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Biochemistry

Reversible effects of chaotropic agents on the proton permeability of *Escherichia coli* membrane vesicles*

(active transport/membrane potential/β-galactosides/amino acids/lipophilic cations/carbodiimides)

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ABSTRACT **Extraction of *E. coli* ML 308-225 membrane vesicles with chaotropic agents causes the vesicles to become specifically permeable to protons. As a result, the vesicles no longer generate a membrane potential, interior negative, and they do not catalyze respiration-dependent lactose or proline transport. Treatment of the extracted vesicles with various carbodiimides decreases the permeability of the vesicle membrane to protons, causing them to regain their ability to generate a membrane potential. By this means, active transport is completely reactivated. Exposure of the vesicles to carbodiimides prior to extraction with chaotropic agents makes transport activity impervious to the effects of the chaotropes.**

Bacterial membrane vesicles catalyze active transport of many different solutes by a respiration-dependent mechanism that does not involve the generation or utilization of ATP or other high-energy phosphate compounds (1–4). In *Escherichia coli* vesicles, most of these transport systems are coupled primarily to the oxidation of D-lactate or reduced phenazine methosulfate (PMS) via a membrane-bound respiratory chain with oxygen or, under appropriate conditions (5–7), fumarate or nitrate as terminal electron acceptor. Recent experiments demonstrate that essentially all of the vesicles catalyze transport (8), and that D-lactate dehydrogenase (D-LDH) and Ca^{++},Mg^{++}-stimulated ATPase are both located on the inner surface of the vesicle membrane (9, 10). These and other findings (1–4, 11–13) demonstrate that essentially none of the vesicles is inverted.

Chemiosmotic phenomena, as postulated by Mitchell (14–16), play an important role in respiration-linked active transport (4, 17–22).† Some of the evidence is derived from studies of certain mutants that are defective in the membranous Ca^{++},Mg^{++}-stimulated ATPase complex (23–25). In certain instances (18, 26, 27), these mutants and vesicles derived from them exhibit a pleiotropic transport defect which is related to an increase in the permeability of the cell membrane to protons. Moreover, the alteration in proton permeability and the defect in active transport can be cured by treatment with *N,N'*-dicyclohexylcarbodiimide (DCC).

Previous experiments from this laboratory (28) demonstrate that chaotropic agents such as guanidine·HCl solubilize D-LDH from *E. coli* membrane vesicles, and enzyme solubilized in this manner has been used to reconstitute active transport in vesicles prepared from mutants defective in D-LDH activity. All attempts to reconstitute the extracted vesicles, however, were unsuccessful presumably because of a physiologic alteration unrelated to the loss of D-LDH. The observations presented here deal with the nature of this physiologic alteration and its reversal by carbodiimides.

Abbreviations: PMS, phenazine methosulfate; D-LDH, D-lactate dehydrogenase; DCC, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDCMI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [^{14}C]methiodide; CMC, 1-cyclohexyl-3-[2-morpholyl-(4)-ethyl]carbodiimide; CMCMI, 1-cyclohexyl-3-[2-morpholyl-(4)-ethyl]carbodiimide [^{14}C]methiodide; DiPC, diisopropylcarbodiimide; and CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

* This is paper XXIX in the series "Active Transport in Isolated Bacterial Membrane Vesicles". Paper XXVIII is S. Schuldiner and H. R. Kaback, submitted for publication.

† S. Schuldiner and H. R. Kaback, submitted for publication.

METHODS

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium A with 1.0% sodium succinate (hexahydrate) as the sole carbon source, and membrane vesicles were prepared as described (10, 29). *S. aureus* U-71 vesicles were also prepared as described (30).

Transport was assayed as described (31). [*1*-^{14}C]Lactose (22 mCi/mmol) was used at a final concentration of 0.4 mM; [*U*-^{14}C]proline (232 mCi/mmol) at a final concentration of 8.3 μM; and [^{3}H]triphenylmethylphosphonium (114 mCi/mmol) at a final concentration of 0.4 mM. [^{3}H]Triphenylmethylphosphonium uptake was assayed using Millipore Cellotate filters†.

Extraction of Membrane Vesicles with Chaotropic Agents. Suspensions of vesicles containing approximately 2.0 mg of membrane protein per ml (final concentration) in 0.05 M potassium phosphate (pH 6.6) were adjusted to a given concentration of a particular chaotropic agent by adding an aliquot of a concentrated solution of the chaotrope. The samples were incubated at 0° for 5–10 min, and centrifuged at approximately 40,000 × *g* for about 30 min. The supernatants were removed for protein determinations; the pellets were washed once in 0.1 M potassium phosphate (pH 6.6), and resuspended to approximately 4.0 mg of protein per ml in 0.1 M potassium phosphate (pH 6.6). Aliquots of these suspensions were then used directly for protein determinations and transport assays. Control preparations were treated in an identical manner, except that the chaotropic agent was omitted.

Discontinuous Polyacrylamide Slab Gel Electrophoresis was carried out in sodium dodecyl sulfate (32).

Oxygen Consumption was determined with a Clark oxygen electrode as described (33).

Materials. Enzyme grade guanidine·HCl and urea were obtained from Schwartz/Mann Biochemicals. Reagent grade sodium perchlorate and sodium thiocyanate were purchased from Fisher Scientific Co.

[*1*-^{14}C]Lactose was obtained from Amersham-Searle, and [*U*-^{14}C]proline from New England Nuclear. Triphenyl-

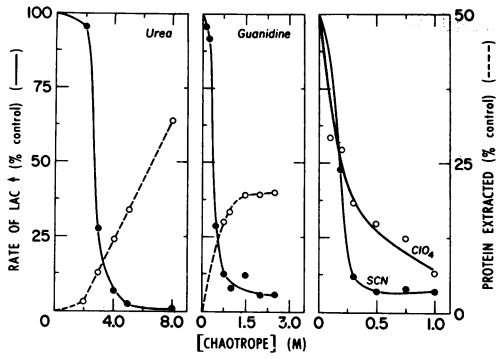

FIG. 1. Effect of chaotropic agents on *E. coli* ML 308-225 membrane vesicles. Vesicles were extracted with given concentrations of urea, guanidine·HCl, sodium thiocyanate (SCN), or sodium perchlorate (ClO_4) as described in *Methods*. Rates of lactose uptake (●—●) were determined for 30 sec under oxygen in the presence of 20 mM sodium ascorbate, 0.1 mM PMS, and 0.4 mM [*1*-^{14}C]lactose (22 mCi/mmol) (31). O- - - -O, protein solubilized by various concentrations of urea or guanidine·HCl.

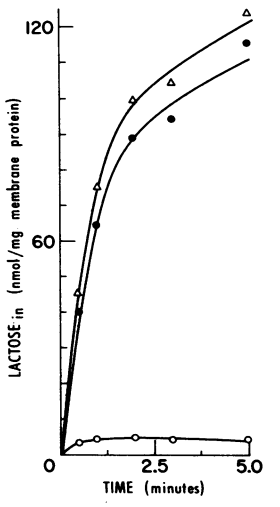

FIG. 2. Reactivation of lactose transport in guanidine·HCl-extracted vesicles by DCC. Membrane vesicles were extracted with 1.0 M guanidine·HCl as described in *Methods* (O). Where indicated (△), samples of this preparation were incubated with 70 μM DCC for 30 min at 25° prior to assaying lactose transport. Lactose transport was assayed at 25° under oxygen in the presence of ascorbate and PMS as described previously (31) and in *Methods*. (●) Lactose uptake by untreated control vesicles. Addition of 70 μM DCC had no significant effect on either the rate or extent of lactose uptake by the control preparation.

methylphosphonium bromide was labeled with tritium†.

DCC was obtained from Calbiochem and *N,N'*-dicyclohexyl[^{14}C]carbodiimide (45 mCi/mmol) was synthesized by The Radiation Synthesis Group of Hoffmann-LaRoche, Inc. under the direction of Dr. A. Leibman‡. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [^{14}C]methiodide (EDCMI) [17 mCi/mmol] (34); 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide (CMC) and 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide [^{3}H]methiodide (CMCMI) [80 mCi/mmol] (35); and diisopropylcarbodiimide (DiPC) were graciously contributed by Dr. James Ofengand of The Roche Institute of Molecular Biology. In each case, the radioactive carbodiimides reactivated active transport in membrane vesicles extracted with chaotropic agents. Non-water-soluble carbodiimides were dissolved in ethanol, and the final concentration of ethanol in the reaction mixtures did not exceed 1.0%.

All other materials were reagent grade obtained from commercial sources.

RESULTS

Effect of Chaotropic Agents on Active Transport. Extraction of ML 308-225 membrane vesicles with increasing concentrations of urea, guanidine·HCl, sodium thiocyanate, or sodium perchlorate leads to drastic inactivation of ascorbate-PMS-driven lactose transport (Fig. 1). Inactivation is dependent upon the concentration of the particular chaotropic agent, and with urea and guanidine·HCl, inactivation is correlated reasonably well with the loss of protein from the vesicles. With 5.0 M urea or 1.0 M guanidine·HCl, transport is virtually completely inactivated and approximately 15–20% of the membrane protein is extracted.

Reactivation of Active Transport by Carbodiimides. Strikingly, the inactivating effects of these harsh denaturants on active transport are completely reversed by treating the extracted vesicles with an appropriate concentration of any of four carbodiimides tested. As shown in Fig. 2, guanidine·HCl-extracted vesicles exposed to 70 μM DCC for 30 min exhibit about a 10–15% increase in specific activity relative to control vesicles. Similar results were obtained with vesicles extracted with 5.0 M urea, 1.0 M sodium perchlorate, or 0.5 M sodium thiocyanate (data not shown), and with 1.0 mM EDC, 2.0 mM CMC, or 3.0 mM DiPC (see below). Although higher concentrations of chaotropes result in some degree of irreversible inactivation, even after extraction with 8.0 M urea or 2.5 M guanidine·HCl, the transport activity of the vesicles can be restored to 20–40% of the control activity by exposure to carbodiimides. Although data will not be shown, it should be emphasized that the reactivating effect of the carbodiimides cannot be reversed by washing the vesicles extensively. Similar results were obtained when proline transport was assayed in ML 308-225 vesicles and when serine transport was assayed in *Staphylococcus aureus* vesicles extracted with 1.0 M guanidine·HCl.

D-Lactate-dependent transport is also reactivated with carbodiimides after extraction with guanidine·HCl or urea, but in this case, restoration to only about 40–50% of the control activity is observed. This is as expected since approximately 50–60% of the membrane-bound D-LDH activity is removed from the vesicles under these conditions (28). Urea or guanidine·HCl-extracted vesicles are completely reconstituted for D-lactate-dependent transport by exposure to DCC, followed by treatment with purified D-LDH (36).

When vesicles are treated with carbodiimides prior to extraction with urea or guanidine·HCl, ascorbate-PMS-driven lactose transport is not significantly affected by extraction with chaotropic agents (data not shown). It should be emphasized though that extraction of carbodiimide-treated vesicles with urea or guanidine·HCl solubilizes the same amount of protein and D-LDH activity as observed with untreated vesicles. These results indicate that carbodiimide-reactive sites in the membrane are accessible to these reagents even before exposure to the chaotropes.

Treatment of vesicles with phospholipase A (bee venom), Triton X-100, pyridine, or high concentrations of lithium or potassium chloride also results in inactivation of active transport, but these effects are not reversed by carbodiimides.

Concentration Dependence of Carbodiimide Reactiva-

‡ A manuscript describing the synthesis is in preparation (C. W. Perry, W. Burger, and A. Leibman).

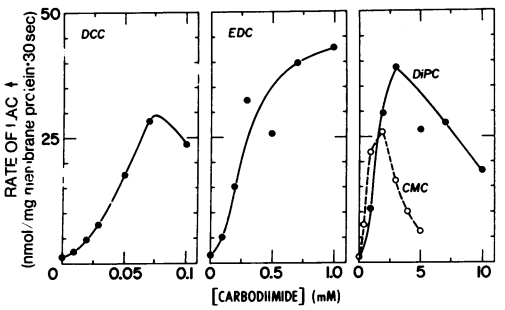

FIG. 3. Concentration dependence of carbodiimide reactivation. Membrane vesicles were extracted with 1.0 M guanidine·HCl as described in *Methods*. Samples were incubated with the designated carbodiimide at the concentration given for 30 min at 25°. At that time, the rate of lactose uptake was measured for 30 sec in the presence of ascorbate and PMS as described previously (31) and in *Methods*.

tion. Each carbodiimide tested reactivates lactose transport, although the concentrations required for optimal reactivation vary considerably (Fig. 3). Maximal rates of transport are observed with 0.07 mM DCC, 1.0 mM EDC, 2.0 mM CMC, and 3.0 mM DiPC, and in most cases, supraoptimal concentrations result in diminished activity. Although the reason for the variation in optimal concentrations for reactivation is not apparent, it seems unlikely that it is directly related to differences in hydrophobicity. Although DCC reactivates at the lowest concentration, DiPC, another hydrophobic carbodiimide, exhibits an optimum that is higher than those observed with EDC and CMC, both of which are water soluble. Treatment of guanidine·HCl-extracted vesicles with various concentrations of oligomycin does not cause reactivation, nor does treatment with dimethylsuberimidate (up to 5 mM). The latter reagent demonstrably cross-links vesicle proteins (data not shown).

Time Course of Reactivation and Carbodiimide Incorporation. With each carbodiimide tested, a marked lag phase is observed in the time course of reactivation (Fig. 4). With DCC, the lag is approximately 10 min; with DiPC, approximately 5 min; with EDC, approximately 2 min; and with CMC, approximately 15 min. In each case, subsequent to the lag, transport activity increases dramatically to a maximum which remains constant over the time course of the experiments. Treatment of the vesicles with any one of the carbodiimides for the time period characteristic of its lag phase, followed by washing of the vesicles, completely abolishes the lag phase when the vesicles are incubated a second time with a different carbodiimide (data not shown). This observation suggests that each of the carbodiimides reacts with the same site(s) in the membrane.

When the vesicles are incubated with radioactive carbodiimides, a lag phase is not observed in the time course of incorporation (Fig. 4). Thus, with DCC, EDCMI, and CMCMI, incorporation increases linearly with time, reaching a plateau prior to or at the same time that maximal transport activity is observed. Although data will not be presented, the time course of incorporation of the carbodiimides into vesicles that were not extracted with chaotropic agents is essentially the same as that shown, a result which is consistent with the conclusion that carbodiimide reactive sites within the vesicles are accessible prior to extraction. It is also noteworthy that vesicles treated with these carbodiimides exhibit disc gel electrophoretic patterns that are identical to guanidine·HCl-extracted vesicles (data not shown).

Mechanism of Inactivation of Active Transport by Chaotropic Agents and Its Reactivation with Carbodi-

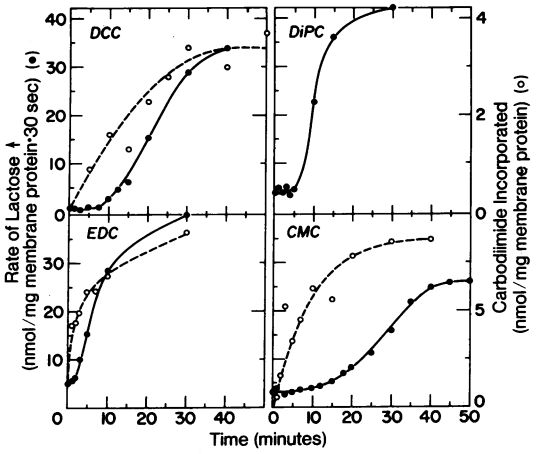

FIG. 4. Time course of carbodiimide reactivation of lactose transport and incorporation of labeled carbodiimides. ML 308-225 membrane vesicles were extracted with 1.0 M guanidine·HCl as described in *Methods*. Samples of these vesicles were incubated with 70 μM DCC, 3.0 mM DiPC, 1.0 mM EDC, or 2.0 mM CMC for the times indicated, and lactose transport was assayed for 30 sec with ascorbate and PMS as described previously (30) and in *Methods* (●). (○) Incorporation of radioactive carbodiimides by guanidine·HCl-extracted ML 308-225 vesicles. Incorporation of EDC[^{14}C]MI (17 mCi/mmol) and CMC[^{3}H]MI (80 mCi/mmol) was determined at final concentrations of 1.0 mM and 2.0 mM, respectively, by rapid filtration and Millipore filtration as described for transport (31), except that the filters were washed five times with 2.0-ml aliquots of 0.1 M LiCl and Millipore Cellotate filters (0.5 μm) were used. Incorporation of [^{14}C]DCC was determined in the following manner: 100-μl reaction mixtures containing 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulfate, 70 μM [^{14}C]DCC (45 mCi/mmol), and about 0.2 mg of membrane protein were incubated for the times shown. The samples were diluted with 2.0 ml of ethanol (100%) and centrifuged. The supernatants were discarded and the pellets resuspended in 100 μl of 0.1 M potassium phosphate (pH 6.6). Aliquots (50 μl) of these suspensions were counted in a liquid scintillation spectrometer. The data were corrected for control samples which were diluted with ethanol prior to addition of [^{14}C]DCC and centrifugation.

imides. Oxidation of D-lactate or reduced PMS by *E. coli* membrane vesicles generates a membrane potential, interior negative, which results from extrusion of protons from the vesicles (17, 19, †). Evidence has also been presented (4, 18–22, †) which indicates that the membrane potential is the driving force for respiration-linked active transport. When ML 308-225 vesicles are extracted with guanidine·HCl, they are unable to generate a membrane potential, interior negative, as judged by their inability to catalyze the uptake of the lipophilic cation triphenylmethylphosphonium (Fig. 5). If the extracted vesicles are exposed to DCC or EDC, their ability to generate a potential in the presence of reduced PMS is completely restored. These effects cannot be attributed to a reversible defect in the respiratory chain, since reduced PMS is oxidized at comparable rates by each of the vesicle preparations (data not shown).

Since any defect resulting in increased ion permeability could abolish the ability of the vesicles to generate and/or maintain an electrical potential, the permeability of the vesicles to various ions was investigated. No significant alteration in the permeability to ^{22}sodium or ^{86}rubidium is observed when the vesicles are extracted with 1.0 M guanidine·HCl or extracted and subsequently treated with DCC (Table 1). Preliminary observations (not shown) also indicate that the permeability of the vesicle membrane to sulfate and arabinose is not affected by these treatments. In contrast, marked differences in proton permeability are observed

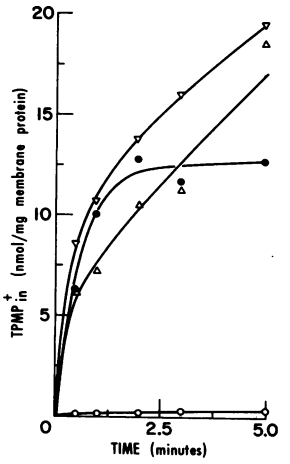

FIG. 5. Reactivation of triphenylmethylphosphonium ($TPMP^+$) uptake in guanidine·HCl-extracted vesicles by carbodiimides. Membrane vesicles were extracted with 1.0 M guanidine·HCl as described in *Methods* (○). Where indicated, samples of this preparation were incubated with 1.0 mM EDC (▽) or 70 μM DCC (△) for 30 min at 25° prior to assaying triphenylmethylphosphonium uptake. [^{3}H]Triphenylmethylphosphonium uptake was assayed at 25° under oxygen in the presence of ascorbate and PMS as described previously† and in *Methods*. (●) Triphenylmethylphosphonium uptake by untreated control vesicles. Addition of 70 μM DCC or 1.0 mM EDC had no significant effect on either the rate or extent of triphenylmethylphosphonium uptake by the control preparation.

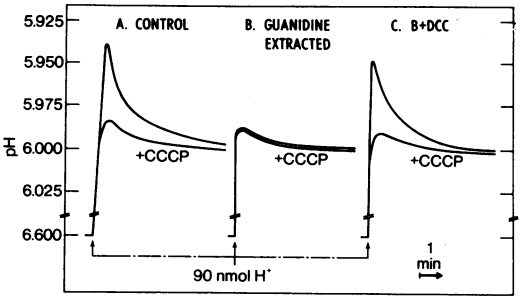

FIG. 6. Effect of chaotropic agents and DCC on proton permeability of ML 308-225 membrane vesicles. Membrane vesicles were extracted with 1.0 M guanidine·HCl as described in *Methods* (B), and a portion of the extracted vesicles was incubated with 70 μM DCC for 30 min at 25° (C). These preparations and untreated control preparations (A) were centrifuged and washed once with 0.66 M KCl containing 2.0 mM potassium phosphate (pH 6.6). The pellets were resuspended in the same medium to a protein concentration of 27.5 mg/ml, and valinomycin was added to a final concentration of 15 μM. Rates of proton equilibration were measured as follows: 1.4-ml aliquots of the suspensions were transferred to a chamber and continually stirred with a small magnetic bar. The pH was monitored with a pH electrode (Radiometer GK 2321C) connected to a Radiometer pH meter (model 26), and the signal was recorded in a double channel Corning recorder (model 845). The experiment was started by rapid addition of 90 nmol of HCl (arrows). Where indicated, CCCP was added to the reaction mixtures at a final concentration of 25 μM.

(Fig. 6). When control vesicles are pulsed with acid, the rate of equilibration of protons across the vesicle membrane exhibits a half-time of about 1–2 min, and is increased dramatically when the proton conductor carbonylcyanide *m*-chlorophenylhydrazone (CCCP) is added. With guanidine·HCl-extracted vesicles, the time course of equilibration is virtually identical to that observed with untreated vesicles in the presence of CCCP, and it is not altered by the proton conductor. Finally, when the guanidine·HCl-extracted vesicles are exposed to DCC, their behavior reverts to that of the control preparation.

DISCUSSION

The results presented in this paper resemble observations made with mitochondrial (37, 38), chloroplast (39), and *E. coli* (25, 40) membrane particles depleted of ATPase. They also resemble observations made with vesicles prepared from certain mutants of *E. coli* which are defective in the membrane ATPase complex (18, 23–27). In these instances, energy-linked transhydrogenase, oxidative phosphorylation, photophosphorylation, and/or respiration-linked active transport are impaired, but can be restored by exposure of the preparations to oligomycin or DCC. The studies of Mitchell and his associates (41, 42) and others (43, 44) indicate that removal of ATPase enhances the proton permeability of their mitochondria and chloroplasts, and that oligomycin restores their proton impermeability. Similarly, vesicles prepared from certain ATPase mutants of *E. coli* exhibit a pleiotropic defect in respiration-linked transport which can be cured by treatment with DCC (18, 26, 27). These effects were also attributed to alterations in the proton permeability of the vesicle membrane.

No evidence has been presented here which indicates that the effect of chaotropic agents is related specifically to the removal or inactivation of a component of the ATPase complex, although this is certainly a possibility. Attempts to reactivate extracted vesicles with chaotropic extracts or with crude preparations of coupling factor (40), however, have been completely unsuccessful. Furthermore, since 60–80% of the cellular Ca^{++},Mg^{++}-stimulated ATPase activity is lost during the preparation of membrane vesicles (10), it is apparent that most of the catalytic subunit of the ATPase complex, at least, is absent from the vesicles even before they are extracted. Two other important points should be mentioned: (*i*) carbodiimide-reactive sites are available prior to extraction of the vesicles with chaotropic agents; and (*ii*) the effects reported here are not specific for DCC, and do not ap-

Table 1. Permeability of ML 308-225 vesicles to sodium and rubidium

Treatment	Efflux rate ($t_{½}$)* (sec) ^{22}Na	^{86}Rb
1. None	27	111
2. Guanidine·HCl-extracted†	20	90
3. Guanidine·HCl-extracted + DCC‡	27	66

Designated preparations of membrane vesicles were adjusted to approximately 40 mg of membrane protein per ml in 50 mM potassium phosphate (pH 6.6). Aliquots (100 μl) of these suspensions were made up to final concentrations of 50 mM ^{22}NaCl (25 μCi/μl) or 40 mM ^{86}RbCl (26.7 μCi/μl), and equilibrated overnight at 0°. Aliquots of the suspensions (2 μl) were then rapidly diluted 100-fold into 50 mM potassium phosphate (pH 6.6) containing either 50 mM NaCl or 40 mM RbCl, respectively, and the samples were assayed at various times by rapid dilution and Millipore filtration (31). After correcting for radioactivity adsorbed to the filters, the results were plotted as the logarithm of the residual radioactivity against time, and the $t_{1/2}$ values were taken directly from these plots. In each case, the loss of radioactivity followed pseudo first-order kinetics.

* $t_{1/2}$ denotes the time required for loss of 50% of the radioactivity present at zero time.

† ML 308-225 vesicles extracted with 1.0 M guanidine·HCl as described in *Methods*.

‡ Guanidine·HCl-extracted vesicles were incubated with 70 μM DCC for 30 min at 25° prior to equilibration with ^{22}Na or ^{86}Rb.

pear to be directly related to the hydrophobicity of the carbodiimides.

With regard to the chemistry of carbodiimide-induced reactivation, very little is known, except that it is highly unlikely that these reagents catalyze intermolecular crosslinking in this system. Given these results and the known carboxyl reactivity of these compounds (45, 46), it seems reasonable to postulate that the carbodiimides may react with carboxyl groups in certain membrane proteins that are involved in proton permeability. Whether these proteins form a carboxyl-lined channel through the membrane or function as proton carriers cannot be answered at present, although it seems intuitively unlikely that a channel could exist that would transmit protons but not sodium or potassium. In any case, the availability of carbodiimide-resistant *E. coli* mutants that lack a specific protein (47) should help to provide a meaningful experimental approach to these problems.

In addition to providing supportive evidence for the essential role of chemiosmotic phenomena in the mechanism of respiration-linked active transport, these studies are remarkable for their implications with respect to the nature of the molecular machinery involved in these transport systems. Not only are the β-galactoside and proline carriers impervious to the effects of these harsh denaturants, but the interactions between most of the components of the respiratory chain are not affected. These conclusions are particularly impressive in view of the fact that approximately 20% of the membrane protein and 50–60% of the D-LDH are solubilized. Essentially all of the D-LDH in these vesicles is located on the inner surface of the vesicle membrane (10).

Finally, these results may be relevant to the development of other vesicle systems for the study of active transport. It is conceivable that some vesicle systems may be nonfunctional because the membrane becomes permeable to protons during preparation. In this context, carbodiimides may prove to be generally useful in studies of active transport in other isolated membrane systems.

We are indebted to The Radiation Synthesis Group of Hoffmann-LaRoche and Dr. A. Leibman for synthesizing [^{14}C]DCC, and to Dr. James Ofengand for providing EDC[^{14}C]MI and CMC[^{3}H]MI.

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