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Studies Concerning the Possible Reconstitution of an Active Cation Pump across an Artificial Membrane

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Summary. The cortical tissue of rat brain was fractionated through zonal centrifugation in a continuous sucrose density gradient, yielding a variety of morphologically distinct membrane fragments derived from nerve-end particles possessing variable levels of activity of Na, K-dependent Mg-sensitive ATPase (Na, K-ATPase) and other enzymes. Upon addition of certain of the zonal fractions, particularly those rich in the ATPase and acetylcholinesterase activities, to one side of planar artificial membranes, formed from mixtures of oxidized cholesterol and alkanes and bathed in a solution containing sodium, potassium, and magnesium ions, direct current membrane resistance fell from one to three orders of magnitude. Subsequent addition of ATP to the same side of the membrane to which the ATPase was added (the *cis* side) led to the development of net short-circuit current flow and open-circuit potential across the membrane (the *cis* side being negative with respect to the *trans* side). Development of the short-circuit current and open-circuit potential is dependent upon the presence of all the substrates of Na, K-ATPase as well as that of the enzyme itself. The net current flow is inhibited and the open-circuit potential discharged by the addition of ouabain to the *trans* side of the membrane, of phospholipase A to the *cis* side, or of trypsin to either side of the membrane. These observations provide circumstantial evidence for the reconstitution of the active cation pump across the artificial bilayer. Efforts to effect a similar reconstitution across membranes of this and other compositions employing Na, K-ATPase preparations from beef heart, beef brain, cat brain, human red cells, rabbit kidney, and rat brain microsomes failed.

It is well established that biological membranes provide the environment for a large number of enzymatic reactions and associated processes including active transport. In these cases, membrane composition appears to be crucial in at least two respects: (1) the membrane may provide specific cofactors required for one or more enzymatic reactions; (2) specific membrane viscoelastic properties, determined by membrane composition, may be essential to proper enzymatic function [29]. These considerations focus

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attention on the desirability of efforts to reconstitute membrane-associated enzymatic processes in artificial membrane systems in which composition of the membrane can be varied and, in principle, viscoelastic properties can be measured. In the report which follows, results of embryonic efforts to achieve these goals are provided; our attention has been directed toward reconstitution of Na, K-dependent Mg-sensitive ATPase (Na, K-ATPase) activity across artificial planar bilayers (black lipid membranes, BLM).

Na, K-ATPase has been implicated in the coupled active transport of sodium and potassium ions across a variety of biomembranes [24, 35–37]. The substrate for the system is ATP; there is a combined effect of Na and K ions on two opposite sides of the membrane; ouabain and other related cardenolides are inhibitory when on the outside of the cell. Several models have been suggested for the molecular events underlying active transport [8, 16, 20, 24, 36, 37, 43]. These models assume that the *transport ATPase* is asymmetrically located across the biomembranes such that: (a) the affinity for potassium and ouabain is high on the outward facing interface; (b) there is high affinity for Na and ATP on the inner interface; (c) the transport process involves extrusion of sodium from the cell and incorporation of potassium into the cell, in accordance with the differential affinities noted above; (d) hydrolysis of ATP involves sodium-dependent formation of a phosphoenzyme intermediate followed by potassium-dependent hydrolysis of this or a subsequent intermediate of the same type; (e) the stoichiometry of sodium and potassium ions transported in mutually opposite directions for each molecule of ATP hydrolyzed is uncertain; however, in quite a few cases the number of K ions taken up is less than the number of Na ions extruded [14, 27, 40]. As the transport process is electrogenic, anion or proton transport need not be implicated in the mechanism.

The study of the cation transport system *in situ* has been complicated by the apparent complexity of the transport system itself as well as difficulties in separating effects on the specific ATPase transport system from those on other transport processes, such as leakage. Our efforts to alleviate these difficulties through reconstitution of the transport system, as noted above, have been published in preliminary form [10]. A more extensive report of these studies emphasizing experimental methodology and including a number of new experiments, follows.

Materials and Methods

Hydrocarbon solvents were purchased from Matheson, Coleman, and Co. They were purified by careful distillation following their passage through a column of basic alumina (activity I). Cholesterol (USP) was recrystallized twice from ethanol. Phospholi-

pase A, phospholipase C, trypsin, ATP and ouabain were purchased from the Sigma Chemical Co. AMPD (2-amino-2-methyl-1,3-propanediol) was purchased from Aldrich Chemical Co. Dodecyl and didodecyl phosphates (Hooker Chemical Co., Niagara Falls) were obtained from Dr. Paul Mueller of Pennsylvania Psychiatric Institute. All other chemicals were analytical grade.

Membrane Assembly and Measurements

The membrane (BLM)-supporting assembly, consisting of a Teflon or polyethylene cup and Lucite chamber, was the same as that described by Mueller and Rudin [21] of the Pennsylvania Psychiatric Institute, Philadelphia, and was kindly provided by these same doctors. The diameter of the hole on which the membrane was formed was 1 mm. For electrical measurements across the BLM, calomel electrodes with agar-saturated KCl bridges were used. The agar used was nonionic (Agarose, Bausch and Lomb) to minimize the possibility of abnormal liquid junction potentials. The agar-KCl bridges were 18 to 24 inches long, made of polyethylene tube: wall thickness +1 mm; ID ~1 mm. The electrical system, salt solutions and the electrode potentials were symmetrical (within 0.1 mV) across the BLM unless stated otherwise.

The electrical measurements utilized the circuitry shown in Fig. 1. The electrometer operational amplifier (Keithley 301) had an input impedance of 10^{12} Ω and a gain of 50,000. The switches utilized were of the momentary shorting type which prevented high voltages from transiently appearing during switching, producing dielectric breakdown of the BLM. The calomel cells were connected in parallel when not in use and kept at constant temperature during the experiment. This procedure kept the imbalance in calo-

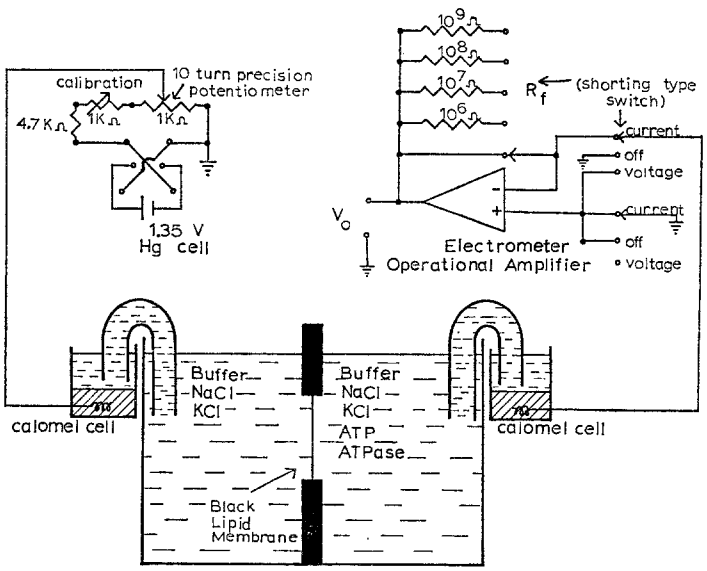

Fig. 1. Schematic diagram of the system employed for the formation of BLM and the electronic circuitry employed in measurements thereon (*see* text for details). The applied potential is added to the *trans* side. The side containing ATPase is referred to as the *cis* side having negative open-circuit potential; the opposite as the *trans* side

mel cell potential to a fraction of a millivolt. To record membrane potential of the BLM, R_f is set to zero and the amplifier set to the voltage-recording mode. The circuit then performs as a unity gain amplifier and the output records the BLM potential in series with any potential added to the *trans* side by the potentiometer.

In the current recording mode, the input to the operational amplifier (from the *cis* side) is held at a virtual ground or zero potential by current feedback through R_f. The membrane current is therefore $I_m = V_0/R_f$. Here, the potential across the BLM is that applied to the *trans* side by the calibrated potentiometer since the calomel half-cell potentials are opposed and cancel. The current mode circuitry is therefore essentially a "voltage clamp." This relation for membrane potential also essentially holds during current flow if the voltage drops across the calomel half-cells and salt bridges (near $10^4\ \Omega$) are considerably less than the potential across the BLM. In the experiments reported here, the error in BLM potential caused by current flow through the calomel half-cells is less than 0.1 %. Corrections in recorded membrane potentials caused by these external voltage drops are therefore unnecessary and a separate four-electrode current and voltage recording system is not required.

The lipid solution for making BLM was prepared as follows (*cf.* ref. [41]). Twice recrystallized cholesterol (5 g) was refluxed in *n*-octane (100 ml) for 6 hr, during which time oxygen gas was bubbled continuously at a rate of approximately 3 bubbles per sec (capillary diameter at the exit 1.5 mm). To the hot solution of *oxidized cholesterol* were added crystallized cholesterol (5 g) and a 1:1 or 2:1 mixture of *n*-decane + *n*-tetradecane (100 ml) and didodecyl or dodecyl phosphate (30 mg). The mixture was allowed to stand at room temperature for at least a week, to prevent formation of a precipitate on the BLM. The clear supernatant solution was used for BLM formation. This lipid mixture is capable of forming stable membranes following storage for 2 to 6 months and can be kept at room temperature exposed to air. A stable BLM could not be formed from a lipid solution in which alkyl phosphate was replaced by phosphatidyl serine. The solution on both sides of the chamber (Fig. 1) consisted of sodium chloride (0.1 M), potassium chloride (20 mM), magnesium chloride (10 mM), *Tris* hydrochloride (20 mM) and $CaCl_2$ (0.01 mM) at pH 7.4 adjusted with HCl. $CaCl_2$ was not used in earlier experiments; however, subsequent studies showed that low concentrations of Ca in the solution improve the stability of the modified BLM (*see below*). All solutions were stored in polyethylene bottles; ground-glass stoppered bottles should be avoided [18]. All measurements on BLM were performed at 36 to 39 °C unless stated otherwise. The solutions in both the compartments were stirred with 7-mm-long Teflon-coated stirring bars at a rate such that the mixing in any one compartment is essentially complete within 30 to 45 sec as evidenced by the addition of some suitable dye. BLM were modified by adding 1 to 100 μg of protein to the solution (in about 5 to 100 μliters aqueous solution usually 0.32 M in sucrose) by a Hamilton microsyringe to one of the compartments separated by the BLM. The volume of solution in each compartment was always kept at 4 to 5 ml. The d-c electrical resistance of the BLM was followed by having the electrometer in the current measuring mode (Fig. 1) and applying a steady step pulse of voltage V_m (clamped). The membrane current is obtained from the electrometer output as $V_0 = I_m R_f$ and the membrane resistance is given as V_m/I_m. Similarly, the open-circuit voltage across the BLM was recorded when the amplifier was in the voltage mode. It was possible to change between these two modes by means of the selector switch. The complete electrical system was enclosed in a Faraday cage. The background noise level in the voltage mode (without BLM) was always less than 0.05 mV, mainly from the stirring motor; with unmodified BLM on the hole the noise level was less than 1 mV.

Teflon chambers employed for the measurements described herein were cleaned by following this sequence of operations: (1) boiling in 10 to 15% KOH or NaOH for 30 to 60 min; (2) washing three times in glass-distilled water; (3) soaking in 0.1 M HCl in

ethanol for several hours followed by 3 or 4 rinsings in distilled water; (4) boiling in absolute ethanol for 10 min followed by soaking at room temperature in fresh ethanol for 30 to 60 min. This procedure appears to be the minimal one required since none of the above steps could be successfully eliminated. Success in formation of a stable BLM does not necessarily assure success in incorporation of protein fragments into that BLM; this has been previously observed by Mueller and Rudin (*see* ref. [22]).

Preparation of Na, K-ATPases

The ATPase from beef heart [31], beef brain [32], cat brain [*cf.* 32], human red blood cells [33] and rabbit kidney [25] were prepared according to published procedures. A Mg-ATPase preparation was obtained from yeast mitochondria [30]. Two Na, K-ATPase preparations were obtained from rat brain by the general procedure described by Mahler and co-workers [17, 19] and outlined in detail below.

The cortices from six rats were obtained by decapitating (without anesthesia) 50 to 60-day-old Sprague Dawley rats. Rat brains were homogenized in an apparatus consisting of a motor-driven Teflon pestle which rotated at 200 rpm in a tight-fitting glass tube. Homogenization was effected on three brain halves at a time in 100 ml of 0.32 M sucrose employing 14 to 15 up-and-down strokes over a period of 1 min. An additional 10 ml of 0.32 M sucrose was then added to the homogenate and mixed through 4 or 5 manual strokes with the pestle motor off. This preparation was then centrifuged for 5 min at 1,000 to 1,500 $\times g$. The supernatant S_1 was decanted carefully so that the loose white flocculant residue remained with the pellet. S_1 was centrifuged at 12,000 $\times g$ for 20 min to yield supernatant S_2 (containing the microsomal and soluble fractions), and pellet P_2 (containing the crude mitochondrial fraction with myelin and nerve end particles or NEP).

P_2 was lysed by adding 50 ml of water $-$ 12.5 ml to each of the four tubes. The mixture was mixed on a vortex apparatus at maximum setting (200 rps) for 30 sec. Five minutes later, the suspension was passed into and out of a 50-ml syringe 5 times through the needle (12 gauge) which was pushed hard against the bottom of the tube to enhance shearing. After 5 min the combined fractions were homogenized with 4 to 5 manual strokes of the homogenizer pestle with the motor off. After another 5 min the mixture was passed through a syringe twice and the sample then loaded into a zonal rotor.

In all experiments with zonal gradient centrifugation a Beckman Spinco model L centrifuge and a Ti-15 rotor with a total volume of 1,660 ml were used. The rotor was thermostated at 4 to 6 °C throughout the procedure. Introduction and recovery of the gradient and loading of the sample and overlay were done while the rotor was spinning at 3,000 rpm. An exponential sucrose gradient (850 ml) was formed from light (15 % w/w) and heavy (45 % w/w) solutions of sucrose at pH 7.4 without any buffer (adjusted with NaOH) by a Beckman High Capacity Gradient Pump. The sample (50 ml) and overlay (100 ml) were injected slowly with a syringe. After centrifugation for 1 hr at 25,000 rpm the rotor was slowed to 3,000 rpm and the density gradient was displaced from the rotor by the gradient pump at a rate of about 20 ml/min with 47 % w/w sucrose. Density measurements were made with an Abbe refractometer. The optical density profile of the gradient was recorded with a continuous flow Unicord Spectrophotometer (LKB Instruments Inc., Rockville, Md.) at 280 nm. Each of the fractions thus collected was diluted with an equal volume of water and spun at 28,000 rpm (70,000 $\times g$) for 30 min. The pellet was resuspended in 30 ml of 0.32 M sucrose or distilled water and stored at $-$25 °C. Adjacent fractions were pooled as zones and stored in small aliquots. This is particularly important since repeated freezing or thawing affected the properties of these preparations significantly.

Enzyme Assay

The ATPase activity was measured by either of the following methods: (a) Colorimetric determination of inorganic phosphate as phosphomolybdate, as described by Mahler and Cotman [17]. (b) Titration of protons liberated in the hydrolysis of ATP: $ATP^{4-} \rightarrow ADP^{3-} + P_i^{2-} + H^+$, employing a Radiometer Titrator-Titrigraph assembly as a pH-stat thermostated at 37 °C. AMPD was employed as the titrating base. In a typical run, the assay mixture contained 10 ml of a solution containing 100 mM NaCl, 30 mM KCl, 5 mM $MgCl_2$, and 3 mM ATP, pH 7.4. Following adjustment of the solution to pH 7.4, the reactions were initiated by the addition of 10 to 100 µliters (1 to 100 µg of protein) of enzyme solution. A continuous record of the course of the reaction was collected for 5 to 15 min with the proportional band control of the pH-stat set at 0.1. Quite generally, the rate of liberation of protons in the ATPase-catalyzed reactions was linear for at least 10 min at 1 mM ATP. The ouabain-insensitive ATPase activity was measured either by adding ouabain to the incubation mixture after the reaction mixture had proceeded for a few minutes (usually 5 to 10 min), *or* by the addition of ouabain to the initial mixture. The amount of inorganic phosphate liberated was computed employing a value of 7.18 as the pK_a for the monophosphate ion [11]. The agreement between the two methods for determining ATPase activity was excellent (*see also* ref. [2]).

Acetylcholinesterase activity was also measured by titration of liberated acetic acid by the pH-stat method as described above for the ATPase activity. In fact, ouabain-sensitive and ouabain-insensitive ATPase activity, as well as acetylcholinesterase activity could be determined in a single sample by starting the reaction with enzyme, adding ouabain after 5 min, and then adding acetylcholine after another 5 min. The results thus obtained agree well with those obtained by conventional methods [15]. Acetylcholinesterase activity was not influenced by the presence of ouabain or Na and K ions. Similarly, acetylcholinesterase inhibitors such as 1,5-*bis*(4-allyldimethylammoniumphenyl)pentan-3-one [BW 24851 dibromide, Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y.], atropine or carbamoyl choline had no effect on ATPase activity.

Results

Following fractionation through zonal centrifugation, the specific activity of ATPase isolated from rat brain nerve-end particles varied from 60 to 500 µM ATP hydrolyzed per hr/mg protein depending on the fraction assayed and the particular preparation employed. The ouabain-insensitive activity ranged from near zero to 80% of the total ATPase activity in different preparations. Frozen preparations kept at −25 °C showed a slight increase (up to 20%) in Na, K-ATPase activity, and a slight decrease in the proportion of ouabain-insensitive ATPase.

The distribution profile of optical density, Mg-ATPase, Na, K-ATPase, and acetycholinesterase in the zonal density gradient was determined and the results are in excellent agreement with related ones previously reported [19]; (*see also* Fig. 2). A comparison of the ATPase activity in the crude homogenate and in zone *E* (encompassing the span between 29.0 and 32% sucrose), which contains the bulk of the ouabain-sensitive ATPase, reveals

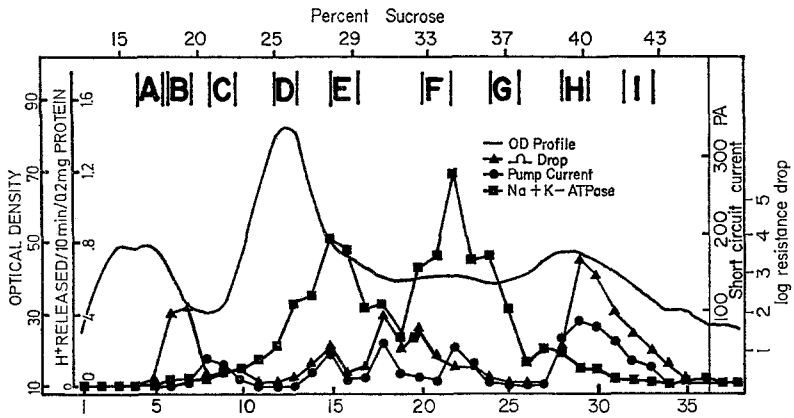

Fig. 2. Analysis of the zonal fractionation of a crude mitochondrial fraction in terms of the optical density profile, the total Na, K-ATPase activity profile, and the profiles for induction of steady-state membrane resistance drop and transmembrane short-circuit current. A peak in SCC at zone *H* is not inhibited by ouabain; however, it correlates well with oligomycin-sensitive ATPase activity. This may correspond to an electrogenic proton pump; it has not been investigated in detail. The values for steady-state resistance drop and the transmembrane SCC varied less than 50% for this preparation. The profiles for Na, K-ATPase activity, steady-state membrane resistance drop, and transmembrane short-circuit current were obtained by a double blind experiment

an increase in specific activity of 7 to 15-fold in various preparations. The yield in this zone is of the order of 30 mg protein from cortices of six rats.

Initial zero-order velocities for Na, K-ATPase-catalyzed hydrolysis of ATP were measured at pH 7.4 as a function of the concentration of ATP for the enzymes present in zones *C* and *E* (Fig. 2). In both cases, the data generated satisfactory double reciprocal Lineweaver-Burke plots from which values of *Km* were obtained graphically: 0.05 mM for zone *C* enzyme and 0.43 mM for zone *E* enzyme. For the enzyme from the latter fraction, a pH-rate profile was obtained at an ATP concentration of 3 mM. A broad ill-defined bell-shaped curve was obtained with maximal velocity observed in the range pH 7.2 to 7.3.

The bulk of the data described below deal with the consequences of interaction of membrane fragments from the zonal preparation with BLM. Employing a single enzyme preparation with a single BLM preparation generally yields good qualitative and quantitative reproducibility of results during the period of a single day and good qualitative but only fair quantitative reproducibility during a period of several days provided the enzyme preparation is not repeatedly frozen and thawed. However, introduction of a new preparation of either membrane fragments or BLM-forming material frequently results in failure to reproduce earlier findings, particularly the

observation of short-circuit current flow across the membrane. Thus, this system possesses an element of irreproducibility reflecting some aspects of the individual preparations. In fact, only about 20% of the preparations accorded completely, although only qualitatively, with the results reported herein. But the important point is that individual preparations do behave consistently and reproducibly and that several different preparations exhibit qualitatively similar behavior. Difficulties in correlating quantitative aspects of the behavior of various "active" preparations and difficulties in understanding the failure of most preparations to exhibit activity stem from our inability to devise a suitable control experiment which could be employed under all experimental conditions. Consequently, experimental difficulties seem to be a technical problem but do not invalidate the qualitative results observed. The nature of the factors responsible for the variable results are not known in their entirety. However, it is certain that the history of the ATPase preparations, BLM solutions, and physical apparatus are all important. Thus, freshly prepared lipid solutions, freshly prepared enzymes or those which have been repeatedly frozen and thawed do not induce transmembrane current flow. Generally, concentrated enzyme preparations which have been frozen for a week or two give active preparations. Traces of detergent which remain following cleaning of the zonal rotor, pump, flow cells, and so forth may have also caused problems in some cases as have trace impurities present even in water doubly distilled from an all-glass apparatus.

Effect of Zonal Fractions on BLM Resistance

The series electrical resistance of the two chambers containing the solution without a formed BLM on the hole and with the calomel electrodes and agar bridges in series was determined to be 7,400 Ω; the resistance with the BLM on the hole was 5 to 10×10^{10} Ω cm^2. When a small quantity of membrane fragments from zonal fractions were added to the solution bathing the BLM, some of the fractions caused a marked decrease in BLM resistance. The resistance drop occurs over an interval of 1 to 15 min after which no further changes are observed. The total resistance drop observed is shown as a function of the concentrations of membrane proteins from zone *F* in Fig. 3. Note that a maximal drop occurs following which further additions cause no further lowering in membrane resistance although the BLM becomes increasingly less stable, both mechanically and to applied electrical pulses. When the membrane preparations at higher concentrations (above 200 μg protein/ml) were added to the aqueous phase before membrane

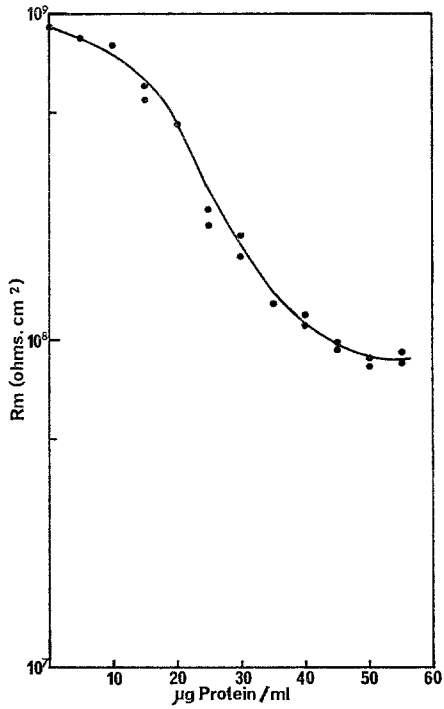

Fig. 3. The logarithm of steady-state membrane resistance (for a 10 mv pulse) plotted as a function of the protein concentration of membrane fragments derived from zone *E* added to one side of the BLM

(BLM) formation, the rate of the spontaneous thinning during BLM formation was markedly decreased. It may also be noted that for different preparations of membrane proteins the maximal resistance drop is different: age of the preparations, freezing-thawing, storage temperature, and the concentration of divalent ions, particularly Ca, all have an effect on the magnitude of the resistance drop. However, for the same fraction from different preparations maximal resistance drop may differ by a factor of 10 to 30. Similarly, for different fractions in the same preparation the maximal resistance drop is different. Such maximal resistance drops, determined with about 50 µg protein/ml on one side of BLM, for various fractions are given in Fig. 3 and discussed below. It is particularly interesting that at lower concentrations of proteins from zones *C* and *E* applied to the chamber with a BLM, the membrane resistance drops in discrete steps. The resistance characteristic of each discrete jump may be calculated from the magnitude of the current *increase* at a specified impressed voltage: these values fall in the range of 2 to 10×10^{10} Ω in solution *A* for the same pre-

paration in any one set of experiments. If NaCl is replaced by LiCl in solution *A*, the value of the resistance associated with the quantal jumps is in the range of 8 to 15×10^{11} Ω. Of course, the resistance change in the total system, calculated from the *total* current flow at the specified voltage, is much smaller than these values.

When membrane fragments from the zonal preparation are added to one side of the BLM in the presence of 1 mM ATP or acetylcholine, the rate of resistance drop of the membrane is increased significantly and the steady-state resistance value is slightly lower than that reached in the absence of these substances. As inferred from the development of open-circuit potential in the presence of a salt gradient (~45 to 55 mV for a 10-fold concentration difference) the conduction pathways formed appear to be permeable to monovalent cations; however, in the presence of ATP at least, there appears to be little discrimination between Na and K. The BLM modified by zone *E* membrane fragments appears to be almost equally permeable to Na^+, K^+ and Cl^- in the presence of acetylcholine whereas it is permeable to cations only in the absence of acetylcholine.

Treatment of membrane fragments separated by the zonal fractionation procedure with various physical and chemical agents significantly changes the ability of the treated preparation to decrease BLM resistance. Thus, treatment of membrane preparations with chaotropic agents [6], ultrasonic irradiation, heat (100 °C for 3 min) glass beads such as *Bio-glas* 1000 and 5000 from Bio. Rad. Labs. [4, 28], sephadex gel, and with various detergents (*see later*) yield preparations which either make the BLM unstable or do not affect BLM resistance detectably. In fact, as shown in Fig. 4, chaotropic agents such as potassium perchlorate and sodium thiocyanate inhibit ATPase activity. For these experiments the membrane fragments from zone *F* were incubated in solution *A* at 37 °C for 10 min in the presence of a specific salt. The ATPase activity was determined in the same mixture by measuring the initial rate of generation of H^+ upon addition of ATP. As shown in this figure, both $KClO_4$ and NaCNS inhibit the ouabain-sensitive Na, K-ATPase activity. Dialysis (25 hr at room temperature against distilled water) of the incubation mixture before addition of ATP yielded a particulate preparation which did not reduce the BLM resistance, although it made BLM very unstable.

Zonal preparations of Na, K-ATPase treated with 0.05% Triton X-100 according to the published procedure [1] were not incorporated into BLM as evidenced by the absence of a resistance drop or development of SCC. The ATPase activity of these detergent-treated preparations was unchanged when compared with that of untreated material. Of the various surfactants

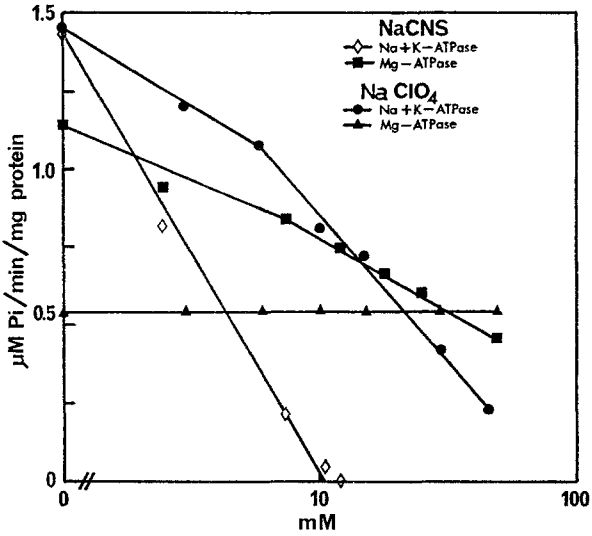

Fig. 4. Specific activity of Na, K-ATPase and Mg-ATPase following incubation of membrane fragments for 10 min at room temperature with various concentrations of sodium thiocyanate and perchlorate. Chaotropic ions were removed by dialysis prior to assay of the enzyme preparations

tested (cholate, deoxycholate, SDS, Tween-40, Tween-80, Sarcosyl NL 97, Lubrol, Triton X-100, and Triton X-114) only Triton X-100 was found to have no effect on BLM resistance (*unpublished observations*) at concentrations at which detergents are known to disperse biomembranes, that is to *solubilize* Na, K-ATPase [1, 12, 42].

Current-Voltage Characteristics of BLM Modified with Various Zonal Fractions

Fig. 5 gives d-c current-voltage curves obtained for a BLM modified with fractions from various regions of the zonal profile. The membrane potential shown is that for the *trans* side with respect to the *cis* side (which is at virtual ground potential in the current recording mode). It may be noted that not only has the resistance of the BLM in the linear range (around zero membrane potential) been lowered by the different protein fractions to varying degrees, but also the shape of the curves show some distinct features at higher voltages. These curves have a characteristic shape which is different for different zonal fractions. Zone *F*, which is rich in ATPase (curve *F*), gives a current-voltage relationship which is almost linear except

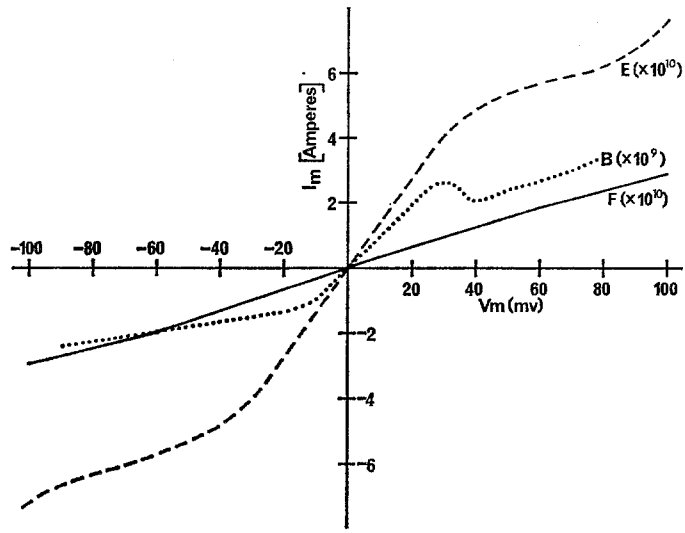

Fig. 5. Steady-state current-voltage curves for BLM modified with membrane fragments derived from three zones of the zonal fractionation preparation (*E*, *B*, and *F*). Each point was obtained by pulsing the membrane to the indicated potential for 5 sec which is several times that of the membrane time constant ($R_m \times C_m$) and the recorder response time. Successive measurements were made at progressively higher values of impressed voltage. The figures in parentheses are proportionality constants relating the indicated values to the current axis which is in amps/mm^2

at higher voltages. The zone high in acetylcholinesterase (zone *E*) dropped BLM resistance by several orders of magnitude (curve *E*) and zone *B* showed a distinct region of differential negative conductance (curve *B*).

In these modified BLM, dielectric breakdown occurred when the impressed voltage was between 40 to 100 mV. In contrast, the dielectric breakdown voltage for unmodified BLM was more than 300 mV. Thus, the points at higher voltages (above 40 mV) in curves shown in Fig. 5 were obtained by pulsing the modified BLM for 5 sec under the voltage clamp.

Reconstitution of the Electrogenic Transport System

Following the addition of membrane fragments from zone *F* (and also other fractions, *see* Fig. 2) to one side of a planar BLM formed in the presence of solution *A*, subsequent addition of dilute solutions of ATP to the same side (the *cis* side) of the membrane results in the development of short-circuit current (SCC) across the membrane. The *trans* side being positive with respect to the *cis* side. The time dependence of development of SCC is shown for a typical case in Fig. 6. The subsequent addition of

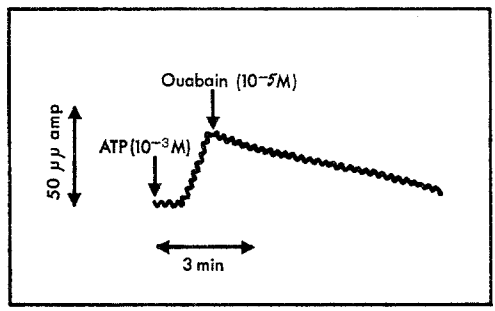

Fig. 6. Time course for the development of transmembrane short-circuit current following the successive addition of dilute solutions of ATP and ouabain to the BLM modified with zone *E* (~10 μg protein/ml). The irregularities in the traces are the result of noise caused by the motor employed to stir the solutions

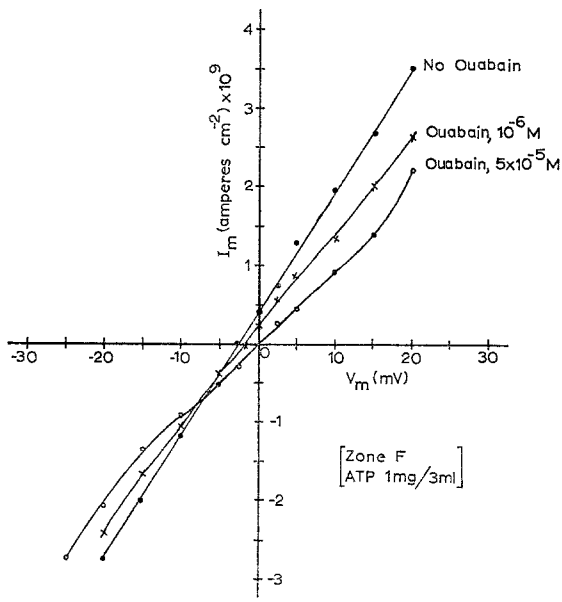

Fig. 7. Steady-state current-voltage curves for BLM modified with membrane fragments derived from zone *F* (~2 μg protein/ml) of the zonal fractionation preparation in the absence and in the presence of two concentrations of ouabain present on the *trans* side

ouabain to the *trans* side of the membrane (that side not containing either ATP or ATPase) causes disappearance of the SCC as shown in Fig. 6; these results are also presented in terms of current-voltage relationships as a function of ouabain concentration in Fig. 7. The ouabain inhibition of SCC may be noted almost immediately after its addition and the effect is usually complete after a few minutes (Fig. 6). Addition of ouabain to the *cis* side prior to the addition of ATP prevents the development of the SCC but not the

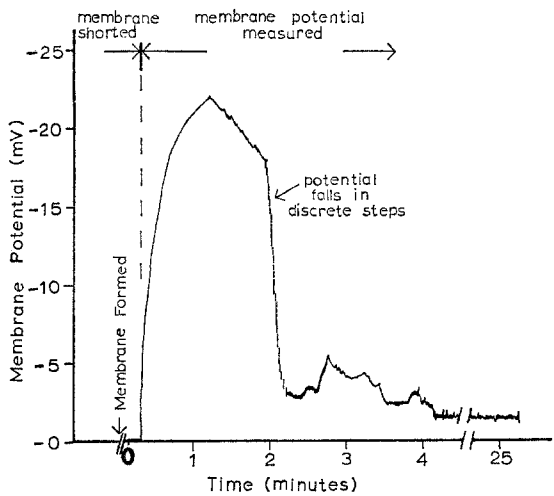

Fig. 8. Time course for development of open-circuit potential across BLM modified with membrane fragments from zone *F* (~1 μg protein/ml) of the zonal fractionation preparation. The BLM was formed with ATP and ATPase on the *cis* side which is negative with respect to the *trans* one

resistance drop; addition of ouabain to the *cis* side following addition of ATP and development of the SCC cause a slow (complete drop in about 10 min) dimunition in the SCC observed. The time course of ouabain-induced loss of SCC, from both the *cis* and *trans* sides, was sufficiently variable from experiment to experiment to preclude a reliable comparison of the kinetics of the inhibition from the two sides. Ouabain at 10^{-4} to 10^{-5} M has little effect on the resistance of unmodified BLM.

Re-examination of the data contained in Fig. 2 reveals that there is some correlation between the ability of certain fractions to induce SCC across the BLM and their capacity to lower the resistance of the same membrane. It is, however, difficult to be certain just how good this correlation is since there is a tendency for the membrane resistance to continue to drop after the SCC has reached a maximal value (or even begun to decrease). This behavior is reflected in the time course of development of open-circuit potential (OCP), the membrane potential with no current flow other than pump currents charging the membrane capacitance, as shown in Fig. 8. Note the sudden rise in OCP which then decays stepwise to a limiting value. There is no data on the behavior of the membrane resistance during the same time period. However, independent checks under similar conditions do suggest that there is a drop in membrane resistance simultaneous with that in OCP. Thus, an initial resistance drop may correspond to an increase

in SCC. (*See also* ref. [10].) However, a further increase in membrane conductance does not apparently affect the SCC, although the OCP is considerably reduced. It has been noticed that addition of ATP prior to the addition of ATPase significantly improves the incorporation of the enzyme into BLM. These results were consistently obtained from at least four zonal preparations.

Na, K-ATPase preparations from beef heart, beef brain, cat brain, human RBC, rabbit kidney and microsomal ATPase from rat brain did not induce SCC or OCP across the BLM under the conditions described above. Three separate preparations of ATPase from each of these sources were tested, usually employing two or more types of BLM.

Effect of Ion Substitution on "Pump" Activity

In the absence of either sodium, potassium, or magnesium in the solution bathing the BLM, no development of SCC across the membrane was observed in any case. Furthermore, in the absence of potassium and magnesium, addition of various ATPase membrane fragments elicited no change in membrane resistance. On the other hand, the change in membrane resistance in the absence of sodium was almost as great as in its presence. When sodium was replaced by an equal concentration of lithium in the membrane bathing solutions, the resistance drop elicited by zone *F* ATPase was diminished two- to threefold. The mechanical stability, however, was increased significantly. No pump current could be detected when sodium was replaced by lithium. In contrast, potassium could be replaced by rubidium in solution without any significant change in SCC or the open-circuit potential.

Effect of Phospholipases and Trypsin on BLM Modified with Zone E Na, K-ATPase

BLM were modified by addition of membrane fractions isolated from zone *E* of the zonal preparations to one compartment bathing the membrane. After waiting until the membrane resistance (for a 10-mV pulse) reached its minimal steady-state value (Fig. 2), a known amount of a hydrolytic enzyme (ca. 2 μg/ml) was introduced on either the *cis* or the *trans* side of the BLM. As a control experiment, the zone *E* membrane fractions were pretreated with the hydrolytic enzymes at a concentration of 1 μg/ml at 35 °C for 10 min and subsequently added to one side of the BLM. The changes in membrane resistance resulting from the treatments with hydrolytic enzymes

Table 1. Effect of hydrolytic enzymes on the resistance of BLM modified with 10 μg/ml of membrane fragments from zone *G*[a]

Enzyme	R_m[b] of the modified BLM (Ω)	R_m after addition of enzyme to the chamber (Ω)	R_m when zone *E* preparation is previously treated with the enzyme (Ω)
Trypsin	4×10^8	10^{10} *cis* and *trans*	5×10^{10}
Phospholipase A (Russell viper)	4×10^8	No effect on *cis* or *trans* side	$> 10^{10}$
Phospholipase C (*B. cereus*)	10^8	No effect on *trans* side; leads to instability on *cis*	$\simeq 10^{10}$, unstable
Phospholipase C (*C. welchii*)	10^8	No effect on *trans* side; leads to instability on *cis*	10^{10}, unstable

[a] BLM resistance in the absence of added membrane fragments was $\sim 10^{10}$ Ω.
[b] R_m: membrane resistance.

are collected in Table 1. Note that neither the hydrolytic enzymes themselves nor the zone *E* fragments pretreated with hydrolytic enzymes have any effect on the BLM resistance. Furthermore, phospholipase C (from both *B. cereus* and *C. welchii*) destabilizes BLM treated with zone *E* membrane fragments much more than these fragments pretreated with the phospholipases. Thus, it appears that the hydrolysis products themselves are not primarily responsible for BLM destabilization. Although, both phospholipase A and trypsin modify conduction pathways, distinctive differences between results obtained with the two enzymes may be noted: phospholipase A affects the membrane properties only from the *cis* side (*see below*) with respect to the zone *E* fragments although trypsin exerts its effects from both the *cis* and *trans* sides. Moreover, treatment with trypsin causes an increase in membrane resistance although that with the phospholipases does not. Mechanical stability and the dielectric breakdown voltage (about 80 to 90 mV) for the trypsin-digested modified BLM also appears to be significantly higher as compared to BLM modified with zone *E* membrane alone (~40 to 50 mV).

Since the modified BLM was found to be stable in the presence of trypsin and phospholipase A, the effect of these enzymes was studied on

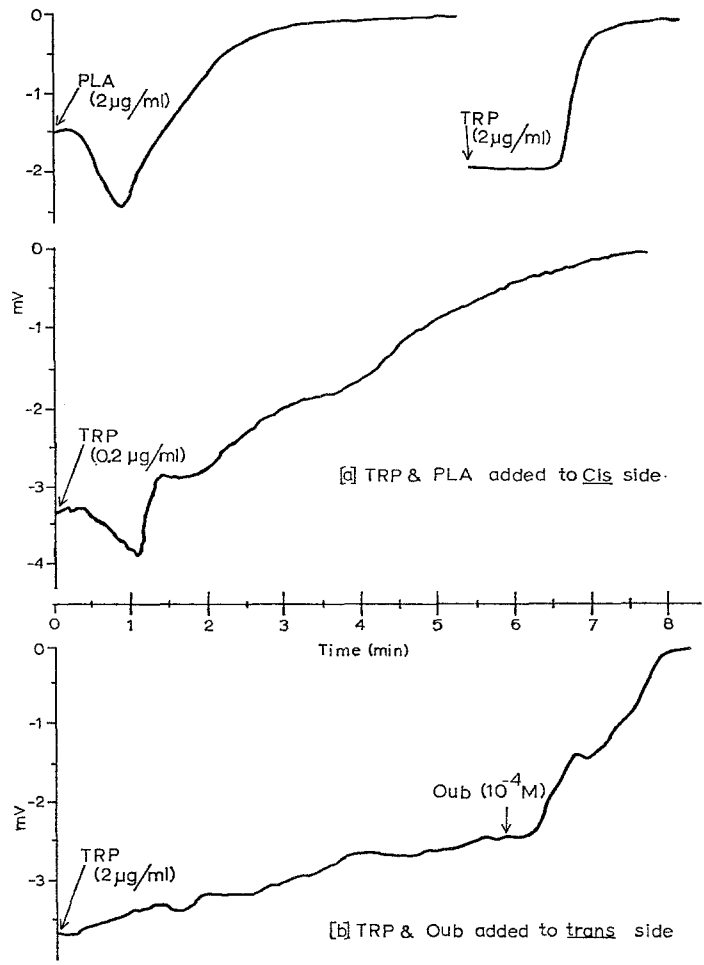

Fig. 9. Time course for the disappearance of open-circuit potential across BLM modified with membrane fragments from zone *F* (~10 μg protein/ml) on the *cis* side (*a*) following addition of phospholipase A (PLA) or trypsin (TRP) on the *cis* side of the membrane and (*b*) following the addition of trypsin and, subsequently, ouabain on the *trans* side

the SCC and OCP. Hydrolytic enzyme was added to either side of a BLM on which the open-circuit potential had developed in the presence of ATP. The results are shown in Fig. 9. In agreement with their effect on the resistance of modified BLM, both trypsin and phospholipase A inhibit the open-circuit potential. However, phospholipase A has its effect only on that side (*cis*) to which ATPase has been added. In contrast, the effect of trypsin is manifest on both sides, although the kinetics of inhibition are different. When added to the side containing ATPase, the effect is visible after a

lag period, and complete inhibition takes place according to first-order kinetics with a halftime less than 1 min. The rate for inhibition by phospholipase is about the same. In contrast, the kinetics for inhibition by trypsin added to the *trans* side is both slower and kinetically more complex. Addition of 10^{-4} M ouabain brings inhibition to completion in about 1 min.

Discussion

The data presented in the preceeding section demonstrate that various particulate membrane fragments isolated from rat brain interact with BLM to produce a 10- to 10^4-fold decrease in membrane resistance. Although the nature of specific components responsible for this resistance drop is far from certain, the following considerations are pertinent.

First, the data in Fig. 3 show that the profile for resistance drop elicited by zonal fractions does not even qualitatively correlate with the optical density profile. The latter profile corresponds approximately to that for protein distribution [19]. Thus the component(s) responsible for resistance drop must not include all membrane proteins but must be restricted to one or more such proteins having a specific set of properties.

Second, a comparison of distribution profiles of various marker enzymes (*cf.* Fig. 2 and ref. [19]) suggests that the ability to cause a resistance drop cannot be attributed to any single marker enzyme. However, the capacity to cause a maximal resistance drop correlates approximately with those fractions in which a peak distribution of acetylcholinesterase, Mg-ATPase, Na, K-ATPase, and mitochondrial ATPase (zone *G*) occurs. Thus, these membrane marker enzymes (and maybe other proteins) appear to be incorporated into the BLM, leading to the resistance drop. In fact, a yeast mitochondrial preparation of Mg-ATPase drops BLM resistance by a factor of 10^3 to 10^4 (M. K. Jain & E. H. Cordes, *unpublished observations*). Similarly, an ATPase preparation from *Streptococcus faecalis* has been shown to drop resistance by several orders of magnitude; cations modify this effect [26]. It may, however, be noted that the converse is not true; that is, not all preparations of Na, K-ATPase or ATPase drop BLM resistance. Similarly, the nature of the conductance pathways created by various zonal fractions may not be the same. In fact as shown in Fig. 5, the current-voltage curves for BLM modified with various zonal fractions suggest that the species incorporated into the BLM from various zonal fractions may be different. Also, a stepwise change in BLM resistance (*cf.* refs. [3, 5]) has been noted

with only membrane fragments from zones *C* and *E*. These observations imply that either there is some component, other than the marker enzymes, responsible for the resistance drop or that the details of structure and composition of these membrane fragments may be responsible for this difference.

To understand more about the incorporation of various proteins into BLM, we looked for more specific functions of the membrane-bound proteins. Thus assuming that the catalytic proteins incorporated into BLM still retain their biological potency, the modified BLM may serve as a suitable site for membrane specific processes such as active transport of cations. As shown in Fig. 6, addition of ATP to one of the compartments (to which zone *F* membrane fragments were originally added) bathing the modified BLM, results in development of a SCC; the resulting OCP is negative on the side containing ATP. The SCC thus induced is inhibited by ouabain added to the *trans* compartment. This experiment accords with the features of an electrogenic ion pump present in the molluscan ganglia of snails [40], frog skin [7], toad bladder [7], and various other tissues [14, 27]. The data shown in Fig. 7 corroborate the conclusions just reached.

Attempts made at detailed characterization of the system responsible for development of transmembrane SCC have yielded little information. However, the following lines of evidence support the conclusion that incorporation of the Na, K-ATPase into BLM yields an electrogenic ion pump across the BLM.

(1) Pump activity is observed only when 20 to 50 ppm of dodecyl or didodecyl phosphate is added to the lipid solution from which BLM is prepared. Both higher and lower concentrations fail to induce SCC. It is possible that this anionic surfactant may introduce proton (or even cationic) conduction pathways across the BLM, and that the SCC may be observed if a pH gradient is introduced across such a BLM. However, SCC is not changed by changing the buffer capacity of the medium, nor does the addition of 50 μmoles of hydrochloric acid to one of the compartments induce any SCC across BLM modified with membrane fractions containing Na, K-ATPase. Moreover, the amount of protons released during the hydrolysis of ATP in the medium surrounding the BLM is not expected to exceed 10 μmoles. The possibility, however, remains that these protons are released in the immediate vicinity of the BLM (say in the unstirred layer) generating considerably larger proton gradients across the BLM. In such a case one has to postulate, however, the presence of a transmembrane proton conduction mechanism which can be shut off by ouabain acting from the other side.

(2) It is particularly interesting to note that deoxycholate-treated Na, K-ATPase from beef brain can be reactivated by several long-chain mono- and dialkyl phosphates, phosphatidylserine and other related phospholipids [38, 39]. Although our data are not extensive and the concentration of various components in the BLM is not certain, there seems to be a qualitative correspondence in the activation of DOC-treated Na, K-ATPase by alkyl phosphate and induction of an electrogenic transport in the BLM containing alkyl phosphate. Even quantitatively, the maximal activity is observed in the same concentration range, although there are differences in the activation curves at lower and higher concentrations of alkyl phosphate. SCC could not be induced across BLM prepared from phosphatidyl serine + decane + tetradecane with or without cholesterol. These results suggest a definite role for alkyl phosphate in the activity of Na, K-ATPase. Furthermore, BLM-cholesterol may provide specific support of electrogenic transport in view of its implication in the activation of Na, K-ATPase from rat brain [23]; it is also possible that oxidized cholesterol may form BLM of low surface pressure (*cf.* ref. [13] facilitating the incorporation of modifying reagents.

(3) A considerable amount of information can be derived from experiments in which one or more components of the system are eliminated. Thus, if the system does not contain Na and K and Mg, no SCC (or OCP) could be observed (Rb will substitute for K). There are several sources of uncertainty in the interpretation of this information: these effects could be due to the lack of suitable charge carriers (ions) in the system, *or* due to the lack of a suitable configuration of "ionophores" (as induced by ions) necessary for their incorporation into BLM, *or* they may result from the lack of a cation participating in the reaction cycle for pump activity. Alternatively, experiments involving an asymmetric ionic or electrical environment could also give some useful information regarding the source of the SCC. However, due to technical problems with lowered dielectric breakdown voltages we have met with little success in this direction.

(4) The results of a double blind experiment correlating Na, K-ATPase activity with the pump activity are shown in Fig. 3. These results clearly indicate that, even though the peak activities of both types fall in the same fractions, the pump activity does not quantitatively correlate with the ATPase activity, nor does the resistance drop correlate with either of these activities.

(5) Zonal fractions pretreated with either ouabain, phospholipase A, or *C* or *D*, or with trypsin do not develop any SCC or OCP.

(6) As shown in Fig. 9, trypsin and phospholipase A block the pump current when added to the side containing the ATPase, and trypsin also blocks OCP when added to the opposite side.

Although the circumstantial evidence just presented is not a proof in itself, and the quantitative reproducibility of results in general is poor, the results do indicate the feasibility of incorporation of fragments of biomembranes into BLM. Furthermore, it seems quite likely that some of the functional properties of biomembranes can be reconstituted by incorporating into BLM a specific active preparation of membrane fragments. The constraints imposed upon the physical state of the fragments are least understood at present. However, a simplistic view regarding formation of membrane fragments would involve fracture of a macroscopic membrane assembly along a plane of weak intermolecular interactions. Thus, depending upon the severity of dispersal techniques used, one may expect to vary both size and composition of the fragments. As the newly exposed faces of the fragments may still be hydrophobic, one would expect them to either vesiculate and/or aggregate so as to minimize the interaction between hydrophobic faces and aqueous medium.

The interaction of membrane fragments with BLM may involve either (a) incorporation of small fragments into BLM, or (b) fusion of vesicles with BLM. In case (a) one would expect that the asymmetry of the plasma membrane of fragments derived from it would be lost during incorporation into BLM except under certain special circumstances when, for example, the fragment is incorporated into only one of the two monolayers of BLM. Alternatively, in case (b), a few large vesicles may coalesce with the BLM and the resulting modified BLM would be such that the built-in asymmetry of the vesicle would be transferred to the modified BLM (Fig. 10). These and other considerations regarding induction of asymmetry into the modified BLM are critical in interpreting the structure and function of the modified BLM and, therefore, of any functional biomembrane.

The "cation pump" shows asymmetry with regard to interaction and uptake of substrates, cofactors, and inhibitors, with regard to discharge of products and cofactors, stoichiometry of the overall process, and with respect to its location in the membrane and its environment. In a biomembrane these asymmetries are built-in properties of the system. In the experimental situation for a reconstituted pump as described in this paper, the asymmetry is induced by the asymmetric distribution of the membrane-fragments and ATP in the aqueous medium across the unmodified BLM. There is no asymmetry with respect to ionic composition and electrical polarity. If how-

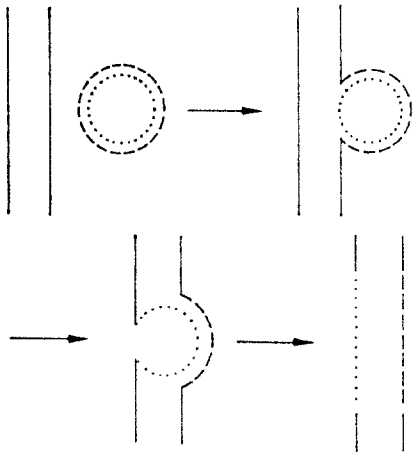

Fig. 10. A possible model for the fusion of a vesicular structure with a planar membrane in which the inside-outside asymmetry of the vesicular membranes is retained in the fusion product

ever, the ATP is added to one compartment and the membrane fragments are added to the opposite compartment, the system shows no detectable SCC, whereas, ATP added to both sides induced almost normal SCC. This suggests that the system is not asymmetric with respect to the distribution of ATP alone. It is, however, possible that asymmetric distribution of ATP induces asymmetry into BLM during the incorporation of membrane fragments. Here, too, it is critical to know whether the vesicles are outside-out or inside-out. The observations that the presence of potassium is necessary for the incorporation of membrane fragments into the BLM and that ATP increases the rate of BLM resistance drop considerably, seem to argue that the vesicles containing "pump molecules" may be inside-out. Consistent with this view is the following observation. Despite several efforts, all attempts to increase the magnitude of the OCP (2 to 5 mV in the steady state) have failed. It may be pertinent that in several nervous tissues including the squid axon [9], lobster axon [34], and others (*see* ref. [27] for a review), the direct contribution to the total membrane potential from a "pump" is generally between 2 and 6 mV. The result is usually ascribed to the high conductance of the membrane. This comparison, therefore, implies that the brain membrane fragments incorporated into BLM may possess both "pump" and "leak" capacities.

Considerations pertaining to the asymmetric distribution of membrane fragments may have some bearing upon the instability of the OCP. As shown in Fig. 8, the open-circuit potential developed across the modified BLM

slowly dissipates. This could arise either from a decrease in BLM resistance or be caused by redistribution and reorientation of "pump molecules" in the BLM phase. A decrease in BLM resistance would provide for an increased rate of discharging the membrane capacitance which is being continuously charged by the cationic pump. A slow decrease in BLM resistance may result either from some component other than the "pump molecule," whose incorporation into BLM is slower. Alternatively, the two possibilities may be the consequence of one and the same phenomenon. It is, for example, conceivable that such reorientation of "pump molecules" results in loss of asymmetry and consequent decrease in SCC (and OCP) and in a decrease in BLM resistance. Here, once again, definitive experiments are not possible until one obtains a "pure" Na, K-ATPase preparation which can also be incorporated into a suitable BLM.

Attempted purification of Na, K-ATPase by conventional methods have yielded preparations of high specific activity [12]. However, rat brain microsomal and zone *E* and *F* preparations similarly treated, or when treated with chaotropic agents and detergents do not induce any SCC under proper conditions; however, some of these preparations did drop resistance, indicating their incorporation into BLM. These observations are consistent with the hypothesis that membrane fragments are incorporated into BLM by a mechanism similar to that shown in Fig. 10.

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