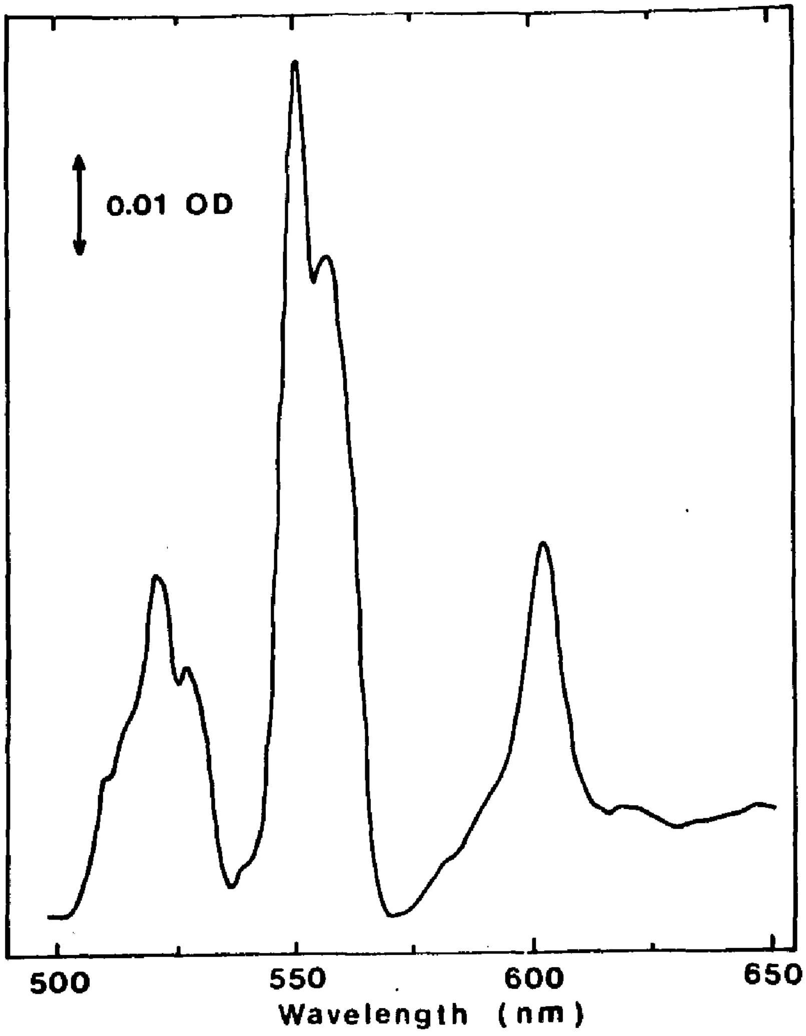

0.01 OD
500
550
600
650
Wavelength (nm)

FIGURE 8

Difference spectra of B. firmus RAB vesicles at pH 9.0

Everted vesicles freshly prepared from *B. firmus* RAB were suspended to 4.1 mg protein/ml in 100 mM potassium carbonate buffer containing 10 mM $MgSO_4$, pH 9.0. Difference spectra were obtained by first fully oxidizing the membrane cytochromes with a few grains of potassium ferricyanide, storing this spectrum in the dual beam spectrophotometer microprocessor and then reducing the membranes in the sample cuvette with the specified reagents. Trace (a) resulted from reduction of cytochromes by 80 mM sodium malate; trace (b) by 40 mM potassium ascorbate plus 0.1 mM PMS; trace (c) by 80 mM NADH; trace (d) by a few grains of sodium dithionite.

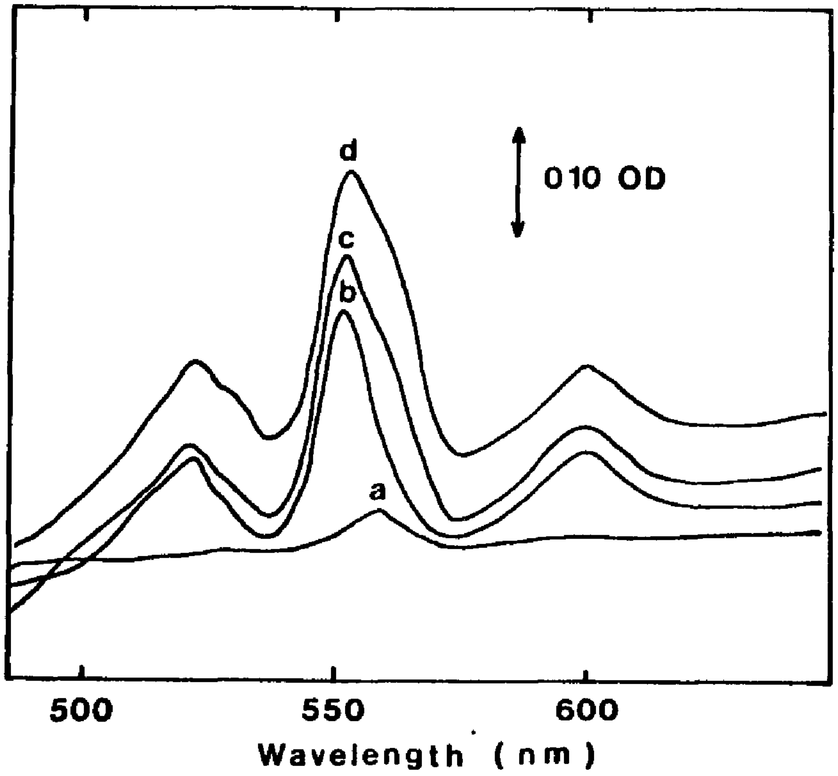

d
c
b
a
0.10 OD
500
550
600
Wavelength (nm)

FIGURE 9

Difference spectra of B. firmus RAB vesicles at pH 7.0

Freshly prepared everted membrane vesicles of B. firmus RAB were washed and suspended to 11 mg protein/ml in 100 mM potassium phosphate buffer containing 10 mM $MgSO_4$, pH 7.0. Difference spectra were obtained as described in the legend to Fig. 8.

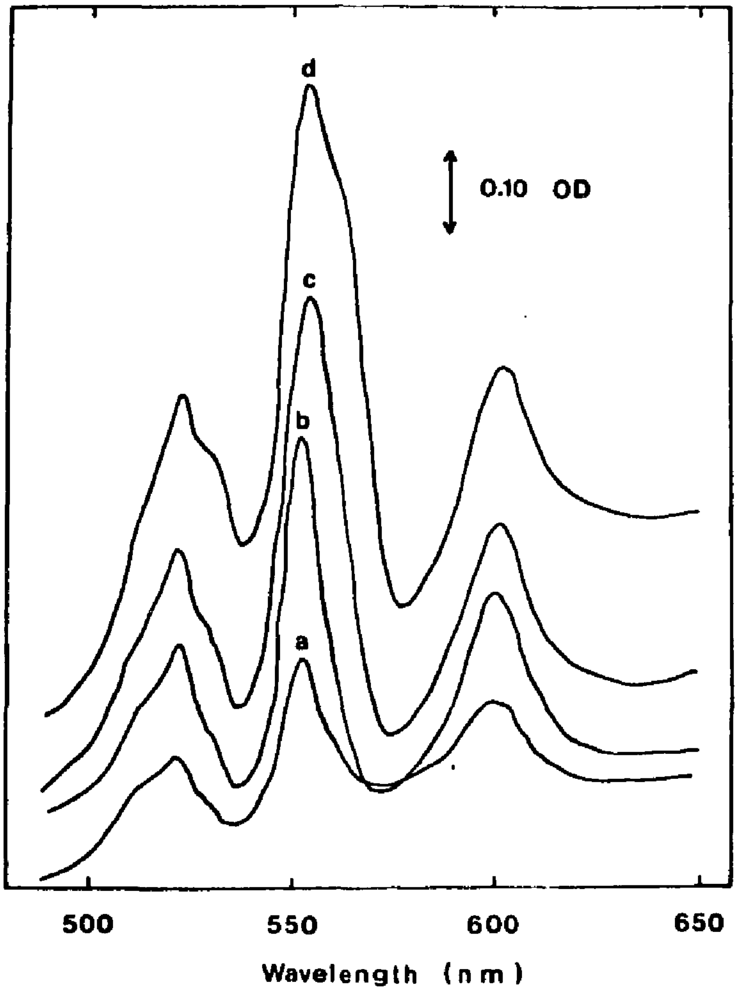
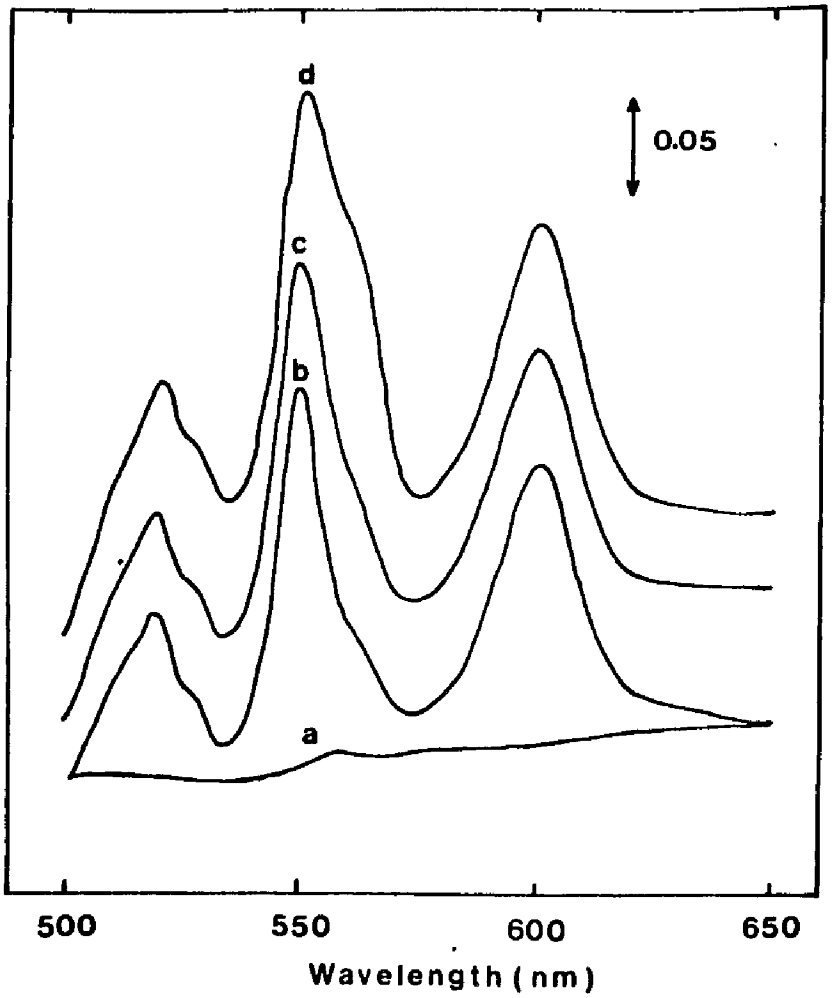

d
c
b
a
0.10 OD
500
550
600
650
Wavelength (nm)

FIGURE 10

Difference spectra of *B. firmus* RABN at pH 7.0

Freshly prepared everted membrane vesicles of *B. firmus* RABN were washed and suspended to 11 mg protein/ml in 100 mM potassium phosphate buffer containing 10 mM $MgSO_4$, pH 7.0. Difference spectra were obtained as described in the legend to Fig. 8.


d
0.05
c
b
a
500
550
600
650
Wavelength (nm)

identify terminal oxidases by their CO-binding properties. A CO-cytochrome complex with absorbance maxima at 588 and 540 nm, with a shoulder at 428 nm and an absorbance minima at 445 nm was observed at room temperature (see Fig. 11A, B). This spectrum was attributed to a cytochrome a_3 consistent with the original description of this species in mitochondria by Keilin (1939). The peaks at 570 and 413 nm and the absorbance minimum at 550 nm, also evident in Figure 11, required about 30 min. to develop fully after bubbling with CO. These properties indicated the presence of a cytochrome o species (Chance, 1953). Taking a CO-difference spectrum at 77° K sharpened the Soret bands and resolved a small additional peak at 426 nm, presumably due to the cytochrome a_3 (Fig. 11C). Attempts to identify CO-binding cytochromes in KM23 and in B. firmus RAB resulted only in broad absorbance troughs at approximately 445 nm. These troughs probably result from an a_3-type of cytochrome oxidase; however, without additional verification such an assignment must remain tentative (Castor and Chance, 1955, 1959).

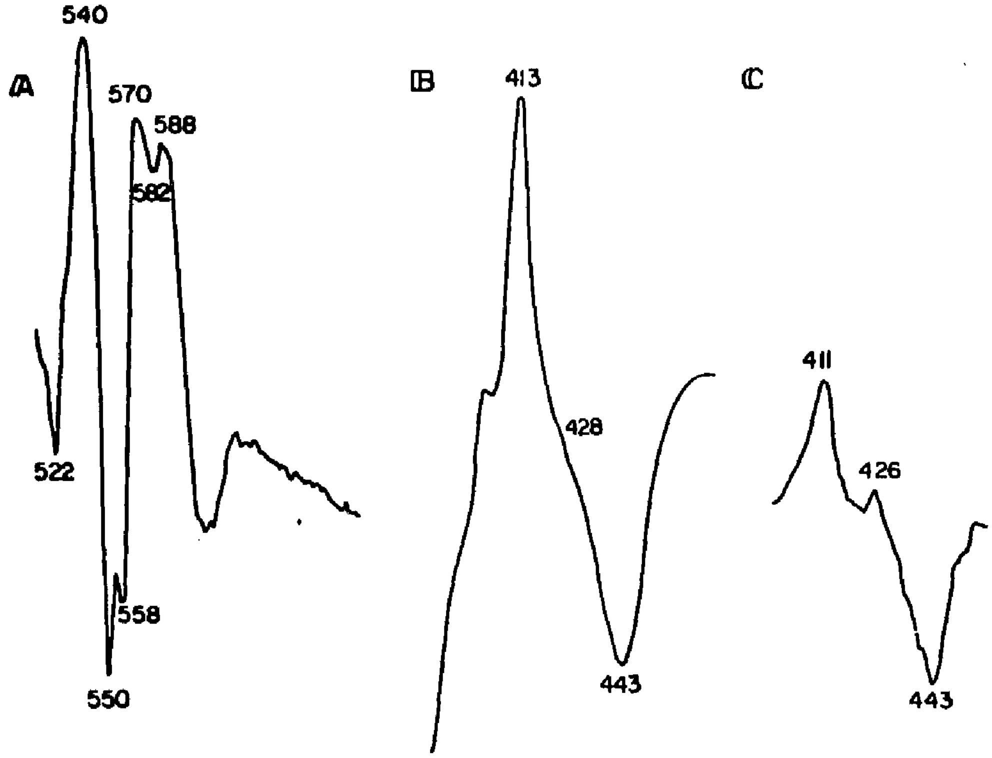
2. Quantification of Cytochromes

The concentrations of membrane cytochromes from both B. alcalophilus and B. firmus RAB, as well as from their non-alkalophilic mutant derivatives, were determined from the room temperature difference spectra using the extinction coefficients detailed in "Materials and Methods". As shown in Table I, membranes from both alkalophiles contain at least 5.5 nmoles of cytochrome heme/mg protein. As a check on these determinations, pyridine hemochromagens were prepared and quantitated. The advantage of measuring cytochrome and heme concentration by the method of heme extraction and pyridine derivatization is twofold. First, the

FIGURE 11

CO difference spectra of *B. alcalophilus* vesicles

CO-reduced, plus dithionite minus dothionite-reduced samples were recorded as described under "Materials and Methods". (A) α and β region at room temperature; (B) Soret bands at room temperature; (C) Soret bands at 77°K.



B. alcalophilus
CO Difference Spectra

TABLE I

CYTOCHROME CONTENTS OF MEMBRANES FROM ALKALOPHILIC BACTERIA AND THEIR NON-ALKALOPHILIC MUTANT DERIVATIVES

Cytochrome contents were assayed in wild-type strains by analysis of difference spectra and by extraction of hemes followed by pyridine hemochromogen preparation, as described in Materials and Methods. For the non-alkalophilic strains only the former method was employed, because acidified ethyl methyl ketone failed to give quantitative extraction from their membranes.

	Concentration of cytochrome (nmoles/mg membrane protein)					
	a-type		*b*-type		*c*-type	
Bacterial Strain	Difference Spectra	Heme Extraction	Difference Spectra	Heme Extraction	Difference Spectra	Heme Extraction
Bacillus alcalophilus	0.51	1.3	2.2	1.7	2.6	2.5
Bacillus alcalophilus KM23	0.23	-	0.54	-	0.50	-
Bacillus firmus RAB	0.57	1.7	2.7	1.1	3.8	2.7
Bacillus firmus RABN	0.43	-	0.50	-	0.53	-

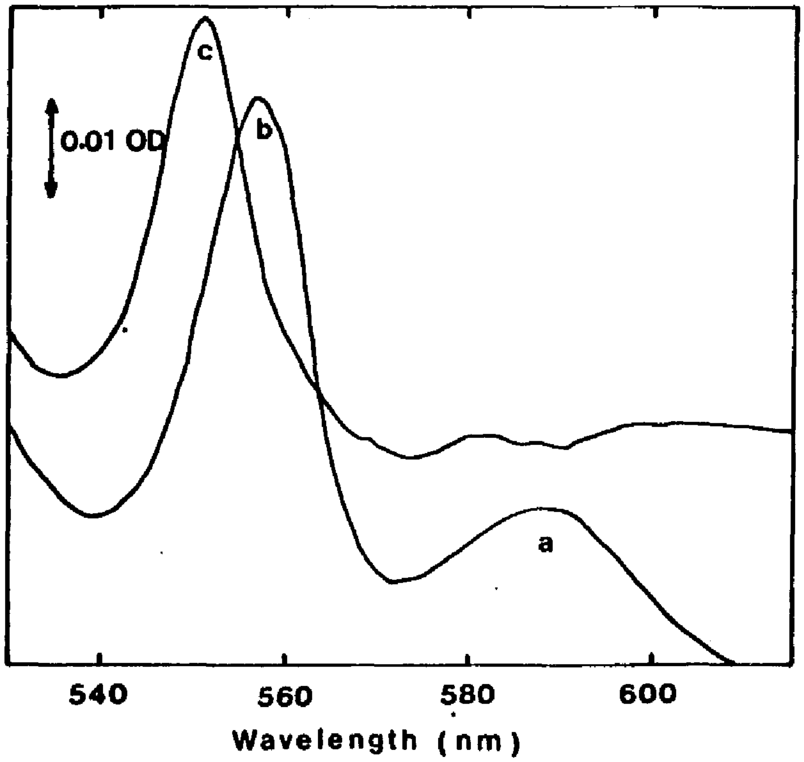
extinction coefficients are more precisely known for each heme derivative, whereas when the heme is attached to the cytochrome protein, small changes in absorbance character are evident from species to species. Second, since the cytochrome c heme is covalently bound to the protein, no heme c extraction takes place; nevertheless, the pyridine hemochromagen derivative can be formed. Thus, a physical separation of the c cytochrome heme from the a and b cytochrome heme is achieved. This allows measurement of absorbance without the complication of overlapping or interfering absorbance bands (see Fig. 12). The hemes were readily extracted from both alkalophiles; however, the hemes from the non-alkalophiles could not be easily extracted and therefore pyridine derivatization failed to provide quantitative information on cytochrome levels in these mutants. A discrepancy between the two methods employed to determine cytochrome concentration was apparent in the cytochrome a measurements. This could most easily be explained as an underestimation of the measurements made by simple difference spectroscopy which are clearly affected by an assumption of extinction coefficient. An analysis of tightly bound copper provided both additional evidence for a "classical" aa_3 type of cytochrome oxidase in B. alcalophilus and supported the larger estimate of a- type cytochrome concentration. In B. alcalophilus membranes 1.16 nmol of Cu/mg of protein was measured. This preparation contained 1.3 nmol of heme a/mg of protein (as determined by heme extraction and derivatization), indicating a Cu:heme a = 0.89 consistent with ratios found in other cytochrome aa_3 systems (Lemberg, 1969).

Even if each of the values represents only a reasonable estimate, the cytochrome contents of the alkalophiles are extraordinarily high. Values reported by other investigators for conventional non-alkalophilic

FIGURE 12

Difference spectra of the pyridine hemochromagen derivatives of B. alcalophilus membrane cytochromes

The hemes from *B. alcalophilus* membrane cytochromes were extracted and the pyridine hemochromagens prepared as described under "Materials and Methods". Dithionite-reduced minus ferricyanide-oxidized difference spectra were immediately recorded for these compounds at room temperature.


c
b
a
0.01 OD
540
560
580
600
Wavelength (nm)

bacteria include 0.72 and 0.76 nmoles heme/mg membrane protein for *Escherichia coli* (Itagaki, 1964) and *Bacillus cereus* (Doi and Halvorson, 1961), respectively. Even unusually high levels found previously, e.g., 4.66 nmoles heme/mg membrane protein in *Nitrosomonas* (Falcone *et al.*, 1962) and 3.88 nmoles heme/mg membrane protein in particles from *Azobacter vinelandii* (Jones and Redfearn, 1966), are exceeded in the alkalophiles. As an additional frame of reference, typical heme contents of whole beef heart mitochondria and rat liver mitochondria are 2.6 and 0.94 nmoles/mg protein (i.e., Green and Wharton, 1963; Chance and Hess, 1962).

Estimations of the cytochrome contents from difference spectra indicated a markedly lower level in the two non-alkalophilic strains than in the parent strains (Table I); the b- and c- type cytochromes were decreased relatively more than a- type cytochromes. No values of cytochrome levels as determined by pyridine derivatization are presented because of the incomplete extraction of hemes from the non-alkalophiles. The difference between the membranes from the alkalophilic and non-alkalophilic strains with respect to the extractability of hemes was interesting, and could relate to changes in various membrane properties.

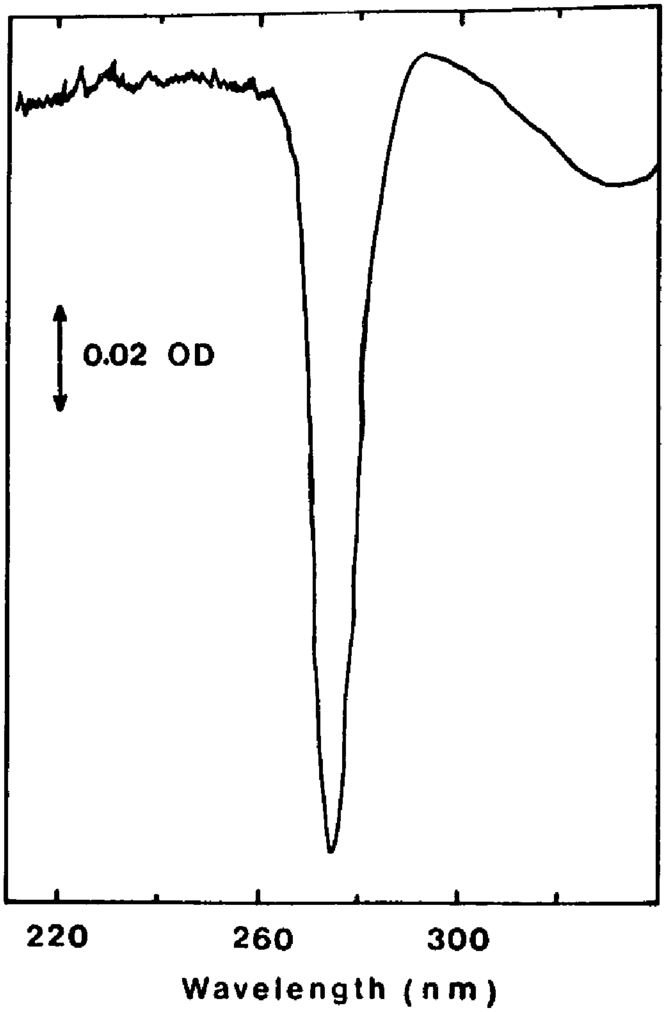
3. Quinone Contents of Membrane Vesicles

Quinones were extracted from membrane vesicles prepared from *B. alcalophilus*, *B. firmus* RAB and their non-alkalophilic derivative strains KM23 and RABN as described in the "Materials and Methods" section. The quinones from *B. alcalophilus* and KM23 were identified as ubiquinone species from their ultraviolet difference spectra (Fig. 13), and were present at concentrations of 6.75 and 6.90 nmole/mg of membrane protein

FIGURE 13

Spectrum of quinones extracted from *B. alcalophilus*

The quinones were extracted from membrane vesicles of *B. alcalophilus* as described under "Materials and Methods". The quinones were resuspended as an ethanolic solution and a ubiquinone $NaBH_4$-reduced minus air-oxidized difference spectrum was recorded at room temperature.


0.02 OD
220
260
300
Wavelength (nm)

in these two strains. Hess *et al*. (1979) have reported the presence of menaquinone in *B*. *alcalophilus*. In their study, Tryptic Soy Broth (Difco) at an unspecified pH was employed as the growth medium. In this laboratory, *B*. *alcalophilus* did not grow in this standard medium until it was modified by increasing the pH above 9.0 with addition of NaOH. Quinones extracted from vesicles prepared from these cultures were also identified as ubiquinone. It is particularly unusual to find ubiquinone in a *Bacillus* species since all *Bacilli* previously examined appear to contain menaquinone only (Watanuki and Aida, 1972; Hess *et al*., 1979). Further characterization of the extracted quinones from *B*. *alcalophilus* was obtained by using reversed-phase chromatography to determine the length of the isoprenoid side chains. Shorter side chains result in greater polarity and therefore increased mobility due to the greater solubility in the polar mobile phase. A *B*. *alcalophilus* extract was resolved into two quinone species with RF values of 0.4 and 0.62. Standard ubiquinone-10, run as a control, had an RF value of 0.25. Lester and Ramasarma (1959) found for ubiquinones -10, -9 and -7, RF values of 0.26, 0.41 and 0.60 respectively. Thus *B*. *alcalophilus* appears to have the quinones, ubiquinone -9 and ubiquinone -7. In contrast, extraction of quinones from membrane vesicles of *B*. *firmus* RAB and *B*. *firmus* RABN resulted in clear spectral identification of menaquinone species only (Fig. 14).

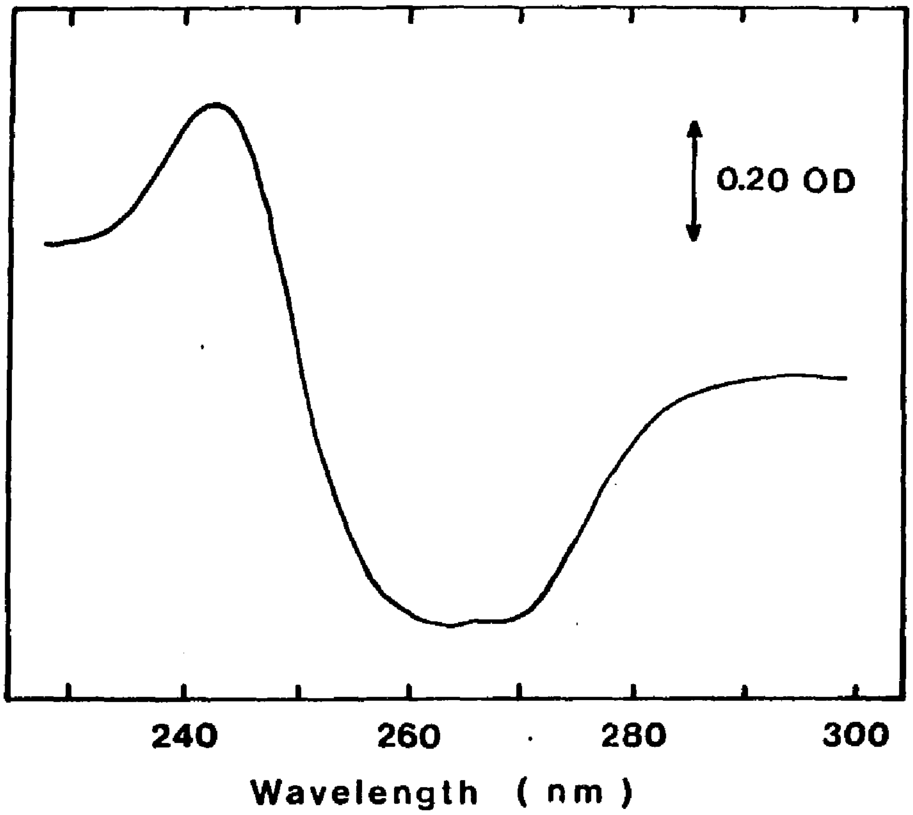
4. Potentiometric Titrations

The cytochromes were further characterized by determinations of their midpoint potentials. The possible dependence of midpoint potentials on pH was of particular interest since such a dependence could be consis-

FIGURE 14

Spectrum of quinones extracted from B. firmus RAB

The quinones of B. firmus RAB were extracted from membranes using the same protocol described for B. alcalophilus under "Materials and Methods". To the 1 ml ethanol solution in the sample cuvette 5 µl $NaBH_4$ (5 mg/ml) was added and a menaquinone-reduced minus oxidized difference spectrum was recorded. Addition of 10 µl acetate buffer (0.5 M, pH 5.0) to the sample cuvette was made to prevent auto-oxidation and maximize absorbance changes.


0.20 OD
240
260
280
300
Wavelength (nm)

tent with a role for the cytochrome in proton pumping. Optical oxidation-reduction titrations were carried out with everted membrane vesicles from B. alcalophilus at room temperature at pH 9.0 and pH 7.0. The oxidation-reduction titrations of the a- type cytochrome were conducted at pH 9.0 using the peak at 600 nm minus a base-line drawn from 615 to 575 nm. This generated a sigmoidal curve which fit the Nernst equation with a midpoint potential at pH 9 of +240 mV with n = 1 (Fig. 15A). At pH 9.0, three b- type cytochromes were resolved using the wavelength pair 560 and 575 nm (Fig. 15B); Em_9 values of +20, -120, and -240 mV were calculated. A fourth very low potential b- type cytochrome was identified using the wavelength pair 564 and 575 nm. This cytochrome titrated with an approximate Em_9 of -320 mV; however, the n value was uncertain (Fig. 15B). The c- type cytochrome titrated with an Em_9 of +70 mV with n = 1 (Fig. 15C).

The oxidation-reduction titrations were repeated at pH 7.0 to see if any additional cytochrome species could be resolved in this manner and to see if any of the cytochromes had pH-dependent midpoint potentials. At pH 7.0, the c- type cytochrome displayed a midpoint potential at +70 mV which was the same as that obtained at pH 9.0 (Fig. 16C). By contrast, both the a- and b- type cytochromes displayed changes in their midpoint potentials at this lower pH. Both quantitative and qualitative analysis of the cytochrome a titrations at pH 7.0 indicated the presence of two oxidation-reduction components, each with n = 1 (Fig. 16A). Em_7 values of +230 and +390 mV were calculated for these species. The titrations of b cytochromes at pH 7.0 were complicated by the large spectral overlap of the cytochrome c. Nevertheless, it is clear that at least one of the b- type cytochromes had a higher midpoint

FIGURE 15

Oxidation-reduction potentiometric titrations of wild-type B. alcalophilus cytochromes at pH 9.0

Optical redox titrations were performed on everted vesicles suspended in 100 mM potassium carbonate, 10 mM $MgSO_4$, pH 9.0 as described in "Materials and Methods". The lines through the data are theoretical n = 1 Nernst curves. (A) titration of a- type cytochromes in everted vesicles (∿5.0 mg of protein/ml). The redox mediators used were 50 μM concentrations of TMPD, DAD, PMS, PES and 2(OH)-1,4 NQ. The absorbance was measured as the difference between 600 nm and a base-line drawn from 615 to 575 nm. (B) titration of b- type cytochromes in everted vesicles (∿6.0 mg of protein/ ml). The redox mediators used were 50 μM concentrations of TMPD, DAD, PMS, PES, 2(OH)-1,4 NQ, phenazine, 2(OH)1,4-AQ and 1 μM concentrations of BV and MV. (C) titrations of cytochrome c in everted vesicles (∿5.0 mg of protein/ml). The redox mediators used were the same as in (A).

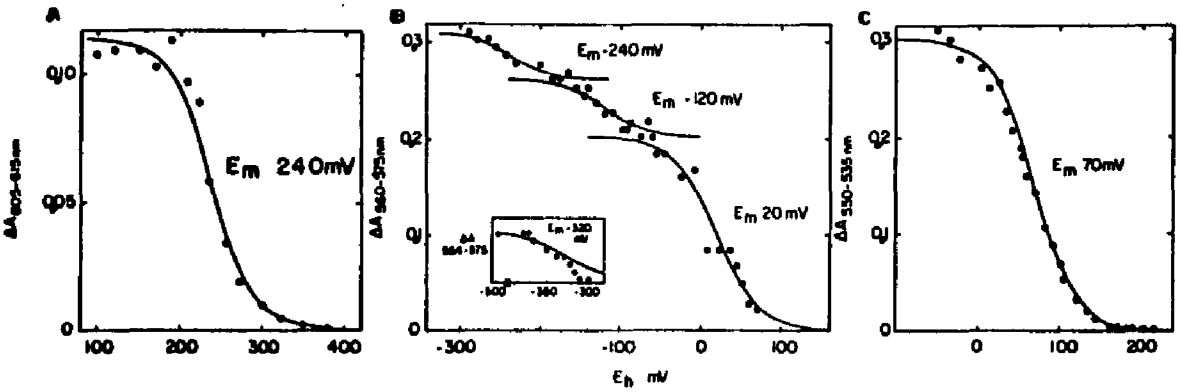
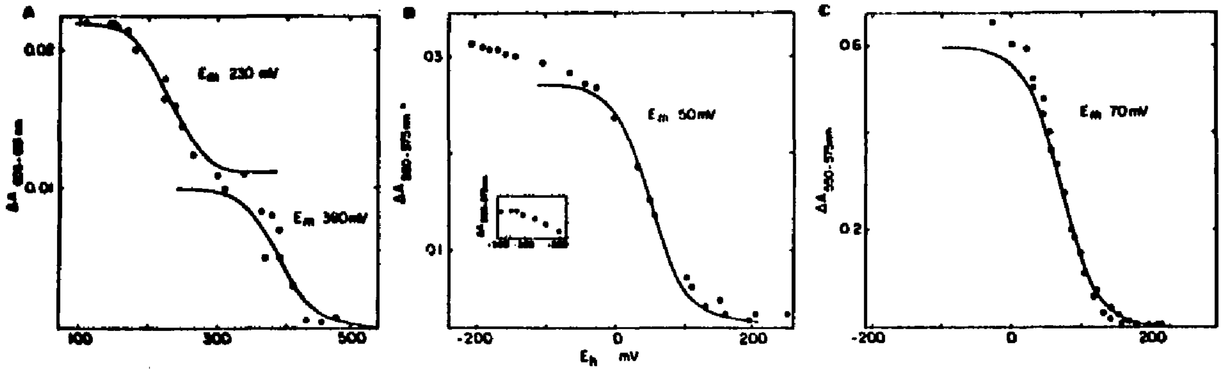

A
0,10
0,05
0
100
200
300
400
E m 240mV
B
0,3
0,2
0,1
0
-300
-100
0
100
E m -240 mV
E m -120 mV
E m 20 mV
E m -320 mV
-300
E h mV
C
0,3
0,2
0,1
0
0
100
200
E m 70mV
ΔA 550-535 nm
ΔA 560-575nm

FIGURE 16

Oxidation-reduction titrations of wild-type B. alcalophilus cytochromes at pH 7.0

Optical redox titrations were performed on everted vesicles suspended in 100 mM KCl, 20 mM MOPS, 5 mM $MgSO_4$, pH 7.0 as described in "Materials and Methods". (A) titration of a- type cytochromes (∿1.0 mg of protein/ml). The redox mediators used were 50 μM concentrations of DAD, PMS, PES, 2(OH)-1,4-AQ. (B) titration of b- type cytochromes (∿5.0 mg of protein/ml). The redox mediators used were 50 μM concentrations of DAD, PMS, PES, 2(OH)-1,4-NQ, phenazine, 2(OH)-1,4-AQ, and 1 μM concentrations of BV and MV. (C) titration of cytochrome c in everted vesicles (∿10 mg of protein/ml). The redox mediators used were the same as in (A).


A
0.02
0.01
E_m 230 mV
E_m 390mV
100
300
500
B
0.3
0.1
E_m 50mV
-200
0
200
E_h mV
C
0.6
0.2
$\Delta A_{550-575nm}$
E_m 70mV
-200
0
200

potential at this pH (Fig. 16B). A midpoint potential value of +50 mV with n = 1 was calculated for this oxidation-reduction species. The levels of the lower potential cytochrome b- species were too small and their potentials were too close together to allow reliable calculation of midpoint potentials at pH 7.0 and have not been pursued. It is important to note, however, that the lower potential b- cytochromes were clearly observed at pH 7.0 in wild type vesicles.

Optical measurements of oxidation-reduction titrations on everted membrane vesicles from the non-alkalophilic strain KM23 were also conducted at pH 7.0, which is near the normal cytoplasmic pH of these cells. Using the wavelength pair 600 and 630 nm, the a- type cytochrome appeared as a single species with an Em_7 value of +100 mV with n = 1 (Fig. 17A). Similarly, only a single b- type cytochrome with an Em_7 value of -120 mV (n = 1) resulted from a cytochrome b titration employing the wavelength pair 557 and 575 nm (Fig. 17B). A c- type cytochrome with Em_7 = +140 mV, n = 1, was titrated using the wavelength pair 550 and 535 nm (Fig. 17C).

EPR spectra were conducted on _B_. _alcalophilus_ membranes that had been poised at selected oxidation-reduction potentials before freezing to 8° K. These spectra provided evidence of ferricytochrome signals as well as numerous signals from FeS clusters. Aasa _et_ _al_. (1976) have shown that the cytochrome a^{+3} in mammalian cytochrome oxidase shows a low spin Fe^{+3} signal with g = 3.03, and cytochrome b^{+3} exhibits EPR signals at g = 3.60 and 3.76 (Siedow _et_ _al_., 1978). Signals typical of low spin hemes were observed at g = 3.1 and 3.4 in wild type _B_. _alcalophilus_ vesicles. At pH 9.0, midpoint potentials of +220 and +20 mV, respectively, were found for these oxidation-reduction species (Fig. 18). These values are in excellent agreement with the potentials found opti-

FIGURE 17

Oxidation-reduction titrations of KM23 cytochromes

Optical oxidation-reduction titrations were performed on everted vesicles (∿5.0 mg protein/ml) suspended in 100 mM KCl, 20 mM MOPS, 5 mM $MgSO_4$, pH 7.0. The redox mediators used were 50 μM concentrations DAD, PMS, PES, and 2(OH)-1,4-NQ. (A) cytochrome a; (B) cytochrome b; (C) cytochrome c.

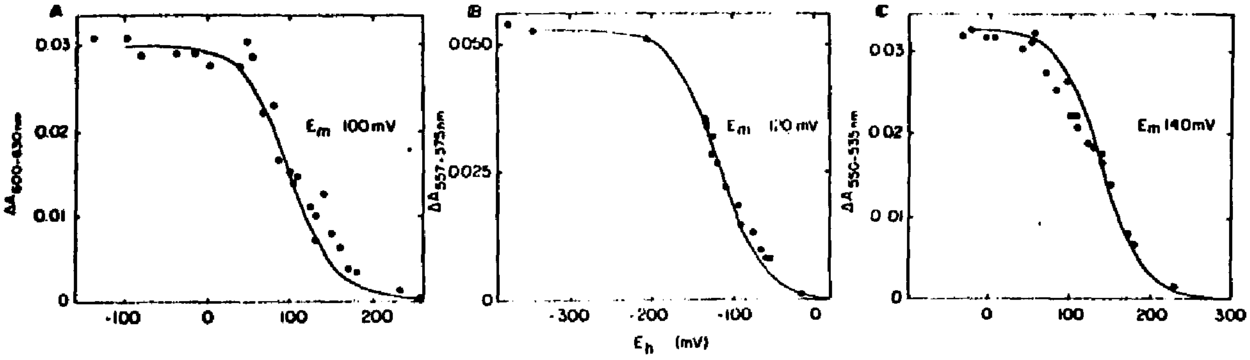
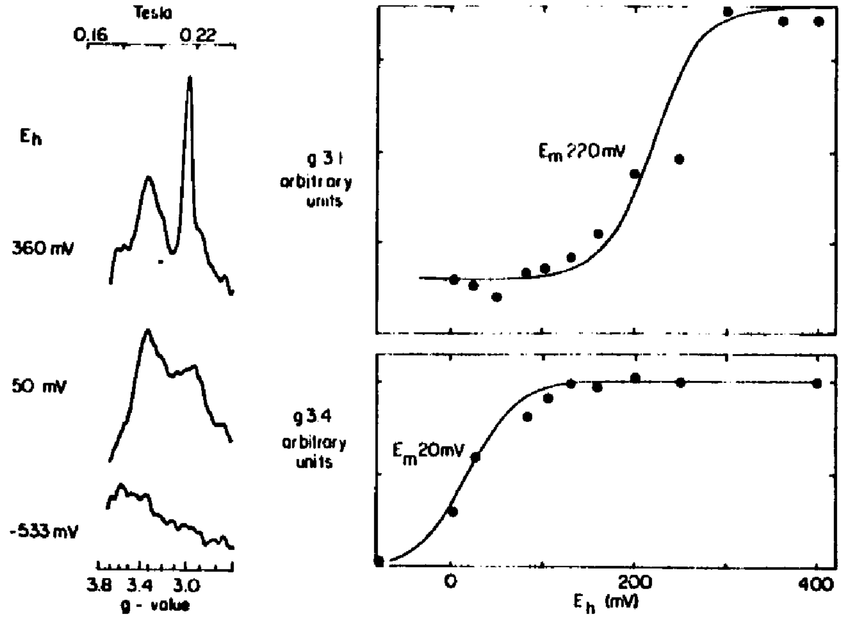
A
0.03
0.02
0.01
0
-100
0
100
200
$\Delta A_{600-630\,nm}$
E_m 100mV
B
0.050
0.0025
0
-300
-200
-100
0
$\Delta A_{557-575\,nm}$
E_m 110 mV
E_h (mV)
C
0.03
0.02
0.01
0
0
100
200
300
$\Delta A_{550-535\,nm}$
E_m 140mV

FIGURE 18

Oxidation-reduction titration of wild-type B. alcalophilus $g = 3.1$ and 3.4 EPR signals

Everted vesicles (∿20 mg of protein/ml) were suspended in 100 mM potassium carbonate, 10 mM $MgSO_4$, pH 9.0, with 50 μM concentrations of TMPD, DAD, PMS, PES, and 20 μM 2(OH)-1,4-AQ. Spectra were taken at 8°K, 2 millitesla, and 5 milliwatts.



Tesla
0.16
0.22
Eh
360 mV
50 mV
-533 mV
3.8
3.4
3.0
g - value
g 3.1
arbitrary
units
Em 220 mV
g 3.4
arbitrary
units
Em 20 mV
0
200
400
Eh (mV)

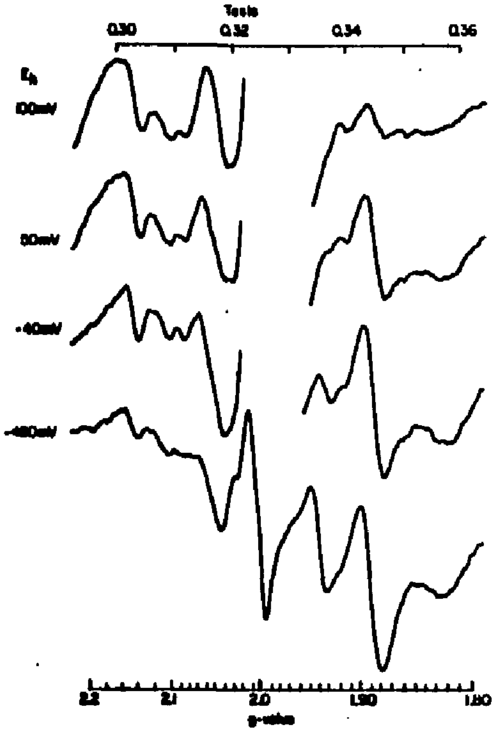
cally for a- and b- type cytochromes. The g = 1.9 signal (characteristic of the Rieske FeS protein) titrated with a midpoint potential of +75 mV at pH 9.0 (Fig. 19) and +150 mV at pH 7.0. While the Rieske protein was not clearly distinguishable in KM23, the FeS clusters, with EPR signal g = 1.94, appeared equally abundant in both strains.

Optical oxidation-reduction titrations were also conducted on everted membrane vesicles of _B. firmus_ RAB and _B. firmus_ RABN. The cytochrome midpoint potentials obtained from these studies revealed respiratory chain profiles for these strains that were similar to those found in _B. alcalophilus_ and KM23 with a few notable differences. Cytochrome a titrations of _B. firmus_ RAB at pH 9.0 indicated the presence of two cytochrome a species (Fig. 20). The major cytochrome a had a midpoint potential of +110 mV. A second a- type cytochrome titrated with a midpoint potential of +210 mV at this pH and contributed 25% of the total cytochrome a absorbance (Fig. 20A). At pH 9.0, four b- type cytochromes present in roughly equal amounts have been identified. Using the wavelength pair of 560 and 575 nm, two cytochromes with Em_9 values of +20 and -120 mV were resolved (Fig. 20B). Two lower potential cytochromes with Em_9 values of -280 and -400 mV were identified using the wavelength pair 563 and 575 nm (Fig. 20B). The c- type cytochrome titrated with an Em_9 of +60 mV (Fig. 20C). All the titrations of cytochromes in _B. firmus_ RAB resulted in data which could be fit to n = 1 Nernst curves. At pH 7.0 the a- type cytochromes titrated with Em_7 values of +130 and +320 mV with the lower potential cytochrome again making up approximately 25% of the total cytochrome a absorbance (Fig. 21A). Only three of the b- type cytochromes could be clearly demonstrated at pH 7.0 (Fig. 21B). They titrated with Em_7 values +20, -120 and -260 mV; pre-

FIGURE 19

Oxidation-reduction titration of Rieske protein in wild-type B. alcalophilus

Everted vesicles (∿10 mg of protein/ml) in 100 mM potassium carbonate, 10 mM $MgSO_4$, pH 9.0 with 50 μM concentrations of TMPD, DAD, PMS, PES and 20 μM 2(OH)-1,4-NQ. Spectra were taken at 8°K, 2 millitesla and 5 milliwatts.


Tesla
0.30
0.32
0.34
0.36
E_h
100mV
80mV
-40mV
-480mV
2.2
2.1
2.0
1.90
1.80
g-value

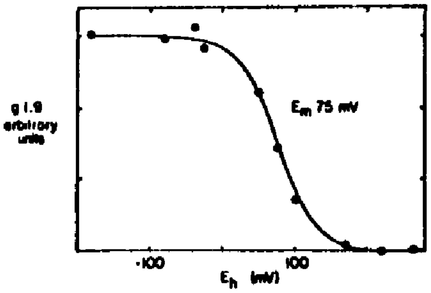
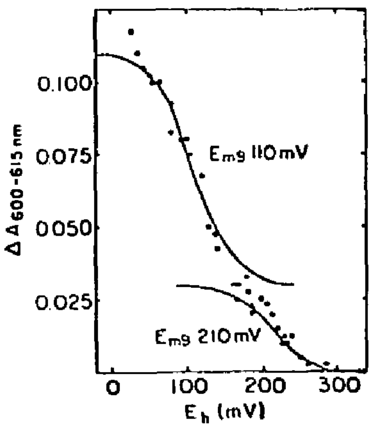
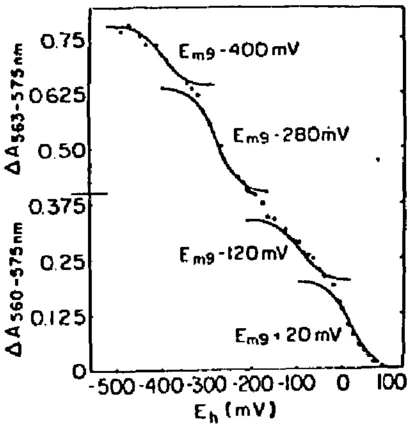

g 1.9
arbitrary
units
E_m 75 mV
-100
100
E_h (mV)

FIGURE 20

Oxidation-reduction titrations of B. firmus RAB cytochromes at pH 9.0

Optical oxidation reduction titrations were performed on everted vesicles suspended in 100 mM potassium carbonate buffer containing 10 mM $MGSO_4$, pH 9.0. (A) titration of a-type cytochromes in everted vesicles (10 mg of protein/ml). The oxidation-reduction mediators employed were 75 μM concentrations of 2,5-$(OH)_2$-1,4 BQ, 1,4 BQ, DAD, 1,2-NQ-4 sulfonate, 1,2-NQ, 5(OH)-1,4-NQ, PMS and PES. The absorbance was measured as the difference between 600 nm and a baseline drawn from 615 to 575 nm. (B) titration of b- type cytochromes in everted vesicles (7.5 mg protein/ml). The redox mediators employed at 60 μM concentration include all those present in (A), plus AQ-1,5-disulfonic acid and AQ-2-sulfonic acid. (C) titration of cytochrome c in everted vesicles (13 mg protein/ml). The mediators used were 50 μM concentration of 2,5-$(OH)_2$-1,4 BQ, DAD, 1,4-NQ, 2-(OH)-1,4-NQ, PMS and PES. The insert shows representative absorbance data at the following potentials: a, +200 mV; b, +100 mV; c, +68 mV; d, +40 mV and e, -40 mV.


ΔA 600-615 nm
0.100
0.075
0.050
0.025
0
100
200
300
E_h (mV)
E_{m9} 110 mV
E_{m9} 210 mV


ΔA 563-575 nm
ΔA 560-575 nm
0.75
0.625
0.50
0.375
0.25
0.125
0
-500
-400
-300
-200
-100
0
100
E_h (mV)
E_{m9} -400 mV
E_{m9} -280 mV
E_{m9} -120 mV
E_{m9} + 20 mV

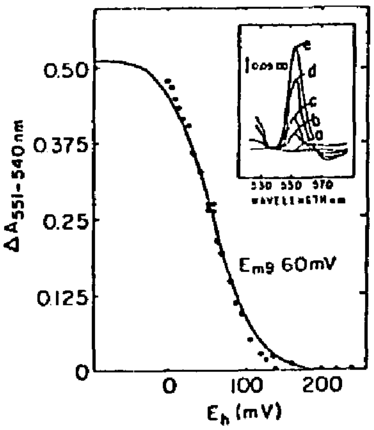
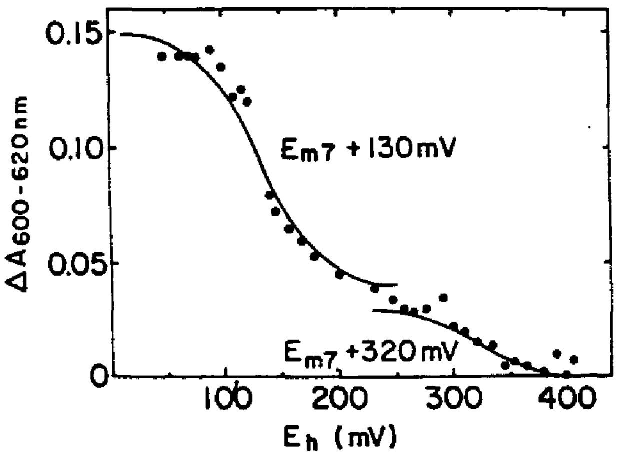
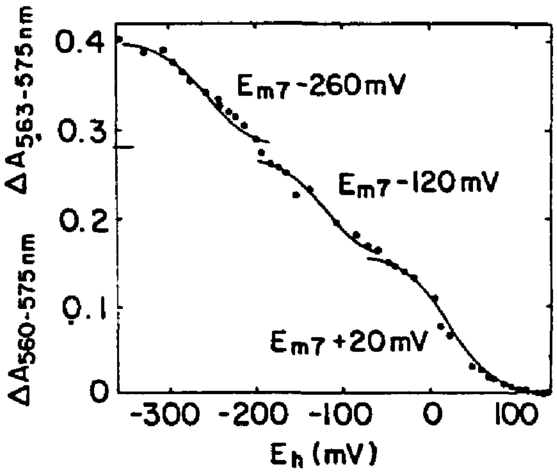

ΔA 551-540 nm
0.50
0.375
0.25
0.125
0
0
100
200
E_h (mV)
E_{m9} 60 mV
0.05 OD
e
d
c
b
a
530
550
570
WAVELENGTH nm

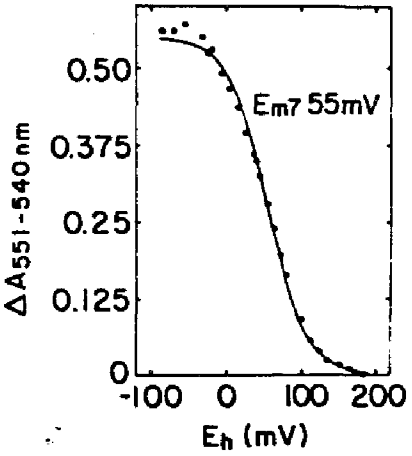
FIGURE 21

Oxidation-reduction titration of B. firmus RAB cytochromes at pH 7.0

Optical oxidation-reduction titrations were performed on everted vesicles suspended in 100 mM potassium phosphate buffer containing 10 mM $MgSO_4$, pH 7.0. (A) titration of a- type cytochromes (5.3 mg protein/ml). The redox mediators used were 67 μM concentration of $2,5(OH)_2$ 1,4 BQ, DAD, 1,2-NQ PMS and PES. (B) titration of b- type cytochromes (6.5 mg protein/ml). The mediators used at 67 μM concentration were DAD, 5(OH) 1,4 NQ, 1,2 NQ, AQ-1,5-disulfonic acid, AQ-2-sulfonic acid, PMS and PES. (C) titration of cytochrome c (6 mg protein/ml). The mediators used at 67 μM concentration were $2,5(OH)_2$-1,4 BQ, DAD, 1,2 NQ-4-sulfonate, 5(OH)-1,4 NQ, 1,2 NQ, PMS and PES.


0.15
0.10
0.05
0
ΔA600-620nm
Em7 +130mV
Em7 +320mV
100
200
300
400
Eh (mV)


0.4
0.3
0.2
0.1
0
ΔA563-575nm
ΔA560-575nm
Em7 -260mV
Em7 -120mV
Em7 +20mV
-300
-200
-100
0
100
Eh (mV)


0.50
0.375
0.25
0.125
0
ΔA551-540nm
Em7 55mV
-100
0
100
200
Eh (mV)

sumably these represent the same three cytochrome b species that showed these midpoint potentials at pH 9.0. The lowest potential cytochrome b, fairly readily titrated at pH 9.0, could not be clearly resolved at the lower pH although additional absorbance at low potentials was observed. The cytochrome c titration resulted in calculation of an Em_7 value of +55 mV for this component (Fig. 21C). Optical oxidation-reduction titrations on everted membrane vesicles from the non-alkalophile _B. firmus_ RABN were all conducted at pH 7.0, a typical cytoplasmic pH for this strain. Using the wavelength pair 600 and 615 nm, only a single cytochrome a species with an Em_7 value of +115 mV could be clearly resolved (Fig. 22A). Cytochrome b titrations were particularly difficult to perform on _B. firmus_ RABN membranes. Only a single b- type cytochrome with $Em_7 = -150$ mV could be clearly identified (Fig. 22B). The possibility of additional cytochrome b species was suggested by an absorbance at higher potentials but accurate titration was severely compromised by the extremely small concentration of these components, if they exist at all, and by the spectral overlap with cytochrome c. The cytochrome c was readily titrated, however, and an Em_7 value of +160 mV was calculated for this species (Fig. 22C).

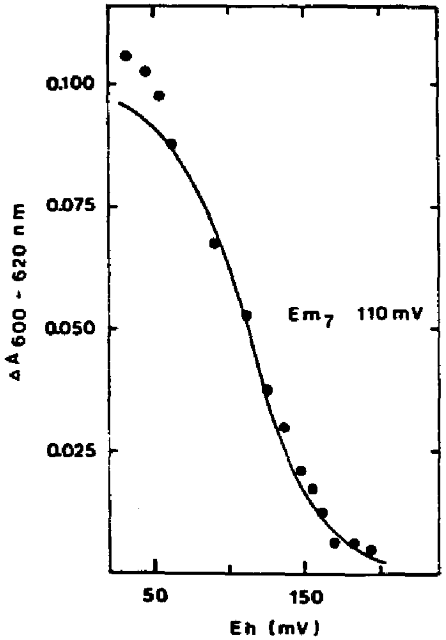
B. Respiratory Rates and Growth Yields

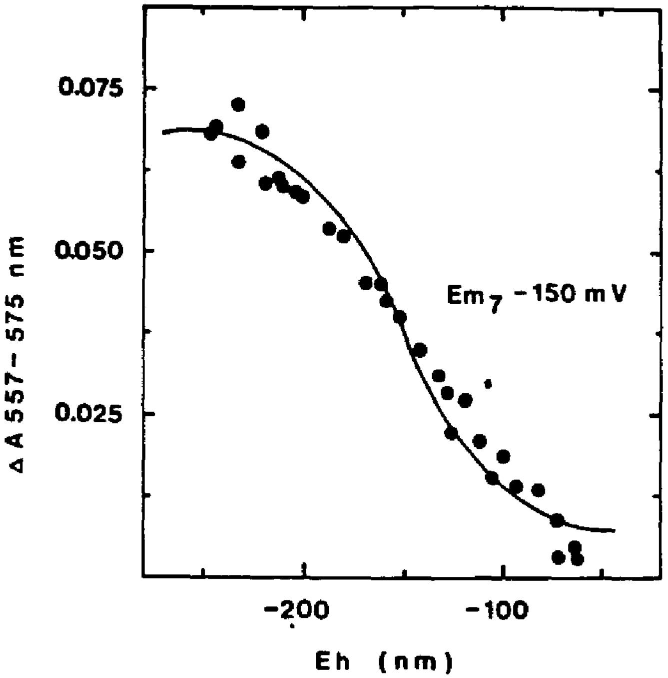
Preliminary to attempts to assess the H^+/O stoichiometries of the alkalophilic respiratory chain, some initial determinations of respiratory rates were made, and growth yields of the alkalophile and non-alkalophile mutants were compared. As shown in Table II the rate of oxygen uptake by L-malate-grown whole cells of the alkalophiles, in the presence of L-malate, was almost 1,000 n-atoms O/min/mg cell protein. This is

FIGURE 22

Oxidation-reduction potentiometric titration of B. firmus RABN cytochromes at pH 7.0

Optical oxidation-reduction titrations were performed on everted vesicles suspended in 100 mM potassium phosphate buffer containing 10 mM $MgSO_4$, pH 7.0. (A) titration of a- type cytochromes (∿6.5 mg protein/ml) The redox mediators used were 67 μM concentrations of $2,5(OH)_2$-1,4-BQ, DAD, 1,2 NQ-4-sulfonate, 5(OH)-1,4 NQ, 1,2 NQ, PMS and PES. (B) titration of a b- type cytochrome (5.7 mg protein/ml). The redox mediators used were 67 μM concentrations of 1,2 NQ-4 sulfonate, 1,4 NQ, 5(OH)-1,4-NQ, 1,2 NQ, AQ 1,5 disulfonic acid, AQ 2 sulfonic acid, PMS and PES. (C) titration of cytochrome c (6.5 mg protein/ml). The oxidation-reduction mediators used were 67 μM concentrations of $2,5(OH)_2$-1,4-BQ, DAD, 1,2 NQ-4 sulfonate, 5(OH)-1,4 NQ, 1,2 NQ, PMS and PES.


0.100
0.075
0.050
0.025
ΔA 600 - 620 nm
50
150
Eh (mV)
Em7 110 mV


0.075
0.050
0.025
ΔA 557 - 575 nm
-200
-100
Eh (nm)
Em7 -150 mV

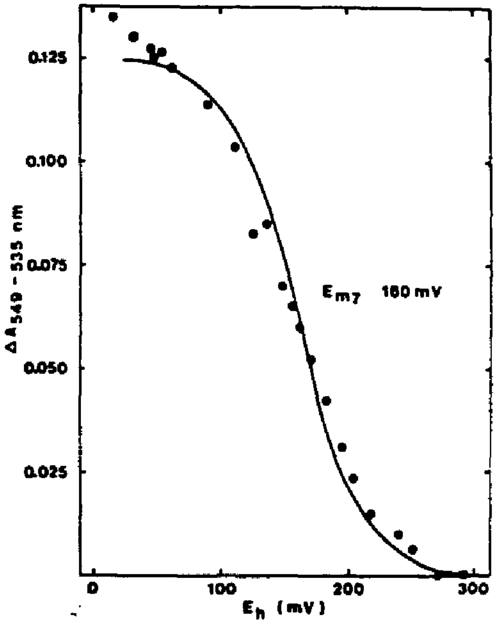

0.125
0.100
0.075
0.050
0.025
ΔA549 - 535 nm
0
100
200
300
Eh (mV)
Em7 180 mV

TABLE II

RESPIRATORY RATES OF WHOLE CELLS OF ALKALOPHILIC BACTERIA AND THEIR NON-ALKALOPHILIC MUTANT DERIVATIVES

Washed L-malate-grown cells of each strain were resuspended in 25 mM buffer at the indicated pH. Oxygen uptake, in the presence of 3.3 mM L-malate, was measured as described under Materials and Methods.

	Oxygen Uptake (natoms O/min/mg cell protein)		
Bacterium	pH 6.8	pH 8.5	pH 10.5
B. alcalophilus	-	996	987
B. alcalophilus KM23	1351	176	-
B. firmus RAB	-	983	979
B. firmus RABN	1404	328	-

very similar to rates reported for *E. coli* (Lawford and Haddock, 1973) in the presence of Krebs cycle intermediates. Moreover, non-alkalophilic KM23 and *B. firmus* RABN exhibited slightly higher rates of oxygen uptake at their optimal pH for growth, pH 6.8, than did the alkalophilic parent strains at their optimum of pH 10.5. At the intermediate pH of 8.5, at which both strains grew suboptimally, the alkalophilic strains retained a greater rate of respiration.

The endogenous rates of oxygen uptake by freshly prepared everted vesicles of both *B. alcalophilus* and KM23 were less than 10 nanoatoms of O/min/mg of membrane protein. Upon the addition of NADH (5 mM), at pH 9.0, wild type vesicles took up oxygen at a rate of 928 nanoatoms of O/min/mg of membrane protein. Lowering the pH to 7.0 caused at least a 10-fold lowering in the rate of oxygen consumption. Various respiratory inhibitors affected oxygen uptake in the wild type everted vesicles at pH 9.0. Cyanide (3 mM) produced 90% inhibition; antimycin A (30 μM), 70%; HOQNO (15 μM), 36%; rotenone (100 μM), 27%. The rate of oxygen uptake by everted vesicles of KM23 at pH 7.0 using NADH as substrate was 914 nanoatoms of O/min/mg of membrane protein. Cyanide (5 mM) reduced oxygen uptake by over 90%; antimycin A (30 μM), by 62%; rotenone (100 μM), by 30%. Addition of 10 μM gramicidin or 30 μM CCCP had no effect on the respiratory rates of either wild type or KM23 membranes.

The lack of correlation between respiratory rates and membrane cytochrome contents is notable. Moreover, the growth yields of *B. alcalophilus* and *B. firmus* RAB on L-malate, Ymal, were found to be 42 and 38 mg dry weight/mmole of L-malate, respectively. These yields were completely comparable to, if not slightly higher than, those obtained for *Bacillus* megaterium and Arthrobacter pyridinolis in parallel determinations. Thus, neither substrate nor oxygen consumption is particularly elevated in the alkalophiles, whereas the cytochrome content is unusually high. Ymal values of 24 and 12 mg dry weight/mmole of L-malate were obtained for KM23 and B. firmus RABN. Thus relative to their alkalophilic parents, the growth yields and cytochrome complements are reduced whereas the oxygen consumption per se is comparable. It seemed possible, therefore, that the alkalophiles might have a more effective respiratory chain than the non-alkalophilic mutants, i.e., might have higher H^+/O stoichiometries so that more bioenergetic work could be conducted per O atom consumed.

C. H^+/O Stoichiometries

Whole cells of B. firmus RAB harvested from cultures in their mid-logarithmic phase were washed and resuspended in 120 mM KCl, 1-2 mM Tris-HCl at pH 9.0 and pH 7.0. In order to detect the very small changes in pH resulting from proton ejection, very low buffering strength was essential. The high potassium concentration supplied the needed charge-compensating cation to dissipate the transmembrane electrical potential ($\Delta\psi$) which results from proton translocation. In the absence of valinomycin and utilizing endogenous substrates, minimal proton ejection could be detected following an oxygen pulse (Fig. 23). When, however, 0.5 µg/ml of the K^+-ionophore, valinomycin, was included in the reaction medium, acidification of the medium was clearly observed following an oxygen pulse (Fig. 24). Higher valinomycin concentrations tended to slow respiration and inhibited proton ejection. Calibration of pH traces was accomplished with injections of a few precisely measured µl of commercially prepared

FIGURE 23

H^+/O in B. firmus RAB at pH 9.0 as a result of an oxygen pulse in the absence of valinomycin

The upper trace is a recording of oxygen tension and the lower trace indicates pH. At the point indicated by the arrow an oxygen pulse in the form of 100 µl of air saturated buffer was added to a suspension of *B. firmus* RAB cells in 120 mM KCl, 1.5 mM Tris-Cl, pH 9.0.

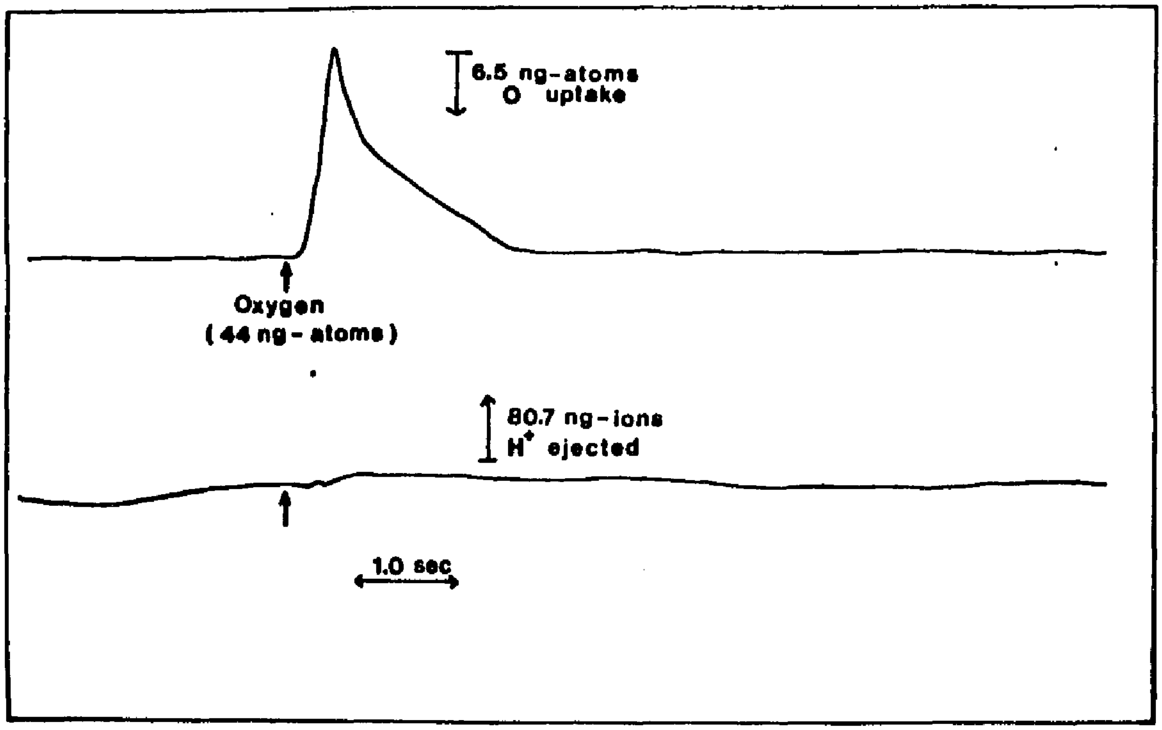
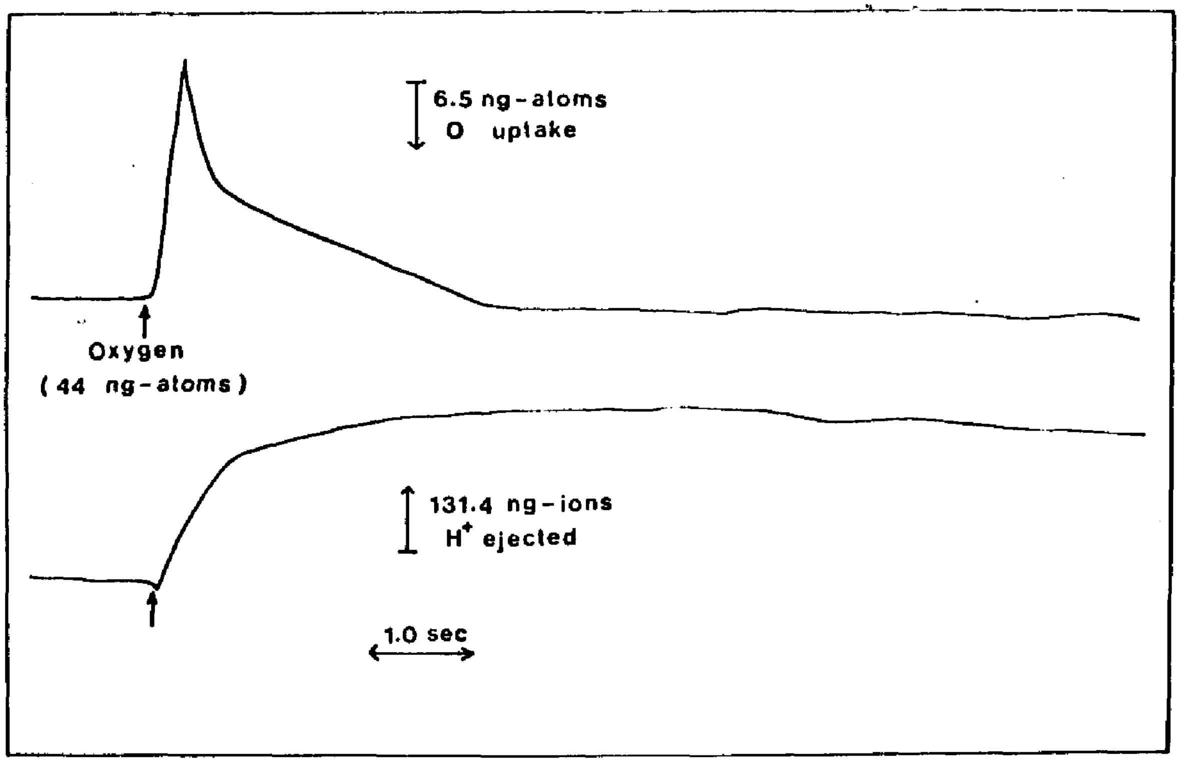

6.5 ng-atoms
O uptake
Oxygen
(44 ng-atoms)
80.7 ng-ions
H^+ ejected
1.0 sec

FIGURE 24

H^+/O in B. firmus RAB at pH 9.0 as a result of an oxygen pulse in the presence of valinomycin

The upper trace is a recording of oxygen tension and the lower trace indicates pH. Valinomycin (0.5 μg/ml final concentration) was added as an ethanolic solution to a suspension of *B. firmus* RAB cells in 120 mM KCl, 1.5 mM Tris-Cl, pH 9.0. An oxygen pulse in the form of 100 μl of air saturated buffer was added to the reaction medium at the point indicated by the arrow. The resultant oxygen and pH curves appear to be biphasic in nature. Initially, there is a rapid phase of respiration-linked proton ejection which is then followed by a longer, slow phase of reduced proton ejection. As the pH trace indicates, minimal proton backflow occurs and the acidification of the media is sustained.


6.5 ng-atoms
O uptake
Oxygen
(44 ng-atoms)
131.4 ng-ions
H^+ ejected
1.0 sec

0.100N HCl ("Baker Analyzed" Reagent). The oxygen traces were similarly calibrated by addition of the air-saturated buffer which has a known oxygen solubility. Tangents could be drawn to the oxygen and proton traces and, from the ratio of their slopes, H^+/O stoichiometries were calculated.

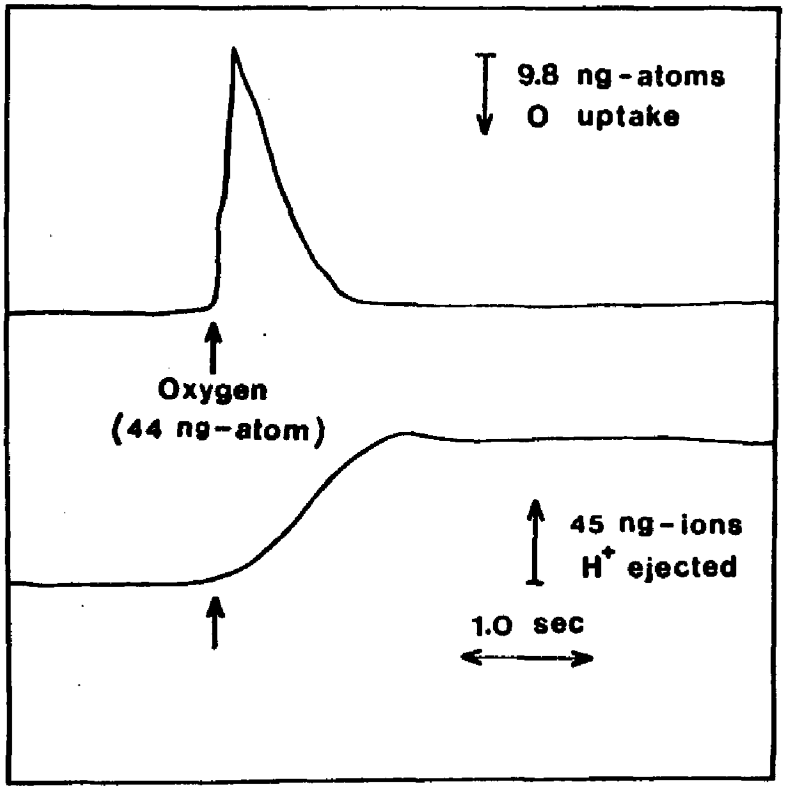
At pH 9.0, both oxygen disappearance and medium acidification exhibited biphasic kinetics (Fig. 24). The initial period immediately following addition of an oxygen pulse lasted for approximately 400 msec and was associated with an almost linear phase of very rapid oxygen consumption. Coupled to this early fast phase of very rapid oxygen consumption was an intial phase of very rapid proton ejection. The fast phase was followed by less rapid oxygen consumption with much slower rates of medium acidification. H^+/O values calculated from the slopes of tangents drawn to the initial, fast phase portion of both oxygen and proton traces resulted in stoichiometries which ranged from 6 to 13. The average and most frequent values obtained were close to 9. H^+/O values for the slower phase were invariably lower, ranging from 2 to 7 and averaging about 4. With several preparations, the high H^+/O stoichiometries and the biphasic nature of the oxygen and proton traces were lost with aging of the preparation. In these instances, proton pumping was clearly compromised more than respiratory rates. Also of note is the very low rate of proton backflow (Fig. 24). Even after several minutes following an oxygen pulse, a net acidification of the reaction medium remained evident.

When H^+/O measurements on B. firmus RAB were conducted at pH 7.0 only a single phase exhibiting exponential decay was observed for both oxygen consumption and proton ejection (Fig. 25). Tangents drawn to early portions of these curves allowed calculation of H^+/O stoichiom-

FIGURE 25

H^+/O in B. firmus RAB at pH 7.0 as a result of an oxygen pulse in the presence of valinomycin

The upper trace is a recording of oxygen tension and the lower trace indicates pH. Valinomycin (0.5 μg/ml final concentration) was added as an ethanolic solution to a suspension of B. firmus RAB cells in 120 mM KCl, 1.5 mM Tris-Cl, pH 7.0. An oxygen pulse in the form of 100 μl of air saturated buffer was added to the reaction medium at the point indicated by the arrow. The resultant oxygen and proton traces exhibited simple exponential decay and proton backflow or "leak" can be seen in the pH trace.


9.8 ng-atoms
O uptake
Oxygen
(44 ng-atom)
45 ng-ions
H⁺ ejected
1.0 sec

etries between 3 and 6, with an average value of approximately 4. Again, there was little proton backflow evident during these experiments and, after a few hours, H^+/O values no higher than 3 were obtained under these conditions.

H^+/O measurements were also made on whole cells of the non-alkalophilic mutant B. firmus RABN. As was found for B. firmus RAB at pH 7.0, only simple exponentially decaying oxygen consumption and proton ejection traces were obtained (Fig. 26). Stoichiometries calculated from the slopes of tangents drawn to the early portions of these curves were generally between 3 and 4. A much more rapid rate of proton backflow was observed for this strain. After only 10 seconds following an oxygen pulse, the pH of the reaction medium had practically returned to its initial value whereas, in experiments with the wild type, H^+ backflow was still incomplete after several minutes.

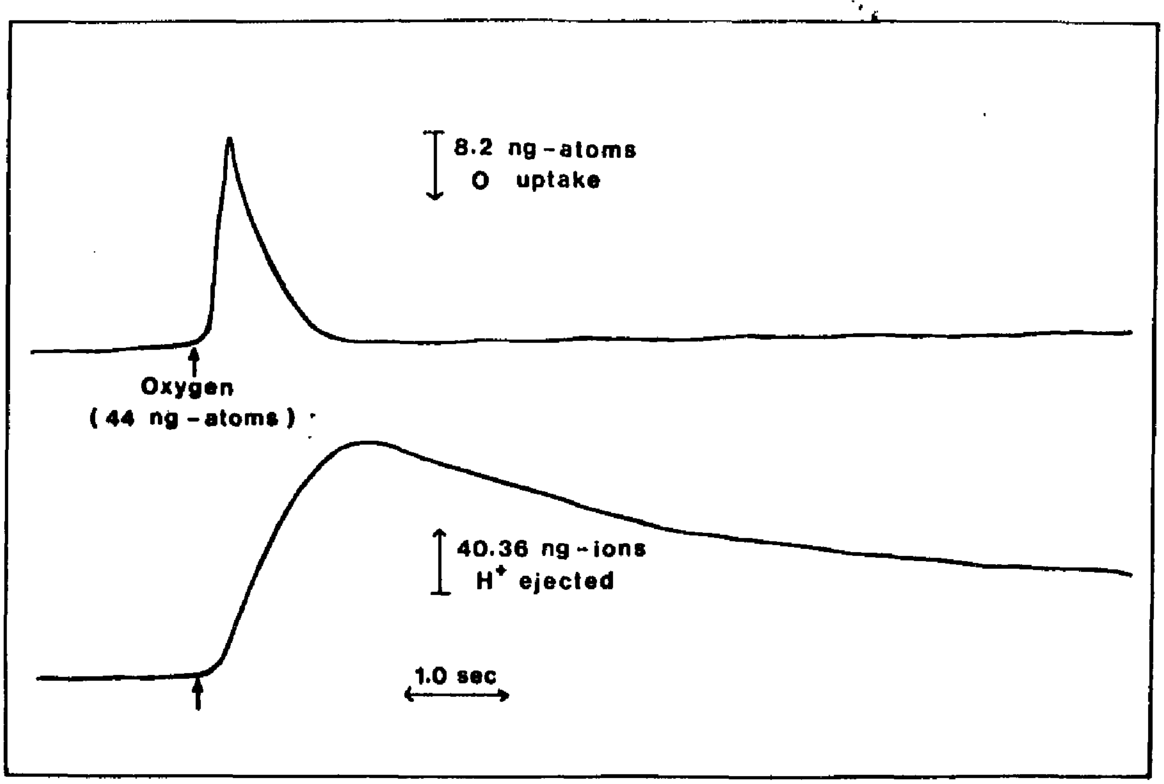
D. Pleitropic Properties of Mutation to Non-Alkalophily

In addition to both the quantitative and qualitative differences in respiratory chain components and the alterations in function evidenced by H^+/O stoichiometries, other changes result from mutation of the wild type alkalophiles to non-alkalophily. Earlier work by members of this laboratory had demonstrated that loss of Na^+/H^+ antiport activity and loss of Na^+-dependent solute transport systems accompanied the loss of alkalophily by mutants of B. alcalophilus and B. firmus RAB (Krulwich et al., 1979; Mandel et al., 1980; Guffanti et al., 1980). Several lines of evidence indicate that in B. alcalophilus both the loss of Na^+-coupling and antiporter activity are primary effects of the mutation (Guffanti et al., 1981). Similar findings have been reported in Escherichia coli (Zilberstein et al., 1980). It has been suggested that the mutation to

FIGURE 26

H^+/O in RABN as a result of an oxygen pulse in the presence of valinomycin

The upper trace is a recording of oxygen tension and the lower trace indicates pH. Valinomycin (0.5 µg/ml final concentration) was added as an ethanolic solution to a suspension of RABN cells in 120 mM KCl, 1.5 mM Tris-Cl, pH 7.0. An oxygen pulse in the form of 100 µl of air saturated buffer was added to the reaction mixture at the point indicated by the arrow. Significant proton backflow is revealed by the rapidity with which the pH trace returns to its initial value before the pulse.


8.2 ng-atoms
O uptake
Oxygen
(44 ng-atoms)
40.36 ng-ions
H⁺ ejected
1.0 sec

non-alkalophily might effect a Na^+-translocating element that is common to Na^+-coupled symport and antiport systems in *B. alcalophilus* (Guffanti *et al.*, 1981). Thus, the finding of profound effect of the mutation on the respiratory chain was surprising.

In addition, during spectrophotometric studies of the membranes it was observed that vesicles from wild-type *B. alcalophilus* contained a chromophore that exhibits an absorption band at 526 nm (Fig. 27). Absorption at this wavelength was unchanged by reduction with dithionite or oxidation by ferricyanide. Non-alkalophilic strains such as KM23 showed little or no light absorption at 526 nm (Fig. 27). While perhaps unrelated, it was notable that after several days of growth of non-alkalophilic mutants, a brown pigment appeared in the culture medium; no such pigment was found in cultures of the wild type. A similar absorption band was observed in absolute spectra of oxidized *B. firmus* RAB vesicles (Fig. 28). These bands were absent from membrane preparations of *B. firmus* RABN. Curiously, this non-alkalophilic strain also released a brown pigment into the culture medium.

Are all the effects of the mutation to non-alkalophily really the result of a single mutational event? Dozens of independently isolated non-alkalophilic strains (KM strains) and independently isolated revertants of KM23 (KMR strains) were studied. As shown in Table III for three alkalophilic strains (wild-type *B. alcalophilus* and two KMR strains) and three non-alkalophilic strains, the pleiotropic properties are expressed in each strain. That is, Na^+/H^+ antiport activity (as assayed by energy- and Na^+-dependent acidification of the intravesicular space) (Fig. 29), Na^+-dependent uptake of AIB (Fig. 30), cytochrome contents above 5 nmol (mg membrane protein)$^{-1}$ and high levels of the

FIGURE 27

Absolute absorption spectra of vesicles from B. alcalophilus and KM23

Membrane vesicles of *B. alcalophilus* and KM23 were suspended to 3.0 mg protein/ml. Absolute absorption spectra of oxidized vesicles at room temperature were obtained using a Perkin-Elmer 557 spectrometer operated in the dual beam mode with buffer alone in the reference cuvette. A few grains of potassium ferricyanide were added to the membrane suspension to prevent reduction of cytochromes. Trace (a) is of vesicles prepared from wild-type *B. alcalophilus* and trace (b) is of vesicles prepared from KM23.

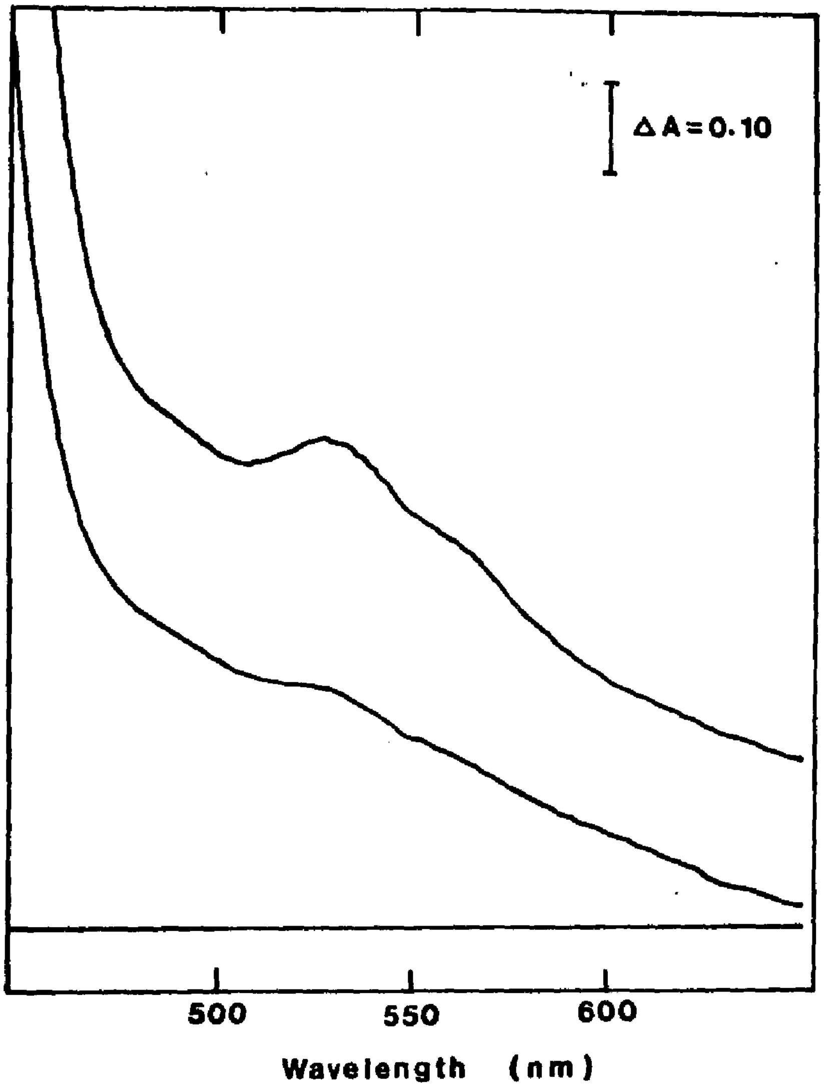

ΔA=0.10
500
550
600
Wavelength (nm)

FIGURE 28

Absolute absorption spectra of vesicles from *B. firmus* RAB and *B. firmus* RABN

Suspensions of membrane vesicles of *B. firmus* RAB (A) and RABN (B) were oxidized with a few grains of potassium ferricyanide and absolute spectra were recorded as described in the legend to Fig. 15.

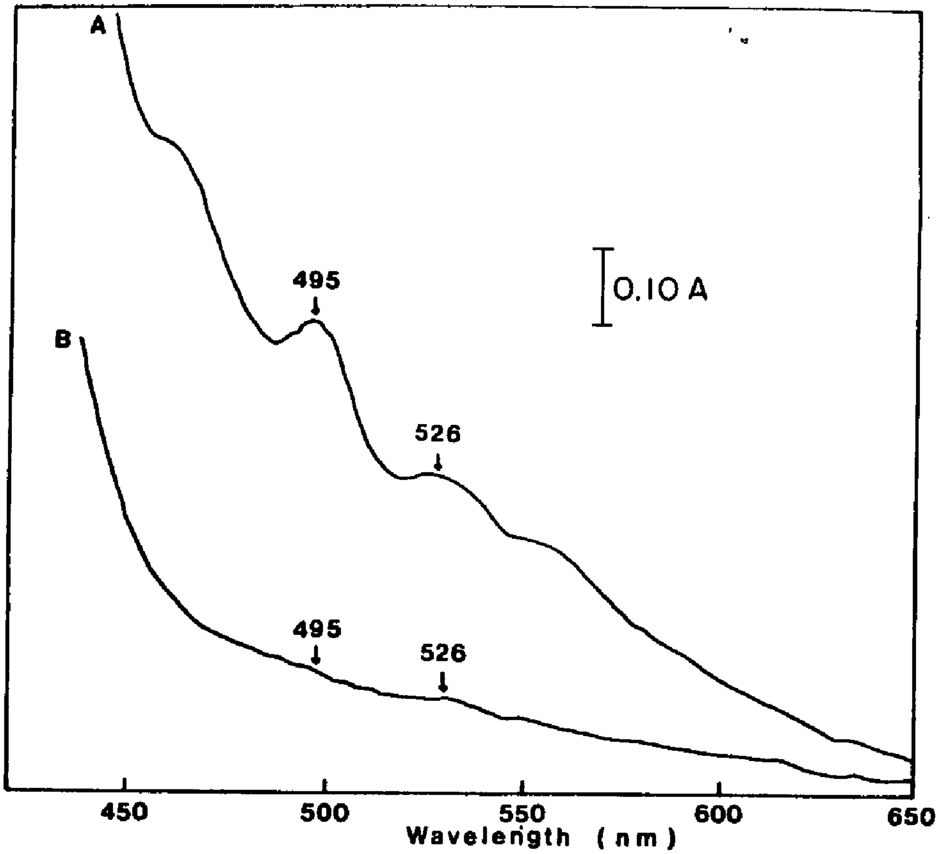

A
B
495
526
495
526
0.10 A
450
500
550
600
650
Wavelength (nm)

FIGURE 29

Methylamine uptake by right-side-out membrane vesicles

Right-side-out membrane vesicles of the alkalophilic revertant KMR4 and the non-alkalophilic mutant KM41 were prepared and suspended to 10 mg of protein/ml in 100 mM potassium carbonate buffer, pH 9.0 containing 10 mM $MgSO_4$. Uptake of $[^{14}C-]$methylamine was measured by flow dialysis as described under "Materials and Methods". In runs (A) and (B), 0.8 ml of KMR4 vesicles were added to the upper chamber of the flow dialysis apparatus; in (C) 0.8 ml of KM41 vesicles were used. In (A) and (C), 100 mM sodium carbonate buffer, pH 9.0 containing 10 mM $MgSO_4$ was pumped through the lower chamber at 6 ml/min; in (B) 100 mM potassium carbonate buffer, pH 9.0 was pumped through the lower chamber. In all three runs, the assay was initiated by addition of 25 μM $[^{14}C-]$methylamine. The vesicles were energized by addition of 20 mM potassium ascorbate plus 2 mM TMPD at fraction 30 as indicated by the arrows.

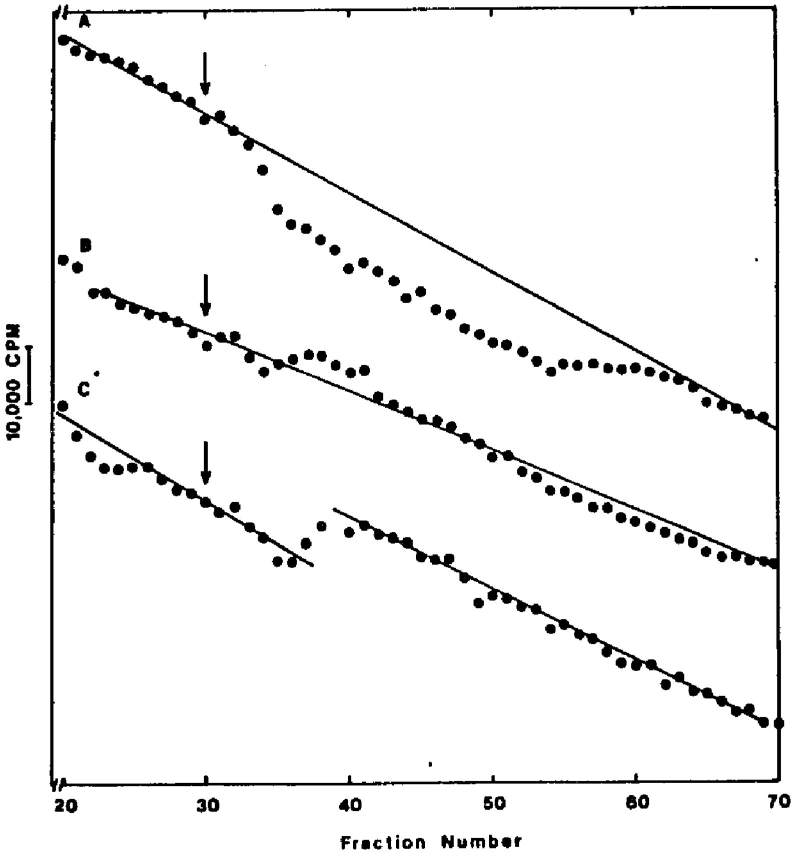

10,000 CPM
A
B
C
20
30
40
50
60
70
Fraction Number

FIGURE 30

AIB uptake by vesicles of non-alkalophilic mutants and alkalophilic revertants of B. alcalophilus

AIB uptake was assayed in right-side-out oriented membrane vesicles prepared from the alkalophilic revertant KMR6 and the non-alkalophile KM41 as described in the "Materials and Methods". Key to figure: the alkalophile KMR6, circles; the non-alkalophile KM41, triangles. Vesicles treated with 100 mM Na^+ are represented by solid symbols; vesicles treated with 100 mM K^+ are represented by the open symbols.

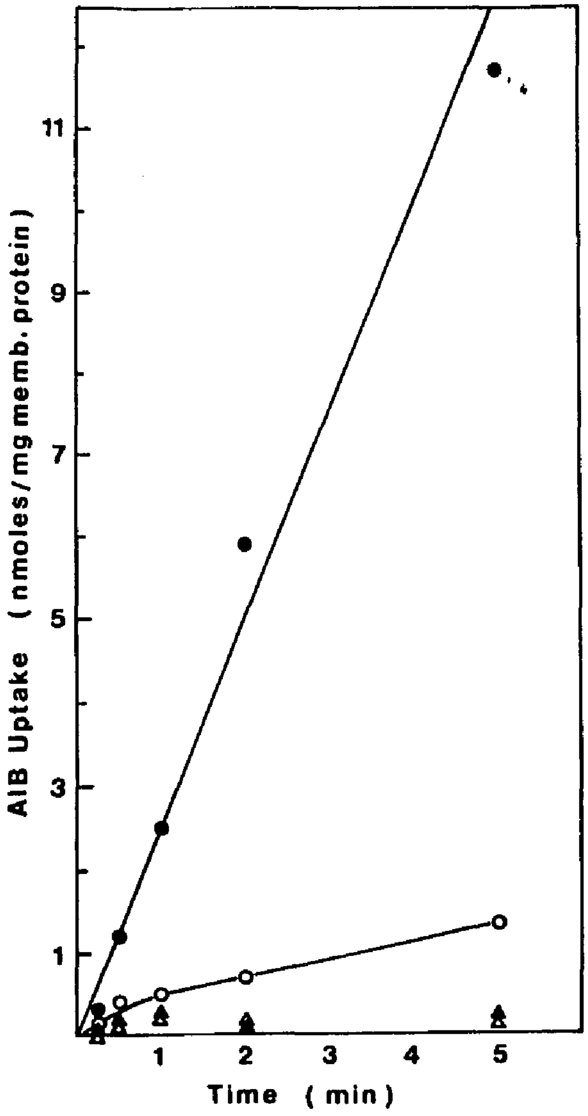

AIB Uptake (nmoles/mg memb. protein)
1
3
5
7
9
11
Time (min)
1
2
3
4
5

TABLE III

BIOENERGETIC CHARACTERISTICS OF MEMBRANE VESICLES FROM NON-ALKALOPHILIC STRAINS OF B. ALCALOPHILUS AND REVERTANTS THEREOF

Strain	Production of pH gradient $[H^+]_{in}>[H^+]_{out}$ upon energization	AIB uptake at pH 9.0 [nmol min^{-1} (mg $protein)^{-1}$]		Total membrane cytochrome content [nmol (mg membrane $protein)^{-1}$]	Absorption band at 526 nm [$\Delta A_{526-505}$ (g membrane $protein)^{-1}$]
		$-Na^+$	$+Na^+$		
Wild type	Yes	0.3	1.4	5.31	11.0
KMR4	Yes	0.5	2.5	5.22	12.2
KMR101	Yes	0.1	1.5	6.50	18.0
KM23	No	0.2	0.2	1.27	<1.0
KM41	No	0.2	0.2	1.66	2.1
KM104	No	0.12	0.12	0.80	<1.0

chromophore that absorbs light at 526 nm were found in all wild-type and revertant strains. By contrast, all KM strains exhibited no Na^+/H^+ antiporter activity, no Na^+-dependent AIB uptake, and much lower cytochrome and chromophore contents (Table III). AIB uptake by vesicles of the KM strains was assayed at pH 7.0 as well as pH 9.0. At the lower pH, appreciable uptake was observed with all three mutants, but this uptake was Na^+-independent, as found both for whole cells and vesicles of KM23 (Krulwich et al., 1979; Bonner et al., 1982).

The frequencies of spontaneous mutation of the wild type to non-alkalophily (1 in 1.6 x 10^9) and reversion of non-alkalophilic strain KM23 to alkalophily (1 in 0.62 x 10^9) are consistent with a single mutation. Again, the distinction between strains was growth of non-alkalophiles at pH 7.0 (but not at pH 10.5) and growth of alkalophiles at pH 10.5 (but not at pH 7.0).

While a single mutation in a Na^+-translocating element could account for simultaneous loss of Na^+-dependent antiport and symport activities, it was less clear how such a mutation could result in the changes in cytochrome content. Since one of the roles of the Na^+/H^+ antiporter is regulation of the cytoplasmic pH, the possibility that perturbations of cytoplasmic pH, upon loss of the antiporter, might mediate changes in cytochrome contents was considered. Growth of wild-type B. alcalophilus at pH 9.0 rather than pH 10.5 resulted in an appreciable decrease in the contents of cytochromes (especially c- type), to 3.5 nmol/mg protein. When lactose served as the carbon source for growth, the medium pH was also approximately 9.0 and only 3.0 nmol heme/mg protein was found. Conversely, growth of strain KM23 at pH 8.5 instead of pH 7.0 resulted in a significant increase in the membrane content of cytochromes, to

2.0 nmol/mg protein as compared to the values in Table II. Importantly, while the cytochrome content was decreased upon growth of the wild type at pH 9.0, Na^+-coupling was still observed; the loss of Na^+-coupling is not, then, a secondary consequence of cytochrome reduction.

It was notable that the content of the chromophore was generally higher at high pH. The possibility that this material was a photoreceptor (for an ion pump?) of the type described in halobacteria (Lanyi, 1981; Stoeckenius *et al.*, 1979) was considered. However, there was no diminution in chromophore content after growth of the wild type in total darkness; moreover, light conditions had no perceptible effect on any of the determinations made. It was also possible that the chromophore was related to the obviously high iron demand for alkalophilic growth. If so, strain KM23 might be induced to produce more of the chromophore under conditions in which iron was growth-limiting. However, growth of strain KM23 in "low iron" medium failed to cause an increase in this material. In the wild type, growth on "low iron" medium caused a decrease in chromophore content as well as in the contents of cytochromes b and c.

DISCUSSION

Respiratory chains containing extraordinary levels of cytochromes and numerous and unusual respiratory chain components have been found in *B. alcalophilus* and *B. firmus* RAB membranes. These special properties may enable the obligate alkalophiles to achieve particularly efficient energy transduction as measured by both proton pumping stoichiometries and molar growth yield determinations. The respiratory chain components that have been identified by spectroscopy and resolved by oxidation-reduction potentiometry in *B. alcalophilus* and its mutant strain KM23 are summarized in Figure 31. The oxidation-reduction species identified in *B. firmus* RAB and RABN are similarly represented in Figure 32. In addition to the large quantitative differences in their cytochrome contents (Table I), striking qualitative differences between the alkalophilic and non-alkalophilic strains are evident in the number of distinguishable oxidation-reduction species and in the array of midpoint potentials of their cytochromes.

The most pronounced qualitative changes between alkalophilic and non-alkalophilic strains were found with respect to their b- type cytochromes. In membrane vesicles from each of the two alkalophiles, four cytochrome b species were distinguishable by oxidation-reduction potentiometry at pH 9.0. The choice of this pH was based on its being a typical cytoplasmic pH for these strains (Guffanti *et al.*, 1978). At pH 9.0, three low potential b- type cytochromes were identified in each alkalophile. In *B. alcalophilus*, cytochromes with Em_9 values of -120, -240 and -320 mV were demonstrated; *B. firmus* RAB had cytochromes with Em_9 values of -120, -280 and, remarkably, -400 mV.

FIGURE 31

Titratable oxidation-reduction components of B. alcalophilus and its non-alkalophilic mutant derivative

Left, oxidation-reduction components of wild-type B. alcalophilus at pH 9.0. The cytochrome b species with Em_9 = +20 mV is the major cytochrome b oxidation-reduction component. Two isopotential cytochrome a components are shown, based on the findings at pH 7.0. Center, oxidation-reduction components of wild-type B. alcalophilus at pH 7.0. Of the multiple b- type cytochromes, only the major oxidation-reduction b component is represented here. Several low potential b cytochromes have been omitted from this figure since accurate resolution of their midpoint potentials at this pH was not obtained. It should be pointed out, however, that these low potential b cytochromes are still visible at pH 7.0 as shown by the redox titration curve in Fig. 4B. Right, oxidation-reduction components of KM23 at pH 7.0. The blocks representing each component indicate the potential range over which the components become 9-91% oxidized or reduced. The lateral positioning is arbitrary.

B. alcalophilus

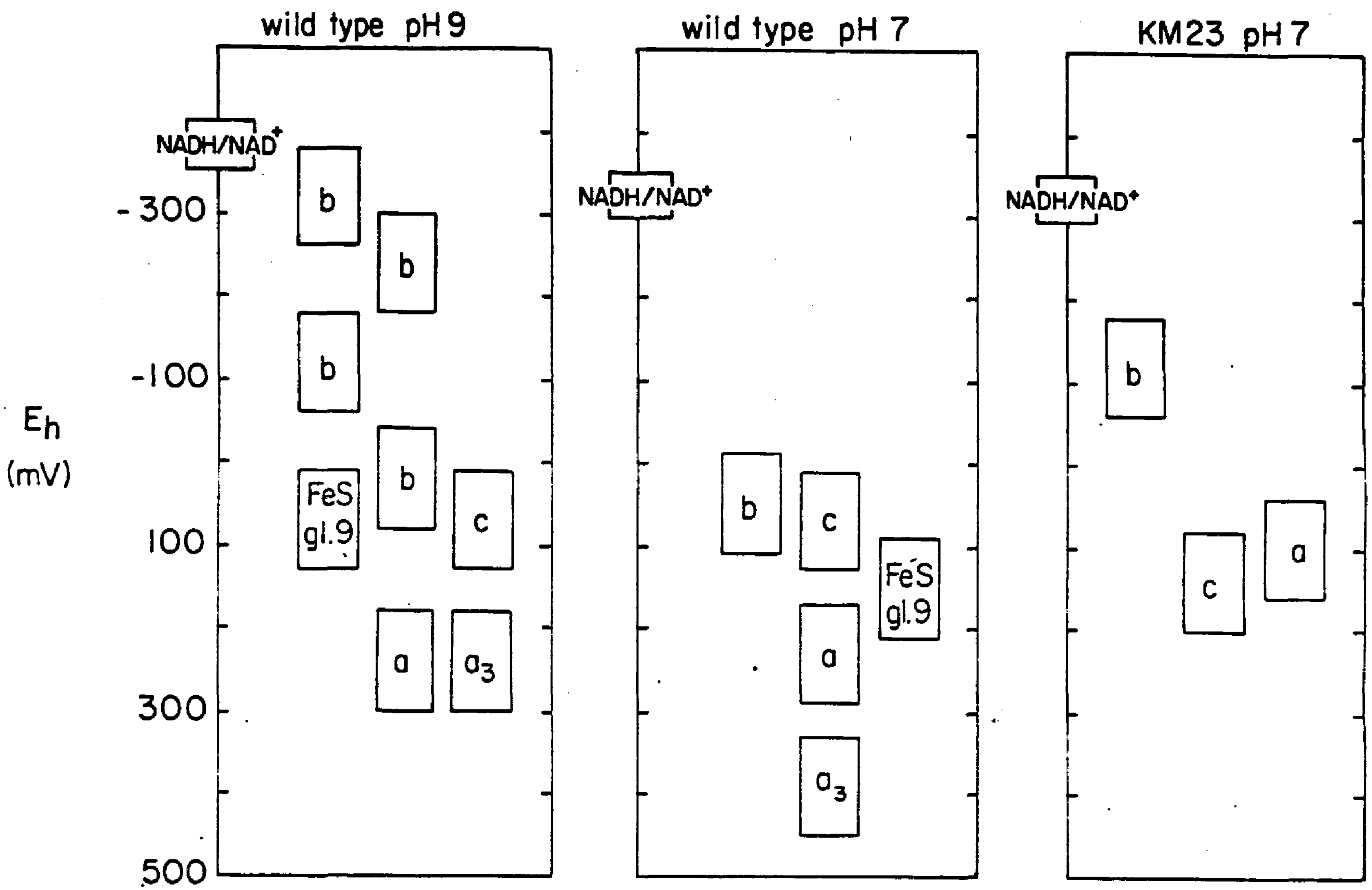
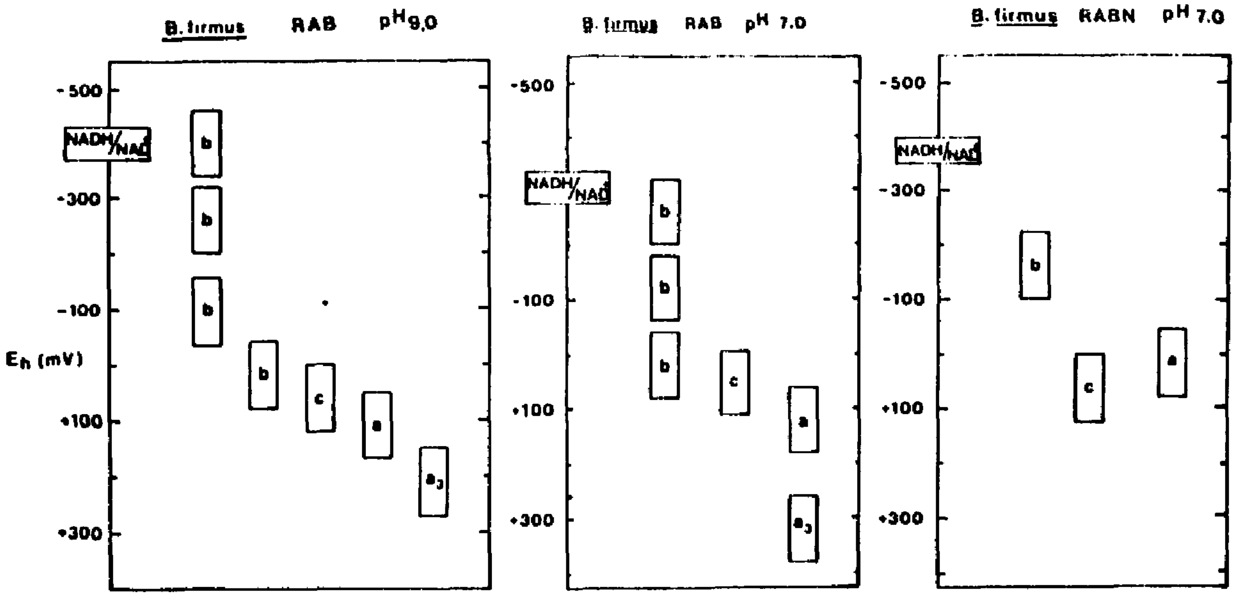


FIGURE 32

Titrable oxidation-reduction components of B. firmus RAB and its non-alkalophilic mutant derivative

Left, oxidation-reduction components of wild-type B. firmus RAB at pH 9.0. The cytochrome a species with Em_9 = +110 mV is the major cytochrome a redox component. Center, cytochromes titrated in B. firmus RAB. At this pH, the two cytochrome a species were more clearly resolved due to the pH dependent nature of the midpoint potention of the cytochrome a_3. Only three cytochrome b species present in approximately equal concentrations could be resolved clearly at this pH; however, the fourth, lowest potential cytochrome resolved at pH 9.0 was qualitatively evident. Right, cytochromes titrated in RABN at pH 7.0. Only a single cytochrome b species with Em_9 = -160 mV was resolved by oxidation-reduction titration in RABN. Additional absorbance was noted at higher potentials but could not be clearly resolved. The blocks representing each component indicate the potential range over which the cytochromes become 9-91% oxidized or reduced. The lateral positioning is arbitrary.


B. firmus RAB pH 9.0
B. firmus RAB pH 7.0
B. firmus RABN pH 7.0
Eh (mV)
-500
-300
-100
+100
+300
NADH/NAD+
b
c
a
a3

When the titrations of these membrane preparations were conducted at pH 7.0 the low potential cytochrome b's in both strains were all qualitatively evident (Fig. 16B, 21B). At this lower pH, however, it was impossible to determine with confidence the midpoint potential of the lowest cytochrome b in *B. firmus* RAB or the precise midpoint potentials for these b- type cytochromes in *B. alcalophilus*. In *B. alcalophilus*, the major cytochrome b species, however, was clearly resolved at both pH 9.0 and pH 7.0. At the more alkaline pH, a midpoint potential of +20 mV was determined. When titrated at pH 7.0, the midpoint potential of this component shifted to +50 mV. This difference in midpoint potentials is appreciably less than the -60 mV/pH unit that is formally expected for an oxidation-reduction species that gains or loses one proton during its oxidation-reduction reaction. There are, however, considerable precedents for this type of observation, which may result from making the determinations at pH values that span the relevant pK values (Petty and Dutton, 1976; Urban and Klingenberg, 1969). In *B. firmus* RAB the highest potential cytochrome b was present in equal amounts to the other b- type cytochromes and its midpoint potential of +20 mV was independent of the pH over the range from 7.0 to 9.0.

In contrast to the multiple b- type cytochromes found at either pH 9.0 or 7.0 in the alkalophiles, titrations of vesicles from KM23 revealed only one cytochrome b species and the midpoint potential of only one cytochrome b could be determined in RABN (an additional b- type cytochrome of higher potential, present only in very small amounts, may also be present in this strain). The midpoint potentials for the cytochrome b's in the non-alkalophiles were -150 mV for RABN and -120 mV for KM23, identical in the latter case to the Em_9 of one of the minor b cytochrome components found at lower potential in _B_. _alcalophilus_. Thus the b cytochrome of KM23 does not correspond, quantitatively or qualitatively, to the major cytochrome b species found in _B_. _alcalophilus_.

It may be of particular significance that multiple cytochrome b species, some with very low oxidation-reduction potentials, are found in the alkalophiles but not in their non-alkalophilic derivatives. At present, we would speculate that these cytochromes may relate to special energy conservation properties of the alkalophile respiratory chain. Numerous models have been proposed for multiple b cytochromes functioning as proton translocators or participating in quinone cycles in the complex III of mitochondria (Mitchell, 1976; Papa, 1976; Trumpower, 1976; Von Jagow and Engel, 1980). The obligate alkalophiles may possess respiratory chains that are more complex than those of typical aerobic non-alkalophiles, including their own non-alkalophilic mutants. Their complex III's may be particularly complicated, i.e., the multiple b cytochromes of the alkalophile may participate in extra oxidation-reduction loops or proton pumping sites, translocating added protons across the membrane. Within the specific theoretical limits to the amount of energy made available from the oxidation of a given substrate, the alkalophilic respiratory chain might facilitate a greater conservation of this energy than generally occurs in non-alkalophilic bacteria. The relationship between a fully functional respiratory chain and the viability of these species may explain the failure of obligate alkalophiles to thrive at pH 7.0.

As presented in Figure 8, NADH was clearly able to reduce cytochromes a, b and c at pH 9.0. At pH 7.0, however, this substrate only caused reduction of cytochrome a and c species. Failure to reduce cytochrome b at pH 7.0 suggests that as the pH is shifted, from pH 9.0 to 7.0, some respiratory chain component(s) exhibits a shift in its midpoint potential or is altered in some fashion compromising alkalophile respiratory chain function. The H^+/O measurements conducted at pH 7.0 also support this hypothesis. In spite of comparable rates of oxygen consumption, very few ejected protons were observed and the biphasic respiration kinetics observed at pH 9.0 were absent at this pH. Some breakdown in the proton pumping mechanism of the alkalophile respiratory chain must take place at lower pH's. This in turn results in generation of a $\Delta\psi$ and total $\Delta\tilde{\mu}_{H^+}$ at pH 7.0 that is too low to support life at pH 7.0 (Kitada _et al_., 1982).

The a- type cytochromes as well as the b- type cytochromes should also be considered within the context of possible proton pumps in view of the still controversial suggestions of others (e.g., Papa, 1976; Wikström and Krab, 1979). A pH-dependent midpoint potential, found for the mitochondrial cytochrome aa_3 (Artzatbgnov _et al_., 1978; Wikström, 1977; Wikström and Krab 1979; Krab and Wikström, 1979) might be an expected property of such a pump. One of the a- type cytochromes from _B. alcalophilus_ had a shift in its midpoint potential from +240 mV at pH 9.0 to +390 mV at pH 7.0. In _B. firmus_ RAB an Em_9 value of +210 mV was obtained for a cytochrome a which shifted to +320 mV at pH 7.0. The pH dependence of the midpoint potential of an a- type cytochrome in both _B. firmus_ RAB and _B. alcalophilus_ makes these oxidation-reduction components additional candidates for proton pump function.

While the cytochrome c's of the alkalophiles did not exhibit pH-dependent shifts in their midpoint potentials, their properties were of interest. The midpoint potentials of +70 mV and +60 mV for *B. alcalophilus* and *B. firmus* RAB are rather low for cytochrome c. In mammalian mitochondria cytochrome c has a midpoint potential of approximately +230 mV (Rodkey and Ball, 1950; Henderson and Rawlinson, 1956, 1961; Dutton *et al.*, 1970). In mitochondria of the yeast *Saccharomyces cerevisiae* and the protist *Crithidia fasciculata*, midpoint potentials of +225 and +230 mV were determined respectively (Coolidge, 1932; Jacobs *et al.*, 1979). Bacterial cytochrome c species also usually have high midpoint potentials, ranging from +140 to +295 mV for a variety of species (Milhaud *et al.*, 1958; Kamen and Vernon, 1960; Vernon and Mangum, 1960; Clark-Walker and Lascelles, 1970; Dutton and Jackson, 1972).

Similarly, most reported values for Rieske midpoint potentials range between +250 and +300 mV. In each organism or organelle where a Rieske protein was identified, a c- type cytochrome was also found approximately isopotential to it at physiological pH. The lowest previously reported value for a Rieske protein, +160 mV, was found for the green sulfur bacterium *Chlorobium limicola f. thiosulfatophilum* (Knaff and Malkin, 1976). A cytochrome c with a correspondingly low midpoint potential of +165 mV was also present (Prince and Olson, 1976). *B. alcalophilus* possesses a Rieske protein with the extraordinarily low midpoint potential of +75 mV at its physiological pH of 9.0. Again, even at this potential, the Em of the Rieske protein is in remarkable proximity to the +70 mV midpoint potential found for the cytochrome c in *B. alcalophilus* at pH 9.0. This relationship may be very important to the energy transducing function of the respiratory chains.

Recent work has shown that the Rieske iron-sulfur protein is the immediate electron donor to ferricytochrome c in mitochondrial complex III

(Trumpower and Edwards, 1979; Trumpower et al., 1980) and in the photosynthetic purple non-sulfur bacterium Rhodopseudomonas sphaeroides (Bowyer et al., 1980). Evidence exists for a similar function for the Rieske iron-sulfur protein in other photosynthetic bacteria (Evans et al., 1974; Prince et al., 1975; Bowyer et al., 1979) and in plant chloroplasts (Malkin and Posner, 1978; Malkin and Chain, 1980). Therefore, it is reasonable to expect the Rieske protein in B. alcalophilus (and no doubt B. firmus RAB, although no determination for iron-sulfur proteins was made in this species) also functions as an electron donor to cytochrome c. If so, the disparity between the midpoint potentials of the Rieske protein ($Em_7 \sim +150$ mV) and cytochrome c ($Em_7 = +70$ mV), when titrated at pH 7.0 rather than at pH 9.0, suggests how the function of this part of the respiratory chain might be compromised at the lower pH. Perhaps of equal interest is the possibility that this segment of the chain might be non-functional in the non-alkalophilic strains at their optimal pH. This possibility is supported both by the preliminary (and admittedly tentative) failure to detect a Rieske protein in KM23 and by the reproducible findings of midpoint potentials for the cytochrome c of KM23 and RABN which are higher than those for the cytochrome a of the non-alkalophiles. Thus KM23 and RABN may not have a full complement of energy coupling sites as postulated for a variety of conventional bacteria by Jones et al. (1977). The presence of moderate amounts of cytochrome c in both KM23 and RABN could be vestigial expressions of the much greater levels found in the parent alkalophiles.

A gross measure of the efficiency with which the alkalophiles transduce and utilize the energy derived from substrate oxidation was provided by growth yield determinations. The Ymal values of 42 and 38 obtained for B alcalophilus and B. firmus RAB respectively are entirely comparable with normal growth of conventional bacteria under optimal conditions. In view of the peculiar energy demands described earlier for alkalophilic bacteria, these growth yields suggested particularly efficient energy conservation. In contrast, the relatively low growth yields (less than half) obtained for the non-alkalophilic mutants indicate species severely compromised in their energy transducing functions, possibly totally lacking some of the energy coupling sites present in the alkalophiles. As noted above the alkalophile may similarly be compromised when operating at pH 7.0.

Determination of H^+/O stoichiometries allowed for a more direct measure of the efficiency with which alkalophile respiratory chains transduce oxidation-reduction energies. One of the greatest complications in accurately determining the proton pumping stoichiometry of the respiratory chain lies in the estimation and correction for proton backflow or leakage. The decision to initiate respiratory chain stoichiometry measurements in B. firmus RAB resulted from observations which indicated that larger concentrations of Na^+ are required to operate its Na^+/H^+ antiporter than that of B. alcalophilus (Krulwich et al., 1982). In fact, under the experimental conditions for H^+/O measurements, there should be no proton re-entry through Na^+/H^+ antiport. In both alkalophiles, the Na^+/H^+ antiporter is electrogenic (Mandel et al., 1980; Guffanti et al., 1980), and the presence of K^+ plus valinomycin dissipates the $\Delta\psi$ needed to drive exchange of Na^+ and H^+. Furthermore, because most solute transport systems are coupled to Na^+ in the alkalophile (Guffanti et al., 1981) rather than to H^+, as is more generally found in bacteria (Harold, 1977), proton backflow should be relatively small in these organisms.

Indeed, as can be seen in Figure 24, after an oxygen pulse, the acidification of the medium due to proton ejection persists. Even after several minutes, addition of alkali was necessary to bring the pH back up to its value prior to the oxygen pulse. In comparison, RABN, the non-alkalophilic mutant, displayed much greater proton backflow (Fig. 26). This also is consistent with the above explanation for the wild-type alkalophile. In contrast to the parent strain, the solute symport systems of the non-alkalophile are coupled to protons (Guffanti *et al.*, 1980; Bonner *et al.*, 1982) and these protons may be a substantial part of the "leak". In the first studies conducted by Mitchell to determine proton extrusion stoichiometries, an exponential decay in the rate of proton ejection due to the build-up of a transmembrane proton gradient was anticipated. The biphasic, respiration-dependent proton ejection kinetics observed in *B. firmus* RAB, however, is not as easily explained.

An initial phase of rapid oxygen utilization which follows the delivery of an oxygen pulse to anaerobic mitochondria has been described recently (Reynefarje and Lehninger, personal communication). This early phase is believed to be caused by the differential rates of oxido-reduction catalyzed by the various elements of the respiratory chain. When mitochondria are left anaerobic for a short period of time, all the respiratory chain components become reduced. The cytochrome oxidase is the most rapidly acting enzyme of the respiratory chain. Therefore, following an oxygen pulse, most of the reduced cytochrome c pool that has been built up is rapidly oxidized by the cytochrome oxidase before there is significant electron transport through the rest of the respiratory chain. Since only the terminal segment of the respiratory chain is functional at this time, H^+/O stoichiometries during this very fast early phase are expected to be considerably lower than afterwards when the rest of the respiratory chain is operational.

If the initial rapid phase of proton-linked respiration in B. firmus RAB was caused by a cytochrome oxidase effect of the type just described, then higher H^+/O ratios should have been found during the subsequent slower phase of respiration. The findings were precisely the opposite. H^+/O stoichiometries averaging approximately 9 were calculated for the fast phase compared with an average value below 6 for the later phase. Perhaps the rate of proton ejection evident at this later stage may be severely reduced by backpressure from the transmembrane gradients established by this time and consequent proton backdecay. Were the H^+/O value obtained during the initial fast phase due entirely to cytochrome oxidase, then a truly incredible stoichiometry would have to exist for this respiratory chain segment in these bacteria. The more likely possibility is that a cytochrome oxidase effect may be partially responsible for the observed early phase and that some protons are pumped from other segments during this phase. Even in this case, since a disproportionate amount of oxygen might be consumed by the terminal oxidase (in terms of a total assessment of the entire electron transport chain and expected oxygen utilization), the H^+/O values calculated during the early phase may yet be an underestimation of the true proton pumping capacity of the alkalophile respiratory system.

Another possible cause for H^+/O underestimation might lie in branching of the respiratory chain in these bacteria. Downs and Jones (1975) were able to demonstrate increased H^+/O stoichiometries in Azotobacter vinelandii respiratory chains upon selectively poisoning electron transport through branching segments leading to alternate terminal oxidases.

Especially in view of the large number of cytochrome species identified (and in particular the evidence for a cytochrome o in B. alcalophilus), there is a high probability that electron transport occurs along branching pathways in the alkalophiles, at least under certain conditions.

In order to make the measurements on the initial fast phase of respiration-coupled proton ejection by B. firmus RAB at pH 9.0, or indeed, to even detect this phase, it was crucial to develop special techniques and apparatus that could respond very rapidly (Reynefarje et al., 1982). The use of a membraneless oxygen electrode and a fast noise-free amplifier, both designed and built for this purpose, allowed for very fast and precise measurements of the oxygen consumption. Recording these signals through use of a digital oscilloscope eliminated problems of pen response and chart speed inadequacies frequently associated with strip-chart recorders. Through very rapid mixing, response times were lowered to a point where events occurring as rapidly as 200 msec following an oxygen pulse could be faithfully resolved and recorded concurrent with acceptable resolution of detail. Without this degree of resolution, the initial fast phase measurements on B. firmus RAB at pH 9.0 could not have been made. Indeed, without such resolution, H^+/O traces of B. firmus RAB, at pH 9.0, might have appeared very similar to those obtained for this strain and for RABN at pH 7.0. In these latter cases, considerably lower stoichiometries were obtained. For B. firmus RAB, average H^+/O stoichiometries measured at pH 7.0 were only approximately 4. In the non-alkalophile, H^+/O values of only 4 were also obtained at pH 7.0 despite the fact that this is the optimal pH for growth of this strain.

It is very important to establish that the medium acidification subsequent to an oxygen pulse is in fact due to net vectorial proton translocation and not to some scaler reaction. No protons were ejected into the medium after an oxygen pulse was administered unless valinomycin was included in the reaction medium. This argues strongly in favor of a vectorial process. Presumably, proton ejection can only occur if the $\Delta\psi$ resulting from this translocation is dissipated. K^+ in the presence of valinomycin becomes free to move across the membrane and acts as a charge compensating ion. If the acidification were due to a scaler reaction, valinomycin would not be expected to influence this process. Further validation of the vectorial nature of the observed proton ejection might be obtained by actually monitoring K^+ influx occurring simultaneously with H^+ ejection following an oxygen pulse. If the protons are truly vectorial a 1:1 stoichiometry should exist for this K^+/H^+ exchange.

Even if the preliminary H^+/O values (6-13 with an average value close to 9) reported in this study for _B. firmus_ RAB at pH 9.0 are not underestimates (or even if the higher values are somewhat inflated), they are substantial compared to other values reported for bacteria. As reviewed earlier (see "Literature Review"), H^+/O values in bacteria are generally much lower; stoichiometries greater than 4-6 are exceptional in bacteria. Studies conducted on _P. denitrificans_ resulted in H^+/O ratio determinations generally falling between 6 and 8 using endogenous or NADH linked substrates (Wimpeny, 1970; Scholes and Mitchell, 1970b; van Verseveld and Stouthamer, 1978a,b; Lawford, 1978,1979). A single study reporting an H^+/O ratio of 10 for this species has also been made (Porte and Vignais, 1980).

The H^+/O stoichiometries obtained with mitochondria are generally larger than those obtained with bacteria and ratios as high as 12 have been reported with NADH linked substrates using a number of techniques (e.g., Brand _et al._, 1976a-d; Pozzan _et al._, 1979). Some factors regarding the coupling of respiration to ATP synthesis should be noted in comparing the values obtained in B. firmus RAB to those found in mitochondria. As previously reviewed (see "Literature Review"), some of the $\Delta\tilde{\mu}_{H+}$ generated by mitochondrial respiratory chains must be utilized to effect electrogenic ATP-ADP exchange in order to move the ATP synthesized by the proton translocating F_1F_0-ATPase out of the mitochondria and into the cytoplasm where it is needed. Some researchers conclude that return of a full proton may be required to energize this exchange (Alexandre et al., 1978). In prokaryotes this nucleotide exchange is not necessary. Therefore, all the protons ejected by the alkalophile respiratory chain can be used to drive Na^+/H^+ antiport which is required for cytoplasmic acidification and ATP synthesis.

These "extra" protons may play a crucial role in the ability of alkalophilic bacteria to survive and grow at elevated pH's. In the original formulations of the chemiosmotic hypothesis (Mitchell, 1961), two protons were thought to be translocated through the mitochondrial F_1F_0-ATPase for the synthesis of ATP. Simple thermodynamics demanded a $\Delta\tilde{\mu}_{H+}$ greater than -200 mV to energize this reaction. All determinations in growing alkalophilic bacteria, however, indicate a $\Delta\tilde{\mu}_{H+}$ significantly smaller than that predicted necessary to drive ATP synthesis by translocation of only 2 protons (Guffanti et al., 1978). Indeed, at pH 9.0, a $\Delta\tilde{\mu}_{H+}$ of only -125 ± 15 mV was obtained which, taken together with a phosphorylation potential (ΔGp) of 12 Kcal/mole (equivalent to ∿ 500 mV) implied that 4 protons might be needed for synthesis of each ATP at this pH. Moreover, at the physiological pH 10.5, a $\Delta\tilde{\mu}_{H+}$ of only 40 ± 20 mV was found while the ΔGp calculated under these conditions was 11 Kcal/mole (equivalent to ∿ 460 mV). Assuming strictly chemiosmotic coupling, where the relevant proton fluxes (i.e., between the respiratory chain, which generates the $\Delta\tilde{\mu}_{H^+}$, and the F_1F_0-ATPase which consumes it) are found only in the bulk aqueous phases separated by the cell membrane, as many as 8 or more protons might be needed to effect ATP synthesis at this pH (Guffanti et al., 1981).

Higher H^+/ATP ratios are not, however, the only possible way to explain the discrepancies between $\Delta\tilde{\mu}_{H^+}$ and ΔGp. Discrepancies between the measured $\Delta\tilde{\mu}_{H^+}$'s and the theoretical ΔGp's have been observed in numerous other systems (for review see Kell, 1979; also Michel and Oesterhelt, 1980 and Guffanti et al., 1981). This has led to the formulation of various modifications of Mitchell's theory with respect to the coupling of respiration to ATP synthesis. Williams suggested a model wherein the protons translocated across the membrane by the respiratory chain do not actually leave the membrane but rather remain membrane-associated (Williams, 1961, 1978 a,b, 1979). Several new proposals for different forms of "localized gradients" have also been made (Azzone et al., 1978; Gould, 1979; Van Dam et al., 1978; Padan and Rottenberg, 1973). Kell et al. (1981) have recently proposed a theory whereby the coupling of oxidative phosphorylation is accomplished through a "protoneural network", i.e., a number of membrane proteinaceous components that act "as conformationally switchable proton conductors, permitting fast controlled lateral proton transfer along the surface of energy transducing membranes..." These theories may account for the differences found between the phosphorylation potentials and $\Delta\tilde{\mu}_{H^+}$ values which are measures of bulk transmembrane gradients. Thus, the measured bulk transmembrane gradients may not be the relevant energy coupling mechanism for ATP synthesis and protons seen in the bulk aqueous phase may be mere reflections of the important coupling processes occurring at the membrane. If these mechanisms for energy coupling are responsible for ATP synthesis, then the alkalophile, with its great abundance of respiratory chain components as revealed by this study, may accomplish this coupling particularly well.

The obligate alkalophiles provide particularly productive systems for studying respiratory chains. They possess extraordinary quantities of respiratory chain components, including sufficient amounts of cytochromes, Rieske protein and several other FeS clusters, facilitating study through use of EPR techniques. Furthermore, substantial respiration-linked proton ejection as well as numerous solute transport systems, some fairly unique, have been identified. In these respects, the alkalophiles are approachable using techniques that have been successfully employed with mitochondria. In addition, they provide mutable systems in which regulatory properties may be examined.

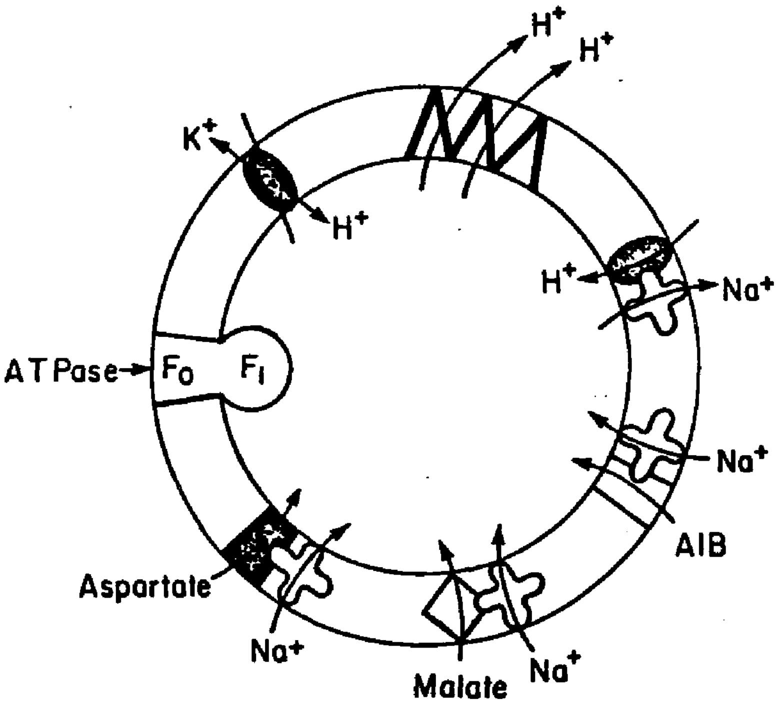
The exact nature of the mutation which changes the alkalophiles into non-alkalophiles remains unknown. Alkalophiles have Na^+/H^+ antiport activity, Na^+-coupled solute transport systems, high levels of membrane cytochromes and a membrane-bound chromophore that absorbs light at 526 nm. Their non-alkalophilic mutant derivatives lack Na^+/H^+ antiport activity, couple transport to protons rather than to Na^+, have decreased cytochrome contents and possess minimal quantities of the chromophore.

A model summarizing the ion and proton movements in membranes of alkalophiles and their non-alkalophilic mutant derivatives is illustrated in Figure 33. Primary proton ejection is the essential function of the respiratory chain. The alkalophile respiratory chain is depicted as being longer than that of the non-alkalophile and also more effective in pumping protons. Proton return, resulting in net acidification of the

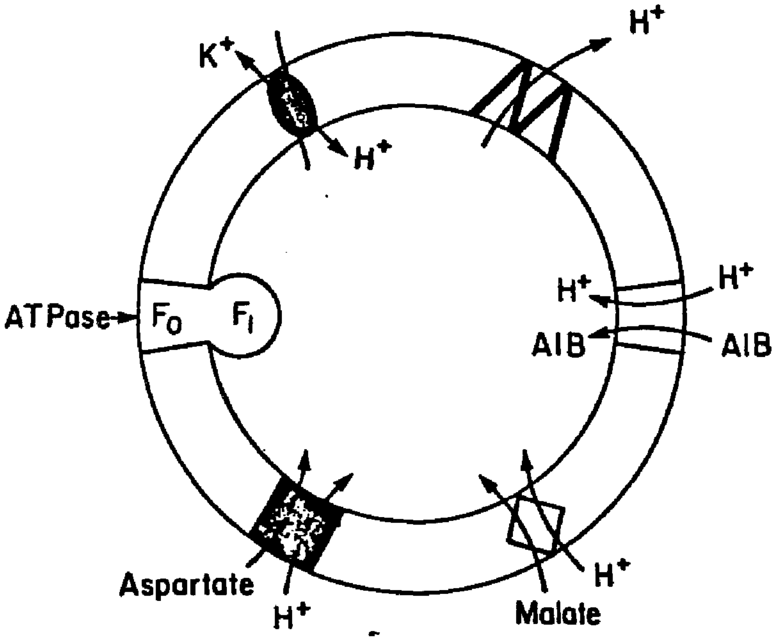
FIGURE 33

A model illustrating several contrasts in bioenergetic properties between alkalophiles and their non-alkalophilic mutants

Longer respiratory chains in the alkalophiles as compared to those in their non-alkalophilic mutants are associated with greater proton pumping. Na^+/H^+ antiport is found only in the alkalophile. Na^+-dependent solute symport is present in the alkalophile. Non-alkalophiles have proton-linked solute transport instead. A Na^+-translocating element common to both the Na^+/H^+ antiporter and each of the solute symporters is proposed. Mutational loss of this suggested element in the non-alkalophile prevents Na^+/H^+ antiport and in some manner allows all of the solute carriers which normally couple to Na^+ in the wild-type alkalophiles, to now couple to protons in these mutants.



ALKALOPHILE



NON-ALKALOPHILE

interior is shown to occur through operation of an electrogenic Na^+/H^+ antiporter. Completion of the Na^+ cycle and solute uptake is accomplished by numerous Na^+/solute symporters. The figures representing Na^+-translocating subunits are shown as common to the Na^+/H^+ antiporter and each Na^+/solute symporter. A mutation in the Na^+ subunit causes loss of Na^+/H^+ antiport, Na^+-coupled solute transport and somehow permits H^+ coupling to solute transport so that Na^+-independent uptake is found at neutral pH in the non-alkalophile.

The observation that all the above properties appeared as an indivisible aggregate, together with spontaneous mutation and reversion frequencies of approximately 1 in 10^9, strongly suggests that a single mutation is responsible for these differences in phenotype. Assuming that a Na^+-translocating subunit couples to both a proton carrier and to each of the individual solute transporters, then it is readily conceivable how mutation in this element could effect both Na^+/H^+ antiport as well as all Na^+-coupled transport systems.

How such a mutation can effect respiratory chain contents is less clear. When grown at pH 10.5, more than 5.5 nmole cytochrome heme/mg membrane protein was measured in the alkalophiles. When the pH for growth was lowered to pH 9.0, however, less than 3.5 nmoles/mg protein was found in *B. alcalophilus* membranes. Of greater significance, the levels of cytochromes in KM23 increased from less than 1.3 nmoles/mg protein to approximately 2 nmoles/mg protein upon shifting the growth pH from 6.8 to 8.5, even though at this higher pH this strain did not grow well. Thus, imposed changes in external pH that presumably lead to some modulation of intracellular pH can have an impact upon cytochrome content. Interestingly, in enteric bacteria, changes in cytoplasmic pH have recently been posited to initiate certain behavioral responses (MacNab and Kihara, 1981; Repaske and Adler, 1981). Perhaps mutational loss of the antiporter changes the typical cytoplasmic pH such that cytochrome contents are then altered as a consequence of changes in gene expression or membrane assembly.

APPENDIX

Oxygen Electrode Amplifier

This solid state amplifier, using a Burr-Brown #3401-A integrated circuit, is based on a design developed by Dr. Philip Davies for use by Dr. Albert Lehninger in his measurements of H^+/O at Johns Hopkins University. It is essentially a low noise amplifier with bucking circuitry and a voltage gain of approximately 1000. Sensitive input and output circuits should be carefully isolated from each other and shielded as necessary. The circuitry associated with the pH reference electrode input, including the 50,000 Ω potentiometer should be thoroughly shielded. These precautions are required to prevent amplifier instability and internal oscillations: All electrode leads must be shielded to avoid pick-up of external electromagnetic fields. All shields must be reliably grounded to the amplifier chassis. A secure, external common ground, e.g., to cold water pipe, is most advisable.

To reduce noise generated by the presence of the stirring magnetic flea and stir plate in close proximity to the O_2 electrode, rejection type electrical filters with maximum rejection frequencies equal to the flea rotations per second can be placed in the leads at the amplifier input and output terminals.

Power requirements:

Regulated DC +15 volts
-15 volts

Ripple frequencies were undetectable when the output of the power supply was placed onto an oscilloscope and amplified to 400 μV full scale.

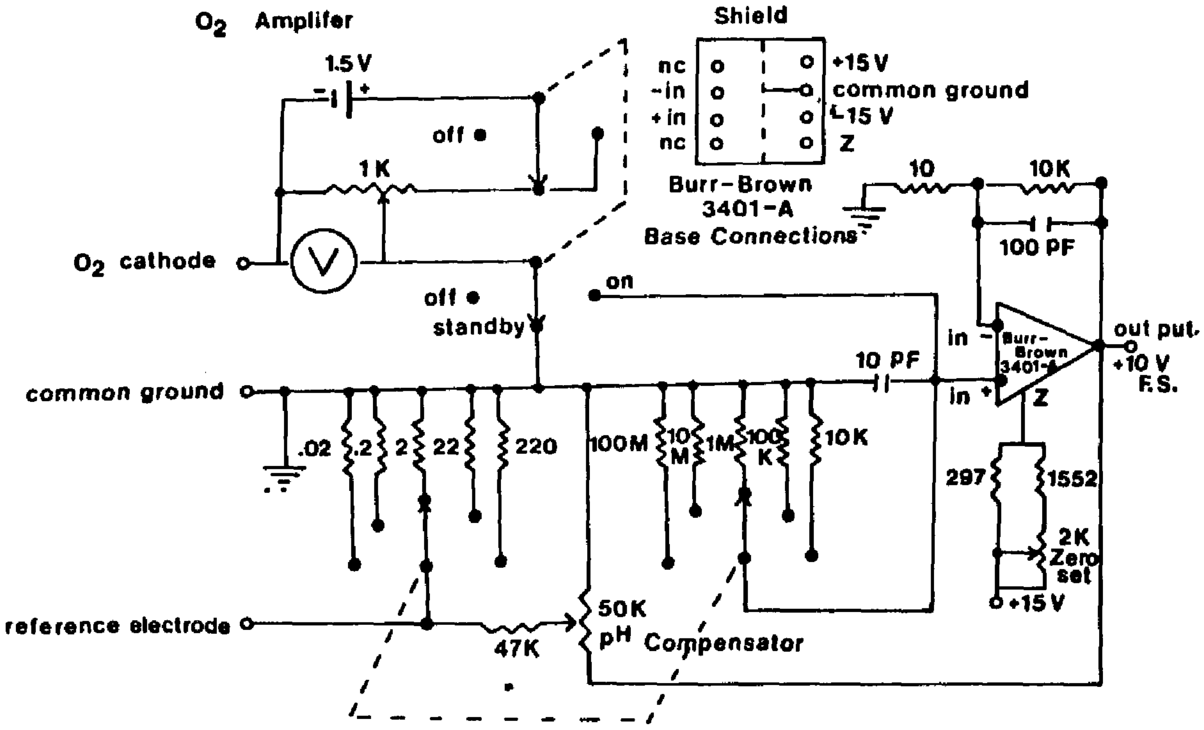


FIGURE 34

Schematic of oxygen amplifier

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