Membrane Potential and Active Transport in Membrane Vesicles from *Escherichia coli*†

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ABSTRACT: Membrane vesicles isolated from *Escherichia coli* ML 308-225 accumulate triphenylmethylphosphonium and safranine O in the presence of appropriate electron donors. Moreover, these cations are accumulated when a potassium diffusion gradient is imposed across the vesicle membrane ($[K^+]_{in} > [K^+]_{out}$), and the vesicles exhibit the same steady-state levels of accumulation for triphenylmethylphosphonium, dimethyldibenzylammonium (in the presence of tetraphenylboron), and rubidium (in the presence of valinomycin). Triphenylmethylphosphonium accumulation by the vesicles is not dependent on the presence of ionophores or lipophilic anions, occurs with vesicles prepared and assayed in either potassium- or sodium-containing media, and does not exhibit certain properties associated with carrier-mediated transport systems. These results provide strong evidence in support of the hypothesis that oxidation of D-lactate or reduced phenazine methosulfate by the vesicles generates an electrical potential, interior negative, across the vesicles membrane. Accumulation of triphenylmethylphosphonium by the vesicles is relatively specific for D-lactate or reduced phenazine methosulfate as electron donors. L-Lactate, succinate, and NADH are oxidized more rapidly than D-lactate, but L-lactate and succinate do not support triphenylmethylphosphonium uptake as well as D-lactate, and NADH is ineffective. These and other observations suggest that there is an energy-coupling site located primarily between D-lactate dehydrogenase and cytochrome b_1 which is responsible for the generation of the membrane potential. Anoxia, various electron transfer inhibitors, and proton conductors block D-lactate dependent triphenylmethylphosphonium accumulation and proton extrusion. However, only proton conductors and electron transfer inhibitors which block electron flow after the energy-coupling site produce efflux of previously accumulated triphenylmethylphosphonium or collapse the proton gradient established as a result of D-lactate oxidation. The observations suggest that the membrane potential may be in equilibrium with the redox state of the respiratory chain at the site of energy coupling. Evidence is also presented which demonstrates that a membrane potential, interior negative, is intimately associated with the ability of the vesicles to catalyze active transport. Steady-state levels of lactose, proline, tyrosine, glutamic acid, and glycine accumulation are directly related to the steady-state level of triphenylmethylphosphonium accumulation. Moreover, addition of lactose to vesicles containing the β-galactoside transport system partially inhibits the uptake of proline and triphenylmethylphosphonium. The effects are not observed in vesicles devoid of the β-galactoside transport system. Although most of the data support a chemiosmotic mechanism for active transport, evidence is presented which indicates that the membrane potential in itself may not be sufficient to account for the totality of active transport. Possible explanations for these inconsistencies are discussed.

Cytoplasmic membrane vesicles isolated from a variety of bacterial cells catalyze active transport of many different solutes by a respiration-dependent mechanism that does not involve the generation or utilization of ATP or other high-energy phosphate compounds (Kaback, 1972, 1973, 1974b; Kaback and Hong, 1973). In *Escherichia coli* and *Salmonella typhimurium* vesicles, most of these transport systems are coupled primarily to the oxidation of D-lactate or reduced phenazine methosulfate via a membrane-bound respiratory chain with oxygen or, under appropriate conditions (Konings and Kaback, 1973; Boonstra et al., 1975), fumarate or nitrate as terminal electron acceptor. Recent experiments demonstrate that essentially all of the vesicles isolated from *E. coli* ML 308-225 catalyze active transport (Short et al., 1974). Moreover, using antibodies against D-lactate dehydrogenase and calcium, magnesium-stimulated ATPase, it has been shown that both of these enzymes are located on the inner surface of the vesicle membrane (Short et al., 1975a, b). These and other findings (Kaback, 1972, 1973, 1974b; Kaback and Hong, 1973; Altendorf and Staehelin, 1974; Konings et al., 1973) demonstrate that essentially none of the vesicles is inverted. It seems apparent therefore that the inability of certain electron donors to drive transport in the vesicle system cannot be attributed to the presence of inverted vesicles in the preparations. These and other observations (Kaback, 1972, 1973, 1974b; Kaback and Hong, 1973; Barnes and Kaback, 1971; Kaback and Barnes, 1971; Stroobant and Kaback, 1975) support the contention that the energy-coupling site for transport is located in a relatively specific segment of the respiratory chain between D-lactate dehydrogenase and cytochrome b_1.

Although it is not known how energy released from the oxidation of D-lactate or other electron donors is coupled to transport, recent experiments (Kaback, 1974b; Hirata et al., 1973; Altendorf et al., 1974, 1975; Schuldiner et al., 1975; Rudnick et al., 1975a,b; Patel et al., 1975) have demonstrated that chemiosmotic phenomena, as postulated by Mitchell (1966, 1973; Harold, 1972), play an important role in respiration-linked active transport. As visualized by this mechanism, oxidation of electron donors is accompanied by expulsion of protons into the external medium, leading to a pH gradient and/or electrical potential across the membrane. This electrochemical gradient is postulated to be the driving force for the inward movement of transport

† From the Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received July 28, 1975.* This is the 28th paper of the series "Mechanisms of Active Transport in Isolated Bacterial Membrane Vesicles". Paper 27 is Rudnick et al. (1975b).

substrates by means of passive diffusion in the case of lipophilic cations, by facilitated diffusion in the case of positively charged substrates such as lysine or potassium, or by coupled movement of protons with a neutral substrate such as lactose or proline (i.e., "symport"). In instances where sodium efflux is observed (Altendorf et al., 1975; Lombardi et al., 1973), the chemiosmotic model invokes sodium-proton "antiport", a mechanism which is thought to catalyze electroneutral exchange of internal sodium with external protons (West and Mitchell, 1974). Moreover, the inhibitory effects of uncoupling agents on transport are attributed to the ability of these compounds to conduct protons across the membrane, thus short-circuiting the "proton-motive force" that drives transport (Mitchell, 1966, 1973; Harold, 1972; Mitchell and Moyle, 1967).

One of the most compelling lines of evidence in favor of the chemiosmotic hypothesis in *E. coli* membrane vesicles stems from the use of lipophilic cations (Bakeeva et al., 1970) to measure the putative membrane potential generated as a result of D-lactate oxidation. Thus, Hirata et al. (1973) and Altendorf et al. (1975) have demonstrated that dimethyldibenzylammonium, under certain conditions, is accumulated by *E. coli* ML 308-225 membrane vesicles. However, many of the properties of this cation are equivocal with respect to the contention that it merely passively equilibrates with the membrane potential (Lombardi et al., 1973, 1974). For this reason, we have undertaken the study of other lipophilic cations in the bacterial membrane vesicle system.

The results presented in this paper provide strong support for the hypothesis that D-lactate and reduced phenazine methosulfate oxidation by *E. coli* ML 308-225 membrane vesicles leads to the generation of a membrane potential, interior negative, and that this potential provides at least part of the driving force for active transport. In addition, evidence is presented which suggests that the membrane potential may be in equilibrium with a segment of the respiratory chain between D-lactate dehydrogenase and cytochrome b_1.

Experimental Section

Methods

Growth of Bacteria and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) and ML 30 ($i^+z^+y^+a^+$) were grown in minimal medium A containing 1.0% sodium succinate (hexahydrate) as the sole source of carbon (Kaback, 1971). Membrane vesicles were prepared from lysozyme–EDTA spheroplasts as described previously (Kaback, 1971) with the exception that the vesicles were not vigorously homogenized with a Teflon–glass homogenizer (Short et al., 1975b). Where indicated, potassium phosphate was replaced with sodium or choline phosphate as described earlier (Lombardi et al., 1973).

When triphenylmethylphosphonium uptake was measured in whole cells the cells were treated with EDTA (Griniuviene et al., 1974) as follows: *E. coli* ML 308-225 grown as described above were centrifuged, washed twice in 0.1 *M* Tris-HCl (pH 8.0), and resuspended to a concentration of 10–20 mg dry weight per ml. The suspension was incubated at 37° for about 5 min, and potassium ethylenediaminetetraacetic acid (pH 7.0) was added to a final concentration of 10 m*M*. Incubation was continued at 37° for 2 min, and the cells were then centrifuged, washed once in 0.1 *M* potassium phosphate (pH 6.6), and resuspended to about 3 mg of protein/ml in the same buffer.

Transport Assays. Uptake of radioactive solutes by membrane vesicles and whole cells was determined as described elsewhere (Kaback, 1974a) with one important difference. Triphenylmethylphosphonium, dimethyldibenzylammonium, and, to a lesser extent, rubidium bind to cellulose nitrate filters (Millipore HA or Schleicher and Schuell Selectron filters) to such an extent that uptake by the membrane vesicles can be completely obscured. This effect probably accounts for the high background radioactivity reported previously (Hirata et al., 1973; Altendorf et al., 1974, 1975) with [^{3}H]dimethyldibenzylammonium. In any case, the problem is alleviated almost completely with the use of cellulose acetate filters (Millipore Cellotate filters). Thus, in all of the experiments reported here, Millipore filters type EH (0.5 μ pore size) were used. Apparent concentration gradients for substrates taken up by the vesicles were calculated using a value of 2.2 μl of intravesicular fluid/mg of membrane protein (Kaback and Barnes, 1971).

Oxygen Consumption. Rates of oxygen uptake were measured with a Clark electrode (YSI Model 53 oxygen monitor) as described previously (Barnes and Kaback, 1971).

Fluorescence Measurements. Fluorescence was measured at an angle of 90° with an Aminco Bowman spectrofluorometer using 1 × 1 cm cuvettes as described previously (Reeves et al., 1973b). The sample chamber was maintained at 25° with a circulating water bath. Light bandpass for excitation and emission was 6 nm. Additions to the cuvette were made with Hamilton microsyringes and mixing was accomplished within 3–5 sec using a small plastic stick.

Materials

Tritiation of Triphenylmethylphosphonium. Triphenylmethylphosphonium was tritiated by The Isotope Synthesis Group of Hoffman-La Roche, Inc., under the direction of Dr. Arnold Liebman. A mixture of 10 mg of triphenylmethylphosphonium bromide and 50 μl of dimethylformamide was frozen to −190°, evacuated to less than 1 μ, thawed, and again evacuated to less than 1 μ. By vacuum transfer, 100 μl of T_2O (10 Ci) and 10 μl of acetic acid were added. The resulting mixture was then stirred at 70° for 140 hr. At that time, all volatile material was removed by vacuum transfer. Labile tritium was removed by treating the residue three times each with 2.0 ml of methanol which was removed by vacuum transfer after equilibrium had taken place. The residue was chromatographed on 20 g of silica gel (E. Merck 7734) packed in methanol containing 0.4% acetic acid, using the same solvent for elution. The procedure was carried out in a 50-ml buret. Fractions containing pure product as determined by thin-layer chromatography on silica gel (carbon tetrachloride–methanol–acetic acid; 30:70:2.5, v/v) were pooled and concentrated to a residue of 7 mg having a specific activity of approximately 114 Ci/mol. The final product exhibited an absorption spectrum which was identical with authentic triphenylmethylphosphonium bromide.

Tritiation of Tetraphenylarsonium. Tetraphenylarsonium chloride was labeled with tritium as described above for triphenylmethylphosphonium. The final product which had a specific activity of 58.8 Ci/mol was at least 99% pure as judged by thin-layer chromatography (see above) and ultraviolet absorption.

Other Lipophilic Cations. [*methyl*-^{14}C]Dimethyldibenzylammonium chloride (6 Ci/mol) and [*methyl*-^{3}H]di-

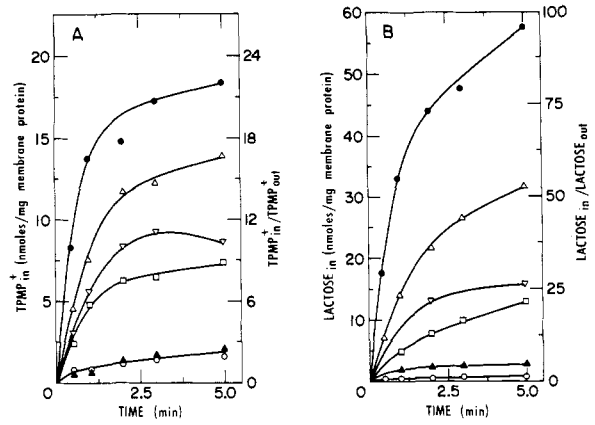

FIGURE 1: (A) Time course of triphenylmethylphosphonium uptake by *E. coli* ML 308-225 membrane vesicles in the presence of various electron donors. Triphenylmethylphosphonium uptake was determined in the presence of the designated electron donor as described previously (Kaback, 1971, 1974a) and in Methods. Sodium ascorbate and phenazine methosulfate were added at final concentrations of 20 and 0.1 m*M*, respectively, and the other electron donors were added at final concentrations of 20 m*M*. The reaction mixtures (50 μl, final volume) contained 0.05 *M* potassium phosphate (pH 6.6), 0.01 *M* magnesium sulfate, 0.1 mg of membrane protein, and 0.4 m*M* [^{3}H]triphenylmethylphosphonium bromide (114 Ci/mol). (B) Time course of lactose uptake by *E. coli* ML 308-225 membrane vesicles in the presence of various electron donors. Lactose uptake was determined exactly as described above with the exception that [1-^{14}C]lactose (22 Ci/mol) was used at a final concentration of 0.4 m*M* in place of triphenylmethylphosphonium. (●) Ascorbate–phenazine methosulfate; (△) D-lactate; (▽) L-lactate; (□) succinate; (▲) NADH; and (○) no added electron donor.

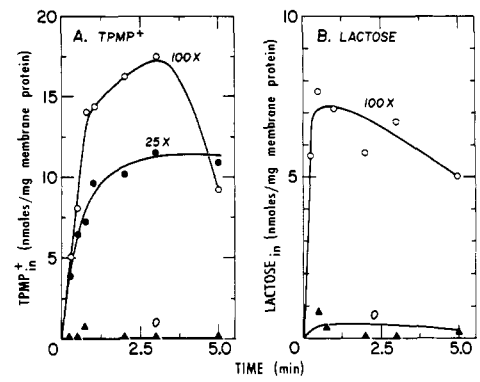

FIGURE 2: Effect of potassium diffusion potentials on triphenylmethylphosphonium (A) and lactose (B) uptake. An aliquot (2 μl) of *E. coli* ML 308-225 membrane vesicles (40 mg of protein/ml) containing 0.1 *M* potassium phosphate (pH 6.6) and valinomycin (1 nmol/mg of membrane protein) was diluted as indicated into 50 or 200 μl of either 0.1 *M* sodium phosphate (pH 6.6) (● and ○, respectively) or 0.1 *M* potassium phosphate (pH 6.6) (▲) containing [^{3}H]triphenylmethylphosphonium (114 Ci/mol) [A] or [1-^{14}C]lactose (22 Ci/mol) [B] at final concentrations of 0.4 m*M*. Both the vesicle suspension and the diluting medium were equilibrated to 25° prior to the start of the experiment. At the times shown, the reactions were terminated and the samples assayed as described previously (Kaback, 1971, 1974a).

methyldibenzylammonium chloride (140 Ci/mol) were synthesized by methods described previously (Lombardi et al., 1973). Safranine O was obtained from Fisher Scientific Company. Triphenylmethylphosphonium bromide, tetraphenylarsonium chloride, dimethyldibenzylammonium chloride, and sodium tetraphenylboron were obtained from K & K Laboratories (Plainville, N.Y.). Sodium phenyldicarbaundecaborane was generously supplied by Dr. Eugene M. Barnes, Jr., of Baylor College of Medicine.

Rubidium-86 chloride was obtained from New England Nuclear Corp. as an aqueous solution in 0.5 *N* HCl. Stock solutions of rubidium-86 chloride (10–30 Ci/ mol) were prepared by neutralizing the commercial product with rubidium hydroxide, and diluting the specific activity with appropriate addition of rubidium chloride. Radioactive amino acids were also obtained from New England Nuclear Corp. [1-^{14}C]Lactose (22 Ci/mol) was obtained from Amersham-Searle Co. Valinomycin was purchased from Calbiochem.

All other materials were of reagent grade obtained from commercial sources.

Results

Uptake of Triphenylmethylphosphonium. The effect of various electron donors on triphenylmethylphosphonium uptake by membrane vesicles isolated from *E. coli* ML 308-225 is shown in Figure 1A. Ascorbate plus phenazine methosulfate stimulate the initial rate of uptake approximately 15-fold over controls incubated in the absence of electron donor. In the presence of this electron donor system, the vesicles accumulate triphenylmethylphosphonium to an apparent steady-state concentration which is approximately 20 times higher than that of the external medium. The initial rate of triphenylmethylphosphonium uptake is stimulated approximately tenfold by D-lactate, and the vesicles accumulate the cation to an intravesicular concentration approximately 16 times higher than that of the external medium. As demonstrated previously for a number of other solutes (Barnes and Kaback, 1971; Lombardi et al., 1973; Lombardi and Kaback, 1972; Reeves et al., 1972), L-lactate and succinate also stimulate triphenylmethylphosphonium uptake, but these electron donors are less effective than ascorbate–phenazine methosulfate or D-lactate. NADH produces no significant stimulation of either the initial rate or extent of triphenylmethylphosphonium uptake. Previous studies also demonstrated that there is no direct relationship between the ability of the vesicles to oxidize these electron donors and their ability to stimulate transport. Similarly, the vesicles used in these experiments exhibit the following oxidase activities toward D-lactate, L-lactate, succinate, and NADH: 200, 224, 240, and 267 ng-atoms of oxygen per mg of membrane protein per minute, respectively (the results represent the average of four independent determinations).

When lactose uptake is measured in the presence of the same electron donors under identical conditions (Figure 1B), there is a reasonably good qualitative correlation between the relative effects of the various electron donors on the initial rates and steady-state levels of lactose and triphenylmethylphosphonium accumulation. Quantitative differences are apparent however, the most obvious of which is that lactose is taken up approximately three to five times better than triphenylmethylphosphonium. Moreover, although ascorbate–phenazine methosulfate stimulates uptake best in both cases, this electron donor system stimulates lactose uptake approximately three times better than D-lactate (Figure 1B) while it stimulates triphenylmethylphosphonium uptake only about 30–40% better than D-lactate (Figure 1A).

The experiments presented in Figure 2A provide an indication that triphenylmethylphosphonium is taken up in response to a membrane potential, interior negative. Thus, when potassium-loaded vesicles containing valinomycin are diluted 25-fold or 100-fold into sodium phosphate buffer, triphenylmethylphosphonium is rapidly taken up, achieving

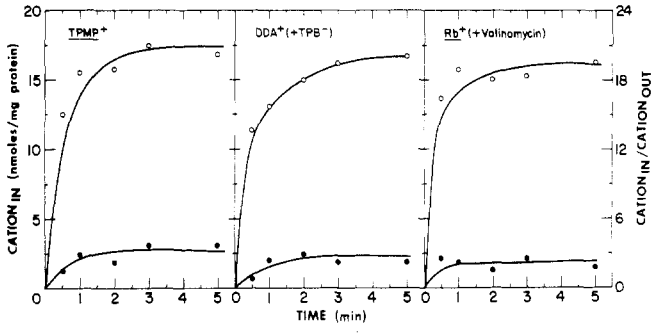

FIGURE 3: Time course of cation uptake by *E. coli* ML 308-225 membrane vesicles. Membrane vesicles prepared from *E. coli* ML 308-225 were washed and resuspended in 0.1 *M* sodium phosphate (pH 6.6) as described previously (Lombardi et al., 1973). Uptake of triphenylmethylphosphonium, dimethyldibenzylammonium, and rubidium was determined in the presence (○) and absence (●) of 20 m*M* lithium D-lactate as described previously (Lombardi et al., 1973; Kaback, 1971, 1974a) and in Methods. The reaction mixtures (50 μl, total volume) contained 0.05 *M* sodium phosphate (pH 6.6), 0.01 *M* magnesium sulfate, and 0.1 mg of membrane protein. Where indicated [^{3}H]triphenylmethylphosphonium (114 Ci/mol), [^{3}H]dimethyldibenzylammonium (140 Ci/mol), or rubidium-86 (14 Ci/mol) were present at final concentrations of 0.4 m*M*. When dimethyldibenzylammonium and rubidium uptake were assayed, sodium tetraphenylboron (10 μ*M*, final concentration) and valinomycin (5 μ*M*, final concentration) were present in the reaction mixtures, respectively.

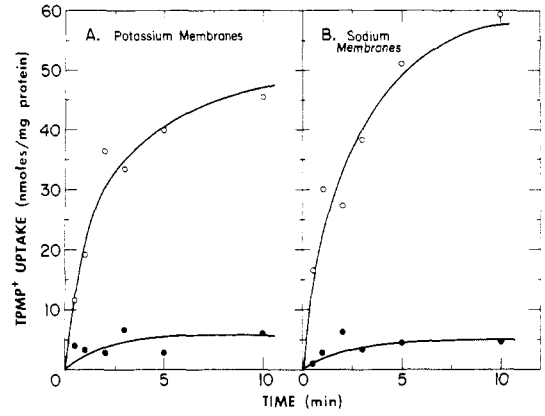

FIGURE 4: Triphenylmethylphosphonium uptake by *E. coli* ML 308-225 membrane vesicles prepared and assayed in potassium (A) or sodium (B). (A) Membrane vesicles were prepared in potassium phosphate as described previously (Kaback, 1971, 1974a) and in Methods. Uptake was determined in the presence (○) and absence (●) of 20 m*M* lithium D-lactate as described in Figure 1A except that [^{3}H]triphenylmethylphosphonium (114 Ci/mol) was used at a final concentration of 1.0 m*M*. (B) The experiment shown was carried out exactly as described in A, except that the vesicles were prepared in sodium phosphate as described previously (Lombardi et al., 1973) and the reaction mixture contained 0.1 M sodium phosphate (pH 6.6) in place of potassium phosphate.

maximum values of approximately 11 and 17 nmol per mg of membrane protein, respectively. The sharp decrease in triphenylmethylphosphonium uptake observed after 3 min under conditions where the vesicles are diluted 100-fold is probably related to the transient nature of the potassium diffusion gradient. In any case, when the vesicles are diluted into media containing an equal concentration of potassium, no triphenylmethylphosphonium uptake is observed. Data for lactose uptake under the same conditions are also presented for comparison (Figure 2B). As shown, lactose is rapidly taken up in response to a 100-fold potassium diffusion gradient, and very little uptake is observed when there is no diffusion gradient. It should be noted that a maximum of only 7–8 nmol of lactose per mg of membrane protein are taken up under conditions where approximately 15 nmol of triphenylmethylphosphonium per mg of membrane protein are accumulated.

Since the rationale behind the use of lipophilic cations is that of passive equilibration with an electrical potential across the membrane (Bakeeva et al., 1970), the steady-state level of accumulation established with this class of compounds should depend solely on the membrane potential and not on the nature of the cation. As shown in Figure 3, this is apparently the case for three different permeant cations—triphenylmethylphosphonium, dimethylbenzylammonium (in the presence of tetraphenylboron), and rubidium (in the presence of valinomycin). Although the initial rates of uptake differ slightly, it is clear that each of these cations is accumulated to essentially the same steady-state level in the presence of D-lactate. Qualitatively similar results were obtained with ascorbate–phenazine methosulfate as electron donor (data not shown). Variations in the initial rate of uptake of these cations are not surprising since the initial rate should reflect the permeability of the individual cation under investigation. In this respect, it should be emphasized that triphenylmethylphosphonium uptake does not require the addition of an ionophore (i.e., valinomycin) [Lombardi et al., 1973; Bhattacharyya et al., 1971] or a lipophilic anion (i.e., tetraphenylboron or phenyldicarbaundecaborane) [Hirata et al., 1974; Altendorf et al., 1974, 1975; Lombardi et al., 1973, 1974].

It has been reported (Hirata et al., 1973; Altendorf et al., 1975; Lombardi et al., 1973, 1974) that dimethyldibenzylammonium uptake in *E. coli* membrane vesicles is dependent upon the presence of sodium, and that maximal uptake is observed only when the vesicles are prepared in sodium-containing buffers. The suggestion has been made (Altendorf et al., 1975) that this effect is due to a requirement for sodium as a counterion for dimethyldibenzylammonium. Were this the case, triphenylmethylphosphonium and rubidium (in the presence of valinomycin) should behave similarly since they are taken up at comparable rates and to the same extent as dimethyldibenzylammonium. As shown previously (Lombardi et al., 1973), valinomycin-dependent rubidium uptake is catalyzed effectively by vesicles prepared and assayed in choline phosphate buffer. Moreover, as shown in Figure 4, ML 308-225 vesicles prepared and assayed in sodium or potassium phosphate buffers take up triphenylmethylphosphonium at essentially the same rate and to the same extent in the presence of D-lactate. It is unlikely therefore that there is a general requirement for sodium as a counterion for potential-induced cation uptake.

It was also reported previously (Lombardi et al., 1974) that exchange of intravesicular dimethyldibenzylammonium with dimethyldibenzylammonium in the external medium is inhibited by *p*-chloromercuribenzenesulfonate, a finding which is not consistent with the hypothesis that this lipophilic cation simply passively equilibrates with the membrane potential. In contrast, when similar experiments are carried out with triphenylmethylphosphonium, *p*-chloromercuribenzenesulfonate does not inhibit the rate of exchange of external triphenylmethylphosphonium with triphenylmethylphosphonium in the intravesicular pool (data not shown).

Uptake of Other Lipophilic Cations. Another cation taken up by mitochondria in response to energization is safranine O (Colonna et al., 1973). When D-lactate is added to a suspension of vesicles in the presence of safranine O, there

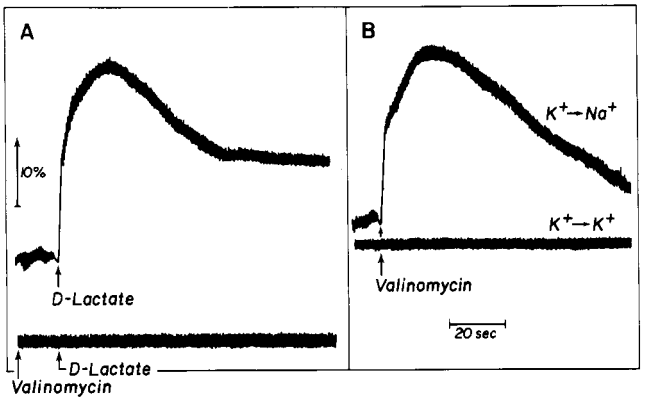

FIGURE 5: Energy-dependent changes in safranine O fluorescence. (A) Lithium D-lactate (20 mM) was added to a cuvette containing safranine O (0.13 μg/ml), 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and membrane vesicles (0.4 mg of protein/ml) in a total volume of 1.5 ml. Fluorescence at 565 nm was recorded (excitation, 495 nm) as described in Methods. Where indicated, the reaction mixtures also contained valinomycin at a final concentration of 7 μM. (B) *E. coli* ML 308-225 membrane vesicles (40 mg of protein/ml) suspended in 0.1 M potassium phosphate (pH 6.6) were diluted 150-fold into a cuvette containing either 0.1 M sodium phosphate (pH 6.6) or potassium phosphate (pH 6.6), as indicated, and safranine O (0.13 μg/ml). The final volume was 1.5 ml. After recording the initial fluorescence value, valinomycin was added to a final concentration of 10 μM, and recording was resumed as described in Methods as rapidly as possible.

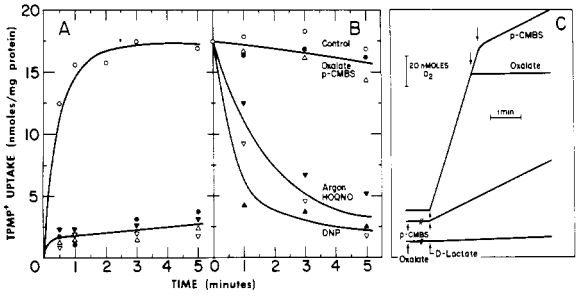

FIGURE 6: Effect of metabolic inhibitors on the rate and extent of D-lactate-dependent triphenylmethylphosphonium uptake and D-lactate oxidation. (A and B) Triphenylmethylphosphonium uptake was determined in the presence of 20 mM lithium D-lactate as described in Figure 1A. Inhibitors were added either 2 min before D-lactate (A) or 5 min after addition of D-lactate (B) to give the following final concentrations: sodium oxalate (●) 20 mM; 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO, ▼), 0.08 mM; p-chloromercuribenzenesulfonate (p-CMBS, △), 0.1 mM. Where indicated (▽) samples were gassed with argon by methods described previously (Kaback and Barnes, 1971). (C) Rates of oxygen uptake were measured at 25° as described in Methods in 1.0 ml (total volume) containing 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and 0.4 mg of protein/ml. lithium D-lactate (20 mM, final concentration) was added as indicated. The inhibitors were added either 2 min prior to addition of D-lactate or as indicated by the arrows to give the following final concentrations: sodium oxalate, 20 mM; p-chloromercuribenzenesulfonate, 0.1 mM. Tracings showing the effects of oxalate and p-chloromercuribenzenesulfonate addition after D-lactate were derived from separate experiments.

is a rapid increase in fluorescence which achieves a maximum within 1–2 min, and subsequently declines to about 50% of the maximum level (Figure 5A). When valinomycin is added, no increase in fluorescence is observed on addition of D-lactate. Although the reason for the "overshoot" observed with D-lactate is not understood, similar enhancement in safranine O fluorescence can be induced by a potassium diffusion gradient such that an electrical potential (interior negative) is imposed across the membrane (Figure 5B). Other experiments (not shown) demonstrate directly that the effects of D-lactate on safranine O fluorescence observed in Figure 5A are due primarily to uptake of the cation and not to a change in the properties of membrane-bound dye. When vesicles are incubated with safranine O in the presence and absence of D-lactate, and subsequently collected by either Millipore filtration or centrifugation followed by extraction of the dye, addition of D-lactate markedly increases the absolute amount of safranine O recovered from the vesicles.

In distinct contrast to the cations described thus far, [^{3}H]tetraphenylarsonium is not taken up to a significant extent by ML 308-225 vesicles in the presence or absence of D-lactate or ascorbate–phenazine methosulfate, and the addition of tetraphenylboron or phenyldicarbaundecaborane has no effect. Moreover, the same negative results were obtained with vesicles prepared in sodium phosphate buffer. This result is puzzling in light of the observation that this cation is an effective inhibitor of D-lactate-dependent active transport but does not affect D-lactate oxidation (i.e., the compound is an effective uncoupling agent) [Lombardi et al., 1973].

Effect of Metabolic Inhibitors on the Rate and Steady-State Level of Triphenylmethylphosphonium Accumulation. The rate and extent of triphenylmethylphosphonium uptake in the presence of D-lactate is markedly inhibited by anoxia, 2-heptyl-4-hydroxyquinoline N-oxide, p-chloromercuribenzenesulfonate, oxalate, and 2,4-dinitrophenol (Figure 6A). Moreover, as shown previously (Barnes and Kaback, 1971; Reeves et al., 1973a), each of these inhibitors, with the exception of dinitrophenol, causes marked inhibition of D-lactate oxidation. 2,4-Dinitrophenol has no effect on D-lactate oxidation, and is presumed to act by increasing the permeability of the membrane to protons (Mitchell, 1966, 1973; Harold, 1972; Mitchell and Moyle, 1967). The sites of inhibition of each of the electron transfer inhibitors used here have been described (Barnes and Kaback, 1971; Kaback and Barnes, 1971; Cox et al., 1970). 2-Heptyl-4-hydroxyquinoline N-oxide inhibits between cytochromes b_1 and a_2, p-chloromercuribenzenesulfonate inhibits between the primary dehydrogenases for D-lactate and succinate and cytochrome b_1, and oxalate is a potent competitive inhibitor of D-lactate dehydrogenase. These observations indicate that the generation of the membrane potential by means of D-lactate oxidation requires the passage of electrons from D-lactate dehydrogenase to oxygen via the membrane-bound respiratory chain, and that cessation of electron flow at any level is sufficient to prevent the generation of the potential.

When the effect of these inhibitors on the steady-state level of triphenylmethylphosphonium accumulation in the presence of D-lactate is studied, a striking difference is observed with the various inhibitors (Figure 6B). In this experiment, each inhibitor was added after the vesicles were loaded with triphenylmethylphosphonium by previous incubation in the presence of D-lactate. As shown, anoxia (argon) and 2-heptyl-4-hydroxyquinoline N-oxide, each of which inhibit electron flow after cytochrome b_1, and 2,4-dinitrophenol cause rapid efflux of triphenylmethylphosphonium. In contrast, oxalate which virtually completely and instantaneously inhibits D-lactate oxidation (Figure 6C) and p-chloromercuribenzenesulfonate which inhibits D-lactate oxidation by approximately 85% (Figure 6C) do not cause significant efflux of triphenylmethylphosphonium from the intravesicular pool. Earlier studies (Kaback and

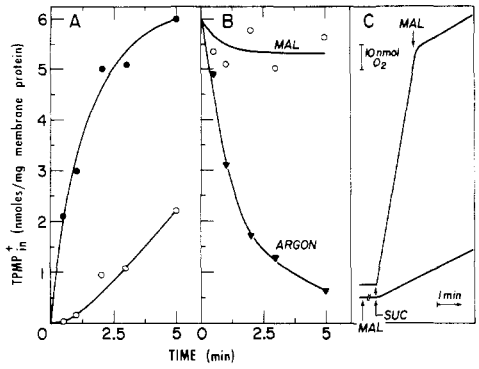

FIGURE 7: Effect of metabolic inhibitors on the rate and extent of succinate-dependent triphenylmethylphosphonium uptake and succinate oxidation. (A and B) Triphenylmethylphosphonium uptake was determined in the presence of 20 m*M* sodium succinate (●) as described in Figure 1A. Sodium malonate (10 m*M*, final concentration) was added either 2 min before succinate (A; ○) or 5 min after addition of succinate (B; ○). Where indicated (▼) samples were gassed with argon by methods described previously (Kaback and Barnes, 1971). (C) Rates of oxygen uptake were measured as described in Figure 6C, except that sodium succinate was used in place of D-lactate. Sodium malonate (10 m*M*, final concentration) was added either 2 min prior to addition of succinate or as indicated by the arrows.

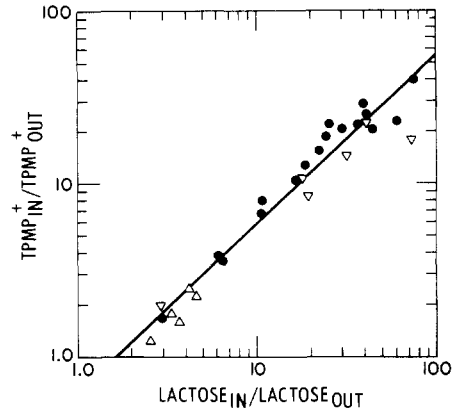

FIGURE 8: Relationship between the membrane potential and lactose accumulation. Triphenylmethylphosphonium and lactose uptake were assayed at 5 min as described in Figure 1 under the following sets of conditions: in the presence of ascorbate-phenazine methosulfate, D-lactate, L-lactate, succinate, NADH, or in the absence of exogenous electron donors (▽); in the presence of ascorbate-phenazine methosulfate and increasing concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (●); and in the presence of D-lactate and increasing concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide (△). The log of the apparent concentration gradient of triphenylmethylphosphonium is plotted as a function of the log of the apparent concentration gradient of lactose.

Barnes, 1971; Lombardi and Kaback, 1972) demonstrate that the vesicles exhibit similar properties with respect to a number of different physiological transport substrates, in addition to rubidium (in the presence of valinomycin) [Lombardi et al., 1973] and dimethyldibenzylammonium (in the presence of tetraphenylboron) [Altendorf et al., 1975], and Reeves et al. (1972) have demonstrated that D-lactate-dependent quenching of 8-anilino-1-naphthalenesulfonate fluorescence exhibits similar properties. These observations suggest that inhibition of electron flow after the energy-coupling site causes rapid dissipation of the membrane potential, while inhibition before this site does not dissipate the potential despite almost complete inhibition of D-lactate oxidation.

As shown by the data presented in Figure 7, these effects are not peculiar to D-lactate-dependent triphenylmethylphosphonium accumulation. Thus, succinate-dependent triphenylmethylphosphonium uptake is markedly inhibited by malonate (Figure 7A), a competitive inhibitor of succinate dehydrogenase which inhibits succinate oxidation by approximately 90% (Figure 7C). However, as shown in Figure 7B, when the vesicles are allowed to accumulate triphenylmethylphosphonium to a steady state in the presence of succinate, followed by addition of malonate, there is little or no efflux of accumulated triphenylmethylphosphonium. On the other hand, when the reaction mixture is gassed with argon, rapid efflux of triphenylmethylphosphonium is observed. Although not shown, similar data have been obtained for succinate-dependent lactose uptake.

Relationship between the Membrane Potential and Active Transport. If the active transport of metabolites such as lactose and amino acids is directly and obligatorily coupled to the membrane potential, and if triphenylmethylphosphonium accumulation reflects that potential, there should be a direct relationship between the accumulation of the physiologic transport substrates and the accumulation of triphenylmethylphosphonium. Moreover, alteration of the membrane potential should be reflected in an appropriate alteration in active transport regardless of the means used to alter the potential. The data presented in Figure 8 are derived from experiments in which the steady-state level of lactose and triphenylmethylphosphonium accumulation with different electron donors was measured at 5 min in the presence and absence of various concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide or carbonyl cyanide *m*-chlorophenylhydrazone. The apparent concentration gradients achieved with each substrate in the presence of each electron donor and at each concentration of inhibitor were calculated, and the log of the triphenylmethylphosphonium concentration gradient was plotted as a function of the log of the lactose concentration gradient. It is clear from the data that the accumulation of lactose is directly related to the magnitude of the membrane potential as determined by triphenylmethylphosphonium accumulation under a wide variety of conditions. Moreover, it is also clear that within experimental error, there is no accumulation of lactose under conditions where there is no accumulation of triphenylmethylphosphonium (i.e., when there is no membrane potential). Although the data will not be presented, similar relationships between triphenylmethylphosphonium accumulation and the accumulation of proline, tyrosine, glutamic acid, and glycine are also apparent. It is noteworthy, however, that lysine accumulation by the vesicles does not exhibit a similar relationship to triphenylmethylphosphonium accumulation. Under conditions in which the concentration gradient for triphenylmethylphosphonium is reduced to approximately 2–3, the vesicles are still able to accumulate lysine against a 28- to 30-fold gradient. It is interesting that lysine transport is also less sensitive to inhibition by carbonyl cyanide *m*-chlorophenylhydrazone relative to other amino acids and lactose (Kaback et al., 1974).

If most of the respiration-linked transport systems in *E. coli* membrane vesicles are tightly coupled to the membrane potential, as suggested by the experiments presented above, it is possible that transport of a solute with a relatively high maximum velocity might inhibit the uptake of a solute which is transported at a much lower rate. In the experiment shown in Figure 9A, D-lactate-dependent proline uptake by ML 308-225 vesicles was assayed in the presence and absence of lactose at a concentration approximately

20-fold higher than the apparent K_m of the *lac* transport system for this substrate. As shown, in the presence of lactose, the rate and extent of proline uptake are inhibited by approximately 50%. When the same experiment is carried out with vesicles prepared from uninduced *E. coli* ML 30 which do not catalyze lactose transport, proline transport is not affected by lactose (Figure 9B). The data presented in Figures 9C and D are consistent with the suggestion that this effect is mediated via the membrane potential. Thus, triphenylmethylphosphonium uptake by ML 308-225 vesicles is inhibited by approximately 30% when the assay is carried out in the presence of lactose (Figure 9C), while triphenylmethylphosphonium uptake by uninduced ML 30 vesicles is unaffected by lactose (Figure 9D).

D-*Lactate-Dependent Proton Efflux and the Properties of the Proton Gradient.* Asymmetric proton movement during electron transfer is one of the basic tenets of the chemiosmotic hypothesis (Mitchell, 1966, 1973; Harold, 1972), and proton efflux during D-lactate oxidation has been described in *E. coli* membrane vesicles (Lombardi et al., 1973; Reeves, 1971), although its significance has been questioned (Lombardi et al., 1973). If the membrane potential is generated by proton extrusion during D-lactate oxidation, the generation and maintenance of a proton gradient by the vesicles should exhibit properties analogous to those described above for triphenylmethylphosphonium accumulation. That is, proton conductors and inhibitors of D-lactate oxidation should inhibit the formation of the proton gradient, and only proton conductors and those inhibitors which block respiration after the energy-coupling site for transport should rapidly dissipate the proton gradient once it has been established. These predictions are borne out by the experiments presented in Figure 10. As shown in panel B, addition of an aliquot of aerated buffer to a sealed suspension of ML 308-225 vesicles which has become anaerobic as a result of D-lactate oxidation results in rapid acidification of the medium. Within about 5–10 sec, the pH trace achieves a steady state which is maintained until the oxygen in the reaction mixture is depleted again at which time the pH returns rapidly to approximately its original value. Thus, generation of a proton gradient in the presence of D-lactate depends upon electron transfer, and the gradient dissipates rapidly when the respiratory chain becomes reduced. In the experiments shown in panels A and C, similar pH measurements were carried out under aerobic conditions. As shown, under these conditions as well, addition of D-lactate results in acidification of the medium, but the generation of the proton gradient takes longer, and it is maintained for a longer period of time (note the difference in the time scale in panel B and panels A and C). When carbonyl cyanide *m*-chlorophenylhydrazone is added to the reaction mixture (panel C) or when it is gassed with argon or 2-heptyl-4-hydroxyquinoline *N*-oxide is added (data not shown), the D-lactate-induced pH change is rapidly reversed. In contrast, however, addition of oxalate at a concentration which virtually completely and instantaneously blocks D-lactate oxidation (cf. Figure 6C) results in slow and incomplete reversal of the D-lactate-induced proton gradient (panel A). Similar results, although not shown, were also obtained with *p*-chloromercuribenzenesulfonate. It should be emphasized that each of these reagents, oxalate and *p*-chloromercuribenzenesulfonate in particular, inhibit D-lactate-induced proton extrusion drastically when added before D-lactate.

Triphenylmethylphosphonium and Lactose Uptake by Intact Cells. Although triphenylmethylphosphonium uptake is not observed with freshly harvested *E. coli* ML 308-225 (data not shown), uptake of the cation can be affected by treatment of the cells with ethylenediaminetetraacetic acid (Griniuviene et al., 1974). As shown by the data presented in Figure 11A, triphenylmethylphosphonium is taken up by appropriately treated cells, and within 20–30 min, the cells approach a steady-state concentration of approximately 17 nmol per mg of cell protein, a value which corresponds to an apparent concentration gradient of 8–9 at an external concentration of 0.4 m*M*. When the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone is added to the reaction mixture, almost all of the accumulated triphenylmethylphosphonium is lost from the cells within 30 sec. Similarly, addition of valinomycin also induces rapid efflux of triphenylmethylphosphonium, although the extent of loss is not so great as that observed with carbonyl cyanide *m*-chlorophenylhydrazone (Figure 11A). Although not shown, 2,4-dinitrophenol also induces rapid efflux of the lipophilic cation. In contrast to these observations with triphenylmethylphosphonium, as described above with vesicles, EDTA-treated cells also do not accumulate tetraphenylarsonium.

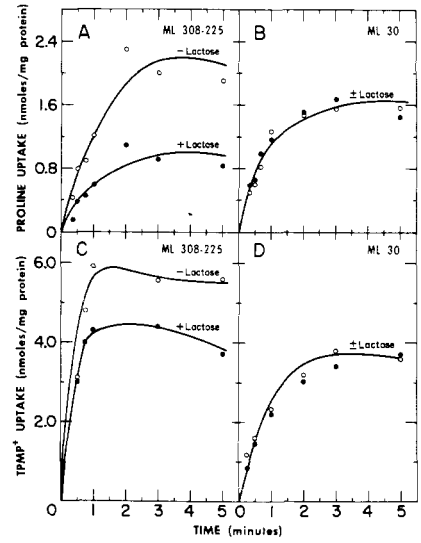

FIGURE 9: Effect of lactose on proline and triphenylmethylphosphonium uptake. Proline and triphenylmethylphosphonium uptake were determined in the presence of 20 m*M* lithium D-lactate as described in Methods. Reaction mixtures (50 μl, total volume) contained 0.05 *M* potassium phosphate (pH 6.6), 0.01 *M* magnesium sulfate, and 0.1 mg of membrane protein. In panels A and C, *E. coli* ML 308-225 membrane vesicles were used; in panels B and D, membrane vesicles from uninduced *E. coli* ML 30 were used. [U-^{14}C]Proline (240 Ci/mol) uptake was assayed at a final concentration of 8.3 μ*M*; [^{3}H]triphenylmethylphosphonium (114 Ci/mol) uptake was assayed at a final concentration of 0.4 m*M*. When lactose was present (●), it was added 2 min prior to the addition of D-lactate to a final concentration of 10 m*M*. (○) Uptake in the absence of lactose.

In comparison to triphenylmethylphosphonium, the cells, like vesicles, take up lactose more rapidly, achieving a steady-state level of about 100 nmol/mg of protein within 5–10 min (Figure 11B). This value corresponds to an apparent concentration gradient of about 80 which is slightly lower than the estimate of Winkler and Wilson (1966) who studied freshly harvested *E. coli* ML 308-225.

Discussion

The results presented in this paper provide convincing evidence that oxidation of D-lactate or reduced phenazine

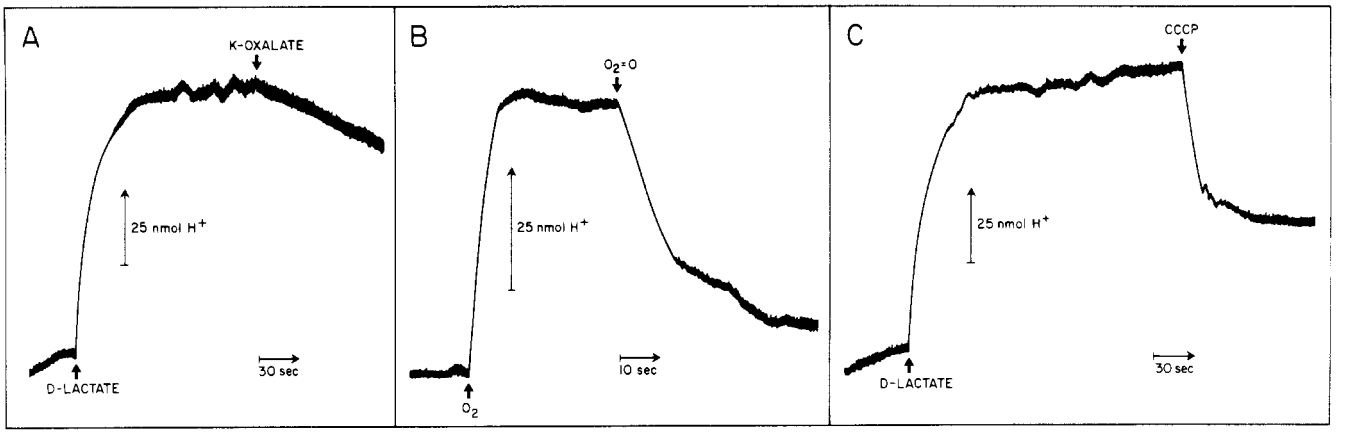

FIGURE 10: Effect of metabolic inhibitors on D-lactate-induced proton release by *E. coli* ML 308-225 membrane vesicles. Membrane vesicles were washed twice in 0.066 *M* potassium chloride containing 2 m*M* potassium phosphate (pH 6.6) and resuspended at a membrane protein concentration of 20 mg/ml. Proton extrusion was assayed in 1.4 ml (total volume) containing 0.066 *M* potassium chloride, 2 m*M* potassium phosphate (pH 6.6), and 2.5 mg of membrane protein/ml. In panels A and C, the experiments were carried out in an open chamber and the reaction was initiated by addition of 10 m*M* lithium D-lactate (final concentration). Where indicated, 10 m*M* potassium oxalate (panel A) or 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [panel C] were added. In panel B, the chamber was sealed with a rubber stopper in which there was a single small portal for addition of samples. Lithium D-lactate (10 m*M*, final concentration) was added to the chamber, and the reaction was allowed to continue until the system became anaerobic (about 6 min). At this time, 25 μl of an oxygen saturated solution of 0.066 *M* potassium chloride was added. The pH changes were calibrated by addition of known amounts of hydrochloric acid, and the pH was monitored with a Radiometer GK 2321C pH electrode connected to a Radiometer pH meter (Model 26). The signal was amplified and recorded in a double channel Corning recorder (Model 845).

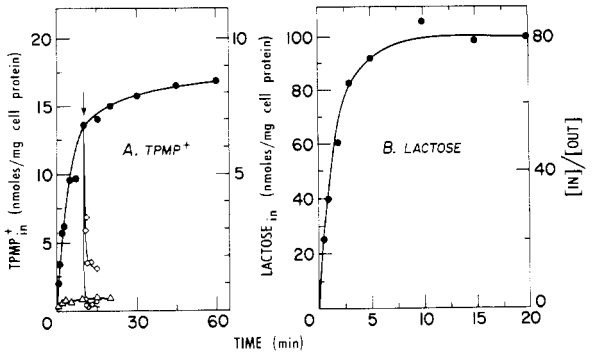

FIGURE 11: Uptake of triphenylmethylphosphonium and lactose by EDTA-treated *E. coli* ML 308-225. *E. coli* ML 308-225 were grown and treated with EDTA as described in Methods. Uptake was measured as described previously (Kaback, 1971, 1974a) and in Methods in 50-μl reaction mixtures (total volume) containing 0.05 *M* potassium phosphate (pH 6.6), 0.01 *M* magnesium sulfate, 0.02 *M* sodium succinate, and 0.075 mg of cell protein. [^{3}H]Triphenylmethylphosphonium (114 Ci/mol), [^{3}H]tetraphenylarsonium (58.8 Ci/mol) [A] or [1-^{14}C]lactose (22 Ci/mol) [B] were used at final concentrations of 0.4 m*M*. Where indicated (arrow in panel A), carbonylcyanide *m*-chlorophenylhydrazone (○) or valinomycin (◇) were added at final concentrations of 10 and 5 μM, respectively. (●) Uptake of triphenylmethylphosphonium (A) or lactose (B); (△) uptake of tetraphenylarsonium. Apparent concentration gradients were calculated using a value of 5.4 μl of intracellular water per mg of cell protein as determined from the data of Winkler and Wilson (1966).

methosulfate by isolated membrane vesicles from *E. coli* results in the generation of a membrane potential, interior negative. Like rubidium (in the presence of valinomycin) and dimethyldibenzylammonium (in the presence of tetraphenylboron or phenyldicarbaundecaborane), triphenylmethylphosphonium and safranine O are accumulated by the vesicles in the presence of appropriate electron donors. Moreover, triphenylmethylphosphonium and safranine O are accumulated when a potassium diffusion gradient is imposed across the vesicle membrane, and the vesicles exhibit the same steady-state level of accumulation of triphenylmethylphosphonium, dimethyldibenzylammonium (in the presence of tetraphenylboron), and rubidium (in the presence of valinomycin). A note of caution should be added, however, with regard to the general use of lipophilic cations to determine potentials in systems which are not amenable to a direct electrophysiological approach. Tetraphenylarsonium, a lipophilic cation which is an effective uncoupling agent in isolated membrane vesicles (Lombardi et al., 1973), is not taken up to any significant extent by either membrane vesicles or EDTA-treated whole cells. Although this anomalous behavior may be related to the net charge on the lipids in the particular membrane under investigation (Haydon and Hladky, 1972), this is probably not the only explanation, as *Staphylococcus aureus* vesicles also do not take up this cation despite a high concentration of anionic phospholipids relative to *E. coli* vesicles (Short and White, 1971).

Regarding the basic contention that the accumulation of lipophilic cations by isolated membrane vesicles reflects the generation of an electrical potential, negative inside, the results presented here with triphenylmethylphosphonium and safranine O are considerably more clear-cut than previous studies with rubidium and valinomycin or dimethyldibenzylammonium and tetraphenylboron (or phenyldicarbaundecaborane). Accumulation of triphenylmethylphosphonium and safranine O occurs in the absence of ionophores or lipophilic anions, is independent of the cation in which the vesicles are prepared or assayed, and does not exhibit properties associated with carrier-mediated transport systems (i.e., exchange of triphenylmethylphosphonium is not inhibited by *p*-chloromercuribenzenesulfonate).

As described previously for a number of solutes including rubidium in the presence of valinomycin (Barnes and Kaback, 1971; Lombardi et al., 1973; Lombardi and Kaback, 1972; Reeves et al., 1972), accumulation of triphenylmethylphosphonium is relatively specific for D-lactate or reduced phenazine methosulfate as electron donors. Since there is an accumulating body of evidence (Kaback, 1972, 1973, 1974a; Kaback and Hong, 1973; Short et al., 1974,

1975a,b; Altendorf and Staehelin, 1974; Konings et al., 1973; Rosen and McClees, 1974) indicating that few, if any, of the vesicles in these preparations are inverted, it is unlikely that the relative inability of other electron donors to drive active transport can be attributed to an artifact of this nature (Mitchell, 1973; Harold, 1972; Hare et al., 1974). Thus, based on previous observations (Kaback and Barnes, 1971; Altendorf et al., 1975; Lombardi et al., 1973; Lombardi and Kaback, 1972; Reeves et al., 1972; Stroobant and Kaback, 1975), as well as those presented in this paper, it appears likely that there is a site located relatively specifically within the portion of the respiratory chain between D-lactate dehydrogenase and cytochrome b_1 which is responsible for the generation of the membrane potential.

Evidence is also presented in this paper which provides a strong indication that the generation of a membrane potential, interior negative, is intimately related to the ability of the vesicles to catalyze active transport of lactose and amino acids. Steady-state levels of lactose, proline, tyrosine, glutamic acid, and glycine accumulation in the presence of D-lactate and ascorbate–phenazine methosulfate are related to the steady-state level of triphenylmethylphosphonium accumulation measured under identical conditions. Assuming that the accumulation of triphenylmethylphosphonium does in fact reflect the presence of a membrane potential, interior negative, these results provide a strong indication that D-lactate and reduced phenazine methosulfate oxidation are coupled to these transport systems primarily via the membrane potential. Further evidence in support of this hypothesis stems from experiments demonstrating that addition of lactose to membrane vesicles containing the *lac* transport system inhibits uptake of both proline and triphenylmethylphosphonium. A reasonable explanation for these observations is that lactose transport partially dissipates the membrane potential in a manner similar to that described for glucose in *Neurospora crassa* by Slayman and Slayman (1974) who measured the potential directly. However, despite the attractiveness of lactose/proton symport as a mechanism for explaining these effects, all efforts to demonstrate this phenomenon directly in isolated membrane vesicles have been negative thus far.

Recent experiments (Schuldiner et al., 1975; Rudnick et al., 1975a, 1975b) indicate that the primary event in the active transport of β-galactosides in *E. coli* ML 308-225 membrane vesicles is the generation of a membrane potential, exterior positive, which results in the appearance of binding sites on the exterior surface of the membrane. To account for the observations, it was postulated that a site in the *lac* carrier protein is negatively charged. In order to translocate such a negatively charged site back to the inner surface of the membrane, the charge would have to be neutralized. This could be accomplished by binding of protons as postulated by Mitchell (1966, 1973) [Harold, 1972] or other cations, or by shielding of the charge by binding of ligand.

The observation that only certain electron transfer inhibitors induce efflux of solutes accumulated by the vesicles has been interpreted as being inconsistent with a chemiosmotic mechanism for active transport (Kaback, 1972, 1973, 1974b; Kaback and Hong, 1973; Lombardi et al., 1973, 1974) because it is not immediately obvious why only certain electron transfer inhibitors should collapse the membrane potential and/or the pH gradient generated by D-lactate oxidation. As demonstrated in this paper, similar effects are observed with D-lactate- and succinate-dependent triphenylmethylphosphonium accumulation, as well as D-lactate-dependent proton extrusion. Other observations have demonstrated that valinomycin-induced rubidium (Lombardi et al., 1973) and tetraphenylboron-dependent dimethyldibenzylammonium uptake (Altendorf et al., 1975; Lombardi et al., 1974) also exhibit similar properties. As shown previously (Barnes and Kaback, 1971; Reeves et al., 1973a), and again in these studies, the inability of oxalate, *p*-chloromercuribenzenesulfonate, and malonate to cause efflux cannot be due to the failure of these compounds to block D-lactate or succinate oxidation. Since this phenomenon has now been observed with protons and with a number of compounds which appear to equilibrate with the membrane potential, it is not unreasonable to suggest that inhibition of electron flow in a manner which leads to reduction of the energy-coupling site leads to dissipation of the membrane potential, while inhibition of electron flow in a manner which leads to oxidation of the energy-coupling site does not result in collapse of the potential. In other words, one explanation that would account for the data is that the membrane per se is relatively impermeable to ions, including protons, and that dissipation of a preexisting potential occurs through a proton "translocator" which is either an integral part of the respiratory chain or in equilibrium with the energy-coupling site. By this means, in the reduced form, the translocator would catalyze net flux of protons across the membrane, and rapid dissipation of the membrane potential would occur. On the other hand, in the oxidized form, the proton translocator would be unable to catalyze net flux, and dissipation of the potential would occur slowly by passive leakage pathways. Assuming that the carriers are in equilibrium with the membrane potential which, in turn, is in equilibrium with the energy-coupling site, certain aspects of theories which have been looked upon as being mutually exclusive would appear to be resolved (Kaback, 1974b).

Despite convincing evidence supporting a chemiosmotic mechanism for active transport in isolated membrane vesicles, a few inconsistencies remain which are not readily explained by the available data. As shown in this paper, membrane vesicles and EDTA-treated whole cells catalyze lactose accumulation at least three to five times better than triphenylmethylphosphonium. Based on a mechanism in which there is one positive charge (i.e., one proton) taken up per mole of lactose (West and Mitchell, 1972, 1973), a potential of approximately -120 mV is required to achieve a lactose concentration gradient of 100. However, based on these studies, the vesicles generate a potential of only about -75 mV as determined with triphenylmethylphosphonium, dimethyldibenzylammonium (in the presence of tetraphenylboron), or rubidium (in the presence of valinomycin). Thus, it seems apparent that although the vesicles generate a membrane potential of the appropriate polarity, the magnitude of the potential is not sufficient to account for the phenomena observed. A similar situation has also been described in mitochondria (Rottenberg, 1970) and in chloroplasts (Schuldiner et al., 1972; Rottenberg et al., 1972). In addition, it is apparent that triphenylmethylphosphonium accumulation in response to an artificially generated membrane potential approximates that observed during D-lactate or reduced phenazine methosulfate oxidation (cf. compare Figure 1A with Figure 2A), while lactose accumulation in response to the artificially generated potential is considerably less than that observed with D-lactate or ascorbate–phenazine methosulfate (compare Figure 1B with Fig-

ure 2B). Insofar as the present studies are concerned, a few possibilities should be entertained as explanations for these inconsistencies. First, and most obvious, the accumulation of lipophilic cations or rubidium (in the presence of valinomycin) may not represent a truly quantitative estimate of the membrane potential. Second, there may be more than one positive charge taken up per mole of solute. In this regard, the data presented in Figure 8 fit best with a mechanism in which two positive charges are taken up with each mole of lactose. Third, there may be another high-energy intermediate in addition to the membrane potential. This hypothetical intermediate would be generated by D-lactate or ascorbate-phenazine methosulfate oxidation, but not be an artificially imposed membrane potential. One candidate for this intermediate which fulfills these criteria is the chemical gradient of protons. However, no significant uptake of 5,5-dimethyloxazolidine-2,4-dione [Waddel and Butler, 1959] is observed when vesicles are incubated in the presence of D-lactate or ascorbate-phenazine methosulfate, although uptake of the weak acid can be induced by an artificially imposed pH gradient. Hopefully, some of these problems will be resolved by more quantitative estimates of the membrane potential and by the development of techniques which allow an assessment of solute/proton symport in the vesicle system.

Acknowledgments

The authors are deeply indebted to Dr. Arnold Liebman of The Isotope Synthesis Group at Hoffmann-La Roche Inc. for preparing tritiated triphenylmethylphosphonium and tetraphenylarsonium. They would also like to thank Dr. E. M. Barnes, Jr., of Baylor College of Medicine for his gift of sodium phenyldicarbaundecaborane.

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Kinetic Light Scattering Studies on the Dissociation of Hemoglobin from *Lumbricus terrestris*†

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ABSTRACT: The kinetics of the pH-induced dissociation of the 3×10^6 mol wt hemoglobin from *Lumbricus terrestris* (the earthworm) have been studied in a light-scattering stopped-flow apparatus. The ligand dependent dissociation data were fit well by a simple sequential model. The data for CO and oxyhemoglobin are consistent with $Hb_{12} \rightarrow 2Hb_6 \rightarrow 12Hb$. Methemoglobin at pH 7 appears to be hexameric and the dissociation is consistent with the model: $Hb_6 \rightarrow 6Hb$. In a sequential decay scheme for which light-scattering changes are monitored, the relative amounts of rapid and slow phase are determined by the rate constants as well as the molecular weights of intermediate species. Assignment of the hexameric intermediate is supported by an investigation of the sensitivity of the theoretical kinetic curves to the molecular weights of the intermediates. This assignment is further supported by the following: (1) the same model will fit the data for oxy- and CO-hemoglobin at all three temperatures (a 24–29-fold variation in rate constants), (2) evidence from electron microscopy shows hexameric forms, and (3) methemoglobin is apparently stable as a hexamer at pH 7. When CO replaces O_2 as the ligand, the dissociation rate *increases* by a factor of four. The met dissociation rate is about 20 times faster than the initial oxyhemoglobin dissociation rate, but perhaps more relevant for comparing dissociation of the hexamer, the met rate was respectively 100 times and 500 times faster than that for the assumed hexameric forms of CO- and oxy-hemoglobin. The activation energies for the dodecamer to hexamer dissociation and for the dissociation of the hexamer to smaller forms were about 30 kcal/mol for oxy-, CO-, and methemoglobin.

The hemoglobin from the earthworm (*Lumbricus terrestris*), one of the largest of the known respiratory proteins, has a molecular weight of about 3×10^6 (Rossi-Fanelli et al., 1970) and is reported to contain 192 hemes (Wiechelman and Parkhurst, 1972). It has been shown to be highly cooperative in binding oxygen, with a Hill number of 5.4 (Cosgrove and Schwartz, 1965). Electron microscopy studies of this protein reveal a structure consisting of 12 subunits, arranged with two regular hexagons face to face (Levin, 1963, Roche, 1965). The molecule has dimensions of 265 Å in length (measured between opposite vertices within a hexagon), 160 Å in width, and 160 Å in thickness (Levin, 1963; Roche, 1965). This oxyhemoglobin structure dissociates at pH 10.2 into the one-twelfth subunits (Levin, 1963) containing 12–16 hemes (Wiechelman and Parkhurst, 1972; Chiancone et al., 1972), and in some cases further dissociation occurs (Levin, 1963; Roche, 1965).

Ligand kinetic studies of this protein were first carried out by Salomon (1941) who measured the rate of oxygen dissociation at pH 8 and 23°. Later Gibson (1955) reported rates for CO combination at pH 6.3 and 9.2 and oxygen dissociation at pH 6.7 and 9.2, all at 20°. Wiechelman and Parkhurst (1972) have studied oxygen dissociation and CO combination as a function of pH and protein concentration and also reported that the deoxyhemoglobin was much less dissociated at pH 10.3 than was the ligand-bound form.

We wish to report here an extensive investigation of the kinetics of the pH-induced dissociation for various ligand forms of the protein.

Experimental Section

Materials

The worms were obtained locally from commercial sources and the hemoglobin was collected as described by Boelts and Parkhurst (1971). The hemoglobin was sedimented by centrifugation in a Spinco Model L ultracentrifuge at 85000*g* for 2.25 hr at 4°C. The hemoglobin pellet was dissolved in 0.001 *M* potassium phosphate buffer (pH 7). Dissociation was induced in the light-scattering, stopped-flow apparatus by flowing equal volumes of the protein in low ionic strength (1 m*M*) pH 7 potassium phosphate buffer against pH 10.7, 0.05 *M* borate buffer ("jump buffer") so that the final pH was 10.3. The initial hemoglobin concentration for dissociation measurements was 120 μM in heme (0.2%). There were no significant changes in

† From the Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588. *Received June 26, 1975.* This work was supported by grants from the Research Council of the University of Nebraska (Biomedical Sciences Support Grant RR-07055-09), Research Corporation, a Nebraska Heart Association Grant-in-Aid, and National Institutes of Health Grant No. HL-15284-03.

‡ Taken in part from a thesis to be presented to the University of Nebraska in partial fulfillment of the requirements of the Ph.D. degree.

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