The Role of Respiration-Dependent Proton Translocation in the Acid Tolerance of \emph{Gluconobacter oxydans}

by

Patrice Anne Boerman

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Master of Science
in
Microbiology

APPROVED:

\underline{D. William Claus}
G. William Claus, Chairman

\underline{Noel R. Krieg}

\underline{Allan A. Jousen}

February 24, 1988
Blacksburg, Virginia
The Role of Respiration-Dependent Proton Translocation in the Acid Tolerance of *Gluconobacter oxydans*  

by  
Patrice Anne Boerman  
G. William Claus, Chairman  
Microbiology  

(ABSTRACT)  

ABSTRACT  

*Gluconobacter oxydans* is characterized by extreme acid tolerance and the ability to carry out rapid, single-step polyol oxidation catalyzed by membrane bound dehydrogenases. Experiments were designed to determine whether acid tolerance is associated with rapid polyol oxidation in this organism. Washed cells were exposed to 0.1 M or 0.5 M NaCl at pH 3.20; subsequent alkalinization of the suspending solution suggested a NaCl-dependent flow of protons (H\(^{+}\)) into the cells. Cells were then exposed to NaCl at pH 3.20 followed by the addition of glycerol to determine whether polyol oxidation resulted in H\(^{+}\) expulsion from the cells. Following glycerol addition, immediate acidification of the suspending solution occurred. To verify that H\(^{+}\) efflux was a result of respiration, experiments were conducted using sodium azide and 2,4-dinitrophenol; both compounds prevented the acidification that otherwise occurred following glycerol addition. Because glycerol oxidation reversed the NaCl-induced flow of H\(^{+}\) into the cell, it appeared that respiration might function to protect acid-labile cell interiors. Cells exposed to NaCl at pH 3.20 in the presence of glycerol maintained cellular viability while loss of viability occurred in the absence of glycerol. To verify the effect of H\(^{+}\) extrusion on pH homeostasis, radioactively labeled organic-acid probes were used to determine intracellular pH in respiring and nonrespiring cells in the presence of 0.1 M NaCl at pH 3.20. No differences in cytoplasmic pH values between respiring and nonrespiring cells were detected. However, because substantial evidence exists for the role of respiration-dependent H\(^{+}\) extrusion in the acid tolerance of *G. oxydans*, use of an alternate method for measurement of internal pH, such as \(^{31}\)P nuclear magnetic resonance spectroscopy, is suggested.
I would first like to express my deep appreciation to
Dr. G. William Claus, for his guidance, encouragement, and seemingly infinite patience during the
course of my research.

I would also like to thank my committee members, Dr. Allan A. Yousten and Dr. Noel R. Krieg for their guidance and support during this project. In addition, I would like to thank Dr. John J. Tyson for his assistance with the mathematics involved in this project.

I would like to express my appreciation to several fellow graduate students. My thanks to
Lori Jones Mason for her help in the lab and, more importantly, for her understanding and en­
couragement. I also wish to thank Edie Shepherd, Pamela Vercellone, Michele Pethel and John Groen for their friendship and encouragement.

I would like to acknowledge the Virginia Academy of Science and Sigma Xi for partial sup­port of this project.

Finally, I dedicate this thesis to my parents, Lee D. and Elizabeth A. Boerman, whose love and confidence in my ability to succeed made this achievement possible.
# Table of Contents

## INTRODUCTION

## LITERATURE REVIEW

- General Characteristics of the Gluconobacters ................................... 3
- Respiration Capabilities ................................................... 4
  - Respiration Rates ...................................................... 4
  - Polyol Dehydrogenases .................................................. 4
  - Function of Rapid Polyol Oxidation ........................................ 7
- Chemiosmotic Theory ..................................................... 8
  - Overview ............................................................ 8
  - Primary H⁺ Pumps ..................................................... 9
- Mechanisms of Internal pH Homeostasis ...................................... 10
  - Controlled Transport of H⁺ ........................................... 10
  - Interconversion of ΔpH and Δψ ...................................... 11
  - Cytoplasmic Buffering Capacity ........................................... 15
  - Metabolic Production of Acids and Bases ........................................... 17
- Determination of Internal pH by the Probe Distribution Technique .............. 17
  - Simplifying Assumptions ................................................ 19
- Probe Requirements ................................................... 20

## MATERIALS AND METHODS

- Reagents ............................................................. 21
- Bacterial Cultures ....................................................... 21
- Media and Growth ...................................................... 22
- Proton Translocation Experiments ........................................... 22
- Determination of Cellular Viability .......................................... 23
- Measurement of Cellular Respiration ........................................... 24
- Internal Volume Measurements ................................................ 24
- Internal pH Measurements ................................................ 25

## RESULTS

- Induced H⁺ Translocation Experiments ........................................... 27
- Cellular Respiration Measurements ........................................... 27
- Effect of Glycerol Respiration on Maintenance of Viability .............. 32
- Internal Water Volume Determinations ........................................... 32
- Internal pH Measurements ................................................ 35

## DISCUSSION

- Induced H⁺ translocation experiments ........................................... 41
- Cellular respiration measurements ........................................... 44
- Effect of glycerol respiration on maintenance of viability .............. 44
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal pH measurements</td>
<td>45</td>
</tr>
<tr>
<td>Recommendations for Future Research</td>
<td>51</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>52</td>
</tr>
<tr>
<td>Appendix A. Effect of NaCl on H⁺ Translocation - Replicate Experiments</td>
<td>57</td>
</tr>
<tr>
<td>Appendix B. Effect of Glycerol on H⁺ Translocation - Replicate Experiments</td>
<td>60</td>
</tr>
<tr>
<td>Appendix C. Effect of NaN₃ on H⁺ Translocation - Replicate Experiments</td>
<td>67</td>
</tr>
<tr>
<td>Appendix D. Effect of Glycerol and NaN₃ on Cellular Respiration - Replicate Experiments</td>
<td>70</td>
</tr>
<tr>
<td>Appendix E. Effect of Glycerol Respiration on Cellular Viability Under Acidic Conditions - Replicate Experiments</td>
<td>76</td>
</tr>
<tr>
<td>Appendix F. Internal Water Volume Measurements of <em>Gluconobacter oxydans</em></td>
<td>81</td>
</tr>
<tr>
<td>Appendix G. Internal pH Values of <em>Gluconobacter oxydans</em> in dH₂O at pH 3.20</td>
<td>84</td>
</tr>
<tr>
<td>Appendix H. Internal pH Values of <em>Gluconobacter oxydans</em> in 0.1 M NaCl at pH 3.20</td>
<td>86</td>
</tr>
<tr>
<td>Appendix I. Internal pH Values of <em>Gluconobacter oxydans</em> in NaCl + KCl at pH 3.20</td>
<td>88</td>
</tr>
<tr>
<td>CURRICULUM VITA</td>
<td>90</td>
</tr>
</tbody>
</table>
List of Illustrations

Figure 1. Effect of NaCl on H\(^+\) movement into \textit{G. oxydans} ........................ 28
Figure 2. Effect of glycerol on H\(^+\) translocation by \textit{G. oxydans} ......................... 30
Figure 3. Effect of NaN\(_3\) on H\(^+\) translocation from \textit{G. oxydans} ....................... 31
Figure 4. Effect of glycerol and NaN\(_3\) on oxygen consumption by \textit{G. oxydans} ............ 33
Figure 5. Effect of respiration on maintaining viability of \textit{G. oxydans} ......................... 34
List of Tables

Table 1. Internal pH values of *G. oxydans* in dH₂O at pH 3.20 ..................... 36
Table 2. Internal pH values of *G. oxydans* in 0.1 M NaCl at pH 3.20 ................. 37
Table 3. Internal pH values of *G. oxydans* in NaCl + KCl at pH 3.20 ................. 39
INTRODUCTION

The genus *Gluconobacter* belongs to the family *Acetobacteraceae* (17) and is composed of chemoorganotrophic, gram negative, rod-shaped cells which are motile by three to eight, or rarely one, polar flagella or are nonmotile (18). The gluconobacters are strictly respiratory organisms for which oxygen serves as the sole terminal electron acceptor (18). Membrane-bound dehydrogenases catalyze rapid oxidation of a wide variety of organic compounds. Of particular interest is their ability to oxidize polyhydroxy (polyol) compounds with stoichiometric release of the oxidation product into the growth medium (5, 18). Polyol oxidation rates by *G. oxydans* are the second highest measured in bacteria (78). Despite the rapid respiratory quotients, *Gluconobacter* are characterized by extremely low P/O ratios (40). This genus is also characterized by extreme acid tolerance, most strains will grow at pH 3.6 (18), and growth has been demonstrated at pH 2.5 (55, 56).

We presume that rapid oxidations, catalyzed by membrane-bound dehydrogenases in *G. oxydans*, provide electrons to the electron transport chain. In accordance with Mitchell’s chemiosmotic theory (25), electron transport through a respiratory chain is accompanied by the outward translocation of $H^+$ from cell interiors. The combination of high respiratory quotients and the extremely low P/O ratios characteristic of *G. oxydans* suggested an inefficient mechanism for
coupling the energy generated from electron transport with oxidative phosphorylation. This led Heefner (31) to speculate that energy generation might not be the sole function of limited polyol oxidation. He questioned whether rapid polyol oxidation, catalyzed by membrane-bound dehydrogenases, was related to acid tolerance in *G. oxydans*.

Heefner (31) found that when washed cells were exposed to 0.15 M NaCl at pH 3.20, an increase in the pH of the reaction mixture occurred that suggested NaCl-dependent entry of protons (H\(^+\)) into cells. Electron microscopy, gel electrophoresis, and enzyme assays indicated congealing of the cytoplasm, loss of intracellular proteins, and loss of soluble enzyme activity under these conditions. When respirable substrate was added to cells in 0.15 M NaCl at pH 3.20, the pH of the reaction mixture decreased, indicating H\(^+\) extrusion from cells. Exposure of respiring cells to acidic NaCl did not result in congealing of cell interiors, denaturation of intracellular proteins, or loss of soluble enzyme activity. Heefner (31) proposed that respiration-driven H\(^+\) extrusion functions, in part, to maintain internal pH near neutrality under the acidic conditions typically occupied by *G. oxydans*.

The goals of the current investigation were to use improved methods to verify the data reported by Heefner (31), and to provide additional evidence for the role of respiration-dependent H\(^+\) translocation in the acid tolerance of *G. oxydans*. Meeting these goals involved (i) repetition of H\(^+\) translocation and cellular viability experiments previously conducted by Heefner (31) (ii) oxygen uptake determinations to verify the link between respiration and H\(^+\) extrusion and (iii) internal pH measurements based upon the transmembrane distribution of radioactively labeled organic-acid probes.
LITERATURE REVIEW

General Characteristics of the Gluconobacters

The genus *Gluconobacter* is placed in the family *Acetobacteraceae* (17) and is composed of chemoorganotrophic, gram-negative, rod-shaped cells which are motile by means of three to eight, or rarely one polar flagella or are nonmotile (18). They are strictly respiratory organisms for which oxygen serves as the only terminal electron acceptor (18). Despite being obligate aerobes, they lack an operational TCA cycle due to the absence of succinate dehydrogenase (23, 24) and use the remaining enzymes exclusively for biosynthesis (24). They oxidize ethanol to acetic acid but do not oxidize acetate to CO₂ and H₂O (18). Catabolism of sugars and polyhydroxy compounds (polyols) is accomplished by means of the hexose-monophosphate pathway, although enzymes of the Entner-Doudoroff pathway have been demonstrated (18). Members of this genus are capable of oxidizing a wide variety of organic compounds (5). Of particular interest is their ability to partially oxidize sugar and polyols with stoichiometric release of the corresponding acids and ketoses into the growth medium (5, 18). Industrially important reactions of this type include the oxidation of sorbitol to sorbose (5) and the conversion of glucose to gluconate and 2-and 5-ketogluconates (77). The optimum growth pH is from 5.5 to 6.0 although most strains will grow at pH 3.6 (18), and
growth has been demonstrated at pH 2.5 (55, 56). *Gluconobacter* species are typically found in fruits, vegetables, cider, wine vinegar, wine, and other environments containing high concentrations of sugars and/or alcohols (18).

**Respiration Capabilities**

**Respiration Rates**

According to White and Claus (79), respiration rates in *G. oxydans* are the second highest found in bacteria, exceeded only by those reported for *Azotobacter* (73). Under optimal conditions for the oxidation of sorbitol to sorbose, oxygen quotients of 1,800 and 3,100 μl O₂/mg dry cell weight/hour were measured in exponential phase and maximum stationary phase cells, respectively. Comparable rates were also found for single-step oxidations of glycerol, glucose, and mannitol. These rapid, partial oxidations, which are associated with extremely high rates of oxygen uptake, are catalyzed by substrate specific, membrane-bound polyol dehydrogenases.

**Polyol Dehydrogenases**

The ability of *G. oxydans* to oxidize a wide variety of substrates derives from two types of polyol dehydrogenases; NAD(P)-dependent dehydrogenases isolated from the soluble fraction of cell-free extracts, and NAD(P)-independent dehydrogenases contained in the particulate fraction of cell-free extracts. The particulate enzymes are integral plasma-membrane proteins and are asso-
associated with cytochromes. Only the particulate dehydrogenases will be discussed since they catalyze the rapid, single-step oxidations associated with unusually high oxygen quotients.

As reviewed by Asai (5), Bertrand determined that the single-step oxidations, characteristic of \textit{G. oxydans} and other acetic acid bacteria, were highly substrate specific. These reactions require sugar alcohols with two \textit{cis} secondary alcohols adjacent to the primary alcohol. Hudson later determined that the pair of secondary alcohols must be in the D-configuration. Together, these requirements are known as the Bertrand-Hudson rule. The majority of oxidations by \textit{G. oxydans} are in accordance with this rule (5).

Glucose dehydrogenases are of major importance to the gluconobacters. Butlin (11, 12) first described a glucose dehydrogenase isolated from the particulate fraction of cell-free extracts. It is acid-stable with a pH optimum of 5.0, and catalyzes the single-step conversion of glucose to gluconic acid. Because this membrane-bound enzyme should be functional in the acid habitats typical of \textit{G. oxydans}, Butlin suggested that these limited oxidations provide energy for growth. Ameyama \textit{et al.} (3), later purified D-glucose dehydrogenase and found it to be active from pH 3.0 to 6.0. These authors suggest that the wide pH range was to be expected since this is a key enzyme for \textit{Gluconobacter} species.

Additional polyol and alcohol dehydrogenases have also been identified from \textit{G. oxydans}. Kersters \textit{et al.}, (39) identified at least one dehydrogenase in the particulate fraction capable of oxidizing polyols with \textit{L-erythro} configurations. These ultramicroscopic membrane particles catalyzed the single-step oxidation of polyols with the Bertrand-Hudson configuration confirming that limited oxidations during growth of \textit{G. oxydans} are catalyzed by the particulate dehydrogenases. Kersters and De Ley (38) found four enzymes involved in the oxidation of aliphatic glycols in the acetic acid bacteria. These include two particulate systems containing one or more polyol dehydrogenases, and primary and secondary alcohol dehydrogenases, all with optimum pH activity at around 6.0. Because the same oxidation products were produced by whole cells
and by the particulate fraction, the authors concluded that the particulate fraction is responsible for
glycol oxidation. Arcus and Edson (4), identified a particulate enzyme from *G. oxydans* that
catalyzes the single-step oxidations of mannitol to fructose and sorbitol to sorbose. This enzyme
has a pH optimum of 5.4 and is cytochrome-linked. Baker and Claus (6) used
*n*-octyl-β-D-glucoside to solubilize the particulate dehydrogenase responsible for oxidation of D-
sorbitol to L-sorbose by *G. oxydans* and determined the pH optimum for this enzyme to be 5.2.
Shinagawa *et al.* (70), isolated a D-sorbitol dehydrogenase bound to a c-type cytochrome with a
pH optimum of 4.5. Adachi *et al.* (1), isolated a particulate alcohol dehydrogenase-cytochrome-ε
complex with a pH optimum of 5.0 to 6.0.

Adachi *et al.* (2), isolated an aldehyde dehydrogenase *G. oxydans* also tightly bound to a c-
type cytochrome with maximum activity at pH 4.0.

Gluconate dehydrogenase and 2-ketogluconate dehydrogenase were isolated from the
particulate fraction of *G. oxydans* by Chiyonobu *et al.* (13). Mowshowitz *et al.* (53), isolated a
particulate fructose-5-dehydrogenase from *Gluconobacter cerinus* which catalyzed the conversion
of D-fructose to 5-keto-D-fructose as low as pH 4.0.

Numerous other particulate dehydrogenases have been identified from *Gluconobacter* species.
These enzymes are also characterized by pH optima in in acid range and by the ability to rapidly
oxidize a wide variety of polyols. The reader is referred to Asai (5) and Kersters and De Ley (38)
for more complete reviews on particulate dehydrogenases in *G. oxydans*. 
Function of Rapid Polyol Oxidation

*G. oxydans* has extremely low P/O ratios of 0.09 to 0.4 (40). The combination of high respiratory quotients and low P/O ratios suggest an inefficient mechanism for coupling the energy generated from electron transport with oxidative phosphorylation. This led to speculation that energy generation might not be the sole function of limited polyol oxidation (31). Heefner (31) questioned whether rapid polyol oxidation, catalyzed by particulate dehydrogenases, was related to acid tolerance in this organism. He found that when washed cells were exposed to 0.15 M NaCl at pH 3.20, an increase in the pH of the reaction mixture occurred that suggested a NaCl-dependent movement of H⁺ into the cells. Electron microscopy, gel electrophoresis, and enzyme assays indicated congealing of the cytoplasm, denaturation of intracellular proteins, and loss of soluble enzyme activity under these conditions. When respirable substrate was added to cells in 0.15 M NaCl at pH 3.20, the pH of the reaction mixture decreased, indicating H⁺ extrusion from cells. However, congealing of cell interiors, denaturation of intracellular proteins and loss of enzyme activity did not occur in respiring cells under the same conditions.

We presume that rapid oxidations catalyzed by the acid-stable, particulate dehydrogenases in *G. oxydans* provide electrons to the respiratory chain. In accordance with Mitchell’s chemiosmotic theory (25), electron transport through a respiratory chain is accompanied by the outward translocation of H⁺ from cell interiors. Heefner (31) postulated that H⁺ extrusion during polyol oxidation functions to maintain internal pH near neutrality under the acidic conditions typical of *G. oxydans*. 

LITERATURE REVIEW 7
**Chemiosmotic Theory**

**Overview**

It is well established that bacteria conserve metabolic energy by means of an electrochemical gradient of (H\(^+\)) across the cytoplasmic membrane. The following information was summarized from Harold's (25) review, and the reader is referred to that paper for a more in-depth presentation. According to Mitchell’s chemiosmotic theory, electron transport through a respiratory chain is accompanied by the outward translocation of H\(^+\) from the cell. This vectorial transport establishes a concentration gradient of H\(^+\) across the membrane. Because the H\(^+\) gradient involves an ion rather than a neutral molecule, it has both a chemical component and an electrical component.

The chemical or pH gradient, results from the difference in H\(^+\) concentration between the cell interior and exterior and is referred to as \(\Delta \text{pH}\). The difference in electrical potential across the membrane results from the outward translocation of the positively charged hydrogen ions and is called \(\Delta \psi\). Both \(\Delta \text{pH}\) and \(\Delta \psi\) exert a force on the extruded H\(^+\) called the proton motive force (\(\Delta p\)) and are related as follows:

\[
\Delta p = \Delta \psi - Z \Delta \text{pH}
\]

where \(\Delta p\) is expressed in mV. The constant Z converts pH units into mV and is equal to 2.303RT/nF where R is the gas constant, T is the absolute temperature, n equals the charge of the transported ion, and F is Faraday’s constant. The value for Z is 59 at 25°C.

According to Mitchell (25), \(\Delta p\) is the means by which energy generated during substrate oxidation is conserved and subsequently coupled to energy requiring membrane-associated proc-
Proton motive force-dependent processes include ATP synthesis via H\(^+\) translocating ATPases, numerous solute-transport systems, and bacterial motility (26, 27).

Energy transduction, the conservation of energy and its subsequent use in endergonic plasma-membrane associated processes, by a chemiosmotic mechanism is dependent upon the following: (i) a topologically-closed cell membrane impermeable to most ions, including H\(^+\) and OH\(^-\) except at specific sites within the membrane (ii) a respiratory, or redox, chain consisting of alternating hydrogen and electron carriers arranged across the cytoplasmic membrane and (iii) a reversible H\(^+\) translocating ATPase located within the cell membrane. Redox chains and ATPases are two examples of primary H\(^+\) pumps.

Primary H\(^+\) Pumps

According to Rosen et al. (66), primary H\(^+\) pumps couple chemical energy directly to the performance of work by means of an electrochemical gradient, or in the reverse mode, couple electrochemical potential to chemical bond formation. Four types of primary pumps are known, two of which will be discussed.

The redox chains located in bacterial plasma membranes are one type of primary pump. These catalyze vectorial dehydrogenation of substrate molecules. Hydrogen is removed from the donor on the inner surface of the cell membrane and carried to an acceptor on the outer surface. Electrons return to the inner surface and H\(^+\) are released to the cell exterior. The H\(^+\)-translocating ATPases also located in bacterial plasma membranes are another type of primary pump. These ATPases catalyze the reversible translocation of H\(^+\) in association with ATP synthesis or hydrolysis according to the following equation:
Mechanisms of Internal pH Homeostasis

It is well established that most bacteria maintain an intracellular pH near neutrality over a wide range of external pH values (8, 33, 34, 54, 57, 58, 61, 62). Typically, the internal pH range of acidophiles is 6.5 to 7.0; neutrophiles vary from 7.5 to 8.0; and alkalophiles range between 8.4 and 9.0 (9, 59). The regulation of internal pH is crucial since the activities of most intracellular enzymes are limited to a narrow pH range. Harold and van Brunt (28) initially demonstrated the requirement for constant intracellular pH for optimum growth of *Streptococcus faecalis*. They found that an alkaline cell interior was required for rapid growth and that lowering internal pH decreased the growth rate in this organism. Padan et al. (58) first demonstrated maintenance of constant internal pH using *Escherichia coli*. They found that respiring, intact cells maintain an approximately constant \( \Delta \rho \) variably composed of \( \Delta \rho \text{H} \) and \( \Delta \psi \). The relative contributions of \( \Delta \rho \text{H} \) and \( \Delta \psi \) were dependent upon the external pH, which ranged from 6.7 to 7.9, and resulted in a constant internal pH.

Controlled Transport of H\(^+\)

Primary H\(^+\) pumps are indicated in the maintenance of internal pH (57, 59). Constant internal pH with variation in external pH has been demonstrated in membrane vesicles (50, 63) as well as intact cells (8, 33, 34, 54, 57, 58, 62, 63). From this, it has been concluded that the mech-
anisms for maintaining pH homeostasis are located within the plasma membrane (57). The use of respiratory inhibitors provides substantial evidence for the involvement of the electron transport chain as a primary H$^+$ pump in internal pH homeostasis since H$^+$ equilibrate across the membrane in the presence of inhibitors (57, 59).

Padan et al. (59) suggest that because the primary H$^+$ pumps maintain the internal pH, they are also involved in the generation of $\Delta \rho$. However, as discussed by Booth (9), the activity of the primary H$^+$ pumps is constrained by the magnitude of the $\Delta \rho$ generated.

**Interconversion of $\Delta pH$ and $\Delta \psi$**

Since $\Delta \rho$ remains approximately constant over the growth range pH in many organisms (20, 33, 37, 57, 58, 59), the relative contributions of $\Delta pH$ and $\Delta \psi$ must vary so as to produce a constant internal pH (35, 57, 59). Therefore, changes in $\Delta pH$ resulting from changes in the external pH must be compensated for by changes in $\Delta \psi$ (35, 57). When the external pH is alkaline, the $\Delta pH$ component of $\Delta \rho$ is small and $\Delta \psi$ is large. As the external pH decreases, $\Delta pH$ will increase while $\Delta \psi$ decreases (35). Dissipation of $\Delta \psi$ at acidic pH is required for the generation of a large $\Delta pH$ (9). The electrogenic translocation of K$^+$ into cells appears to be the means by which $\Delta \psi$ is dissipated when the external pH is acidic (7, 8, 9, 19, 26, 30, 35, 36, 37, 42, 43, 57, 59, 60, 76).

The interdependence of K$^+$ and establishment of $\Delta pH$ was first shown in glycolizing S. faecalis (9). Sodium-loaded cells in K$^+$-free medium formed only minimal $\Delta pH$ at all external pH values. After the addition of K$^+$, $\Delta pH$ was established based upon the external pH. Dependence on K$^+$ transport in S. faecalis was also shown in mutants with reduced H$^+$ extrusion and K$^+$ uptake systems (7). These cells were able to grow at pH 7.5 but not at pH 6.0 suggesting that the
inability to generate $\Delta pH$ interfered with growth under acidic conditions. This occurred whether formation of $\Delta pH$ was prevented directly by the inability to extrude $H^+$ or by the inability to accumulate $K^+$.

Dependence on a constant internal $pH$ of 7.6 to 7.8 for growth has been shown in *S. faecalis* (28, 42, 43). At low external $K^+$ concentrations, however, the internal $pH$ is not regulated normally and is lower than 7.6 (8, 30, 43). A $K^+$ concentration of greater than 0.5 mM is required for normal regulation of internal $pH$ (43).

As reviewed by Booth (9), a mutant of *S. faecalis* with a two-fold decrease in $K^+$ uptake activity has an impaired capacity to generate $\Delta pH$. He also discusses work by Kobayashi *et al.* (43) who used mutants incapable of extruding $Na^+$ but who accumulated $Na^+$ by leakage in response to $\Delta \psi$. In these organisms, $Na^+$ can substitute for $K^+$ in the generation of $\Delta pH$. The authors interpret this to mean an indirect role for cations in the dissipation of $\Delta \psi$. Booth (9) concludes that generation of $\Delta pH$ depends upon the combination of $H^+$-translocating ATPases and $K^+$ transport.

Bakker and Harold (7) determined that $K^+$ transport in *S. faecalis* required ATP and $H^+$ circulation. Internal $K^+$ concentrations up to 50 mM observed, too high to be in response to $\Delta \psi$. They also determined $K^+$ uptake to be electrogenic.

Kobayayashi *et al.* (43), showed that generation of a $pH$ gradient after the addition of glucose to *S. faecalis* was accompanied by internal $K^+$ accumulation. As the glucose supply was exhausted, the acidity of the cell interior increased and $K^+$ accumulation decreased. These investigators also showed that mutants deficient in the ability to accumulate $K^+$ slowly generated a minimal $\Delta pH$. The authors concluded that internal $pH$ is controlled by $H^+$-translocating ATPases but the production of $\Delta pH$ requires cation flux, particularly electrogenic $K^+$ uptake. Subsequent investigations by Kobayashi *et al.* (45) indicate an amplification of ATPase activity under conditions of
low cytoplasmic pH caused by low external K$^+$ concentrations. In two subsequent studies (41, 44), Kobayashi and colleagues confirmed that internal pH is controlled solely by ATPase with a dependence on K$^+$ influx, and that the primary function of the ATPase is the regulation of cytoplasmic pH.

Bakker and Mangerich (8) found that the addition of K$^+$ to K$^+$-depleted S. faecalis resulted in depolarization of the membrane by 60mV. This was compensated for by an increase in $\Delta$pH such that $\Delta$p remained constant at 120 mV. Similar, although somewhat smaller, changes were observed in E. coli. These authors concluded that K$^+$ transport is electrogenic, and, therefore thus depolarizes the plasma membrane and facilitates continued H$^+$ extrusion in both organisms. Using E. coli K12, Padan et al. (58), also demonstrated depolarization of the membrane concomitant with K$^+$ uptake. An increase in the respiratory rate was observed and resulted in an increase in internal pH.

Potassium ions also appeared to be involved in raising the cytoplasmic pH in E. coli after experimental lowering of the external pH (72). After a rapid decrease in external pH from 7.0 to 5.6, the internal pH decreased by approximately 0.4 units in 30 seconds. In K$^+$-replete cells, recovery of internal pH took three to four minutes (71). Recovery was slowed in cells lacking K$^+$ in the external medium and in mutants without K$^+$ transport systems (5). Kroll and Booth (46) also demonstrated the limited ability of E. coli to raise internal pH in the absence of K$^+$. The internal pH was lowered by washing K$^+$-depleted cells pH 5.3 buffer. These cells were incubated with glucose at various external pH values from 6.4 to 7.6 and produced minimal pH gradients. During subsequent K$^+$ uptake, however, the internal pH increased from 6.5 to 7.6 in 45 seconds.

Evidence suggests that K$^+/H^+$ exchange is responsible for maintenance of a constant internal pH in Paracoccus denitrificans (19). In this organism, K$^+$ accumulation occurred in conjunction with H$^+$ extrusion. Potassium-replete, respiring cells acidified the external medium until the oxygen supply was depleted indicating respiration-dependent H$^+$ extrusion. The addition of K$^+$ to K$^+-$
depleted cells was also followed by H\(^+\) extrusion as indicated by a decrease in the external pH. Depolarization of the membrane indicates that K\(^+\) accumulation is electrogenic.

When *Vibrio alginolyticus* was incubated in the presence of K\(^+\), \(\Delta p\)H and \(\Delta \psi\) varied with external pH (76). At acidic external pH, K\(^+\)-depleted cells produced minimal \(\Delta p\)H. The addition of K\(^+\) produced partial dissipation of \(\Delta \psi\) and \(\Delta p\)H dependent on the external pH. The collapse of \(\Delta \psi\) and the magnitude of \(\Delta p\)H decreased with increasing external pH such that these values were approximately the same as obtained in cells with adequate K\(^+\). These authors contend that K\(^+\) transport controls the interconversion of \(\Delta p\)H and \(\Delta \psi\) in accordance with external pH. In addition, they conclude K\(^+\) influx is electrogenic and allows for increased H\(^+\) extrusion.

The concentration of K\(^+\), as well as oxygen tension, regulate the contribution of \(\Delta \psi\) and, therefore \(\Delta p\) in *Bradyrhizobium* species (21). In K\(^+\)-depleted cells, the internal pH was lower than in cells with adequate K\(^+\). After K\(^+\) addition, the intracellular pH increased. The authors contend that K\(^+\) uptake causes a partial dissipation of \(\Delta \psi\) thus facilitating additional H\(^+\) extrusion via the respiratory chain. They also suggest that the decreased \(\Delta \psi\) prevents subsequent reentry of H\(^+\).

The addition of K\(^+\) to nongrowing glucose- or arginine-energized *Streptococcus lactis* caused an increase in internal pH and a decrease in \(\Delta \psi\) (36). The same results were obtained in growing *S. lactis* cells (37). These authors suggest K\(^+\) movement is one of the principal factors regulating the interconversion of \(\Delta p\)H and \(\Delta \psi\) in this organism.

The preceding information provides substantial evidence for the role of cation transport, particularly electrogenic K\(^+\) uptake, in the generation of \(\Delta p\)H at acidic external pH. It can be concluded that, as positively charged ions are translocated inward, dissipation of the transmembrane electrical potential results and allows for additional pumping of H\(^+\) from the cell interior.
The mechanisms by which $K^+$ is transported, however, are not yet known (9, 26, 57, 74). As reviewed by Rosen (65), it has been determined that *E. coli* has at least two $K^+$ transport systems which are thought to be involved in internal pH regulation. According to Stewart *et al.* (74), the Trk system has a low affinity for $K^+$. The Trk system requires $\Delta \psi$ for operation and is regulated by ATP but does not require this compound as the energy source for $K^+$ transport. The Kdp system of *E. coli* appears to be a $K^+$-ATPase that is derepressed by growth in low external $K^+$ concentrations when requirements cannot be met by the Trk system. This transport system has a sufficiently high affinity for $K^+$ that most external solutions contain enough contaminating $K^-$ to saturate the system. The Kdp system requires ATP as an energy source for $K^+$ uptake. Multiple $K^+$ transport systems have been found in other bacteria as well (19, 29, 52) although the mechanisms by which they operate are not completely understood.

According to Harold and Kakinuma (29), bacteria typically use these systems to accumulate $K^+$ to concentrations of 500 mM or greater despite external $K^+$ levels as low as 50 $\mu$M. Requirements for a large internal $K^+$ pool, other than for cytoplasmic pH control, have not been identified (29).

**Cytoplasmic Buffering Capacity**

Although the controlled transmembrane movement of $H^+$ is the primary mechanism by which internal pH homeostasis is maintained, cytoplasmic buffering capacity plays a secondary role (5). The data presented by Booth (9) indicate similar buffering capacities despite wide variations in growth range pH and the different modes of metabolism of the organisms studied. Booth (9) contends that amino acid side chains of cellular proteins are the major component of the internal buffering capacity and that they have only limited ability to offset changes in internal pH.
Sanders and Slayman (68) cite results from various types of whole cells which indicate that internal buffering capacity increases with decreasing internal pH. They suggest the major buffering compounds have pK values much more acidic than the internal pH and that this is consistent with the idea that amino acid side chains are responsible for internal buffering. Since all side chains except histidyl have pK values outside of the range of pH 6 to 8, the minimum internal buffering capacity will be at normal internal pH and the maximum capacities at higher and lower pH. Finally, they suggest that internal buffering is not a true regulator of pH homeostasis except during minor fluctuations of internal acidity or alkalinity.

Roos and Boron (64), in an extensive review of intracellular pH in eucaryotic cells, suggest that acid stress is alleviated both by removal of H+ by metabolic processes and by the action of internal buffering. However, they add that physiochemical buffering functions only in partial and short-term alleviation of acid stress and that buffering is of limited capacity sufficient only to minimize decreases in internal pH.

Krulwich et al. (47), found that the internal buffering capacity of a thermoacidophile, *Bacillus acidocaldarius*, was not significantly higher than *E. coli* or the thermophilic neutrophile *Bacillus stearothermophilus* in the acidic pH range. These authors also found that the buffering capacity measured in *Bacillus subtilis* was by far the highest of those organisms tested. No evidence has been presented suggesting the pH range of *B. subtilis* has been expanded due to high internal buffering capacity (9).

From this evidence, it appears that cytoplasmic buffering capacity does not play a significant role in maintaining constant internal pH in neutrophilic organisms.
Metabolic Production of Acids and Bases

In addition to controlled transport of H⁺ and cytoplasmic buffering, Booth (9) suggests that the metabolic production of H⁺ and OH⁻ may be a mechanism for cytoplasmic pH regulation in bacteria. Although such a system has been demonstrated in plant cells, there is no evidence indicating that H⁺ or OH⁻ generation acts to regulate internal pH in bacterial cells (9). The production of acids and bases appears to be more important in perturbation of intracellular pH than in its regulation (9, 35).

In summary, it is generally accepted that the internal pH of neutrophilic bacteria is regulated predominately by H⁺ extrusion via respiratory chains or H⁺ ATPases and cation influx at low external pH.

Determination of Internal pH by the Probe Distribution Technique

The distribution of weak organic-acid probes is frequently used for determining internal pH when the cell interior is alkaline relative to the exterior, and this technique has been reviewed thoroughly (9, 32, 57, 59, 67). The distribution method is based on the assumption that the membrane is permeable to the lipophilic, undissociated form of the probe but impermeable to the hydrophilic, dissociated form. The undissociated probe equilibrates across the membrane, dissociation occurs in the alkaline cell interior, and the anions are retained within the cell.
After equilibration of the probe, cells are separated from the external media by centrifugation or filtration (32, 57). A disadvantage of the centrifugation method is the development of anaerobic conditions within the pellet (32) which leads to an underestimation of the probe accumulation ratio in respiring cells (57). Aerobic conditions may be maintained during brief centrifugation by adding catalase and \( \text{H}_2\text{O}_2 \) to the reaction mixture (32). An additional disadvantage of the centrifugation method is that significant and variable volumes of medium are trapped within the pellet (32) leading to an overestimation of probe retained by the cells. Rapid separation of cells from external medium by filtration allows cells to remain aerobic unless the cell density is high (32). The sensitivity of the filtration method is limited, however, because the filters are clogged by bacterial concentrations as low as 100 \( \mu \text{g} \) cell protein (57). In addition, significant and inconsistent volumes of medium are retained on the filters (32).

In order to determine the concentration ratios necessary for calculating \( \Delta p\text{H} \), knowledge of the internal cell volume is required (32, 57, 67). Internal cell volume measurements are typically made using the dual radioisotope labeling method of Stock (75). This technique is based upon the distribution of \( ^3\text{H}_2\text{O} \) and a \( ^{14}\text{C} \)-labeled plasma-membrane impermeant polymer which represent the total and extracellular volumes, respectively. The difference between the two values is the internal cell volume.

If the external \( p\text{H} \) is more than one \( p\text{H} \) unit above the \( pK_a \) of the weak-acid probe, essentially all of the acid is dissociated both inside and outside of the cell. This allows calculation of the \( \Delta p\text{H} \) according to the following equation:

\[
\Delta p\text{H} = \log \left( \frac{[A^-]_{\text{in}}}{[A^-]_{\text{out}}} \right)
\] (1)

According to Krulwich and Guffanti (48), the choice of probe is especially important when measuring the internal \( p\text{H} \) in acidophiles. This consideration necessarily extends to measuring the internal \( p\text{H} \) of neutrophiles under very acidic conditions as well. The external \( p\text{H} \) should be
sufficiently greater than the pKa of the probe to allow 90-99% dissociation of the probe both in and out of the cell (57). However, when the pH of the reaction mixture is near or below the pKa of the acid, the concentration of undissociated acid is significant and must be corrected for (59) by means of the following equation (14):

$$pH_{in} = pK_a + \log \left( \frac{[HA + A^-]_{in}}{[HA + A^-]_{out}} \times (10^{PH_0 - pK_a} + 1) - 1 \right)$$  \hspace{1cm} (2)

When the external pH is within one pH unit of the pKa or is below the pKa of the acid, the accumulation ratio of the acid probe inside the cells to that outside of the cells, is less sensitive to \(\Delta pH\). This insensitivity to internal pH results from a differential increase in the undissociated acid that accumulates inside the cell in response to the chemical potential (57). In addition, the accumulation ratio will be small and difficult to measure accurately under conditions of limited dissociation (48). The problem is compounded since any error in the accumulation ratio is magnified as the value of the correction factor increases (5).

**Simplifying Assumptions**

When using the distribution of organic-acid probes to measure  \(\Delta pH\), the following simplifying assumptions are made (32): (i) equilibrium of the probe across the membrane is assumed to exist, (ii) because ion concentrations are used in calculations rather than ion activities, the two are assumed to be equal, (iii) the probes are assumed to be free in solution, (iv) one cellular compartment is assumed to exist.
Probe Requirements

According to Kashket (32) and Krulwich and Guifanti (48), the probes used in determining \( \Delta \text{pH} \) must (i) be nonmetabolizable, (ii) diffuse passively across the cytoplasmic membrane, (iii) be used at \( \mu \text{M} \) concentrations in order to avoid dissipation of the gradient, and (iv) should not be bound by cellular components. Nonspecific binding must be corrected for if it occurs. If one or more of these requirements are not met under the conditions in which internal pH is being measured, inaccuracies in the values obtained will result.

During the current investigation, the distribution of weak organic-acid probes, as well as other procedures, have been used in attempt to determine whether internal pH homeostasis is maintained by \( G. \text{oxydans} \) and, if so, by what mechanisms this is accomplished.
MATERIALS AND METHODS

Reagents

Glycerol and sorbitol were purchased from Sigma Chemical Co., St. Louis, Mo. Yeast extract and peptone were purchased from DIFCO Laboratories, Detroit, Mi. The [carboxyl-\(^{14}\)C] inulin, [methoxy-\(^{3}\)H] inulin, [carboxyl-\(^{14}\)C] dextran, and [\(^{3}\)H] water were obtained from New England Nuclear, Boston, Ma. Ecoscint scintillation fluid was purchased from National Diagnostics, Somerville, New Jersey.

Bacterial Cultures

The organism used in this study, *Gluconobacter oxydans* ATCC strain 621, was obtained from the American Type Culture Collection, Rockville, Maryland. Stock cultures were kept for
long term storage in 15% glycerol under liquid nitrogen. Working cultures were maintained as liquid suspensions in 66% glycerol at -10°C (79).

**Media and Growth**

Cells were grown in a liquid medium containing (w/v) 1% yeast extract, 1% peptone, and 5% of either glycerol or sorbitol. Sorbitol was the preferred substrate since cell aggregation, which was observed in glycerol medium, did not occur. Cultures were incubated in Bellco Nephelo culture flasks at 28°C on a reciprocal shaker at 200 reciprocations per minute (RPM) and growth was monitored spectrophotometrically at 620 nm. Middle exponential-phase cultures (0.55 to 0.80 O.D.) or late-exponential phase cultures (0.80 to 1.00 O.D.) were centrifuged, and washed twice in double-distilled deionized water (ddH₂O) before use.

**Proton Translocation Experiments**

Cells were grown in 250 ml of glycerol medium to late-exponential phase (LEP), centrifuged at 5860 x g for 10 minutes, and washed twice in 180 ml of ddH₂O. The pellet was suspended in 5 ml of ddH₂O and held on ice until needed.

A Fisher Pencil Combination pH Electrode was inserted into a 10 ml flat-bottomed test tube that contained 4.5 ml of one of the following reaction mixtures, each adjusted to pH 3.20:
dddH₂O, 0.1 M NaCl, 0.5 M NaCl, or 0.5 M NaCl plus 0.005 M NaN₃. The pH adjustments were made with 1.0 N HCl. Three-tenths ml of cell suspension was then added and the reaction mixture was stirred continuously. Changes in the pH of the reaction mixture were monitored by means of an Orion Digital Ionalyzer, Model 610, and values were recorded at 30 second intervals.

**Determination of Cellular Viability**

Ten ml of LEP, sorbitol-grown cells were centrifuged at 4000 RPM for 10 minutes, washed twice in 20 ml of sterile dddH₂O, and resuspended in 4 ml of sterile dddH₂O. One ml of this suspension was added to either 50 ml of sterile 0.1 M NaCl adjusted to pH 3.20 or to 50 ml of sterile 0.1 M NaCl containing 1% glycerol adjusted to pH 3.20 in 500 Erlenmeyer flasks. The pH adjustments were made with 1.0 N HCl. These mixtures were incubated at 28°C on a reciprocal shaker at 200 RPM. Twenty μl aliquots were removed from each reaction mixture at 30 minute intervals, and serially diluted in 1.5 ml micro test tubes containing 180 μl of dddH₂O. Petri dishes containing 5% glycerol medium were divided into eight sections and each section was inoculated with 20 μl of a given dilution. Three replicates of each dilution were plated. After incubation at 28°C for 48 hours, the number of colonies within each 20 μl inoculum was counted and expressed as colony forming units per ml. Colonies were counted using a dissecting microscope.
**Measurement of Cellular Respiration**

Twenty ml samples of LEP culture were removed from a growth flask, centrifuged at 4000 RPM for 10 minutes, and washed twice in 20 ml of dddH$_2$O. The pellets were suspended in 20 ml of the following reaction mixtures, each of which had been adjusted to pH 3.20: dddH$_2$O; 0.1 M NaCl; 0.1 M NaCl plus 0.005 M NaN$_3$; 0.5 M NaCl; or 0.5 M NaCl plus 0.005 M NaN$_3$. The pH adjustments were made with 1.0 N HCl.

Respiration rates were determined using a Clark cell electrode, YSI Model 53 Oxygen Monitor, and a Fisher Series 5000 Recordall chart recorder. Each cell suspension was added separately to the Clark cell and allowed to reach 100% oxygen saturation. The endogenous respiration rate was determined for each reaction mixture. In separate samples of each reaction mixture, respiration was measured after injection of 100 µl of 50% (v/v) glycerol into the Clark cell, and the percent oxygen saturation was recorded continuously.

**Internal Volume Measurements**

A 250 ml volume of culture was grown to mid-exponential phase (MEP), centrifuged at 10,400 x g for 10 minutes, washed twice in 180 ml of dddH$_2$O, and suspended in either 10 or 15 ml of dddH$_2$O. This produced suspensions containing 5 to 10 mg dry weight of cells per ml. One ml samples of this concentrated cell suspension were transferred to each of two 1.5 ml microcentrifuge tubes. One of these tubes received 100 µl of both [³H] water (specific activity 1 mCi/g) and either [carboxyl-¹⁴C] inulin (specific activity 1.3 mCi/g) or [carboxyl-¹⁴C] dextran (specific activity 1.2 mCi/g) while no isotopes were added to the other tube. After either zero, 5,
or 30 minute incubations at room temperature, both of these reaction mixtures were centrifuged for
5 to 15 minutes at 13,000 x g. The supernatant fluid was then aspirated from the unlabeled pellet,
and 50 µl of the labeled supernatant was transferred to the unlabeled pellet. The remaining labeled
supernatant was discarded, and both pellets were suspended in 0.5 ml of dddH₂O. Three 100 µl
samples were removed from each tube and transferred separately to four ml capacity scintillation
vials each containing 2 ml of Ecoscint scintillation fluid. Radioactivity was assayed with a TM
Analytic BetaTrac 6895 scintillation counter. The tritium window was adjusted to count between
0 and 255, the ¹⁴C range was 350 to 620. Both polymers were purified immediately prior to use,
the inulin by by paper chromatography, and the dextran by means of Amicon 30,000 molecular
weight cutoff filters.

**Internal pH Measurements**

Ten ml of cells were grown to MEP, centrifuged at 3800 RPM for 10 minutes, washed twice
in 25 ml of sterile dddH₂O, and suspended in 4 ml of sterile dddH₂O. One ml samples of this
suspension were transferred to 500 ml Erlenmeyer flasks containing 50 ml of one of the following
reaction mixtures each adjusted to pH 3.20: dddH₂O; dddH₂O plus 1% glycerol; 0.1 M NaCl; 0.1
M NaCl containing 1% glycerol; 0.1 M NaCl plus 750 µM KCl; 0.1 M NaCl plus 750 µM KCl and
1% glycerol; 0.05 M NaCl; 0.05 M NaCl plus 750 µM KCl and 1% glycerol. The pH adjustments
were made with 1.0 M HCl. These mixtures were incubated at 28°C on a reciprocal shaker at 200
RPM.

After approximately 3 hours, 5 ml samples were removed from each reaction mixture and
transferred to 500 ml Erlenmeyer flasks. Either [carboxyl-¹⁴C] acetylsalicylic acid (specific activity
56.6 mCi/mmol; final concentration 0.5 or 1 µM) or [carboxyl-¹⁴C] salicylic acid (specific activity
56.6 mCi/mmol; final concentration 0.5 µM) was added and, the mixtures were incubated from 2 to 15 minutes to allow for equilibration of the probe. One-ml samples were removed and filtered through 0.22 µm or 0.45 µm nitrocellulose filters. The filters were transferred to 4 ml capacity scintillation vials containing 4 ml of Ecoscint scintillation fluid. Radioactivity was assayed with a BetaTrac 6895 scintillation counter with 14C window settings of 000 to 255 and 90 to 350.

To correct for nonspecific binding of probe to cell constituents, cells were suspended in the various reaction mixtures to which 5% toluene or 5% butanol had been added. The counts per minute (cpm) obtained from toluene or butanol disrupted cells were attributed to nonspecific probe binding and were subtracted from the total cpm on the filters.

To correct for radioactivity trapped in extracellular space between cells, [methoxy-3H] inulin (specific activity 353.63 mCi/g) was added to 5 ml of reaction mixture. One-ml samples were removed, filtered, and the radioactivity was counted. The microliter volume of extracellular fluid was then calculated based upon a proportion of the total cpm per one µl of isotope to the cpm on the filter. This value was subtracted from the total µl of probe retained on the filter. Tritium channels were set to count from 000 to 350.

Dry cell weights were required for internal pH calculations. These values were determined by adding one ml of the concentrated cell suspension used for internal pH measurements to 50 ml sterile ddH2O. One ml of this suspension was then transferred to a predried, tared aluminum weighing boat, and dried at 105°C to a constant weight.

After correcting for nonspecific binding of the probe to cellular components and for radioactivity trapped in extracellular space on the filters, the internal pH was calculated according to the equation:

\[
pH_{in} = pK_a + \log \left( \frac{[HA + A^-]_in}{[HA + A^-]_{out}} \times 10^{pH_{in} - pK_a + 1} - 1 \right)
\]
Induced $H^+$ Translocation Experiments

To acidify cell interiors for $H^+$ translocation experiments, concentrated cell suspension was added to either 0.1 M NaCl at pH 3.20 or 0.5 M NaCl at pH 3.20. The pH of the 0.1 M NaCl and 0.5 M NaCl-containing reaction mixtures increased from 3.20 to 3.45 and 3.67, respectively (Fig. 1). Alkalinization of the NaCl-containing reaction mixtures was attributed to movement of $H^+$ into cells. Because this alkalinization occurred to a greater extent in 0.5 M NaCl than in 0.1 M NaCl, the inward translocation of $H^+$ appeared dependent upon the NaCl concentration of the reaction mixtures. The mechanism by which NaCl fostered the movement of $H^+$ into the cells was not investigated. The pH increase occurring in dddH$_2$O (Fig. 1) was approximately one-tenth of a pH unit and was attributed to dilution of the reaction mixture by cell suspension.

If rapid respiration functions to protect $G. oxydans$ in acidic environments, it was expected that polyol oxidation should result in the expulsion of $H^+$ from artificially acidified cell interiors. Glycerol was the polyol chosen because its oxidation product, dihydroxyacetone, is neutral and had no effect on the pH of the reaction mixtures. Concentrated cell suspension was first added to either
Figure 1. Effect of NaCl on H⁺ movement into G. oxydans: Cells grown in 250 ml of 5% glycerol medium were harvested during late exponential phase, washed twice in dddH₂O, and suspended in 5 ml dddH₂O. At time zero, 0.3 ml of concentrated cell suspension was added to 4.5 ml of either dddH₂O adjusted to pH 3.20 (○), 0.1 M NaCl adjusted to pH 3.20 (△), or 0.5 M NaCl adjusted to pH 3.20 (□). The pH of each reaction mixture was monitored continuously and values were recorded at 30 second intervals. Each data point is the average value from three replicates, and range bars indicate the variation between replicates. This figure is representative of 3 separate experiments, replicate data are shown in Appendix A.
0.1 M NaCl at pH 3.20 or 0.5 M NaCl at pH 3.20. Following the influx of H⁺ into cells, glycerol was added and immediate acidification of the reaction mixtures occurred (Fig. 2). The pH of the 0.1 M NaCl-containing reaction mixture decreased 0.25 unit, from 3.60 to 3.35, while the 0.5 M NaCl-containing reaction mixture decreased 0.35 unit from pH 3.80 to 3.45. This acidification was attributed to the extrusion of H⁺ from cells as a result of glycerol oxidation and electron transport.

If acidification of the reaction mixture was due to efflux of H⁺ associated with glycerol oxidation and electron transport, it was expected that the electron-transport inhibitor sodium azide (NaN₃) should interfere with this acidification. Concentrated cell suspension was added to solutions containing either 0.1 M NaCl plus 0.005 M NaN₃ at pH 3.20 or 0.5 M NaCl plus 0.005 M NaN₃ at pH 3.20 (Fig. 3). After movement of H⁺ into cell interiors, glycerol was added but no acidification of the reaction mixtures occurred. This was attributed to electron transport inhibition and the cessation of outward H⁺ translocation from cells.

**Cellular Respiration Measurements**

To verify that the glycerol effect (Fig. 2) was due to respiration, oxygen uptake was measured under the same conditions used in the H⁺ translocation experiments. Endogenous respiration did not occur in reaction mixtures containing washed cells and 0.1 M NaCl at pH 3.20 (Fig. 4). Oxygen uptake by cells in 0.1 M NaCl at pH 3.20 began immediately after the addition of glycerol, as evidenced by a 40% decrease in oxygen saturation in six minutes. In contrast, a 4% decrease in oxygen saturation was observed during the same period after the addition of glycerol to reaction mixtures containing NaN₃, washed cells, and 0.1 M NaCl at pH 3.20. Since rapid oxygen uptake (Fig. 4) occurred under the same conditions as H⁺ extrusion (Fig. 2), and since *G. oxydans* is
Figure 2. Effect of glycerol on H\(^{+}\) translocation by *G. oxydans*: At time zero, 0.3 ml of concentrated cell suspension, prepared as described in Figure 1, was added to 4.5 ml of either ddH\(_2\)O at pH 3.20, (○), 0.1 M NaCl at pH 3.20 (△), or 0.5 M NaCl at pH 3.2 (□). Following H\(^{+}\) translocation into cells, 0.1 ml of 50% (v/v) glycerol was added to initiate respiration and electron transport (arrow). The pH of each reaction mixture was monitored continuously and values were recorded at 30 second intervals. Each data point is the average value from three replicates, and range bars indicate the variation between replicates. This figure is representative of 6 separate experiments, replicate data are shown in Appendix B.
Figure 3. Effect of NaN₃ on H⁺ translocation from *G. oxydans*: At time zero, 0.3 ml of concentrated cell suspension, prepared as described in figure 1, was added to 4.5 ml of either ddH₂O adjusted to pH 3.20 (○) or a solution containing 0.5 M NaCl and 0.005 M NaN₃ adjusted to pH 3.20 (△). Following the NaCl-induced influx of H⁺ into cells, 0.1 ml of 50% (v/v) glycerol was added as a substrate for respiration (arrow). The pH of each reaction mixture was monitored continuously and values were recorded at 30 second intervals. Each curve represents the average of three replicates, and range bars indicate the variation between replicates. This figure is representative of 3 separate experiments, replicate data are shown in Appendix C.
strictly respiratory, it was assumed that H⁺ extrusion is driven by glycerol oxidation coupled with electron transport.

**Effect of Glycerol Respiration on Maintenance of Viability**

Because glycerol oxidation reversed the NaCl-induced movement of H⁺ into cells, experiments were designed to determine whether respiration by *G. oxydans* functioned to protect acid-labile cell interiors under acidic conditions. Cells held in a solution of 0.1 M NaCl at pH 3.20 containing glycerol remained viable during a 3.5 hour incubation (Fig. 5). The number of viable cells remained at approximately 1 x 10⁷ CFU/ml throughout this incubation period. In contrast, when cells were incubated in 0.1 M NaCl at pH 3.20 in the absence of glycerol, no viable cells remained at the end of the incubation. These data suggest that H⁺ extrusion associated with glycerol oxidation and electron transport functions to maintain internal pH homeostasis and cellular viability under acidic conditions.

**Internal Water Volume Determinations**

The H⁺ extrusion, oxygen uptake, and viability experiments suggest a role for respiration-dependent H⁺ extrusion in internal pH homeostasis. To verify the effect of H⁺ extrusion, internal pH was determined based upon the distribution of weak organic-acid probes. This technique is based upon the extent of intracellular probe accumulation and requires knowledge of the internal
Figure 4. Effect of glycerol and NaN₃ on oxygen consumption by *G. oxydans*. Twenty ml of late-exponential phase culture grown in 5% sorbitol medium was centrifuged, washed twice in dddH₂O, and the pellet was suspended in 20 ml of 0.1 M NaCl at pH 3.20 or 0.1 M NaG at pH 3.20. Samples of each reaction mixture were transferred individually to a Clark cell and allowed to reach 100% oxygen saturation. The endogenous rate of respiration was determined for cells suspended in 0.1 M NaCl adjusted to pH 3.20 (○). The arrow indicates the addition of 100 µl of a solution containing 50% (v/v) glycerol to reaction mixtures containing cells and either 0.1 M NaCl at pH 3.20 (△) or 0.1 M NaCl plus 0.005 M NaN₃, also adjusted to pH 3.20 (□). Oxygen uptake was monitored and recorded continuously. Each curve is representative of at least nine separate experiments, replicate data are shown in Appendix D.
Figure 5. Effect of respiration on maintaining viability of *G. oxydans*: Ten ml of glycerol-grown cells were centrifuged, washed twice with dddH₂O, and suspended in 4 ml of dddH₂O. One ml of this suspension was added to either 50 ml of 0.1 M NaCl adjusted to pH 3.20 (○), or to 50 ml of a solution containing 0.1 M NaCl and 1% glycerol, adjusted to pH 3.20 (△). The reaction mixtures, contained in 500 ml Erlenmeyer flasks, were incubated at 28° C, and shaken at 200 RPM. Samples were removed periodically, serially diluted, and plated. The data points are average values from triplicate platings from one experiment. Range bars indicate the variation between replicates. The curves presented here are representative of five viability experiments, replicate data are shown in Appendix E.
water volume. The internal water volume of G. oxydans was determined by measuring the distribution of $^3$H$_2$O and one of two membrane-impermeable polymers, $[^{14}C]$ dextran or $[^{14}C]$ inulin. The mean value from 14 measurements was $2.70 \pm 0.10 \mu l/mg$ dry cell weight. Estimates of the internal volume of G. oxydans by other investigators are not available in the literature. However, this estimate is similar to the values obtained for a comparable sized organism, Escherichia coli. Winkler and Wilson (80), Collins and Hamilton (15), and Booth et al. (10) measured internal volumes of E. coli and obtained values of 2.70, 2.4, and 2.68 $\mu l/mg$ dry cell weight, respectively.

Internal pH Measurements

Internal pH was measured to determine whether respiration-dependent H$^+$ extrusion by G. oxydans resulted in cytoplasmic pH homeostasis under extremely acidic conditions. These measurements were made by determining the transmembrane distribution of either $[^{14}C]$ acetylsalicylic acid or $[^{14}C]$ salicylic acid as described by Rottenberg (66). The results from internal pH measurements are summarized in Tables 1 through 3.

As shown in Figure 1, a one-tenth unit increase in the pH of the reaction mixture occurred when cells were added to dddH$_2$O at pH 3.20. This was attributed to dilution of the reaction mixture by cell suspension rather than to a significant influx of H$^+$ into cells. Table 1 lists the internal pH values for cells suspended in dddH$_2$O or in dddH$_2$O containing glycerol, both adjusted to pH 3.20. The data in Table 1 indicate that glycerol had no effect on internal pH since the maximum difference between respiring and nonrespiring cells was 0.1 unit.
Table 1. Internal pH values of *G. oxydans* in dH₂O at pH 3.20

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Probe</th>
<th>Accumulation Ratio²</th>
<th>Internal pH³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dH₂O</td>
<td>dH₂O + Glycerol</td>
<td>dH₂O</td>
</tr>
<tr>
<td>1</td>
<td>Acetylsalicylic acid</td>
<td>257</td>
<td>241</td>
</tr>
<tr>
<td>2</td>
<td>Acetylsalicylic acid</td>
<td>434</td>
<td>411</td>
</tr>
</tbody>
</table>

¹ Washed cells were incubated for approximately three hours in 0.1 M NaCl or 0.1 M NaCl plus glycerol at pH 3.2. Five ml samples were removed and radioactively labeled organic-probe was added to a final concentration of 0.5 μM. Cells were then separated from the reaction mixture by filtration. Radioactivity in the cells and filtrate were determined.

² Ratio of the internal probe concentration in μM of acid/μl internal volume to μM of acid / μl of filtrate; 

³ Internal pH was calculated according to the following equation:

\[ \text{pH}_{\text{in}} = \text{pK}_a + \log \left( \frac{[HA + A^-]_{\text{in}}}{[HA + A^-]_{\text{out}}} \times 10^{\text{pH}_0 - \text{pK}_a + 1} \right) \]
Table 2. Internal pH values of *G. oxydans* in 0.1 M NaCl at pH 3.20

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Probe</th>
<th>Accumulation Ratio&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Internal pH&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td>NaCl + Glycerol</td>
</tr>
<tr>
<td>1</td>
<td>Acetylsalicylic acid</td>
<td>41</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>Acetylsalicylic acid</td>
<td>116</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td>104</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td>Acetylsalicylic acid&lt;sup&gt;*&lt;/sup&gt;</td>
<td>394</td>
<td>398</td>
</tr>
<tr>
<td>4</td>
<td>Salicylic acid</td>
<td>168</td>
<td>187</td>
</tr>
<tr>
<td>5</td>
<td>Salicylic acid</td>
<td>41</td>
<td>84</td>
</tr>
<tr>
<td>6</td>
<td>Salicylic acid</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>Salicylic acid</td>
<td>177</td>
<td>274</td>
</tr>
</tbody>
</table>

<sup>1</sup> Washed cells were incubated for approximately three hours in 0.1 M NaCl or 0.1 M NaCl plus glycerol at pH 3.2. Five ml samples were removed and radioactively labeled organic probe was added to a final concentration of 0.5 μM (* <sup>*</sup> indicates a final probe concentration of 1 μM). Cells were then separated from the reaction mixture by filtration. Radioactivity in the cells and filtrate was determined.

<sup>2</sup> Ratio of the internal probe concentration in μM of acid / μl of internal volume to μM of acid / μl of filtrate; 

\[ \frac{[HA + A^-]_{in}}{[HA + A^-]_{out}} \]

<sup>3</sup> Internal pH was calculated according to the following equation:

\[ pH_{in} = pK_a + \log \left( \frac{[HA + A^-]_{in}}{[HA + A^-]_{out}} \times \left( 10^{pH_{out} - pK_a} + 1 \right) \right) \]
The intracellular accumulation of salicylic acid was expected to be greater than acetylsalicylic acid due to the higher pKₐ of the latter. Since the pKₐ of the probe is accounted for in the internal pH calculations (see Materials and Methods), the values obtained were expected to be similar regardless of which probe was used, the data presented in Table 2 indicate that this was the case.

As demonstrated in the H⁺ translocation experiments (Fig. 1), it appeared that H⁺ move into cells in the presence of NaCl. Acidification of the external medium after the addition of glycerol indicated respiration-dependent H⁺ extrusion (Fig. 2). If H⁺ extrusion were occurring, one would expect to find higher internal pH values in respiring cells than in nonrespiring cells under the same conditions. Therefore, internal pH measurements were made on cells incubated for approximately three hours in 0.1 M NaCl at pH 3.20 either in the presence or absence of glycerol (Table 2). Data from 7 experiments indicate that respiring cells were a maximum of 0.3 pH unit more alkaline inside than nonrespiring cells. However, in seven measurements, the internal pH values of respiring and nonrespiring cells were identical or varied by only 0.1 unit. Under these conditions, large differences in internal pH between respiring and nonrespiring cells were not detected.

A substantial body of evidence indicates that K⁺ uptake is required for the generation of a pH gradient (alkaline inside) at acidic external pH. For this reason, the effect of increased external K⁺ concentrations on internal pH was determined (Table 3). These internal pH values were obtained from cells which were incubated for approximately three hours in either 0.1 M NaCl plus 750 μM KCl or 0.05 M NaCl plus 750 μM KCl, both of which were adjusted to pH 3.20. An average internal pH of 6.1 ± 0.1 was determined from four experiments conducted on respiring cells with added K⁺ (Table 3) compared to an average internal pH of 5.5 ± 0.4 from respiring cells in acidic NaCl without supplemental K⁺ (Table 2). Thus, large differences in the internal pH of respiring cells due to the presence of supplemental K⁺ were not detected. Neither were internal pH differences detected between respiring and nonrespiring cells when both were incubated in NaCl plus KCl. In four experiments conducted in the absence of supplemental K⁺ (Table 2), internal
Table 3. Internal pH values of G. oxydans in NaCl + KCl at pH 3.20

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Probe</th>
<th>Accumulation Ratio&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Internal pH&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NaCl + KCl</td>
<td>NaCl + Glycerol + KCl</td>
</tr>
<tr>
<td></td>
<td>Acetylsalicylic acid</td>
<td>404</td>
<td>313</td>
</tr>
<tr>
<td>2</td>
<td>Acetylsalicylic acid</td>
<td>179</td>
<td>194</td>
</tr>
<tr>
<td>3</td>
<td>Acetylsalicylic acid</td>
<td>301</td>
<td>287</td>
</tr>
</tbody>
</table>

1 Washed cells were incubated for approximately three hours in 0.1 M NaCl and 750 μM KCl or 0.1 M NaCl plus 750 μM KCl and glycerol at pH 3.2. Five ml samples were removed and radioactively labeled organic-acid probe was added to a final concentration of 0.5 μM. Cells were separated from the reaction mixture by filtration. Radioactivity in the cells and filtrate was determined.

2 Ratio of the internal probe concentration in μM of acid / μl of internal volume to μM of acid / μl of filtrate;

\[
\frac{[HA + A^-]_{\text{in}}}{[HA + A^-]_{\text{out}}}
\]

3 Internal pH was calculated according to the following equation:

\[
\text{pH}_{\text{in}} = pK_a + \log \left( \frac{[HA + A^-]_{\text{in}}}{[HA + A^-]_{\text{out}}} \times \left( 10^{\text{pH}_{\text{in}} - pK_a} + 1 \right) - 1 \right)
\]
pH values were identical or varied by 0.1 unit. Internal pH values from respiring cells with supplemental K\(^+\) (Table 3) ranged from 6.0 to 6.2 while nonrespiring cells with supplemental K\(^+\) varied from 5.9 to 6.2.
DISCUSSION

The goals of the current investigation were to use improved methods to verify data reported by Heefner (31), and to provide additional evidence for the role of respiration-dependent proton $H^+$ translocation in the acid tolerance of Gluconobacter oxydans. Meeting these goals involved (i) repetition of $H^+$ translocation and cellular viability experiments (ii) oxygen uptake determinations to verify the link between respiration and $H^+$ extrusion and (iii) measurement of intracellular pH in respiring and nonrespiring cells under acidic conditions.

Induced $H^+$ translocation experiments.

Heefner (31) developed a method by which cells could be loaded with $H^+$ for subsequent use in translocation studies. He suspended washed cells in 0.1 M NaCl or 0.5 M NaCl at pH 3.20. This resulted in marked alkalinization of the reaction mixture and was attributed to $H^+$ influx into cells. He found that the effect of NaCl on external pH was similar to that seen with 2,4-dinitrophenol (2,4-DNP) thus providing additional evidence for $H^+$ influx. When washed cells
were suspended 0.0006 M 2,4-DNP at pH 3.20, alkalinization of the reaction mixture followed. This compound is known to transport H\(^+\) across bacterial plasma membranes. Because the effects on external pH were similar, he concluded that alkalinization of reaction mixtures in the presence of NaCl was also due to the inward translocation of H\(^+\). Heefner's method of artificially acidifying cell interiors was used in the present investigation.

The effect of NaCl on inward H\(^+\) translocation seen in this study (Fig. 1) was identical to that reported by Heefner (31). Immediately after washed cells were suspended in NaCl initially adjusted to pH 3.20, the pH of the reaction mixture increased. The 0.1 M NaCl-containing reaction mixture increased to pH 3.48 and the 0.5 M NaCl suspension increased to pH 3.70. These increases suggested an influx of H\(^+\) into cells. Because alkalinization of the reaction mixture occurred to a greater extent in 0.5 M NaCl than in 0.1 M NaCl, the H\(^+\) influx appeared dependent upon the concentration of NaCl present. The pH increase of both reaction mixtures in this study were similar to values reported by Heefner (31). He measured external pH values of up to 0.15 unit in 0.1 M NaCl and 1.0 unit in 0.5 M NaCl.

The pH increase in dddH\(_2\)O (Fig. 1) was attributed largely to dilution of the reaction mixture by cell suspension, since the impermeability of the plasma membrane to H\(^+\) made a large influx of these ions unlikely. Data obtained by Heefner (31) also suggest that significant H\(^+\) influx into cells was not occurring under these conditions. When he incubated cells in dddH\(_2\)O with or without glycerol at pH 3.20 for approximately three hours, neither the respiring nor the norespiring cells lost viability. The maintenance of viability seen in this study (Fig. 5) by nonrespiring cells despite the acidic external pH, also suggests that significant H\(^+\) influx was not occurring in the absence of NaCl.

If rapid respiration functions to protect G. oxydans under acidic conditions, it was expected that polyol oxidation would result in the expulsion of H\(^+\) from artificially acidified cell interiors. Glycerol was chosen as the substrate in the current study since its oxidation product is neutral and
had no effect on the pH of the reaction mixture. Washed cells were first suspended in 0.1 M NaCl or 0.5 M NaCl at pH 3.20, to allow for H⁺ influx (Fig. 2) after which glycerol was added. This resulted in pH decreases in the 0.1 M NaCl and 0.5 M NaCl containing reaction mixtures (Fig. 2) from pH 3.55 to 3.35 and from 3.80 to 3.45, respectively. It seems reasonable to attribute this H⁺ efflux to glycerol oxidation and electron transport. Both the occurrence of H⁺ extrusion and the magnitude of the external pH decreases (Fig. 2) were similar to those observed by Heefner (31) who measured pH decreases from 0.17 to 1.0 units.

If acidification of the reaction mixtures (Fig. 2) was due to H⁺ extrusion associated with glycerol oxidation and electron transport, it was expected that an electron-transport inhibitor would interfere with this acidification. In order to verify the link between acidification of the external medium and glycerol oxidation, H⁺ movement was followed in reaction mixtures containing 0.5 M NaCl and 0.005 M sodium azide (NaN₃) as shown in Figure 3. Under these conditions, acidification of the reaction mixture did not follow glycerol addition. This was attributed to inhibition of electron transport and concomitant cessation of H⁺ extrusion.

The H⁺ efflux described above is similar to that seen by Collins and Hamilton (15) after adding oxygen to anaerobic suspensions of Escherichia coli. The external pH fell rapidly and was attributed to H⁺ extrusion associated with respiration of endogenous substrate. According to these authors, when the oxygen supply was exhausted, H⁺ diffused back into cells. Acidification of the reaction mixture was also noted after anaerobic suspensions of Micrococcus denitrificans (69) received oxygen pulses. The authors attributed acidification of the reaction mixture to bursts or respiratory activity which resulted from the addition of oxygen. Lawford and Haddock (49) found that inclusion of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) prevented acidification of the reaction mixture after the addition of oxygen to anaerobic suspensions of E. coli. Zychlinsky and Matin (81) found that inhibition of respiration in Thiobacillus acidophilus, by anaerobiosis or by the addition of azide or cyanide, resulted in alkalinization of the external medium due to interference with respiration-dependent H⁺ extrusion. Goulbourne et al. (22) followed H⁺

DISCUSSION
movement in an acidophilic bacterium identified as PW2 and observed alkanlization of the external solution after adding azide or a protonophore, either 2,4-DNP or CCCP, to respiring cells.

Cellular respiration measurements

To verify that the effect of glycerol was due to respiration, oxygen uptake was measured in the current study under the same conditions used in the H\textsuperscript+ translocation experiments. Endogenous respiration did not occur in reaction mixtures containing washed cells and 0.1 M NaCl at pH 3.20 (Fig. 4). Oxygen uptake by cells in 0.1 M NaCl at pH 3.20 began immediately after the addition of glycerol, as evidenced by a 40% decrease in oxygen saturation in six minutes. In a reaction mixture containing washed cells, 0.1 M NaCl, and NaN\textsubscript{3} at pH 3.20, a 4% decrease in oxygen saturation was observed during the same period. The initiation of oxygen uptake after glycerol addition, as well as its inhibition in the presence of NaN\textsubscript{3}, provides evidence that glycerol respiration was occurring. Since rapid oxygen uptake (Fig. 4) occurred under the same conditions as H\textsuperscript+ extrusion (Fig. 2), and since G. oxydans is strictly respiratory, it was assumed that H\textsuperscript+ extrusion (Fig. 2) is driven by glycerol oxidation coupled with electron transport.

Effect of glycerol respiration on maintenance of viability

Because glycerol oxidation reversed the NaCl-induced movement of H\textsuperscript+ into cells, experiments were designed to determine whether respiration by G. oxydans functioned to maintain vi-
ability under acidic conditions. Cells incubated in 0.1 M NaCl at pH 3.20 in the presence of glycerol remained viable during a 3.5 hour incubation (Fig. 6). In contrast, when cells were incubated in 0.1 M NaCl at pH 3.20 in the absence of glycerol, viable numbers decreased logarithmically. Work by White (78) with G. oxydans indicates that the decrease in viable numbers was not a result of the inability to survive periods of starvation. He incubated cells in dddH₂O for 50 hours during which time samples were removed periodically for serial dilution and plating. No significant decrease in viable numbers was observed during that time. After a 100 hour incubation in dddH₂O, he found that viable numbers decreased from 10¹⁰ colony forming units/ml to 10⁹ colony forming units/ml. White's data suggest that cells incubated at 0.1 M NaCl at pH 3.20 for three hours in the absence of substrate did not lose viability due to starvation. The H⁺ translocation and viability experiments conducted in the current study, in combination with White's data, indicate that loss of viability by nonrespiring cells in the presence of 0.1 M NaCl at pH 3.20 was due to the lethal accumulation of H⁺ within acid-labile cell interiors.

**Internal pH measurements**

By definition, G. oxydans is a neutrophile since its optimum growth pH is 5.5 to 6.0 (18). It is, however, capable of growth at pH 3.6 (18) and, therefore, is considerably more acid tolerant than most neutrophiles. Neutrophiles generally maintain internal pH values within the range of 7.5 to 8.0 while acidophiles vary from 6.5 to 7.0 and alkalophiles range from 8.4 to 9.0 (9).

In order to determine the ability of G. oxydans to maintain cytoplasmic pH homeostasis under acidic conditions, internal pH measurements were made on nonrespiring cells in each of the following reaction mixtures adjusted to pH 3.20: dddH₂O, 0.1 M NaCl, 0.1 M NaCl and 750 μM
KCl, and 0.05 M NaCl and 750 μM KCl. In order to determine the effect of substrate oxidation on internal pH, measurements were made in these reaction mixtures after the addition of glycerol.

Internal pH measurements were based upon the transmembrane distribution of radioactively labeled weak organic-acid probes. Ideally, the external pH at which these measurements are made should be at least one pH unit greater than the pKₐ of the organic-acid probe (9, 32, 57). Under these conditions, essentially all of the acid is in the dissociated form both inside and outside of the cell. Specifically, 90 to 99% dissociation of the probe is optimum for internal pH measurements (57). The ΔpH is then calculated according to equation 1 (14, 59, 67). When the external pH is within one unit of the pKₐ however, a significant portion of the probe remains in the undissociated form and must be corrected for mathematically, as shown in equation 2 (14).

Commonly used probes include: 5,5,-dimethyl-2,4-oxazolidinedione (DMO), pKₐ 6.3; acetic acid, pKₐ 4.75; benzoic acid, pKₐ 4.2; acetylsalicylic acid, pKₐ 3.48; and salicylic acid, pKₐ 3.0. Because of their low pKₐ values, only the latter two are suitable for use at very low external pH. In the current study, [¹⁴C] acetylsalicylic and [¹⁴C] salicylic acids were used in determining the internal pH of G. oxydans at an external pH of 3.2. In the case of acetylsalicylic acid, the difference between the external pH and the pKₐ is less than one unit and would result in approximately 30% dissociation. Use of salicylic acid, with a pKₐ below the external pH, produces somewhat less dissociation. Because of the limited dissociation occurring with these probes, equation 2 was used in calculating internal pH values.

No differences in the internal pH of respiring and nonrespiring cells incubated in dddH₂O at pH 3.20 were detected (Table 1). This was not surprising since translocation experiments indicated H⁺ influx under these conditions was minimal (Fig.1). Maintenance of viability by nonrespiring cells incubated in dddH₂O at pH 3.20 (32) also suggested that significant H⁺ influx into cells was not occurring under these conditions. The low H⁺ permeability of the plasma membrane due to the lipid bilayer and the mechanisms for control of ion translocation through
proteins in the membrane, also suggest that significant H\(^+\) influx would not have occurred under these conditions. The similarity in internal pH values shown in Table 1 may have resulted from the fact that the cytoplasm of nonrespiring cells was not being acidified by H\(^+\) influx. However, even in the absence of NaCl-dependent H\(^+\) influx, H\(^+\) extrusion by rapidly respiring cells was expected to produce a more alkaline internal pH than in nonrespiring cells. The similarity in internal pH values between respiring and nonrespiring cells more likely results from the insensitivity of the organic-acid distribution technique when used at low external pH.

Significant differences in internal pH were expected between respiring and nonrespiring cells incubated in 0.1 M NaCl at pH 3.20 since H\(^+\) translocation experiments provided substantial evidence for acidification of the cytoplasm in the presence of NaCl (Fig. 1). Nonrespiring cells accumulated H\(^+\), indicated by alkalinization of the external medium (Fig. 1), presumably resulting in a lowered cytoplasmic pH. Viability studies indicated that this decrease in internal pH was sufficient to cause cell death (Fig. 6). In contrast, respiring cells extruded H\(^+\), as evidenced by acidification of the external medium after glycerol addition (Fig. 2). Retention of viability by cells incubated in 0.1 M NaCl at 3.2 in the presence of glycerol (Fig. 6), suggested that respiration and electron transport function to expel sufficient H\(^+\) to maintain internal pH homeostasis.

Despite substantial evidence indicating respiration-dependent H\(^+\) extrusion in the maintenance of pH homeostasis, significant differences in cytoplasmic pH between respiring and nonrespiring cells were not detected (Tables 1,2,3). The apparent similarities in internal pH values probably reflect problems inherent in the probe-distribution technique under the conditions of low external pH rather than being an accurate indication of the internal pH of G. oxydans.

The probe distribution technique is commonly used for measuring intracellular pH when the external pH is more than one unit above the pK\(_a\) of the probe. Under these conditions, there is extensive dissociation of the probe and the accumulation ratio, calculated from the quantity of probe retained within the cell to that outside, is large. In contrast, under conditions of low external
pH and limited dissociation, the accumulation ratio is relatively small and difficult to measure accurately (48). This problem is compounded since as the value of the correction factor relating the external pH and pKa of the probe increases, any error in the accumulation ratio is magnified (9). The correction factor is relatively high when the pKa of the acid is below the external pH, and decreases as the pKa rises above the external pH. As a result, the correction factor for salicylic acid, pKa 3.0, is higher than that required for acetylsalicylic acid, pKa 3.48. However, the internal pH values in Table 1 were similar regardless of which probe was used.

When probe dissociation is less than 90%, the accumulation ratio of acid retained inside the cell to that outside, is less sensitive to internal pH (57). This may be an additional reason for the similarity in the internal pH values between respiring and nonrespiring cells in acidic NaCl. Under these conditions, there is a differential increase in the undissociated acid that accumulates inside the cell in response to the ΔpH (57). Data from the current study suggested that accumulation ratios are insensitive to cytoplasmic pH since large changes in these values translate into minor variations in internal pH (Tables 1,2,3).

Insensitivity in internal pH measurements may also arise from use of the filtration method for separating cells from the reaction mixture. Because the filters are clogged by low bacterial concentrations, the sensitivity of this method is low (9). Frequently, the radioactivity due to the probe retained in cells is not much greater than the radioactivity due to medium trapped in the extracellular space on the filter (57). An additional disadvantage of filter separation is variability in the quantity of fluid trapped on the filters (32). However, the filtration method does offer an advantage when used for measuring the internal pH of actively respiring cells because aerobic conditions are maintained during the separation process. The alternative procedure, separation by centrifugation, results in the development of anaerobic conditions within the pellet and, therefore, diminution of the pH gradient. The filtration method was used in the current study for separation of cells from external medium.

DISCUSSION
Substantial evidence exists for the role of cation influx, particularly $K^+$, for the interconversion of $\Delta p$H and $\Delta \psi$ and, consequently, the generation of a large pH gradient under acidic conditions (7, 8, 9, 19, 26, 35, 36, 37, 42, 43, 57, 59, 60, 76). Presumably, electrogenic $K^+$ uptake decreases $\Delta \psi$ by depolarizing the electrical charge gradient across the plasma membrane. Since many cells maintain an approximately constant $\Delta p$ (20, 33, 37, 57, 58, 59), a decrease in $\Delta \psi$ facilitates additional $H^+$ translocation from cell interiors and an increase in internal pH (8).

Because of the importance of $K^+$ influx in cytoplasmic pH homeostasis in other bacteria, the effect of external $K^+$ concentration was determined in $G. oxycdans$. The presence of supplemental KCl did not appear to affect either the formation or magnitude of the pH gradients in this organism. The internal pH values measured in the presence of supplemental $K^+$ (Table 3) were similar to those obtained from cells incubated in acidic NaCl without added $K^+$ (Table 2). This was not surprising since, according to Rosen (65), contaminating $K^+$ in the external environment is usually sufficient to saturate the $K^+$ uptake system, and, presumably for generation of a pH gradient (interior alkaline). Neither were significant differences were observed between the internal pH values from respiring and nonrespiring $G. oxycdans$ in acidic NaCl plus KCl (Table 3). The similarity of cytoplasmic pH values for respiring and nonrespiring cells in the current study was attributed to the use of organic-acid probes at external pH values near their $pK_a$, as discussed previously.

According to Booth (9), use of pH probes below their $pK_a$ values is very sensitive to small errors in cell volume, cell mass, and nonspecific probe binding. In the current study, error may have been introduced into internal pH measurements by overestimation of the internal water volume. In making these determinations, [3H] water was used as a measure of the total water volume, and one of two cell wall impermeable polymers, [14C] dextran or [14C] inulin, were used to determine extracellular volume. The difference between the two values was taken as the internal water volume. Due to the large molecular weights of dextran and inulin, these polymers should have been excluded by the cell wall and unable to penetrate the outer membrane of $G. oxycdans$. As a result, the values for internal volume represent both the cytoplasm and the periplasmic space. Dissociated
internal pH probes, however, should have accumulated within the cytoplasm only. Calculation of the internal probe concentration based upon cytoplasmic and periplasmic volume would lead to an underestimate of the internal pH. Controversy exists as to the extent that internal pH is affected by inaccuracies in cell volume measurements. According to Padan and Schuldiner (57), the final internal pH value will be relatively insensitive to small inaccuracies in cell volume measurements due to the logarithmic relationship between the accumulation ratio and the internal pH. In contrast, Booth (9) contends that use of probes at external pH values below their pKₐ is very sensitive to small errors in cell volume. In the current study, overestimation of internal volume may partially account for the cytoplasmic pH values measured for respiring cells being consistently lower than the range of 6.5 to 7.0 which is characteristic of neutrophiles (9).

Booth (9) also contends that use of pH probes below their pKₐ values is sensitive to small errors arising from nonspecific binding of probe to internal cell components. Nonspecific binding results in overestimation of the quantity of probe retained in cells and, therefore, erroneously high accumulation ratios. In certain experiments performed in the current study, the amount of radioactivity detected in toluene or butanol disrupted cells was subtracted from the radioactivity in intact cells as a correction for nonspecific probe binding. However, binding corrections were not made in the initial internal pH determinations. The values shown in Table 2 are similar regardless of whether probe binding corrections were made. For this reason, it appears that inaccuracies in the accumulation ratios due to probe binding did not translate into consistently higher internal pH values than in those experiments in which binding corrections were made.

DISCUSSION
Recommendations for Future Research

The organic-acid distribution technique has been used successfully by other investigators to determine internal pH at low external pH (14, 16, 51). It would be my recommendation, however, that $^{31}$P nuclear magnetic resonance spectroscopy be considered for future determinations of the internal pH of *G. oxydans* under acidic conditions. This technique is based upon the sensitivity of chemical shifts in resonances of intracellular phosphorus-containing compounds, most commonly inorganic phosphorus. According to Padan and Schuldiner (57), $^{31}$P NMR is noninvasive, nondestructive, and the sensitivity and time resolution are superior to other techniques. However, because the quantity of the phosphorus-containing compound must be sufficiently high for detection, the cell concentrations required are much higher than under physiologic conditions (57). Despite this, results for internal cell pH obtained by means of $^{31}$P NMR have been found to agree with those measurements obtained from the distribution of organic-acid probes (32, 57, 59). Nuclear magnetic resonance techniques avoid the complications arising from use of organic-acid probes at low external pH. The use of $^{31}$P NMR eliminates the need for internal volume and dry weight measurements, and for mathematical manipulations to correct for limited probe dissociation, retention of probe in extracellular space on filters, and nonspecific binding.


LITERATURE CITED
Appendix A. Effect of NaCl on H$^+$ Translocation

- Replicate Experiments
Appendix A. Effect of NaCl on H⁺ Translocation - Replicate Experiments
Appendix A. Effect of NaCl on H⁺ Translocation - Replicate Experiments
Appendix B. Effect of Glycerol on H\(^+\) Translocation

- Replicate Experiments
Appendix B. Effect of Glycerol on H⁺ Translocation - Replicate Experiments
Appendix B. Effect of Glycerol on H⁺ Translocation - Replicate Experiments
Appendix B. Effect of Glycerol on H⁺ Translocation - Replicate Experiments
Appendix B. Effect of Glycerol on H⁺ Translocation - Replicate Experiments
Appendix B. Effect of Glycerol on H⁺ Translocation - Replicate Experiments
Appendix B. Effect of Glycerol on $H^+$ Translocation - Replicate Experiments
Appendix C. Effect of NaN₃ on H⁺ Translocation - Replicate Experiments
Appendix C. Effect of \( \text{NaN}_3 \) on \( \text{H}^+ \) Translocation - Replicate Experiments
Appendix C. Effect of NaN₃ on H⁺ Translocation - Replicate Experiments
Appendix D. Effect of Glycerol and $\text{NaN}_3$ on Cellular Respiration - Replicate Experiments
Appendix D. Effect of Glycerol and NaN₃ on Cellular Respiration - Replicate Experiments
Appendix D. Effect of Glycerol and NaN₃ on Cellular Respiration - Replicate Experiments
Appendix D. Effect of Glycerol and NaN₃ on Cellular Respiration - Replicate Experiments
Appendix D. Effect of Glycerol and NaN₃ on Cellular Respiration - Replicate Experiments
Appendix D. Effect of Glycerol and NaN₃ on Cellular Respiration - Replicate Experiments
Appendix E. Effect of Glycerol Respiration on Cellular Viability Under Acidic Conditions - Replicate Experiments
Appendix E. Effect of Glycerol Respiration on Cellular Viability Under Acidic Conditions - Replicate Experiments
Appendix E. Effect of Glycerol Respiration on Cellular Viability Under Acidic Conditions - Replicate Experiments
Appendix E. Effect of Glycerol Respiration on Cellular Viability Under Acidic Conditions - Replicate Experiments
Appendix E. Effect of Glycerol Respiration on Cellular Viability Under Acidic Conditions - Replicate Experiments
Appendix F. Internal Water Volume

Measurements of *Gluconobacter oxydans*
Appendix F. Internal water volume measurements of *G. oxydans*¹

<table>
<thead>
<tr>
<th>Incubation Date</th>
<th>Labeled Compound</th>
<th>Radioactivity²</th>
<th>Internal water volume³</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-24-87</td>
<td>H₂O</td>
<td>6,652 ± 97</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>27,321 ± 789</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>1,741 ± 90</td>
<td></td>
</tr>
<tr>
<td>6-25-87</td>
<td>H₂O</td>
<td>6,472 ± 1,009</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>23,889 ± 3,006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>187 ± 36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>1,696 ± 433</td>
<td></td>
</tr>
<tr>
<td>7-20-87</td>
<td>H₂O</td>
<td>14,408 ± 2,055</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>28,471 ± 337</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>74 ± 26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>293 ± 19</td>
<td></td>
</tr>
<tr>
<td>6-23-87</td>
<td>H₂O</td>
<td>7,457 ± 145</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>28,005 ± 99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>115 ± 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>895 ± 13</td>
<td></td>
</tr>
<tr>
<td>7-21-87</td>
<td>H₂O</td>
<td>10,245 ± 675</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>30,181 ± 1,457</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>88 ± 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>552 ± 20</td>
<td></td>
</tr>
<tr>
<td>7-23-87</td>
<td>H₂O</td>
<td>7,743 ± 1,007</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>17,560 ± 109</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>629 ± 157</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>3,006 ± 162</td>
<td></td>
</tr>
<tr>
<td>7-30-87</td>
<td>H₂O</td>
<td>13,176 ± 70</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>28,270 ± 1,382</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inulin</td>
<td>115 ± 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inulin</td>
<td>533 ± 31</td>
<td></td>
</tr>
<tr>
<td>8-7-87</td>
<td>H₂O</td>
<td>762 ± 21</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>1,835 ± 82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>614 ± 16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>2,440 ± 222</td>
<td></td>
</tr>
<tr>
<td>8-8-87</td>
<td>H₂O</td>
<td>16,831 ± 510</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>21,028 ± 333</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>1,372 ± 90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>5,111 ± 67</td>
<td></td>
</tr>
<tr>
<td>8-9-87</td>
<td>H₂O</td>
<td>16,024 ± 160</td>
<td>2.62</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>21,480 ± 198</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>1,847 ± 104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>6,854 ± 168</td>
<td></td>
</tr>
<tr>
<td>8-10-87</td>
<td>H₂O</td>
<td>16,526 ± 683</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>22,161 ± 248</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>2,031 ± 133</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>7,944 ± 77</td>
<td></td>
</tr>
<tr>
<td>Incubation Date</td>
<td>Labeled Compound</td>
<td>Radioactivity(^a)</td>
<td>Internal water volume(^b)</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------</td>
<td>---------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>6-23-87</td>
<td>H(_2)O</td>
<td>(C_p): 6,809 ± 247</td>
<td>2.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C_s): 27,607 ± 1,850</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>(C_p): 96 ± 14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C_s): 929 ± 35</td>
<td></td>
</tr>
<tr>
<td>8-3-87</td>
<td>H(_2)O</td>
<td>(C_p): 10,245 ± 675</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C_s): 4,522 ± 39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inulin</td>
<td>(C_p): 58 ± 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C_s): 13 ± 2</td>
<td></td>
</tr>
<tr>
<td>8-7-87</td>
<td>H(_2)O</td>
<td>(C_p): 743 ± 17</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C_s): 1,888 ± 82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dextran</td>
<td>(C_p): 606 ± 31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C_s): 2,498 ± 40</td>
<td></td>
</tr>
</tbody>
</table>

1 One ml of concentrated cell suspension containing 5 to 10 mg dry cell weight/ml was added to each of two 1.5 ml microcentrifuge tubes. \([\text{carboxyl-}^{14}\text{C}]\) dextran or \([\text{carboxyl-}^{14}\text{C}]\) inulin were added to one tube and no isotopes were added to the other tube. Both tubes were incubated at room temperature for the time periods specified, then centrifuged. Supernatant from the nonradioactively labeled pellet was discarded and 50 \(\mu\)l of the labeled supernatant was transferred to the tube containing the nonlabeled pellet. The remaining labeled supernatant was discarded and both pellets were suspended in 0.5 ml dH\(_2\)O. Three 100 \(\mu\)l samples were removed from each tube and the radioactivity was determined.

2 \(C_p\) = counts / minute in 100 \(\mu\)l of labeled pellet suspension. \(C_s\) = counts / minute in 100 \(\mu\)l labeled supernatant.

3 Internal water volume was calculated according to the equation in Stock (75).
Appendix G. Internal pH Values of *Gluconobacter oxydans* in dH$_2$O at pH 3.20
Appendix G. Internal pH values of *G. oxydans* in dH$_2$O at pH 3.20

<table>
<thead>
<tr>
<th>Date</th>
<th>Probe</th>
<th>Dry Weight$^2$ (mg/ml)</th>
<th>Time$^3$</th>
<th>Corrected CPM$^4$</th>
<th>Accumulation Ratio$^5$</th>
<th>Internal pH$^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>dH$_2$O</td>
<td>dH$_2$O + Glycerol</td>
<td>dH$_2$O</td>
<td>dH$_2$O + Glycerol</td>
<td>dH$_2$O</td>
</tr>
<tr>
<td>10-19-87</td>
<td>Acetylsalicylic acid</td>
<td>0.17</td>
<td></td>
<td>4410</td>
<td>3956</td>
<td>257</td>
</tr>
<tr>
<td>10-23-87</td>
<td>Acetylsalicylic acid</td>
<td>0.09</td>
<td>187</td>
<td>10174</td>
<td>964</td>
<td>434</td>
</tr>
</tbody>
</table>

1 Washed cells were incubated in dddH$_2$O or dddH$_2$O plus glycerol at pH 3.20. Five ml samples were removed at the times specified, and radioactively labeled organic-acid probe was added to a final concentration of 0.5 μM. Cells were then separated from the reaction mixture by filtration. Radioactivity in the cells and filtrate was determined.

2 Dry weight of cells retained on filters.

3 Number of minutes cells were incubated in acidic NaCl before samples were removed for internal pH measurements.

4 $C_i$ = cpm from cells on filters. Values were corrected for probe trapped in the extracellular space on the filter. $C_o$ = cpm / 10 μl of filtrate.

5 Ratio of the internal probe concentration in μM of acid / μl of internal volume to μM of acid / 1 μl of filtrate; $([HA + A^-]_{in}) / [HA + A^-]_{out}$.

6 Internal pH was calculated according to the following equation:

$$pH_{in} = pK_a + \log \left( \frac{[HA + A^-]_{in}}{[HA + A^-]_{out}} \times (10^{pH_{o} - pK_a + 1}) - 1 \right)$$
Appendix H. Internal pH Values of *Gluconobacter oxydans* in 0.1 M NaCl at pH 3.20
Appendix H. Internal pH values of *G. oxydans* in 0.1 M NaCl at pH 3.20

<table>
<thead>
<tr>
<th>Date</th>
<th>Probe</th>
<th>Dry Weight (^2) (mg/ml)</th>
<th>Time (min) (^3)</th>
<th>Corrected CPM (^4)</th>
<th>Accumulation Ratio (^5)</th>
<th>Internal pH (^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td>NaCl + Glycerol</td>
<td>NaCl</td>
<td>NaCl + Glycerol</td>
<td>NaCl</td>
</tr>
<tr>
<td>10-03-87</td>
<td>Acetylsalicylic acid</td>
<td>0.56</td>
<td>5</td>
<td>8</td>
<td>34044</td>
<td>34790</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86</td>
<td>88</td>
<td>49881</td>
<td>49408</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>158</td>
<td>167</td>
<td>4781</td>
<td>7065</td>
<td>55</td>
</tr>
<tr>
<td>10-18-87</td>
<td>Acetylsalicylic acid</td>
<td>0.36</td>
<td>23</td>
<td>30</td>
<td>21257</td>
<td>21286</td>
</tr>
<tr>
<td></td>
<td></td>
<td>178</td>
<td>183</td>
<td>20123</td>
<td>20252</td>
<td>104</td>
</tr>
<tr>
<td>11-01-87</td>
<td>Acetylsalicylic acid</td>
<td>0.09</td>
<td>198</td>
<td>177</td>
<td>10142</td>
<td>9625</td>
</tr>
<tr>
<td>11-17-87</td>
<td>Salicylic acid</td>
<td>0.12</td>
<td>203</td>
<td>191</td>
<td>21257</td>
<td>1256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118</td>
<td>120</td>
<td>154</td>
<td>201</td>
<td>454</td>
</tr>
<tr>
<td>11-21-87</td>
<td>Salicylic acid</td>
<td>0.12</td>
<td>206</td>
<td>196</td>
<td>344</td>
<td>499</td>
</tr>
<tr>
<td>11-23-87</td>
<td>Salicylic acid</td>
<td>0.20</td>
<td>178</td>
<td>171</td>
<td>1312</td>
<td>2100</td>
</tr>
<tr>
<td>11-25-87</td>
<td>Salicylic acid</td>
<td>0.20</td>
<td>178</td>
<td>171</td>
<td>1312</td>
<td>2100</td>
</tr>
</tbody>
</table>

---

1. Washed cells were incubated for approximately three hours in 0.1 M NaCl or 0.1 M NaCl plus glycerol at pH 3.20. Five ml samples were removed at the times specified and a radioactively labeled organic-acid probe was added to a final concentration of 0.5 μM (* indicates 1 μM probe concentration). Cells were then separated from the reaction mixture by filtration. Radioactivity in the cells and filtrate were determined.

2. Dry weight of cells retained on filters.

3. Number of minutes cells were incubated in acidic NaCl before samples were removed for internal pH measurements.

4. C\(_i\) = cpm from cells on filters. Values were corrected for probe trapped in the extracellular space on the filters. C\(_o\) = cpm / 10 μl of filtrate.

5. Ratio of the internal probe concentration in μM of acid / μl of internal volume to μM of acid / 1 μl of filtrate;

   \( \frac{[HA + A^-]_i}{[HA + A^-]_o} \)

6. Internal pH was calculated according to the following equation:

   \[
   \text{pH}_i = \text{pK}_a + \log \left( \frac{[HA + A^-]_i}{[HA + A^-]_o} \right) \times (10^{[H^+_o - \text{pK}_a + 1]} - 1)
   \]
Appendix I. Internal pH Values of *Gluconobacter oxydans* in NaCl + KCl at pH 3.20
### Appendix I. Internal pH values of *G. oxydans* in NaCl + KCl at pH 3.20

<table>
<thead>
<tr>
<th>Date</th>
<th>Probe</th>
<th>Dry Weight(^a) (mg/ml)</th>
<th>Time(^b)</th>
<th>Corrected CPM(^c)</th>
<th>Accumulation Ratio(^d)</th>
<th>Internal pH(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-28-87*</td>
<td>Acetylsalicylic acid</td>
<td>0.09</td>
<td>90</td>
<td>80</td>
<td>C(_i) 6298</td>
<td>6266</td>
</tr>
<tr>
<td>10-28-87</td>
<td>Acetylsalicylic acid</td>
<td>0.09</td>
<td>127</td>
<td>120</td>
<td>C(_i) 8850</td>
<td>7881</td>
</tr>
<tr>
<td>10-30-87</td>
<td>Acetylsalicylic acid</td>
<td>0.16</td>
<td>175</td>
<td>168</td>
<td>C(_i) 8366</td>
<td>8047</td>
</tr>
<tr>
<td>11-01-87</td>
<td>Acetylsalicylic acid</td>
<td>0.09</td>
<td>174</td>
<td>164</td>
<td>C(_i) 7429</td>
<td>7687</td>
</tr>
</tbody>
</table>

\(^a\) Washed cells were incubated in 0.1 M NaCl + KCl, 0.1 M NaCl + KCl + glycerol, 0.05 M NaCl + KCl, or 0.05 M NaCl + KCl + glycerol at pH 3.20. Five ml samples were removed at the times specified, and radioactively labeled organic-acid probe was added to a final concentration of 0.5 \(\mu\)M. Cells were then separated from the reaction mixture by filtration. Radioactivity in the cells and filtrate were determined.

\(^b\) Dry weight of cells on filters.

\(^c\) Number of minutes cells were incubated in acidic NaCl before samples were removed for internal pH measurements.

\(^d\) C\(_i\) = cpm from cells on filters. Values were corrected for probe retained in extracellular space on filters. C\(_o\) = cpm / 10 \(\mu\)l of filtrate.

\(^e\) Internal pH was calculated according to the following equation:

\[
\text{pH}_{\text{in}} = \text{pK}_a + \log \left( \frac{[\text{HA} + \text{A}^-]_{\text{in}}}{[\text{HA} + \text{A}^-]_{\text{out}}} \times (10^{\text{pH}_0 - \text{pK}_a} + 1) - 1 \right)
\]
CURRICULUM VITA
of
Patrice Anne Boerman

BUSINESS ADDRESS:

Microbiology Section Department of Biology Virginia Polytechnic Institute and State University Blacksburg, Virginia 24061 (703) 961-5196

PERSONAL DATA:

Birth Date and Place: July 30, 1958, Evanston, Illinois
Family Status: Single
Permanent Address: 743 East Church Street
Williamston, Michigan
Telephone (517) 655-3318
Present Position: Graduate Teaching Assistant
Department of Biology

EDUCATION:

1976 - 1980 Michigan Technological University
Houghton, Michigan
B.S., 1980, Forestry

1985 - Present Virginia Polytechnic Institute and State University, Blacksburg, Virginia
M.S. Candidate, Microbiology

POSITIONS HELD:

1985 - Present Graduate Teaching Assistant,
Department of Biology, Virginia Tech,
Blacksburg, Virginia

1980 - 1985 Environmental Technologist
Virginia Iron, Coal and Coke Company
Coeburn, Virginia
M.S. RESEARCH:

The Role of Respiration-Dependent Proton Translocation in the Acid Tolerance of *Gluconobacter oxydans*

TEACHING EXPERIENCE:

1985 - 1987
Taught three sections each of college level first, and third quarter General Biology Laboratory. Taught two sections of second quarter General Biology Laboratory.

Taught three sections second quarter Principles of Biology Laboratory.

Taught five quarters of college level General Microbiology Laboratory, two sections each quarter.

AWARDS AND HONORS:

Xi Sigma Pi Forestry Honor Fraternity

RESEARCH GRANTS:

Virginia Academy of Science $500
Sigma Xi $450

MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS:

The American Society for Microbiology
The Society for Industrial Microbiology

PERTINENT COURSES:

UNDERGRADUATE:

- Principles of Biology (lecture and laboratory)
- General Botany (lecture and laboratory)
- General Chemistry (lecture and laboratory)
- Organic Chemistry (lecture and laboratory)
- General Microbiology (lecture and laboratory)
- Advanced Microbiology (lecture and laboratory)
- Soil Microbiology (lecture and laboratory)
- Cell Biology
- Sanitary Bacteriology (lecture and laboratory)
- Industrial Microbiology

CURRICULUM VITA
Virology
Virology Laboratory
Techniques in Microscopy
Physiology of Vascular Plants
Principles of Statistical Methods
Technical and Scientific Writing

GRADUATE:

Biochemistry for the Life Sciences
Physiology of Microorganisms
Foundation in Microbial Genetics (lecture and laboratory)

[Signature]

Anne Barman