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# Ionophoric Material Derived From Eel Membrane Preparation

## I. Chemical Characteristics

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*Summary.* Prolonged digestion with trypsin released ionophoric material from a preparation of microsomes obtained from *Electrophorus electricus*. The material induced a large increase in the conductance of a black lipid membrane prepared from oxidized cholesterol. The induced conductance of cations was greater than that of anions. There was no discrimination between monovalent inorganic cations, neither with respect to conductance nor with respect to rate of incorporation. Gel filtration indicated a molecular weight of about 2000 for the active material. The material was 50- to 200-fold more effective when applied to the membrane on both sides than when applied on only one side. The material was unstable to storage, particularly at pH levels far from enutrality, but was partially reactivated in some cases by lyophilization or by a high concentration of dithiothereitol.

It is an accepted fact that the enzyme $(Na^+ + K^+)$-ATPase is involved in the active transport of $Na^+$ (Skou, 1965; Siegel & Albers, 1970). What is not yet determined is whether this enzyme contains the whole machinery for the active transport of $Na^+$ or just part of the machine. The $(Na^+ + K^+)$-ATPase splits the $\gamma$-phosphate of the ATP molecule which provides the energy required for $Na^+$-transport (Skou, 1965; Siegel & Albers, 1970; Shamoo & Brodsky, 1972). No conclusive data are yet available to demonstrate that the enzyme contains the site at which $Na^+$ resides for the purpose of being transported. It is not yet known how the chemical energy released from hydrolysis of ATP by $(Na^+ + K^+)$-ATPase is coupled to the movement of $Na^+$ from inside the cell to the outside of the cell.

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We have recently (Shamoo & Albers, 1973) shown that the acid-soluble peptides of a one-hour tryptic digest of the microsomal fraction of the electric organ of *Electrophorus* contains a $Na^+$-dependent ionophore. The $Na^+$-dependent ionophore is defined as that material which causes an increase in conductance of a black lipid membrane in the presence of $Na^+$. The increase in conductance in the presence of $Na^+$ occurs at a faster rate and at lower concentrations of the ionophore than in the presence of other monovalent cations. Incidental to our study of the one-hour tryptic digest of eel electroplax microsomal fraction, we prepared a 24-hour tryptic digest and carried out the procedure in a similar fashion to that described for the one-hour digestion (Shamoo & Albers, 1973). We observed that the peptides from a 24-hour tryptic digest retain the ability to increase the bilayer conductance in the presence of a monovalent cation, but the specificity for $Na^+$ is absent. The bilayer in the presence of the ionophore (24-hour digest) is partially cation selective.

The purpose of this paper is to report the properties and characteristics of the 24-hour digest peptides. It is possible that the ionophoric activity of the 24-hour tryptic digestion of eel electroplax microsomes either (1) comes from the basic peptide of the $Na^+$-dependent ionophore which lost its $Na^+$-requirement due to prolonged digestion, or (2) is an independent ionophore from the $Na^+$-dependent ionophore.

## Materials and Methods

### *Tryptic Digestion of Eel Microsomes*

The microsomal fraction of the electric organ of *Electrophorus electricus* has been described (Albers, Fahn & Koval, 1963). The reaction mixture for the tryptic digestion contained 4 mg/ml eel microsomal preparation and 10 μg/ml trypsin in 40 mM Tris-HCl (pH 8) at room temperature (24 °C). The standard incubation time was 24 hr. The reaction was terminated by the addition of 5% trichloroacetic acid. The mixture was centrifuged for 45 min at 20,000 × *g*. The supernatant was removed and passed through a DEAE-cellulose column previously equilibrated with 0.2 M ammonium acetate, pH 5.1.

The column was washed with distilled water and the entire eluate was collected and lyophilized. The lyophilized material was considered the ionophoric material to be tested. (For further detail *see* Shamoo and Albers, 1973.) The protein concentrations in this report were determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

### *Black Lipid Membrane and Conductance Measurements*

The oxidized cholesterol preparation for the formation of black lipid membrane has been described (Tien, Carbone & Davidowicz, 1966). The electronic circuitry has also been described (Lattore, Ehrenstein & Lecar, 1972). The standard electrolyte solution for the measurements of conductance was 0.1 M NaCl, 5 mM histidine, pH 7.4, at 21 °C.

[In the previous report (Shamoo & Albers, 1973), the temperature should have been stated as 15 °C instead of 19 °C.] The ionophoric material was introduced into the solution inside the Teflon cup (unless otherwise indicated). The system was under +50 mV voltage clamp, the solution inside the Teflon cup being positive with respect to the outside chamber (i.e., the side of the ionophore was positive). The material used here will not incorporate into the membrane and change conductance at all if we do not have a positive voltage or at least initiate the incorporation with a positive voltage.

## *Chemical Treatments*

We applied several chemical treatments to the final DEAE-eluate of the 24-hour tryptic digest of the microsomal fraction.

*Organic Solvent Extraction.* An aliquot of 15 mg of the 24-hour tryptic digest of eel microsomes was dried and extracted thoroughly with 10 ml of chloroform. After centrifugation at 5,000 × *g* for 10 min, the chloroform fraction was removed. The residue was dried with nitrogen gas and extracted in 10 ml of 2 : 1 chloroform/methanol. After centrifugation at 5,000 × *g* for 10 min, the chloroform/methanol fraction was removed. The residue was dried with nitrogen gas and dissolved in 1 ml of distilled water. A similar procedure was followed using ether alone and the fraction was called the ether fraction.

*Dansylation.* An aliquot of the tryptic digest of the microsomes, 2 mg/ml, was incubated with 0.25% DNS-Cl (1-Dimethylaminonaphthalene-5-sulfonyl chloride) in 0.1 M Triethylamine, pH 8 to 9, at 24 °C for 16 hr (Zamura, Nakajimia, Nakayama, Pisano & Udenfriend, 1973). DNS-Cl was first prepared as 0.5% (w/v) in dioxane.

*NEM-Treatment.* An aliquot of 2 mg/ml of 24-hour tryptic digest of eel microsomes was incubated with 10 mM N-ethylmaleimide, 40 mM Tris-HCl, pH 7.5, at 24 °C. The reaction was terminated by an excess (25 mM) of DTT (dithiothreitol) (Riordan & Valee, 1972).

*DTNB-Treatment.* An aliquot of 2 mg/ml of 24-hour tryptic digest of eel microsomes was incubated with 1.25 mg/ml of Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 40 mM Tris-HCl, pH 7.5, at 24 °C. The reaction was terminated by an excess (25 mM) of DTT (Habeeb, 1972).

*Photooxidation.* An aliquot of 2 mg/ml of 24-hour tryptic digest of eel microsomes was incubated with 0.01% final concentration methylene blue, phosphate buffer, pH 8.0, and exposed to fluorescent light for 1.5 hr at 24 °C. The reaction was terminated by an excess of DTT (Westhead, 1972).

A similar experiment was performed except the pH was 2.0 using 10 mM HCl instead of phosphate buffer.

*$H_2O_2$-Treatment.* An aliquot of 2 mg/ml of 24-hour tryptic digest of eel microsomes was incubated with 8% final concentration of $H_2O_2$ at room temperature (24 °C) for 2 hr. The reaction was terminated by an excess of DTT (Neumann, 1972).

*Air-$O_2$-Oxidation.* An aliquot of 2 mg/ml of 24-hour tryptic digest of eel microsomes was diluted 1 : 10 with 5 mM Tris-HCl, at pH 8.0 or 7.0, or with 10 mM HCl, pH 2.0 at 24 °C. Oxygen gas was bubbled for 2 min and the beaker left open overnight (White, 1972).

*Pronase-Digestion.* An aliquot of 2 mg/ml of 24-hour tryptic digest was incubated with 0.2 mg/ml pronase at pH 8.0 for 19 hr at 25 °C. The reaction was terminated by boiling for 5 min and centrifuging at 20,000 × *g* to remove pronase.

## Results

Many kinds of conductance changes have been induced in bilayers by incorporation of biological materials (Jain, White, Strickholm, Williams & Cordes, 1972; Shamoo & Albers, 1973; Sachs, Spenney, Saccomani & Goodall, 1974). Great care must be exercised to define experimentally the constituents responsible for these observations. One approach we took was to examine other tissues for ionophoric activity. We isolated sarcoplasmic reticulum (SR) of dog heart according to the method of Harigaya and Schwartz (1969). SR membranes were submitted to 24-hour tryptic digestion and carried through a procedure similar to that described in Materials and Methods for the electroplax microsomal fraction. Thus, we obtained the DEAE-eluate of dog heart SR. The test showed no evidence of any monovalent cation ($Na^+$, $K^+$, $Li^+$, $Cs^+$, $Rb^+$ and $NH_4^+$) ionophoric properties (Shamoo & Kirshberger, *unpublished data*). However, the data on divalent ionophoric activity of this fraction are not conclusive at this time. Since SR are known to have little if any ($Na^+ + K^+$)-ATPase (Katz, Toda, Repke, Iorio & Kirchberger, 1974), the data therefore suggest that not any tryptic digest of a membrane would yield ionophores with cationic selectivity.

### *Ionophore as a Peptide*

Ionophore is defined as that material which causes an increase in the conductance of black lipid membrane. We have three consistent lines of evidence that suggest that the ionophore (which may be assumed to be heterogeneous and impure) extracted as the acid-soluble tryptic digest (24-hour) of eel electroplax microsomes is a peptide rather than a lipid or carbohydrate, but we have not excluded the possibility that the ionophore may contain carbohydrate or lipid moieties. The evidence is: first, that 18-hour pronase digestion of a nonspecific peptide bond or bonds caused a complete inactivation of the ionophoric material; second, the dansylation (DNS-Cl) of ionophore, a reaction known to involve the free $\alpha$-amino group of proteins among other groups also caused a complete inactivation of the ionophoric material; and third, organic solvent extraction employing chloroform or chloroform/methanol or with ether caused no inactivation of the ionophoric material. No ionophoric material was found in the organic solvent fraction. If the ionophoric material was lipid-dependent, we should have an inactivation of ionophore left behind in the aqueous fraction, but this was not found. However, the chloroform/methanol fraction (*see* Materials and Methods) showed ionophoric activity, similar per mg protein (Lowry

protein) to that found in the aqueous fraction. This may indicate either that ionophore is closely attached to some lipid or that we have both highly polar and partially polar ionophoric materials.

*Effect of Monovalent Cations on the Effect of the Active Material on BLM*

Fig. 1 presents data on BLM conductance *vs.* NaCl concentration. Similar data were obtained with KCl, LiCl, CsCl, $NH_4Cl$ and RbCl. The electrolyte solution was 0.1 M NaCl, 5 mM histidine, pH 7.4, at 24 °C. The ionophoric material was added into the 5-mM histidine (pH 7.4) solution before the addition of the salts. No increase in conductance was observed until the above-mentioned monovalent cations were introduced. There is

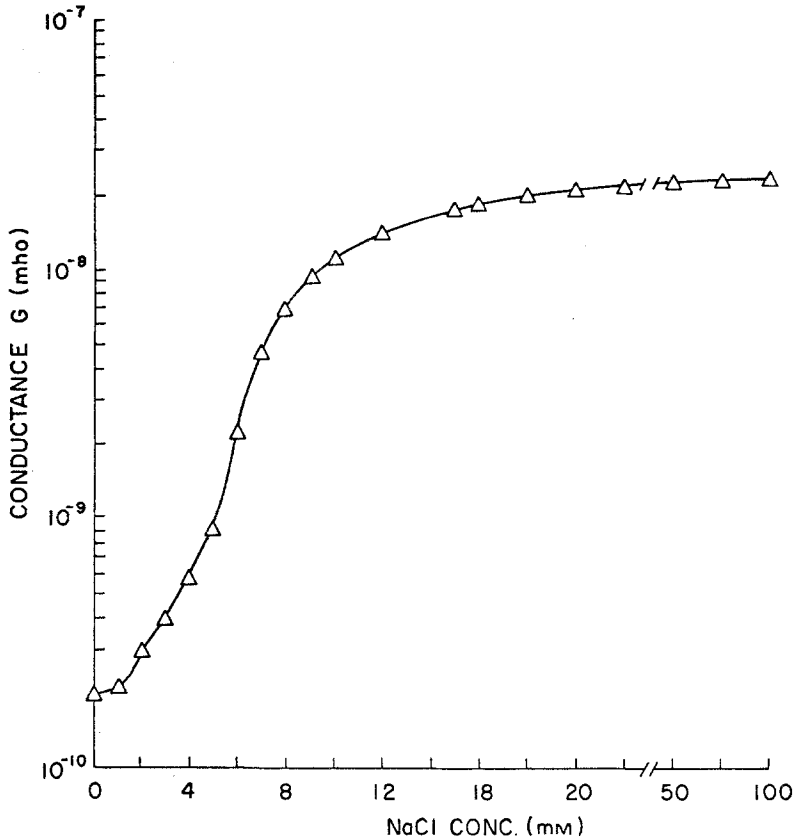


Fig. 1. Oxidized cholesterol BLM conductance versus NaCl concentration. The BLM conductance was measured in the presence of 5 mM histidine, pH 7.0, and also in the presence of a concentration of $7 \times 10^{-4}$ mg/ml eel electroplax ionophoric material. The ionophoric material was obtained as previously described. The concentration of NaCl was increased by the addition of small volumes of highly concentrated NaCl (3 M) to both sides of the membrane bathing fluid. The conductance reached a steady state within 3 min after each salt addition

clearly a saturation phenomenon for NaCl concentrations greater than 50 mM. Visual inspection of the figure shows that the apparent $K_m$ for $Na^+$ is close to 6 mM. The log of conductance versus the log of sodium chloride concentration is a complicated curve. No simple power relationship exists between the conductance and ionophore concentration. The bilayer in the presence of ionophore was partially cationic selective, resulting in a transmembrane potential of 45 mV/decade of salt concentration. The linear part of mV *vs.* salt concentration curve was between 10 and 50 mM. No significant selectivity among the monovalent cations was found.

*Estimate of Molecular Weight of the Ionophore*

G-25 Sephadex column was calibrated by the appropriate standards with known molecular weights. Fractionation of the active material by G-25 Sephadex column indicated that the active ionophore (or ionophores) has a molecular weight between 6000 (insulin peak) and 1411 (bacitracin peak) as shown in Fig. 2. At low molecular weight peptide adsorption into the column produces errors in the estimate of molecular weights. We can, however, make a rough estimate for the molecular weight of the active material. Thus, reference to Fig. 2 suggests that the molecular weight of the ionophore or ionophores may be approximately 2000.

*Inactivation of the Ionophoric Material*

Several days handling of the ionophoric material caused inactivation. Attempts were made to identify the cause of inactivation and subsequently to identify the type of chemical bonds that are modified to cause inactivation.

Table 1 presents data on different chemical treatment of the ionophoric material (*see* Materials and Methods for details). The first column represents the type of treatment and the second column represents percent activity recovered after the treatment. Control experiments with zero time of incubation with each of the different treatments indicated that the starting material possessed full ionophoric activity. N-ethylmaleimide, known to covalently bond with SH groups (Riordan & Valee, 1972) caused no significant inactivation of ionophoric activity. Neither DTNB, $H_2O_2$ nor the air-$O_2$ oxidation at pH's 2, 7 or 8 [all convert $-$SH bonds to $-$S$-$S bonds (Habeeb, 1972; Neumann, 1972; Westhead, 1972; White, 1972)] caused any significant inactivation of ionophoric activity. Thus the inactivation of the ionophoric material cannot be attributed to either oxidation or reduction of the accessible peptide $-$SH or $-$S$-$S groups. Photooxidation with methylene blue at pH 2.0, and at pH 8.0 (Westhead, 1972) caused about 80% inactivation. Therefore, the photooxidation was not pH-dependent and

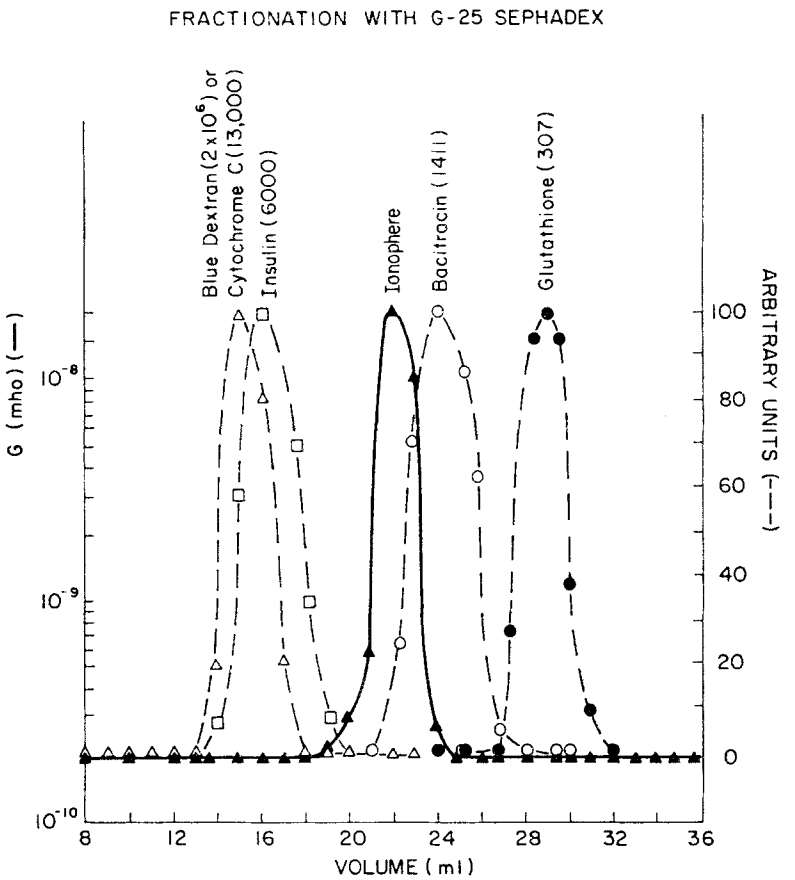


Fig. 2. Fractionation with G-25 Sephadex column; conductance and concentration of the molecular weight standards versus fraction number. A 32-ml G-25 Sephadex column was equilibrated with 0.5 M ammonium acetate at pH 7.0. The elution pattern of several standards with known molecular weights was determined on the column. The column was then eluted by 50 ml of 0.5 M ammonium acetate, pH 7.0. The presence of the standards in fractions of 1 ml of the eluate was determined by either direct optical density ($A_{280}$, $A_{580}$) measurements or by Lowry protein determination. A one-ml fraction showed maximal amounts of the standard and was taken arbitrarily as 100. Other determinations of the standard were calculated as percentages of the maximal amount. After calibration of the column, 0.6 ml (2 mg/ml) of the eel ionophoric material was added to the column. The column was eluted by 50 ml of 0.5 M ammonium acetate, pH 7.0. One-ml fractions after lyophilization were then tested individually for activity on the conductance of oxidized cholesterol BLM at $5 \times 10^{-4}$ mg/ml final concentration. The protein concentration of each fraction was determined prior to testing. Then variable volumes (usually from 1 to 10 ml) were introduced to give approximately $5 \times 10^{-4}$ mg/ml. The solid line represents the ionophoric activity (as an increase in conductance) versus the fraction number. The broken lines represent the location of the standards versus the fraction number

this may imply that a histidine moiety does not play an important role in the ionophoric activity. This is in contradistinction to the fact that changing the pH to $< 6.0$ converts the ionophore from cationic selective to anionic

Table 1. Effect of chemical treatments on the ionophoric activity[a]

| Treatment | % Activity |
|---|---|
| None | 100[b] |
| NEM ($10^{-2}$ M) | 93 |
| DTNB (1.25 mg/ml) | 94 |
| Air-$O_2$, pH 8.0 | 95 |
| $H_2O_2$ (8%) | 96 |
| Photooxidation, pH 2.0 | 20 |
| Photooxidation, pH 8.0 | 18 |

[a] The standard electrolyte solution bathing the black lipid membrane for conductance measurements is 0.1 M NaCl, 5 mM histidine, pH 7.0, 24 °C. (*See* Materials and Methods for the concentrations and conditions of different treatments.)
[b] 100% activity is defined as the steady-state increase in conductance obtained in 10 min by $1.0 \times 10^{-3}$ mg/ml untreated 24-hour tryptic digest of the electroplax microsomes. Thus, 20% activity indicates that five times ($5 \times 10^{-3}$ mg/ml) the amount of the untreated material is needed to reach the same steady-state increase in conductance within 10 min.

selective (Blumenthal & Shamoo, 1974). Histidine is the best candidate to fit such data. Histidine has an $-NH^+$ in its side residue with pK of 6.0.

### *Dissociation of the Active Material into Subunits*

During several days storage of the active material at 4 °C in dilute form (0.2 mg/ml and at pH's between 2 and 3, the normal pH of the final product due to the extraction procedure) we observed complete inactivation of the material with respect to increasing the conductivity of a bilayer membrane. Several explanations occurred to us: (1) some form of hydrolysis of the active material occurs during prolonged storage; (2) oxidation through air oxygen converts the sulfhydryl SH group to S – S bonds resulting in its inactivation; and (3) dissociation of the active material into inactive subunits may occur. To test the first possibility we reconcentrated the active material by lyophilization and were able to restore over 75% of the ionophoric activity of the material. This strongly suggests that cleavage due to hydrolysis is not the underlying cause of inactivation. The second possibility was excluded because of the data of Table 1 and Fig. 3 described below. If the inactivation was due to oxidation, the exposure of the inactive material to low concentration of a strong reducing agent such as DTT should have restored the activity (Cleland, 1964). Fig. 3 presents the data on conductivity versus DTT concentration. Low concentrations of DTT (up to 30 mM) had no appreciable effect, whereas high dosages (> 100 mM) completely restored the activity. An explanation of such reactivation of the ionophoric activity at high dosages is not clear to us at this time. However, high DTT

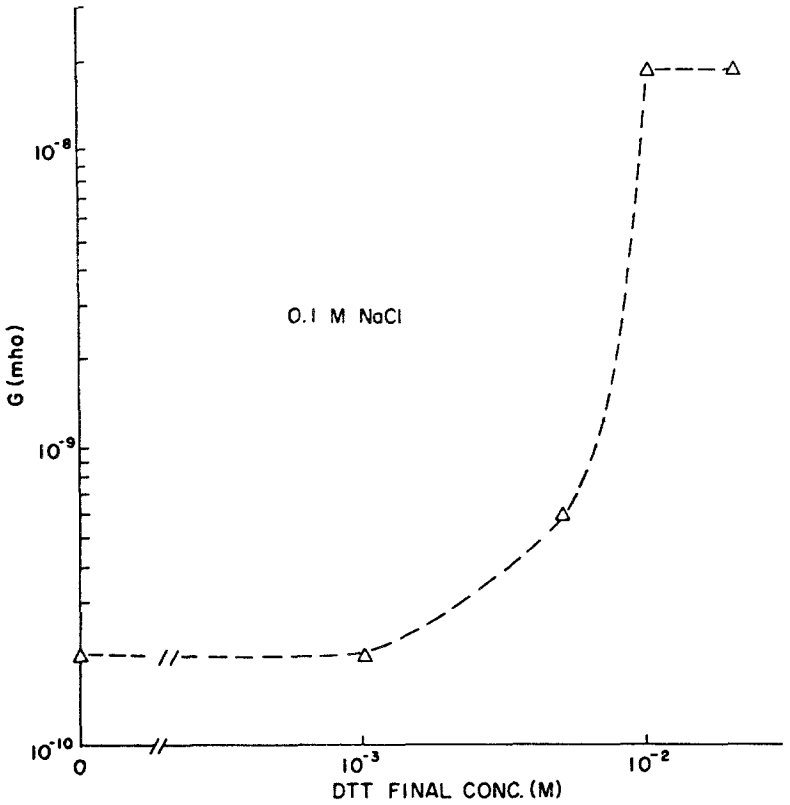


Fig. 3. Oxidized cholesterol BLM conductance versus various concentrations of dithiothreitol (DTT). The active material was stored at 0.2 mg/ml for a two-week period. Final concentrations of $2 \times 10^{-3}$ mg/ml were completely inactive in attempting to increase the BLM conductance. Prior to testing, aliquots of the active material were subjected to varied concentrations of DTT. The test on BLM conductance was then performed in the presence of 0.1 M NaCl + 5 mM histidine and $8 \times 10^{-4}$ mg/ml DTT-treated material

concentrations may reduce a partially hidden disulfide bond. DTT alone at the highest concentrations used here had no effect on BLM conductance.

To test the third possibility we stored the active material for five days at concentrations of 0.2 mg/ml in buffers at different pH's ranging from pH 2.0 to 10. The introduction of ionophoric material into the bathing solution did not change the pH of the bathing solution. Fig. 4 presents the data on conductance versus the pH of storage (triangles). It appears that storage at high or low pH's causes the material to be inactive when tested on BLM conductance at pH 7.0. The simplest explanation is that the active material dissociates into inactive subunits at extreme pH's. There is no immediate effect when varying the pH ($> 4.0$) of the bathing fluid on the oxidized cholesterol BLM conductance. Reconcentration of the inactive

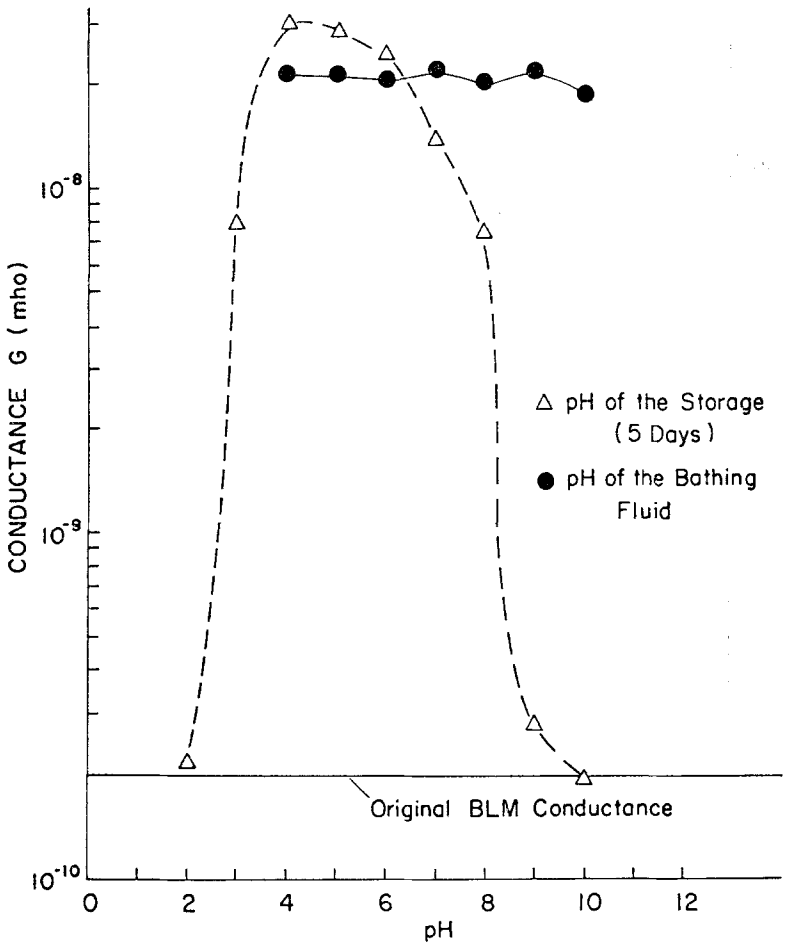


Fig. 4. Oxidized cholesterol BLM conductance versus pH. In reference to the pH of the bathing fluid, conductance was measured in the presence of 0.1 M NaCl and 5 mM buffer at the appropriate pH. The bilayer membrane became unstable when the pH of the bathing medium was below 4.0. In reference to the pH of the five-day storage medium, the active material was stored for five days at the indicated pH with 50 mM of buffer. The conductance was measured in the presence of 0.1 M NaCl + 5 mM histidine, pH 7.0, in the presence of $8 \times 10^{-4}$ mg/ml pH-treated eel ionophoric material. The ionophore was obtained as previously described. The solutions were: 10 mM HCl, pH 2.0; 1 mM HCl, pH 3.0; 50 mM sodium acetate, pH 4.0, 5.0, 5.6 and 6.0; 50 mM MES (2[N-morpholino]-ethane sulfonic acid), pH 7.0; 50 mM Tris-HCl, pH 8.0 and 9.0; and 50 mM AMP (2-amino-2-methyl-1-propanol), pH 10.0

material at pH $< 4.0$ by lyophilization restored about 75% of the activity. No experiments were performed on reconcentrations of the inactive material at pH $> 6.0$.

*Oligomer Formation*

Previous work, which measured the conductance response of membranes versus amount of ionophore (when added on one side only), indicated that

six molecules of the ionophoric material were required to form a conductive unit (Shamoo & Albers, 1973). Not shown were similar results obtained with the material in this report (i.e., the 24-hour tryptic digest). The amount of the one-hour digest ionophore required to be added on both sides of the membrane to achieve the same increase in conductance as compared to the addition of ionophore on one side of the membrane was five- to 10-fold less. In the case of the 24-hour tryptic digest ionophore, the amount required to achieve steady-state increase in conductance (up to $3 \times 10^{-1}$ $\Omega^{-1}/cm^2$) on both sides of the membrane was 50 to 200 times less than that required on one side of the membrane. This indicates that the smaller the ionophore, the more likely it is to penetrate faster from either side of the aqueous solution into the membrane or from within the membrane itself and form a conductive unit. This supports the idea that an oligomer may be required for the conductive unit. This is similar to what is known for gramicidin A (Urry, Goodall, Glickson & Mayers, 1971). Also, polyene antibiotics nystatin and amphotericin B behave in the same way (Cass, Finkelstein & Krespi, 1970).

## Discussion

The loss of $Na^+$-requirement for ionophore after 24 hr of tryptic digestion may indicate either that we have partially destroyed ionophore which led to loss of $Na^+$-requirement or that the 24-hour tryptic digest produced an overwhelming amount of nonspecific ionophores resulting in masking the $Na^+$-requirement. One way to resolve this is by purification of the ionophores separately or to repeat tryptic digestion of the partially purified ionophoric material obtained from a one-hour digest.

If the 24-hour tryptic digest is a different ionophore than the $Na^+$-specific ionophore, one may speculate on its physiological significance. The most obvious one would be that a monovalent cationic-selective sieve may serve in series with the $Na^+$-specific ionophore and/or the $Na^+$ transporting system. To answer questions of this kind we need further purifications.

*Note Added in Proof*: We have recently extracted a $Ca^{++}$-dependent and selective ionophore from $(Ca^{++}+Mg^{++})$-ATPase of sarcoplasmic reticulum (Shamoo & Mac Lennan, Sept. 1974. *Proc. Nat. Acad. Sci., In press*).

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