parable strength to the visible band(s), even though the oscillator strengths of the former are much smaller.

One feature of the CD spectrum of the ternary bromphenol blue–NADH–H_4 LDH complex (Figure 8) which is puzzling is that the contribution of the NADH 340-nm band appears to be very small. The binary LDH–NADH complex has a positive CD band centered at about 340 nm for bovine H_4 (J. F. Towell, unpublished experiments) and porcine M_4 (Gurevich et al., 1972), which has ~70% of the amplitude of the ternary LDH–NADH–oxamate complex. There is no indication of a positive band of this intensity in the ternary complex with bromphenol blue. It may be that the NADH binds in a different way in the ternary complex with bromphenol blue than it does in the ternary complex with oxamate or in the binary complex. It is possible that the dye bound at the substrate-binding site partly overlaps the nicotinamide binding site and that NADH is bound primarily through the AMP moiety. Such a binding mode, with the nicotinamide ring relatively free, would be expected to lead to negligible CD in the 340-nm reduced nicotinamide band.

References

Adams, M. J., Liljas, A., & Rossmann, M. G. (1973) *J. Mol. Biol. 76*, 519–31.

Anderson, S. R., & Weber, G. (1965) *Biochemistry 4*, 1948–57.

Cassim, J. Y., & Yang, J.-T. (1969) *Biochemistry 8*, 1947–51.

Cornish-Bowden, A., & Koshland, D. E., Jr. (1970) *Biochemistry 9*, 3325–36.

DiSabato, G., & Kaplan, N. O. (1963) *Biochemistry 2*, 776–81.

Foster, R., Hammick, D. L., & Wardley, A. A. (1953) *J. Chem. Soc.*, 3817–20.

Franglen, G. T., & Martin, N. H. (1954) *Biochem. J. 57*, 626–30.

Geiger, R. E., & Wagnière, G. H. (1973) in *Wave Mechanics: The First Fifty Years* (Price, W. C., Chissick, S. S., & Ravensdale, T., Eds.) pp 292–314, Butterworths, London.

Glazer, A. N. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 1057–63.

Gurevich, V. M., Kurganov, B. I., Sugrobova, N. P., & Yakovlev, V. A. (1972) *Biochemistry (Engl. Transl.) 37*, 856–8.

Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes, 3rd Ed. 11*, 191–292.

Jacobsberg, L. B., Kantrowitz, E. R., & Lipscomb, W. N. (1975) *J. Biol. Chem. 250*, 9238–49.

Klotz, I. M. (1974) *Acc. Chem. Res. 7*, 161–8.

Kosheleva, G. N. (1956) *Tr. Vses. Nauchno-Issled. Inst. Khim. Reakt. Osobo Chist. Khim. Veshchestv 21*, 57–61; (1959) *Chem. Abstr. 53*, 8919*h*.

Kragh-Hansen, U., Moller, J. V., & Lind, K. E. (1974) *Biochim. Biophys. Acta 365*, 360–71.

McPherson, A., Jr. (1970) *J. Mol. Biol. 51*, 39–46.

Moscowitz, A. (1960) in *Optical Rotatory Dispersion* (Djerassi, C., Ed.) p 150, McGraw-Hill, New York.

Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D., & Kaplan, N. O. (1964) *J. Biol. Chem. 239*, 1753–61.

Pfeiffer, P., & Quehl, K. (1931) *Ber. Dtsch. Chem. Ges. B 64*, 2667–71.

Towell, J. F. (1977) Ph.D. Thesis, Colorado State University, Fort Collins, CO.

Wassarman, P. M., & Lentz, P. J., Jr. (1971) *J. Mol. Biol. 60*, 509–22.

Wilson, J. B. (1951) *J. Assoc. Off. Agric. Chem. 34*, 343–4.

Mechanism of the Melibiose Porter in Membrane Vesicles of *Escherichia coli*†

Dunell E. Cohn and H. Ronald Kaback*

ABSTRACT: The melibiose transport system of *Escherichia coli* catalyzes sodium–methyl 1-thio-β-D-galactopyranoside (TMG) symport, and the cation is required not only for respiration-driven active transport but also for binding of substrate to the carrier in the absence of energy and for carrier-mediated TMG efflux. As opposed to the proton–β-galactoside symport system [Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691], efflux and exchange of TMG occur at the same rate, implying that the rates of the two processes are limited by a common step, most likely the translocation of substrate across the membrane. Furthermore, the rate of exchange, as well as efflux, is influenced by imposition of a membrane potential ($\Delta\Psi$; interior negative), suggesting that the ternary complex between sodium, TMG, and the porter may bear a net positive charge. Consistently, energization of the vesicles leads to a large increase in the V_{max} for TMG influx, with little or no change in the apparent K_m of the process. It is proposed that the sodium gradient ($Na^+_{out} > Na^+_{in}$) and the $\Delta\Psi$ (interior negative) may affect different steps in the overall mechanism of active TMG accumulation in the following manner: the sodium gradient causes an increased affinity for TMG on the outer surface of the membrane relative to the inside and the $\Delta\Psi$ facilitates a reaction involved with the translocation of the positively charged ternary complex to the inner surface of the membrane.

A large body of evidence has accumulated indicating that chemiosmotic phenomena, as postulated by Mitchell (1961, 1966, 1968, 1973, 1977), are responsible for respiration-dependent active transport in membrane vesicles isolated from *Escherichia coli* (Kaback, 1974, 1976; Harold, 1976; Konings & Boonstra, 1977). According to this hypothesis, oxidation of electron donors that drive substrate accumulation leads to the development of a transmembrane electrochemical gradient

† From the Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received April 8, 1980.*

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of protons ($\Delta\bar{\mu}_{H^+}$)[1] that is the immediate driving force for active transport. In 1963, Mitchell (1963) proposed explicitly that accumulation of β-galactosides by *E. coli* occurs via coupled movement with protons, i.e., symport, and this concept has received strong experimental support (West, 1970; West & Mitchell, 1972, 1973; Schuldiner & Kaback, 1975; Ramos & Kaback, 1977a,b; Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979).

Although many bacterial transport systems catalyze proton–substrate symport, certain other bacterial systems utilize sodium as the symported ion [Stock & Roseman, 1971; Lanyi et al., 1976; Tokuda & Kaback, 1977; Lopilato et al., 1978; for review, see Lanyi (1979)]. For these systems, it has been proposed that the driving force is an electrochemical gradient of sodium ($\Delta\bar{\mu}_{Na^+}$), created from $\Delta\bar{\mu}_{H^+}$ by exchange of protons for sodium by an antiport mechanism (West & Mitchell, 1974; Tokuda & Kaback, 1977; Schuldiner & Fishkes, 1978; Reenstra et al., 1980). One transport system that catalyzes sodium–substrate symport in this fashion is the melibiose transport system of *Salmonella typhimurium* (Stock & Roseman, 1971; Tokuda & Kaback, 1977) and *E. coli* (Lopilato et al., 1978), where it has been demonstrated convincingly that methyl 1-thio-β-D-galactopyranoside (TMG) uptake is coupled to the uptake of sodium (or lithium).

Although it is generally agreed that cation–substrate symport occurs obligatorily during respiration-linked active transport, mechanistic studies have been initiated only recently. In a novel approach toward understanding proton–β-galactoside symport in *E. coli*, passive lactose movements were used to drive turnover of the *lac* carrier under a variety of conditions in isolated membrane vesicles (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979). From studies on efflux and exchange and the effects of $\Delta\bar{\mu}_{H^+}$ on these processes and on the kinetics of lactose uptake, the following results and conclusions were presented. (1) Efflux of lactose is much slower than exchange, indicating that the rate-limiting step for efflux is associated with the return of the unloaded carrier to the inner surface of the membrane. (2) Rates of efflux, but not exchange, are altered by imposition of $\Delta\Psi$, ΔpH, or $\Delta\bar{\mu}_{H^+}$, implying that the unloaded carrier may catalyze a reaction involving the movement of a negative charge and that the loaded carrier is neutral, as proposed previously (Schuldiner et al., 1975; Rottenberg, 1976; Ramos & Kaback, 1977b,c). (3) Energy, in the form of $\Delta\bar{\mu}_{H^+}$ or a lactose concentration gradient ($\Delta\mu_{Lac}$), decreases the apparent K_m for lactose uptake by about 2 orders of magnitude with little effect on the maximum velocity of transport. Lastly, Padan et al. (1979) demonstrated that treatment of the vesicles with the histidine-specific reagent diethyl pyrocarbonate (DEPC) causes an increase in the apparent K_m for $\Delta\bar{\mu}_{H^+}$- and $\Delta\mu_{Lac}$-driven lactose influx with no effect on energy-independent processes such as facilitated diffusion of lactose or binding of *p*-nitrophenyl α-D-galactopyranoside (NPG).

In this paper, analogous studies were carried out with the sodium-dependent melibiose transport system in membrane vesicles from *E. coli*. The results indicate that the melibiose porter differs from the *lac* carrier in each of the points summarized above and indicate that many aspects of the two mechanisms are different.

[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; $\Delta\bar{\mu}_{Na^+}$, electrochemical gradient of sodium; TMG, methyl 1-thio-β-D-galactopyranoside; $\Delta\Psi$, membrane potential; ΔpH, pH gradient; $\Delta\mu_{Lac}$, concentration gradient of lactose; DEPC, diethyl pyrocarbonate; NPG, *p*-nitrophenyl α-D-galactopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* RA11 was generously provided by Dr. T. H. Wilson. This strain contains a deletion for the *lac y* gene, has a temperature-stable, inducible melibiose transport system, and is α-galactosidase negative (Lopilato et al., 1978). Cells were grown at 37 °C in Medium 63 (Cohen & Rickenberg, 1956) supplemented with a 1% pancreatic digest of casein (Bactotryptone, Difco), 0.5 mg/L thiamin, and 10 mM melibiose and harvested during late exponential phase. Membrane vesicles were prepared from these cells by osmotic lysis as described previously (Kaback, 1971; Short et al., 1975) except that lysozyme and sucrose were used at final concentrations of 50 μg/mL and 30%, respectively, for the preparation of spheroplasts. Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6) and stored in liquid nitrogen.

For studies with buffers other than potassium phosphate, vesicles stored in liquid nitrogen were thawed, resuspended in a 20-fold excess of the desired buffer, and incubated for 20 min at room temperature. The suspension was then centrifuged at 40000*g* for 30 min, and the pellet was resuspended and washed once in a similar volume of the desired buffer. The final pellet was resuspended to an appropriate protein concentration in the same buffer and stored on ice until use.

Transport Assays. Uptake of [^{14}C]TMG was assayed by filtration as described previously (Kaback, 1974), except that the reactions were terminated by adding 2.0 mL of 0.1 M potassium chloride containing 50 mM potassium phosphate (pH 5.5), and the filters (nitrocellulose, 0.45 μm) were washed 1 time with 2.0 mL of the same buffer.

For efflux and exchange, vesicles were concentrated to approximately 30 mg of protein/mL, a small aliquot of [^{14}C]TMG was added, and the suspension was incubated at room temperature for 3 h in order to allow TMG to equilibrate with the intravesicular space. Reactions were initiated by diluting 2.0 μL of the suspension, 200- or 400-fold as indicated, into a given buffer, followed by agitation on a vortex mixer. After incubation for given times at 25 °C, 2.0 mL of the dilution buffer was added, the vesicles were filtered immediately, and the filter was washed once with 2.0 mL of the same buffer. Radioactivity was determined by liquid scintillation spectrometry.

Binding of p-Nitrophenyl α-D-[6-^{3}H]Galactopyranoside ([^{3}H]NPG). Binding of [^{3}H]NPG to membrane vesicles under nonenergized conditions was assayed by flow dialysis (Rudnick et al., 1976; Tokuda & Kaback, 1978; Padan et al., 1979). Vesicles were present in the upper chamber at a concentration of 12.5 mg of protein/mL, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to a final concentration of 10 μM to ensure complete deenergization. Specifically bound [^{3}H]NPG was determined from the increase in the dialyzable concentration of [^{3}H]NPG following addition of excess unlabeled NPG (Rudnick et al., 1976).

Treatment with Diethyl Pyrocarbonate (DEPC). Melibiose-induced *E. coli* RA11 membrane vesicles were treated with DEPC as described previously (Padan et al., 1979). Vesicles suspended in 0.1 M potassium phosphate (pH 6.6) were titrated to pH 6.0 with 0.1 M monobasic potassium phosphate and adjusted to a final concentration of about 2.0 mg of protein/mL by adding appropriate volumes of 0.1 M potassium phosphate (pH 6.0). Small aliquots of 2.0 M DEPC (freshly prepared in absolute ethanol) were added to the membrane suspensions to give the final concentrations of DEPC desired. The suspensions were immediately vortexed

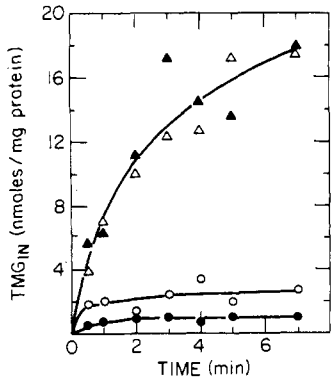

FIGURE 1: Time course of TMG uptake by *E. coli* RA11 membrane vesicles in the presence of various cations. Aliquots (25 μL) of membrane vesicles containing about 0.1 mg of membrane protein were diluted to a final volume of 50 μL containing, in final concentrations, 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulfate, and 10 mM potassium (○), lithium (△), or sodium (▲) chloride. After incubation at 25 °C for 5 min, potassium ascorbate (pH 6.6) and PMS were added to final concentrations of 20 and 0.1 mM, respectively, and the samples were incubated for an additional 30 s. At this time, uptake was initiated by addition of [^{14}C]TMG (15 mCi/mmol) to a final concentration of 0.5 mM. Alternatively, uptake was assayed with 10 mM potassium, sodium, or lithium chloride added, but without ascorbate and PMS (●). All assays were carried out in an atmosphere of water-saturated oxygen (Kaback, 1974), terminated and assayed by filtration at the times indicated as described under Methods.

to achieve complete mixing and incubated at room temperature for 10 min. Reactions were terminated by a fivefold dilution with ice-cold 0.1 M potassium phosphate (pH 7.0) and immediate centrifugation at 40000*g* for 30 min. The supernatants were discarded and the pellets resuspended in at least a 100-fold excess (v/v) of 0.1 M potassium phosphate containing 0.01 M sodium ion (pH 7.0). Following centrifugation at 40000*g* for 30 min, the supernatants were again discarded and the pellets resuspended in the same buffer to an appropriate concentration.

Calculations. The internal concentration of solute accumulated by the vesicles was calculated by using a value of 2.2 μL of intravesicular fluid/mg of membrane protein (Kaback & Barnes, 1971).

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

Materials

[^{14}C]Methyl 1-thio-β-D-galactopyranoside ([^{14}C]TMG) was obtained from New England Nuclear, and DEPC was purchased from Sigma Chemical Co. [^{3}H]NPG was synthesized and purified as described (Rudnick et al., 1976). Valinomycin and CCCP were obtained from Calbiochem. All other materials were reagent grade obtained from commercial sources.

Results

Effects of Cations on TMG Accumulation. Respiration-driven accumulation of TMG by membrane vesicles from melibiose-induced *E. coli* RA11 is highly dependent on the presence of sodium or lithium (Figure 1). Thus, addition of ascorbate and phenazine methosulfate (PMS) leads to a marked uptake of TMG in the presence of sodium or lithium, but not potassium. The small effect observed in the absence of sodium or lithium (i.e., in potassium) is due most likely to contamination by sodium (as determined by atomic absorption spectroscopy, 100 mM potassium phosphate contains approximately 100 μM sodium). These results agree with other observations in intact cells and membrane vesicles from melibiose-induced *E. coli* and *S. typhimurium* (Stock & Roseman, 1971; Tokuda & Kaback, 1977; Lopilato et al., 1978).

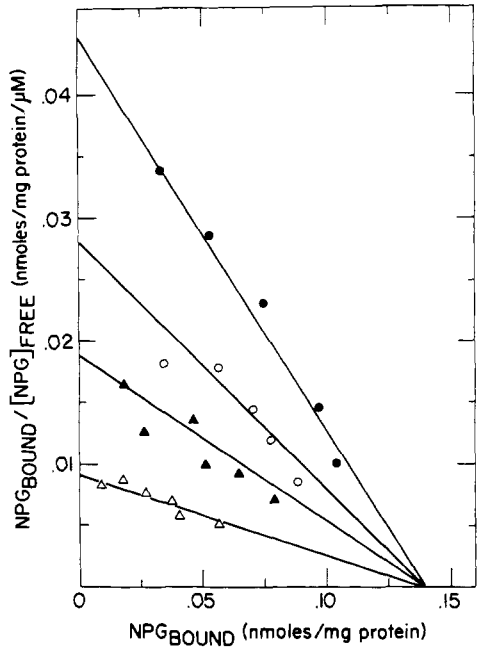

FIGURE 2: Effect of sodium on NPG binding in the absence of energy. *E. coli* RA11 (melibiose-induced) membrane vesicles were washed twice and resuspended to 12.5 mg of membrane protein/mL in 0.1 M potassium phosphate (pH 6.6) without (△) or with sodium sulfate added to final sodium concentrations of 0.3 (▲), 1.0 (○), and 10 mM (●). In addition, the reaction mixtures contained 10 μM CCCP to ensure complete deenergization. Binding of [^{3}H]NPG was assayed at concentrations ranging from 1 to 15 μM. For each experimental point shown, a separate flow dialysis experiment was carried out as described under Methods. In all cases, the sodium concentration in the buffer pumped from the lower chamber was the same as that added to the vesicles in the upper chamber. Results were quantitated as described previously (Rudnick et al., 1976; Tokuda & Kaback, 1978) and plotted according to Scatchard (1949).

As shown previously (Stock & Roseman, 1971; Tokuda & Kaback, 1977; Lopilato et al., 1978), sodium and lithium lower the apparent K_m for TMG transport with little or no effect on V_{max}. Similarly, in *E. coli* RA11 vesicles, the apparent K_m for TMG uptake is 1.8 mM without added sodium or lithium (data not shown) and 0.25 mM in the presence of 10 mM sodium (cf. Figure 6), while V_{max} is increased marginally [26 nmol/(min mg of protein) without added sodium or lithium compared to 31 nmol/(min mg of protein) with 10 mM sodium].

Effect of Sodium on NPG Binding. Sodium-dependent [^{3}H]NPG binding to the melibiose porter is observed in *S. typhimurium* vesicles in the presence of CCCP by means of flow dialysis (Tokuda & Kaback, 1978), and in RA11 vesicles [^{3}H]NPG bound under these conditions is completely displaced by addition of excess melibiose, TMG, or unlabeled NPG (data not shown). Furthermore, when binding under nonenergized conditions is studied as a function of NPG concentration and the results are plotted according to Scatchard (1949), a linear function is obtained, indicating that there is a single class of binding sites. Such data are presented in Figure 2 for RA11 vesicles that were preequilibrated with given concentrations of sodium, and it is readily apparent that the cation effects a decrease in the K_D for NPG (15.5 μM without added sodium and 7.4, 5.0, and 3.1 μM in the presence of 0.3, 1.0, and 10 mM sodium, respectively) without altering the number of binding sites (0.14 nmol/mg of membrane protein).

Efflux and Exchange. The effect of sodium on the passive, carrier-mediated efflux of TMG down a concentration gradient

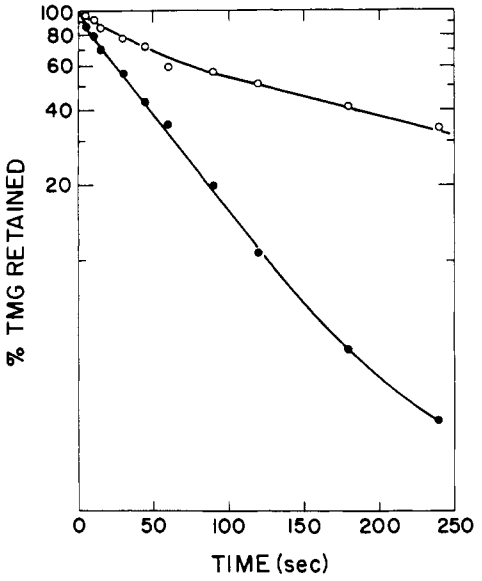

FIGURE 3: Effect of sodium on TMG efflux. Membrane vesicles prepared from melibiose-induced *E. coli* RA11 were washed twice and resuspended to about 30 mg of membrane protein/mL in 0.1 M potassium phosphate (pH 6.6) with (●) and without (○) 10 mM sodium. A small aliquot of [^{14}C]TMG (4.5 mCi/mmol) was added to each suspension to a final concentration of 10 mM. After the samples were equilibrated for 3 h at room temperature, 2-μL aliquots were rapidly diluted 200-fold into the same buffer as that used for resuspension except that TMG was omitted. At times shown, samples were rapidly diluted with 2.0 mL of 0.1 M potassium phosphate (pH 6.6), immediately filtered, and assayed as described under Methods. The percentage of TMG retained was determined by comparison with the zero time points (22 nmol/mg of protein ±10%). Each time point represents the average of two assays.

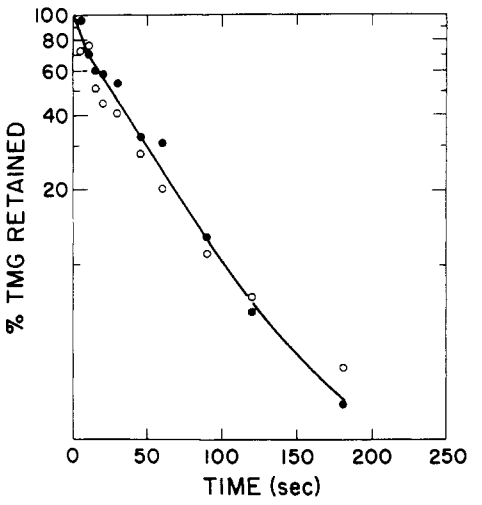

FIGURE 4: Comparison of efflux and exchange of TMG. Membrane vesicles from melibiose-induced *E. coli* RA11 were washed twice, resuspended to 30 mg of protein/mL in 0.1 M potassium phosphate (pH 6.6) containing 10 mM sodium, and equilibrated with 10 mM [^{14}C]TMG as described under Methods and in Figure 3. Assays were conducted as described in Figure 3, but in the absence (●) and presence (○) of 10 mM unlabeled TMG in the dilution buffer. Each point is the average of two assays.

was investigated by examining the rate of TMG efflux in the presence and absence of added sodium. Concentrated vesicle suspensions were equilibrated with 10 mM [^{14}C]TMG with and without 10 mM sodium and then diluted 200-fold into the same buffer without TMG (Figure 3). Clearly, the rate of efflux is enhanced in the presence of the cation ($t_{1/2}$ = 180 s in the absence of sodium and 40 s in the presence of 10 mM sodium). Although data will not be presented, treatment of vesicles with 40 μM *N*-naphthylmaleimide, which completely inhibits the melibiose porter (unpublished information), leads to very slow rates of efflux ($t_{1/2}$ = 360 s). Similarly, slow rates of efflux are observed with *E. coli* ML 30 vesicles, which lack the melibiose carrier and were not induced for the β-galactoside transport system ($t_{1/2}$ = 450 s). Finally, the rate of TMG efflux from loaded RA11 vesicles is not affected by addition of CCCP in the presence or absence of sodium, indicating that the efflux rate is not limited by the generation of a membrane potential.

For exchange studies, vesicles were equilibrated with [^{14}C]TMG in a similar manner but diluted into buffer containing an equimolar concentration of unlabeled TMG. Although not shown, loss of intravesicular TMG under these conditions is also sodium dependent ($t_{1/2}$ = 80 and 40 s in the absence and presence of sodium, respectively). Importantly, moreover, when sodium-dependent efflux and exchange are contrasted, both reactions proceed at the same rate within experimental error (Figure 4). This finding implies that the rate-limiting step for both processes is associated with the translocation of substrate across the membrane.

Effect of $\Delta\Psi$ on TMG Efflux and Exchange. It is well established that a transient $\Delta\Psi$ (interior negative) is generated in *E. coli* membrane vesicles by imposition of a potassium diffusion gradient ($K^+_{in} > K^+_{out}$) in the presence of valinomycin and that a $\Delta\Psi$ generated in this manner drives the accumulation of various substrates (Hirata et al., 1973, 1974; Lombardi et al., 1974; Schuldiner & Kaback, 1975; Tokuda & Kaback, 1977; LeBlanc et al., 1980). In RA11 vesicles, transient accumulation of TMG is observed under the same conditions in the presence of sodium (data not shown).

The effect of imposed $\Delta\Psi$'s on rates of substrate efflux and exchange can be used to assess whether or not the rate-limiting step for either process involves the translocation of a charged species across the membrane (Kaczorowski et al., 1979; LeBlanc et al., 1980). As shown in Figure 5A, when vesicles equilibrated with [^{14}C]TMG and potassium are diluted 400-fold into buffer devoid of potassium, the rate of efflux is diminished on addition of valinomycin [i.e., when a $\Delta\Psi$ (interior negative) is imposed] and the $t_{1/2}$ for efflux increases from about 1 to 2 min. This effect is unchanged in the presence of 0.5 μM monensin but is completely abolished when 10 μM CCCP is present in the dilution medium, indicating that the observed effect is due to the imposed potential and not to a change in sodium ion distribution across the vesicle membrane. Valinomycin causes no change in the rate of efflux when potassium-loaded vesicles are diluted into media containing equimolar potassium concentrations (data not shown).

Identical experiments, performed under exchange conditions (i.e., with equimolar unlabeled TMG in the dilution medium), are presented in Figure 5B. Importantly, the rate of loss of TMG from the vesicles via exchange is also diminished when $\Delta\Psi$ (interior negative) is imposed across the membrane. Since imposition of $\Delta\Psi$ alters the rates of efflux as well as exchange, a step involving the translocation of charge across the membrane may be rate limiting for both processes.

Effect of $\Delta\bar{\mu}_{H^+}$ on the Kinetics of TMG Uptake. When initial rates of sodium-dependent TMG transport by melibiose-induced *E. coli* RA11 vesicles are determined as a function of TMG concentration in the presence and absence of $\Delta\bar{\mu}_{H^+}$, the results presented in Figure 6 are obtained. As shown in the inset, in the absence of $\Delta\bar{\mu}_{H^+}$, the system exhibits an apparent K_m of 0.24 mM and a V_{max} of 2.5 nmol/(min mg of protein). In marked contrast, in the presence of $\Delta\bar{\mu}_{H^+}$, the apparent K_m remains constant at 0.24 mM, while V_{max} increases more than 10-fold to 33 nmol/(min mg of protein) (cf.

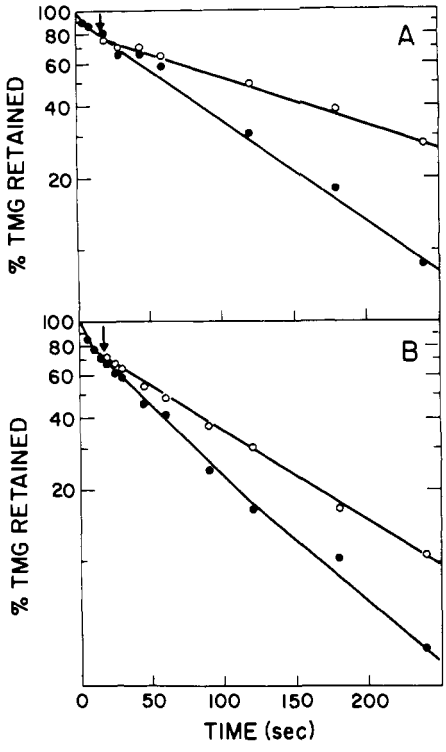

FIGURE 5: Effect of imposed $\Delta\Psi$ (interior negative) on TMG efflux (A) and exchange (B). Vesicles were washed and resuspended to 30 mg of protein/mL in 0.1 M potassium phosphate (pH 6.6) containing 10 mM sodium and equilibrated with 20 mM [^{14}C]TMG (3.6 mCi/mmol) for 3 h at room temperature. Panel A: Aliquots (2 μL) of the suspension were diluted 400-fold into 0.1 M choline phosphate (pH 6.6) containing 10 mM sodium. At the time indicated by the arrow, either valinomycin was added to a final concentration of 4.0 μM (○) or an equivalent volume (0.5 μL) of ethanol was added (●). At the times indicated, the samples were diluted with 2.0 mL of the choline phosphate buffer and immediately filtered, and the filter was washed once with an additional 2.0 mL of the same buffer. Panel B: Vesicles were treated as described in panel A, except that they were diluted into 0.1 M choline phosphate (pH 6.6) containing 10 mM sodium and 20 mM unlabeled TMG in addition. At the time indicated by the arrow, either valinomycin (○) or ethanol (●) was added. Each point is the average of two assays, and the percentage TMG retained was calculated by comparison with zero time points (44 nmol/mg of protein ±10%).

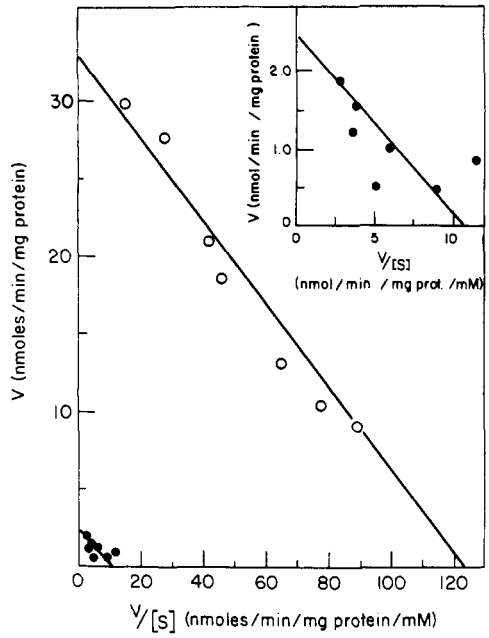

FIGURE 6: Kinetic analysis of TMG influx under energized and nonenergized conditions. Vesicles were assayed for TMG uptake by filtration as described previously (Kaback, 1974) and under Methods. For assay under energized conditions (○), the reaction mixtures (50 μL, final volume) contained 50 mM potassium phosphate (pH 6.6), 10 mM sodium, 10 mM magnesium sulfate, 0.1 mg of membrane protein, 20 mM potassium D-lactate (pH 6.6), and concentrations of [^{14}C]TMG (45 mCi/mmol) ranging from 0.1 to 2.0 mM. The vesicles were preincubated at 25 °C with the D-lactate for 3 min before addition of [^{14}C]TMG. Initial rates were determined from time points taken during the first 15 s of the reaction and were linear over this time period. For assay of TMG influx under nonenergized conditions (●; expanded in inset), vesicles were washed and resuspended to 10 mg of membrane protein/mL in 0.1 M potassium phosphate (pH 6.6) containing 10 mM sodium and 10 mM magnesium sulfate and CCCP was added to 10 μM final concentration. Aliquots (100 μL) were preincubated at 25 °C, and [^{14}C]TMG (20 mCi/mmol) was added at concentrations ranging from 0.05 to 2.0 mM. Initial rates were determined from time points taken during the first 10 s of the reaction and were linear over this time period. Because of the high protein concentration, 47 mm diameter filters were used for the assays. All data were corrected for nonspecific, passive influx by subtracting values obtained from identical experiments using vesicles pretreated with 40 μM *N*-naphthylmaleimide for 30 min at room temperature or vesicles prepared from *E. coli* ML 30 that were not induced for either the melibiose or β-galactoside transport systems. Data are presented as Eadie–Hofstee plots.

the body of Figure 6). Clearly, therefore, with this system, in contradistinction to the *lac* transport system (Kaczorowski et al., 1979), $\Delta\bar{\mu}_{H^+}$ causes a marked increase in V_{max} with no significant change in apparent K_m.

Inhibition of TMG Accumulation by DEPC. Treatment of melibiose-induced RA11 vesicles with 2.0 mM DEPC causes inhibition of the initial rate and steady-state level of TMG accumulation (Figure 7, inset). Moreover, as shown in the body of the figure, DEPC treatment reduces the V_{max} of active transport (intercept with the *y* axis) with no significant effect on the apparent K_m (the slope of the functions). This result differs from that obtained with the *lac* transport system (Padan et al., 1979) where DEPC treatment increases the apparent K_m with no effect V_{max}. It should be emphasized, moreover, that with the present system, as shown with the *lac* system, DEPC treatment has no significant effect on the $\Delta\Psi$ generated in the presence of reduced PMS (−127 and −115 mV without and with DEPC treatment, respectively) and no effect on NPG binding in the absence of $\Delta\bar{\mu}_{H^+}$ (data not shown).

Discussion

The results presented in this paper support the concept (Stock & Roseman, 1971; Tokuda & Kaback, 1977; Lopilato et al., 1978) that active transport of TMG catalyzed by the melibiose transport system involves the coupled movement of sodium (or lithium). In addition, the data demonstrate that sodium is necessary for high-affinity binding of NPG to the porter and for maximal rates of carrier-mediated efflux of TMG down a concentration gradient. It should be emphasized that the effects of sodium on the affinity for NPG and on TMG efflux are due to the presence of sodium per se and not to a sodium gradient, since the vesicles were equilibrated with the cation and the external concentration was maintained during the assays. The sodium-induced increase in affinity (decrease in K_D) is about fivefold, a value that is in good agreement with the effect of sodium on the apparent K_m for active TMG uptake [a four- to eightfold decrease, as reported here and by Tokuda & Kaback (1977) and Lopilato et al. (1978)].

Recently, a unique approach toward elucidating the mechanism of proton–lactose symport in *E. coli* membrane vesicles was reported (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979), and a similar approach was adopted here. Although the symported ions differ, there are many superficial similarities between the melibiose transport system

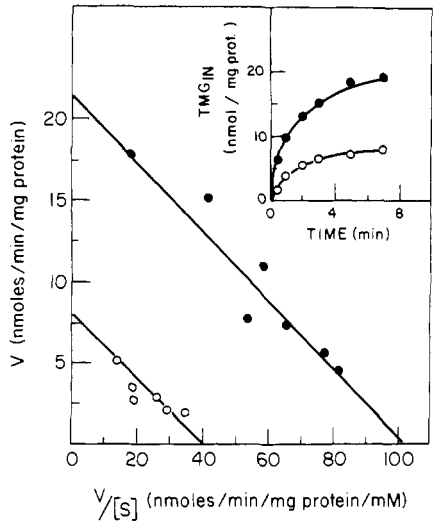

FIGURE 7: Effect of DEPC on the kinetics of reduced PMS driven TMG uptake. Vesicles were treated with 2.0 mM DEPC (○) or with an equivalent volume (0.1%) of ethanol (●) as described previously (Padan et al., 1979) and under Methods. After washing and resuspension, initial rates of TMG influx were determined as in Figure 6, except that the vesicles were energized with 20 mM potassium ascorbate (pH 7.0) and 0.1 mM PMS instead of D-lactate and the assays were conducted at pH 7.0. Incubation with ascorbate and PMS was carried out for 30 s prior to addition of [^{14}C]TMG, and the reaction mixtures were maintained under an atmosphere of water-saturated oxygen at all times. Data were analyzed and corrected for nonspecific influx as described in Figure 6 and are presented as Eadie–Hofstee plots. Inset: Time course of TMG uptake by control vesicles (●) and vesicles treated with 2 mM DEPC (○). Vesicles were energized with reduced PMS as described above and assayed with [^{14}C]TMG at a final concentration of 0.5 mM.

and the *lac* transport system in *E. coli*. Both porters catalyze active accumulation of many of the same substrates (e.g., TMG and melibiose) and both bind NPG in the absence of energy with similar numbers of binding sites. Furthermore, the kinetic parameters of active transport are similar in both cases. Despite the similarities, however, clear and important differences are apparent.

There is a marked difference between the two systems, for instance, when rates of efflux and exchange are compared. With the *lac* system, exchange is at least 10-fold faster than efflux (Kaczorowski & Kaback, 1979), indicating that the latter process is limited by a step associated with return of the unloaded carrier to the inner surface of the membrane. In contrast, the melibiose porter catalyzes efflux and exchange at essentially identical rates, suggesting that both processes are limited by a common step, either binding or release of substrate or translocation of substrate across the membrane.

The observation that TMG efflux is retarded by imposition of $\Delta\Psi$ (interior negative) is consistent with the notion that passive, carrier-mediated flux of TMG down a concentration gradient occurs in symport with sodium, making the overall process electrogenic. A similar effect of $\Delta\Psi$ on lactose efflux was reported for the β-galactoside transport system (Kaczorowski et al., 1979), but this finding, in itself, does not provide insight into what partial reaction(s) in the overall process involves the translocation of charge. With the *lac* system, however, imposed $\Delta\Psi$'s have no effect on the exchange reaction, indicating that the reactions catalyzed by the loaded carrier do not involve the translocation of a charged species [i.e., that the ternary complex between the *lac* carrier, proton(s), and substrate is neutral (Kaczorowski et al., 1979)]. Given this conclusion and the polarity effects of the imposed $\Delta\Psi$'s, it was suggested that the unloaded *lac* carrier catalyzes a reaction associated with the translocation of a net negative charge [see also, Schuldiner et al. (1975), Rottenberg (1976), and Ramos & Kaback (1977b,c)]. In contrast to these observations, exchange of TMG via the melibiose porter, a process that must be electroneutral as an overall reaction, is retarded by an imposed $\Delta\Psi$ (interior negative), indicating that either binding and release of substrate or translocation of the ternary complex across the membrane involves the movement of charge. Since it seems unlikely that binding and release of substrate involve charge movement, the simplest explanation for the observations as a whole is that the ternary complex between the melibiose porter, sodium, and TMG is positively charged.

If sodium–TMG symport via the melibiose porter involves a positively charged ternary complex and translocation of this complex is rate limiting for active transport, generation of $\Delta\Psi$ (interior negative) would be expected to increase the maximum velocity of transport with little or no effect on the apparent K_m of the reaction. Clearly, the kinetic experiments presented in Figure 6 support this idea. Addition of reduced PMS, which leads to the generation of a large $\Delta\Psi$, causes a dramatic increase in V_{max} and no observable change in apparent K_m. Since the rate of efflux is also decreased by imposition of $\Delta\Psi$ (interior negative), the coupling of $\Delta\Psi$ to active TMG accumulation may be viewed largely as an effect of the electric field on the positively charged ternary complex. Again, it should be emphasized that these results are markedly different from those obtained with the *lac* transport system where $\Delta\bar{\mu}_{H^+}$ causes a dramatic decrease in the apparent K_m for lactose (by 2 orders of magnitude) with little effect on V_{max}.

Treatment of RA11 vesicles with DEPC causes a decrease in V_{max} for $\Delta\Psi$-driven TMG accumulation without significant effects on the apparent K_m, the $\Delta\Psi$ generated in the presence of reduced PMS, or binding of NPG. With the *lac* system the reagent also inhibits $\Delta\bar{\mu}_{H^+}$-driven lactose transport without altering $\Delta\bar{\mu}_{H^+}$ or the energy-independent parameters of the system, but in this case DEPC treatment leads to an increase in apparent K_m with no change in V_{max} (Padan et al., 1979). Mechanistic considerations aside, it is noteworthy that in both cases, DEPC treatment interferes with the primary kinetic response of these transport systems to the electrochemical ion gradients that drive substrate accumulation. That is, with the *lac* system, DEPC treatment inhibits the decrease in apparent K_m induced by $\Delta\bar{\mu}_{H^+}$, while with the melibiose system, the reagent interferes with the increase in V_{max} caused by $\Delta\bar{\mu}_{Na^+}$.

The immediate driving force for sodium–substrate symport is $\Delta\mu_{Na^+}$, which is composed of two components: (i) the membrane potential ($\Delta\Psi$) generated by proton extrusion through the membrane-bound respiratory chain and (ii) the chemical gradient of sodium ($Na^+_{out} > Na^+_{in}$) established by means of sodium–proton antiport. In view of the results presented here, it is proposed that the two components of $\Delta\bar{\mu}_{Na^+}$ affect different steps in the overall transport mechanism. Since the affinity of the porter for NPG is increased in the presence of sodium, maintenance of a low internal sodium concentration would result in higher affinity for substrate on the outer surface of the membrane relative to the inner surface, thus promoting accumulation of substrate inside of the vesicles. On the other hand, $\Delta\Psi$ (interior negative) causes an increase in V_{max} for influx and a decrease in the rate of efflux, and it is suggested that these manifestations are due to an effect of the electric field on a positively charged ternary complex between the porter, sodium, and substrate. Finally, it should be emphasized that regardless of the validity of these speculations, it is apparent that, despite many superficial similarities, proton–

lactose symport and sodium–TMG symport are different processes mechanistically.

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References

Cohen, G. N., & Rickenberg, H. V. (1956) *Ann. Inst. Pasteur, Paris 91*, 693.
Harold, F. M. (1976) *Curr. Top. Bioenerg. 6*, 83.
Hirata, H., Altendorf, K., & Harold, F. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1804.
Hirata, H., Altendorf, K., & Harold, F. M. (1974) *J. Biol. Chem. 249*, 2939.
Kaback, H. R. (1971) *Methods Enzymol. 22*, 99.
Kaback, H. R. (1974) *Science (Washington, D.C.) 186*, 882.
Kaback, H. R. (1976) *J. Cell. Physiol. 89*, 575.
Kaback, H. R., & Barnes, E. M. (1971) *J. Biol. Chem. 246*, 5523.
Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691.
Kaczorowski, G. J., Robertson, D. E., & Kaback, H. R. (1979) *Biochemistry 18*, 3697.
Konings, W. N., & Boonstra, J. (1977) *Curr. Top. Membr. Transp. 9*, 177.
Lanyi, J. K. (1979) *Biochim. Biophys. Acta 559*, 377.
Lanyi, J. K., Renthal, R., & MacDonald, R. I. (1976) *Biochemistry 15*, 1603.
LeBlanc, G., Rimon, G., & Kaback, H. R. (1980) *Biochemistry 19*, 2522.
Lombardi, F. J., Reeves, J. P., Short, S. A., & Kaback, H. R. (1974) *Ann. N.Y. Acad. Sci. 227*, 312.
Lopilato, J., Tsuchiya, T., & Wilson, T. H. (1978) *J. Bacteriol. 134*, 147.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Mitchell, P. (1961) *Nature (London) 191*, 144.
Mitchell, P. (1963) *Biochem. Soc. Symp. 22*, 142.
Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc. 41*, 445.
Mitchell, P. (1968) in *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Ltd., Bodmin, England.
Mitchell, P. (1973) *J. Bioenerg. 4*, 63.
Mitchell, P. (1977) *FEBS Lett. 78*, 1.
Padan, E., Patel, L., & Kaback, H. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6221.
Ramos, S., & Kaback, H. R. (1977a) *Biochemistry 16*, 848.
Ramos, S., & Kaback, H. R. (1977b) *Biochemistry 16*, 854.
Ramos, S., & Kaback, H. R. (1977c) *Biochemistry 16*, 4271.
Reenstra, W. W., Patel, L., Rotenberg, H., & Kaback, H. R. (1980) *Biochemistry 19*, 1.
Rottenberg, H. (1976) *FEBS Lett. 66*, 159.
Rudnick, G., Schuldiner, S., & Kaback, H. R. (1976) *Biochemistry 15*, 5126.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14*, 5451.
Schuldiner, S., & Fishkes, H. (1978) *Biochemistry 17*, 706.
Schuldiner, S., Kerwar, G., Weil, R., & Kaback, H. R. (1975) *J. Biol. Chem. 250*, 1361.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1975) *J. Biol. Chem. 250*, 4291.
Stock, J., & Roseman, S. (1971) *Biochem. Biophys. Res. Commun. 44*, 132.
Tokuda, H., & Kaback, H. R. (1977) *Biochemistry 16*, 2130.
Tokuda, H., & Kaback, H. R. (1978) *Biochemistry 17*, 698.
West, I. C. (1970) *Biochem. Biophys. Res. Commun. 41*, 655.
West, I. C., & Mitchell, P. (1970) *J. Bioenerg. 3*, 445.
West, I. C., & Mitchell, P. (1973) *Biochem. J. 132*, 587.
West, I. C., & Mitchell, P. (1974) *Biochem. J. 144*, 87.