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multiple conformations during thermal unfolding.

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Supplementary Material Available

The derivation of eq 8 and 9 of the biallosteric model, the equations for model II, and the derivations, results, and discussion of model IV (17 pages). Ordering formation is given on any current masthead page.

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Potassium Ion Is Required for the Generation of pH-Dependent Membrane Potential and ΔpH by the Marine Bacterium *Vibrio alginolyticus*[†]

Hajime Tokuda,* Tatsunosuke Nakamura, and Tsutomu Unemoto

ABSTRACT: The electrochemical potential gradient of protons in the marine bacterium *Vibrio alginolyticus* was measured as a function of external pH. In K$^+$-containing cells, the membrane potential ($\Delta\psi$) and ΔpH vary with external pH as reported in other bacteria. On the other hand, K$^+$-depleted cells show little pH dependence in the magnitude of $\Delta\psi$ from pH 6.0 to 8.5. The cytoplasmic pH in these cells varies depending on external pH, resulting in the generation of a small ΔpH at acidic pH. Addition of K$^+$ to K$^+$-depleted cells leads to partial dissipation of $\Delta\psi$ and concomitant generation of ΔpH. Strikingly, this effect of K$^+$ is dependent on external pH. Collapse of $\Delta\psi$ and generation of ΔpH by the addition of K$^+$ decrease with increasing external pH. Thus, the $\Delta\psi$ and ΔpH obtained after addition of K$^+$ are essentially the same as those determined in K$^+$-containing cells, and cytoplasmic pH becomes less dependent on external pH. The results suggest that the variation of $\Delta\psi$ and ΔpH with external pH is controlled by K$^+$ transport.

The gram-negative marine bacterium *Vibrio alginolyticus* requires Na$^+$ for growth, and 0.3–0.5 M NaCl is optimal. When cells are grown on medium containing 0.3 M NaCl, the internal K$^+$ concentration is about 0.4 M, which is about 30-fold higher than that of the medium. On the other hand, the internal concentration of Na$^+$ is only about 0.1 M. Apparently, energy is required to maintain the internal ion compositions against their concentration gradients. Recently, evidence demonstrating the involvement of the proton motive force in K$^+$ uptake (Rhoads & Epstein, 1977; Wagner et al., 1978; Bakker & Harold, 1980) and Na$^+$ extrusion (West & Mitchell, 1974; Lanyi et al., 1976; Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978; Bhattacharyya & Barnes, 1978; Brey et al., 1978; Niven & MacLeod, 1978; Krulwich et al., 1979) has been presented for various bacteria. Therefore, it was of interest to examine the role of the proton motive force in the maintenance of internal ionic environment and, conversely, the effect of ionic environment on the formation of the proton motive force in *V. alginolyticus*.

According to the chemiosmotic hypothesis of Mitchell (Mitchell, 1968; Harold, 1977; Rosen & Kashket, 1978),

vectorial proton ejection leads to the formation of a proton motive force (Δp)[1] which is described by (in mV)

$$\Delta p = \Delta\psi - \frac{2.3RT}{F}\Delta pH \qquad (1)$$

where $\Delta\psi$ is the electrical component across the membrane, ΔpH is the chemical difference in proton concentration across the membrane, and $2.3RT/F$ equals 59 at room temperature. Recently, the proton motive force and its components have been determined in various bacteria and membrane vesicles (Ramos et al., 1976; Tokuda & Kaback, 1977; Zilberstein et al., 1979; Friedberg & Kaback, 1980) by flow dialysis, a technique (Colowick & Womack, 1969; Ramos et al., 1979) that allows continuous monitoring of $\Delta\psi$ and ΔpH. The experiments revealed the interesting phenomenon that intracellular pH is maintained near neutral over a wide range of external pH values not only in *Escherichia coli* but also in cells grown under extreme acidic (Krulwich et al., 1978) or alkaline (Guffanti et al., 1978) conditions. Thus, it is apparent that there is a mechanism for the regulation of internal pH. Furthermore, the mechanism seems to be present in membrane vesicles since intravesicular pH is independent of external pH in membrane vesicles prepared from *E. coli* (Ramos et al., 1976), *S. typhimurium* (Tokuda & Kaback, 1977), and, very recently, *M. lysodeikticus* (Friedberg & Kaback, 1980). However, it is also clear that $\Delta\psi$ in membrane vesicles differs from that in cells in the sensitivity to external pH (Friedberg & Kaback, 1980). In membrane vesicles, $\Delta\psi$ is relatively insensitive to external pH (Ramos et al., 1976; Tokuda & Kaback, 1977; Friedberg & Kaback, 1980), while in cells, $\Delta\psi$ increases by about ~20 mV per pH unit from pH 5.5 to 7.5 (Zilberstein et al., 1979; Friedberg & Kaback, 1980). These discrepancies in the response of $\Delta\psi$ to external pH in cells and membrane vesicles are not due to technical artifacts since direct measurement of $\Delta\psi$ in *E. coli* giant cells with microelectrodes and lipophilic cations like TPP^+ and $TPMP^+$ gave essentially the same results (Felle et al., 1980).

During the course of our study on the relationship between the ionic environment and generation of proton motive force in *V. alginolyticus*, we found that K^+ depletion renders the cells insensitive to external pH with regard to the generation of $\Delta\psi$ and ΔpH. The evidence for the requirement of K^+ in the generation of pH-sensitive $\Delta\psi$ and ΔpH is presented in this paper.

Materials and Methods

Growth of Cells. Vibrio alginolyticus 138-2 was grown aerobically on synthetic medium containing 0.3 M NaCl, 10 mM KCl, 2 mM K_2HPO_4, 0.01 mM $FeSO_4$, 15 mM ($NH_4)_2SO_4$, 5 mM $MgSO_4$, 1% glycerol, and 50 mM Tris-HCl (pH 7.5) at 37 °C to the late logarithmic phase of growth. For studies with K^+-containing cells, the cells were harvested by centrifugation at 4 °C, washed twice with either 0.4 M choline chloride or 0.1 M potassium phosphate (pH as specified) containing 0.3 M KCl, and resuspended in the same solution to a final concentration of 60–80 mg of cell protein per mL.

K$^+$ Depletion. Harvested cells were treated twice with 50 mM diethanolamine hydrochloride (pH 9.3) containing 0.4 M NaCl for 10 min at 25 °C. For assays at various pH values, alkali-treated cells were washed twice with 0.1 M sodium phosphate at the desired pH containing 0.3 M NaCl and resuspended in the same buffer to a final concentration of 60–80 mg of cell protein per mL.

Determination of $\Delta\psi$. $\Delta\psi$ (negative inside) was determined at room temperature from the equilibrium distribution of [^{3}H]tetraphenylphosphonium by using flow dialysis (Ramos et al., 1979). Oxygenated buffer was pumped from the lower chamber at a flow rate of 2 mL/min. Fractions of 2 mL were collected in a Gilson fraction collector, and 0.4 mL of each fraction was mixed with 3 mL of Triton X-100/toluene scintillation liquid for the determination of radioactivity.

Determination of ΔpH. ΔpH (interior alkaline) was determined from the equilibrium distribution of [^{14}C]acetylsalicylic acid[2] (Maloney, 1977; Krulwich et al., 1978) by using flow dialysis as described above for measurement of $\Delta\psi$.

Calculations. Steady-state concentration gradients of [^{3}H]TPP$^+$ and [^{14}C]acetylsalicylic acid were calculated from flow dialysis experiments by using a value of 3.3 μL of internal water space per mg of cell protein (Unemoto et al., 1973; Unemoto & Hayashi, 1979). $\Delta\psi$ was determined from the Nernst equation ($\Delta\psi = 59 \log [TPP^+]_{in}/[TPP^+]_{out}$). Internal pH was calculated from the distribution of acetylsalicylic acid ($pK = 3.49$) as described (Schuldiner et al., 1972; Rottenberg, 1979), and ΔpH was calculated from the difference between internal and external pH.

In all calculations, corrections were made for changes in the volume of the upper chamber of flow dialysis due to addition of cell suspensions.

The proton motive force (Δp) was calculated by substitution in eq 1.

Determination of K^+. For routine assay, a K^+ electrode (F2312K, Radiometer, Copenhagen, Denmark) was used with a calomel electrode (K701, Radiometer) as the reference electrode. In order to determine K^+ content of cells, an appropriate volume of a concentrated cell suspension in 0.4 M choline chloride or 0.1 M sodium phosphate containing 0.3 M NaCl was diluted into 2 mL of the same salt solution, and the K^+ content before and after rupture of cells by sonication was recorded. From the difference in K^+ concentrations before and after cell rupture, the internal K^+ concentration was calculated on the basis of a calibration performed under identical conditions with standard KCl solutions. The values obtained by this method were reproducible and found to be essentially the same as those obtained by using atomic absorption spectrophotometry (Unemoto & Hayashi, 1979).

For the determination of K^+(Rb^+) uptake, the concentrated cell suspension was diluted into 2 mL of 0.1 M sodium phosphate containing 0.3 M NaCl, 10 mM K(Rb)Cl, and 20 mM glycerol at a concentration of 1–1.5 mg of protein per mL. K^+ or Rb^+ uptake was measured from the decrease in ion concentration as determined with a K^+ electrode.

Oxygen Uptake. Rate of oxygen uptake was determined at 25 °C with an oxygen electrode (Yellow-Springs, OH) attached to a Hitachi 056 recorder.

Protein Determination. Protein was determined as described by Lowry et al. (1951) by using bovine serum albumin as a standard.

Materials. [^{3}H]TPP$^+$ (bromide salt), synthesized by the Isotope Synthesis Group of Hoffmann-La Roche, Inc., under the direction of Arnold Liebman, was a generous gift from H. R. Kaback, Roche Institute of Molecular Biology, Nutley,

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[1] Abbreviations used: Δp, proton motive force; $\Delta\psi$, membrane potential; TPP$^+$, tetraphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Tris, tris(hydroxymethyl)aminomethane.

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[2] In order to check degradation, [^{14}C]acetylsalicylic acid was recovered after flow dialysis experiments and analyzed on a silica gel G thin-layer chromatogram using the solvent system hexane/acetic acid/CHCl$_3$ (85:15:10). Essentially all the radioactivity was found in a position of authentic acetylsalicylic acid.
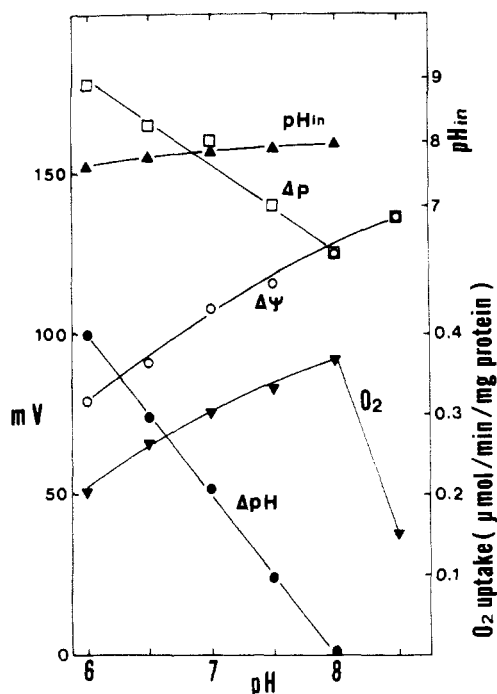
FIGURE 1: Effect of external pH on $\Delta\psi$, ΔpH, Δp, and the rate of oxygen uptake in *Vibrio alginolyticus*. *V. alginolyticus* was harvested, washed with either 0.4 M choline chloride alone (those assayed at pH 7.0 to 8.5) or 0.1 M potassium phosphate (pH 6.0 to 8.0) containing 0.3 M KCl, and suspended in the same solution to give final concentrations of 60–80 mg of protein per mL as described under Materials and Methods. $\Delta\psi$ and internal pH were determined in 0.1 M potassium phosphate (pH 6.0–8.5) containing 0.3 M KCl, 0.05 M NaCl, and 20 mM glycerol by flow dialysis (Ramos et al., 1979) by using the equilibrium distribution of [³H]TPP⁺ and [¹⁴C]-acetylsalicylic acid across the membrane, respectively. Cells were used at a final concentration of 2–5 mg of protein per mL in each experiment. Δp and ΔpH were calculated as described under Materials and Methods. Values presented were an average of several experiments. The rate of oxygen uptake was assayed as described under Materials and Methods by using cells washed with 0.1 M potassium phosphate (pH 6.0–8.5) containing 0.3 M KCl and assayed in 0.1 M potassium phosphate (pH 6.0–8.5) containing 0.3 M KCl, 0.05 M NaCl, and 20 mM glycerol. Values shown represent µmol of O_2 consumed per min per mg of protein. (O) $\Delta\psi$; (●) ΔpH; (□) Δp; (▲) internal pH; (▼) rate of oxygen uptake.

NJ. [¹⁴C]Acetylsalicylic acid was purchased from New England Nuclear. Carbonyl cyanide *m*-chlorophenylhydrazone was obtained from Sigma Chemical Co.

Results

$\Delta\psi$ and ΔpH in K^+-Containing Vibrio alginolyticus. The results shown in Figure 1 were obtained with cells washed with 0.4 M choline chloride or buffer containing a high concentration of K⁺ and assayed in the presence of K⁺.[3] The internal K⁺ concentration of these cells was found to be around 0.3–0.4 M and did not decrease significantly after repeated washing. $\Delta\psi$, determined from the steady-state distribution of [³H]TPP⁺ by flow dialysis (Ramos et al., 1979), increased by about –20 mV/pH unit from pH 6.0 to 8.5. On the other hand, ΔpH, calculated from the distribution of [¹⁴C]acetylsalicylic acid,[2] decreased linearly from 1.7 pH units (100 mV) at pH 6.0 to 0 at pH 8.0 and above. This alteration in ΔpH with external pH results from the fact that cytoplasmic pH remains essentially constant at around pH 8.0 over the range of pH values
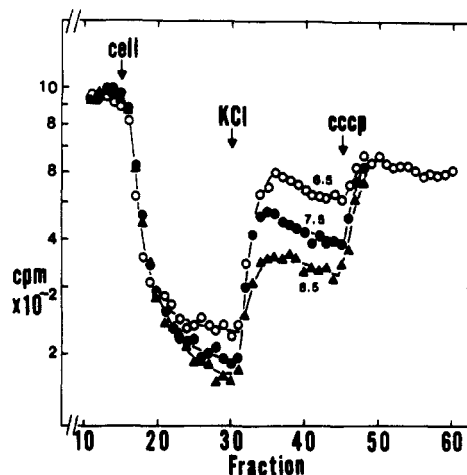

FIGURE 2: Effect of K⁺ and external pH on the generation of $\Delta\psi$ in K⁺-depleted *V. alginolyticus*. K⁺-depleted *V. alginolyticus* was prepared and washed with 0.1 M sodium phosphate (pH as specified) containing 0.3 M NaCl. $\Delta\psi$ was determined in 0.1 M sodium phosphate containing 0.3 M NaCl and 20 mM glycerol at the pH indicated in the figure by the flow dialysis method as described under Materials and Methods. Experiments were started by the addition of [³H]TPP⁺ (244 µCi/µmol) at a final concentration of 19 µM at fraction 1. Concentrated cell suspension, KCl, and CCCP were added at arrows indicated at final concentrations of 2.9 mg/mL at pH 6.5, 2.9 mg/mL at pH 7.5, and 3.3 mg/mL at pH 8.5, for the cell suspension and 10 mM and 10 µM, respectively, for KCl and CCCP. In all cases, fractions 1–10 were not shown in the figure, and in the case of experiments at pH 7.5 and 8.5, fractions 49–60 were omitted. (O) pH 6.5; (●) pH 7.5; (▲) pH 8.5.

tested (closed triangles). As a result of these variations in $\Delta\psi$ and ΔpH, Δp varies from about –180 mV at pH 6.0 to –140 mV at pH 8.5. Although the absolute magnitude of $\Delta\psi$ and ΔpH differ slightly, the overall pattern observed here is similar to that reported for *E. coli* (Zilberstein et al., 1979) and *M. lysodeikticus* (Friedberg & Kaback, 1980).

The rate of oxygen uptake was also determined as a function of external pH, and results were given in Figure 1. An almost linear increase in the rate of oxygen uptake was observed with the increase in external pH up to 8.0. Although the rate of oxygen uptake was measured in the presence of 20 mM glycerol, the activity shown was mainly due to the endogenous energy pool since the omission of glycerol did not change the rate of oxygen uptake.

K^+ Depletion by Alkali Washing. Although detailed experimental conditions will be reported elsewhere,[4] washing cells with alkaline buffer containing salts other than K⁺ causes depletion of internal K⁺ without reducing the capacity of the cells to generate Δp. Internal K⁺ in alkali-washed cells was replaced by ions present in the washing medium. Thus, the internal ion composition of these cells can be manipulated by washing the cells with the alkaline buffer of desired ionic composition. The cells washed with alkaline buffer containing 0.4 M NaCl were found to retain less than 1 mM K⁺. On the other hand, the cells washed with neutral buffer or unbuffered salt solution still retained 0.3–0.4 M K⁺.

Generation of $\Delta\psi$ and ΔpH by K^+-Depleted Cells. K⁺-Depleted cells were prepared in the presence of NaCl, and $\Delta\psi$ and ΔpH were determined after washing the cells with 0.1 M sodium phosphate (pH as specified) containing 0.3 M NaCl. The data presented in Figure 2 show [³H]TPP⁺ uptake assayed by flow dialysis at pH 6.5 (open circles), 7.5 (closed circles), and 8.5 (closed triangles). After addition of cells at fraction 15, the radioactivity in the dialysate decreases to a steady state,

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[3] The assay buffer also contained 50 mM NaCl since under weak alkaline conditions (pH 8.0–8.5), Na⁺ stabilized the integrity of membrane structure. The effect was specific to Na⁺ and not observed with K⁺, choline⁺, Li⁺, or Mg²⁺ (unpublished observation).

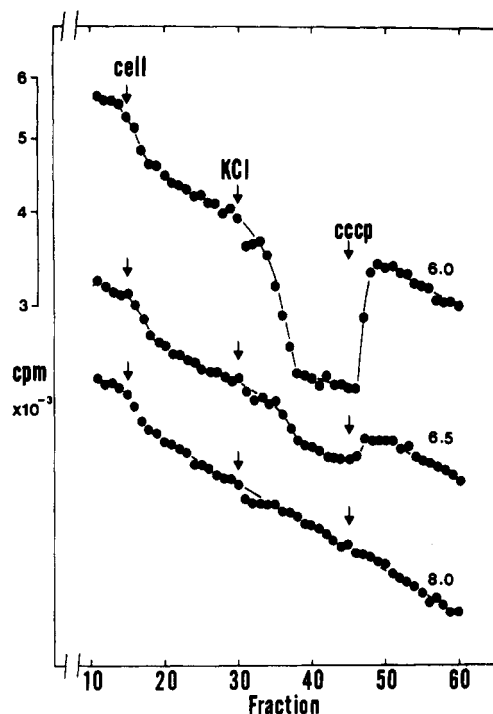[4] T. Nakamura, T. Unemoto, and H. Tokuda, unpublished results.

FIGURE 3: Effect of K$^+$ and external pH on the generation of ΔpH in K$^+$-depleted *V. alginolyticus*. ΔpH in K$^+$-depleted cells was assayed by using [^{14}C]acetylsalicylic acid (33 μCi/μmol, 38 μM as a final concentration) by flow dialysis in 0.1 M sodium phosphate containing 0.3 M NaCl and 20 mM glycerol at pH indicated in the figure. Concentrated cell suspension was added at fraction 15 to give final concentrations of 5.8 mg/mL at pH 6.0, 5.9 mg/mL at pH 6.5, and 5.9 mg/mL at pH 8.0. KCl and CCCP were added as indicated by arrows at final concentrations of 10 mM and 10 μM, respectively. Although results shown are displayed in decreasing order, the levels of radioactivity obtained initially in the dialysates (fraction 11) were approximately 5600 cpm in each case.

and, surprisingly, the extent of the decrease is similar at each pH value tested. The decrease in radioactivity represents uptake but not binding since addition of CCCP caused complete release of accumulated [^{3}H]TPP$^+$ at pH 6.5 (results not shown). $\Delta\psi$'s of -142, -147, and -151 mV at pH 6.5, 7.5, and 8.5, respectively, can be calculated. By use of K$^+$-containing cells, the $\Delta\psi$'s obtained were -91, -116, and -137 mV at pH 6.5, 7.5, and 8.5, respectively (Figure 1). Comparing these figures, it is clear that $\Delta\psi$ in K$^+$-depleted cells is considerably less sensitive to external pH than that in K$^+$-containing cells.

For examination of the effect of K$^+$ on the generation of $\Delta\psi$ at various pH values, KCl was added to the K$^+$-depleted cells at fraction 30 (Figure 2). Clearly, addition of KCl causes rapid release of accumulated [^{3}H]TPP$^+$ and establishment of a new steady state that is now dependent on external pH. Furthermore, $\Delta\psi$'s calculated from the TPP$^+$ distribution ratio achieved after addition of KCl are -88, -110, and -122 mV at pH 6.5, 7.5, and 8.5, respectively. Although not shown, cells treated with 2 mM KCN and 10 μM CCCP generated no $\Delta\psi$.

The results shown in Figure 3 represent the flow dialysis experiments in which accumulation of [^{14}C]acetylsalicylic acid was used to measure ΔpH in K$^+$-depleted cells at pH 6.0, 6.5, and 8.0. At pH 6.0 and 6.5, addition of K$^+$-depleted cells at fraction 15 causes a small decrease in dialyzable radioactivity (over that expected from dilution) which is indicative of the presence of a relatively small ΔpH. When K$^+$ is added at fraction 30, however, there is a marked increase in acetylsalicylate uptake, reflected by a decrease in the dialyzable radioactivity. A new steady state is observed, and all of the accumulated weak acid is released by CCCP. Furthermore, the ΔpH calculated from the new level observed after addition
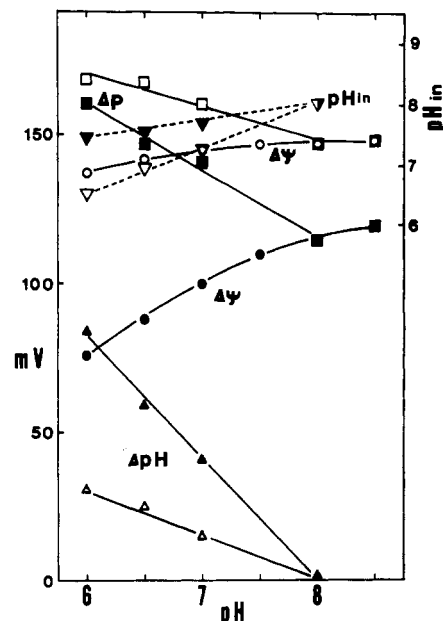

FIGURE 4: $\Delta\psi$, ΔpH, Δp, and internal pH in K$^+$-depleted *V. alginolyticus* as a function of external pH. $\Delta\psi$, ΔpH, Δp, and internal pH were determined at various external pHs in K$^+$-depleted *V. alginolyticus* by flow dialysis as described in Figures 2 and 3. Results obtained in the absence (open symbols) and the presence (closed symbols) of K$^+$ were calculated as mentioned under Materials and Methods and were replotted as a function of external pH. (○, ●) $\Delta\psi$; (△, ▲) ΔpH; (□, ■) Δp; (▽, ▼) internal pH.

of KCl (fraction 40) is comparable to that obtained in K$^+$-containing cells (Figure 1) and dependent upon external pH. Thus, when KCl is added at pH 8.0, no detectable change is observed, and an intermediate effect is observed at pH 6.5.

By use of K$^+$-depleted cells, $\Delta\psi$, intracellular pH, ΔpH, and Δp were determined in the presence and the absence of K$^+$ as described, and the results are expressed as a function of external pH in Figure 4. In the absence of K$^+$ (open circles), $\Delta\psi$ is -140 to -150 mV and essentially independent of external pH, while in the presence of the cation (closed circles), $\Delta\psi$ varies from about -75 mV at pH 6.0 to a maximum of about -120 mV at pH 8.5 with a linear increase of about -20 mV per pH unit between pH 6.0 and 7.0. In the absence of K$^+$, the cells are unable to maintain a constant internal pH, and, as a result, ΔpH exhibits a maximum value of only 30 mV at pH 6.0. In marked contrast, in the presence of K$^+$, internal pH is maintained at pH 7.5–8.0, and the cells exhibit a ΔpH of about 84 mV at pH 6.0 that decreases linearly to 0 at pH 8.0. In other words, K$^+$ allows the cells to maintain a constant internal pH, and at relatively acid pH, a large ΔpH is generated. Finally, it is apparent that in the absence of K$^+$ Δp is -10 to -30 mV higher than that observed in the presence of the cation over the range of pH values tested.

Although the results presented were obtained by adding 10 mM KCl, no additional effects were observed when 0.3 M KCl was used. Furthermore, the steady-state levels of TPP$^+$ and acetylsalicylate accumulation were maintained for at least 30 min at both K$^+$ concentrations.

Oxygen Uptake by K$^+$-Depleted Cells. Rates of oxygen uptake in K$^+$-depleted cells were measured in the presence of glycerol as a function of external pH (Figure 5). In the absence of K$^+$, the cells show much smaller activity than that observed in K$^+$-containing cells (see Figure 1). Addition of K$^+$, however, greatly stimulates the rate of oxygen uptake over the pH range from 6.0 to 8.5, and the activity obtained in the presence of K$^+$ is comparable to that in K$^+$-containing cells. Washing cells with alkaline buffer led not only to K$^+$ depletion
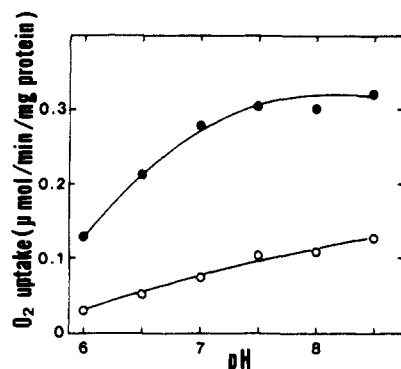
FIGURE 5: Stimulation of the rate of oxygen uptake in K^+-depleted cells by the addition of K^+. Depletion of K^+ was performed as mentioned in Figure 2 and under Materials and Methods. The rate of oxygen uptake was measured in 0.1 M sodium phosphate (pH 6.0–8.5) containing 0.3 M NaCl and 20 mM glycerol with (●) or without (O) 10 mM KCl. The oxygen uptake obtained in the presence of K^+ showed a biphasic time course, initial rapid uptake, and subsequent slower and nonlinear uptake. The results shown were calculated from the initial rapid phase. Values indicated represent μmol of O_2 consumed per min per mg of cell protein.

Table I: Effect of Various Ions on $\Delta\psi$ and ΔpH in K^+-Depleted V. alginolyticus[a]

ions added	$\Delta\psi$ (mV)	ΔpH (mV)
none	−137	31
KCl	−76	84
RbCl	−122	50
choline chloride	−141	29
LiCl	−138	30
CsCl	−137	26

[a] K^+ depletion was performed as described under Materials and Methods. $\Delta\psi$ and ΔpH in K^+-depleted cells were determined in 0.1 M sodium phosphate (pH 6.0) containing 0.3 M NaCl and 20 mM glycerol by flow dialysis as described in Figures 2 and 3. Values shown were calculated at the steady state obtained after the addition of indicated salt at a final concentration of 10 mM.

but also to significant diminution of the endogenous energy pool since omission of glycerol reduced the rate of oxygen uptake (results not shown).

Effect of Various Ions on $\Delta\psi$ and ΔpH in K^+-Depleted Cells. The ability of various ions to replace K^+ with respect to the phenomena described above was examined in K^+-depleted cells at pH 6.0 (Table I). Besides K^+, Rb^+ is the only ion that collapses $\Delta\psi$ with a concomitant increase in ΔpH among ions tested, and its effect is much smaller than that of K^+.

pH Dependence of K^+ Transport by K^+-Depleted Cells. The initial rate of K^+ uptake determined with K^+-depleted cells had a pH optimum from 7.5 to 8.0. The activity at pH 6.0 was approximately 50% of that at pH 7.5. On the other hand, the rate at pH 8.5 was only slightly lower than that at pH 8.0. It was also found that the cells actively accumulate Rb^+ at a slower rate than K^+.

Discussion

The results presented in this paper clearly indicate that the generation of ΔpH in *Vibro alginolyticus* is dependent on K^+. Increased proton ejection, which is necessary for establishment of ΔpH, may become possible by electrogenic influx of K^+ which serves as a "counterion". Such a mechanism has been speculated in *Streptococcus faecalis* (Harold et al., 1970) and *Escherichia coli* (Padan et al., 1976). Stimulation of oxygen uptake by the addition of K^+ to K^+-depleted cells (Figure 5) supports this idea. Strikingly, however, the "electrogenicity" of K^+ uptake is dependent on external pH. Thus K^+-depleted

cells in the absence of K^+ generate a $\Delta\psi$ that is essentially constant as a function of pH, while the $\Delta\psi$ in untreated cells and in K^+-depleted cells in the presence of K^+ increases with external pH. However, it is not easy to explain the mechanism of pH-dependent effect of K^+ on $\Delta\psi$ and ΔpH from the rate of K^+ movement alone since the initial rate of K^+ uptake has a pH optimum from 7.5 to 8.0.

The observations presented here give a possible explanation to the different responses of $\Delta\psi$ to external pH in intact cells and in membrane vesicles (Ramos et al., 1976; Tokuda & Kaback, 1977; Friedberg & Kaback, 1980). Since it is well-known that membrane vesicles are defective in their ability to accumulate K^+ (Bhattacharyya et al., 1971; Lombardi et al., 1973; Sprott & MacLeod, 1974), it seems likely that the insensitivity of $\Delta\psi$ to external pH in isolated membrane vesicles may be related to the lack of K^+ transport activity. The observations that membrane vesicles generate a ΔpH of comparable magnitude to that in cells in the absence of K^+ uptake may suggest the occurrence of flux of ion(s) across the vesicular membrane which can serve as a "counterion".

Much attention has been paid recently to the mechanism that regulates internal pH, and evidence suggesting the participation of Na^+/H^+ antiport (Krulwich et al., 1979; Zilberstein et al., 1980) and K^+/H^+ antiport (Plack & Rosen, 1980) have been reported. These systems are thought to prevent the cytoplasm from extensive alkalinization by allowing the entry of protons in exchange for cytoplasmic Na^+ or K^+. Although the present results clearly indicate the K^+ requirement for the generation of ΔpH, i.e., alkalinization, further studies are necessary in order to define precisely the role of ions in the regulation of internal pH.

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Papovavirus Chromatin Associated Cellular Endonuclease Which Introduces One Double-Strand Cut in Superhelical Deoxyribonucleic Acid[†]

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ABSTRACT: Nuclear extracts from SV40-infected CV-1 monkey kidney cells and from polyoma-infected 3T3 mouse cells contain an endonucleolytic activity which cleaves circular viral DNA within the chromatin to full-length linear rods [Waldeck, W., Föhring, B., Chowdhury, K., Gruss, P., & Sauer, G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5964–5968; Scott, W. A., & Wigmore, D. J. (1978) *Cell (Cambridge, Mass.) 15*, 1511–1518]. Sedimentation of the nuclear extracts through sucrose density gradients revealed a preferential binding of the endonuclease to the viral chromatin. Deproteinized exogenous covalently closed superhelical DNA substrates such as SV40 and polyoma as well as Col E$_1$ and PM2 DNAs were linearized by the endonuclease by introduction of one double-strand break per molecule. The reaction products, FOIII unit length rods, were shown to be devoid of single-strand nicks by electrophoresis in denaturing agarose gels. The double-strand break was randomly located within the various substrates since redigestion of the FOIII with single-cut restriction endonucleases failed to generate discrete pairs of reaction products. Neither linear double-stranded nor nicked circular FOII DNA structures were accepted as substrates. The endonucleolytic activity does not require the presence of ATP but is sensitive to EDTA. The enzyme activity is of cellular origin since nuclear extracts from uninfected CV-1 cells converted exogenous superhelical DNA to FOIII structures with the same properties as those described above. The biological properties of the endonuclease are discussed in the light of its possible function in permitting genetic exchange between different circular genomes. Further, it may play an essential role late during the replication of papovavirus DNA when the catenated daughter molecules are liberated from each other by an as yet unidentified mechanism.

We have recently shown that the origin of DNA replication in Simian virus 40 (SV40) and polyoma chromatin is particularly accessible to endogenous chromatin-bound endonuclease which cleaves open the covalently closed circular viral DNA to form linear rods of unit length (Waldeck et al., 1978). Scott & Wigmore (1978) also reported that the origin of DNA replication in SV40 chromatin is exposed to the action of DNase I as well as endogenous endonuclease.

The reasons why this part of the viral chromatin is relatively vulnerable to nuclease action remain, at present, entirely speculative. The nature of the endogenous nucleases which are capable of cleaving the viral DNA at or close to the origin

of replication is also unknown.

The endonucleolytic activity associated with SV40 chromatin in crude nuclear extracts from infected cells eventually degraded the endogenous SV40 DNA by randomly introducing single-strand and double-strand cuts. Protein-free exogenous SV40 FOI DNA,[1] when added as a substrate to unpurified SV40 chromatin preparations, was converted to FOIII and to FOII (Scott & Wigmore, 1978; Waldeck et al., 1978). Partial

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[1] Abbreviations used: FOI DNA, covalently closed double-stranded superhelical DNA; FOII DNA, circular double-stranded DNA bearing at least one nick in one of the strands; FOIII DNA, double-stranded linear rods of unit length; FOIV DNA, covalently closed relaxed double-stranded DNA; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; pfu, plaque-forming units.