Sodium–Proton Antiport in Isolated Membrane Vesicles of *Escherichia coli*†

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ABSTRACT: Sodium movements are studied in isolated membrane vesicles of *Escherichia coli*. The addition of D-lactate to membrane vesicles previously equilibrated with $^{22}Na^+$ induces a rapid efflux of $^{22}Na^+$ against its electrochemical gradient. This energy-dependent efflux can be detected under conditions (pH 6.6) in which both a pH gradient and an electrical potential are formed across the membrane, or under conditions (pH 7.5) in which only an electrical potential is formed. In the former case, valinomycin at a concentration of 2.5 μM completely abolishes the membrane potential and lowers the total proton electrochemical gradient by 70%; however, it has no effect on the D-lactate-induced $^{22}Na^+$ efflux. At pH 7.5, valinomycin inhibits the $^{22}Na^+$ efflux concomitantly with the inhibition of the electrical potential. These results are consistent with a change in the Na^+/H^+ antiport following an alteration in the external pH. It is proposed that at low pH values, the exchange is electroneutral, whereas at high pH values the exchange is electrogenic. As is to be expected, sodium movements induce proton movements in the opposite direction. When Na^+ containing vesicles are diluted into a medium devoid of Na^+, acidification of the interior of the vesicle, dependent on the existence of a sodium gradient, can be measured. Upon supply of an energy source, inverted membrane vesicles generate a large pH gradient. Under such conditions the addition of Na^+ to the medium evokes a rapid proton efflux. Only Li^+ can replace the Na^+; potassium, choline, or guanidine cannot. The possible physiological implications of the functioning of a Na^+/H^+ antiport are discussed.

A high internal potassium to sodium ratio is characteristic of the metabolizing prokaryotic as well as eukaryotic organism. In recent years it has become clear that in bacteria the basic energy transducing process is proton extrusion which generates an inwardly directed proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$), composed of a chemical component (ΔpH) and an electrical one ($\Delta\Psi$). The high internal potassium concentration can be best explained by its passive equilibration with the existing membrane potential (Rottenberg, 1973; Harold & Altendorf, 1974). Mitchell (1966) has suggested that the internal sodium concentration is maintained at a low level by a tightly coupled sodium–proton-exchange mechanism. Evidence supporting the presence of such an antiport has been presented for several systems. In mitochondria, pH measurements show catalysis of H^+ equilibration of Na^+ (Mitchell & Moyle, 1967). Osmotic swelling experiments indicate the electroneutral entry of Na^+ by exchange with H^+ (Brierley et al., 1968; Mitchell & Moyle, 1969). Harold & Papineau (1972) have demonstrated that, in *Streptococcus faecalis*, sodium extrusion is inhibited by a proton conducting uncoupler. In the same vein, West & Mitchell (1974) described an outward translocation of H^+ in *Escherichia coli* induced by the addition of Na^+. In isolated membrane vesicles, Lombardi et al. (1973) demonstrated an energy-dependent sodium efflux, although they did not interpret it as due to a Na^+/H^+ antiport mechanism. Tokuda & Kaback (1977) have recently shown energy-dependent Na^+ extrusion in membrane vesicles from *S. typhimirium*. Lanyi & MacDonald (1976), Lanyi et al. (1976), and Eisenbach et al. (1977) have suggested a Na^+/H^+ antiport in membrane vesicles of the halophile *H. halobium*.

In this work, we describe and characterize the Na^+/H^+ antiport of *E. coli* in isolated membrane vesicles. We present results supporting the notion that the stoichiometry of the exchange process is altered with changes in the external pH: at low pHs (6.6), the exchange is electroneutral, at higher pH values (7.5), more than one proton is translocated per sodium ion. The system is relatively specific and, of the ions tested, it translocates only sodium and lithium.

Experimental Procedure

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 30 ($i^+ z^+ y^+ a^+$) or *E. coli* ML 308-225 ($i^- z^- y^+ a^+$) were grown on minimal medium A (Davis & Mingioli, 1950) containing 1% disodium succinate (hexahydrate) or 0.5% sodium glucuronate. "Right-side-in" membrane vesicles were prepared as previously described (Kaback, 1971; Short et al., 1975) except that 25% sucrose was added. Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6) and stored at −70 °C.

Inverted membrane vesicles were prepared as described by Tsuchiya & Rosen (1975), omitting the last wash. Vesicles were suspended at a final protein concentration of 6–12 mg/mL in a solution containing Tris-HCl, 10 mM, pH 7.5, magnesium sulfate, 10 mM, potassium chloride, 0.15 M, and glycerol, 55%, and stored in liquid nitrogen.

For studies at various pHs, membranes were treated essentially as previously described (Ramos et al., 1976): Membrane suspensions containing about 4 mg of protein per mL were thawed rapidly at 46 °C, diluted at least tenfold with 0.1 M potassium phosphate at the desired pH, and incubated for 10 min at 25 °C. The suspension was centrifuged at 40 000*g* for 30 min and the pellet resuspended and washed once in a similar volume of the same buffer. The final pellet was resuspended to a protein concentration of about 4 mg per mL.

Sodium Loading and Transport Assays. Membrane vesicles at a final protein concentration of 2 mg/mL were resuspended

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in 50 mM potassium phosphate containing 10 mM magnesium sulfate and 10 mM ^{22}NaCl (4000–6000 cpm/nmol) at pH 6.6 or 7.5. The samples were incubated for 2–14 h at 4 °C whereupon aliquots were transferred to a series of reaction vessels and incubated at 25 °C for given periods of time after the proper additions had been made. Incubation was terminated by the addition of 2 mL of 0.1 M LiCl; the samples were rapidly filtrated [Schleicher and Schuell cellulose nitrate filters (0.45-μm pore size)] and washed with an additional 2 mL of the above solution. Similar results were obtained when the incubation was terminated by the addition of 0.1 M choline chloride. The whole procedure (termination, filtration, and washing) did not last for more than 7–10 s. With any stopping solution used, a slower filtration rate causes leakage of sodium from the vesicles. Samples were run in triplicate and background values, obtained from samples diluted with 2 mL of LiCl solution and incubated for 2 h at 25 °C before filtration, were subtracted.

Fluorescence Measurements. Fluorescence was measured with an Eppendorf fluorimeter. The exciting light was filtered through a 366-nm filter. Emission was measured via a Corning CS 4-96 filter, a Strand Electric Co. Cinemoid filter no. 62, and Wratten no. 58 filter. Calculations and treatment of the results were carried out as previously described (Schuldiner et al., 1972).

Protein Determinations. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Materials

^{22}NaCl (carrier free) and [^{14}C]glucuronic acid (76 Ci/mol) were obtained from The Radiochemical Centre, Amersham. Valinomycin was purchased from Sigma; FCCP[1] was from DuPont. All other materials were of reagent grade and were obtained from commercial sources.

Results

Energy-Dependent Sodium Efflux. The data presented in Figure 1 illustrate a typical experiment in which sodium efflux is followed. In this experiment, the vesicles had been passively loaded with ^{22}Na until equilibration. If no additions are made, the sodium content is stable for several hours (not shown). Upon addition of 20 mM lithium D-lactate to the medium, the ^{22}Na content decreases dramatically. Although not shown, similar results were obtained upon addition of reduced PMS (under aeration) or potassium succinate. Addition of the proton conductor FCCP inhibited the D-lactate induced efflux. These findings indicate an active extrusion process whose energy source is the proton electrochemical gradient. Upon oxidation of D-lactate, membrane vesicles generate a pH gradient as well as a membrane potential across the membrane (Ramos et al., 1976; Schuldiner & Kaback, 1975). The results presented above do not allow us to determine which component is the major contributor of the driving force for the sodium efflux. However, this question can be elucidated through the use of the appropriate ionophore. Thus the addition of 2.5 μM valinomycin in a potassium containing medium completely collapses the electrical potential across the membrane and only slightly increases the pH gradient. As a result, the $\Delta\bar{\mu}_{H^+}$ is decreased by about 70% (data not shown; Ramos et al., 1976; Ramos & Kaback, 1977a,b). Under these conditions, valinomycin does not inhibit Na^+ efflux (Figure 1A). Therefore,

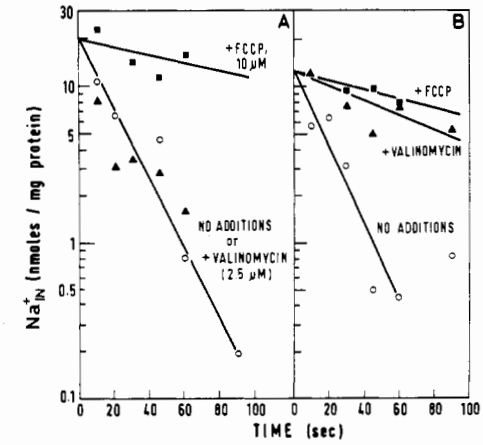

FIGURE 1: Effect of valinomycin on the D-lactate-dependent sodium extrusion. Membrane vesicles were prepared from *E. coli* ML-308-225 according to Kaback (1971). The vesicles were passively loaded with ^{22}NaCl and assayed for ^{22}Na content as described under Experimental Procedure. When the experiment was performed at pH 7.5 (Figure 1B), the membranes were previously brought to that pH, as described (Ramos et al., 1976). FCCP (10 μM) or valinomycin (2.5 μM) was added 3 min before the reaction was begun. Lithium D-lactate was added at a final concentration of 20 mM to all the vessels at zero time.

it may be concluded that the pH gradient and not the total protonmotive force drives the sodium efflux.

However, it has been reported that, even at pH 7.5, conditions under which the only component of the proton electrochemical gradient is the membrane potential (Padan et al., 1976), the internal sodium concentration of logarithmic *E. coli* cells is lower than the external one (Schultz & Solomon, 1961). Therefore, it was of interest to follow the Na^+ efflux in membrane vesicles at this external pH. As can be seen from the data in Figure 1B, under these conditions as well there is a D-lactate induced ^{22}Na efflux, which is inhibited by FCCP. However, at this pH, valinomycin inhibits the above process, indicating that the Na^+/H^+ exchange is an electrogenic one.

Generation of ΔpH by a Na^+ Gradient. The functioning of a sodium proton exchange mechanism predicts that, if a sodium concentration gradient is artificially set up across the membrane, protons will move in the opposite direction upon its decay, generating a pH gradient. It is possible to monitor pH gradients by several means (for a review, see Rottenberg, 1975). The basic principle involved in most of the known techniques is that certain weak acids or amines freely permeate the membrane only in their un-ionized state. Therefore their distribution across the membrane will be ultimately determined by the H^+ concentration gradient.

A technique introduced to measure pH gradients in chloroplasts (Schuldiner et al., 1972) and since extensively used in many systems (Rottenberg, 1975) is based on the measurement of the fluorescence of 9-aminoacridine. The data in Figure 2 illustrate the results obtained in an experiment in which the generation of ΔpH was followed utilizing the above technique. Thus, when Na^+ loaded membranes are diluted into a medium devoid of Na^+, proton entry may be detected by changes in 9-aminoacridine fluorescence. No fluorescence changes are detected if the dilution is made in a Na^+ containing medium.

Collapse of the ΔpH by Na^+. Inside-out membrane vesicles from *E. coli* possess the ability to generate a pH gradient (acid inside) when supplied with either ATP or an oxidizable substrate. The data in Figure 3A illustrate the effect of potassium succinate on the fluorescence of 9-aminoacridine. A large decrease in the fluorescence can be observed immediately upon

[1] Abbreviations used are: FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; ATP, adenosine triphosphate; PMS, phenazine methosulfate.

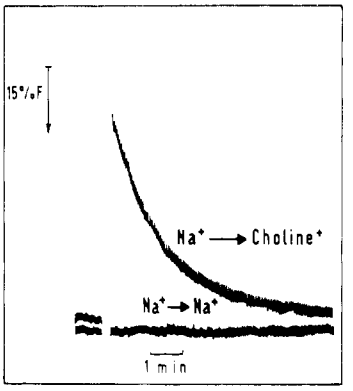

FIGURE 2: Generation of ΔpH by a sodium gradient. Membrane vesicles prepared as described in Figure 1 were loaded with sodium as follows: a membrane suspension containing 4 mg of protein per mL was removed from storage and thawed rapidly at 46 °C, diluted tenfold with 0.08 M sodium phosphate, 0.02 M potassium phosphate (pH 7.5), and incubated for 10 min at 25 °C. The suspension was centrifuged at 27 000*g* for 30 min and the pellet resuspended and washed once in a similar volume of the same buffer. The final pellet was resuspended at a final protein concentration of 40 mg/mL. The experiment was started by addition of 25 μL of the above membrane suspension to a cuvette containing in a final volume of 2.5 mL: 10 μM 9-aminoacridine, 0.02 M potassium phosphate (pH 7.5), and either of the following, as indicated: 0.08 M choline phosphate (pH 7.5) or 0.08 M sodium phosphate (pH 7.5). The direction of the arrow (F) indicates the direction of the fluorescence increase.

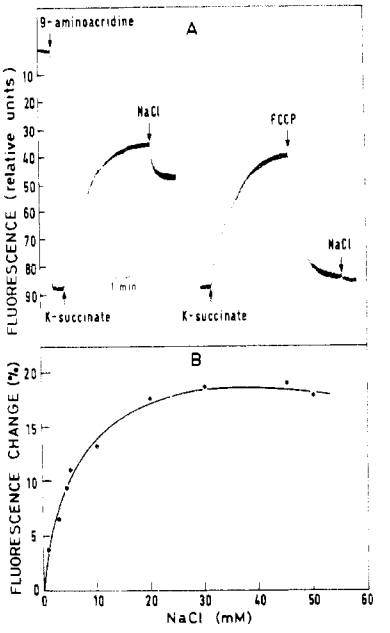

FIGURE 3: Effect of sodium on ΔpH. (A) Membrane vesicles were prepared from *E. coli* ML-308-225 according to Tsuchiya & Rosen (1975). Membrane vesicles (200 μg of protein) were added to a cuvette containing the following in a final volume of 2.5 mL: Tris-Cl, 10 mM (pH 8.0), $MgSO_4$, 10 mM, and KCl, 0.14 M. After a 1-min incubation, 9-aminoacridine was added to a final concentration of 10 μM. Where indicated, potassium succinate was added to a final concentration of 10 mM, NaCl to 15 mM, and FCCP to 7×10^{-7} M. Assuming an internal volume of 5 μL/mg of protein, the succinate-induced quenching corresponds to a ΔpH of 3.5 units, the decrease induced by Na^+, to 0.2 unit. (B) Membrane vesicles and conditions were as in A except that the final concentration of NaCl was as indicated. The effect of NaCl is expressed as the percent of the change in fluorescence obtained upon succinate addition.

addition of the energy source. This change in fluorescence indicates the generation of a large ΔpH across the membrane due to the action of the respiration-driven H^+ "pump". The fluorescence levels off after 3–5 min and, as would be expected, it is sensitive to the proton conductor FCCP. Addition of a pulse of 15 mM NaCl to the cuvette after the steady state has been attained causes an increase in the 9-aminoacridine fluorescence. This increase may be explained by a decrease in the pH gradient as a result of H^+ efflux upon the addition of Na^+ to the medium. Addition of NaCl before generation of the gradient (not shown) or after inhibition by FCCP causes no change in the fluorescence. As would be expected, the decrease of the ΔpH depends on the concentration of the externally added Na^+. As shown in Figure 3B, at adequately high Na^+ concentrations, a saturation level is obtained.

Specificity of the Na^+/H^+ Antiport. The data presented in Figure 4A illustrate the effects of various ions on the ΔpH. Na^+ is the most efficient in the induction of the H^+ efflux. Guanidine, which has been reported to replace sodium in nerve systems (Moore et al., 1967; Tasaki et al., 1965), is ineffective. Choline as well as K^+ do not induce any efflux. Moreover, addition of Na^+ after supplying either of the above ions causes an efflux similar in degree to that obtained by Na^+ in the absence of any other ion (not shown). Lithium, however, causes a considerable H^+ efflux under these conditions. It is assumed that lithium and sodium are translocated through one common antiporter. This hypothesis finds support in the data presented in Figure 4B. Thus, the addition of Li^+ to a system which already contains Na^+ does not produce further H^+ efflux. Vice versa, Na^+ has no effect if it is added to a Li^+-containing cuvette.

Effect of Valinomycin on the Na^+-Induced Proton Efflux. At pH 8.0, inverted vesicles generate a proton electrochemical gradient which seems to be composed mainly of a large pH gradient. Assuming an internal volume of the vesicles of 5 μL/mg protein (data not shown), it can be calculated (Schuldiner et al., 1972) that the interior of the vesicle is 2.5–3.0 pH units more acidic than the exterior. Under these conditions, valinomycin has no effect on the extent of the ΔpH

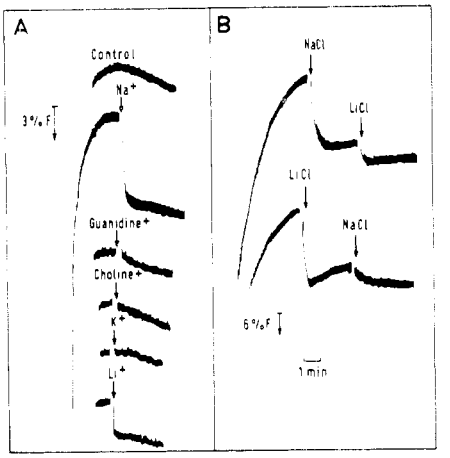

FIGURE 4: Specificity of the sodium proton antiport. (A) Membrane vesicles and conditions were as in Figure 3. The ΔpH was generated by the addition of ATP to a final concentration of 0.4 mM. The different ions were added as chloride salts at a final concentration of 15 mM. (B) Conditions were as in A except that consecutive additions were made as indicated and that potassium succinate (10 mM) was used to generate the ΔpH.

(Figure 5), thus lending support to the possibility that the electrical potential present across the membrane is but of a small magnitude.

In the presence of valinomycin the addition of NaCl causes a decrease in the ΔpH two- to threefold bigger than the decrease achieved in the absence of valinomycin. This is indeed to be anticipated if the Na^+/H^+ exchange is electrogenic at this high pH. Thus at every cycle one or more charges are

TABLE I: Apparent Na^+ Dependency of Glucuronate Accumulation.[a]

Ion in medium	Concn ratio $[(glucuronate)_{in}/(glucuronate)_{out}]$ at pH of medium					
	5.0	6.4	6.6	6.8	7.0	7.6
$Choline^+$	8.5	7.4	3.7	1.7	0	0
Na^+	10.2	9.5	6.8	5.0	2.0	0
Ratio $Na^+/choline^+$	1.2	1.28	1.84	2.9	∞	

[a] Membrane vesicles were prepared from *E. coli* ML30 grown on 0.5% sodium glucuronate as a carbon source and concentrated to 40 mg of protein/mL in 0.1 M potassium phosphate (pH 6.6). The membranes were diluted 100-fold into a medium containing in a final volume of 200 μL: 10 mM $MgSO_4$ and either 50 mM choline phosphate or 50 mM sodium phosphate as indicated. (The pH of the buffer was as indicated.) The reaction was initiated after 30 s by the addition of [^{14}C]glucuronate (12.2 μM, 76 Ci/mol) and 1 μM valinomycin. Transport assays were terminated at different times as previously described. The maximal concentrations attained were calculated assuming an internal osmotic volume of 2.2 μL/mg protein.

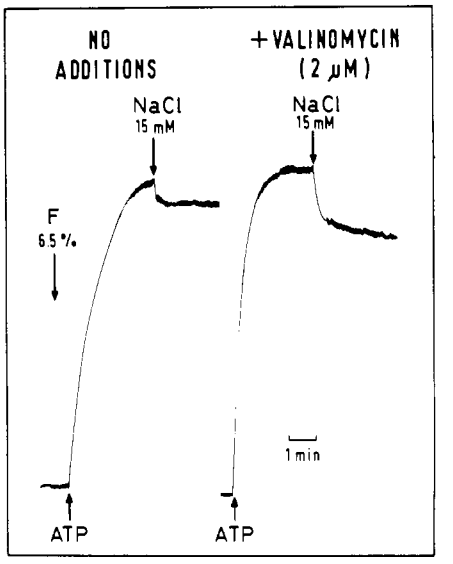

FIGURE 5: Effect of valinomycin on sodium-induced proton efflux. Membrane vesicles and conditions were as in Figure 3. The ΔpH was generated by the addition of ATP to a final concentration of 0.4 mM. Where indicated, 5 μL of an ethanolic solution of valinomycin (mM) was added 1 min prior to the ATP. Note that, although the extent of fluorescence quenching is very similar in the presence or in the absence of valinomycin, the rate of quenching is much faster in its presence.

translocated. An agent such as valinomycin can provide a means of dissipating this charge and may therefore accelerate the exchange process.

Apparent Sodium Dependency of the Accumulation of Substrates. It is widely accepted that accumulation of many substrates in bacteria occurs through the action of a substrate–proton symport (Mitchell, 1966, 1976; Harold, 1972; Kaback, 1974b, 1976). According to this hypothesis, the electrochemical gradient of protons is generated either by oxidation of electron donors through the respiratory chain or by the membrane-bound, proton-translocating adenosine triphosphatase (ATPase). It is this electrochemical gradient which is postulated to be the immediate driving force for the inward movement of transport substrates.

In the context of the present work, it was interesting to determine whether the action of the Na^+/H^+ antiport could sustain a H^+ gradient large enough to drive the active transport of substrates. Respiration-dependent accumulation of substrate in membrane vesicles isolated from *E. coli* is generally independent of the presence of Na^+ in the medium (Kaback, 1974b, 1978). However, when active transport of a substrate such as glucuronate is driven by an artificial membrane potential, some stimulation by external Na^+ is discerned (Table I). This transient potential will be dissipated by incoming protons; external Na^+ may exchange with the above, thus enabling further glucuronate accumulation. When a pH gradient (acid outside) is superimposed on the membrane potential, a higher degree of accumulation is achieved. Under these conditions the effect of external Na^+ becomes less apparent. However, when the electrochemical gradient of protons is limiting (alkaline pH outside), the effect of the externally added Na^+ becomes more and more noticeable. Thus, the ratio of the concentration gradient obtained when Na^+ is added to the medium as compared with the choline cation, increases from 1.2 (at an external pH of 5.5) to 2.9, and >3.0 at pH 6.8 or higher.

Discussion

The results presented in this paper provide evidence that supports the existence of a sodium/proton exchange system in isolated *E. coli* membrane vesicles. The data presented support the existence of an electroneutral exchange mechanism at low external pHs and an electrogenic mechanism at high pHs. In the latter case, sodium is extruded from vesicles whose potential is interior negative. It is thus assumed that more than one proton is exchanged per sodium ion. This change in stoichiometry as a function of the external pH is particularly interesting in view of the report that the stoichiometry of many H^+-solute symports changes with shifts in pH (Ramos & Kaback, 1977b; Ramos et al., 1976; Rottenberg, 1976). In all cases, an increase in the number of protons translocated is reported as the external pH increases. The results are consistent with the existence of an ionizable group on the carrier protein. Its degree of titration will be expressed as a change in the stoichiometry.

Our results regarding the specificity of the antiport system show that lithium and sodium ions are translocated by a common mechanism. This is in agreement with reports which provide evidence that sodium can be replaced by lithium in some, but not all, sodium-requiring symport systems. A prerequisite for the functioning of the symport mechanism is the build up of a lithium gradient through the action of the antiport.

It is evident that the mechanism by which prokaryotic and eukaryotic cells maintain a low Na^+ concentration is basically different. Eukaryotic organisms possess ATP-driven Na^+ pumps which actively extrude the ion. In bacteria, there is no known primary pump for sodium; the basic energy yielding process is proton extrusion which generates an inwardly directed proton electrochemical gradient. Sodium extrusion is achieved by a sodium–proton exchange diffusion system as proposed by Mitchell (1966).

The functioning of such a system in the bacterium poses some questions regarding its physiological importance: (1) a low internal sodium concentration naturally implies the existence of a sodium gradient which may serve as an energy source. Accordingly, several symport systems have been described in which Na^+ acts as a co-ion. It is interesting to note that sodium symports are widespread only in those organisms in whose natural environment there are high Na^+ concentrations, e.g., halophilic or marine microorganisms (Drapeau et

al., 1966; Wong et al., 1969; Thompson & MacLeod, 1971; Sprott & MacLeod, 1972; Lanyi & MacDonald, 1975; Lanyi et al., 1976). In many other microorganisms, the sodium symports seem to be an exception rather than the general rule (Stock & Roseman, 1971; Frank & Hopkins, 1969; Miner & Frank, 1974; Halpern et al., 1973; Kahane et al., 1975; Willis & Furlong, 1975; Tokuda & Kaback, 1977). In this context it should be stressed that in mitochondria, which seem to be evolutionally related to bacteria, proton symports are widespread (Harold, 1972). The evolutionary trends toward the Na^+ symports known in animal systems (Crane, 1977; Christensen, 1970) are still to be investigated. (2) The observation that sodium gradients can serve as an energy source for active transport under stringent conditions, in which the $\Delta\bar{\mu}_{H^+}$ is limiting, may hint of a possible function: Under some conditions (e.g., starvation) the $\Delta\bar{\mu}_{H^+}$ will decay at a certain rate as will the Na^+ gradient. However, the *capacity* of the latter could, under many conditions, be greater than the *capacity* of the H^+ gradient. Since the decay of the Na^+ gradient involves extrusion of protons (in the whole cell), the sodium gradient could be regarded as a possible energy buffer. (3) Another question is whether the antiport has any function in controlling the microorganism's internal pH. At high external pHs (>8.0), the interior of the cell is more acidic than the medium, although the main energy yielding process remains proton extrusion and the membrane potential is positive outside (Padan et al., 1976). This control of the internal pH has been attributed to the functioning of the Na^+/H^+ antiport, although no detailed mechanism has been proposed (Padan et al., 1976). In order to explain the fact that the inside of the cell is more acidic than the medium, one could take advantage of the electrogenicity of the antiport. In this case the rate of proton leakage back to the cell through the antiport could be higher than the rate of proton movement outward through the proton "pump". However, the number of net charges retranslocated into the cell should be smaller than the charge translocated by the proton "pump", so that the membrane potential does not completely collapse during this process. Although no direct data to support a certain stoichiometry are presented in this communication, one could speculate that such requirements may be met if, during each cycle in which $n - 1$ protons are actively extruded by the respiration-linked "pump", n protons are retranslocated through the antiport at the same time that $n - 1$ Na ions are extruded ($n \geq 3$). Thus, if n is 3, each cycle will result in the net movement of one proton into the cell therefore causing the interior to be more acidic. However, one net charge will still be pumped to the outside of the cell.

However, the actual physiological importance of the antiport and its role in the control of the internal pH cannot be decided at the present time. It awaits a more detailed study, probably involving isolation of mutants defective in this function. Moreover, the detailed molecular mechanism by which the exchange occurs has not been described as yet and will probably require isolation of the component(s) involved.

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Interaction of Choleragen with the Oligosaccharide of Ganglioside G_{M1}: Evidence for Multiple Oligosaccharide Binding Sites†

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ABSTRACT: The oligosaccharide moiety of the monosialoganglioside galactosyl-*N*-acetylgalactosaminyl[*N*-acetylneuraminyl]galactosylglucosylceramide (G_{M1}) induced a blue shift in the fluorescence spectra of choleragen and its B protomer similar to that observed previously with G_{M1} [Mullin, B. R., et al. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1679-1683; Moss, J., et al. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74,* 74-78]. The decrease in λ_{max} was maximal when the molar ratio of oligosaccharide to choleragen was 5.6. The circular dichroic spectra of choleragen and its A and B protomers indicated that the secondary structures were composed predominantly of β-pleated sheets. In the presence of oligosaccharide, the mean residue ellipticity of choleragen and its B protomer decreased between 220 and 240 nm; a maximal effect occurred when the molar ratio of oligosaccharide to choleragen was 5.7. Multiple oligosaccharide binding sites on choleragen were confirmed by equilibrium dialysis and gel permeation chromatography. With either procedure, each toxin molecule was observed to bind between 5 and 6 molecules of oligosaccharide. In addition, the apparent weight average molecular weight of choleragen and its B protomer, obtained from sedimentation equilibrium measurements, was increased by approximately 5500 in the presence of oligosaccharide; these results are consistent with 5-6 binding sites for the oligosaccharide (M_w = 1021). The determinants for the binding of choleragen to its ganglioside receptor reside in the B protomer of the toxin and the carbohydrate chain of the ganglioside; presumably, there is one binding site on each of the polypeptide chains that comprise the B protomer. The multivalent nature of choleragen and the observed perturbations of the toxin molecule induced by the oligosaccharide portion of its cell membrane receptor may be involved in the mechanism by which choleragen eventually activates adenylate cyclase within the cell.

Choleragen, an exotoxin of *Vibrio cholerae,* is responsible for the clinical manifestations of cholera and appears to exert its effects on vertebrate cells through activation of adenylate cyclase (Finkelstein, 1973). The toxin is a protein (M_w = 84 000) composed of two protomeric species, A and B (Finkelstein, 1973; Lönnroth & Holmgren, 1973; Cuatrecasas et al., 1973; van Heyningen, 1974; Sattler et al., 1975). The A protomer contains two nonidentical polypeptide chains, A_1 and A_2, linked by a single disulfide bridge. A_1 can activate adenylate cyclase in cell-free systems independent of the B protomer (Gill & King, 1975; Bitensky et al., 1975; Sahyoun & Cuatrecasas, 1975) or the toxin receptor (Moss et al., 1976a).

The B protomer is believed to contain five identical polypeptide chains (Gill, 1976; Kurosky et al., 1977; Lai et al., 1977) and binds the toxin to its cell membrane receptor, the monosialoganglioside G_{M1}[1] (Cuatrecasas, 1973a-c; Holmgren et al., 1973; King & van Heyningen, 1973; van Heyningen, 1974; Staerk et al., 1974). Although the B protomer is polymeric, choleragen can be precipitated and inactivated by equimolar amounts of G_{M1} (Holmgren et al., 1973, 1974).[2] The ability of choleragen to induce redistribution of ganglioside receptors on lymphocytes indicated that choleragen is functionally multivalent (Révész & Greaves, 1975; Craig & Cuatrecasas, 1975; Sedlacek et al., 1976).

Determining the stoichiometry of the choleragen-ganglioside interaction is complicated by the self-association of gangliosides in solution (Gammack, 1963; Yohe & Rosenberg, 1972; King et al., 1976). Since the oligosaccharide chains of gangliosides would not be expected to form micelles and the specificity of G_{M1} in the choleragen-ganglioside interaction resides in the carbohydrate moiety (Holmgren et al., 1974; Staerk et al., 1974), we examined the interaction of oligosaccharides derived from gangliosides with choleragen. Our results indicate that choleragen and its B protomer contain multiple binding sites for the oligosaccharide portion of G_{M1}.[3]

Experimental Procedure

Materials. G_{M1} and G_{D1a} were purified from bovine brain by silicic acid column chromatography (Penick et al., 1966).

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[1] Abbreviations used are: oligo-G_{M1}, Galβ1→3GalNAcβ1→4-[AcNeuα2→3]Galβ1→4Glc; gangliosides are designated by the nomenclature of Svennerholm (1963a).

[2] At low concentrations of choleragen, equimolar concentrations of G_{M1} precipitated the toxin whereas at higher concentrations, molar ratios of 2:1 or more were required (Holmgren et al., 1974; Staerk et al., 1974). Thus the precipitation and inactivation of choleragen by G_{M1} appear to be complex and may be influenced by the self-association of gangliosides in solution.

[3] A preliminary report of this work was presented at the annual meeting of the American Society of Biological Chemists, April 3-7, 1977 (Fishman et al., 1977b).