It is important to remark that at equilibrium the population of any state of A is independent of the path molecules A take. For example, if the in plane reaction is forbidden, it does not change any of our results. Only the kinetics would be modified.

Added in Proof

An equation similar to eq A1 has been proposed recently by Parry et al. (1976).

References

Brisson, A. D. (1975), Thèse de 3ème cycle, Université Paris VII.

Brisson, A. D., Devaux, P. F., and Changeux J. P. (1975b), *C. R. Hebd. Seances Acad. Sci., Ser. C 280,* 2153-3156.

Brisson, A. D., Scandella, C. J., Bienvenüe, A., Devaux, P. F., Cohen, J., and Changeux, J. P. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1087-1091.

Butler, K. W., Tattrie, N. H., and Smith, I. C. P. (1974), *Biochim. Biophys. Acta 363,* 351-360.

Cartaud, J., Benedetti, E. L., Cohen, J. B., Meunier, J. C., and Changeux, J. P. (1973), *FEBS Lett. 33,* 109-113.

Cohen, J. B., Weber, M., Huchet, M., and Changeux, J. P. (1972), *FEBS Lett. 26,* 43-47.

Devaux, P. F., Bienvenüe, A., Lauquin, G., Brisson, A. D., Vignais, P. M., and Vignais, P. V. (1975a), *Biochemistry 14,* 1272-1280.

Devaux, P. F., Bienvenüe, A., Lauquin, G., Colbeau, A., Zachowski, A., Brisson, A. D., Changeux, J. P., Vignais, P. M., and Vignais, P. V. (1975b), *Biol. Membr. Neurochem. 41,* 17-24.

Duguid, J. R., and Raftery, M. A. (1973), *Biochemistry 12,* 3593-3597.

Dupont, Y., Cohen, J. B., and Changeux, J. P. (1974), *FEBS Lett. 40,* 130-133.

Eldefrawi, M. E., Britten, A. G., and O'Brien, R. D. (1971), *Pestic. Biochem. Physiol. 1,* 101-108.

Griffith, O. H., Jost, P. C. (1976), in Spin Labeling, Berliner, L. J., Ed., New York, N.Y., Academic Press.

Karlin, A. (1969), *J. Gen. Physiol. 54,* 245-264.

Lowry, O. H., Rosebrough, N. H., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265-275.

Nickel, E., and Potter, L. T. (1973), *Brain Res. 57,* 508-517.

Parry, G., et al. (1976); *FEBS Lett. 67,* 123-129.

Rouser, G., Fleisher, S., and Yamamoto, A. (1969), *Lipids 5,* 494-496.

Seelig, J. (1976), Spin Labeling, Berliner, L., Ed., New York, N.Y., Academic Press, p 403.

Vignais, P. M., Devaux, P. F., Colbeau, A. (1975), in Biomembranes: Lipids, Proteins and Receptors, Barton, R. M., and Packer, L., Ed., Webster Grove, Miss., BI Science.

Weber, M., and Changeux, J. P. (1974), *Mol. Pharmacol. 10,* 1-14; 15-34.

The Electrochemical Proton Gradient in *Escherichia coli* Membrane Vesicles†

Sofia Ramos*,‡ and H. Ronald Kaback

ABSTRACT: Membrane vesicles isolated from *Escherichia coli* grown under various conditions generate a transmembrane pH gradient (ΔpH) of about 2 pH units (interior alkaline) under appropriate conditions when assayed by flow dialysis. Using the distribution of weak acids to measure ΔpH and the distribution of the lipophilic cation triphenylmethylphosphonium to measure the electrical potential ($\Delta\Psi$) across the membrane, the vesicles are demonstrated to develop an electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) of almost -200 mV (interior negative and alkaline) at pH 5.5 in the presence of reduced phenazine methosulfate or D-lactate, the major component of which is a ΔpH of about -120 mV. As external pH is increased, ΔpH decreases, reaching 0 at about pH 7.5 and above, while $\Delta\Psi$ remains at about -75 mV and internal pH remains at pH 7.5-7.8. The variations in ΔpH correlate with changes in the oxidation of reduced phenazine methosulfate or D-lactate, both of which vary with external pH in a manner similar to that described for ΔpH. Finally, ΔpH and $\Delta\Psi$ can be varied reciprocally in the presence of valinomycin and nigericin with little change in $\Delta\bar{\mu}_{H^+}$ and no change in respiratory activity. These data and those presented in the following paper (Ramos and Kaback, 1976) provide strong support for the role of chemiosmotic phenomena in active transport and extend certain aspects of the chemiosmotic hypothesis.

Membrane vesicles isolated from *Escherichia coli* retain the same polarity as the membrane in the intact cell and catalyze active transport of various solutes by a respiration-dependent mechanism that does not involve the generation or utilization of ATP or other high-energy phosphate intermediates (Kaback, 1972, 1973, 1974b; Kaback and Hong, 1973; Stroobant and Kaback, 1975). Although the precise means by which energy released from the oxidation of D-lactate or reduced phenazine methosulfate (PMS)[1] is coupled to transport in this system are unknown, an increasing accumulation of evidence indicates that chemiosmotic phenomena play a central role in the process (Hirata et al., 1973; Altendorf et al., 1975; Kaback, 1974b; Schuldiner and Kaback, 1975; Patel et al., 1975). As visualized by the Mitchell hypothesis (Mitchell,

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, the electrochemical gradient of protons; ΔpH, the proton gradient across the membrane; $\Delta\Psi$, the electrical potential across the membrane; PMS, phenazine methosulfate; DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium (bromide salt); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; glucose-6-P, glucose 6-phosphate; Tris, tris(hydroxymethyl)aminomethane.

1961, 1966, 1973, 1976; Greville, 1971; Harold, 1972; Hamilton, 1975), oxidation of electron donors is accompanied by the expulsion of protons into the external medium, leading to an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) which is composed of an electrical and a chemical parameter according to the following relationship:

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

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 room temperature). Moreover, according to this hypothesis, it is the electrochemical gradient of protons or one of its components which is postulated to be the immediate driving force for the inward movement of transport substrates.

Using lipophilic cations and rubidium (in the presence of valinomycin), it has been demonstrated that *E. coli* membrane vesicles generate a $\Delta\Psi$ (interior negative) of approximately −75 mV in the presence of reduced PMS or D-lactate (Hirata et al., 1973; Altendorf et al., 1975; Schuldiner and Kaback, 1975). Furthermore, it has been shown that the potential causes the appearance of high affinity binding sites for dansyl and azidophenyl galactosides on the surface of the vesicle membrane (Reeves et al., 1973; Kaback, 1974b; Schuldiner et al., 1975a–c, 1976a,b; Rudnick et al., 1975a,b), and that the potential is partially dissipated as a result of lactose accumulation (Schuldiner and Kaback, 1975). Although these findings support the chemiosmotic concept, it is apparent that $\Delta\Psi$ in itself is insufficient to account for the magnitude of solute accumulation by the vesicles if it is assumed that the stoichiometry between protons and solute is 1:1 (Schuldiner and Kaback, 1975). This deficiency, in addition to the apparent absence of a transmembrane pH gradient, left reasonable doubt as to the quantitative relationship between $\Delta\bar{\mu}_{H^+}$ and solute accumulation. Recent experiments (Ramos et al., 1976) demonstrate, however, that *E. coli* vesicles generate a large ΔpH under appropriate conditions, and that this component of $\Delta\bar{\mu}_{H^+}$ (but not $\Delta\Psi$) varies dramatically with external pH. With this demonstration, it became apparent that, under certain conditions, $\Delta\bar{\mu}_{H^+}$ is sufficient thermodynamically to account for the magnitude of solute accumulation by the vesicles. Furthermore, it was shown that $\Delta\Psi$ and ΔpH can be varied reciprocally through the use of appropriate ionophores, thus providing a framework within which to test other more specific aspects of the chemiosmotic hypothesis.

The observations presented in this and the following paper (Ramos and Kaback, 1977) provide almost unequivocal evidence for the primary role of chemiosmotic phenomena in the process of active transport.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium A (Davis and Mingioli, 1950) containing 1.0% disodium succinate (hexahydrate) or 1.0% sodium glucuronate as indicated, *E. coli* ML 30 ($i^+z^+y^+a^+$) on minimal medium A containing 1.0% sodium gluconate, and *E. coli* GN-2 ($i^-z^+y^+a^+$; enzyme I^-) on medium 63 (Cohen and Rickenberg, 1956) containing 0.2% disodium glucose-6-P. Membrane vesicles were prepared from these cells as described previously (Kaback, 1971; Short et al., 1975), except that lysozyme and sucrose were used at 50 μg/mL and 30%, respectively, for the preparation of spheroplasts from *E. coli* GN-2. Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6) and stored in liquid nitrogen.

For studies at various pH's and with buffers other than potassium phosphate, membrane suspensions containing about 4 mg of protein per mL were thawed rapidly at 46 °C, diluted at least tenfold with 0.1 M buffer at the desired pH, and incubated for 10 min at 25 °C. The suspension was centrifuged at 40 000*g* for 30 min and the pellet resuspended and washed once in a similar volume of the same buffer. The final pellet was then resuspended to an appropriate protein concentration in 0.1 M buffer at the same pH.

Transport Assays. Filtration assays (Kaback, 1974a; Schuldiner and Kaback, 1975) were carried out using Millipore Cellotate filters (0.5-μm pore size). Electron donors and isotopically labeled solutes were used as indicated. Flow dialysis was performed as described (Ramos et al., 1976) with the upper chamber of the apparatus open to the atmosphere, and the reaction mixtures were gassed with oxygen. The upper and lower chambers were separated by Spectropor 1 dialysis tubing (6000–8000 molecular weight cut-off; Fisher Scientific), and both chambers were stirred with magnetic bars. Membrane vesicles suspended in 0.05 M buffer at a given pH containing 0.01 M magnesium sulfate (unless stated otherwise) were added to the upper chamber (total volume 0.8 mL), and electron donors, isotopically labeled solutes, and ionophores were used as indicated. The same buffer (0.05 M at the same pH as the upper chamber) was pumped from the lower chamber at a rate of 6.0 mL per min using a Pharmacia pump (Model P3). Fractions of about 1.7 mL were collected and assayed for radioactivity by liquid scintillation spectrometry.

Determination of ΔpH. ΔpH was determined by measuring the accumulation of acetate, propionate, or 5,5-dimethyloxazolidine-2,4-dione (DMO) using flow dialysis (Ramos et al., 1976). Data were quantitated assuming that dialysis rates obtained after addition of nigericin represent 0 ΔpH.

Determination of $\Delta\Psi$. $\Delta\Psi$ was determined by measuring the accumulation of [^{3}H]triphenylmethylphosphonium ($TPMP^+$) using filtration (Schuldiner and Kaback, 1975) or flow dialysis (Ramos et al., 1976). As shown previously (Ramos et al., 1976), similar results are obtained with both techniques.

Calculations. Concentration gradients for solutes taken up by the vesicles were calculated using a value of 2.2 μL of ultravesicular volume per mg of membrane protein (Kaback and Barnes, 1971). Internal pH was determined by difference. In this regard, it is important that the pH of the external medium becomes significantly alkaline during oxidation of ascorbate–PMS (Figure 1B), and that this alteration in pH has been taken into account in the calculations. Similar considerations are not necessary with D-lactate, as no change in external pH is observed with this electron donor (Figure 1B). The electrical potential ($\Delta\Psi$) was calculated from the Nernst equation ($\Delta\Psi = 58.8 \log [TPMP^+]_{in}/[TPMP^+]_{out}$) using steady-state concentration values obtained from $TPMP^+$ uptake experiments. The proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) was calculated by substituting values for $\Delta\Psi$ and ΔpH into eq 1.

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, Ohio) as described previously (Barnes and Kaback, 1971).

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

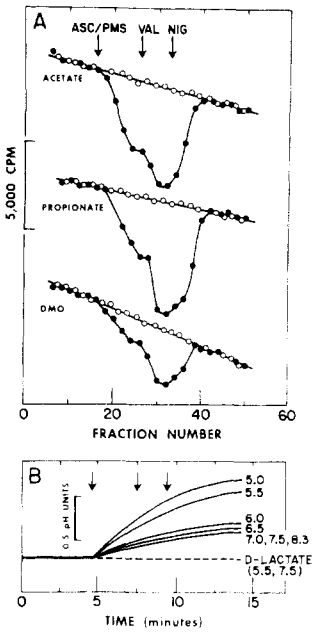

FIGURE 1: (A) Flow dialysis determinations of acetate, propionate, and DMO uptake by membrane vesicles prepared from *E. coli* ML 308-225 grown on succinate. The assays shown were carried out with membrane vesicles (about 2.5 mg of protein per mL) suspended in 0.05 M potassium phosphate at pH 5.5 as described previously (Ramos et al., 1976) and in Methods. Sodium [1-^{14}C]acetate (54 mCi/mmol), sodium [1-^{14}C]propionate (53 mCi/mmol), or [2-^{14}C]DMO (11 mCi/mmol) was used as indicated at final concentrations of 37.5, 37.5, and 200 μM, respectivly. As shown by the arrows, sodium ascorbate and phenazine methosulfate (ASC/PMS), valinomycin (VAL), and nigericin (NIG) were added to the upper chamber at final concentrations of 20 mM, 0.1 mM, 1 μM, and 0.1 μM, respectively (closed symbols). Open symbols were obtained from an identical experiment carried out in the absence of ascorbate and PMS. Although the three curves are displayed in decreasing order, the levels of radioactivity obtained initially in the dialysate (fraction 8) were similar in each case (i.e., 12 000 cpm for acetate, 11 000 cpm for propionate, and 12 300 cpm for DMO). (B) Change in external pH during oxidation of ascorbate-PMS. The tracings shown are recordings of the pH of the upper chamber of the flow dialysis apparatus containing ML 308-225 membrane vesicles (about 2.5 mg of protein per mL) suspended in 0.05 M potassium phosphate. Potassium phosphate (0.05 M) at the same pH as the initial pH of the upper chamber was pumped through the lower chamber as described previously (Ramos et al., 1976) and in Methods. At the time indicated by the arrow, sodium ascorbate (20 mM) and PMS (0.1 mM) or lithium D-lactate (20 mM) were added to the upper chamber, and the pH was recorded continuously with a Radiometer 2321C pH electrode connected to a Radiometer pH meter (Model PHM64). The signal was recorded with a double-channel Corning recorder (Model 845). The pH values given represent the initial pH of the suspension prior to addition of ascorbate-PMS or D-lactate.

Materials

[^{3}H]Triphenylmethylphosphonium bromide was prepared by the Isotope Synthesis Group at Hoffmann-LaRoche, Inc., under the direction of Dr. Arnold Liebman as described (Schuldiner and Kaback, 1975). Other isotopically labeled materials were purchased from New England Nuclear and Amersham/Searle. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. Nigericin was the generous gift of Dr. J. Berger of Hoffmann-La Roche, Inc.

Results

Determination of ΔpH. The data presented in Figure 1A illustrate typical flow dialysis experiments carried out in 0.05 M potassium phosphate buffer at pH 5.5 with acetate, propionate, and DMO, three weak acids which are not metabolized by the vesicles (Ramos et al., 1976). Shortly after addition of a given isotopically labeled weak acid to the upper chamber containing membrane vesicles, radioactivity appears in the dialysate, increases linearly for about 2-3 min (not shown), and reaches a maximum which then decreases at a slow rate (open symbols). When ascorbate and PMS (closed symbols) or D-lactate (not shown) are added, the vesicles accumulate each weak acid, and the concentration in the dialysate decreases relative to the level observed in the absence of electron donor. Addition of valinomycin, an ionophore which specifically increases the potassium permeability of the membrane (Harold, 1970), causes the vesicles to accumulate more of each weak acid, and the concentrations in the dialysate decrease still further. The effect of valinomycin is dependent upon the presence of potassium (data not shown). Thus, vesicles prepared in Tris or choline phosphate buffer at pH 5.5 take up similar amounts of acetate, propionate, or DMO in the presence of ascorbate-PMS, but no increase in accumulation is observed on addition of valinomycin unless potassium is also added to the reaction mixtures. Finally, when nigericin is added (Figure 1A), the acids are released from the vesicles and the external concentrations return to the control levels, an observation which is consistent with the notion that nigericin catalyzes an electrically neutral exchange of potassium for protons and, thus, collapses ΔpH (Ashton and Steinrauf, 1970).

Using the equilibrium concentration of each weak acid in the dialysate subsequent to the addition of ascorbate-PMS (i.e., fraction 25 or 7 min), the amount of each weak acid taken up by the vesicles can be calculated (in nmol per mg of membrane protein, 3.9 for acetate; 3.5 for propionate; and 6.9 for DMO). With these values, together with the external concentration of each acid and its pK value (acetate, 4.75; propionate, 4.87; and DMO, 6.30) and the intravesicular volume of 2.2 μL per mg of membrane protein, it can be calculated (Schuldiner et al., 1972; Rottenberg, 1975) that the intravesicular pH with all three acids is 7.8. Similarly, using the equilibrium concentrations recovered in the dialysate after addition of valinomycin (i.e., fraction 25 or 8.5 min), it can be calculated that the intravesicular pH is 8.3 in all three cases. Given the external pH values at the analogous times (Figure 1B), the ΔpH obtained after addition of ascorbate-PMS is about 2.0 pH units (7.8 inside minus 5.8 outside), corresponding to −118 mV, and the ΔpH obtained after addition of ascorbate-PMS and valinomycin is about 2.2 pH units (8.3 inside minus 6.1 outside), corresponding to −130 mV. Similar results were obtained with butyrate (Ramos et al., 1976).

Effect of External pH on ΔpH, ΔΨ, Internal pH, $\Delta\bar{\mu}_{H^+}$, and Respiration. As reported initially with whole cells (Padan et al., 1976) and subsequently with ML 308-225 membrane vesicles (Ramos et al., 1976), ΔpH varies markedly with external pH. With ascorbate-PMS (Figure 2A), ΔpH remains almost constant at −115 to −120 mV from pH 5.0 to pH 5.5, decreases drastically above pH 5.5, and is negligible at pH 7.5 and above. Significantly, despite marked variation in ΔpH as a function of external pH, internal pH and ΔΨ remain essentially constant at pH 7.8 and −75 mV, respectively. As a result of the variation in ΔpH, $\Delta\bar{\mu}_{H^+}$ exhibits a maximal value of about −195 mV at pH 5.5 (−120 mV ΔpH + −75 mV ΔΨ) and a minimal value of about −75 mV at pH 7.5 and above (0 ΔpH + −75 mV ΔΨ). When the rate of oxidation of reduced PMS is studied as a function of external pH, a relationship similar to that observed for ΔpH and $\Delta\bar{\mu}_{H^+}$ is obtained. The rate of reduced PMS oxidation is relatively high at pH 5.0, increases to a maximum at pH 5.5, decreases sharply above pH 5.5, and remains constant at about 35% of maximal activity from pH 7.5 to pH 8.3.

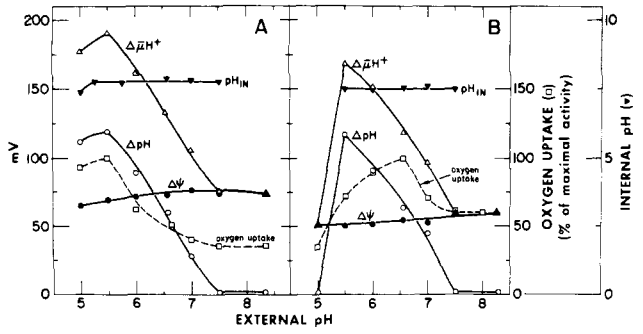

FIGURE 2: Effect of external pH on internal pH, ΔpH, ΔΨ, $\Delta\bar{\mu}_{H^+}$, and oxidation of reduced PMS (A) and D-lactate (B). The experiments shown were carried out as described in Figure 1 and in Methods using vesicles prepared from *E. coli* ML 308-225 grown on succinate. Internal pH (▼—▼) was calculated from flow dialysis experiments with [1-^{14}C]acetate performed at each pH given using sodium ascorbate and PMS (A) or lithium D-lactate (B) at final concentrations of 20, 0.1, and 20 mM, respectively. ΔpH values (○—○) were calculated as described in Methods after correcting the external pH for the change induced by ascorbate-PMS oxidation (see Figure 1B). No correction was necessary when D-lactate was used as electron donor. The external pH values given in panel A represent the initial pH prior to addition of ascorbate-PMS. ΔΨ values (●—●) were calculated from filtration assays carried out with [^{3}H]TPMP$^+$ (1.33 Ci/mmol) at a final concentration of 24 μM (Schuldiner and Kaback, 1975; Ramos et al., 1976). Assays were carried out after 10-min incubations using ascorbate and PMS (A) or D-lactate (B) as electron donors. $\Delta\bar{\mu}_{H^+}$ values (△—△) were calculated from ΔpH and ΔΨ as described in Methods. Rates of oxidation of reduced PMS (A, □—□) and D-lactate (B, □—□) were determined as described previously (Barnes and Kaback, 1971; Konings et al., 1971) and in Methods using sodium ascorbate and PMS or lithium D-lactate at final concentrations of 20 and 0.1 or 20 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 membrane vesicles. The maximum rate (100% observed at pH 5.5) was about 480 ng-atoms of oxygen taken up per min per mg of membrane protein. Similar corrections for D-lactate oxidation were not necessary since there was no significant nonenzymatic oxidation of this electron donor, and the maximum rate of oxidation (100% observed at pH 6.6) was about 200 ng-atoms of oxygen taken up per min per mg of membrane protein.

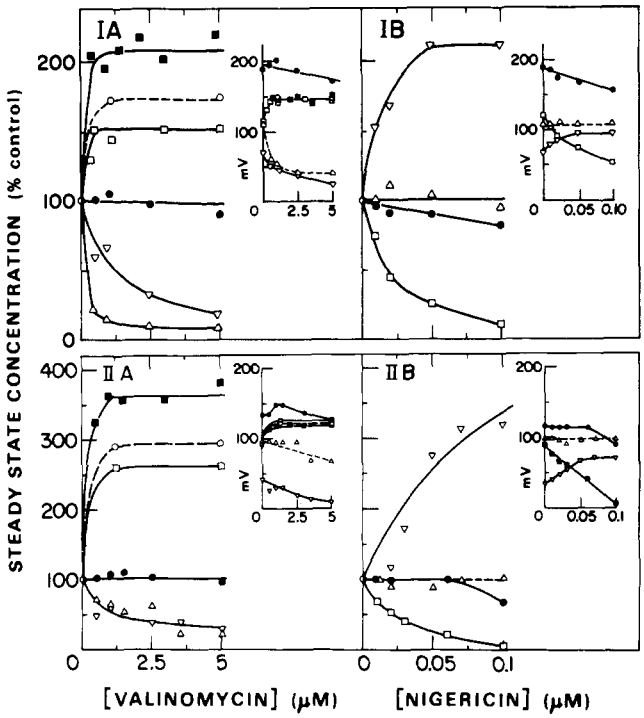

FIGURE 3: Effect of valinomycin (panels A) and nigericin (panels B) on ΔpH, ΔΨ, and $\Delta\bar{\mu}_{H^+}$ in membrane vesicles prepared from *E. coli* ML 308-225 grown on succinate (I) and *E. coli* GN-2 grown on glucose-6-P (II). ΔpH was determined by flow dialysis in the presence of [1-^{14}C]acetate (□—□), [1-^{14}C]propionate (○- - -○), and [2-^{14}C]DMO (■—■) and ascorbate—PMS at an initial pH of 5.5 (i.e., prior to addition of ascorbate-PMS) as described in Figure 1 and Methods. Values calculated in millivolts (see insets) were corrected for the change in external pH induced by reduced PMS oxidation (see Figure 1B). Where indicated, given concentrations of valinomycin (panels A) or nigericin (panels B) were added to the upper chamber containing membrane vesicles. Steady-state levels of TPMP$^+$ accumulation (ΔΨ) were determined at pH 5.5 (▽—▽) and pH 7.5 (△—△) in the presence of given concentrations of valinomycin and nigericin as described in Figure 2. The effect of the ionophores on $\Delta\bar{\mu}_{H^+}$ at pH 5.5 (●—●) was calculated from ΔpH and ΔΨ values obtained at each ionophore concentration. The percentage of $\Delta\bar{\mu}_{H^+}$ remaining at each ionophore concentration is the percentage of $\Delta\bar{\mu}_{H^+}$ measured in the absence of ionophores. The following control values (100%) were obtained in the absence of ionophores (nmol per mg of membrane protein). (I) Acetate uptake, 3.9; propionate uptake, 3.5; DMO uptake, 7.7; TPMP$^+$ uptake, 0.80 (at pH 5.5), 0.91 (at pH 7.5). (II) Acetate uptake, 1.6; propionate uptake, 1.5; DMO uptake, 2.7; TPMP$^+$ uptake, 0.26 (at pH 5.5), 2.5 (at pH 7.5). The millivolt (mV) values plotted in the insets were calculated from the experimental values presented.

With D-lactate as electron donor (Figure 2B), similar variations in ΔpH, $\Delta\bar{\mu}_{H^+}$, and oxidation rates are observed with two important differences. Although ΔpH and, as a result, $\Delta\bar{\mu}_{H^+}$ exhibit maximal values of about −115 and −170 mV, respectively, at pH 5.5 and minimal values of 0 and −55 mV, respectively, at pH 7.5 and above, no ΔpH is observed at pH 5.0 and $\Delta\bar{\mu}_{H^+}$ consists entirely of a ΔΨ component of about −55 mV. This variation is consistent with the relatively low rate of D-lactate oxidation observed at pH 5.0 (in addition, see Kohn and Kaback, 1973). Moreover, D-lactate oxidation exhibits a maximum at about pH 6.6, approximately one pH unit higher than the optimum for ΔpH and $\Delta\bar{\mu}_{H^+}$.

Although data will not be presented, the shape of the ΔpH curve, in particular the point at which ΔpH reaches zero, can be modified to some extent by varying the pK of the buffer in which the vesicles are suspended. With reduced PMS as electron donor and vesicles prepared in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (pK = 8.1) or 0.05 M potassium 2-(N-morpholino)ethanesulfonate (pK = 6.1), ΔpH achieves a value of zero at pH 8.0 and pH 7.0, respectively, while the optimum for ΔpH remains at pH 5.0–6.0 under all conditions tested.

Effect of Valinomycin and Nigericin on ΔpH, ΔΨ, $\Delta\bar{\mu}_{H^+}$, and Oxidation of Reduced PMS and D-Lactate. The experiments presented in Figure 3 were carried out with vesicles prepared from *E. coli* ML 308-225 grown on succinate (section I) and *E. coli* GN-2 grown on glucose-6-P (section II). Similar experiments were also carried out with vesicles prepared from *E. coli* ML 308-225 grown on glucuronate and *E. coli* ML 30 grown on gluconate (not shown) in order to investigate the range of variability of the parameters determined in different vesicle preparations and because certain important correlations between active solute accumulation and $\Delta\bar{\mu}_{H^+}$, ΔΨ, and ΔpH in these vesicles will be discussed in the following paper (Ramos and Kaback, 1977). The results (Figure 3) are presented in millivolts (see insets) and, in addition, as a percentage of control values obtained in the absence of ionophores so that the full magnitude of the experimental variations induced by the ionophores can be visualized.

Although there are quantitative differences among the vesicle preparations, certain important qualitative similarities are immediately apparent. As increasing concentrations of valinomycin are added at pH 5.5 and pH 7.5 (panels A), ascorbate-PMS-dependent TPMP$^+$ uptake (i.e., ΔΨ in the insets) decreases and, at pH 5.5, there is an increase in the uptake of DMO, propionate, and acetate (i.e., ΔpH in the insets).

TABLE I: Effect of Valinomycin, Nigericin, and Carbonyl Cyanide *m*-Chlorophenylhydrazone on Oxidation of Reduced PMS and D-Lactate.[a]

Compd Added	Concn (μM)	Rel. Rate of Oxygen Uptake: ASC-PMS (%)	Rel. Rate of Oxygen Uptake: D-Lactate (%)
Valinomycin	1.00	120	95
	2.50	115	90
	5.00	120	85
Nigericin	0.01	90	85
	0.05	90	85
	0.10	85	86
CCCP[b]	0.10	90	100
	0.50	90	98
	1.00	85	97

[a] Rates of oxygen uptake were measured with membrane vesicles prepared from *E. coli* ML 308-225 grown on succinate as described previously (Barnes and Kaback, 1971) and in Methods. Assay mixtures (1.0 mL) contained 50 mM potassium phosphate (pH 5.5), 10 mM magnesium sulfate, membrane protein (0.2 mg), 20 mM sodium ascorbate, and 0.1 mM PMS or 20 mM lithium D-lactate, and given concentrations of valinomycin, nigericin, or CCCP. Ionophores were added as aliquots of ethanolic solutions such that the final concentration of ethanol was less than 0.4% in the reaction mixtures. At the concentrations of ethanol used, there was no effect on respiration. All assays were carried out at 25 °C and rates of oxygen consumption were linear with time for a minimum of 5 min. Rates of oxygen uptake are expressed as a percentage of the rate of a control sample incubated without ionophores. Samples incubated with ascorbate-PMS were corrected for nonenzymatic oxidation of reduced PMS by subtracting values obtained in the absence of membrane vesicles. [b] CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone.

Although data is not presented, it is also noteworthy that valinomycin does not induce the uptake of these permeant acids at pH 7.5. As shown here and in Figure 1, the percent increase in the uptake of each acid in the presence of valinomycin varies, although the same ΔpH values are obtained with all three acids (compare the data presented in the body of Figure 3, IA and IIA, with the millivolt values presented in the appropriate insets). Furthermore, with each vesicle preparation, the effect of valinomycin on $\Delta\bar{\mu}_{H^+}$ is almost negligible.

In contrast to the results obtained with valinomycin, nigericin induces the opposite effects at pH 5.5 (Figure 3, panels B). With each preparation, acetate uptake (ΔpH) decreases as nigericin is increased from 0 to 0.1 μM, while $TPMP^+$ uptake ($\Delta\Psi$) increases, and $\Delta\bar{\mu}_{H^+}$ remains essentially unchanged over the same concentration range. It is also apparent that this ionophore has no effect on $TPMP^+$ uptake at pH 7.5, a finding which is consistent with the absence of ΔpH at this external pH (see Figure 2 and Ramos et al., 1976).

Although mitochondria and chloroplasts generally exhibit an increase in respiration in the presence of ionophores and proton conductors, this is not the case with bacterial membrane vesicles (Barnes and Kaback, 1971; Lombardi et al., 1973). As shown in Table I, valinomycin, nigericin, or CCCP do not elicit significent changes in D-lactate or reduced PMS oxidation at pH 5.5. Although there is a small apparent increase in the oxidation of reduced PMS in the presence of valinomycin, this effect is unrelated to the increase in ΔpH observed under these conditions, as a similar increase in ΔpH is observed with D-lactate (data not shown) even though valinomycin has essentially no effect on the oxidation of this electron donor (Table I). These compounds also have no significant effect on reduced PMS or D-lactate oxidation at pH 7.5 (data not shown).

Discussion

In addition to providing a framework of comparison for findings to be presented in the following paper (Ramos and Kaback, 1977), a number of these observations are worthy of discussion in their own right. Each weak acid utilized in Figures 1 and 3 yields the same value for intravesicular pH, and the relative uptake of each acid after addition of ascorbate-PMS (or D-lactate) varies with its pK value. Acetate which has a pK of 4.75 is accumulated best on addition of ascorbate-PMS but exhibits the least change on addition of valinomycin (i.e., 145% of the value obtained after addition of ascorbate-PMS), DMO (pK 6.30) exhibits the least accumulation on addition of ascorbate-PMS and the most dramatic effect on addition of valinomycin (i.e., 210% of the value obtained after addition of ascorbate-PMS), and propionate (pK 4.87) exhibits intermediate properties. These differences can be explained by theoretical considerations (Waddel and Butler, 1959; Rottenberg, 1975). Weak acids equilibrate with a transmembrane pH gradient (interior alkaline) in the following manner: The undissociated acid is permeant and enters the intramembranal pool in this form. If the intramembranal compartment is sufficiently alkaline relative to the pK of the weak acid, the acid dissociates, and the anion accumulates in the intramembranal pool. It follows, therefore, that the greater the differential between the internal pH and the pK of the acid, the higher the percentage of the acid in the ionized form in the intramembranal pool, and the higher the level of accumulation. Thus, at an intramembranal pH of 7.8 (the intramembranal pH after addition of ascorbate-PMS), essentially all of the acetate and propionate and significantly less DMO will be ionized in the intravesicular pool. Therefore, DMO will accumulate to a relatively lesser extent than acetate or propionate. It is also clear that an increase in the intravesicular pH (such as occurs on addition of valinomycin) will have a greater relative effect on DMO accumulation because it will increase the concentration of the anionic form of DMO inside of the vesicles to a more significant extent.

The variation in ΔpH (and as a result $\Delta\bar{\mu}_{H^+}$) with external pH correlates fairly well with variations in the rates of oxidation of reduced PMS and D-lactate (Figure 2). Since the relatively low rates of reduced PMS oxidation observed above pH 5.5 produce increasingly lower ΔpH values, but constant $\Delta\Psi$ values, it seems reasonable to suggest that relatively low rates of electron flow through the energy-coupling site are sufficient to generate $\Delta\Psi$, while relatively high rates of electron flow are necessary to support a significant ΔpH. It is also important to realize, however, that in order to generate a significant ΔpH across the vesicle membrane without producing an increase in $\Delta\Psi$, an anion(s) must move with the protons that are ejected from the vesicles or alternatively, a cation(s) must move in the opposite direction (i.e., into the vesicles). Although these possibilities have not been investigated exhaustively, a number of observations suggest that the compensatory ion movement(s) that must take place at pH 5.5 are probably not related to the movement of a particular ionic species. Thus, the relatively high rates of oxidation (Figure 2) and the high rate of proton extrusion observed at relatively acid pH (see Figure 12 in Lombardi et al., 1973) may cause an increase in ΔpH with no change in $\Delta\Psi$ because of compensatory movements of ions in both directions across the membrane. It also seems likely that ion movements may play an important role in the diminution and ultimate loss of ΔpH at progressively alkaline pH since similar effects of external pH on ΔpH have been observed in intact *E. coli* (Padan et al., 1976), *Halobacterium halobium* (Bakker et al., 1976), and in vesicles from *Pseudomonas*

capsulata (Rottenberg, personal communication) without corresponding changes in respiration. In this regard, recent experiments (Eisenbach et al., 1977; Ramos, Rottenberg, and Kaback, unpublished information) suggest the operation of a mechanism which catalyzes the exchange of external protons for intravesicular sodium or potassium at relatively alkaline pH. It seems clear, in any event, that the effect of external pH on ΔpH may be a complicated phenomenon and will require further study.

As shown previously (Ramos et al., 1976) and in Figure 3, at pH 5.5, valinomycin abolishes $\Delta\Psi$ and simultaneously causes an increase in ΔpH; while nigericin induces the opposite effects. Furthermore, the proton conductor CCCP dissipates both ΔpH and $\Delta\Psi$, providing evidence that protons are the electrogenic species (Ramos et al., 1976). It is also apparent that the compounds elicit these changes with no significant effect on respiration (Table I). The explanation for the valinomycin effects may be conceptualized by considering a theoretical membrane which is completely impermeable to ions. In such a membrane, a small amount of proton extrusion will result in a large $\Delta\Psi$ with an immeasurably small ΔpH. If such a membrane becomes permeable to ions (e.g., potassium after addition of valinomycin), the result will be a diminution in $\Delta\Psi$ coincident with the appearance of a measurable ΔpH. With bacterial membrane vesicles at pH 5.5, as discussed above, there should be a finite permeability to ions (regardless of whether these pathways are specific or nonspecific) in order to account for the ΔpH that is present at this external pH. Moreover, if this suggestion is correct, the ion permeability of the membrane is still limiting for ΔpH since valinomycin increases ΔpH in the presence of potassium. Thus, valinomycin, by allowing potassium to move as a counterion to protons, could cause an increase in ΔpH at the expense of $\Delta\Psi$ without an increase in oxidation (i.e., proton extrusion). The finding that the ionophore does not cause the appearance of ΔpH at pH 7.5 may result from relatively low oxidation rates at this pH which are not increased when the vesicles are made permeable to potassium or other ions (i.e., the vesicles do not exhibit respiratory control).

Nigericin, on the other hand, catalyzes an electrically silent exchange of protons for potassium (Ashton and Steinrauf, 1970), and it is not immediately obvious why the decrease in ΔpH caused by this ionophore should result in an increase in $\Delta\Psi$ without a commensurate increase in respiration (i.e., proton extrusion). Returning to the analogy of the membrane which is completely impermeable to ions, it is apparent that a small increase in proton extrusion in such a theoretical membrane would result in a large increase in $\Delta\Psi$. Therefore, it seems possible that a small increase in reduced PMS or D-lactate oxidation which is within the limits of detection of the oxygen electrode could account for the increase in $\Delta\Psi$ observed with nigericin at pH 5.5. In any case, regardless of the precise explanation of these effects, the data, especially when considered in conjunction with the observations to be presented in the following paper (Ramos and Kaback, 1977), provide direct evidence for the primary importance of chemiosmotic phenomena in the mechanism of active transport and, in addition, allow an explanation for many earlier observations which seemed to contradict the validity of this contention.

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References

Altendorf, K., Hirata, H., and Harold, F. M. (1975), *J. Biol. Chem. 249,* 4587.

Ashton, R., and Steinrauf, L. K.)1970), *J. Membr. Biol. 49,* 547.

Bakker, E. P., Rottenberg, H., and Caplan, S. R. (1976), *Biochim. Biophys. Acta 440,* 557.

Barnes, E. M., Jr., and Kaback, H. R. (1971), *J. Biol. Chem. 246,* 5518.

Cohen, G. N., and Rickenberg, H. W. (1956), *Ann. Inst. Pasteur, Paris 91,* 693.

Davis, G. D., and Mingioli, E. S. (1950), *J. Bacteriol. 60,* 17.

Eisenbach, M., Cooper, S., Garty, H., Johnstone, R. M., Rottenberg, H., and Caplan, S. R. (1977), *Biochim. Biophys. Acta* (in press).

Greville, G. D. (1971), *Curr. Top. Bioenerg. 3,* 1.

Hamilton, W. A. (1975), *Adv. Microbiol. Physiol. 12,* 1.

Harold, F. M. (1970), *Adv. Microbiol. Physiol. 4,* 45.

Harold, F. M. (1972), *Bacteriol. Rev. 36,* 172.

Hirata, H., Altendorf, K., and Harold, F. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1804.

Kaback, H. R. (1971), *Methods Enzymol. 22,* 99.

Kaback, H. R. (1972), *Biochim. Biophys. Acta 265,* 367.

Kaback, H. R. (1973), in Bacterial Membranes and Walls, Leive, L., Ed., New York, N.Y., Marcel Dekker, p 241.

Kaback, H. R. (1974a), *Methods Enzymol. 31,* 698.

Kaback, H. R. (1974b), *Science 186,* 882.

Kaback, H. R., and Barnes, E. M. Jr. (1971), *J. Biol. Chem. 246,* 5523.

Kaback, H. R., and Hong, J.-S. (1973), in CRC Critical Reviews in Microbiology, Laskin, A. I., and Lechevalier, H., Ed., Cleveland, Ohio, CRC Press, p 333.

Kohn, L. D., and Kaback, H. R. (1973), *J. Biol. Chem. 248,* 7012.

Konings, W. N., Barnes, E. M., Jr., and Kaback, H. R. (1971), *J. Biol. Chem. 246,* 5857.

Lombardi, F. J., Reeves, J. P., and Kaback, H. R. (1973), *J. Biol. Chem. 248,* 3551.

Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mitchell, P. (1961), *Nature (London) 191,* 144.

Mitchell, P. (1966), *Biol. Rev. Cambridge Philos. Soc. 41,* 445.

Mitchell, P. (1973), *J. Bioenerg. 4,* 63.

Mitchell, P. (1976), *Biochem. Soc. Trans. 4,* 399.

Padan, E., Zilberstein, D., and Rottenberg, H. (1976), *Eur. J. Biochem. 63,* 533.

Patel, L., Schuldiner, S., and Kaback, H. R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3387.

Ramos, S., and Kaback, H. R. (1977), *Biochemistry 16* (following paper in this issue).

Ramos, S., Schuldiner, S., and Kaback, H. R. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1892.

Reeves, J. P., Shechter, E., Weil, R., and Kaback, H. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2722.

Rottenberg, H. (1975), *J. Bioenerg. 7,* 61.

Rudnick, G., Weil, R., and Kaback, H. R. (1975a), *J. Biol. Chem. 250,* 1371.

Rudnick, G., Weil, R., and Kaback, H. R. (1975b), *J. Biol. Chem. 250,* 6847.

Schuldiner, S., and Kaback, H. R. (1975), *Biochemistry 14,* 5451.
Schuldiner, S., Kerwar, G. K., Weil, R., and Kaback, H. R. (1975a), *J. Biol. Chem. 250,* 1361.
Schuldiner, S., Kung, H., Weil, R., and Kaback, H. R. (1975b), *J. Biol. Chem. 250,* 3679.
Schuldiner, S., Rottenberg, H., and Avron, M. (1972), *Eur. J. Biochem. 25,* 64.
Schuldiner, S., Rudnick, G., Weil, R., and Kaback, H. R. (1976b), *Trends Biochem. Sci. 1,* 41.
Schuldiner, S., Spencer, R. D., Weber, G., Weil, R., and Kaback, H. R. (1975c), *J. Biol. Chem. 250,* 8893.
Schuldiner, S., Weil, R., and Kaback, H. R. (1976a), *Proc. Natl. Acad. Sci. U.S.A. 73,* 109.
Short, S. A., Kaback, H. R., and Kohn, L. D. (1975), *J. Biol. Chem. 250,* 4291.
Stroobant, P., and Kaback, H. R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3970.
Waddel, W. J., and Butler, T. C. (1959), *J. Clin. Invest. 38,* 720.

The Relationship between the Electrochemical Proton Gradient and Active Transport in *Escherichia coli* Membrane Vesicles†

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ABSTRACT: In the previous paper [Ramos, S., and Kaback, H. R. (1977), *Biochemistry 16* (preceding paper in this issue)], it was demonstrated that *Escherichia coli* membrane vesicles generate a large electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) under appropriate conditions, and some of the properties of $\Delta\bar{\mu}_{H^+}$ and its component forces [i.e., the membrane potential ($\Delta\Psi$) and the chemical gradient of protons (ΔpH)] were described. In this paper, the relationship between $\Delta\bar{\mu}_{H^+}$, $\Delta\Psi$, and ΔpH and the active transport of specific solutes is examined. Addition of lactose or glucose 6-phosphate to membrane vesicles containing the appropriate transport systems results in partial collapse of ΔpH, providing direct evidence for the suggestion that respiratory energy can drive active transport via the pH gradient across the membrane. Titration studies with valinomycin and nigericin lead to the conclusion that, at pH 5.5, there are two general classes of transport systems: those that are driven primarily by $\Delta\bar{\mu}_{H^+}$ (lactose, proline, serine, glycine, tyrosine, glutamate, leucine, lysine, cysteine, and succinate) and those that are driven primarily by ΔpH (glucose 6-phosphate, D-lactate, glucuronate, and gluconate). Importantly, however, it is also demonstrated that at pH 7.5, all of these transport systems are driven by $\Delta\Psi$ which comprises the only component of $\Delta\bar{\mu}_{H^+}$ at this external pH. In addition, the effect of external pH on the steady-state levels of accumulation of different solutes is examined, and it is shown that none of the pH profiles correspond to those observed for $\Delta\bar{\mu}_{H^+}$, $\Delta\Psi$, or ΔpH. Moreover, at external pH values above 6.0–6.5, $\Delta\bar{\mu}_{H^+}$ is insufficient to account for the concentration gradients established for each substrate unless the stoichiometry between protons and accumulated solutes is greater than unity. The results confirm many facets of the chemiosmotic hypothesis, but they also extend the concept in certain important respects and allow explanations for some earlier observations which seemed to preclude the involvement of chemiosmotic phenomena in active transport.

Chemiosmotic coupling was initially suggested by Mitchell (1966, 1968, 1970a,b; Greville, 1969) as a mechanism for oxidative phosphorylation in mitochondria and photophosphorylation in chloroplasts and, more recently, has been implicated in active transport in bacteria (Mitchell, 1973, 1976; Harold, 1972; Hamilton, 1975). According to this hypothesis, oxidation of electron donors via a membrane-bound respiratory chain or hydrolysis of ATP catalyzed by the membraneous calcium, magnesium-stimulated ATPase complex is accompanied by electrogenic explusion of protons into the external medium, leading to an electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) which is composed of electrical and chemical components. Transport of organic acids is postulated to be dependent upon the chemical gradient of protons (ΔpH) (i.e., the undissociated acid is transported through the membrane and is presumed to accumulate in the ionized form due to the relative alkalinity of the internal milieu), while the transport of positively charged compounds such as lysine or potassium is purportedly coupled to the electrical component ($\Delta\Psi$) of $\Delta\bar{\mu}_{H^+}$. The uptake of neutral substrates such as lactose is thought to be coupled to $\Delta\bar{\mu}_{H^+}$ and to occur via symport with protons (i.e., cotransport). Although not explicitly stated, the chemiosmotic hypothesis also implies that the macromolecular carriers (i.e., porters) which mediate the flow of transport substrates across the membrane play the relatively passive role of allowing solutes to equilibrate with $\Delta\bar{\mu}_{H^+}$ or one of its components.

In addition to providing direct support for some of the general predictions of the chemiosmotic hypothesis, previous work from this laboratory (Ramos et al., 1976; Ramos and Kaback, 1977) establishes a powerful experimental framework within which to test other more specific aspects of the concept. In this paper, the relationship between $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\Psi$ and the accumulation of specific metabolites is examined.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$), ML 30 ($i^+z^+y^+a^+$), and

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