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Quantitative Measurements of Membrane Potential in *Escherichia coli*†

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ABSTRACT: By use of giant cells of *Escherichia coli* induced by growth in the presence of 6-amidinopenicillanic acid, membrane potentials have been measured by two completely independent techniques: *directly* with intracellular microelectrodes and *indirectly* from the steady-state distribution of [^{3}H]tetraphenylphosphonium. Under a variety of conditions, the two methods yield values that agree very closely. Thus, with both techniques, the membrane potential approximates −85 mV (interior negative) at pH 5.0 and −142 mV at pH 8.0, with an average slope of −22 mV/pH unit over the range pH 5.0–7.0. A parallel study of membrane vesicles prepared from giant cells was undertaken using tetraphenylphosphonium distribution alone as a measure of membrane potential. The vesicles were found to exhibit a much smaller slope of membrane potential vs. extracellular pH (about −6 mV/pH unit) than intact giant cells. The results indicate that distribution studies with these lipophilic cations provide an excellent measure of membrane potential and are discussed in relation to calculations of H^+/substrate stoichiometry for proton-symport systems in *E. coli*.

According to the generalized chemiosmotic hypothesis (Mitchell, 1961, 1966, 1968, 1973, 1979), energy derived from respiration, photochemical reactions, or ATP[1] hydrolysis is transformed into a transmembrane difference of electrochemical potential for protons ($\Delta\bar{\mu}_{H^+}$) that is the immediate driving force for a wide range of processes in energy-transducing cells and organelles. Since both the chemical concentration of protons and the electric field acting on them enter into the electrochemical potential, it is obvious that demonstration of the validity of the chemiosmotic hypothesis depends upon quantitative measurement of the membrane potential ($\Delta\psi$), as well as the transmembrane pH difference (ΔpH). The formal relationship is

$$\Delta\bar{\mu}_{H^+} = (\psi_i - \psi_0) + \frac{RT}{F} \ln ([H^+]_i/[H^+]_0) = \Delta\psi - \frac{2.3RT}{F}\Delta\text{pH} \quad (1)$$

where both differences ($\Delta\psi$ and ΔpH) are taken in the same direction, cytoplasm minus cell exterior.

Although large quantities of data have been reported which support the generalized chemiosmotic hypothesis, few energy-transducing membranes can be subjected to direct electrophysiological study, and most estimates of both $\Delta\psi$ and ΔpH have relied on the use of permeant ions and permeant weak acids or bases as distribution indicators. As far as *Es-*

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient; V_m, the measured potential difference across the cell membrane; $\Delta\psi$, actual membrane potential; ΔpH, pH difference across the membrane; TPP^+, tetraphenylphosphonium (bromide salt); $TPMP^+$, triphenylmethylphosphonium (bromide salt); EDTA, ethylenediaminetetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ATP, adenosine 5′-triphosphate.

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cherichia coli is concerned, correlative support for the weak-acid determinations of intracellular or intravesicular pH (Padan et al., 1976; Zilberstein et al., 1979; Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977) has come from high-resolution ^{31}P nuclear magnetic resonance spectroscopy (Navon et al., 1977; Ogawa et al., 1978). The main purpose of the present report is to demonstrate a separate corroboration of the lipophilic cation determinations of $\Delta\psi$, by making use of newly developed electrophysiological techniques with giant *E. coli* (Adler et al., 1968; Long et al., 1978) and hydrophobically coated microelectrodes (Felle et al., 1978).

Experimental Procedures

Materials

[^{3}H]TPP$^+$ and [^{3}H]TPMP$^+$ were prepared by the Isotope Synthesis Group of Hoffmann-La Roche under the direction of Dr. Arnold Liebman. 5,5-Dimethyl[2-^{14}C]oxazolidine-2,4-dione (DMO) was purchased from New England Nuclear, and 6-amidinopenicillanic acid (mecillinam) was kindly provided by Leo Pharmaceutical Products, Ballerup, Denmark. Ascorbic acid and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem, phenazine methosulfate was obtained from Sigma Chemicals, and Spectrapor 2 dialysis tubing was obtained from Fisher Scientific. All other materials were of reagent grade and were obtained from commercial sources.

Methods

Growth of Bacteria and Preparation of Giant Cells. In all experiments, *E. coli* K-12, strain W1485 (F$^+$, λ^-), was used. Cells were grown in giant cell broth (4.5% nutrient broth, 0.4% yeast extract, 1% NaCl, and 20 mM potassium phosphate at pH 7.0) with gentle shaking at 25 °C. For preparation of giant cells, 6-amidinopenicillanic acid (mecillinam, at 3–5 μg/mL) was added to the cultures at the onset of log-phase growth. The swollen, spherical cells were harvested from these cultures several hours later (see Figure 1) when they had reached average diameters of 3–5 μm. For electrophysiological experiments, a drop of growth suspension was added to the recording chamber and diluted with 10 mM potassium phosphate at the desired pH, containing 1 mM NaCl, 0.5 mM magnesium chloride, 0.5 mM calcium chloride, 5% sucrose, and 0.5% glycerol. Cells to be punctured were caught and held, by vacuum-assisted capillarity, on cup-shaped pipets made with a de Fonbrune microforge. For distribution measurements, cells were harvested by centrifugation at 800*g* for 15 min, washed once in 120 mM Tris-HCl (pH 8.0) containing 12% sucrose, and resuspended in the same solution at a concentration of 9–10 mg of cell protein per mL. The cells were then treated with 0.1 mM ethylenediaminetetraacetic (EDTA; Padan et al., 1976), harvested again by centrifugation, and washed once and resuspended in 100 mM potassium phosphate at the desired pH, containing 10 mM magnesium chloride, 0.5 mM calcium chloride, 12% sucrose,[2] and 0.5% glycerol. Final suspensions contained 50–70 mg of cell protein per mL and were stored on ice for not more than 8 h before use.

Microelectrode Preparation and Use. Except for the preparation of the microelectrodes, standard electrophysiological techniques (Gradmann & Slayman, 1975) were used. Micropipets, pulled (from fiber-filled borosilicate tubing) to tip diameters of 0.08–0.2 μm, were silylated by exposure to the vapor of 1% dichlorodimethylsilane in benzene, followed by baking at 150 °C for 1 h. The pipets were subsequently filled with 1 M KCl by capillary displacement and then dip-coated with embedding paraffin (1% in xylene) and/or dioleoylphosphatidylcholine (1% in chloroform).

Under the conditions of these experiments, the microelectrode tips stabilized in the cells with a sealing resistance (r_L) of ~14 Gohms (14×10^9 ohms). This value and an average specific resistance (R_m) for the giant-cell membrane equal to $(2.0–2.3) \times 10^3$ ohms·cm^2 were obtained from a plot of the total recorded input resistance against cell diameter, varied over the range 2.5–5.0 μm (Felle et al., 1978). From these results, plus a simple electrical equivalent circuit in which the "true" membrane voltage ($\Delta\psi$) and membrane resistance are taken as series elements shunted by the sealing resistance, $\Delta\psi$ can be calculated as

$$\Delta\psi = V_m \frac{R_{m/d^2\pi} + r_L}{r_L} \quad (2)$$

where d is the cell diameter and V_m is the actual measured potential. Cells reported in the present experiments averaged 4.6 ± 0.2 μm in diameter, so that the average correction factor of 1.24 was used to convert observed voltages to $\Delta\psi$.

Measurement of $\Delta\psi$ and ΔpH by Flow Dialysis. Flow dialysis was performed essentially as described by Ramos et al. (1979), using [^{3}H]tetraphenylphosphonium (TPP$^+$) and [^{3}H]triphenylmethylphosphonium (TPMP$^+$) distribution to measure $\Delta\psi$ and [2-^{14}C]DMO distribution to measure ΔpH (Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977). At the inception of the assays, [^{3}H]TPP$^+$ (2.5 Ci/mmol), [^{3}H]TPMP$^+$ (1.0 Ci/mmol), or [2-^{14}C]DMO (49.8 mCi/mmol) at a final concentration of 10, 10, or 18 μM, respectively, was added to 0.74 mL of 100 mM potassium phosphate (at a given pH), 12% sucrose, 0.5% glycerol, 10 mM magnesium chloride, and 0.5 mM calcium chloride contained in the upper chamber of a flow-dialysis apparatus. After a constant rate of dialysis was achieved (8–9 min, fraction 30), 2.5 mg of cell protein or 1.7 mg of vesicle protein was added to the upper chamber in a volume of 50 μL, followed immediately by potassium ascorbate (20 μL of a 2.5 M solution) and phenazine methosulfate (2 μL of a 100 mM solution) to final concentrations of about 60 and 0.25 mM, respectively. Once a new steady state was achieved (fraction 60), CCCP was added to a final concentration of 160 μM. Potassium phosphate (100 mM, at the same pH as the solution in the upper chamber) containing 12% sucrose, 0.5% glycerol, 10 mM magnesium chloride, and 0.5 mM calcium chloride was pumped from the lower chamber at a rate of 6.0 mL/min, and 1.7-mL fractions were collected. Radioactivity in the dialysate was assayed by liquid scintillation spectrometry.

Preparation of Vesicles from Giant Cells. Giant cells were prepared, harvested, and treated wtih EDTA as described above. The cells were then washed once in 50 mM potassium phosphate (pH 6.6) containing 20% sucrose and 20 mM magnesium sulfate and resuspended in the same solution to a final protein concentration of about 10 mg/mL. This suspension was dialyzed overnight at 4 °C in Spectrapor 2 dialysis tubing against 100 volumes of 50 mM potassium phosphate (pH 6.6). The buffer was changed once every 2 h for the first 6 h and then once more after 10 h. The viscous dialysate was collected in a beaker, and EDTA, DNase, and RNase were added to final concentrations of 10 mM, 10 μg/mL, and 10 μg/mL, respectively. The mixture was stirred on a magnetic

[2] The concentrations of sucrose and magnesium chloride were increased for the flow-dialysis experiments because the giant cells were found to be somewhat unstable to mechanical agitation under the conditions used for electrophysiological measurements.

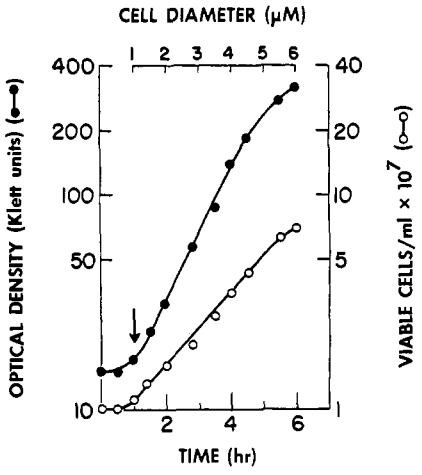

FIGURE 1: Growth of *E. coli* W1485 in the presence of mecillinam. Cells were inoculated into giant-cell broth (cf. Methods) at a concentration of ~1 × 10^7 cells/mL (15 Klett units) and incubated on a rotary shaker at 25 °C. After initiation of log-phase growth, mecillinam was added to the culture at a final concentration of 5 μg/mL (arrow at 1 h), and incubation was continued. The increase in cell diameter (upper scale) was followed by phase contrast microscopy, the increase in optical density by means of a Klett–Summerson photoelectric colorimeter with a no. 60 filter (●), and the increase in viable cells (○) by serial dilution and plating in the absence of mecillinam.

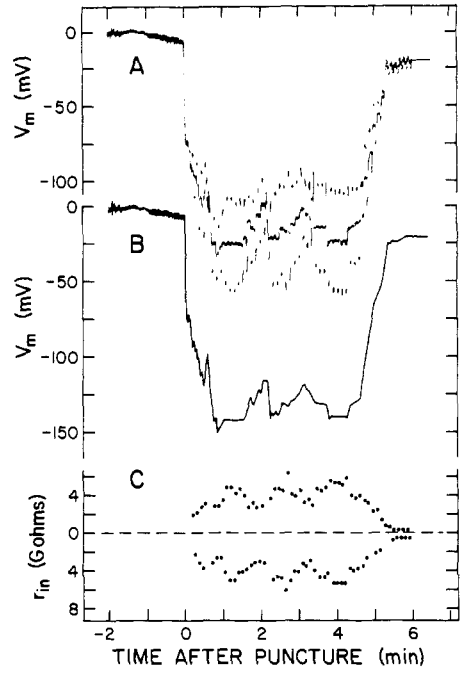

FIGURE 2: Sample electrical record from giant *E. coli* W1485. (A) Photograph of record, with pips representing peak voltage displacements (ΔV) in response to square pulses of current (0.5 s, ±6.2 pA). (B) Tracing of (A) to show the resting membrane potential clearly. (C) Plot of recorded input resistance ($r_{in} = \Delta V/6.2$ pA) vs. time. No correction has been made for the slight drift in electrode resistance observable as the residual pulses at the end of the record and amounting to ~0.5 Gohm.

stirrer at room temperature for 30 min, magnesium sulfate was added to a final concentration of 20 mM, and stirring was continued for another 15 min. Unlysed giant cells were removed by centrifugation at 500*g* for 20 min, and the vesicles present in the supernatant were harvested by sedimentation at 29000*g* for 10 min. The vesicles were then washed twice in 50 mM potassium phosphate (pH 6.6) and resuspended in 100 mM potassium phosphate at the desired pH, containing 12% sucrose, 0.5% glycerol, 10 mM magnesium chloride, and 0.5 mM calcium chloride, to a concentration of 25–40 mg of protein per mL. Vesicles prepared from giant cells in this fashion were larger in diameter than those prepared by the usual procedure (Kaback, 1971; Short et al., 1975) (i.e., 1.0–2.0 μm in diameter compared to 0.5–1.0 μm for vesicles prepared by the standard method).

Determinations of Internal Volume. The internal volumes of EDTA-treated *E. coli* W1485, giant cells, and vesicles prepared from giant cells were determined with [^{14}C]inulin and [^{3}H]water as described (Kaback, 1970). Values of 5.85, 7.06, and 2.85 μL/mg of protein ± 15% were obtained from cells, giant cells, and vesicles prepared from giant cells, respectively.

Calculations. Calculations of ΔpH and $\Delta\psi$ were made as described (Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977; Ramos et al., 1979) by using values for the internal volume given above. Values for $\Delta\bar{\mu}_{H^+}$ were calculated by substituting values for ΔpH and $\Delta\psi$ into eq 1.

Protein Determinations. Protein was measured according to Lowry et al. (1951) with bovine serum albumin as the standard.

Results

Growth of Giant Cells. On a mass basis, growth of *E. coli* W1485 in the presence of mecillinam closely resembles that of normal cells. In giant-cell broth at 25 °C, log-phase growth lasts 4–5 h (Figure 1). During that time, cell diameters increase to the range of 4–5 μm; potassium content, ATP content, and respiration are stable or decline only slightly (Felle et al., 1978). The number of viable cells per unit volume of suspension increases with a doubling time of about 1.5 h (lower curve, Figure 1), compared with about 0.9 h for total mass doubling (upper curve, Figure 1), which presumably means that some mechanical fragmentation of cells occurs, along with swelling, after normal cell division is blocked by the drug. In shaking liquid cultures, the cells are of spherical geometry, and in stationary plate cultures, they form thin circular disks (Long et al., 1978).

The transition to the stationary phase, with growth in mecillinam beyond 5 h, progresses slowly and with profound physiologic changes: declining intracellular ATP and potassium, loss of motility, cessation of cell division, vacuolation, and degradation of the cell wall complex (Felle et al., 1978). Although individual cells continue to grow during this period, many lyse, leaving identifiable ghosts. Thus, cells 4–5 μm in diameter are the largest that are likely to be physiologically "normal", and the electrical experiments reported below used almost exclusively cells in this narrow size range. Slightly smaller cells (see Methods) were used for the distribution measurements.

Microelectrode Measurements of $\Delta\psi$. A typical intracellular recording showing both voltage and resistance data is given in Figure 2A. The pips are voltage displacements (ΔV) resulting from the injection of 0.5-s square pulses of current (±6.2 pA). Although the record is somewhat noisy, it can be separated for easier inspection into the voltage trace (Figure 2B) and the recorded input resistance ($r_{in} = \Delta V/6.2$ pH; Figure 2C). In addition to a resting potential (V_m) of −140 mV, Figure 2B displays several recording problems: slow "sealing in" of the microelectrode, evidenced by the irregular transit of potential from −60 to −140 mV, associated with increasing resistance; spontaneous withdrawal or cell death; some instability of the resting potential. For the most part, these problems can be attributed to an unstable leakage resistance around the microelectrode, but that interpretation is

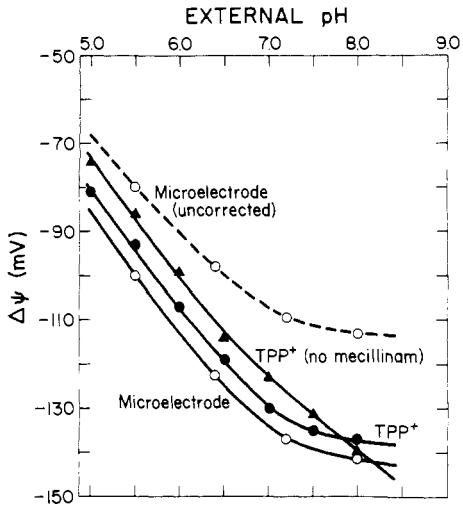

FIGURE 3: Effect of external pH on the membrane potential ($\Delta\psi$) of *E. coli*. (Open symbols) Microelectrode data; (filled symbols) [^{3}H]TPP$^+$ data. For the microelectrode data, the upper plot represents average measured potentials (V_m; 6–15 cells at each pH); the lower plot has been corrected for current leakage through the membrane seal at the point of electrode insertion ($=V \times 1.24$). For TPP$^+$ data, the lower plot (●) was obtained from giant cells prepared as described under Methods; the upper plot (▲) was obtained from normal *E. coli* handled in the same way as the giant cells, except without mecillinam. Plotted points are averages for three separate experiments. SEM's of the data points: 9 mV for microelectrodes and 10 mV for TPP$^+$.

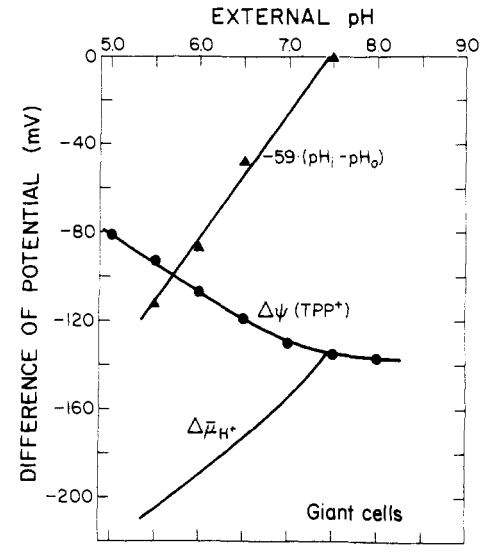

FIGURE 4: Dependence of ΔpH and $\Delta\bar{\mu}_{H^+}$ on extracellular pH. ΔpH (▲) was estimated from the distribution of [2-^{14}C]DMO and multiplied by −59 to give millivolts. Points are averages for three separate determinations. The fitted line was drawn by least squares and has a slope of −57 mV/pH unit and an abscissal intercept of 7.46. $\Delta\psi$ (●) is replotted from Figure 3 and the curve drawn by eye. $\Delta\bar{\mu}_{H^+}$ (lower curve) was drawn as the sum of the other two curves, according to eq 1.

not completely satisfactory for the depolarizing wobbles at 2 and 3 min, since the corresponding resistance changes are disproportionately large compared with the voltage shifts. This cell was one of a group for which the sealing resistance around the micropipet was estimated to be 60 Gohms and for which the correction factor that should be applied to obtain $\Delta\psi$ from V_m is 1.1 (rather than 1.24; see Methods). Hence, for this cell, $\Delta\psi$ = −154 mV.

The results from a systematic investigation of the effect of extracellular pH on $\Delta\psi$ in mecillinam-induced giant cells are shown by the open circles in Figure 3. Average measured potentials (V_m) are plotted in the upper (dashed) curve, and values corrected ($\Delta\psi = 1.24V_m$) for current leakage are plotted in the lower (solid) curve. The average $\Delta\psi$ increases from −100 mV at pH 5.5 to −142 mV at pH 8.0; up to pH 7.0, $\Delta\psi$ can be approximated by a straight line with a slope of −22 mV/pH unit.

Measurement of $\Delta\psi$ by Distribution of Lipophilic Cations. It is obvious from the filled points plotted in Figure 3 that calculations of $\Delta\psi$ in EDTA-treated giant cells of *E. coli*, based on the steady-state distribution of [^{3}H]TPP$^+$ at several different external pH values, yield a curve identical in shape with that obtained with the microelectrode technique. Although the TPP$^+$ curve is displaced 6 mV positive to that from the microelectrode measurements, both the scatter of data (SEM at each point = 9 mV for the electrical data and 10 mV for the flow-dialysis data) and the very different nature of the assumptions which underlie the two kinds of calculations (see Methods) make it clear that this 6-mV offset is not significant. Estimation of $\Delta\psi$ in EDTA-treated *E. coli* W1485 grown in the absence of mecillinam again gives a curve of similar shape (at least up to pH 7.5) but displays an additional 7-mV offset positive to that for giant cells. The difference between the two TPP$^+$ curves should be regarded as barely significant.

ΔpH and $\Delta\mu_{H^+}$ in Giant Cells of E. coli. For comparison with previous studies on intact cells and vesicles from normal *E. coli*, we also measured ΔpH in giant cells. With external pH varied over the range 5.5–7.5, internal pH is essentially constant, and the results (converted to millivolts) define a straight line with an actual slope of 57 mV/pH unit (Figure 4, filled triangles) which we take to be identical with the theoretical slope of 59 mV/pH unit. Also replotted in Figure 4 is the pH dependence of $\Delta\psi$, determined with TPP$^+$ in EDTA-treated giant cells. Added together according to eq 1, these two curves yield the total difference of electrochemical potential for protons across the plasma membrane of EDTA-treated giant *E. coli* (bottom curve, Figure 4). Obviously, $\Delta\bar{\mu}_{H^+}$, the total driving force acting to push protons across the membrane, does not change as steeply with pH as does ΔpH because of the compensatory increase in $\Delta\psi$. The significance of this point will be considered in the Discussion.

Behavior of Giant-Cell Vesicles. Since there is reportedly a significant difference in the response of $\Delta\psi$ to external pH in intact cells and right-side-out membrane vesicles that is critical to calculations of H$^+$/substrate transport stoichiometry (Zilberstein et al., 1979; Ramos & Kaback, 1977b,c; Letellier & Shechter, 1979), [^{3}H]TPP$^+$ distribution was also examined in giant-cell vesicles. The procedure used to make giant-cell vesicles is considerably more mild (see Methods) than that normally employed, since it seemed possible that this might affect $\Delta\psi$ and the pH sensitivity of $\Delta\psi$ in vesicles. The results are plotted in Figure 5 (open circles) and compared with redrawn data from intact giant cells (filled circles). It is clear that vesicles behave differently from intact cells, with respect to the pH sensitivity of $\Delta\psi$. All points fall near a straight line with a slope of −6 mV/pH unit, and $\Delta\psi$'s in vesicles and intact giant cells coincide at only one pH, ~5.5, where $\Delta\psi$ equals −93 to −95 mV. For data previously published (Ramos et al., 1976) on vesicles from normal *E. coli*, the slope was also −6 mV/pH unit over the range pH 5–7.

Discussion

Although some improvements in the microelectrode technique for recording from giant bacteria clearly would be desirable, as has been discussed elsewhere (Felle et al., 1978), it is evident that *E. coli*—and probably other gram-negative bacteria that are sensitive to mecillinam [see, e.g., Lorian &

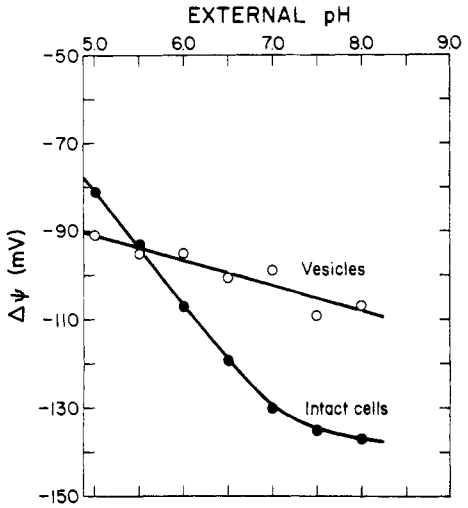

FIGURE 5: Effect of external pH on $\Delta\psi$ in giant-cell vesicles. Vesicle data (○) represent averages for three separate experiments. The fitted line was drawn by least squares, with a slope of −5.7 mV/pH unit and a value of −102 mV at pH 7.0. A comparison plot for intact giant cells (●) is redrawn from Figure 3.

Atkinson (1977)]—is now accessible to direct electrophysiological investigation.

Experiments presented in this paper are only an introduction to the detailed quantitative analysis of charge transport which remains to be carried out on bacterial membranes, but they do make two important points. *First*, in respiring giant cells of *E. coli*, $\Delta\psi$ is large, oriented with the cell interior negative, and becomes increasingly negative as the external pH is raised. In all these respects, giant *E. coli* closely resembles eukaryotic microorganisms, chiefly *Neurospora* and the characean algae, which have been studied more extensively by electrophysiologic techniques (Slayman, 1977; Hope & Walker, 1975). *Second*, at least under the conditions of these experiments, the microelectrode measurements closely confirm estimates of $\Delta\psi$ in giant *E. coli* based on the steady-state distribution of the lipophilic cation [^{3}H]TPP$^+$ (and also [^{3}H]TPMP$^+$; data not shown). This finding strongly supports the previous use of lipophilic cations with intact cells (Schuldiner & Kaback, 1975; Zilberstein et al., 1979) and membrane vesicles (Schuldiner & Kaback, 1975; Ramos et al., 1976; Ramos & Kaback, 1977a–c) of *E. coli*. The agreement between the two methods, despite the EDTA treatment for the TPP$^+$ method, may be regarded at present as a consequence of bacterial indifference to the absence of calcium.

Apart from providing strong support for one of the basic tenets of the chemiosmotic hypothesis, the overall results presented in this paper shed light on the specific problem of stoichiometry ratios for proton-symport mechanisms in *E. coli*. Because of the inapplicability of tracer-flux techniques to proton-transport problems, estimates of H$^+$/substrate stoichiometry must be based almost exclusively on measurements of $\Delta\bar{\mu}_{H^+}$ and steady-state levels of substrate accumulation. In our hands, the most reliable data have come from flow-dialysis experiments with right-side-out membrane vesicles of *E. coli*. Ramos et al. (1976) showed that intravesicular pH is almost independent of extravesicular pH so that ΔpH (see eq 1) is extremely sensitive to external pH, exhibiting maximal values of 2 units at acid pH values and decreasing to zero at pH ~7.5. The same authors, studying the distribution of TPMP$^+$, also concluded that $\Delta\psi$ in vesicles is rather insensitive to extravesicular pH (a slope of −6 mV/pH unit; see Results). Somewhat later, Ramos & Kaback (1977b,c) also found that steady-state accumulation levels for various transport substrates, especially proline and lactose, exceed $\Delta\bar{\mu}_{H^+}$ at alkaline pH and suggested that a pH-dependent increase of H$^+$/substrate stoichiometry (Rottenberg, 1976) might account for the observations.

More recently, however, studies on EDTA-treated whole cells (Zilberstein et al., 1979; Letellier & Shechter, 1979) revealed that $\Delta\psi$ increases much more steeply with pH than had been observed earlier with intact cells (Padan et al., 1976) or vesicles (Ramos et al., 1976; Ramos & Kaback, 1977a). The present results, including both the microelectrode data and the lipophilic ion data, confirm the findings of Zilberstein et al. (1979) and extend them to cells that have not been treated with EDTA (i.e., those studied with microelectrodes). Present experiments with TPP$^+$ alone also reconfirm the smaller pH dependence of $\Delta\psi$ in vesicles. The conclusion to be drawn from all of these measurements is that, because of the larger $\Delta\psi$ in intact cells at alkaline pH, $\Delta\bar{\mu}_{H^+}$ is indeed large enough to account for the steady-state level of lactose accumulation with a stoichiometry of 1 H$^+$/1 lactose. Therefore, only in vesicles and only at alkaline pH is there a need to postulate either a change in stoichiometry or an error in the TPMP$^+$ measurements to account for steady-state levels of lactose accumulation. It is hoped that direct electrophysiological measurements on giant-cell vesicles will clarify the cause and functional significance of their anomalous behavior at alkaline pH.

Acknowledgments

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Electron Paramagnetic Resonance Detectable States of Cytochrome P-450$_{cam}$†

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ABSTRACT: Cytochrome P-450$_{cam}$ is a low-spin Fe^{3+} hemoprotein (g = 2.45, 2.26, and 1.91) which is made 60% high spin (g = 7.85, 3.97, and 1.78) at 12 K by the addition of 1 mol of substrate per mol of enzyme. Low-temperature EPR spectra show that the low-spin fraction of substrate-bound P-450$_{cam}$ contains two magnetic species. The majority species has an unusual EPR spectrum (g = 2.42, 2.24, and 1.97) which cannot be simulated by using the range of crystal field parameters known for other heme proteins. The minority species has the same g values as substrate-free enzyme. Both low-spin species show Curie law temperature dependence below 50 K and have similar saturation behavior. Above 50 K the g = 2.42, 2.24, and 1.97 species rapidly loses signal intensity. The distribution of low-spin species is pH dependent (apparent pK_a = 6.2) with the g = 2.42, 2.24, and 1.97 magnetic species favored at high pH. The substrate binding stoichiometry and the equilibria observed in the low-spin fraction suggest that there are not multiple protein forms of cytochrome P-450$_{cam}$. Putidaredoxin and other effector molecules which specifically catalyze hydroxylation convert either the high-spin or the g = 2.42, 2.24, and 1.97 low-spin species to another new magnetic species (g = 2.47, 2.26, and 1.91). This species is only seen in the presence of substrate, and its stability reflects the catalytic potency of the effector molecule. The EPR and UV–visible spectra of cytochrome P-420 depend upon the manner in which the P-420 is generated. Incubation with acetone or reaction with *N*-ethylmaleimide or diethyl pyrocarbonate generates P-420 with different spectral characteristics. Through identification of active-site amino acids by chemical modification and comparison with porphyrin model complexes, the range of ligands likely to participate in each of the EPR detectable species is assigned. Mechanisms of interconversion of these species and their bearing on catalysis are discussed.

Cytochrome P-450$_{cam}$ (cytochrome m)[1] is the central enzyme of a three-component mixed-function oxidase which converts the 5-methylene group of camphor to the 5-exo alcohol (Katagiri et al., 1968). Dramatic changes in the magnetic resonance spectra of the heme iron accompany each step in the catalytic cycle (Gunsalus et al., 1973). In particular, Mössbauer spectroscopy has been valuable in detailing the electronic properties in each of the semistable ferric and ferrous intermediates of the cycle (Sharrock et al., 1973, 1976), and EPR spectroscopy of the ferric intermediates has demonstrated the sensitivity of the iron environment to substrates. At 12 K the native cytochrome m (m^o) is low spin ($S = {}^1/_2$) with EPR g values at 2.45, 2.26, and 1.91, but addition of substrate (m^{os}) is accompanied by a 60% conversion to a rhombically distorted high-spin ($S = {}^5/_2$) form with g = 7.85, 3.97, and 1.78 (Tsai et al., 1970). Substrate binding also causes a 26-nm blue shift in the optical Soret band which has been associated with the EPR detectable high-spin transition. Quantitation of the cytochrome absorption at 25 °C in the Soret region implies that only 6% of the substrate-bound cytochrome is in the low-spin form (Sligar, 1976). The low-spin fraction increases with decreasing temperature according to the usual Arrhenius relationship to a maximum of 40% at ~200 K. Below this temperature, Mössbauer spectroscopy shows that the Arrhenius dependence is not observed and there is only a small change in the high-/low-spin distribution. (Sharrock et al., 1973). At temperatures above 100 K, the EPR spectrum of the low-spin m^{os} magnetic species is identical with that observed for m^o; however, at lower temperatures an anomalous signal at g = 1.97 is observed (Tsai et al., 1970). This signal was postulated to arise from either an excited state or a contaminant. We present evidence in this report which shows that the signal represents in fact a new low-spin form of cytochrome m.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; NEM, *N*-ethylmaleimide; DEP, diethyl pyrocarbonate; cytochrome P-450$_{cam}$ is shortened to cytochrome m or m (for monoxygenase) (Sligar, 1976) to facilitate presentation of its various redox and substrate-bound states.

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