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Proton Motive Force and the Physiological Basis of Delta pH Maintenance in *Thiobacillus acidophilus*

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At optimal growth pH (3.0) *Thiobacillus acidophilus* maintained an internal pH of 5.6 (ΔpH of 2.6 units) and a membrane potential (Δψ) of some +73 mV, corresponding to a proton motive force (Δp) of −83 mV. The internal pH remained poised at this value through external pH values of 1 to 5, so that the ΔpH increased with decreasing external pH. The positive Δψ increased linearly with ΔpH: above a ΔpH of 0.6 units, some 60% of the increase in ΔpH was compensated for by an opposing increase in Δψ. The highest magnitude of ΔpH occurred at an external pH of 1.0, where the cells could not respire. Inhibiting respiration by CN^- or azide in cells at optimal pH decreased ΔpH by only 0.4 to 0.5 units and caused a corresponding opposite increase in Δψ. Thus, a sizable ΔpH could be maintained in the complete absence of respiration. Treatment of cells with thiocyanate to abolish the Δψ resulted in a time-dependent collapse of ΔpH, which was augmented by protonophores. We postulate that *T. acidophilus* possesses unusual resistance to ionic movements. In the presence of a large ΔpH (>0.6 pH units), limited diffusion of H^+ into the cell is permitted, which generates a positive Δψ because of resistance to compensatory ionic movements. This Δψ, by undergoing fluctuations, regulates the further entry of H^+ into the cell in accordance with the metabolic state of the organism. The effect of protonophores was anomalous: the Δp was only partially collapsed, and respiration was strongly inhibited. Possible reasons for this are discussed.

It is firmly established that acidophilic bacteria, which grow optimally at pH values of around 3.0, maintain a near-neutral cytoplasmic pH and possess a membrane potential (Δψ) of an orientation reversed from that found in neutrophilic bacteria (1, 5–8, 16, 21). There is disagreement, however, on whether the ΔpH is maintained passively or through active chemiosmotic mechanisms. The idea that a passive mechanism is involved was first proposed by Hsung and Haug (5), who reported that the ΔpH in *Thermoplasma acidophila* was not affected by treatment with 2,4-dinitrophenol (DNP) or azide or even when the cells were subjected to boiling; the particular passive mechanism which they postulated was a Donnan potential of positively charged membrane-impermeable molecules that presumably kept H^+ out of the cell. This view is supported by the studies of Oshima et al. (16) with *Bacillus acidocaldarius* and those of Cox et al. (1) with *Thiobacillus ferrooxidans*, which showed that a large ΔpH was maintained in these organisms in the presence of protonophores or respiratory inhibitors. In contrast, Krulwich et al. (8) reported that DNP completely abolished ΔpH in *Bacillus acidocaldarius*, and the protonophores as well as respiratory chain inhibitors stopped proton motive force (Δp)-linked transport functions; these authors concluded that ΔpH in this organism was actively maintained.

We report here on the Δp and the mechanism of cytoplasmic pH maintenance in a recently described (4) acidophilic bacterium, *Thiobacillus acidophilus*, which is unique (11) among acidophiles in having both autotrophic and hetrotrophic growth potentials. It grows optimally at 29°C and at a pH of 3.0 to 3.5 and can be cultivated in simple mineral salts media. Consequently, it is a more suitable experimental organism than the acidophiles studied hitherto: *T. acidophila* and *B. acidocaldarius* require high growth temperatures, and *T. ferrooxidans* is an obligate chemolithotroph which, due to its obligatory dependence on reduced iron and CO_2 (11), is difficult to grow to high cell densities.

MATERIALS AND METHODS

Organism and growth conditions. *T. acidophilus* was obtained from American Type Culture Collection, Rockville, Md. It was cultivated heterotrophically in glucose-mineral salts medium of the composition described by Guay and Silver (4), except that the glucose concentration was 0.4%. The mineral salts base, the

trace elements, and the glucose solutions were autoclaved separately and mixed aseptically. Cultures were grown at 29°C either in a chemostat run under glucose limitation (9) or in Erlenmeyer flasks that were incubated on a rotary shaker. In the latter case, the cells were harvested in late log phase. Before harvesting, culture purity was checked by microscopic examination and by streaking on glucose agar plates. *Escherichia coli* K-12 was obtained from our departmental culture collection and was grown in Difco nutrient broth.

Preparation of cell suspensions and flow dialysis. *T. acidophilus* cells were harvested by centrifugation (9,000 × *g*, 20 min, 4°C) and washed once in 0.1 M β-alanine buffer (pH 3.0); in experiments designed to check the effect of external pH on various parameters, the pH of this buffer was changed appropriately. The washed cells were taken up in buffer of the same composition and pH values as the wash buffer to a cell density of approximately 20 mg of protein per ml. In the experiment conducted to check the effect of exposure of *E. coli* to pH 3.0, *E. coli* cells were washed in 0.1 M potassium phosphate buffer (pH 7.0) and then taken up in 0.1 M β-alanine buffer (pH 3.0).

The ΔpH and Δψ values were measured at 29°C by determining the distribution of ^{14}C-labeled aspirin and KSCN, respectively, using a flow-dialysis apparatus (17) modified by the method of Feldman (2). Hsung and Haug (5), Rottenberg (19), and Cox et al. (1) have presented evidence that SCN^- distribution does indeed reflect Δψ values in acidophilic bacteria, and Cox et al. have discussed why the use of a probe like aspirin (pK_a = 3.48) with a relatively low pK_a value is preferable for ΔpH measurement in these organisms. The reaction mixture in the upper chamber of the apparatus contained, in a total volume of 0.8 ml, 0.1 M β-alanine buffer at appropriate pH, whole cells to a final concentration of 2 mg of protein per ml, and either 62.5 μM ^{14}C-aspirin (20 mCi/mmol), or 40 μM [^{14}C]KSCN (31 mCi/mmol). These probe concentrations were found to be optimal in control experiments in which a range of concentrations (20 to 82.5 μM for aspirin, 20 to 100 μM for KSCN) were employed; up to 80 μM aspirin and 200 μM KSCN had no effect on the respiratory capacity of this bacterium. Additional control experiments established that the measured values of Δψ and ΔpH were independent of the cell protein concentration employed in the reaction mixture in the range of 1.4 to 2.8 mg of protein per ml. β-Alanine buffer (0.1 M) at appropriate pH was pumped through the lower chamber of the apparatus at the rate of 2 ml/min; 1-ml fractions were collected and assayed for radioactivity by mixing 0.5 ml of each fraction with 3.0 ml of Packard Instagel scintillation cocktail and counting in a Beckman LS 8000 liquid scintillation counter. The upper and lower chambers of the apparatus were separated by spectropore dialysis tubing, and the upper chamber was kept stirred with a magnetic stirring bar. The typical procedure was to add the probe to the upper chamber 3 to 4 min before the addition of cells; the rate of removal of the probe from the upper chamber during this time, after correcting for dilution caused by the addition of the cells, was used as the base line (see text for further details). Studies with heated cells, or those treated with appropriate reagents (e.g., SCN^- plus carbonyl cyanide-*m*-chlorophenyl hydrazone [CCCP]; see Fig. 6), which did not possess ΔpH or Δψ, established that there was no nonspecific binding of either of the probes to the cells. Δψ and ΔpH were calculated from the ionic distributions as discussed by Ramos et al. (17).

EDTA treatment of cells. Since the outer cell envelope of these gram-negative bacteria could present a barrier to the influx of the probe molecules and other agents employed in this study, EDTA-treated cells were employed in several experiments. For EDTA treatment, washed cells were taken up in 0.1 M β-alanine buffer at appropriate pH to a density of 7 mg of protein per ml. EDTA was added to a final concentration of 100 mM, and the suspension was shaken for 3 min at 29°C. The cells were then washed and suspended to a density of approximately 20 mg of cell protein per ml in buffer of the same composition and corresponding pH.

Incubation with various agents. In some experiments, the effect of prolonged incubation of cells with various agents (protonophores, respiratory inhibitors, etc.) was investigated. Incubation was done by using 2 to 5 ml of cell suspensions (20 mg of cell protein per ml) to which the desired agent was added and which were held at 29°C with magnetic stirring. Control cells (without any agents) were incubated in parallel under identical conditions. At appropriate intervals sufficient volumes of these suspensions to give a final concentration of 2 mg of protein per ml in the flow-dialysis reaction mixture were removed for ΔpH and Δψ measurements. Enough of the appropriate agent(s) was added to the reaction mixture, as well as the dialysis buffer to ensure that the desired concentration of the agent was maintained in the reaction mixture during the course of flow dialysis.

Cell water determination. Cell water determination was done as described by Rottenberg (20) by using a ^{14}C source (360 μCi/mmol) as a marker for extracellular water. One milligram of cell protein corresponded to 2.04 ± 0.29 μl of cell water (average of eight individual determinations).

Miscellaneous. Oxygen uptake was measured by using a Clarke-type oxygen electrode (Yellow Spring Instrument Co., Yellow Spring, Ohio) as described by Matin and Konings (12), except that measurements were made at 29°C. The reaction mixture (3-ml volume) contained 0.1 M β-alanine buffer (pH 3.0), 0.5 mg of cell protein, and 10 mM glucose. Endogenous uptake rate was measured in the absence of glucose and was found to be only some 5 to 10% lower than the rate observed after the addition of glucose. Protein was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard.

[^{14}C]KSCN and ^{14}C-aspirin were purchased from Amersham/Searle Corp., Arlington Heights, Ill.

RESULTS

ΔpH, Δψ, and Δp values in *T. acidophilus*. The flow-dialysis experiments to measure ΔpH were initiated by adding ^{14}C-aspirin to the upper chamber of the apparatus. By the time the first fraction was obtained, the radioactivity appearing in the dialysate had reached a maximum level and then decreased gradually at a constant rate (Fig. 1A). Addition of EDTA-treated cells (between fraction 7 and 8) caused a sharp de-

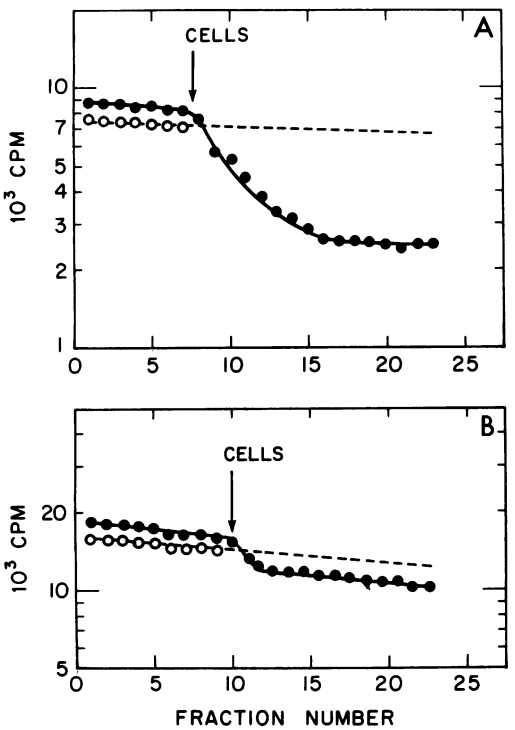

FIG. 1. Flow-dialysis determination of transmembrane distribution of ^{14}C-aspirin (A) and [^{14}C]KSCN (B) in *T. acidophilus*. Thle upper chamber of the apparatus contained, in a total volume of 0.8 ml, 0.1 M β-alanine buffer (pH 3.0) and 62.5 μM ^{14}C-aspirin (A), or 40 μM [^{14}C]KSCN (B); EDTA-treated cells (0.1 ml) were added at the point indicated by arrows. See text for further details. Symbols: ●, experimental points; ○, values (counts per minute) for fractions 1 through 7 corrected for the dilution due to the addition of cell suspension (0.875-fold). pH_{in} and $\Delta\psi$ were calculated by using the following equations: (i) $pH_{in} = pK_a + \log_{10} \{[(aspirin)_{in}/(aspirin)_{out}](1 + 10^{pH_{out} - pK_a}) - 1\}$ and (ii) $\Delta\psi = 59.9 \times \log_{10} [(SCN^-)_{in}/(SCN)_{out}]$.

crease in the dialysate radioactivity, which was, of course, due to both the dilution of the probe in the upper chamber and its uptake by the cells. After correction for dilution (broken line), the transmembrane distribution of the probe was determined, and cellular pH was calculated. The experiment shown in Fig. 1A gave a value of 5.96 for the cytoplasmic pH corresponding to a ΔpH of 2.96; in 25 individual measurements an average ΔpH value of 2.60 ± 0.34 was found.

Attempts were made to determine the uptake of [^{14}C]triphenylmethylphosphonium by the cells in several individual experiments by using the flow-dialysis technique as described above. None was ever found, indicating the absence of a negative $\Delta\psi$. However, [^{14}C]KSCN was taken up readily, and in the experiment shown in Fig. 1B, a $\Delta\psi$ value of +78 mV was found; additional measurements (17 in all) gave an average value of +73 ± 12 mV. These values of ΔpH and $\Delta\psi$ correspond to a Δp value of −83 mV.

The effect of various changes in the experimental procedure on these measurements was investigated. Results of 7 to 10 independent measurements for each variation showed that omission of EDTA treatment of cells, inclusion in the reaction mixture of a balanced salts mixture (of the composition and concentration employed in the growth medium), use of cells that had been kept frozen in liquid nitrogen for up to 3 weeks, or maintenance of a constant stream of water-saturated oxygen over the upper chamber of the flow-dialysis apparatus made no difference to the measured values of ΔpH and $\Delta\psi$. Similarly, there appeared to be no difference in the value of these parameters between chemostat- and batch culture-grown cells. However, inclusion of glucose (10 mM) in the reaction mixture collapsed ΔpH by some 20%. The last observation suggests that the uptake of glucose in this bacterium occurs through a symport with protons, as appears to be the case in the other thiobacilli (13, 18). However, all previous indications of H^+ symport of glucose are based on negative evidence, namely, the lack of involvement of the phosphotransferase system in glucose transport. This is the first time that a direct demonstration is made of the collapse of ΔpH accompanied by the addition of glucose. In all subsequent work, the experimental protocol as specified in the legend to Fig. 1 was followed; although liquid nitrogen-frozen cells appeared to be as good as fresh cells, the freshly harvested cells were nevertheless used. Similarly, although EDTA-treatment appeared to make no difference to the measured value of Δp, the effect of this treatment on every parameter investigated was determined. It made no difference to any of the parameters examined. Both ΔpH and $\Delta\psi$ were abolished by prior heating (90°C for 5 min) of cells, and such cells exhibited no binding of either of the probe molecules.

Effect of change in external pH on various parameters. The incentive to investigate the effect of change in external pH came from the observation that the respiratory capacity of *T. acidophilus* was greatly influenced by the pH at which the cells were suspended (Fig. 2). At or below pH 1.5, the bacterium could not respire. As the external pH increased there was a sharp rise in the oxygen uptake rate, reaching a peak value at between pH 2.5 and 3.5, the pH range of optimal growth. Raising the pH above these values led to a gradual decline in oxygen uptake capacity. We reasoned that if respiration were essential for the maintenance of ΔpH, then the ΔpH at different external pH values should

exhibit changes in parallel to the changes in the respiratory potential; in particular, there should be no ΔpH at external pH values of 1.5 or lower, where the organism cannot respire. The results (Fig. 2) show, however, that the ΔpH attains its highest value—some 4.5 pH units—at an external pH of 1.0, and that in general there is no correlation at all between the respiratory capacity and the capacity to maintain a ΔpH. The internal pH remained poised at 5.5 to 5.8 throughout the range of the external pH values examined, leading to a linear increase in ΔpH with decreasing external pH. It is noteworthy that the ΔpH slope extrapolates to zero at an external pH value of 5.8 (Fig. 2), which is consistent with the measured values of cytoplasmic pH at different external pH values.

Since the cytoplasmic pH remained unaffected by external pH, it was of interest to determine whether the Δψ values were affected by changes in external pH. As regards the causation of Δψ, one possibility is that it arises from the passive diffusion into the cells of H^+ down their electrochemical gradient, and if this is so, and since ΔpH (i.e., H^+ diffusion potential) changed with external pH, some sort of parallel change in Δψ can be expected. The results (Fig. 3) revealed an inverse linear relationship between Δψ and external pH; plotted in another way (Fig. 4), the data indicate a direct linear relationship between Δψ and ΔpH. At external pH of 5.0, the cells took up neither [^{14}C]triphenylmethylphosphonium nor [^{14}C]KSCN, indicating the absence of a measurable Δψ. Decreasing the external pH increased the Δψ (Fig. 3),

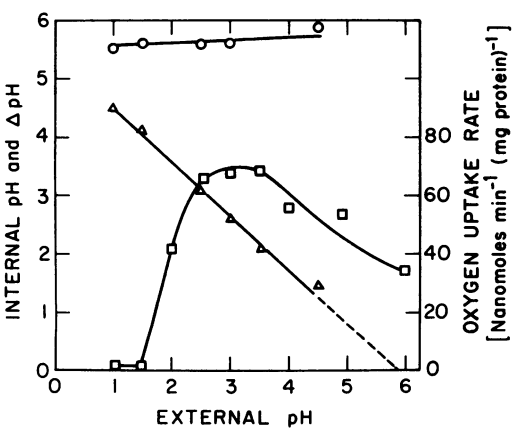

FIG. 2. Effect of external pH on internal pH (○), ΔpH (△), and respiratory potential (□) of *T. acidophilus*. Freshly harvested cells were washed once in 0.1 M β-alanine buffer at appropriate pH value and were suspended in buffer of the same composition and pH as described in the text. See the text for procedures used in different measurements.

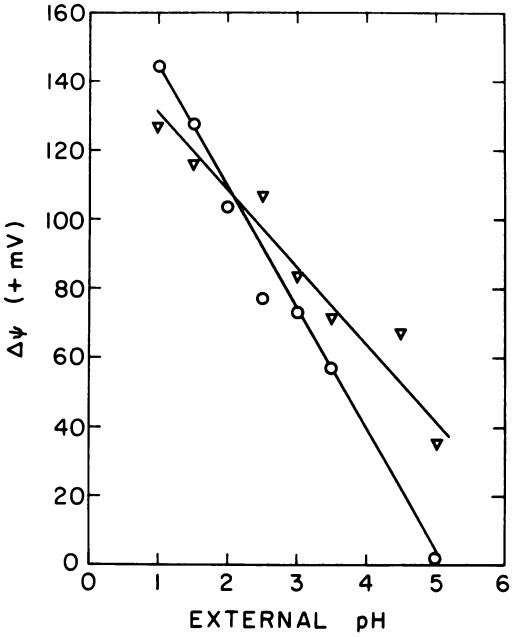

FIG. 3. Effect of external pH on Δψ (○), and Δp (▽) in *T. acidophilus*. Cell suspensions were prepared as described in legend to Fig. 2 and in the text.

and at an external pH of 1.0 the Δψ was some +140 mV. The slope relating Δψ and ΔpH (Fig. 4) extrapolated to zero Δψ at a ΔpH value of −37 mV (~0.6 pH units). Above this value of ΔpH, some 60% (or 37 mV) of the increase in ΔpH was compensated for by an opposing increase in Δψ. The data indicate that if the positive Δψ is indeed due to the diffusion of H^+, then the permeability barrier of the cytoplasmic membrane is such that no diffusion of H^+ is permitted up to a ΔpH value of some −37 mV; above this ΔpH, about 40% of the extant H^+ diffusion potential is resisted. These points are reflected in the changes in Δp in response to external pH (Fig. 3).

Effect of inhibitors of respiration. An alternate approach to investigating the importance of respiratory potential to ΔpH maintenance is, of course, to make use of respiratory inhibitors. At the optimal external pH of 3.0, cyanide or azide at 10 mM concentration or above inhibited respiration in *T. acidophilus* by 95%, and we therefore investigated their effect on ΔpH. The presence of CN^- or azide in the reaction mixture decreased ΔpH by some 0.5 pH units (~30 mV) and caused a corresponding increase in the positive Δψ so that the Δp was reduced to −36 mV (Table 1).

It is clear that a sizable ΔpH can be maintained in the absence of respiration. The ques-

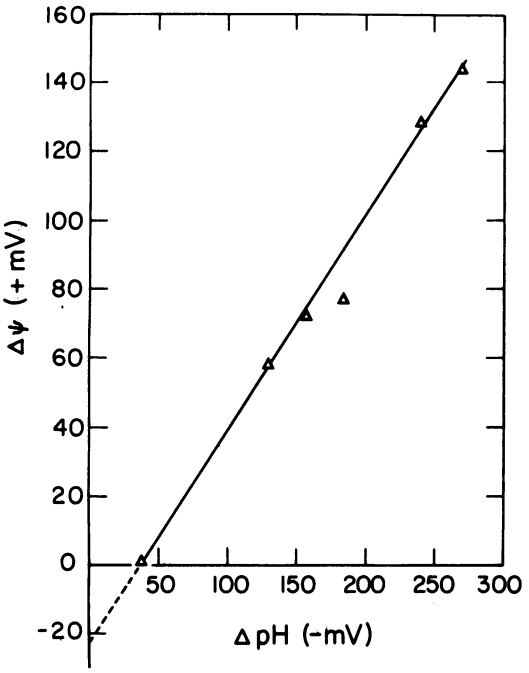

FIG. 4. $\Delta\psi$ in *T. acidophilus* plotted as a function of ΔpH at different external pH values.

tion we now posed was, for how long? This is an ecologically important question since in their natural milieu the acidophiles must often encounter conditions that preclude respiration for extended time periods. To seek an answer to this question, we incubated resting cell suspensions of *T. acidophilus* in the presence of CN^- or azide and measured Δp parameters for up to 48 h. Compared with cells just exposed to the respiratory inhibitor, there was no further change in ΔpH, $\Delta\psi$, and Δp values in those incubated with the inhibitor for 24 h; only after 48 h of incubation was there a further significant decrease in ΔpH (Table 1). Thus, a ΔpH of considerable magnitude can be maintained in *T. acidophilus* for prolonged time periods in the absence of respiration.

Control cell suspensions prepared from the same batch of cells used in the above experiments, but incubated in parallel without the addition of the respiratory inhibitor, exhibited no change in Δp parameters up to 24 h, and even after 48 h of incubation the changes observed were small (Table 1). Electron micrographs of thin sections of *T. acidophilus* cells have revealed the presence of large amounts of inclusions that resemble (14) poly-β-hydroxybutyric acid (unpublished observation), and it may be surmised that an H^+ pump energized by the respiration of this reserve polymer accounts for the different capacities of these and respiration-inhibited cells to maintain ΔpH and Δp values. We have extended such long-term studies to the measurement of cell viability, cellular ATP levels, and other parameters and will present the results in a separate communication (E. Zychlinsky and A. Matin, manuscript submitted).

For comparative reasons, we determined the capacity of *E. coli*, which is of course neutrophilic, to maintain a ΔpH at an external pH of 3.0. *E. coli* had no respiratory capacity at pH 3.0, so that any ΔpH that it can maintain at this external pH value will be respiration independent. *E. coli* cells washed once in phosphate buffer (0.1 M, pH 7.0) were suspended in 0.1 M β-alanine buffer at pH 3.0 and incubated at 37°C, as described above. After 20 min of incubation, these cells possessed a cytoplasmic pH of 5.2, corresponding to a ΔpH of 2.2. The cells also possessed a $\Delta\psi$ of +120 mV, so that a small Δp of some −30 mV was retained after this time period. Neither the ΔpH nor the $\Delta\psi$ could be detected after incubation at pH 3.0 of 2.5 h or longer (24 h). It is evident that nonrespiring *T. acidophilus* cells possess a much greater capacity to maintain ΔpH at acidic external pH values than do nonrespiring *E. coli* cells.

Effect of protonophores. As mentioned above, the effect of protonophores on Δp parameters in acidophiles has been a matter of some contro-

TABLE 1. Effect of incubation with respiratory inhibitors on components of Δp in *T. acidophilus*[a]

Time of incubation	Control cells			Cells plus respiratory inhibitor[b]		
	ΔpH	$\Delta\psi$ (mV)	Δp (mV)	ΔpH	$\Delta\psi$ (mV)	Δp (mV)
0[c]	2.5	+60	−90	2.1	+90	−36
1 min	2.5	+60	−90	2.1	+94	−32
15 min	2.6	+63	−93	2.1	+92	−34
24 h	2.7	+70	−92	2.1	+94	−32
48 h	2.4	+90	−54	1.7		

[a] The procedure was as described in the text and in the legend to Fig. 5; external pH was 3.0.
[b] Respiration was inhibited by 10 mM cyanide or azide.
[c] Values in cells immediately after exposure to the inhibitor.

versy, and we therefore investigated their effects in *T. acidophilus*. All five protonophores tested with cells suspended at external pH of 3.0 had only a minimal effect on ΔpH, even though the concentration used was higher than the known effective concentrations of these agents in neutrophilic bacteria (Table 2). All of them, however, brought about a significant increase in Δψ (>20 mV). The Δp decreased but remained at significant values of −37 to −54 mV in the presence of the protonophore. Protonophores are believed to be most effective at pH values close to their pK_a values (3, 15), and for this reason picric acid, which has a pK_a of 0.3, was included in these studies. Since its effectiveness was no different from that of the other protonophores, the pK_a effect has probably little to do with the observed results.

The effect of one protonophore (100 μM CCCP) on ΔpH and Δψ was investigated in *T. acidophilus* cells suspended also at an external pH of 2.0. There was no change in ΔpH, which remained poised at 3.4; a slight increase in Δψ—from +103 to +118 mV—was observed; and a Δp of some −86 mV remained in the presence of the protonophore. Thus, at this lower external pH value the protonophore was even less effective than at pH 3.0.

As was discussed above, *E. coli* cells exposed to pH 3.0 transiently possess ΔpH as well as a positive membrane potential. We wondered whether the protonophores would abolish this ΔpH, and therefore we tested the effect of 100 μM CCCP in *E. coli* cells incubated at pH 3.0 for 20 min. The protonophore was completely ineffective in collapsing the ΔpH (Fig. 5). Thus, under these conditions, CCCP is ineffective in *E. coli* as well, although control experiments established that the same solution of the protonophore at 5 μM was effective in completely collapsing ΔpH in *E. coli* cells at pH 6.5.

Effect of anion addition. It is evident that under all conditions, but especially under those where the cells are poisoned by a respiratory

TABLE 2. Effect of protonophores on components of Δp in *T. acidophilus*[a]

Protonophore[b]	Concn (μM)	ΔpH	Δψ (mV)	Δp (mV)
None		2.6	+70	−86
CCCP	10 to 100	2.2	+95	−37
FCCP	25 and 50	2.4	+93	−51
TCS	20	2.6		
DNP	50	2.4	+90	−54
Picric acid	50	2.3	+94	−44

[a] Washed cell suspensions at pH 3.0 were used.
[b] Abbreviations: FCCP, *p*-trifluoromethoxycarbonyl-cyanidephenylhydrazone; TCS, tetrachlorosalicyalanilide.

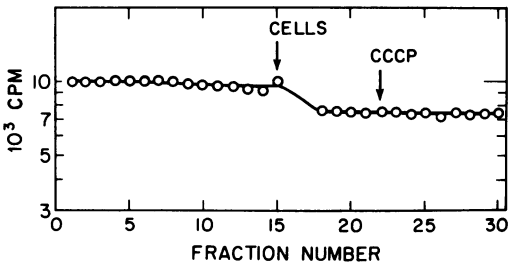

FIG. 5. Effect of CCCP on ΔpH in *E. coli* suspended at pH 3.0. See the text and the legend to Fig. 1 for procedure; values of counts per minute in dialysate before the addition of cells have been corrected for the dilution effect.

chain inhibitor or a protonophore, a significant part of the extant ΔpH is counterbalanced by a Δψ of opposite polarity. What happens to ΔpH when this Δψ is abolished? We approached this question by treating the cells with the anion thiocyanate, which has been shown to be lipophilic and an effective agent for collapsing a positive membrane potential (8, 19). However, when 1 mM thiocyanate was included in the reaction mixture for cytoplasmic pH measurement, no effect on this parameter was observed. This lack of effect could have been due to the impermeability of the cell membrane to H^+. Even if SCN^- had instantaneously abolished the Δψ in the above experiment, an effect on the internal pH would be delayed if the cytoplasmic membrane hampers the influx of H^+. Consequently, the effect on ΔpH of incubation of cells with SCN^- for several minutes was investigated. It is evident (Fig. 6) that SCN^--exposed cells exhibited a time-dependent collapse of ΔpH; at 15 min, the SCN^--exposed cells possessed only about 45% ΔpH compared with control cells incubated without SCN^- under otherwise identical conditions and length of time. Krulwich et al. (8) also showed that SCN^- abolishes ΔpH in *B. acidocaldarius* although, unlike results presented here, the effect they reported was instantaneous.

If the time dependence of the SCN^- effect on ΔpH is indeed due to the permeability barrier to H^+, then addition of protonophores might augment this effect. This was found to be the case (Fig. 6). Cells incubated with CCCP and SCN^- showed complete collapse of ΔpH by 8 min; by contrast, those incubated with SCN^- alone showed only a 25% collapse in ΔpH at a corresponding time interval. Incubation with CCCP alone had only a minor effect on ΔpH during the course of the experiment, in agreement with the data presented in Table 2. Precisely analogous results were obtained when SCN^- concentration was increased to 10 mM and picric acid (50

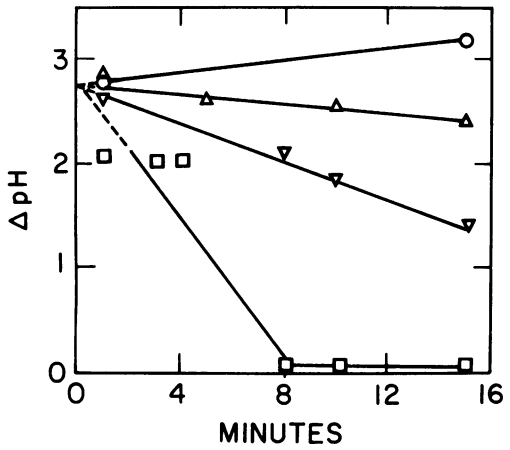

FIG. 6. Effect of incubation of *T. acidophilus* cells with various agents on their ΔpH values. Washed cell suspensions in 0.1 M β-alanine buffer (pH 3.0) (cell density, 20 mg/ml) were incubated with the indicated agent at 29°C with magnetic stirring. Samples were removed at appropriate time intervals to measure ΔpH. Symbols: ○, control cells, no agent added; △, cells incubated with 100 μM CCCP; ▽, cells incubated with 1 mM KSCN; □, cells incubated with 100 μM CCCP plus 1 mM KSCN. Reaction mixtures employed in ΔpH determination as well as the inflow buffer for the lower chamber of the flow-dialysis apparatus contained the appropriate agents at the indicated concentrations.

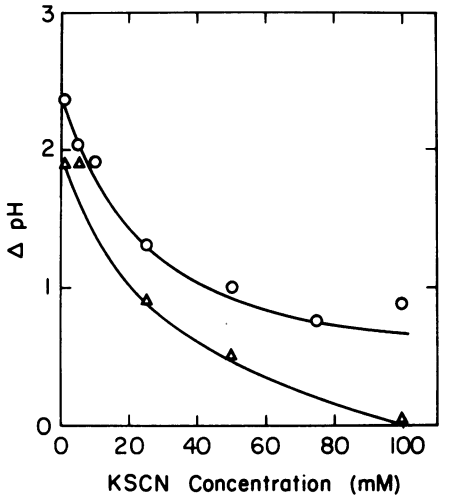

FIG. 7. Effect of 5 min of incubation with different concentration of KSCN without (○) and with 100 μM CCCP (△) on ΔpH in *T. acidophilus*. The method used was as described in legend to Fig. 5 and in the text.

μM) replaced CCCP in the experiment of the above type (data not shown).

Apart from the resistance to H^+ influx, the time dependence of the SCN^- effect could be due also to the permeability barrier to SCN^- itself leading to a gradual collapse of $\Delta\psi$. This possibility was supported by the experiment presented in Fig. 7, which shows that the effectiveness of SCN^- alone in collapsing ΔpH increased as its concentration in the incubation mixture was increased. It is noteworthy, however, that in agreement with the results discussed above, at each concentration tested, the concurrent presence of CCCP augmented the SCN^- effect.

Effect of protonophores on respiration. In bacteria as well as in mitochondria, protonophores abolish Δp and in general cause an increase in the rate of respiration. As we have seen (Table 2), the protonophores did partially collapse Δp in *T. acidophilus*, and we therefore wondered whether this collapse had any effect on the respiratory rate of this bacterium. We found to our surprise (Table 3) that the different protonophores, at concentrations comparable to those at which they had partially collapsed Δp (Table 2), acted as strong inhibitors of respiration in this bacterium: the respiratory rate was inhibited up to 80 to 100%. To our knowledge this is the first instance of inhibition of respiration by protonophores.

One of the experimental observations presented by Cox et al. (1) to support the idea that SCN^- distributes in accordance with $\Delta\psi$ in *T. ferrooxidans* was that DNP greatly augmented the SCN^--caused inhibition of respiration in this organism. No information was provided on the effect of DNP alone on respiration, but if what we have shown for *T. acidophilus* applies also to *T. ferrooxidans*, then their conclusion would appear to be unwarranted on the basis of the evidence presented here.

DISCUSSION

We have demonstrated that, like other acidophilic bacteria so far examined (1, 5–8, 16, 21),

TABLE 3. Effect of protonophores on respiratory rate of *T. acidophilus*[a]

Protonophore[b]	Concn (μM)	% Inhibition[c]
CCCP	10 to 100	65 to 80
TCS	50 to 100	75
FCCP	5	75
	15 to 100	85
DNP	50 to 200	70
	>200 to 1,000	100
Picric acid	50	90

[a] Washed cell suspensions at pH 3.0 were employed.

[b] Abbreviations as in footnote *b* of Table 2.

[c] Control value was 72 nmol of oxygen taken up per min per mg of protein.

T. acidophilus, suspended at its optimal growth pH, maintains a near-neutral cytoplasmic pH and a $\Delta\psi$ of polarity reversed from that found in neutrophilic bacteria. The magnitude of Δp was -83 mV. This is similar to values reported in other acidophiles based on probe concentrations comparable to those employed here: -83 to -100 mV in *B. acidocaldarius* (8), -143 mV in *T. ferrooxidans* (1), and -120 mV in *T. acidophila* (5). These values are lower than those reported for neutrophilic bacteria, and it may be that the acidophiles can drive their energy-requiring functions at lower values of Δp. However, it is not known how valid is the flow-dialysis method in measuring the absolute magnitude of Δp in intact cells of acidophilic bacteria. It is conceivable that due to permeability impediments and other factors the probes do not fully equilibrate with Δp—indeed, experimental evidence presented in this paper (Fig. 7) suggests that permeability barrier exists to SCN^-. We are therefore attempting to measure Δp parameters in *T. acidophilus* spheroplasts as well as to employ fluorimetric methods to measure these parameters in intact cells, spheroplasts, and membrane vesicles of this bacterium.

It is evident that at the optimal growth pH, respiration contributes, albeit to a small degree, to the maintenance of ΔpH in this organism. Treatment of cells with CN^- or azide at concentrations at which these inhibitors abolished respiration instantaneously decreased ΔpH by some 0.4 to 0.5 units, indicating that this component of the ΔpH depended on the respiration-linked extrusion of H^+. However, this represents <20% of the total ΔpH, and it is patently clear that a large ΔpH, corresponding to some -126 mV at optimal pH, can be maintained for prolonged periods in the complete absence of respiration. That respiration is not essential for the maintenance of ΔpH is indicated also by the lack of any correlation between the respiratory capacity and the capacity to hold ΔpH and by the fact that the highest ΔpH—of some -270 mV—was found at an external pH which did not permit respiration. Our results in this respect agree with those of Hsung and Haug (5), Searcy (21), and Cox et al. (1), but differ from those of Krulwich et al. (8), who implied that the lack of respiration abolished ΔpH in *B. acidocaldarius*.

What enables the bacterium to maintain this large ΔpH in the absence of respiration-linked extrusion of H^+? Experiments designed to check the effect of SCN^- on normal cell suspensions (Fig. 6 and 7) strongly suggest that the positive $\Delta\psi$ plays a role in opposing the entry of H^+ into the cells; since the positive $\Delta\psi$ is increased in azide- or CN^--treated cells, this role is most likely enhanced under these conditions of inhibited respiration. Still, the increased $\Delta\psi$ compensates only partially for the extant ΔpH, and a net force of some -35 mV remains impelling the H^+ to move into the cells. Whether this force is passively resisted cannot at present be decided. Although the relationship between $\Delta\psi$ and ΔpH (Fig. 4) suggests that an H^+ diffusion potential of some -37 mV can be passively resisted, it cannot be ruled out that active extrusion of H^+, powered by an ATPase(s), continues in respiration-inhibited cells. Preliminary studies (E. Zychlinsky and A. Matin, unpublished observations) show that cells incubated with CN^- (or azide) plus *N,N'*-dicyclohexylcarbodiimide for prolonged time periods do not show any lower ΔpH value than those incubated with CN^- (or azide) alone, suggesting lack of involvement of ATPases. However, further work involving direct determination of ionic movements in respiration-inhibited cells is required before firm conclusions can be drawn on this point.

The question as to how the positive $\Delta\psi$ arises is of central importance. Hsung and Haug (5) ascribed it to a Donnan potential of charged macromolecules, but this view is hard to reconcile with the observed large changes in $\Delta\psi$ in response to manipulations (change in external pH, treatment with respiratory poisons, etc.) that caused only a minimal change in the cytoplasmic pH. We believe it more plausible that the diffusion of H^+ into the cells plays a role in the generation of $\Delta\psi$. This idea is consistent with the experimental evidence: the positive $\Delta\psi$ increases with decreasing external pH; a direct relationship exists between the positive $\Delta\psi$ and ΔpH, and treatment of cells with respiratory inhibitors or protonophores causes equivalent and opposite changes in ΔpH and $\Delta\psi$. It must be emphasized, however, that movement of some other ion(s) is probably also involved. For instance, when external pH was decreased from 3.0 to 1.0, there was an increase in $\Delta\psi$ of some $+67$ mV (Fig. 3). If all of it were due to H^+ influx into the cell, a concurrent drop of ca. 1 pH unit would have occurred in the cytoplasmic pH. In fact, however, a drop of only 0.3 pH unit was observed (Fig. 2). Thus, uptake of a cation other than H^+ or expulsion of an anion probably occurred under these conditions.

The capacity to maintain a positive $\Delta\psi$ for prolonged time periods implies an unusual capacity to hold cellular cations. For instance, like other bacteria, *T. acidophilus* possesses a high concentration of intracellular K^+ (0.4 M; E. Zychlinsky and A. Matin, unpublished observations) whose compensatory exit from the cells could have neutralized the positive $\Delta\psi$. We do not know whether the sequestering of cellular cations is achieved through passive membrane properties or through active mechanisms. The fact that respiration-inhibited cells (Table 1) as

well as those treated with CN^- and *N,N'*-dicyclohexylcarbodiimide (E. Zychlinsky and A. Matin, unpublished observations) can maintain the positive $\Delta\psi$ of the order of +100 mV for prolonged periods of time would tend to implicate passive resistance, but further work is required on this point. By contrast, *E. coli* could not hold a positive $\Delta\psi$ for more than a few hours. When exposed to pH 3.0 over short time periods, the bacterium exhibited a ΔpH and a positive $\Delta\psi$ that indicated an influx of H^+ into the cells without counterbalancing movement of other ions. However, within 2.5 h of exposure to pH 3.0 both the ΔpH and the $\Delta\psi$ were completely eliminated, indicating presumably compensatory exit of cellular cations, such as K^+.

As regards the effect of protonophores, again our results agree with most of the previous work except that of Krulwich et al. (8). All the five protonophores tested in this study caused only a slight (7 to 15%) collapse in ΔpH. A roughly corresponding increase in $\Delta\psi$ of opposite polarity took place, but a Δp of up to -54 mV remained. We do not know why the protonophores failed to abolish the residual Δp. It is conceivable that the combination of low extracellular pH and the presence of a large $\Delta\psi$ of +90 to +100 mV somehow impairs the effectiveness of these agents. This suggestion is consistent with three observations reported in this paper: First, elimination of $\Delta\psi$ (by the use of SCN^-) greatly augmented the effectiveness of the protonophores (Fig. 6); second, *T. acidophilus* cells suspended at pH 2.0 which possessed a $\Delta\psi$ of $>$ +100 mV were even less sensitive to CCCP than the cell at pH 3.0, which possessed a much lower positive $\Delta\psi$; and third, CCCP had no effect also on *E. coli* cells, when they possessed a $\Delta\psi$ of some +100 mV.

It is very striking that even though the protonophores caused little change in intracellular pH, they strongly inhibited respiration in *T. acidophilus*. This is, of course, in complete contrast to the effect of protonophores on neutrophilic bacteria, mitochondria, etc., where generally they stimulate respiration. Again, we can only speculate as to the mechanism of this inhibition. It may be that the elevation of the $\Delta\psi$ to the value of +90 to +100 mV that invariably results from the exposure to protonophores (Table 2) leads to a physical alteration in the spatial orientation of the respiratory chain, which obstructs the flow of electrons down the chain. The fact that the respiratory capacity is rather abruptly eliminated at or below an external pH of 3.0 (Fig. 2) would appear to be consistent with this view: it is around this external pH value that the $\Delta\psi$ acquires a magnitude of around +100 mV.

We postulate the following mechanism for the maintenance of ΔpH in *T. acidophilus*. We believe that an unusual resistance to ionic movements, presumably mainly to the efflux of cellular cations, holds the key to acidophilism. In the presence of a large ΔpH (>0.6 units), limited diffusion of H^+ into the cell is permitted, which generates a positive $\Delta\psi$ because of absence of compensatory ionic movements. In respiring cells, this $\Delta\psi$ limits further entry of H^+ into the cell to a rate compatible with the cell's energy requirements and its capacity to actively pump H^+ out. In the absence of respiration, further net influx of H^+ occurs which, without decreasing the cytoplasmic pH to non-physiological levels does, nevertheless, increase the positive $\Delta\psi$, so that the Δp is decreased to a relatively low level. This low level of Δp is maintained either because of the passive impermeability of the cell membrane to H^+ or because ATPases, energized by endogenous reserves, continue to pump H^+ out of the cell. Work is in progress to test the various tenets of this postulate.

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