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Electrochemical Proton Gradient in Inverted Membrane Vesicles from *Escherichia coli*†

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ABSTRACT: Inverted membrane vesicles prepared from *Escherichia coli* ML 308-225 generate a transmembrane electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$; interior positive and acid) during oxidation of D-lactate, succinate, reduced phenazine methosulfate, or NADH or hydrolysis of ATP. Using the distribution of the lipophilic anion thiocyanate to measure the membrane potential ($\Delta\Psi$) and the permeant weak base methylamine to measure the pH gradient (ΔpH), maximal values for $\Delta\bar{\mu}_{H^+}$ of approximately +160 mV are obtained. Many of the properties of $\Delta\bar{\mu}_{H^+}$ in inverted vesicles are similar to those described previously in right-side-out vesicles [Ramos, S., & Kaback, H. R. (1977) *Biochemistry 16*, 848]: (1) the magnitude of the $\Delta\Psi$ (interior positive) generated in the presence of D-lactate or reduced phenazine methosulfate is similar to that observed in right-side-out vesicles but of opposite polarity and independent of pH from 5.5 to 8.0; (2) plots of ΔpH vs. internal pH in inverted vesicles and external pH in the right-side-out vesicles are similar with D-lactate as the electron donor; (3) as observed with right-side-out vesicles, dissipation of $\Delta\Psi$ or ΔpH leads to a concomitant increase in the other parameter without a change in the rate of respiration; (4) inverted vesicles catalyze Na^+ accumulation, and it is apparent that the process can be driven by either $\Delta\Psi$ (interior positive) or ΔpH (interior acid).

According to the chemiosmotic hypothesis of Mitchell (1961, 1966, 1968, 1976; Greville, 1971; Harold, 1972), oxidation of electron donors via a membrane-bound respiratory chain or hydrolysis of ATP by the Ca^{2+},Mg^{2+}-stimulated ATPase complex is accompanied by expulsion of protons into the external medium, leading to the generation of an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$)[1] that is the immediate driving force for active transport in many bacterial systems (Mitchell, 1973; Hamilton, 1975; Harold, 1976; Kaback, 1976). Moreover, recent evidence with right-side-out cytoplasmic membrane vesicles prepared from *Escherichia coli* (Kaback, 1971; Short et al., 1975; Owen & Kaback, 1978, 1979a,b) provides virtually unequivocal evidence that chemiosmotic phenomena play a central, obligatory role in the energetics and mechanism of active transport (Ramos et al., 1976; Ramos & Kaback, 1977a–c; Boonstra & Konings, 1977).

The proton electrochemical gradient is composed of electrical and chemical parameters according to the relationship

$$\Delta\bar{\mu}_{H^+} = \Delta\Psi - \frac{2.3RT}{F}\Delta\text{pH} \qquad (1)$$

where $\Delta\Psi$ represents the electrical potential across the membrane and ΔpH is the chemical difference in proton concentrations across the membrane ($2.3RT/F$ is equal to 58.8 mV at 25 °C). Over the past few years, methods have been developed (Schuldiner & Kaback, 1975; Ramos et al., 1976, 1979; S. Ramos, L. Patel, and H. R. Kaback, unpublished experiments) that allow quantitation of $\Delta\bar{\mu}_{H^+}$ and its components in right-side-out vesicles, and it has been demonstrated that oxidation of certain electron donors leads to the development of $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) that approaches −200 mV at pH 5.5. This value is very similar to that observed in intact cells under the same conditions (Padan et al., 1976; Zilberstein et al., 1979).

Strong support for the quantitative validity of the measurements has been obtained recently. Electrophysiologic techniques have been applied to *E. coli* giant cells induced by growth in 6-amidinopenicillanic acid, providing a means of verifying $\Delta\Psi$ measurements made with premeant lipophilic cations. Using *E. coli* W1485 giant cells and recording intracellularly, Felle et al. (1978) measured a $\Delta\Psi$ (interior negative) under a variety of conditions, and the results agree to within 10 mV with values obtained by using [^{3}H]tetraphenylphosphonium (Porter et al., 1979). In addition, it has been demonstrated that distribution measurements with this

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; $\Delta\Psi$, electrical potential; ΔpH, pH gradient; CCCP, carbonyl cyanide (*m*-chlorphenyl)hydrazone; PMS, phenazine methosulfate; Q_1, ubiquinone 1; Δ_{Na^+}, chemical gradient of sodium.

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lipophilic cation provide an excellent quantitative measure of $\Delta\Psi$ in cultured neuroblastoma/glioma NG108-15 hybrid cells (Lichtshtein et al., 1979). Finally, Navon et al. (1977) and Ogawa et al. (1978) have utilized high-resolution ^{31}P nuclear magnetic resonance spectroscopy to measure ΔpH (interior alkaline) in intact *E. coli*, and the results are very similar to those obtained from distribution studies with permeant weak acids in right-side-out plasma membrane vesicles (Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977).

One of the most important and interesting aspects of the protonmotive force in *E. coli* cells (Padan et al., 1976; Navon et al., 1977) and right-side-out vesicles (Ramos et al., 1976; Ramos & Kaback, 1977a) is the propensity of the system to maintain internal pH at pH 7.5. Thus, at an external pH of 5.5, ΔpH is interior alkaline and exhibits a maximum value of about −120 mV (i.e., 2 pH units). As external pH is increased, ΔpH decreases and approaches zero at pH 7.5. Although the reasons for this phenomenon may be complicated [cf. Ramos & Kaback (1977a)], an explanation that could account for the data is the operation of a mechanism that catalyzes the exchange of external H^+ for internal Na^+ at relatively alkaline pH (Padan et al., 1976; Ramos & Kaback, 1977a). In part, the studies to be presented here were initiated to investigate this possibility.

Rupture of cells by passage through a French pressure cell at low shear forces (Hertzberg & Hinkle, 1974; Rosen & McClees, 1974) yields a population of small vesicles whose membrane is inverted relative to the membrane of the intact cell (Futai, 1974; Altendorf & Staehelin, 1974). Inverted vesicles prepared in this manner exhibit the following activities: (1) oxidative phosphorylation (Hertzberg & Hinkle, 1974); (2) respiration- and ATP-dependent Ca^{2+} transport (Rosen & McClees, 1974; Tsuchiya & Rosen, 1975, 1976); (3) $\Delta\bar{\mu}_{H^+}$ (interior positive and acid)-dependent lactose efflux and lactose accumulation in the presence of an artificially imposed $\Delta\Psi$ (interior negative) (Lancaster & Hinkle, 1977); (4) respiration- and ATP-dependent generation of ΔpH (interior acid) (Singh & Bragg, 1976; Schuldiner & Fishkes, 1978; Brey et al., 1978; Brey & Rosen, 1979) and $\Delta\Psi$ (interior positive) (Singh & Bragg, 1979). Since the polarity of these vesicles is opposite to that of vesicles prepared by osmotic lysis (Kaback, 1971; Short et al., 1975; Owen & Kaback, 1978, 1979a,b), studies of $\Delta\bar{\mu}_{H^+}$ and its components in the inverted preparations should provide the basis for an interesting comparison. In addition, Schuldiner & Fishkes (1978), Brey et al. (1978), and Beck & Rosen (1979) have reported that addition of Na^+ leads to a decrease in ΔpH in inverted vesicles. Given these observations, it is apparent that inverted vesicles should provide a viable system for studying the relationship between H^+/Na^+ antiport and the components of $\Delta\bar{\mu}_{H^+}$.

In this paper, the $\Delta\bar{\mu}_{H^+}$ generated by inverted vesicles is quantitated under a variety of conditions and compared to the $\Delta\bar{\mu}_{H^+}$ generated by right-side-out vesicles. In addition, it is demonstrated that inverted vesicles catalyze Na^+ accumulation and that this process is coupled to both components of $\Delta\bar{\mu}_{H^+}$.

Experimental Section

Methods

Growth of Cells. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown at 37 °C on minimal medium A (Davis & Mingioli, 1950) containing 1.0% disodium succinate (hexahydrate).

Preparation of Membrane Vesicles. Inverted vesicles were prepared as described by Hertzberg & Hinkle (1974), frozen, and stored in liquid nitrogen. Except where stated, all experiments were performed with inverted vesicles prepared by a slightly modified procedure using phosphate buffer. Cells (14 L) were grown to late log phase and harvested by centrifugation at 13000*g* for 10 min. The pellet was resuspended in 1.5 L of 50 mM potassium phosphate (pH 7.5) containing 5 mM magnesium sulfate, centrifuged, and resuspended to 20% (wet weight/volume) in 50 mM potassium phosphate (pH 7.5), 5 mM magnesium sulfate, and 1 mM dithiothreitol. The cells were then broken by a single passage through an American Instrument Co. French pressure cell at 8000 psi. Unbroken cells and large debris were removed by centrifugation at 48000*g* for 15 min, and inverted vesicles were collected by centrifuging the supernatant at 210000*g* for 120 min. The pellet was resuspended in 50 mM potassium phosphate (pH 7.5) containing 5 mM magnesium sulfate by gentle homogenization to a final concentration of 10–15 mg of protein per mL, and 1.0-mL aliquots were frozen and stored in liquid N_2. Prior to use, samples were thawed rapidly at 46 °C. Under these conditions, the vesicles were stable for at least 6 months.

Right-side-out vesicles were prepared by osmotic lysis of lysozyme–ethylenediaminetetracetic acid induced spheroplasts as described (Kaback, 1971; Short et al., 1975).

For studies at various pH values and with buffers other than potassium phosphate, membrane suspensions were diluted at least 20-fold with a given buffer at the desired pH and incubated at 4 °C for at least 30 min. The suspensions were then centrifuged at 210000*g* for 2 h, the supernatants were discarded, and the pellets were resuspended in the same buffer at the same pH.

Oxygen Uptake. Rates of oxygen uptake were measured with the Clark electrode of a YSI Model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) as described previously (Barnes & Kaback, 1971).

ATPase Assays. Aliquots (2 μL) of inverted vesicles at 15 mg of protein per mL were diluted 25-fold into 50 mM potassium phosphate buffer at a given pH containing 10 mM magnesium sulfate and incubated at 25 °C. Reactions were then initiated by adding 1 μL of a stock solution of [γ-^{32}P]ATP (final concentration 5 mM, containing about 70000 cpm per reaction mixture) and terminated at a given time by addition of 1 mL of 2.5% ammonium molybdate in 1 N hydrochloric acid. Inorganic phosphate was extracted into 1.8 mL of water-saturated isobutyl alcohol–benzene (50:50 v/v; Martin & Doty, 1947), and 1-mL aliquots were counted by liquid scintillation spectrometry. Results were corrected for inorganic ^{32}P present at zero time and for nonenzymatic hydrolysis of ATP.

Transport Assays. Steady-state levels of solute accumulation were determined by flow dialysis (Ramos et al., 1976, 1979; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977). The upper chamber of the apparatus was open to the atmosphere, and both the reaction mixture (in the upper chamber) and the dialysis buffer were gassed with oxygen. The upper and lower chambers of the flow dialysis apparatus were separated by Spectropor 2 dialysis tubing (12000–14000 molecular weight cutoff; Fisher Scientific Co.), and both chambers were stirred with magnetic bars. Membrane vesicles suspended in 50 mM buffer at a given pH containing 10 mM magnesium sulfate were added to the upper chamber (total volume 0.8 mL), and electron donors, isotopically labeled solutes, and ionophores were added as indicated. The same buffer (50 mM at the same pH as the upper chamber) was pumped through the lower chamber at 6 mL/min by using a Pharmacia pump (Model P3). Fractions of 1.7 mL were collected and assayed by liquid scintillation spectrometry.

Intravesicular Volume. The intravesicular volume of inverted vesicles was determined from the difference in [^{3}H]-water and [^{14}C]inulin spaces as described by Kaback (1970), with the exception that the inverted vesicle suspensions were centrifuged at 210000*g* for 2 h. A value of 1.09 μL/mg of protein ± 10% was obtained.

Sonication. Vesicles were sonicated in a Laboratory Supplies Co. sonicator as described previously (Short et al., 1975).

Determination of ΔpH. ΔpH was determined by measuring the accumulation of [^{14}C]methylamine, [^{14}C]ethylamine, *N*-[^{14}C]methylmorpholine, or 9-aminoacridine using flow dialysis (Ramos et al., 1976, 1979; Ramos & Kaback, 1977a) as described. Concentrations of [^{14}C]methylamine, [^{14}C]-ethylamine, and *N*-[^{14}C]methylmorpholine in the dialysate were determined by liquid scintillation spectrometry, and the concentration of 9-aminoacridine was determined by fluorescence using a Perkin-Elmer MPF-4 spectrophotofluorometer (excitation 420 nm; emission 500 nm). Values for ΔpH were also determined from quenching of 9-aminoacridine fluorescence (Schuldiner et al., 1972). The reaction mixtures (1.5-mL total volume) contained 50 mM potassium phosphate at a given pH, 10 mM magnesium sulfate, 3 μM 9-aminoacridine, and a given amount of protein. Samples were incubated at 25 °C, and the reactions were initiated by addition of D-lactate to a final concentration of 20 mM.

Determination of ΔΨ. ΔΨ was determined by measuring the accumulation of [^{14}C]thiocyanate by using flow dialysis (Ramos et al., 1976, 1979; Ramos & Kaback, 1977a) as described.

Calculations. Concentration gradients for solutes taken up by vesicles were calculated from semilog plots of counts per minute or relative fluorescence against fraction number (Ramos et al., 1979) using a value of 1.09 μL of intravesicular fluid per mg of protein. Internal pH was calculated from the steady-state concentration gradients established for given weak bases, and ΔpH was calculated from the difference between internal pH and external pH (Waddel & Butler, 1959; Schuldiner et al., 1972; Rottenberg, 1979). The electrical potential (ΔΨ) was calculated from the Nernst equation ($\Delta\Psi = 58.8 \log [SCN^-]_{in}/[SCN^-]_{out}$) using steady-state concentration values obtained from flow dialysis experiments. The proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) was calculated by substituting calculated values for ΔΨ and ΔpH into eq 1. Values obtained under a given set of experimental conditions were reproducible to within 10–15%.

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

Materials

[^{14}C]Methylamine, [^{14}C]ethylamine, [^{14}C]acetate, ^{22}Na, ^{42}K, and ^{86}Rb were obtained from New England Nuclear, and [^{14}C]thiocyanate was from Amersham/Searle. *N*-[^{14}C]-Methylmorpholine was generously provided by Peter Hinkle of Cornell University, Ithaca, NY. [^{3}H]Tetraphenylphosphonium bromide was prepared by the Isotope Synthesis Group at Hoffmann-La Roche Inc. under the direction of Arnold Liebman. Carbonyl cyanide (*m*-chlorophenyl)-hydrazone (CCCP) was purchased from Calbiochem.

Results

Physical Properties of Inverted Vesicles. Although electron micrographs will not be presented, inverted vesicles prepared according to Hertzberg & Hinkle (1974) or by the slightly modified procedure described here and negatively stained with phosphotungstic acid reveal essentially identical populations of unilamellar vesicles 120–150 nm in diameter. In addition, the preparations contain some nonvesicular membrane fragments (<10% of the total membrane) that presumably represent pieces of outer membrane.

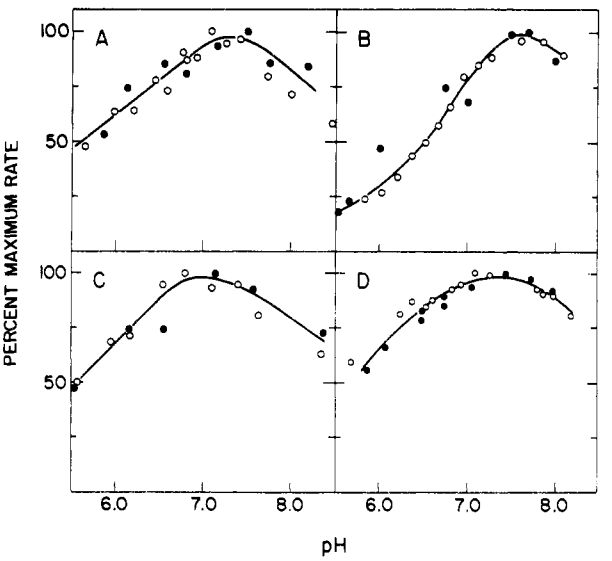

FIGURE 1: pH–rate profiles for the oxidation of (A) D-lactate, (B) succinate, (C) reduced PMS, and (D) NADH, by inverted (●) and right-side-out (○) vesicles from *E. coli* ML 308-225. Rates of oxidation were determined with a Clark electrode as described under Methods. Measurements were performed in 50 mM potassium phosphate at given pH values containing 10 mM magnesium sulfate at 25 °C. The concentrations of D-lactate, succinate, ascorbate, PMS, and NADH were 20, 20, 20, 0.0025, and 5 mM, respectively. Values given for reduced PMS oxidation were corrected for nonenzymatic oxidation of reduced PMS by subtracting control values obtained at each pH in the absence of vesicles. Corrections for nonenzymatic oxidation of other electron donors were not necessary. The maximum rates of oxidation of each electron donor (i.e., 100%) were (in nmol per mg of protein per min) as follows: D-lactate, 45 and 170; succinate, 55 and 120; reduced PMS, 175 and 400; NADH, 130 and 135 (for inverted and right-side-out vesicles, respectively).

As opposed to intact cells and vesicles prepared by osmotic lysis (Kaback, 1971; Short et al., 1975) which accumulate permeant lipophilic cations and/or weak acids (Schuldiner & Kaback, 1975; Padan et al., 1976; Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977), inverted vesicles accumulate thiocyanate and/or methylamine, ethylamine, *N*-methylmorpholine, and 9-aminoacridine (cf. below), indicating that they generate a $\Delta\bar{\mu}_{H^+}$ of opposite polarity (i.e., interior positive and acid). Importantly, moreover, under the conditions described, the preparations do not accumulate detectable quantities of [^{3}H]tetraphenylphosphonium or [^{14}C]-acetate (not shown), indicating that they are not contaminated to a significant extent with right-side-out vesicles.

Effect of pH on Substrate Oxidation and ATP Hydrolysis. The ability of inverted vesicles to catalyze the oxidation of D-lactate, succinate, NADH, and reduced phenazine methosulfate (PMS)[2] is dependent upon the pH of the reaction mixtures, and, in each instance, maximum rates of oxidation are observed between pH 7.0 and pH 8.0 (Figure 1, solid circles). However, the shapes of the pH–rate profiles clearly differ, and the absolute rates of oxidation vary with the substrate utilized. Specifically, the maximum rates of NADH and reduced PMS oxidation [130 and 175 nmol/[min (mg of

[2] The pH profile for reduced PMS oxidation by right-side-out vesicles shown in Figure 1C is significantly different from that shown in Figure 2A of Ramos & Kaback (1977a) because the PMS concentration used here was only 2.5 μM, as opposed to 100 μM.

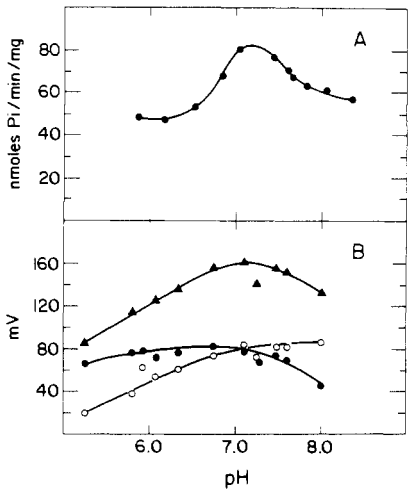

FIGURE 2: Effect of external pH on (A) the rate of ATP hydrolysis and (B) $\Delta\Psi$ (interior positive), ΔpH (interior acid), and $\Delta\bar{\mu}_{H^+}$ (interior positive and acid) generated in the presence of ATP. All experiments were performed with inverted *E. coli* ML 308-225 membrane vesicles suspended in 50 mM potassium phosphate at given pH values, 10 mM magnesium sulfate, and 5 mM ATP. Hydrolysis of ATP was measured as described under Methods with vesicles at a final concentration of 0.6 mg of protein per mL. Values for $\Delta\Psi$ (○) and ΔpH (●) were calculated from flow dialysis experiments carried out as described under Methods, using [^{14}C]thiocyanate distribution to measure $\Delta\Psi$ and [^{14}C]methylamine distribution to measure ΔpH (vesicles were used at a concentration of 8 mg of protein per mL in both cases). Values for $\Delta\bar{\mu}_{H^+}$ (▲) were computed with eq 1.

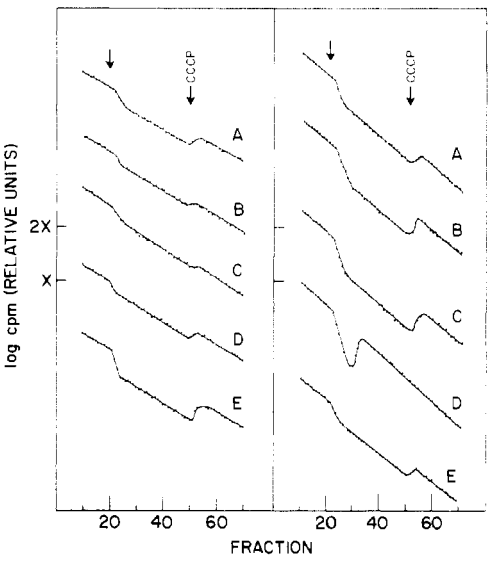

FIGURE 3: Flow dialysis measurements of methylamine (left-hand panel) and thiocyanate (right-hand panel) uptake by inverted vesicles from *E. coli* ML 308-225 in the presence of various energy sources. Assays were carried out with vesicles suspended at a final concentration of 10 mg of protein per mL in 50 mM potassium phosphate (pH 7.5 for ascorbate/PMS and ATP, pH 8.1 for succinate and NADH, and pH 6.9 for D-lactate) containing 10 mM magnesium sulfate as described under Methods. At a time indicated by the first arrow, D-lactate (A), succinate (B), ascorbate/PMS (C), NADH (D), or ATP (E) was added to concentrations of 20, 20, 20/0.2, 5, or 5 mM, respectively. At a time indicated by the second arrow, CCCP was added to a final concentration of 20 μM. In the experiments shown in the left-hand panel, [^{14}C]methylamine (50 mCi/mmol) was added to the upper chamber of the flow dialysis apparatus at a final concentration of 10 μM; in the right-hand panel, [^{14}C]thiocyanate (60 mCi/mmol) was added at 13 μM. Although the curves are displayed in decreasing order of magnitude, the level of radioactivity obtained in the initial fraction was similar (approximately 10^4 cpm).

protein)]] are twofold to fourfold greater than the rates of D-lactate and succinate oxidation [45 and 55 nmol/[min (mg of protein)]]. It is also highly significant that oxidation of these electron donors by right-side-out vesicles (Figure 1, open circles) exhibits very similar pH–rate profiles and that the rates of D-lactate, succinate, and NADH oxidation are not altered significantly when right-side-out vesicles are sonicated (data not shown).

The pH–rate profile for ATP hydrolysis by inverted vesicles exhibits an optimum at pH 7.0–7.5 (Figure 2A). Although data will not be presented, right-side-out vesicles also hydrolyze ATP over the pH range shown, and the rate is stimulated two- to fourfold when the preparations are sonicated (Short et al., 1975).

Generation of $\Delta\bar{\mu}_{H^+}$ by Inverted Membrane Vesicles and the Effect of pH. Vesicles prepared by osmotic lysis (Kaback, 1971; Short et al., 1975) exhibit the same polarity and configuration as the membrane in the intact cell (Owen & Kaback, 1978, 1979a,b) and catalyze the accumulation of permeant lipophilic cations and/or weak acids (Schuldiner & Kaback, 1975; Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977), demonstrating that they generate a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline). In contrast, vesicles prepared as described here generate a $\Delta\bar{\mu}_{H^+}$ of the opposite polarity (interior positive and acid), as shown by the observations presented in Figure 3. In these experiments, accumulation of the permeant weak base methylamine was used as a probe for ΔpH (interior acid; Figure 3, left-hand panel), and accumulation of the permeant lipophilic anion thiocyanate was used to monitor the generation of $\Delta\Psi$ (interior positive; Figure 3, right-hand panel). In each case, changes in the external concentration of the probes were monitored in the presence of given energy sources by using flow dialysis (Ramos et al., 1979). It is apparent that accumulation of both methylamine and thiocyanate is observed when D-lactate, succinate, NADH,[3] reduced PMS, or ATP is added to the vesicles and that the accumulated probe is released when the protonophore CCCP is added to the reaction mixtures. In addition, although data are not shown, control experiments demonstrate that the uptake of both methylamine and thiocyanate is directly proportional to vesicle concentration in the range 4.5–10 mg of protein per mL (i.e., ΔpH and $\Delta\Psi$ are constant over this range of protein concentrations).

When generation of $\Delta\Psi$, ΔpH, and $\Delta\bar{\mu}_{H^+}$ by inverted vesicles is measured as a function of pH with ATP (Figure 2B), D-lactate (Figure 4A), succinate (Figure 4B), reduced PMS (Figure 4C), or NADH (Figure 4D), the pH profiles for $\Delta\Psi$, ΔpH, and $\Delta\bar{\mu}_{H^+}$ exhibit considerable variation. On the other hand, the pH profile for $\Delta\bar{\mu}_{H^+}$ in the presence of each electron donor or ATP is similar to the pH–rate profile for the oxidation of the particular electron donor (compare Figures 1 and 4) or the hydrolysis of ATP (compare panels A and B in Figure 2). It should be noted, however, that the rate of oxidation is not directly proportional to the magnitude of $\Delta\bar{\mu}_{H^+}$. For example, at pH 7.5, the rate of NADH oxidation is 2.8 times faster than the rate of D-lactate oxidation, while the respective values of $\Delta\bar{\mu}_{H^+}$ are +152 and +124 mV. Lastly, unlike

[3] Thiocyanate uptake by inverted vesicles in the presence of NADH is transient and returns to the control level before CCCP is added. Although the reason for this effect is unknown, it is not due to oxygen limitation or to the generation of NAD^+ which could behave as a permeant cation in this system. Thus, the effect is observed over a range of protein concentrations, and addition of NAD^+ in the presence of D-lactate or reduced PMS does not lead to a decrease in thiocyanate uptake. In any case, in all of the experiments presented, the maximum values for thiocyanate uptake in the presence of NADH are given.

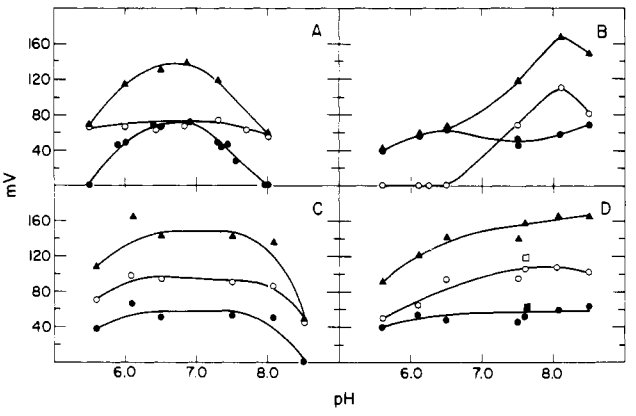

FIGURE 4: Effect of external pH on ΔpH (interior acid), ΔΨ (interior positive), and $\Delta\bar{\mu}_{H^+}$ (interior acid and positive) generated in the presence of (A) D-lactate, (B) succinate, (C) reduced PMS, and (D) NADH. All experiments were performed with inverted vesicles from *E. coli* ML 308-225 (10 mg of protein per mL) in 50 mM potassium phosphate at a given pH containing 10 mM magnesium sulfate, and D-lactate, succinate, ascorbate/PMS, or NADH was added to final concentrations of 20, 20, 20/0.2, or 5 mM, respectively. Values for ΔpH (●) and ΔΨ (○) were determined from flow dialysis experiments using [^{14}C]methylamine and [^{14}C]thiocyanate, respectively, as described in Figure 3 and under Methods. $\Delta\bar{\mu}_{H^+}$ (▲) was calculated from the values obtained for ΔpH and ΔΨ according to eq 1. Oxidation of reduced PMS (panel C) resulted in a small alkalinization of the reaction mixture (Ramos & Kaback, 1977a); the indicated pH was measured at the end of the reaction and was never more than 0.2 pH unit higher than the pH of the dialysis buffer. In panel D, (□) and (■) represent values of ΔΨ and ΔpH, respectively, measured in the presence of 5 mM NADH and 80 μM ubiquinone 1.

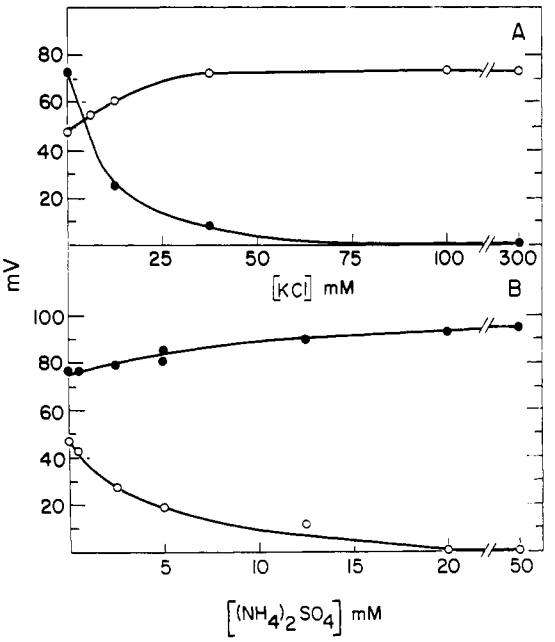

FIGURE 5: Effect of chloride (A) and ammonia (B) on ΔpH (interior acid) and ΔΨ (interior positive) in inverted vesicles from *E. coli* ML 308-225. All experiments were performed with vesicles suspended to 10 mg of protein per mL in 50 mM potassium phosphate (pH 7.3) containing 10 mM magnesium sulfate, and potassium D-lactate (20 mM final concentration) was used as the electron donor. Values for ΔpH (○) and ΔΨ (●) were determined from flow dialysis experiments using [^{14}C]methylamine and [^{14}C]thiocyanate, respectively, as described in Figure 3 and under Methods. Chloride (A) or ammonia (B) was added to given concentrations as potassium and sulfate salts, respectively.

right-side-out vesicles where NADH oxidation is coupled to the generation of $\Delta\bar{\mu}_{H^+}$ only when ubiquinone 1 (Q_1) is added to the vesicles (Stroobant & Kaback, 1975, 1979; Ramos et al., 1976), inverted vesicles generate $\Delta\bar{\mu}_{H^+}$ in the presence of NADH without added quinones. Moreover, addition of Q_1 to inverted vesicles results in only a marginally significant increase in $\Delta\bar{\mu}_{H^+}$ (Figure 4D).

Reciprocal Relationship between ΔΨ and ΔpH. With right-side-out vesicles (Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977), dissipation of ΔΨ (interior negative) at pH 5.5 by addition of valinomycin in the presence of K^+ leads to a reciprocal increase in ΔpH (interior alkaline), and, conversely, dissipation of ΔpH by nigericin leads to a reciprocal increase in ΔΨ. Moreover, these effects occur without an increase in the rate of substrate oxidation (Ramos & Kaback, 1977a). In analogy to these observations, when chloride (K^+ salt) is added to inverted vesicles over concentrations ranging from 0 to 100 mM, ΔΨ (interior positive) decreases from +73 mV to zero, and ΔpH (interior acid) increases from +47 to +74 (Figure 5A). Similarly, when the ammonia concentration of the reaction mixtures is increased progressively from 0 to 100 mM, ΔpH (interior acid) decreases from +47 mV to zero, while ΔΨ (interior positive) increases from +73 mV to +94 mV (Figure 5B). Although not shown, it is important that no change in the rate of D-lactate oxidation is observed over the range of chloride or ammonia concentrations tested.

Quantitation of ΔpH (Interior Acid). Using fluorescence quenching of 9-aminoacridine to quantitate ΔpH (Schuldiner et al., 1972), both Singh & Bragg (1976) and Schuldiner & Fishkes (1978) have reported values for ΔpH of +200 mV or more in inverted membrane vesicles from *E. coli.* Since ΔpH values obtained from methylamine distribution studies appear to be considerably lower (Figures 4 and 5), possible reasons for the discrepancy were evaluated. It is apparent that the differences are related in part to the use of high chloride concentrations by Singh & Bragg (1976) and Schuldiner & Fishkes (1978). However, it is equally clear that the ΔpH determined by methylamine accumulation does not exceed +75 mV even at 300 mM chloride (Figure 5A).

Table I: Comparison of Methods for Determination of ΔpH (Interior Acid) in Inverted *E. coli* Vesicles[a]

method of determination	probe	protein (mg/mL)	[Cl^-] (mM)	ΔpH (mV)
flow dialysis	methylamine	4.5-10	0	+47
	methylamine	10.0	300	+73
	ethylamine	10.0	0	+49
	N-methylmorpholine	10.0	0	+53
	9-aminoacridine	10.0	0	+70
	9-aminoacridine	10.0	300	+111
		4.5	0	+95
		0.5	0	+150
fluorescence quenching	9-aminoacridine	0.5	0	+150
	9-aminoacridine	0.5	300	+189
		1.5	0	+135

[a] Inverted vesicles were suspended in 50 mM potassium phosphate (pH 7.3) and 10 mM magnesium sulfate, and potassium D-lactate was added to a final concentration of 20 mM. Chloride, when present, was added as the potassium salt. ΔpH (interior acid) was measured by either flow dialysis or fluorescence quenching as described under Methods. Where indicated, [^{14}C] methylamine (50 mCi/mmol), [^{14}C]methylmorpholine (20 mCi/mmol), or 9-aminoacridine was used at final concentrations of 10, 40, 375, or 3 μM, respectively.

In order to assess this discrepancy more completely, ΔpH generated by D-lactate oxidation at pH 7.3 was investigated using methylamine, ethylamine, or *N*-methylmorpholine accumulation and quenching of 9-aminoacridine fluorescence (Table I). In addition, uptake of 9-aminoacridine was monitored directly by flow dialysis. As discussed above (cf.

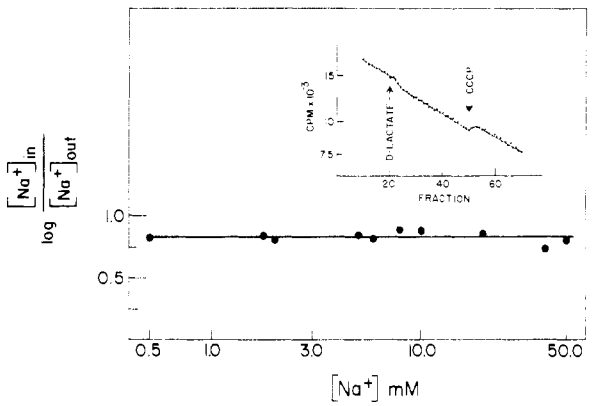

FIGURE 6: Effect of Na^+ concentration on the steady-state level of Na^+ accumulation in inverted vesicles from *E. coli* ML 308-225. In each experiment, vesicles were suspended to a final concentration of 9 mg of protein per mL in 50 mM potassium phosphate (pH 7.3) containing 10 mM magnesium sulfate, and potassium D-lactate (20 mM final concentration) was used as the electron donor. Na^+ was added to indicated concentrations as the sulfate salt with approximately 1 μCi of $^{22}Na^+$ per reaction mixture. Inset: Na^+ accumulation by inverted vesicles as described above in the presence of 10 mM Na^+. At the time indicated by the first arrow, potassium D-lactate was added to a final concentration of 20 mM; at the second arrow, CCCP was added to a final concentration of 20 μM.

Figure 5A), methylamine accumulation assayed by flow dialysis is directly proportional to protein concentration from 4.5 to 10 mg of vesicle protein per mL and yields ΔpH values of +47 and +73 mV, respectively, in the absence and presence of 300 mM chloride. Furthermore, similar ΔpH values are obtained in the absence of chloride with ethylamine and *N*-methylmorpholine. In contrast, fluorescence quenching of 9-aminoacridine at 0.5 mg of protein per mL (Schuldiner et al., 1972) yields ΔpH values of +150 mV in the absence of chloride and +189 mV in 300 mM chloride. However, when ΔpH is determined with 9-aminoacridine using either fluorescence quenching or flow dialysis, the magnitude of ΔpH in the absence of chloride decreases from +150 mV at 0.5 mg of protein per mL to +75 mV at 10 mg of protein per mL (a value of +95 mV is obtained at 4.5 mg of protein per mL). Clearly, therefore, there is a serious quantitative discrepancy between ΔpH values obtained with methylamine, ethylamine, or *N*-methylmorpholine and those obtained with 9-aminoacridine regardless of the assay method employed. In addition, the observation that ΔpH as determined with 9-aminoacridine is not constant over any range of vesicle concentrations tested suggests that the probe cannot be used to quantitate ΔpH in this system.

Accumulation of Na^+ by Inverted Vesicles. Since right-side-out vesicles catalyze energy-dependent Na^+ extrusion (Lombardi et al., 1973; Lanyi et al., 1976; Lanyi & MacDonald, 1976; Eisenbach et al., 1977; Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978), it follows that inverted vesicles should catalyze Na^+ accumulation in a manner similar to that observed for Ca^{2+} (Rosen & McClees, 1974; Tsuchiya & Rosen, 1975, 1976; Beck & Rosen, 1979; Reenstra et al., 1979). As shown in the inset of Figure 6, when D-lactate is added to inverted vesicles in the presence of $^{22}Na^+$, the concentration of the cation in the external medium decreases, and the effect is completely reversed by addition of CCCP. Moreover, as demonstrated in the body of the figure, the magnitude of the Na^+ gradient established under these conditions is independent of Na^+ concentration from 0.5 to 50 mM. In addition, the steady-state level of Na^+ accumulation is independent of external pH from values of 5.7–8.0 (Table II).

Table II: Cation Gradients and ΔpH (Interior Acid) in Inverted Vesicles[a]

cation	external pH	buffer	$\frac{[\text{cation}]_{in}}{[\text{cation}]_{out}}$	ΔpH (mV) (+) cation	ΔpH (mV) (−) cation
Na^+	5.7	potassium phosphate	58	+43	+41
Na^+	6.4	potassium phosphate	57	+65	+62
Na^+	7.0	potassium phosphate	54	+66	+69
Na^+	7.3	potassium phosphate	58	+49	+47
Na^+	8.0	potassium phosphate	59	ND[b]	0
Na^+	7.3	choline phosphate	47	+49	+47
K^+	7.3	choline phosphate	0	+47	+47
Rb^+	7.3	choline phosphate	0	+46	+47

[a] Cation gradients and ΔpH (methylamine accumulation) were measured by flow dialysis as described in Figures 3 and 6 and under Methods with vesicles suspended to a final concentration of 10 mg of protein per mL in 50 mM potassium or choline phosphate at a given pH and 10 mM magnesium sulfate. Potassium D-lactate (20 mM final concentration) was used as the electron donor except in experiments where $^{42}K^+$ or $^{86}Rb^+$ accumulation was tested. In these instances, the choline salt of D-lactate was used. For experiments in potassium phosphate, the sulfate salt of Na^+ was added to a final concentration of 10 mM; for experiments in choline phosphate, the sulfate salts of Na^+, K^+, or Rb^+ were added to final concentrations of 25 or 50 mM. In determinations of radioactive cation accumulation, sulfate salts of $^{22}Na^+$ (6.2 mCi/mmol), $^{42}K^+$ (24 mCi/mmol), or $^{86}Rb^+$ (0.48 mCi/mmol) were used at the concentrations given above. [b] ND, not determined.

Studies with right-side-out vesicles suggest that Na^+ extrusion is driven by $\Delta\bar{\mu}_{H^+}$ (Lanyi et al., 1976; Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978), and recent work with inverted vesicles describes partial reversal of 9-aminoacridine (Schuldiner & Fishkes, 1978) and quinacrine (Brey et al., 1978) fluorescence quenching on addition of Na^+. Since both probes respond to ΔpH (interior acid), these effects of Na^+ have been taken to imply that Na^+/H^+ antiport plays a specific role in regulating internal pH in intact cells and right-side-out vesicles. However, these experiments (Schuldiner & Fishkes, 1978; Brey et al., 1978) were carried out in the presence of chloride, thus obscuring a potential contribution by $\Delta\Psi$. Given these and other considerations (cf. Table I), we studied the effect of Na^+ on ΔpH (interior acid) using methylamine rather than 9-aminoacridine or quinacrine, and the experiments were performed in the absence of chloride so that $\Delta\Psi$ (interior positive) was maintained (Table II). When 10 mM Na^+ is added to the vesicles under these conditions, ΔpH (interior acid) remains constant from pH 5.7 to 7.3. Furthermore, the absence of an effect of Na^+ on ΔpH cannot be due to a competitive effect of the K^+ present in the medium, since the same results are obtained when Na^+ at concentrations up to 50 mM is added to vesicles suspended in choline phosphate. The effect of Na^+ on ΔpH was studied by comparing the pH gradients formed by vesicles in the absence and presence of Na^+ and also by adding Na^+ to vesicles after ΔpH was established during flow dialysis. In both cases, no change in ΔpH was observed so long as $\Delta\Psi$ was not dissipated. Finally, it is apparent that no detectable accumulation of either $^{42}K^+$ or $^{86}Rb^+$ occurs under these conditions, suggesting that the Na^+/H^+ antiporter is relatively specific for Na^+. It is also noteworthy that $^{86}Rb^+$ accumulation is not observed when the

Table III: Coupling of the Na^+ Gradient to Components of $\Delta\bar{\mu}_{H^+}$[a]

additions	$\Delta\mu_{Na^+}$ (mV)	ΔpH (mV)	ΔΨ (mV)
none	45	+47	+72
100 mM potassium chloride	48	+74	0
20 mM ammonium sulfate	31	0	+94

[a] Na^+ gradients, ΔpH (methylamine accumulation), and ΔΨ (thiocyanate accumulation) were measured by flow dialysis as described under Methods and in Figures 3 and 6 with 20 mM potassium D-lactate as the electron donor. Inverted vesicles were suspended in 50 mM potassium phosphate (pH 7.3) and 10 mM magnesium sulfate at a final concentration of 12 mg of protein per mL, and ^{22}Na (6.2 mCi/mmol) (sulfate salt) was used at 10 mM (final concentration).

vesicles are suspended in choline phosphate containing 100 mM choline chloride (i.e., under conditions where ΔΨ is dissipated and ΔpH is enhanced) and when ATP hydrolysis is used to generate $\Delta\bar{\mu}_{H^+}$ (data not shown).

Direct support for the contention that Na^+ accumulation can be driven by either ΔpH (interior acid) or ΔΨ (interior positive) is presented in Table III. In these experiments, steady-state levels of Na^+ accumulation were determined in the presence of D-lactate under control conditions and under conditions where ΔΨ or ΔpH was dissipated by addition of chloride or ammonia, respectively. Clearly, although Na^+ accumulation is not observed when $\Delta\bar{\mu}_{H^+}$ is abolished (i.e., in the presence of CCCP; Figure 6 inset), $\Delta\mu_{Na^+}$ remains when either ΔΨ or ΔpH is dissipated selectively. Similar data were obtained by using ATP (K^+ salt) (data not shown).

Discussion

Physical Properties of Inverted Vesicles. Although the conclusion may be surprising, it is apparent from some of the studies described here that passage of cells through a French pressure cell under conditions described initially by Hertzberg & Hinkle (1974) yields a preparation of small inverted vesicles that is not contaminated to a significant extent with right-side-out vesicles. A possible explanation for the uniformity of the preparation may be related to the size of the inverted vesicles which is at least 10-fold smaller in diameter than that of right-side-out vesicles prepared by osmotic lysis (Kaback, 1971; Short et al., 1975). Since the great majority of the membrane-bound proteins in the *E. coli* membrane are embedded in the cytoplasmic surface (Owen & Kaback, 1978, 1979a,b) an inverted configuration may be favored thermodynamically in very small vesicles. That is, when the membrane is sheared to sufficiently small pieces, the membrane may reseal in such a manner that the surface on which the proteins are most densely packed faces the exterior. In other words, in very small membrane vesicles, the difference in radius of curvature between the inner and outer leaflets of the membrane is significant and may cause resealing in an inverted conformation because the outer surface which has the larger radius of curvature will accommodate a higher density of proteins.

$\Delta\bar{\mu}_{H^+}$ in Inverted Vesicles. Results presented in this paper demonstrate clearly that inverted vesicles generate a $\Delta\bar{\mu}_{H^+}$ (interior positive and acid) that is of opposite polarity but comparable magnitude to that generated by right-side-out vesicles (Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977). With D-lactate or reduced PMS as electron donors, inverted vesicles exhibit maximum ΔΨ values that compare favorably with those generated by right-side-out vesicles in the presence of the same electron donors. In addition, the ΔΨ generated in both preparations is relatively

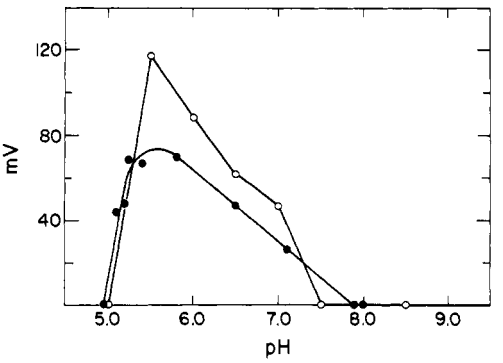

FIGURE 7: Effect of pH on the periplasmic side of the membrane on ΔpH generated by right-side-out (○) and inverted (●) vesicles prepared from *E. coli* ML 308-225. Data for right-side-out vesicles are from Figure 2B or Ramos & Kaback (1977a); data for inverted vesicles are from the experiments shown in Figure 4A with ΔpH (calculated from [^{14}C]methylamine distribution) plotted as a function of internal pH.

independent of pH. Values for ΔpH in inverted and right-side-out vesicles are also comparable, but the data cannot be compared directly since, of necessity, the ΔpH generated in the respective systems is studied as a function of pH on opposite sides of the membrane. Direct comparison can be made, however, when the ΔpH generated by inverted vesicles in the presence of D-lactate (Figure 3A) is replotted as a function of internal pH (cf. Figure 7, solid symbols). When the data are presented in this manner and compared to those of Ramos & Kaback (1977a) with right-side-out vesicles (Figure 7, open symbols), the magnitude of ΔpH and the shape of the pH profiles are reasonably similar.

The components of $\Delta\bar{\mu}_{H^+}$ in inverted vesicles can be altered reciprocally (Figure 5) with no change in respiration, as shown previously with right-side-out vesicles (Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977). These phenomena can be rationalized by considering the interrelationship between the components of $\Delta\bar{\mu}_{H^+}$. At steady state, under conditions where both ΔΨ and ΔpH are present across the membrane, the magnitude of $\Delta\bar{\mu}_{H^+}$ is determined by the efficiency of the proton pump and the back-leak of protons through the membrane, and each parameter of $\Delta\bar{\mu}_{H^+}$ limits the magnitude of the other. With right-side-out vesicles, for instance, ΔΨ (interior negative) will tend to pull protons toward the interior of the vesicles and thus act to limit the pH gradient. Similarly, ΔpH (interior alkaline) will act to limit ΔΨ (interior negative) because protons will tend to diffuse into the vesicles down their concentration gradient, thus decreasing the net extrusion of positive charge and decreasing ΔΨ. Therefore, by dissipation of either ΔΨ or ΔpH, a force that is limiting for the other parameter is removed, allowing it to increase without a corresponding increase in the rate of proton pumping. Clearly, the same explanation in reverse can be applied to inverted vesicles.

Quantitation of ΔpH (Interior Acid). Although some systems exhibit excellent correlation between fluorescence quenching of 9-aminoacridine and distribution of weak bases (Deamer et al., 1972; Schuldiner et al., 1972; Casadio & Melandri, 1977), the former technique appears to overestimate ΔpH in certain instances (Michels & Konings, 1978; Elema et al., 1978). It is apparent from the data presented here that inverted *E. coli* vesicles fall into the latter category. Although the precise reason for the discrepancies is unknown, in a more extensive study of the problem with chromatophores from *Rhodopseudomonas spheroides*, Elema et al. (1978) have suggested that 9-aminoacridine, in addition to accumulating

in the intravesicular space, may bind to the "energized" membrane. While fluorescence quenching of 9-aminoacridine is a convenient, diagnostic probe for the presence of ΔpH (interior acid), caution should be exercised in interpreting the results quantitatively in this system.

In this context, it should be noted that Brey et al. (1978) and Brey & Rosen (1979) claimed to demonstrate K^+/H^+ antiport in inverted *E. coli* membrane vesicles based on the observation that addition of K^+ to the preparation leads to a decrease in fluorescence quenching of quinacrine. Moreover, using a similar experimental approach, these workers have drawn conclusions regarding the stoichiometry of various antiport mechanisms. In view of the problems inherent in the interpretation of fluorescence quenching data and preliminary observations indicating that inverted vesicles accumulate neither $^{42}K^+$ nor $^{86}Rb^+$ (Table II), the purported demonstration of K^+/H^+ antiport in these preparations should be questioned. Furthermore, it is obvious that stoichiometry estimates based entirely on fluorescence quenching data are highly tenuous at best.

H^+/Na^+ Antiport. Evidence consistent with the presence of a H^+/Na^+ exchange system in *E. coli* was first presented by West & Mitchell (1974), who suggested an electrically neutral antiport mechanism and thus implied that the process is coupled to ΔpH specifically. With the demonstration that intact cells (Padan et al., 1976) and right-side-out membrane vesicles (Ramos et al., 1976; Ramos & Kaback, 1977a; Tokuda & Kaback, 1977) generate a large ΔpH (interior alkaline) at pH 5.5 that decreases with increasing external pH and approaches zero at pH 7.5, it was suggested that a H^+/Na^+ antiporter, by operating at relatively alkaline pH, might account for this behavior (Padan et al., 1976; Ramos & Kaback, 1977a; Schuldiner & Fishkes, 1978). Subsequently, however, it was demonstrated that right-side-out vesicles catalyze Na^+ extrusion equally well at pH 6.6 and 7.5 (Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978). In addition, other evidence was presented (Schuldiner & Fishkes, 1978) indicating that H^+/Na^+ antiport is electrically neutral at pH 6.6 (i.e., coupled to ΔpH) but electrogenic at pH 7.5 (i.e., coupled to $\Delta\Psi$), as demonstrated previously for the transport of a number of organic acids (Ramos & Kaback, 1977b,c). It should also be emphasized that Lanyi & MacDonald (1976) have presented evidence for electrogenic H^+/Na^+ antiport in right-side-out vesicles from *Halobacterium halobium*.

In analogy to right-side-out vesicles, inverted vesicles catalyze Na^+ accumulation when a $\Delta\bar{\mu}_{H^+}$ (interior positive and acid) is present across the membrane. However, the properties of the system make it difficult to conclude that H^+/Na^+ antiport, in itself, can account for the absence of ΔpH (interior alkaline) at pH 7.5 in intact cells and right-side-out vesicles. Thus, the concentration gradient of Na^+ established by inverted vesicles is constant with pH from pH 5.7 to 8.0 (cf. Table II), Na^+ accumulation by inverted vesicles can be driven by either ΔpH (interior acid) or $\Delta\Psi$ (interior positive) (cf. Table III), and, most importantly, Na^+ accumulation does not lead to dissipation of ΔpH (interior acid) over the pH range studied (cf. Table II). Finally, since ΔpH is relatively high at alkaline pH in inverted vesicles, it is difficult to envisage how H^+/Na^+ antiport, by functioning at alkaline pH specifically, can be responsible for the collapse of ΔpH in intact cells and right-side-out vesicles.

Acknowledgments

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References

Altendorf, K. H., & Staehelin, L. A. (1974) *J. Bacteriol. 117*, 888.
Barnes, E. M., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5518.
Beck, J. C., & Rosen, B. P. (1979) *Arch. Biochem. Biophys. 194*, 208.
Boonstra, J., & Konings, W. N. (1977) *Eur. J. Biochem. 78*, 361.
Brey, R. N., & Rosen, B. P. (1979) *J. Biol. Chem. 254*, 1957.
Brey, R. N., Beck, J. C., & Rosen, B. P. (1978) *Biochem. Biophys. Res. Commun. 83*, 1588.
Casadio, R., & Melandri, B. A. (1977) *J. Bioenerg. 9*, 17.
Davis, G. D., & Mingioli, E. S. (1950) *J. Bacteriol. 60*, 17.
Deamer, D. W., Prince, R. C., & Crofts, A. R. (1972) *Biochim. Biophys. Acta 274*, 323.
Eisenbach, M., Cooper, S., Garty, H., Johnstone, R. M., Rottenberg, H., & Caplan, S. R. (1977) *Biochim. Biophys. Acta 465*, 599.
Elema, R. P., Michels, P. A. M., & Konings, W. N. (1978) *Eur. J. Biochem. 92*, 381.
Felle, H., Stetson, D. L., Long, W. S., & Slayman, C. L. (1978) in *Frontiers of Biological Energetics* (Dutton, P. L., Leigh, J. J., & Scarpa, A., Eds.) Vol. II, p 1399, Academic Press, New York.
Futai, M. (1974) *J. Membr. Biol. 15*, 15.
Greville, G. D. (1971) *Curr. Top. Bioenerg. 3*, 1.
Hamilton, W. A. (1975) *Adv. Microb. Physiol. 12*, 1.
Harold, F. M. (1972) *Bacteriol. Rev. 36*, 172.
Harold, F. M. (1976) *Curr. Top. Bioenerg. 6*, 83.
Hertzberg, E., & Hinkle, P. (1974) *Biochem. Biophys. Res. Commun. 53*, 178.
Kaback, H. R. (1970) *Curr. Top. Membr. Transp. 1*, 35.
Kaback, H. R. (1971) *Methods Enzymol. 22*, 99.
Kaback, H. R. (1976) *J. Cell. Physiol. 89*, 575.
Lancaster, J., & Hinkle, P. (1977) *J. Biol. Chem. 252*, 7657.
Lanyi, J. K., & MacDonald, R. E. (1976) *Biochemistry 15*, 4608.
Lanyi, J. K., Renthal, R., & MacDonald, R. E. (1976) *Biochemistry 15*, 1603.
Lichtshtein, D., Kaback, H. R., & Blume, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 650.
Lombardi, F. J., Reeves, J. P., & Kaback, H. R. (1973) *J. Biol. Chem. 248*, 3551.
Lowry, O. H., Rosebrough, N. J., Farr, A. J., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Martin, J. B., & Doty, D. M. (1947) *Anal. Chem. 21*, 965.
Michels, P. A. M., & Konings, W. N. (1978) *Eur. J. Biochem. 85*, 147.
Mitchell, P. (1961) *Nature (London) 191*, 144.
Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc. 41*, 445.
Mitchell, P. (1968) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Ltd., Bodmin, England.
Mitchell, P. (1973) *J. Bioenerg. 4*, 63.
Mitchell, P. (1976) *Biochem. Soc. Trans. 4*, 399.
Navon, G., Ogawa, S., Shulman, R. B., & Yamane, T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 888.
Ogawa, S., Shulman, R. B., Glynn, P., Yamane, T., & Navon, G. (1978) *Biochim. Biophys. Acta 502*, 45.
Owen, P., & Kaback, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3148.
Owen, P., & Kaback, H. R. (1979a) *Biochemistry 1*, 1413.
Owen, P., & Kaback, H. R. (1979b) *Biochemistry 18*, 1423.

Padan, E., Zilberstein, D., & Rottenberg, H. (1976) *Eur. J. Biochem. 63*, 533.
Porter, J. S., Slayman, C. L., Kaback, H. R., & Felle, H. (1979) *Proc. Annu. Meet. Am. Soc. Microb., 79th*, 175 (Abstr. K2).
Ramos, S., & Kaback, H. R. (1977a) *Biochemistry 16*, 848.
Ramos, S., & Kaback, H. R. (1977b) *Biochemistry 16*, 854.
Ramos, S., & Kaback, H. R. (1977c) *Biochemistry 16*, 4271.
Ramos, S., Schuldiner, S., & Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1892.
Ramos, S., Schuldiner, S., & Kaback, H. R. (1979) *Methods Enzymol. 55*, 680.
Reenstra, W. W., Patel, L., & Rottenberg, H. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 248.
Rosen, B. P., & McClees, J. S. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5042.
Rottenberg, H. (1979) *Methods Enzymol. 55*, 547.
Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14*, 5451.
Schuldiner, S., & Fishkes, H. (1978) *Biochemistry 17*, 706.
Schuldiner, S., Rottenberg, H., & Avron, M. (1972) *Eur. J. Biochem. 25*, 64.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1975) *J. Biol. Chem. 250*, 4291.
Singh, A. P., & Bragg, P. D. (1976) *Eur. J. Biochem. 67*, 177.
Singh, A. P., & Bragg, P. D. (1979) *Arch. Biochem. Biophys. 195*, 74.
Stroobant, P., & Kaback, H. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3970.
Stroobant, P., & Kaback, H. R. (1979) *Biochemistry 18*, 226.
Tokuda, H., & Kaback, H. R. (1977) *Biochemistry 16*, 2130.
Tsuchiya, T., & Rosen, B. (1975) *J. Biol. Chem. 250*, 7687.
Tsuchiya, T., & Rosen, B. (1976) *J. Biol. Chem. 251*, 962.
Waddel, W. J., & Butler, T. C. (1959) *J. Clin. Invest. 38*, 720.
West, I., & Mitchell, P. (1974) *Biochem. J. 144*, 87.
Zilberstein, D., Schuldiner, S., & Padan, E. (1979) *Biochemistry 18*, 669.

Stoichiometry and Stereochemistry of Deuterium Incorporated into Fatty Acids by Cells of *Escherichia coli* Grown on [*methyl*-$^2H_3$]Acetate†

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ABSTRACT: The incorporation of deuterium into the C_{12}, C_{14}, and C_{16} saturated fatty acids biosynthesized by *Escherichia coli* grown in a medium containing [*methyl*-$^2H_3$]acetate was studied. The incorporation of deuterium into these saturated fatty acids from three distinct populations of deuterium offers the best explanation for the observed isotopic distribution. The most enriched of these populations consisted of the terminal methyl group in which 80% of each fatty acid contained a methyl group with three deuteriums. This deuterium population would be derived directly from the acetate methyl group with no isotope exchange. The second population (45% deuterated) consisted of one labeled hydrogen for each even carbon. This second population was derived from acetate via malonyl-CoA. The third population (19% deuterated) consisted of one labeled hydrogen for each odd carbon and was derived indirectly from acetate via NADPH. Desaturation of the deuterated C_{16} saturated fatty acid using the *Corynebacterium diphtheriae* desaturation system produced palmitoleic acid which lost hydrogen equivalent to one proton containing ~22% deuterium. Chemical and mass spectrometric analyses of derivatives of this palmitoleic acid clearly showed that the NADPH-derived hydrogen on C_9 was lost in the desaturation and that the acetate-derived hydrogen on C_{10} was retained. Thus, the hydrogen incorporated from acetate at C_{10} has an *L* configuration and that derived from NADPH at C_9 has a *D* configuration.

Information on the origins and stereochemistries of the hydrogen atoms that are incorporated into fatty acids during their biosynthesis has been limited. Experimental evidence has shown that hydrogens from the following sources are incorporated into fatty acids to varying degrees: methyl hydrogen atoms of acetyl-CoA (Bressler & Wakil, 1961; D'Adamo et al., 1961; Foster & Bloom, 1962), methylene hydrogens of malonyl-CoA (Bressler & Wakil, 1961; Arnstadt et al., 1975), methylene hydrogens of malonyl-ACP (Arnstadt et al., 1975), hydride ions from NADPH (Foster & Bloom, 1961; Seyama et al., 1977a,b, 1978), and the protons from water (Schoenheimer & Rittenberg, 1936; Jungas, 1968; Patton & Lowenstein, 1979). In only a few of these studies has the stoichiometry of the incorporated label at specific carbons been determined. These include the work of Jungas (1968), who determined, using adipose tissue incubated in tritiated water, that 0.96 atom and 0.71 atom of tritium/carbon atoms are incorporated on the even and odd carbons of the biosynthesized fatty acids, respectively. Particularly noteworthy is the work of Seyama et al. (1978), who have shown, using purified fatty acid synthetases, the position of deuterium incorporation into fatty acids from both deuterated water and stereospecifically deuterated pyridine nucleotides. Although these works establish the positions of the hydrogens that are incorporated into fatty acids from water and from pyridine nucleotides, they say little about the efficiencies of these incorporations and nothing about the stereochemistries of the incorporated hydrogens.

In this paper I describe the chemical, mathematical, and biosynthetic approaches which have been used to establish both the stereochemistry and stoichiometry of hydrogens incorporated in vivo into fatty acids. In the paper which follows

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