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Transmembrane pH Gradient and Membrane Potential in *Clostridium acetobutylicum* during Growth under Acetogenic and Solventogenic Conditions

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The proton motive force and its electrical and chemical components were determined in *Clostridium acetobutylicum*, grown in a phosphate-limited chemostat, using [^{14}C]dimethyloxazolidinedione and [^{14}C]benzoic acid as transmembrane pH gradient (ΔpH) probes and [^{14}C]triphenylmethylphosphonium as a membrane potential ($\Delta\psi$) indicator. The cells maintained an internal-alkaline pH gradient of approximately 0.2 at pH 6.5 and 1.5 at pH 4.5. The ΔpH was essentially constant between pH 6.5 and 5.5 but increased considerably at lower extracellular pH values down to 4.5. Hence, the intracellular pH fell from 6.7 to 6.0 as the external pH was lowered from 6.5 to 5.5 but did not decrease further when the external pH was decreased to 4.5. The transmembrane electrical potential decreased as the external pH decreased. At pH 6.5, $\Delta\psi$ was approximately -90 mV, whereas no negative $\Delta\psi$ was detectable at pH 4.5. The proton motive force was calculated to be -106 mV at pH 6.5 and -102 mV at pH 4.5. The ability to maintain a high internal pH at a low extracellular pH suggests that *C. acetobutylicum* has an efficient deacidification mechanism which expresses itself through the production of neutral solvents.

The growth and metabolism of *Clostridium acetobutylicum* in batch culture at initial pH values near neutrality is characterized by two distinct physiological phases (19). A predominantly acetate-butyrate fermentation occurs at neutral pH, whereas acetone and butanol are formed in substantial quantities when the pH of the medium falls below 5.0 (19). Recently, several attempts have been made to understand the regulatory mechanism governing this physiological transition (1, 2, 5, 7). The evidence suggests that the switch to solventogenesis is triggered not only by the fall in external pH but also by the increased fatty acid concentration in the medium (16, 17). Both factors were correlated with a critical role of the level of the undissociated acidic products, especially butyric acid, which were shown to be able to penetrate the cell membrane (12, 17). Therefore, it may be suggested that perturbations in intracellular pH (pH_{in}) caused by the production of fatty acids would lead to modification of cell metabolism and initiation of solvent formation (20).

The objective of this investigation was to determine the magnitude of the proton motive force ($\Delta\tilde{\mu}_{H^+}/F$) and its components, the transmembrane pH gradient (ΔpH) and the membrane potential ($\Delta\psi$), in *C. acetobutylicum* grown under acetogenic and solventogenic conditions.

MATERIALS AND METHODS

Organism and growth conditions. *C. acetobutylicum* ATCC 824 was used in this study. The bacterium was maintained in reinforced clostridial medium (Oxoid Ltd., Basingstoke, Hampshire, England) at 4°C. For inoculum preparation, mixtures of spores and cells were heated at 80°C for 45 min and transferred to chemically defined medium. Cells growing exponentially were used to inoculate a phosphate-limited chemostat. The chemically defined culture medium contained the following components (per liter of distilled water): glucose, 30 g; $NaH_2PO_4 \cdot H_2O$, 0.06 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 0.01 g; KCl, 0.051 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.015 g; $FeSO_4 \cdot 7H_2O$, 0.012 g; biotin, 0.1 mg; thiamine-HCl, 2 mg; *p*-aminobenzoic acid, 2 mg; and resazurin, 1 mg. The continuous-culture experiments were performed at 35°C in a Bioflo chemostat (model C-30; New Brunswick Scientific Co., Inc., Edison, N.J.) with a 350-ml culture volume. The agitation speed was 200 rpm. The dilution rate was maintained at 0.13 h^{-1}. The pH of the medium was controlled by the automatic addition of 5 N NaOH. The culture and the fresh medium was kept anaerobic by sparging with sterile oxygen-free N_2 gas at a flow rate of 25 ml/min. A minimum of 7 culture volume changes were used for the transition from one steady-state growth condition to another. Culture purity in the chemostat was determined at weekly intervals by Gram stain and colony morphology on reinforced clostridial agar plates.

Fermentation product analysis. The concentrations of acetate, butyrate, acetone, butanol, ethanol, and acetoin were measured by gas chromatography as follows. A sample from the growing culture was centrifuged at 34,800 × *g* for 10 min to sediment the cells. A 0.2-ml sample of cell-free culture fluid was removed, and 25 μl each of 0.5 M propionic acid (to act as internal standard in the gas chromatographic analysis) and 2.5 N H_2SO_4 were added. After mixing, 1 μl of the sample was injected into a gas chromatograph (Hewlett-Packard model 5721A) equipped with a flame ionization detector and connected to a Hewlett-Packard model 3390 recording integrator. The fatty acids and solvents were separated in a glass column (2 mm [inside diameter] by 2 m [length]) packed with Chromosorb 101 (80/100 mesh). Nitrogen was used as the carrier gas. The injector and detector temperatures were 200 and 250°C, respectively. The oven temperature was programmed from 150 to 185°C at the rate of 12°C/min.

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Reagents. [^{14}C]5,5-dimethyl-2,4-oxazolidinedione (DMO; 50.0 mCi/mmol), [^{14}C]benzoic acid (18.8 mCi/mmol), [*methyl*-^{14}C]triphenylmethylphosphonium iodide (TPMP$^+$; 19.5 mCi/mmol), and [^{3}H]raffinose (7.8 Ci/mmol) were purchased from New England Nuclear of Canada, Lachine, Quebec, Canada. Triphenylmethylphosphonium bromide and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co., St. Louis, Mo., and *N*,*N*′-dicyclohexylcarbodiimide (DCCD) was purchased from Fisher Scientific Ltd., Toronto, Ontario, Canada. All other materials were of reagent grade or the highest grade available.

Measurement of ΔpH. The ΔpH was calculated from the distribution of the weak acid [^{14}C]DMO (0.67 μM; 50 mCi/mmol) or [^{14}C]benzoic acid (2.29 μM; 18.8 mCi/mmol) (15). [^{3}H]raffinose (2 μM; 124.6 mCi/mmol) was also incorporated into the sample to allow estimation of the cytoplasmic volume required for calculation of the internal-to-external concentration ratio of the ΔpH probes. In this experiment, growing culture was withdrawn from a chemostat operating under steady-state conditions, and 10-ml samples were dispensed into Corex centrifuge tubes (15 ml) while flushing with O_2-free N_2 gas. After the addition of [^{3}H]raffinose and [^{14}C]DMO or [^{14}C]benzoic acid, the tubes were capped with recessed rubber stoppers (designed for Wheaton 400 serum bottles [Fisher Scientific]) and incubated with shaking in a water bath at 35°C. At 10 min, the samples were centrifuged (34,800 × *g* for 10 min at room temperature), and the supernatant fluid was removed. The radioactivity in samples (100 μl) of the supernatant was determined by dispersing each sample in 5 ml of ACS scintillation fluid (Amersham Canada Ltd., Oakville, Ontario, Canada) contained in glass vials and counting in a Beckman LS-3150 T scintillation counter. The walls of the centrifuge tubes were wiped dry, and 1 M perchloric acid was added to the pellet to a final volume of 2 ml. After extraction of the cells at room temperature for 15 min, the debris was pelleted, and a 400-μl sample of the supernatant fluid was counted as described above. The distribution of [^{14}C]DMO or [^{14}C]benzoic acid across the cell membrane was determined as described by Jarrell and Sprott (8). The pK_a values of DMO and benzoic acid at 35°C were taken to be 6.21 (8) and 4.21 (13), respectively. The pH of the cell-free supernatant fluid from the culture after incubation with the probes was used as the external pH value and was within 0.1 of the original pH of the culture in the chemostat.

Metabolism of DMO and benzoic acid. The ability of growing *C. acetobutylicum* cells to metabolize DMO and benzoic acid was examined as described by Jarrell and Sprott (8), with some modifications. The growing culture was withdrawn from a phosphate-limited chemostat operating at pH 4.5, which, of the pH values tested, resulted in maximum uptake of the probe compounds. The possible metabolism of DMO was checked by transferring duplicate samples (10 ml) of the phosphate-limited culture into Corex centrifuge tubes and adding [^{14}C]DMO to a final concentration of 5.03 μM (50.0 mCi/mmol). Incubation was performed at 35°C in a water bath. After 10 min, one of the tubes was centrifuged (34,800 × *g* for 10 min at room temperature), and the supernatant (S_1) was removed. The other tube was similarly treated after 2 h of incubation. The pellets were incubated with 0.1 ml of *n*-butanol for 15 min at 80°C. Water (0.2 ml) was added, and the incubation continued for another 15 min at 80°C. The suspensions were then centrifuged (34,800 × *g* for 10 min), and the supernatant fluid (S_2) was removed. Samples of S_1 and S_2 were spotted on precoated silica gel GHL plates (Analtech Inc., Newark, Del.) and developed in hexane-diethyl ether-acetic acid (70:30:4, by volume). The plates were examined for radioactivity with a thin layer scanner (model RTLS-1A; Panax Equipment Ltd., Redhill, Surrey, United Kingdom).

For benzoic acid metabolism studies, the same method was used, except that [^{14}C]benzoic acid, instead of [^{14}C]DMO, was added to the culture samples to a final concentration of 11.47 μM (18.8 mCi/mmol).

Sensitivity of ΔpH to the presence of an uncoupler and an ATPase inhibitor. The ΔpH of the cells taken from the chemostat set at pH 4.5 was measured as described above after incubation at 35°C for 15 min in the presence of CCCP or DCCD at concentrations specified in Table 1. [^{14}C]benzoic acid was used as ΔpH probe in this experiment.

Measurement of Δψ. The transmembrane electrical potential was calculated from the distribution of TPMP$^+$ by using the Nernst equation. A sample (5 ml) of the growing culture from the chemostat was transferred, by anaerobic techniques, to a 60-ml serum bottle preflushed with O_2-free N_2 gas. [^{14}C]TPMP$^+$ was added to a final concentration of 20 μM (3.22 mCi/mmol). The bottle was then capped with a butyl rubber seal and incubated, with shaking, in a water bath at 35°C. At timed intervals, duplicate 0.5-ml samples were removed and filtered through 0.45-μm (pore size) Metricel filters (Gelman Sciences, Inc., Montreal, Quebec, Canada) and washed immediately with 5 ml of prewarmed 0.1 M LiCl. The filtration and washing procedure lasted for about 15 s. The filters were inserted into glass scintillation vials and dried overnight at 40°C. Each filter was moistened with 0.1 ml of distilled water and solubilized with 1 ml of NCS tissue solubilizer (Amersham) for 1 h. The NCS tissue solubilizer in each vial was neutralized with 34 μl of glacial acetic acid, 5 ml of ACS scintillation fluid was added, and the radioactivity in the sample was measured in the scintillation counter. The maximum value obtained for the [^{14}C]TPMP$^+$ uptake was used to calculate the Δψ. A 0.1-ml sample of the culture, taken from the serum bottle after incubation, was also solubilized with an identical volume of NCS, neutralized with acetic acid, and counted in ACS scintillation fluid to provide a measure of total [^{14}C]TPMP$^+$ in the reaction mixture. As a control for nonspecific binding of [^{14}C]TPMP$^+$ to the filter and cellular materials, the cells were incubated with 100 μM CCCP for 15 min at 35°C before the addition of [^{14}C]TPMP$^+$ to depolarize the cell membrane. After another 20 min of incubation, samples were filtered and counted. Intracellular space values obtained from the ΔpH measurements, described above, were used to estimate the internal TPMP$^+$ concentration. The Δψ was then calculated as described by Jarrell and Sprott (8).

Estimation of proton motive force. The proton motive force

TABLE 1. Effect of CCCP and DCCD on ΔpH[a]

Condition (μM)	Benzoic acid distribution ratio (in:out)	ΔpH
Control	29.2	1.67
CCCP (100)	2.8	0.56
CCCP (250)	2.5	0.50
DCCD (100)	19.0	1.45
DCCD (500)	3.4	0.64

[a] The growing culture from the chemostat at pH 4.5 was incubated with CCCP or DCCD for 15 min at 35°C before the addition of [^{14}C]benzoic acid (2.29 μM; 18.8 mCi/mmol). The ΔpH was determined in triplicate as described in the text.

was calculated from its two components (i.e., the ΔpH and Δψ) by using the following equation: $\Delta\bar{\mu}_{H^+}/F = \Delta\psi - 2.3\ (RT/F)\ \Delta pH$ (8).

RESULTS

Influence of pH on fermentation products of phosphate-limited culture. The change in the fermentation products produced by *C. acetobutylicum* as the culture pH was lowered (Fig. 1) indicated the presence of two different fermentation patterns, with the changeover point lying between pH 5.0 and 5.5. The solvents acetone and butanol were the predominant fermentation products at low pH but were present only in trace amounts at high pH. Fatty acids, on the other hand, were the major products formed at high pH. Measurements of the proton motive force and its components in the cells grown under these conditions, therefore, permitted investigation of their possible role in directing the pattern of metabolism of the cells.

pH_{in} of growing cells. As an experiment preliminary to the measurement of the pH_{in}, the penetration of DMO and benzoic acid into growing cells was studied. DMO completely equilibrated during the short period of mixing and centrifugation before incubation, and no further uptake of the label was observed during the subsequent incubation period of 30 min. Similarly, benzoic acid penetrated rapidly into the cells and equilibrated within 5 min of incubation. In addition, the distribution of both DMO and benzoic acid was not affected by initial external concentrations of the compounds 20 times higher than those used in the ΔpH determinations, suggesting that DMO and benzoic acid were not extensively bound to the cellular materials. Furthermore, no metabolism of either DMO or benzoic acid by growing *C. acetobutylicum* cells could be detected even after they had been incubated with the labeled compounds for up to 2 h. The techniques used in this experiment would have permitted the detection of 1% conversion of the labeled compounds in both the S_1 and S_2 samples.

The distribution of raffinose, the extracellular space marker, was found to be independent of raffinose concentration up to 10 mM and of incubation time between 0 and 20 min. The values for intracellular space in the pellets of cells grown in the chemostat with different pH settings were similar, and, based on five separate experiments, they were calculated to be 1.24 (±0.16) μl/mg (dry weight) at pH 6.5 and 1.34 (±0.06) μl/mg (dry weight) at pH 4.5.

From the distribution of DMO and benzoic acid, the pH_{in} values obtained for *C. acetobutylicum* cells grown under phosphate-limiting conditions in a chemostat ranged from 4.5 to 6.5 (Fig. 2). The pH_{in} dropped rapidly from approximately 6.7, at external pH 6.5, to approximately 6.2 at an external pH of 6.0 and then declined slowly to pH 6.0, at which it remained relatively stable, even when the external pH was lowered to 4.5. A small pH gradient was therefore present at higher external pH values, whereas a much larger pH gradient was generated at lower external pHs. It is noteworthy that the pH_{in} values were almost the same whether the external pH was maintained at 4.5 or 5.5, but solvents were produced only at pH 4.5. This result was obtained with either probe. However, the use of benzoic acid as an indicator of ΔpH provided greater precision than was possible with DMO, especially at low external pH values (e.g., pH 4.5), due to the difference in their pK_a values.

Sensitivity to ΔpH to DCCD and CCCP. The ΔpH was measured in the cells taken from the chemostat at pH 4.5, after incubation at 35°C for 15 min in the presence of the proton conductor CCCP or the ATPase inhibitor DCCD (Table 1). The pH gradient was diminished by CCCP at the concentrations tested. However, ΔpH was only slightly affected by 100 μM DCCD but was decreased substantially when 500 μM DCCD was added. It appears, therefore, that the pH gradient was more sensitive to the presence of the uncoupler, CCCP, than to the ATPase inhibitor, DCCD.

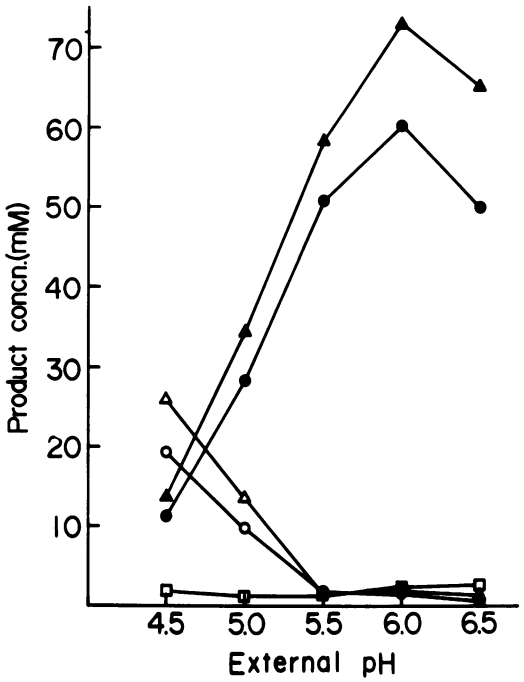

FIG. 1. Effect of the pH of a phosphate-limited culture of *C. acetobutylicum* ATCC 824 on steady-state concentrations of fermentation products. Symbols: △, butanol; ○, acetone; □, ethanol; ▲, butyrate; ●, acetate. Dilution rate, 0.13 h^{-1}. Cell density ranged from 0.93 g/liter at pH 4.5 to 1.06 g/liter at pH 6.5.

Measurement of Δψ. As an experiment preliminary to the measurement of Δψ, the kinetics of $TPMP^+$ entry into cells grown in the chemostat at pH 6.5 and 4.5 was examined. The cells grown at pH 6.5 were found to concentrate this lipophilic cation, with maximum accumulation achieved 20 min after its addition. The observed uptake of $TPMP^+$ was eliminated if the cells were first depolarized by exposure to 100 μM CCCP for 15 min. In contrast, there was no uptake of $TPMP^+$ into cells grown in the chemostat at pH 4.5. It was reported that the presence of 2 μM tetraphenylboron was required for uptake or maximum uptake of $TPMP^+$ in some bacteria (9). However, no such enhancement was detectable in *C. acetobutylicum* ATCC 824 (data not shown). The optimal concentration of $TPMP^+$ for uptake experiments was approximately 20 μM.

The Δψ was calculated from the ratio of concentrations of $TPMP^+$ inside and outside the cells under steady-state conditions. Corrections for nonspecific binding of the label to the filter and to the cellular materials were determined by using CCCP-poisoned cells. The results (Table 2) indicated that the magnitude of the Δψ (interior negative) increased with the external pH. At external pH 6.5, Δψ was found to be approximately −90 mV. However, at external pH 4.5, $TPMP^+$ did not concentrate in the cells, suggesting that there was no negative Δψ.

The proton motive force consists of the Δψ and ΔpH and was shown to be relatively constant in growing *C.*

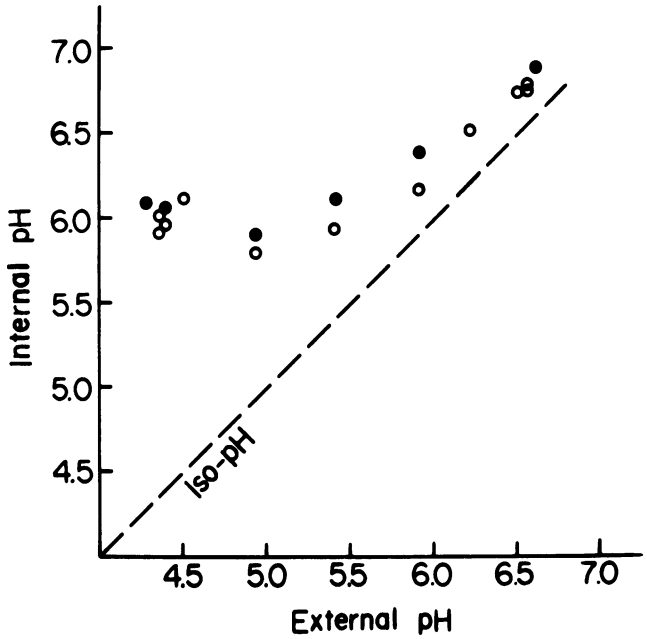

FIG. 2. pH_{in} of *C. acetobutylicum* grown in a phosphate-limited chemostat maintained at different pH values. Growing culture was withdrawn from the chemostat at various pHs. Internal pH values were calculated from the distribution of [^{14}C]DMO (○) and [^{14}C]benzoic acid (●) as described in the text. Each point is the mean of quadruplicate determinations.

acetobutylicum ATCC 824 cells. At an external pH of 6.5, the $\Delta\tilde{\mu}_{H^+}/F$ was approximately −106 mV and was contributed to predominantly by the $\Delta\psi$; at pH 4.5, however, ΔpH became the only component of the $\Delta\tilde{\mu}_{H^+}/F$ (as $\Delta\psi$ was 0 [Table 2]) and was calculated to be −102 mV. It seems that, in the present system, a change in one of the components of the $\Delta\tilde{\mu}_{H^+}/F$ was accompanied by a compensatory change in the other, so that the $\Delta\tilde{\mu}_{H^+}/F$ remained constant.

DISCUSSION

The present study was undertaken to investigate the involvement of the proton motive force ($\Delta\tilde{\mu}_{H^+}/F$) and its components, the $\Delta\psi$ and ΔpH, in the regulation, in *C. acetobutylicum* ATCC 824, of the transition from acetogenic to solventogenic fermentation. This was accomplished by varying the extracellular pH of a phosphate-limited culture and determining the ΔpH, $\Delta\psi$, and fermentation products

TABLE 2. Proton motive force in *C. acetobutylicum* grown in a phosphate-limited chemostat culture[a]

pH of chemostat	61.1 ΔpH (mV)	Δψ (mV)	$\Delta\tilde{\mu}_{H^+}/F$ (mV)
4.5	102 ± 1	0[b]	−102
5.0	59 ± 1	−40 ± 4	−99
5.5	43 ± 2	−52 ± 7	−95
6.0	28 ± 2	−56 ± 5	−84
6.5	16 ± 2	−90 ± 3	−106

[a] Proton motive force was calculated from its two components, ΔpH and Δψ. ΔpH values obtained from [^{14}C]benzoic acid distribution were used here. Δψ was determined from [^{14}C]TPMP$^+$ accumulation as described in Materials and Methods. In each experiment, 4 samples were taken for ΔpH measurement, and 12 samples were taken for Δψ measurement. Each value represents the mean ± the standard deviation.

[b] No uptake of TPMP$^+$ above the blank value.

over a range of steady-state pH values from 6.5 to 4.5. It was observed that the pH_{in} dropped fairly sharply from approximately 6.7 to 6.0 when the external pH was lowered from 6.5 to 5.5 but that it was maintained constant at 6.0 when the external pH was decreased to 4.5. This was in marked contrast to the parallel decrease in pH_{in} and extracellular pH observed in *Clostridium pasteurianum* and *Clostridium thermoaceticum* (3, 18). It was reported that if during growth of *C. thermoaceticum* in batch culture the external pH fell below 5.0, the ΔpH collapsed (3). The consequent acidification of the cytoplasm was attributed to acetic acid production and resulted in the inhibition of pH-sensitive cellular reactions, leading to cessation of both growth and fermentation (3). Because fatty acids are known to behave as uncouplers (4, 6) which transport protons into the cells, the ability of *C. acetobutylicum* to maintain a high pH_{in}, even at pH 4.5, points to a very efficient deacidification system. Such a system is presumed to have been responsible for the transition from acetogenesis to solventogenesis in *C. acetobutylicum* ATCC 824 when the medium pH dropped below 5.5, observed in the present study.

It has been found that activities of some of the enzymes involved in the metabolism of *C. acetobutylicum* are pH sensitive. Among them, hydrogenase, which is responsible for the disposal of excess protons as hydrogen gas, is inactive in vivo in solvent-producing cells grown at pH 4.5 (1). The loss of hydrogenase activity may result in more reducing power becoming available for solvent production. Another enzyme, acyl coenzyme A transferase, which converts fatty acids to the corresponding coenzyme A derivatives, shows higher in vitro activity when cells are grown at low pH (1). Modification of these enzyme activities as a result of the pH decline seems to favor solventogenesis. However, such change in activity may not function as a triggering factor in the initiation of this process, because pH_{in} values were found to be quite similar at external pHs of 4.5 and 5.5, even though solvents were formed only at pH 4.5. This conclusion is in agreement with the recent finding that *C. acetobutylicum* could produce solvents at neutral pH in the presence of added fatty acids (7).

The attainment of a critical concentration of undissociated butyric acid was reported to be required for the initiation of solvent formation, and this value was calculated to be approximately 5.7 mM for *C. acetobutylicum* (16, 17). However, it is not clear why only the undissociated form of butyric acid was considered to be important in this process. In fact, Holt et al. (7) found that solvent production by *C. acetobutylicum* occurred at pH 7.0 in the presence of added fatty acids. At the pH and butyric acid concentration used by Holt et al. (7), the concentration of undissociated butyric acid was only 0.5 mM, considerably lower than the critical value of 5.7 mM previously reported (16). The results obtained in the present study provide a new perspective from which the fatty acid effect can be considered in terms of the total intracellular concentration of fatty acids, without distinguishing between their dissociated and undissociated forms. As shown in the present study, the ΔpH of the cells was small and relatively constant between external pH 6.5 and 5.5 but increased sharply when the external pH was further decreased to 4.5. Correspondingly, it could be expected that the internal-to-external concentration ratios of the fatty acids would increase slowly, and then dramatically, when the external pH is lowered from 6.5 to 4.5. This expectation is based on the assumption that these acids passively diffuse across the cell membrane and distribute themselves according to the ΔpH, as has been generally

accepted (3). Therefore, although the external fatty acid concentrations at the transition phase decreased with the decreasing external pH, the high intracellular fatty acid levels at that point could always be established because of the increasing ΔpH. Although the high intracellular fatty acid level may be required for initiation of solvent formation, the mechanism of the fatty acid induction of solventogenesis remains unclear and is unlikely to be explicable in terms of the lowering of pH_{in} of this bacterium.

The results of this investigation show *C. acetobutylicum* to have a large ΔpH at low pH. The involvement of the H^+-translocating ATPase in the maintenance of the ΔpH and thus in the deacidification of the cytoplasm of the cells was suggested by the effect of DCCD, an inhibitor of this enzyme. However, the sensitivity of the ΔpH to DCCD did not seem to be high. The ΔpH was only slightly affected by 100 μM DCCD, although it was substantially decreased in the presence of 500 μM DCCD. The uncoupler (CCCP), on the other hand, was found to reduce the ΔpH effectively. In contrast, in *C. pasteurianum*, the ΔpH was reduced by CCCP (200 μM) and even inverted to an internal-acidic ΔpH by DCCD (200 μM) (18). The ΔpH of *C. thermoaceticum* was also shown to be considerably reduced, although not completely dissipated, by 100 μM DCCD (3). The results obtained in the present study indicate, therefore, that other proton extrusion mechanism(s), in addition to the H^+-ATPase, may contribute to the deacidification of the cytosol of *C. acetobutylicum* grown at low pH. The production of solvents may provide a major pathway for the removal of protons from the cytoplasm. It has also been reported that the NADH-rubredoxin oxidoreductase, which has a very high activity at low pH, may participate in proton extrusion (14). The actual contribution of each of these deacidification mechanism remains to be determined.

The Δψ in *C. acetobutylicum* was found to decrease with the decreasing external pH such that at pH 4.5 the Δψ approached 0 or was positive. A similar decline in Δψ with decreasing external pH has been reported for other bacteria, such as *Streptococcus lactis*, *Staphylococcus aureus* (10), *C. thermoaceticum* grown under anaerobic conditions (3), and *Escherichia coli* grown under aerobic conditions (11). It seems that the influence of growth of the cells at different pH values was not on the magnitude of the proton motive force but on the relative contributions made to the proton motive force by the ΔpH and the Δψ.

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