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Sodium-Dependent Methyl 1-Thio-β-D-galactopyranoside Transport in Membrane Vesicles Isolated from *Salmonella typhimurium*†

Hajime Tokuda and H. Ronald Kaback*

ABSTRACT: Membrane vesicles isolated from *Salmonella typhimurium* G-30 grown in the presence of melibiose catalyze methyl 1-thio-β-D-galactopyranoside (TMG) transport in the presence of sodium or lithium, as shown initially with intact cells by Stock and Roseman (Stock, J., and Roseman, S. (1971), *Biochem. Biophys. Res. Commun. 44,* 132). TMG-dependent sodium uptake is also observed, but only when a potassium diffusion potential (interior negative) is induced across the vesicle membrane. Cation-dependent TMG accumulation varies with the electrochemical gradient of protons generated as a result of D-lactate oxidation, and the vesicles catalyze D-lactate-dependent sodium efflux in a manner which is consistent with the operation of a proton–sodium exchange mechanism. Although the stoichiometry between sodium and TMG appears to be 1:1 when transport is induced by a potassium diffusion potential, evidence is presented which indicates that the relationship may exceed unity under certain conditions. The results are explained in terms of a model in which TMG–sodium (lithium) symport is driven by an electrochemical gradient of protons which functions to maintain a low intravesicular sodium or lithium concentration through proton–sodium (lithium) antiport.

Recent studies confirm the hypothesis that chemiosmotic phenomena, as postulated by Mitchell (1961, 1966, 1968, 1973), are responsible for respiration-linked active transport in membrane vesicles isolated from *Escherichia coli* (for reviews, see Harold, 1972, 1976; Ramos et al., 1976; Ramos and Kaback, 1977a,b; Kaback, 1976). Oxidation of electron donors which drive transport in the vesicles leads to the development of an electrochemical gradient of protons across the membrane, and it has been shown that this thermodynamic entity is composed of interconvertible electrical and chemical parameters according to the following relationship:

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

where $\Delta\bar{\mu}_{H^+}$ represents the electrochemical gradient of protons, $\Delta\Psi$ denotes the electrical potential across the membrane, and ΔpH is the chemical difference in proton concentrations across the membrane ($2.3RT/F$ = 58.8 mV at room temperature).

Evidence has also been presented (Harold, 1972, 1976; Ramos and Kaback, 1977a,b; Kaback, 1976) which indicates that $\Delta\bar{\mu}_{H^+}$ or one of its components is the immediate driving force for the accumulation of a variety of different solutes and that accumulation of certain solutes most probably occurs via coupled movements with protons (i.e., symport). By this means, a substrate-specific membrane protein (i.e., a porter or carrier) translocates substrate with one or more protons, the substrate moving against and the proton(s) with their respective electrochemical gradients. The net result, substrate accumulation, is accomplished energetically by removing protons from the internal space.

One attractive conceptual aspect of the chemiosmotic hypothesis for bacterial active transport is its analogy to the mechanism suggested for sugar and amino acid transport in many eucaryotic cells (Crane, 1977). In these systems, an electrochemical gradient of sodium, rather than protons, is generated through the action of the membraneous sodium, potassium-dependent ATPase, and accumulation of sugars and amino acids occurs via coupled movements with sodium (this process is referred to traditionally as cotransport rather than symport).

Although it is almost certain that many bacterial transport systems catalyze proton–substrate symport, several instances have been reported in which the transport of a specific solute is dependent upon the presence of sodium or lithium ion (Drapeau et al., 1966; Wong et al., 1969; Thompson and MacLeod, 1971; Sprott and MacLeod, 1972; Harold and Baarda, 1967; Harold et al., 1970; Stock and Roseman, 1971;

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Gale, 1971; Eagon and Wilkerson, 1972; Frank and Hopkins, 1969; Miner and Frank, 1974; Halpern et al., 1973; Kahane et al., 1975; Willis and Furlong, 1975; MacDonald and Lanyi, 1975; Lanyi et al., 1976a,b). Moreover, some of these studies, in particular those of Stock and Roseman (1971) and Lanyi et al. (1976b), indicate that symport or cotransport mechanisms may be operative. Since the basic energy-yielding process in bacteria is thought to be proton extrusion and bacteria apparently do not possess a sodium, potassium-dependent ATPase or a primary sodium pump, the existence of such transport systems presents certain obvious problems, among which are: (1) The relationship between the proton electrochemical gradient and these transport systems; (2) the mechanism by which the internal sodium concentration is maintained at a low level.

In this communication, the sodium-dependent α-galactoside transport system (TMG permease II (Prestidge and Pardee, 1965)) is examined in membrane vesicles isolated from *Salmonella typhimurium*. The results suggest that sodium- or lithium-dependent methyl 1-thio-β-D-galactopyranoside (TMG) transport is driven by the proton electrochemical gradient through the mediation of a proton–sodium (lithium) antiport mechanism. A similar mechanism has been proposed recently to explain light-dependent glutamate transport in vesicles from *Halobacterium halobium* (Lanyi et al., 1976b).

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *S. typhimurium* G-30 (*Gal* E^-), obtained from Dr. M. J. Osborn, was grown on minimal medium A (Davis and Mingioli, 1950) supplemented with 0.5% yeast extract and 0.5% melibiose (unless indicated otherwise). Spheroplasts were prepared with lysozyme and ethylenediaminetetraacetate essentially as described by Osborn et al. (1972), and membrane vesicles were prepared as described previously for *E. coli* (Kaback, 1971; Short et al., 1975). Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6) and stored in liquid nitrogen.

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

Transport Assays. Filtration assays (Kaback, 1974; Schuldiner and Kaback, 1975) were carried out using Millipore Cellotate filters (0.5-μm pore size). Electron donors and isotopically labeled solutes were used as described.

Flow dialysis was performed as described (Ramos et al., 1976; Ramos and Kaback, 1977a,b) with the upper chamber of the apparatus open to atmosphere, and the reaction mixtures were gassed with oxygen. The upper and lower chambers were separated by Spectrapor 1 dialysis tubing (6000–8000 molecular weight cutoff; Fisher Scientific), and both chambers were stirred with magnetic bars. Membrane vesicles suspended in 0.05 M buffer at a given pH containing 0.01 M magnesium sulfate were added to the upper chamber (total volume was 0.8 mL), and electron donors, isotopically labeled solutes, and ionophores were used as indicated. The same buffer (0.05 M at the same pH as the upper chamber) was pumped from the lower chamber at a rate of 6.0 mL/min using a Pharmacia pump (Model P3). Fractions of about 1.7 mL were collected and assayed for radioactivity by liquid scintillation spectrometry.

Determination of ΔpH. ΔpH was determined by assaying the accumulation of [1,2-^{14}C]acetate using flow dialysis (Ramos et al., 1976; Ramos and Kaback, 1977a,b).

Determination of $\Delta\Psi$. The electrical potential across the membrane ($\Delta\Psi$) was determined by measuring the accumulation of [^{3}H]triphenylmethylphosphonium ($TPMP^+$) (bromide salt) using flow dialysis (Schuldiner and Kaback, 1975; Ramos et al., 1976; Ramos and Kaback, 1977a,b).

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). Internal pH was calculated as described by Waddel and Butler (1959) and Schuldiner et al. (1972), and ΔpH was determined by difference. Corrections for external pH were not necessary in the experiments reported here, since D-lactate was used as electron donor (Ramos and Kaback, 1977a). The electrical potential ($\Delta\Psi$) was calculated from the Nernst equation ($\Delta\Psi = 58.8 \log [TPMP^+]_{in}/[TPMP^+]_{out}$) using steady-state concentration values obtained from $TPMP^+$ uptake experiments. The proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) was calculated by substituting values for ΔpH and $\Delta\Psi$ into eq 1.

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

Materials

[^{3}H]Triphenylmethylphosphonium bromide was prepared by the Isotope Synthesis Group at Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman as described (Schuldiner and Kaback, 1975). [^{14}C]Methyl 1-thio-β-D-galactopyranoside was obtained from New England Nuclear. Other isotopically labeled materials were purchased from New England Nuclear and Amersham-Searle. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. Nigericin was the generous gift of Dr. J. Berger of Hoffmann-La Roche, Inc.

Results

Cation-Dependence of TMG Transport. Membrane vesicles prepared from *S. typhimurium* G-30 grown in the presence of melibiose catalyze the accumulation of TMG with D-lactate (Figure 1) or reduced phenazine methosulfate (not shown) as electron donors. However, as opposed to the β-galactoside transport system in *E. coli* vesicles, transport of the galactoside here is almost absolutely dependent upon the addition of sodium or lithium ion. With potassium, choline, or ammonium D-lactate as electron donor, very little TMG uptake is observed relative to control samples incubated in the absence of electron donors. On the other hand, with sodium D-lactate or particularly lithium D-lactate, marked stimulation of the initial rate and steady-state level of TMG accumulation is observed. Since similar effects are evident with 20 mM lithium or sodium chloride in the presence of potassium, choline, and ammonium D-lactate (data not shown), it is clear that the effect is due specifically to sodium and lithium cation. It is also noteworthy

[1] Abbreviations used are: TMG, methyl 1-thio-β-D-galactopyranoside; $TPMP^+$, triphenylmethylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

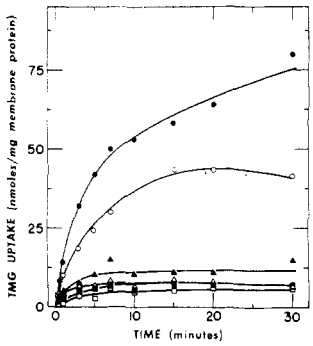

FIGURE 1: Time course of TMG uptake by *S. typhimurium* G-30 membrane vesicles in the presence of various cations. Aliquots (25 μL) of membrane vesicles containing about 0.1 mg of membrane protein were diluted to a final volume of 50 μL containing, in final concentrations, 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate. After a 30 s to 1 min incubation at 25 °C, lithium (●), sodium (○), or potassium (▲) D-lactate was added to a final concentration of 20 mM, and immediately thereafter, [^{14}C]TMG (7.1 mCi/mmol) to a final concentration of 1.3 mM. Alternatively, 20 mM lithium chloride (△), 20 mM sodium chloride (■), or nothing (□) was added prior to addition of [^{14}C]TMG. Incubations were continued at 25 °C for the times indicated, terminated, and assayed as described (Kaback, 1974), except that 100 mM potassium phosphate (pH 6.6) rather than 100 mM lithium chloride was used to dilute and wash the samples. Although not shown, similar results were obtained with potassium, choline, or ammonium D-lactate (20 mM final concentrations) in the presence and absence of lithium or sodium chloride (20 mM final concentrations).

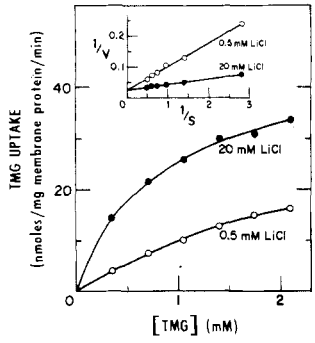

FIGURE 2: Effect of lithium chloride on the kinetics of TMG transport. Initial rates of TMG uptake were measured at 15, 30, 45, and 60 s in the presence of 0.5 mM (○) and 20 mM lithium chloride (●) and given concentrations of [^{14}C]TMG (7.1 mCi/mmol) as described in Figure 1. The values obtained are presented as a double-reciprocal plot in the inset.

that addition of lithium or sodium chloride in the absence of D-lactate causes only minimal stimulation over the control values (Figure 1) and that D-lactate-dependent accumulation of proline, alanine, and tyrosine in the same vesicles is not sodium or lithium dependent (not shown). Moreover, although data will not be presented, sodium and lithium have no significant effect on the rate of D-lactate oxidation by the vesicles and TMG uptake is not observed under any conditions with vesicles prepared from *S. typhimurium* G-30 grown in the absence of melibiose.

The stimulatory effect of lithium exhibits saturation kinetics when the initial rate of TMG uptake is measured as a function of increasing lithium chloride concentrations (data not shown), and the lithium concentration required to produce half-maximal stimulation is approximately 0.5 mM. Similar titration studies with sodium chloride demonstrate that half-maximal stimulation is achieved at about 0.4 mM sodium chloride (data not shown). The effect of lithium chloride on the apparent K_M and V_{max} for TMG uptake was also investigated (Figure 2).

TABLE I: Effect of Sodium Preloading on the Initial Rate of TMG Uptake.[a]

Preloading	Preincubation with D-lactate	Initial rate of uptake [nmol min^{-1} (mg of protein)$^{-1}$]	% control
−	−	11.9	100
+	−	6.8	57
−	+	12.7	107
+	+	12.8	108

[a] Aliquots (25 μL) of membrane vesicles prepared from *S. typhimurium* G-30 containing 0.15 mg of membrane protein were diluted to a final volume of 50 μL, containing, in final concentrations, 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate. Sodium chloride (50 mM, final concentration) was added to designated samples, and these samples as well as the controls were incubated at 25 °C for 2 h. At this time, initial rates of TMG uptake were assayed by filtration (Methods) at 15, 30, 45, and 60 s after addition of 50 mM sodium chloride (to those samples which were not preloaded) and 20 mM potassium D-lactate. Where indicated, preloaded and control samples were incubated with potassium D-lactate for 5 min prior to addition of [^{14}C]TMG (7.1 mCi/mmol) at a final concentration of 1.3 mM. Other details of the assay are described in Figure 1.

In these experiments, the initial rate of transport was determined as a function of TMG concentration in the presence of 0.5 mM and 20 mM lithium chloride, and the results are presented in double-reciprocal fashion (inset). Clearly, lithium enhances the apparent affinity of the system for TMG without a significant effect on the maximum rate of uptake. The apparent K_M for TMG uptake is 3.8 and 0.8 mM in the presence of 0.5 and 20 mM lithium chloride, respectively, while the apparent V_{max} is approximately 46 nmol per mg of membrane protein per min at both lithium chloride concentrations.

As shown by the data presented in Table I, when the sodium chloride concentration across the vesicle membrane is allowed to equilibrate by preincubating the vesicles with sodium chloride for 2 h, the intitial rate of TMG uptake in the presence of D-lactate is diminished by about 50% relative to vesicles assayed in the usual manner. On the other hand, when the equilibrated vesicles are incubated with D-lactate for 5 min prior to addition of TMG, the initial rate of uptake is identical to that observed in the control samples within experimental error. The results are consistent with the interpretation that the effects of sodium and lithium on TMG transport are not due merely to the presence of the cations, but to a concentration gradient ($[Na]_{out} > [Na]_{in}$) established during D-lactate oxidation. Further support for this interpretation will be presented below.

TMG-Dependent Sodium Uptake. The experiments presented thus far define one parameter of a symport system, sodium-dependent solute accumulations. The other parameter, solute-dependent cation uptake, is not observed with vesicles incubated with ^{22}Na and TMG in the presence of D-lactate or ascorbate and phenazine methosulfate for reasons that will be discussed. In any event, imposition of a potassium diffusion gradient across the *E. coli* vesicle membrane in the presence of valinomycin causes the generation of a membrane potential (interior negative) which drives the transport of a number of solutes (Hirata et al., 1973, Altendorf et al., 1975; Schuldiner and Kaback, 1975), and a similar phenomenon is observed with this transport system (Figure 3). When valinomycin-treated *S. typhimurium* vesicles prepared in potassium phosphate are diluted into choline phosphate in the presence of sodium

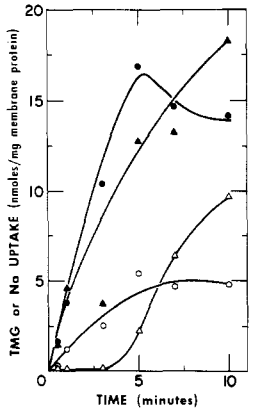

FIGURE 3: TMG-dependent ^{22}Na uptake and sodium-dependent [^{14}C]TMG uptake induced by a potassium diffusion potential. *S. typhimurium* G-30 membrane vesicles, prepared as described under Methods, were suspended in 100 mM potassium phosphate (pH 6.6) to a final concentration of 27 mg of protein/mL, and valinomycin was added to a final concentration of 1 nmol/mg of protein. Aliquots of this suspension (2 μL) were then diluted into 100 μL of 100 mM choline phosphate (pH 6.6) and 10 mM magnesium sulfate containing the following additional constituents: (○) 1.3 mM [^{14}C]TMG (7.1 mCi/mmol); (●) 1.3 mM [^{14}C]TMG (7.1 mCi/mmol) plus 4 mM sodium chloride; (▲) 4 mM ^{22}NaCl (2970 cpm/nmol); or (▲) 4 mM ^{22}NaCl (2970 cpm/nmol) plus 1.3 mM TMG. Samples were terminated and assayed as described (Kaback, 1974), except that 100 mM choline phosphate (pH 6.6) was used to dilute and wash the samples. Although not shown, sodium-dependent [^{14}C]TMG uptake and TMG-dependent ^{22}Na uptake under these conditions were not observed in the absence of valinomycin or when the vesicles were diluted into 100 mM potassium phosphate (pH 6.6).

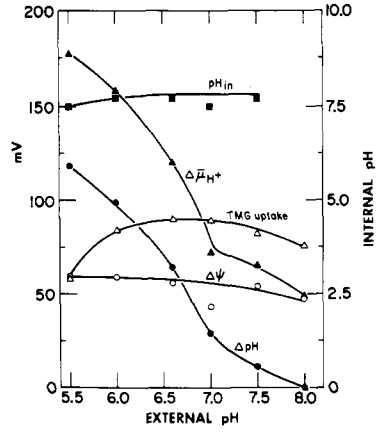

FIGURE 4: Effect of external pH on ΔpH, $\Delta\Psi$, $\Delta\bar{\mu}_{H^+}$, and the steady-state level of TMG accumulation. The experiments shown were carried out using flow dialysis as described under Methods and by Ramos et al. (1976) and Ramos and Kaback (1977a,b). *S. typhimurium* G-30 membrane vesicles (approximately 3.7 mg of protein/mL, final concentration) were suspended in 50 mM potassium phosphate buffer at a given pH containing 10 mM magnesium sulfate and 20 mM lithium chloride. Potassium D-lactate at a final concentration of 20 mM was used as electron donor. Internal pH (■) and $\Delta\Psi$ (○) were measured using [1,2-^{14}C]acetate (54 mCi/mmol) at a final concentration of 37.5 μM and [^{3}H]TPMP$^+$ (4.36 Ci/mmol) at a final concentration of 12.5 μM, respectively. ΔpH (●) and $\Delta\bar{\mu}_{H^+}$ (▲) were calculated as described under Methods. [^{14}C]TMG (3.6 mCi/mmol) was used at a final concentration of 1.3 mM, and steady-state levels of accumulation (△) were calculated and converted into millivolts by substituting appropriate values into the Nernst equation (mV = 58.8 log $[TMG]_{in}/[TMG]_{out}$).

chloride (or lithium chloride (data not shown)), TMG uptake is stimulated relative to control samples from which sodium and lithium are omitted. Moreover, when TMG transport is induced under these conditions, stimulation of ^{22}Na uptake is also observed (Figure 3). Thus, when potassium-loaded vesicles are treated with valinomycin and diluted into choline phosphate containing ^{22}NaCl in the presence of TMG, the rate and extent of ^{22}Na uptake are enhanced relative to samples incubated in the absence of TMG. Although data are not shown, neither sodium-dependent TMG uptake nor TMG-dependent sodium uptake is observed in the absence of a potassium diffusion gradient (i.e., when the vesicles are diluted into potassium phosphate) or when valinomycin is omitted. Moreover, it is apparent from the data presented in Figure 3 that approximately equimolar amounts of TMG and sodium are taken up under these conditions, an observation which is consistent with the suggestion (Stock and Roseman, 1971) that the stoichiometry between TMG and sodium is 1:1.

The Electrochemical Proton Gradient and Its Relationship to Cation-Dependent TMG Accumulation. As shown previously with *E. coli* vesicles (Ramos et al., 1976; Ramos and Kaback, 1977a,b), D-lactate or reduced phenazine methosulfate oxidation leads to the generation of a large ΔpH under certain conditions, and this component of $\Delta\bar{\mu}_{H^+}$ varies considerably with external pH. Similarly, with *S. typhimurium* G-30 vesicles, the magnitude of ΔpH is markedly dependent upon external pH (Figure 4). At pH 5.5, ΔpH exhibits a value of about −120 mV (2 pH units); above pH 5.5, ΔpH decreases drastically, and becomes negligible at pH 7.5–8.0. Clearly, this variation in ΔpH is related to the propensity of the vesicles to maintain their internal pH at pH 7.5–8.0 over the range of external pH values tested. The electrical potential ($\Delta\Psi$) remains relatively constant at −50 to −60 mV from pH 5.5–8.0, while $\Delta\bar{\mu}_{H^+}$ (the sum of ΔpH and $\Delta\Psi$) exhibits a value of about −180 mV at pH 5.5 (−120 mV ΔpH + −60 mV $\Delta\Psi$) which decreases sharply to approximately −70 mV at pH 7.0 and −50 mV at pH 8.0. Although these data differ somewhat from those obtained previously with *E. coli* vesicles (Ramos et al., 1976; Ramos and Kaback, 1977a), as in the previous case, it should be noted that at acid pH (i.e., pH 5.5) $\Delta\bar{\mu}_{H^+}$ is relatively high and consists primarily of a ΔpH component, while at more alkaline pH values (i.e., pH 7.5 and above) $\Delta\bar{\mu}_{H^+}$ is relatively low and consists almost entirely of a $\Delta\Psi$ component.

When steady-state levels of lithium-dependent TMG accumulation are examined under identical conditions, it is apparent that variations in this parameter do not parallel the variations in $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\Psi$. Optimal accumulation is observed at pH 6.0–7.0, with lower values above and below this pH range. Moreover, the TMG concentration gradients exceed the values obtained for $\Delta\bar{\mu}_{H^+}$ beginning at approximately pH 6.8. These findings are similar to those presented for a number of proton symport systems in *E. coli* vesicles (Ramos and Kaback, 1977b) and will be discussed in more detail.

As increasing concentrations of valinomycin are added to vesicles at pH 6.6 (Figure 5A) or pH 7.5 (Figure 5B), $\Delta\Psi$ decreases linearly from about −50 mV in the absence of valinomycin to about −15 mV in the presence of 3 μM valinomycin. Under the same conditions, ΔpH increases from about −65 mV in the absence of valinomycin to almost −90 mV at 1 μM valinomycin, and then decreases progressively to approximately −50 mV at 3 μM valinomycin. A decrease in ΔpH at valinomycin concentrations exceeding 1 μM is not observed with *E. coli* vesicles at pH 5.5 (Ramos et al., 1976; Ramos and Kaback, 1977a) and occurs for unknown reasons in this case. In any event, as a result of the alterations in $\Delta\Psi$ and ΔpH, $\Delta\bar{\mu}_{H^+}$ increases slightly from about −115 to −120 mV from 0 to 1 μM valinomycin, and subsequently decreases to about −60 mV as the valinomycin concentration is raised to 3 μM.

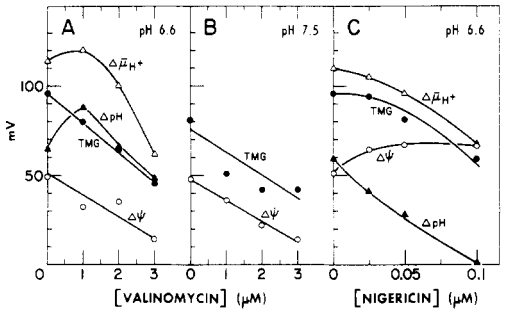

FIGURE 5: Effect of valinomycin (A and B) and nigericin (C) on ΔpH, Δψ, $\Delta\bar{\mu}_{H^+}$, and the steady-state level of TMG accumulation. ΔpH (▲) was determined by flow dialysis in the presence of [1,2-^{14}C]acetate, potassium D-lactate, and given concentrations of valinomycin or nigericin at pH 6.6 (A and C) as described under Methods and in Figure 5. Steady-state levels of $TPMP^+$ accumulation (Δψ, O) were measured under the same conditions at pH 6.6 (A and C) and pH 7.5 (B). $\Delta\bar{\mu}_{H^+}$ (Δ) was calculated as described under Methods. Steady-state levels of TMG accumulation (●) at each concentration of ionophore were determined as described under Methods and in Figure 5.

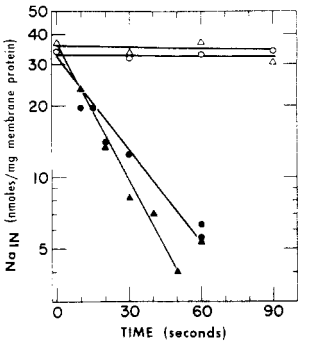

FIGURE 6: D-Lactate induced sodium efflux at pH 6.6 and 7.5. *S. typhimurium* G-30 membrane vesicles were resuspended in 50 mM potassium phosphate at pH 6.6 (Δ, ▲) or pH 7.5 (O, ●) containing 10 mM magnesium sulfate and 15 mM ^{22}NaCl (1400 cpm/nmol) to a final concentration of 2 mg of protein/mL, and the samples were incubated overnight at 4 °C. Aliquots (50 μL) were then transferred to a series of reaction vessels which were incubated at 25 °C for given times after addition of the following constituents (in final concentrations): filled symbols, 20 mM potassium D-lactate; open symbols, no addition or 20 mM potassium D-lactate plus 10 μM CCCP. The values presented were corrected for background radioactivity which was obtained with samples that were boiled for 5 min prior to assay. Incubations were terminated and samples were assayed as described in Figure 1.

As shown in Figure 5C, nigericin induces an effect on ΔpH at pH 6.6 which is opposite to that of valinomycin. As the nigericin concentration is increased from 0 to 0.1 μM, ΔpH decreases linearly from about −60 mV to 0. In contrast, Δψ increases from about −50 mV to about −65 mV at 0 and 0.025 μM nigericin, respectively, and continues to increase slightly as the nigericin concentration is raised to 0.1 μM. Despite the marked effect on ΔpH, as a result of the increase in Δψ, the ionophore elicits a relatively mild change in $\Delta\bar{\mu}_{H^+}$, causing only about 35% inhibition at 0.1 μM. At pH 7.5, nigericin causes essentially no change in $\Delta\bar{\mu}_{H^+}$ (i.e., Δψ), a finding which is consistent with the almost complete absence of ΔpH at this external pH [cf. Figure 4] (Ramos et al., 1976; Ramos and Kaback, 1977a) (not shown).

The corresponding effects of increasing concentrations of valinomycin and nigericin on lithium-dependent TMG accumulation are also shown in Figure 5A–C. In each case, the steady-state level of TMG accumulation is slowly and progressively inhibited with increasing concentrations of each ionophore in a manner that correlates reasonably well with their effects on $\Delta\bar{\mu}_{H^+}$. Although not shown, nigericin has no significant effect on TMG accumulation at pH 7.5. Furthermore, it is very likely that the inhibition of TMG uptake observed at pH 6.6 with nigericin is due specifically to collapse of ΔpH secondary to proton–potassium exchange (as opposed to collapse of a lithium gradient), since this ionophore does not catalyze proton–lithium exchange (Ashton and Steinrauf, 1970).

Proton–Sodium Antiport. Although West and Mitchell (1974) have presented evidence with intact *E. coli* which is consistent with the presence of a proton–sodium antiport mechanism, this phenomenon has not been demonstrated in *E. coli* or *S. typhimurium* membrane vesicles and its potential importance with respect to sodium (or lithium)-dependent solute accumulation in bacterial systems has been appreciated only recently (Lanyi et al., 1976b). As shown in the experiments presented in Figure 6, when *S. typhimurium* vesicles equilibrated with ^{22}Na are exposed to D-lactate, rapid efflux of ^{22}Na occurs at both pH 6.6 and 7.5. Moreover, when the proton conductor CCCP is added, sodium efflux is completely abolished. Although these experiments in and of themselves do not provide unequivocal evidence for proton–sodium antiport, other preliminary experiments utilizing flow dialysis, which will not be presented in detail here, support this interpretation. In these experiments, *S. typhimurium* vesicles at a high protein concentration (20 mg of protein/mL) were equilibrated with [^{14}C]acetate, and, at a given time, sodium, lithium, or potassium chloride was added to the upper chamber of the flow dialysis apparatus. Shortly after the addition of sodium or lithium salts, the concentration of acetate in the dialysate decreases sharply and achieves a new equilibrium, indicating that a ΔpH (interior alkaline) has been established. Addition of potassium chloride, alternatively, causes no change in the concentration of acetate in the dialysate. It should also be emphasized that evidence for light-driven proton–sodium antiport in *H. halobium* vesicles has been presented by Lanyi et al. (1976b), Lanyi and MacDonald (1976), and Eisenbach et al. (1977). Finally, the observation that sodium efflux occurs at pH 7.5 where there is essentially no ΔpH (cf. Figure 4) is consistent with the suggestion of Lanyi and MacDonald (1976) that proton–sodium antiport can function electrogenically.

Discussion

The experiments presented in this paper document essentially four phenomena: (1) Respiration-linked TMG accumulation by *S. typhimurium* membrane vesicles isolated from appropriately grown cells is probably dependent upon the presence of a sodium or lithium gradient; (2) sodium (or presumably lithium) is taken up simultaneously with TMG under certain conditions; (3) sodium (lithium)-dependent TMG accumulation varies with the $\Delta\bar{\mu}_{H^+}$ generated as a result of D-lactate oxidation; and (4) the vesicles catalyze sodium efflux in the presence of $\Delta\bar{\mu}_{H^+}$ in a manner which is consistent with the operation of a proton–sodium antiport mechanism. A schematic diagram which integrates these phenomena into a working model for sodium (lithium)-dependent TMG accumulation is presented in Figure 7. The model draws freely from the sodium gradient hypothesis for active transport in eucaryotic systems (Crane et al., 1961; Crane, 1962; Christensen, 1970; Schultz and Curran, 1970) and the chemiosmotic hypothesis of Mitchell (1961, 1966, 1968, 1973), and is similar basically to the proposal of Lanyi et al. (1976b) and Lanyi and MacDonald (1976) for light-driven glutamate transport in *H. halobium* vesicles.

Depicted in the upper right-hand corner of the scheme is a

putative mechanism for the generation of $\Delta\bar{\mu}_{H^+}$, the ultimate driving force for sodium-dependent TMG accumulation. Although little evidence is available concerning the precise mechanism of proton extrusion with D-lactate or reduced phenazine methosulfate as electron donors, the mechanism shown represents a classic "loop" as postulated by Mitchell (1961, 1966, 1968, 1973). Accordingly, the proton and/or electron carriers comprising the respiratory chain (or a part of it) are disposed asymmetrically across the membrane in such a fashion that, in the first arm of the loop, 2 protons and 2 electrons pass vectorially from one carrier on the inner surface of the membrane to the next carrier on the outer surface. The second arm of the loop involves the vectorial transfer of electrons (but not protons) from the carrier on the outer surface of the membrane to a third carrier on the inner surface, resulting in the appearance of 2 protons in the external medium. The sum of the two processes (i.e., extrusion of protons into the external medium and vectorial flow of electrons from the outer to the inner surface of the membrane) results in the generation of a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline).

The next process to be considered is exchange of external protons for internal sodium, a reaction which is presumed to be catalyzed by a proton–sodium antiporter as suggested by West and Mitchell (1974). By this means, $\Delta\bar{\mu}_{H^+}$ generated by D-lactate oxidation provides the driving force for maintaining a low internal sodium concentration. Clearly, the mechanism provides a means for accomplishing this aim without the intervention of more classical ATP-driven sodium "pumps". On the other hand, it should be evident that generation of $\Delta\bar{\mu}_{H^+}$ through the action of the membraneous calcium, magnesium-stimulated ATPase complex would accomplish the same end. It should also be recognized that the relationship between proton–sodium antiport and sodium–TMG symport is the weakest aspect of the model experimentally. Without specific inhibitors or mutants defective in this exchange mechanism, the suggested relationship will be difficult to establish more firmly.

Finally, accumulation of TMG is mediated by a solute-specific symporter which catalyzes the coupled movements of TMG and sodium (or lithium) across the membrane. As suggested for many eucaryotic transport systems (Crane et al., 1961; Crane, 1962; Christensen, 1970; Schultz and Curran, 1970), the immediate driving force for TMG accumulation is provided by the concentration gradient of sodium. Once sodium and TMG are released into the internal space, the cation is removed by exchange with external protons, while the galactoside accumulates to high internal concentrations. Thus, the symport mechanism depicted here is similar conceptually to that proposed for proton–solute symport which appears to be more common in bacterial systems. The latter systems, however, are coupled directly to $\Delta\bar{\mu}_{H^+}$ or one of its components, while the former is coupled indirectly via proton–sodium antiport.

In addition to providing a rationale for sodium-dependent TMG transport, the model allows an explanation for an ostensibly puzzling finding discussed above. TMG-dependent sodium uptake is not observed when the vesicles are incubated in the presence of D-lactate or reduced phenazine methosulfate, but only when a $\Delta\Psi$ (interior negative) is induced artificially by means of a potassium diffusion gradient in the presence of valinomycin. If the rate of sodium–proton exchange is much more rapid than the rate of TMG-dependent sodium influx, it is evident that sodium uptake will not be observed when $\Delta\bar{\mu}_{H^+}$ is present (i.e., in the presence of D-lactate or reduced phenazine methosulfate). On the other hand, under the conditions

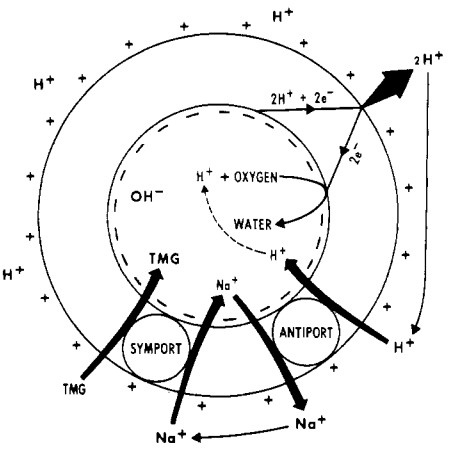

FIGURE 7: Schematic representation of sodium-dependent TMG accumulation in *S. typhimurium* G-30 membrane vesicles.

described in Figure 3, $\Delta\Psi$ is generated in the absence of ΔpH and proton–sodium exchange should not function at maximum efficiency. Another plausible explanation is that a pH gradient (interior acid) is induced by the potassium diffusion potential under these conditions. If this were the case, electroneutral sodium extrusion would be inhibited, and uptake of the cation would be observed in the presence of TMG. Either or both of these explanations might also account for the marked differences in the time courses of TMG and sodium uptake observed by Stock and Roseman (1971) in their experiments with intact cells.

Regarding the stoichiometry between sodium and TMG transport, the data presented in Figure 3 are consistent with a 1:1 relationship. However, if a 1:1 stoichiometry is constant under all conditions and the sodium gradient is in equilibrium with $\Delta\bar{\mu}_{H^+}$, the concentration gradient of TMG (expressed in millivolts) should never exceed $\Delta\bar{\mu}_{H^+}$, and this is clearly not the case. As shown in Figure 4, at external pH values above approximately pH 7.0 TMG accumulation exceeds $\Delta\bar{\mu}_{H^+}$. At pH 8.0, for instance, TMG is accumulated to an intravesicular concentration which is equivalent to approximately −75 mV, while $\Delta\bar{\mu}_{H^+}$ is only about −45 mV. As discussed previously (Rottenberg, 1976; Ramos and Kaback, 1977b), this observation is consistent with the suggestion that an increase in stoichiometry to values higher than unity may occur at relatively alkaline pH values. It should be noted furthermore that the putative increase in stoichiometry with this transport system may not necessarily involve an additional sodium ion, but rather a proton. In other words, at alkaline pH, the sodium–TMG symporter might catalyze the translocation of a proton in addition to sodium (or lithium) and TMG.

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