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

Sofia Ramos‡ and H. Ronald Kaback*

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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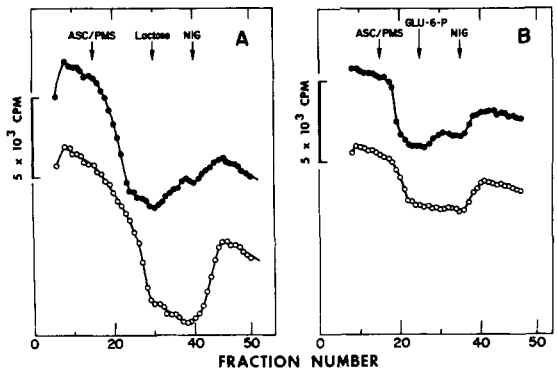

FIGURE 1: Effect of lactose (A) and glucose-6-P (B) transport on pH. Flow dialysis assays were carried out with ML 308-225 (A, ●—●), ML 30 (A and B, O—O), and GN-2 (B, ●—●) membrane vesicles (about 2.5 mg of protein per mL) suspended in 0.05 M potassium phosphate at pH 5.5 containing 0.01 M magnesium sulfate and 1 μM valinomycin as described previously (Ramos et al., 1976; Ramos and Kaback, 1977). Sodium [^{3}H]acetate (685 mCi/mmol) at a final concentration of 18.5 μM was used in A and sodium [1-^{14}C]acetate (54 mCi/mmol) at a final concentration of 37 μM was used in B. As indicated by the arrows, sodium ascorbate and phenazine methosulfate (ASC/PMS), lactose (LAC) [panel A] or glucose-6-P (G-6-P) [panel B], and nigericin (NIG) were added to the upper chamber at final concentrations of 20 mM and 0.1 mM, 2.5 mM or 2.5 mM, and 0.1 μM, respectively. Although the curves shown in panels A and B are displayed in decreasing order, the levels of radioactivity obtained initially in the dialysates (fraction 10) were approximately 25 000 cpm in panel A and approximately 10 000 cpm in panel B.

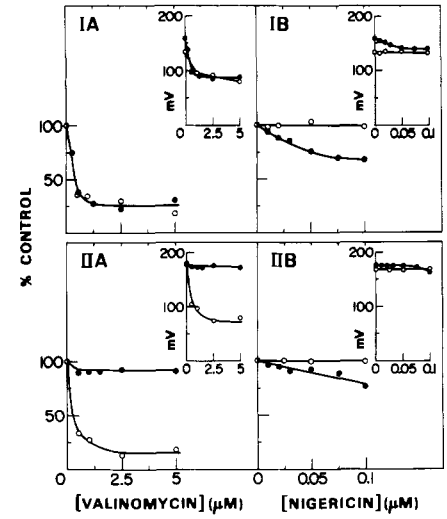

FIGURE 2: Effect of valinomycin (A) and nigericin (B) on steady-state levels of accumulation of lactose (top) and proline (bottom). Membrane vesicles prepared from *E. coli* ML 308-225 grown on succinate (Methods) were transferred into 0.1 M potassium phosphate at pH 5.5 (●—●) and pH 7.5 (O—O) as described in Methods. Aliquots (0.4 mL containing about 6 mg of protein) were diluted to a final volume of 0.8 mL in the upper chamber of the flow dialysis apparatus. The reaction mixtures contained, in final concentrations, 0.05 M potassium phosphate (pH 5.5 or pH 7.5, as indicated) and 0.01 M magnesium sulfate, and valinomycin (A) or nigericin (B) was added to given final concentrations. Flow dialysis was performed as described (Ramos et al., 1976; Ramos and Kaback, 1977) using sodium ascorbate (20 mM), PMS (0.1 mM), and either [1-^{14}C]-lactose (22 mCi/mmol) [top panels] or [U-^{14}C]proline (236 mCi/mmol) [bottom panels] at final concentrations of 400 μM or 8.3 μM, respectively. Data are presented as a percentage of control samples incubated in the absence of ionophores and in millivolts (insets). The control values obtained at pH 5.5 and pH 7.5, respectively, were as follows (in nmol/mg membrane protein): lactose, 108 and 74; proline, 1.8 and 1.7. Although not shown, generally similar results were obtained using flow dialysis and/or filtration assays with the following isotopically labeled transport substrates: [U-^{14}C]serine (156 mCi/mmol) at 12.8 μM; [U-^{14}C]glycine (97 mCi/mmol) at 22 μM; [U-^{14}C]tyrosine (404 mCi/mmol) at 3.6 μM; [U-^{14}C]glutamate (229 mCi/mmol) at 17.4 μM; [U-^{14}C]leucine (280 mCi/mmol) at 14 μM; [U-^{14}C]lysine (306 mCi/mmol) at 6.5 μM; [U-^{14}C]cysteine (271 mCi/mmol) at 10.8 μM; and [2,3-^{14}C]succinate (5.18 mCi/mmol) at 200 μM.

GN-2 ($i^-z^+y^+a^+$; enzyme I^-) were grown, and membrane vesicles were prepared as described in the previous paper (Ramos and Kaback, 1977).

For studies at various pH's, membrane vesicles which had been prepared and frozen in 0.1 M potassium phosphate (pH 6.6) were thawed and transferred to 0.1 M potassium phosphate at the desired pH as described previously (Ramos et al., 1976; Ramos and Kaback, 1977). The protein concentration of the suspensions was adjusted to approximately 6.0 mg per mL.

Transport Assays. Filtration assays (Kaback, 1971, 1974a) and flow dialysis experiments (Ramos et al., 1976; Ramos and Kaback, 1977) were performed as described using sodium ascorbate and phenazine methosulfate (PMS)[1] at final concentrations of 20 and 0.1 mM, respectively. Isotopically labeled solutes were used as indicated.

Calculations. Concentration gradients for solutes taken up by the vesicles were calculated using a value of 2.2 μL of intravesicular fluid per mg of membrane protein (Kaback and Barnes, 1971). The values were converted into millivolts (mv) using the Nernst equation (mV = 58.8 log concentration gradient).

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

Materials

Isotopically labeled materials were purchased from New England Nuclear and Amersham/Searle. Valinomycin was obtained from Calbiochem and nigericin was the generous gift of Dr. J. Berger of Hoffmann-La Roche Inc.

Results

ΔpH and Solute Accumulation. If accumulation of metabolites is coupled to the ΔpH across the membrane, and if the passive accumulation of weak acids reflects that ΔpH, it follows that acetate accumulation should be diminished in the presence of physiological transport substrates such as lactose or glucose-6-P which are accumulated in relatively large amounts by the vesicles. This prediction is borne out by the data presented in Figure 1. As shown in panel A, when ML 308-225 membrane vesicles are allowed to accumulate acetate in the presence of ascorbate-PMS and 1 μM valinomycin at pH 5.5 [at this valinomycin concentration, there is only about 60% inhibition of lactose uptake (Figure 2)], addition of lactose causes the release of about 50% of the accumulated acetate, and the concentration of the weak acid in the dialysate increases appropriately. When the same experiment is carried out with uninduced ML 30 vesicles which do not transport lactose (Barnes and Kaback, 1971), acetate accumulation is not affected by addition of lactose. Similarly, when glucose-6-P is added to vesicles which transport this metabolite, acetate is released from the vesicles, and no effect is observed on addition of the hexose-P to vesicles which do not contain this transport system (Figure 1B).

Effect of Valinomycin and Nigericin on the Steady-State Level of Accumulation of Transport Substrates. In order to provide the appropriate background for the experiments to be presented in this section, a few salient points from preceding

[1] Abbreviations used: DMO, 5,5-dimethyloxazolidine-2,4-dione; PMS, phenazine methosulfate; glucose-6-P, glucose 6-phosphate.

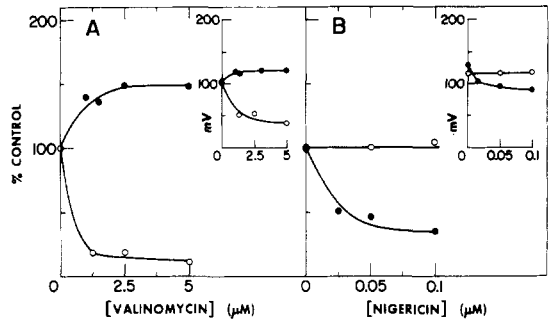

FIGURE 3: Effect of valinomycin (A) and nigericin (B) on the steady-state level of glucose-6-P accumulation at pH 5.5 (●—●) and pH 7.5 (○—○). Experiments were performed by flow dialysis with membrane vesicles prepared from *E. coli* GN-2 grown on glucose-6-P (Methods) as described in Figure 2 except that [U-^{14}C]glucose-6-P (38.7 mCi/mmol) was used at a final concentration of 70 μM. Control values obtained without ionophores at pH 5.5 and pH 7.5, respectively, were 12.7 and 7.8 nmol per mg of membrane protein. Although not shown, generally similar results were obtained using flow dialysis and/or filtration assays with D- [1-^{14}C] lactate (9.1 mCi/mmol) at a final concentration of 200 μM using vesicles prepared from *E. coli* ML 308-225 grown on succinate (Methods); with [6-^{14}C]-glucuronate (44 mCi/mmol) at a final concentration of 100 μM using vesicles prepared from *E. coli* ML 308-225 grown on glucuronate (Methods); and with [U-^{14}C]gluconate (4.1 mCi/mmol) at a final concentration of 400 μM using vesicles prepared from *E. coli* ML 30 grown on gluconate (Methods).

papers (Ramos et al., 1976; Ramos and Kaback, 1977) will be reemphasized: (i) $\Delta\bar{\mu}_{H^+}$ is maximal at pH 5.5 where approximately two-thirds of the total driving force is ΔpH and one-third is $\Delta\Psi$. (ii) At pH 7.5, $\Delta\bar{\mu}_{H^+}$ is reduced by approximately two-thirds and consists solely of a $\Delta\Psi$ component. (iii) At pH 5.5, ΔpH and $\Delta\Psi$ can be manipulated reciprocally by valinomycin and nigericin with little or no effect on $\Delta\bar{\mu}_{H^+}$. (iv) Nigericin has no effect on $\Delta\Psi$ (and therefore no effect on $\Delta\bar{\mu}_{H^+}$) at pH 7.5.

The data presented in Figures 2 and 3 are representative results derived from experiments in which the steady-state level of accumulation of 14 individual solutes was studied in the presence of ascorbate-PMS at pH 5.5 (●—●) and pH 7.5 (○—○) as a function of increasing concentrations of valinomycin (panels A) and nigericin (panels B). Results for lactose and proline (Figure 2) and for glucose-6-P (Figure 3) only are presented in the interests of space, and the data are plotted as a percentage of control values obtained in the absence of valinomycin and nigericin and in millivolts (insets).

Direct quantitative correlations between variations in the accumulation of a particular substrate and variations in $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\Psi$ are observed in but a few instances at pH 5.5. Nevertheless, certain qualitative statements are clearly justified when the experiments are considered as a whole: (i) Accumulation of lactose and proline (Figure 2), serine, glycine, tyrosine, leucine, and, surprisingly, lysine, glutamate, and succinate at pH 5.5 responds to increasing concentrations of valinomycin and nigericin in a manner that is similar to the effect of these ionophores on $\Delta\bar{\mu}_{H^+}$ (cf. Figure 3 in Ramos and Kaback, 1977). Accumulation of each of these solutes is progressively inhibited or relatively unaffected by increasing concentrations of both ionophores. (ii) Accumulation of glucose-6-P (Figure 3) and D-lactate, glucuronate, and gluconate at pH 5.5 is stimulated by valinomycin and inhibited by nigericin in a manner clearly reminiscent of the effects of the ionophores on DMO, propionate, and acetate uptake (cf. Figure 3 in Ramos and Kaback, 1977). (iii) Regardless of whether the accumulation of a particular solute is stimulated, inhibited, or unaffected by valinomycin or nigericin at pH 5.5, in each and every case, valinomycin causes marked inhibition of accumulation at pH 7.5 and nigericin has no effect whatsoever at this external pH (Figures 2 and 3). It is generally clear therefore that, at pH 5.5, the transport systems fall into two categories: Those that are driven preferentially by $\Delta\bar{\mu}_{H^+}$ and those that are driven preferentially by ΔpH. Moreover, it is patently obvious that all of the systems are driven by $\Delta\Psi$ at pH 7.5 where $\Delta\Psi$ comprises the only component of $\Delta\bar{\mu}_{H^+}$.

Since the coupling between the accumulation of a particular solute and $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\Psi$ apparently varies with external pH, it should not be surprising that strict correlations between $\Delta\bar{\mu}_{H^+}$ or ΔpH are observed in only a few instances at pH 5.5. With proline (Figure 2), tyrosine, and lysine, for example, there is reasonably good correlation between the effects of valinomycin and nigericin on $\Delta\bar{\mu}_{H^+}$ and the effects of the ionophores on the accumulation of these amino acids. However, lactose (Figure 2), glycine, and serine accumulation is also coupled preferentially to $\Delta\bar{\mu}_{H^+}$ at pH 5.5, but there is an apparent bias toward $\Delta\Psi$ in these instances since valinomycin inhibits lactose, glycine, and serine uptake more effectively than it inhibits $\Delta\bar{\mu}_{H^+}$ (compare Figure 2 with Figure 3, section IA in Ramos and Kaback, 1977). Similarly, although accumulation of glucose-6-P (Figure 3), D-lactate, glucuronate, and gluconate is coupled to ΔpH at pH 5.5, in only one case (i.e., D-lactate) is the accumulation of these substrates in complete equilibration with ΔpH.[2] It is also clear that nigericin does not inhibit the accumulation of these solutes as effectively as it dissipates ΔpH at pH 5.5 (compare Figure 3 with Figure 3, section IIB, in Ramos and Kaback, 1977).

Concentration Gradients of Specific Solutes as a Function of External pH. Given the response of $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\Psi$ to external pH (Ramos et al., 1976; Ramos and Kaback, 1977), pH titrations of various transport systems take on new and important significance. Assuming a stoichiometry of 1:1 between protons and most transport substrates as suggested by Mitchell (Mitchell, 1973; West and Mitchell, 1972, 1973), those transport systems which are driven by $\Delta\bar{\mu}_{H^+}$ or ΔpH should exhibit maxima for accumulation at approximately pH 5.5, and those systems which are driven by ΔpH should exhibit little or no activity at pH 7.5 and above. Finally, systems driven by $\Delta\Psi$ should exhibit relatively little variation with external pH from pH 5.0 to pH 8.5.

The data presented in Figure 4 compare the effect of external pH on $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\Psi$ with the effect of external pH on the accumulation of various transport substrates. Each experimental point was obtained from flow dialysis experiments carried out under identical conditions. Clearly, with few

[2] Intravesicular pH was calculated from flow dialysis experiments in which accumulation of acetate (pK = 4.75), glucose-6-P (pK_1 = 0.94; pK_2 = 6.11), D-lactate (pK = 3.86), glucuronate (pK = 3.02), and gluconate (pK = 3.63) was measured in the presence of ascorbate-PMS at pH 5.5. Determinations were made as described in Figure 1 of Ramos and Kaback (1977) using the appropriate membrane vesicle preparations (i.e., vesicles from *E. coli* ML 308-225 grown on either succinate or glucuronate, from *E. coli* ML 30 grown on gluconate, and from *E. coli* GN-2 grown on glucose-6-P) and isotopically labeled substrates as described in Figure 3. Intravesicular pH values of 7.31 (minus valinomycin) and 7.47 (plus 1 μM valinomycin) were obtained with glucose-6-P, as opposed to values of 7.22 and 7.90, respectively, with acetate in the same vesicle preparation. Values of 7.64 (minus valinomycin) and 8.22 (plus valinomycin) were obtained with D-lactate, and values of 7.80 and 8.20, respectively, with acetate in the same vesicle preparation. Values of 7.75 (minus valinomycin) and 8.40 (plus valinomycin) were obtained with glucuronate, as opposed to values of 7.30 and 7.80, respectively, with acetate in the same vesicle preparation. Values of 7.20 (*minus valinomycin*) and 7.80 (plus valinomycin) were obtained with gluconate, as opposed to values of 7.30 and 8.10, respectively, with acetate in the same vesicle preparation.

exceptions, none of the predictions are fulfilled. In no case is there a correlation between the accumulation of a given substrate and $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\Psi$ as a function of external pH. Strikingly, moreover, although the magnitude of $\Delta\bar{\mu}_{H^+}$ is sufficient to account thermodynamically for solute accumulation at pH 5.0 and 5.5, it is apparent that, above pH 6.0 or pH 6.5, most of the solutes are accumulated to an extent which is in excess of the value determined for $\Delta\bar{\mu}_{H^+}$.[3] Using proline as a specific example, this amino acid is accumulated to an intravesicular concentration which is approximately 2000-fold higher than that of the external medium at pH 5.5 (i.e., equivalent to about −195 mV) and is therefore apparently in complete equilibration with $\Delta\bar{\mu}_{H^+}$. However, at pH 7.5–8.0 where $\Delta\bar{\mu}_{H^+}$ is only about −80 mV, proline is concentrated to the extent of −150 to −160 mV. It is also especially noteworthy that two organic acids, glucose-6-P and succinate, are accumulated against large concentration gradients in the pH range where ΔpH is zero.

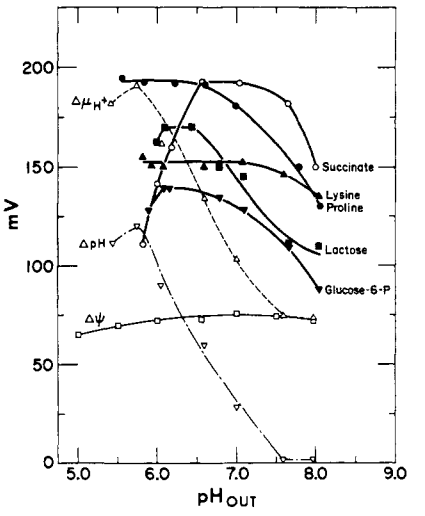

FIGURE 4: Effect of external pH on $\Delta\bar{\mu}_{H^+}$ (Δ), ΔpH (▽), and $\Delta\Psi$ (□) and on steady-state levels of accumulation of lactose (■), proline (●), lysine (▲), succinate (○), and glucose-6-P (▼). ΔpH and $\Delta\Psi$ were determined by flow dialysis at given external pH values as described in Figure 2 of Ramos and Kaback (1977) using [1-^{14}C]acetate (54 mCi/mmol) at a final concentration of 37.5 μM and [^{3}H]TPMP$^+$ (1.33 Ci/mmol) at a final concentration of 24 μM, respectively. $\Delta\bar{\mu}_{H^+}$ was calculated from these values as described in Methods. Concentration gradients for lactose, proline, lysine, succinate, and glucose-6-P were determined by flow dialysis at given external pH values as described in Figures 1 and 2 of Ramos and Kaback (1977) using sodium ascorbate (20 mM), PMS (0.1 mM), and isotopically labeled transport substrates at the following specific activities and final concentrations: [1-^{14}C]lactose (22 mCi/mmol) at 400 μM; [U-^{14}C]proline (236 mCi/mmol) at 8 μM; [U-^{14}C]lysine (306 mCi/mmol) at 6.5 μM; [2,3-^{14}C]succinate (5.18 mCi/mmol) at 200 μM; and [U-^{14}C]glucose-6-P (38.7 mCi/mmol) at 70 μM. Calculations were made as described in Methods. Vesicles prepared from *E. coli* ML 308-225 grown on succinate (Methods) were used for the studies with acetate, TPMP$^+$, lactose, proline, lysine, and succinate; and vesicles prepared from *E. coli* GN-2 grown on glucose-6-P (Methods) were used for the studies with glucose-6-P. Although not shown, it is important that $\Delta\Psi$ with GN-2 membranes is −45 mV at pH 5.5 and −100 mV at pH 7.5 (see insets in Figure 3, sections IIA and IIB in Ramos and Kaback, 1977).

Discussion

The observations presented here and in previous papers (Ramos et al., 1976; Ramos and Kaback, 1977) directly support the contention that chemiosmotic phenomena play an obligatory role in the mechanism of respiration-linked active transport in isolated bacterial membrane vesicles. In addition, the findings extend the chemiosmotic theory in certain important respects and, finally, they provide explanations for earlier observations which seemed to exclude the importance of chemiosmotic phenomena in the mechanism of active transport.

Evidence is presented which shows unequivocally that the ΔpH (interior alkaline) generated across the vesicle membrane is intimately associated with the ability of the vesicles to catalyze active transport. Thus, addition of lactose or glucose-6-P to membrane vesicles which transport these compounds causes partial collapse of pH (Figure 1), demonstrating that oxidation of reduced PMS (or D-lactate) is coupled to these transport systems via the pH gradient under the conditions of the experiments. Previous studies (Schuldiner and Kaback, 1975) demonstrate that lactose accumulation causes partial collapse of $\Delta\Psi$ as well. A reasonable explanation for both of these effects is that lactose and glucose-6-P are taken up in conjunction with protons as suggested by the chemiosmotic hypothesis (Mitchell, 1973, 1976; Harold, 1972; Hamilton, 1975).

The differential effects of valinomycin and nigericin on $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\Psi$ and the effects of these ionophores on the accumulation of various solutes (Figures 2 and 3) also support some of the general aspects of the chemiosmotic formulation. At pH 5.5, there are two classes of transport systems: those that respond primarily to changes in $\Delta\bar{\mu}_{H^+}$ (lactose, proline, serine, glycine, tyrosine, glutamate, leucine, lysine, cystine, and succinate) and those that respond primarily to changes in ΔpH (glucose-6-P, D-lactate, glucuronate, and gluconate). However, each of these transport systems is driven by $\Delta\Psi$ at pH 7.5 where $\Delta\Psi$ comprises the only component of $\Delta\bar{\mu}_{H^+}$. The latter conclusion is particularly striking with respect to the transport systems that are coupled primarily to ΔpH at pH 5.5 which exhibit dramatic stimulation by valinomycin at this pH and dramatic inhibition by the same ionophore at pH 7.5 (Figure 3). Consistently, nigericin inhibits accumulation of these substrates at pH 5.5 but has no effect at pH 7.5.

Although the results as a whole confirm the overall validity of the chemiosmotic hypothesis, many of the observations are not readily explained by the hypothesis as it is presently formulated. For instance, according to current notion, accumulation of organic acids is obligatorily dependent on the relative alkalinity of the internal pH. Organic acids are presumably transported in their undissociated form and accumulate in the internal pool in the ionized form. Since there is no ΔpH across the membrane at pH 7.5 in either whole cells (Padan et al., 1976) or isolated membrane vesicles (cf. Fig. 4 and Ramos et al., 1976; Ramos and Kaback, 1977), the putative mechanism cannot account for acid accumulation at high external pH. It is also apparent that, if the stoichiometry between protons and transport substrates remains constant at 1:1 at all values of external pH, the concentration gradients observed at relatively high pH (Figure 4) are impossible to accommodate thermodynamically with the present chemiosmotic model. There is at least one simple explanation for these observations within the bounds of the chemiosmotic framework, however. Possibly, the stoichiometry between protons and transport substrates varies as a function of external pH in such a manner that it is 1:1 at pH 5.5, but increases to higher values as external pH is increased. If, for instance, the stoichiometry between protons

[3] In the experiments shown in Figure 4, glucose-6-P accumulation was measured with membrane vesicles prepared from *E. coli* GN-2, while the determinations of ΔpH and $\Delta\Psi$ were carried out with vesicles prepared from *E. coli* ML 308-225. As demonstrated in the preceding paper [Ramos and Kaback, 1977, Figure 3, inset in section IIB], GN-2 vesicles exhibit a $\Delta\Psi$ of about −100 mV at pH 7.5. It is apparent therefore that, at pH 7.5, glucose-6-P accumulation by GN-2 vesicles is essentially in equilibrium with $\Delta\bar{\mu}_{H^+}$ (i.e., $\Delta\Psi$).

and proline were 2:1 at pH 7.5 rather than 1:1, the concentration gradient of proline would be thermodynamically compatible with $\Delta\bar{\mu}_{H^+}$ at pH 7.5 [i.e., if the stoichiometry is 2:1, the concentration gradient would vary as the square of the charge gradient (Mitchell, 1973; Rottenberg, 1976)]. In a similar vein, it is conceivable that, at pH 5.5, transport of organic acids occurs by the standard chemiosmotic mechanism (i.e., one proton—two if the acid is glucose-6-P—is taken up per mole of undissociated acid), while at pH 7.5 two or more protons are taken up per mole of acid, one (two with glucose-6-P) in association with the substrate itself and one in association with the carrier molecule. By this means, the transport of glucose-6-P,[3] D-lactate, glucuronate, and gluconate at pH 7.5 would become electrogenic, having become symport mechanisms. Rottenberg (1976) has also considered these possibilities recently in a theoretical manner.

The proposition that carrier molecules themselves respond to $\Delta\bar{\mu}_{H^+}$ or one of its components and do not function merely as portals which allow the equilibration of substrates with a given driving force is also consistent with a number of other observations. When the accumulation of a large number of transport substrates is examined as a function of external pH (Figure 4), in no single case does the accumulation of substrate exhibit a pH optimum which correlates with the pH optima for $\Delta\bar{\mu}_{H^+}$ or ΔpH. Moreover, none of the transport systems exhibits the flat pH profile of $\Delta\Psi$, and all of the systems manifest considerable activity at pH 7.5 where there is a negligible ΔpH. In addition, it is important to realize that certain predictions derived from the notion that carriers play a passive role in the transport mechanism are flatly inconsistent with some of the observations presented here. The transport of succinate and glutamate and lysine, for instance, should be coupled theoretically to ΔpH and $\Delta\Psi$, respectively, and this is clearly not the case. Thus, it seems inescapable that carrier molecules must play a dynamic role in modulating the response of a given transport substrate to the electrochemical proton gradient. Finally, it should be emphasized that direct evidence supporting this conclusion has been presented (Reeves et al., 1973; Schuldiner et al., 1975a–c, 1976a,b; Rudnick et al., 1975a,b, 1977). Generation of $\Delta\Psi$ and/or $\Delta\bar{\mu}_{H^+}$ across the vesicle membrane results in a perturbation of the *lac* carrier protein that causes the appearance of high affinity binding sites for dansyl galactosides, azidophenyl galactosides, and *p*-nitrophenyl α-D-galactopyranoside on the surface of the membrane.

A number of initial observations from this laboratory (see Lombardi et al., 1974, for a review) appeared to be in direct conflict with the proposal that chemiosmotic phenomena play a central role in active transport in isolated bacterial membrane vesicles. Although some of these inconsistencies were resolved in subsequent publications (Kaback, 1974b; Schuldiner and Kaback, 1975); Stroobant and Kaback, 1975), some of the more inexplicable observations can be resolved only in light of the findings presented here, in Ramos et al. (1976) and in Ramos and Kaback (1977). One of the most difficult of these observations to accommodate is the variation in the ability of different electron donors to drive different transport systems relative to D-lactate or reduced PMS. In chemiosmotic theory, oxidation of any particular electron donor (i.e., reduced PMS, D-lactate, succinate, or NADH) would presumably be associated with a unique value of $\Delta\bar{\mu}_{H^+}$. Accordingly, the order of effectiveness of the various electron donors in stimulating steady-state uptake would be expected to be the same for all respiration-linked transport systems, this order reflecting their relative effectiveness in generating $\Delta\bar{\mu}_{H^+}$. As shown previously (Lombardi et al., 1974), the observations do not support the theory. However, the inconsistency can now be resolved. At pH 5.5, some transport systems are driven by $\Delta\bar{\mu}_{H^+}$, others by $\Delta\bar{\mu}_{H^+}$ with a bias toward $\Delta\Psi$, and others by ΔpH, while at pH 7.5 all of the systems are driven by $\Delta\Psi$. Moreover, some electron donors (i.e., succinate) generate a significant $\Delta\Psi$ but no ΔpH (Ramos et al., 1976). Therefore, those transport systems with a bias toward $\Delta\Psi$ will be stimulated more effectively by succinate at pH 5.5 than the systems which are in equilibrium with $\Delta\bar{\mu}_{H^+}$ or ΔpH.

Another apparent inconsistency that is resolved by the present findings is the variation in the inhibitory potency of valinomycin with different transport systems. As shown by Lombardi et al. (1973), the half-maximal inhibitory concentrations of this ionophore vary over three orders of magnitude when its effect on various transport systems is examined. As demonstrated here, depending on the particular transport system and the external pH [the previous studies were carried out at pH 6.6 where ΔpH is only about 60–70 mV (Figure 2 in Ramos et al., 1976 and Ramos and Kaback, 1977)], valinomycin may stimulate, inhibit, or have essentially no effect. It is apparent therefore that, in light of the current observations, most of the earlier contradictory findings, if not all, can be accommodated within the confines of a chemiosmotic formulation.

Acknowledgments

We express our appreciation to Dr. Shimon Schuldiner for stimulating discussion during the course of much of this work and to Dr. Hagai Rottenberg for sending us his manuscript prior to publication.

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Actin and Myosin-Linked Calcium Regulation in the Nematode *Caenorhabditis elegans.* Biochemical and Structural Properties of Native Filaments and Purified Proteins†

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ABSTRACT: Calcium regulation of actomyosin activity in the nematode, *Caenorhabditis elegans,* has been studied with purified proteins and crude thin filaments. Actin and tropomyosin have been purified from *C. elegans* and shown to be similar in most respects to actin and tropomyosin from rabbit skeletal muscle. The actin comigrates with rabbit actin on polyacrylamide–sodium dodecyl sulfate gel electrophoresis, forms similar filaments and paracrystals, and activates the Mg^{2+}-ATPase of rabbit myosin heads as efficiently as rabbit actin. Nematode tropomyosin has a greater apparent molecular weight (estimated by mobility on polyacrylamide–sodium dodecyl sulfate gels) than the rabbit protein, yet it forms Mg^{2+}-paracrystals with a slightly shorter periodicity. Native thin filaments extracted from nematodes activate rabbit myosin subfragment 1 Mg^{2+}-ATPase in a calcium sensitive manner; the extent of activation is threefold greater in 0.2 mM $CaCl_2$ than in the absence of calcium. This observation suggests that the thin filaments contain components which are functionally equivalent to vertebrate troponins. Calcium is also required for maximal activation of the Mg^{2+}-ATPase of purified nematode myosin by pure rabbit F-actin. *C. elegans* therefore has both myosin and thin filament-linked calcium regulatory systems. The origin of the actin, tropomyosin, and myosin from different tissues and the use of genetic analysis to answer questions about assembly and function in vivo are discussed.

In muscle, thick and thin filaments are organized in parallel arrays. During contraction, the filaments slide past each other, to give overall shortening of the sarcomeres (Huxley and Niedergerke, 1954; Hanson and Huxley, 1954). The relative movement of the filaments is effected by cross bridges protruding from the thick filaments, which interact with the thin filaments in cycles of attachment and detachment (Huxley, 1969). In all muscles, contraction is regulated by intracellular calcium levels. The site of action of Ca^{2+} may be on either the thick filaments, or the thin filaments, or both, depending on the organism and muscle type (Lehman et al., 1972; Lehman and Szent-Györgyi, 1975). For both thick and thin filament linked regulatory mechanisms, it appears that interactions between cross bridges and thin filaments are blocked at very low calcium concentrations.

It is clear that efficient, controlled contraction is dependent on the structural integrity of a highly complex, multicomponent system. Little is known about the processes regulating the assembly and maintenance of the muscle filaments or their organization into mixed lattices. The use of specific mutants has been extremely helpful in understanding analogous questions in virus assembly (Casjens and King, 1974; Katsura and Kuhl, 1974) and bacterial ribosome functions (Davies and Nomura, 1972). This approach appears feasible in the nematode, *Caenorhabditis elegans,* because paralyzed mutants, in which the body wall muscle cells exhibit disrupted filament lattices, have been found (Epstein and Thomson, 1974; Epstein et al., 1974). There is also a need for a better understanding of the relationship between biochemical reactions in vitro and

† From the Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305. *Received July 14, 1976.* This work was performed during the tenure of a Science Research Council of Great Britain Fellowship to H.E.H., a McCormick Fellowship through Stanford University School of Medicine to M.-Y.W.T., and a National Genetics Foundation (through the Grant Foundation) and Mellon Foundation Fellowships to H.F.E. The research was supported by grants from the National Institute of Aging (No. AG-00448), the Muscular Dystrophy Associations of America, Inc., the American Heart Association, Inc. through the Santa Clara County Chapter (No. 73-950), and the American Cancer Society through an institutional grant to Stanford University.