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Electrogenesis from an ATPase-ATP-Sodium Pseudo Pump

Edward S. Hyman

Touro Research Institute, New Orleans, Louisiana

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Summary. The short circuit current and the open circuit voltage responses of membranes to ATP, which have been attributed to membrane ATPase acting as a sodium pump, have been reproduced not only in a lipid membrane containing solubilized ATPase but also in membranes formed of the phospholipids contained in ATPase. The response is greatest with cardiolipin, but occurs with other acidic phospholipids. This observation of electrogenesis without hydrolysis is a surface phenomenon probably due to the alignment of ATP on the phospholipid by ion association at its interface with the water phase. The finding constitutes a precaution for interpreting studies of membrane Na-K-ATPase or for its incorporation into an artificial membrane. The substances necessary for electrogenesis are present at the mitochondrial membrane, and the particular orientation of the ATP on the phospholipids in vitro suggests a role for this ion association in the function of Na-K-ATPase.

If purified membrane sodium-potassium dependent ATPase (Na-K-ATPase) is incorporated into an in vitro membrane, the preparation serves as a model to study the electrochemical and transport properties of the enzyme which is likely to be responsible for the ubiquitous sodium pump. To this end Jain *et al.* (1972) reproduced many of the phenomena of Na transport in an inert artificial membrane of oxidized cholesterol in aliphatic hydrocarbons that had been doped with membrane fragments from rat brain containing Na-K-ATPase which had been isolated by density gradient fractionation. This preparation often responded to ATP with a brief short circuit current (SCC) and a brief open circuit voltage (OCV), each in the direction expected if Na^+ were transported away from the ATP. Redwood, Muldner, and Thompson (1969) found that an easily solubilized bacterial ATPase would bind to a "black" lecithin membrane and sharply lower its resistance. Shamoo and Albers (1973) isolated an anionic fragment of proteolysis of the ATP-ase molecule which formed nonselective pores through a black membrane, but which would do so only in the presence of sodium.

In this study some electrical responses of a membrane to ATP, attributed to Na-K-ATPase by previous investigators, were found in an artificial lipid membrane containing the enzyme solubilized from the canine outer renal medulla. However, these responses were also found to occur without the enzyme in membranes which were made of the phospholipids extracted from ATPase or which contained only pure acidic phospholipids. Since the electrogenesis is not dependent on hydrolysis of ATP, the system is not a sodium ATPase pump; however, the observation is relevant to the interpretation of attempts to reconstitute electrogenic pumps in membranes, and the orientation of ATP on an acidic membrane may have significance.

Materials and Methods

ATPase was solubilized from the outer medulla of a dog kidney by a modification of the method of Schwartz *et al.* (1971). The water-solubilized enzyme hydrolyzed 90 μmoles of P_i per mg of protein per hr.

In order to be sure that the enzyme would be incorporated in the lipid of an experimental lipid membrane, the enzyme was solubilized in the lipid solution used to form the membranes. First the water-solubilized enzyme was absorbed onto a sol of sphingomyelin which was made by sonicating 40 mg of sphingomyelin in 1.5 ml of 25 mM imidazole buffer at pH 7.1. After centrifuging, the complex was freeze dried *in vacuo* and stored. This material made an optically clear dispersion in chloroform containing cardiolipin, giving the sphingomyelin-cardiolipin mixture found to form K^+ selective membranes. (Hyman, 1973; Buzhinsky, 1974)

Lipid membranes were formed across a 1 mm-hole in a Teflon membrane separating two chambers of aqueous solutions. The solutions were electrically connected by way of matched calomel half cells to a high impedance (2×10^{14} ohms) voltmeter to measure OCV, and connected in series with a Kiethley picoammeter, model 414A, to measure SCC. The output of either instrument was recorded on a 10-inch servorecorder. Overall calibration and imposed bias were supplied from a Leeds and Northrop K 3 Potentiometer appropriately placed in the circuit.

Results

The ATPase in the freeze dried sphingomyelin-ATPase complex, assayed by redispersing it in buffer, was found to liberate 50 μM P per mg protein per hr. This value fell to 12 μM P after 5 days of storage and rose to 20 μM P by the 15th day, where it remained for several weeks. When the freeze-dried material was dispersed in chloroform for 1 hr and then redispersed in buffer, it assayed as low as 2 μM P per mg protein per hour, 93% of which activity was ouabain sensitive. This was considered sufficient for electrical purposes.

Membranes formed of the phospholipid-enzyme complex had a resistance little different from those formed from the phospholipid alone, about 10^8 ohms/cm^2. With 100 mM NaCl in each aqueous chamber the enzyme containing membrane was challenged with 0.5 mM Na_2ATP in 100 mM NaCl. The typical OCV and SCC response is shown in Fig. 1*a*. When open circuited, the side to which ATP had been added quickly became 90 to 110 mV negative compared to the opposite chamber, and when short circuited a peak current as large as 2.5×10^{-7} Å/cm^2 rapidly appeared in the direction of Na^+ leaving the chamber containing ATP. The current decayed in 15 to 25 min and a second application of ATP had no effect. The membrane response to ATP was symmetrical. The response was not much greater with 5 or 50 mM Na_2ATP. It was not significantly affected by ouabain or digoxin.

With 5 mM Na_2SO_4 on one (cis) side of the enzyme membrane and 50 mM Na_2SO_4 on the other (trans) side, the cis side is 40–45 mV positive when open circuited. When short circuited a significant net Na^+ current flows. In a typical experiment, Fig. 1*b*, ATP added to the cis side reverses both the OCV and SCC, the quantitative response being like the previous (Fig. 1*a*) except for the shift of coordinates.

As a control the same experiments were repeated with the sphingomyelin-cardiolipin membrane without enzyme. The responses to ATP were similar. A membrane of sphingomyelin alone was inert, but one of cardiolipin was fully active. In Fig. 1*c* a black lipid membrane made of cardiolipin in chloroform/decane 3:1 gave a similar SCC response. This membrane had an impedance of about 1×10^6 ohms per cm^2 and it would develop only 30–40 mV when open circuited.

The OCV and SCC responses of membranes of net neutral phosphatidyl choline and phosphatidyl ethanolamine were negligible. The greatest response occurred with the strongly acidic cardiolipin or diisooctyl phosphate. The response of a phosphatidyl-serine membrane was significantly less. Differences between Na^+ and K^+ were not significant. Similar responses were obtained with GTP and ITP. There was no response to Na_3 citrate, Na_2SO_4 or $Na_5P_3O_{10}$.

Lipid Analysis of Solubilized ATPase

As determined by the Folin-Ciocalteu reagent, using bovine albumen as a standard, the solubilized enzyme was 53% protein by weight. The major lipids were resolved in duplicate thin layer silicic acid chromato-

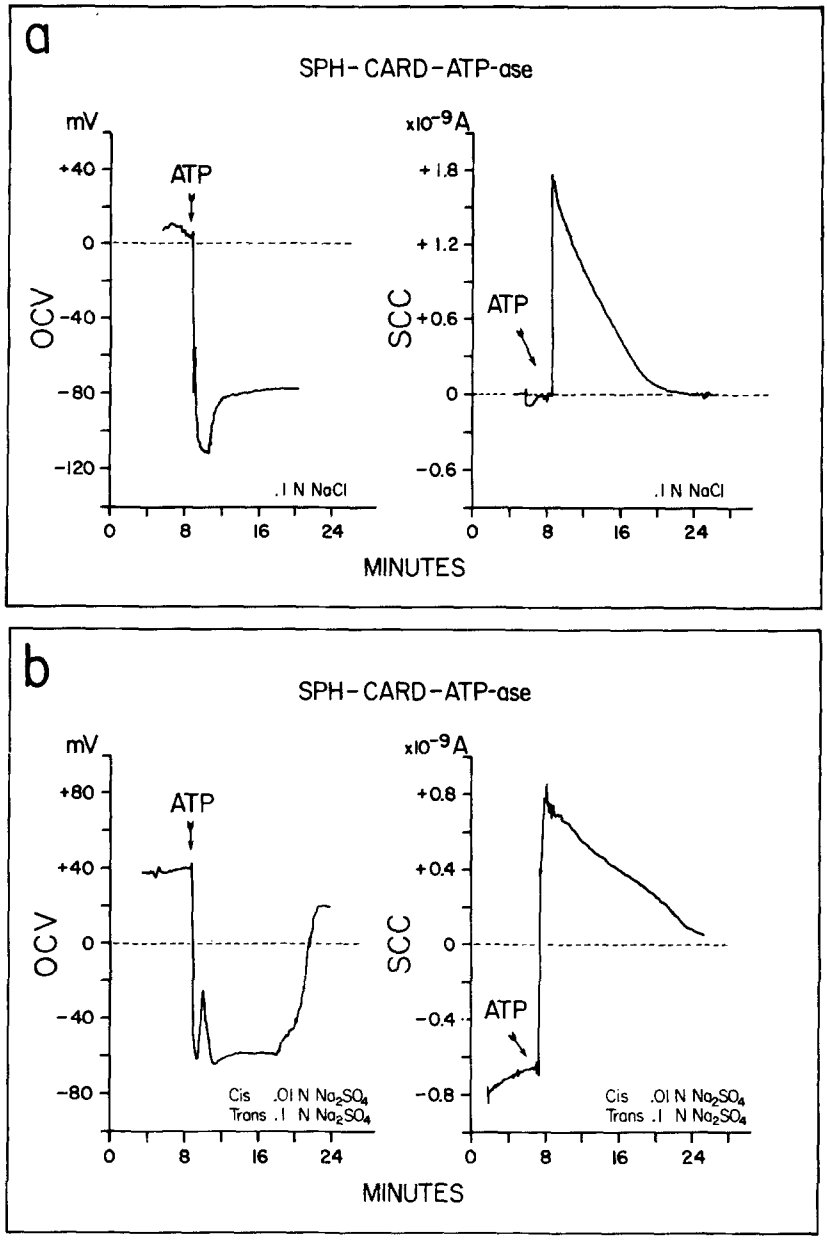
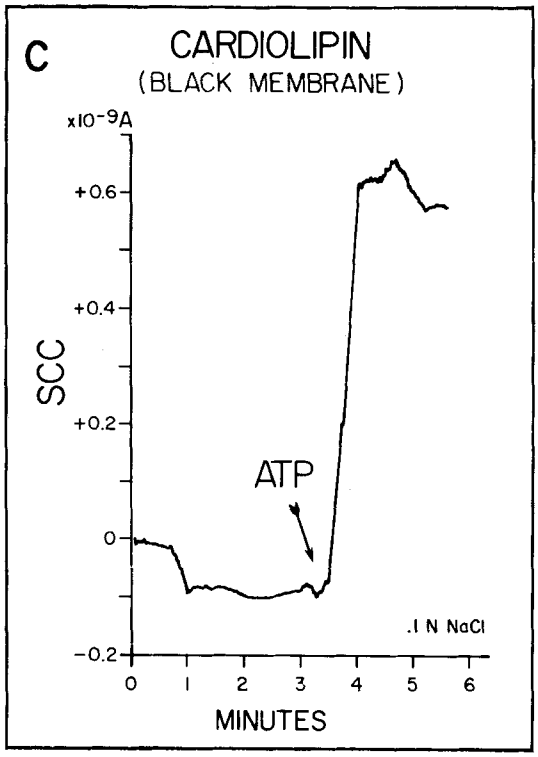
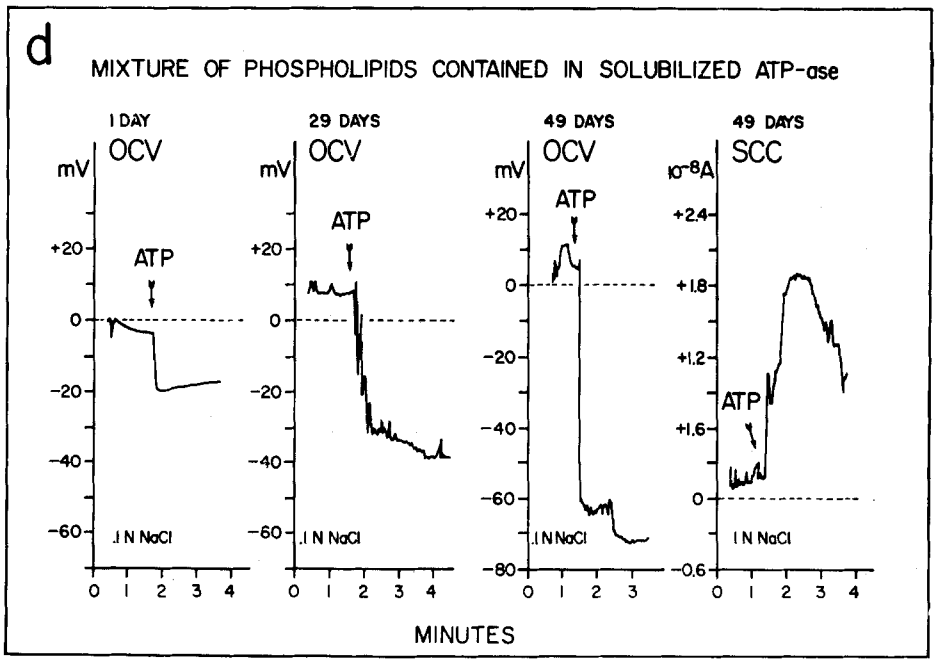


Fig. 1. (*a*): Electrical responses of a sphingomyelin-cardiolipin-ATPase membrane to ATP. A flat, but not "black", lipid membrane occluded a 1-mm hole in a Teflon partition between two chambers each containing 0.1 N NaCl. The membrane is challenged with 0.001 N Na_2ATP on one (cis) side. If open circuited the cis side becomes negative with respect to the trans side, and if short circuited a current flows in the external circuit in the direction of sodium leaving the cis side. The lipid is 20 mg cardiolipin, 40 mg sphingomyelin, and solubilized Na-K-ATPase (0.2 mg per protein per ml) in chloroform/decane 3:1. (*b*): Electrical response of a biased sphingomyelin-cardiolipin-ATPase membrane to ATP. Same as *a*, except the cis chamber contains 0.01 N Na_2SO_4 and the trans chamber contains 0.1 N Na_2SO_4. Since the membrane is cation selective the cis chamber is positive to the trans on OCV, and a "sodium" current flows from trans to cis on SCC. Addition of 0.001 N Na_2ATP to the cis chamber reverses both the OCV and the SCC. The magnitude





of the response is the same as in *a*. (*c*): A classic "bi-molecular" membrane made of cardiolipin, 40 mg per ml, in chloroform/decane responded similarly to ATP. The OCV only reached 30 mV. (*d*): Response to ATP of a membrane of a mixture of chromatographically pure phospholipids in the same proportions as in the analysis in Table 1. After 7 weeks storage at 20 °C with antioxidant the response is maximal. The change with time is thought to be due to a rearrangement of the aggregates of lipids in solution

Table 1. Quantitative analysis of the lipids of Na-K-ATPase which had been solubilized in water

	Analysis of solubilized ATPase		
	mg	mg/mg protein	% total weight
ATPase	49	1.9	100
Protein	26	1.0	53
Phospholipids[a]	11	0.42	22.4
P. serine	1.3 1.14	0.047	2.5
Sphingomyelin	3.0 3.0	0.115	6.1
P. choline	3.3 3.3	0.127	6.7
P. ethanolamine	2.9 2.9	0.114	5.9
Cardiolipin	0.53 0.53	0.02	1.1
Cholesterol	3.3	0.13	6.7
Lubrol	6.3	0.24	12.9
Other (glycolipids, etc.)			5.1

[a] duplicate chromatograms.

grams using two solvent systems. The silicic acid at each spot was etched away from the plate and placed into a test tube. The lipid was eluted with chloroform-menthol and dried *in vacuo*. Phospholipids were digested with perchloric acid and quantitated as inorganic phosphorus. Weights of the phospholipids were calculated based upon an arbitrary assignment of palmitic acid for all fatty acids. Cholesterol was determined by Liebermann-Burchardt reaction. Eluted Lubrol (a nonionic detergent used to solubilize the enzyme) was found to form a clathrate with alkali cations and it was quantitated by a modification of a method devised to quantitate discrete ionophores. (Hyman, 1970) The analysis is given in Table 1.

A mixture of phospholipids in the same proportions as Table 1 was compounded of chromatographically pure phospholipids from commercial sources. A trace of alpha tocopherol was added as an antioxidant. This mixture contained very little cardiolipin as compared to the active cardiolipin membranes above, and it contained no cholesterol, lubrol,

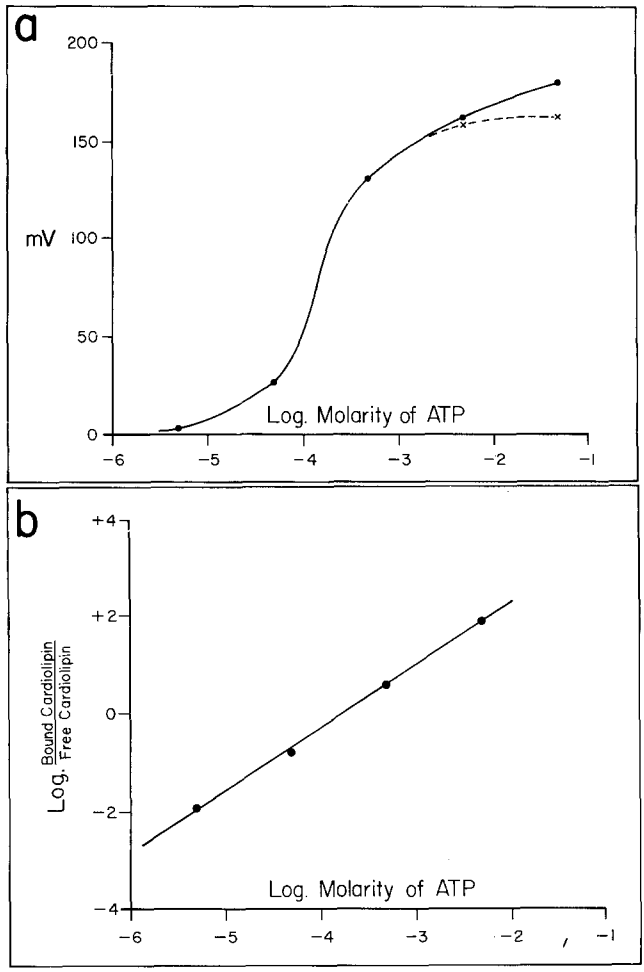


Fig. 2. (*a*): A jet stream of Na_2ATP in 0.1 M NaCl is applied to a flat membrane formed of cardiolipin in decane/chloroform 2:1 between identical solutions of 0.1 M NaCl. Voltage observed across the membrane *vs.* logarithm of the molarity of Na_2ATP. (*b*): The voltage generated when 0.05 M Na_2ATP is applied to the cardiolipin membrane in *a* is assumed to be due to maximal binding of ATP by cardiolipin. Since the bound ion adds a charge to the membrane as a capacitor, voltage is assumed to be a linear function of binding. The voltages of the remaining points in *a* are replotted according to the mass action equation log [ATP]=log [bound cardiolipin/free cardiolipin]−log K (assoc)

glycolipids, or proteins. In Fig. 1*d* the OCV response of membranes of this mixture was minimal on the first day, but increased with time as the mixture was stored at 20 °C. At 7 weeks the response was comparable to those of the most active membranes. A similar mixture of lipids devoid of cardiolipin, similarly aged, became active but not as active as the membrane containing cardiolipin. It contained phosphatidyl serine.

In another experiment a series of flat membranes thicker than bilayers made of cardiolipin in decane/chloroform 2:1, formed between solutions of 100 mM NaCl, were each challenged with a jet of Na_2ATP of varying concentration in 100 mM NaCl. In Fig. 2*a* the immediate maximal OCV response was plotted against the logarithm of the molarity of ATP so as to include a wide range of concentrations. Since the molarity of sodium was doubled at the highest concentration of ATP added and the membrane is cation permeable, the emf response corrected for the theoretical Nernst potential is represented by the broken line. Subtracting the estimated Nernst contribution, the maximal response of 181 mV obtained with 50 mM Na_2ATP became 163 mV. There was a 26 mV response to 5×10^{-5} M and a distinct response to 5×10^{-6} M ATP. A similar membrane using phosphatidyl glycerol instead of cardiolipin gave only one tenth of the response of the cardiolipin membrane, and one made of phosphatidic acid failed to respond.

Discussion

Ouabain sensitive Na-K-ATPase, solubilized from microsomes of the outer medulla of canine kidney, adsorbed onto a sol of sphingomyelin, centrifuged, and freeze dried, can be stored at $-20\,°C$ for long periods of time with retention of considerable hydrolytic activity. Since the measured activity fell and rose again, the measurement is probably a function of the orientation of the enzyme with respect to the lipid matrix. The complex dispersed in chloroform retains significant ouabain-sensitive activity, and it serves to incorporate the enzyme into the organic solution used to form lipid membranes. The surface of these membranes becomes cloudy because of the protein contained. These membranes reacted electrically to ATP and reproduce some of the phenomena observed with intact fragments of biological membranes. The OCV and SCC responses observed were larger, longer in duration, and more consistent than those reported by Jain *et al.* (1972) using a preparation of intact membrane fragments. As expected, the membrane containing solubilized enzyme was electrically symmetrical. Unlike the preparation containing intact membrane fragments, the electrical responses of these membranes were not affected by ouabain or by digoxin, although the hydrolytic function of the enzyme was 93% ouabain sensitive after dispersion in chloroform. This indicated that the electrical response did not involve hydrolysis of ATP. Further experiments clearly showed that the electrogenesis did

not depend upon the presence of the enzyme, but it would occur in membranes made either of acidic phospholipids or a mixture of phospholipids in the proportions found on analysis of ATPase preparation. This mixture contained very little cardiolipin. In another experiment electrogenesis occurred to a lesser extent when cardiolipin was omitted from the mixture, but phosphatidyl serine was still present. Mixtures required time to become active, perhaps due to a requirement for reaggregation. The electrical signal produced by interaction of ATP with the phospholipids of ATPase must be taken into account in all electrometric studies of ATPase reconstitution in a lipid membrane.

The Nature of the Electrogenesis

In its simplest form a large electrical signal is generated when 0.5 mM Na_2ATP in 100 mM NaCl is applied to one side of a membrane formed of only cardiolipin and decane between identical solutions of 100 mM NaCl. Although the sodium concentration was raised only 1% and the chloride concentration was unchanged, a 130 mV change appeared across the membrane with the side containing ATP becoming negative. The change in emf is too large to be due to a change in the Donnan equilibrium or to a change in the potential of the electrical double layer at the membrane surface (MacDonald & Bangham, 1972), and another explanation must be found.

In other experiments the selection of Cl^- or any other small anion makes little difference, perhaps because the membrane is predominantly cation permeable. Since no detectable inorganic phosphate can be found after incubation of ATP for 1 hr in an appropriately buffered aqueous emulsion of cardiolipin, the electrogenesis is not likely to depend upon the energy of hydrolysis of ATP. This is consistent with the finding that the Na^+ or K^+ salts of another polyanion, cellulose sulfate, gave a similar electrical signal. Cellulose sulfate is hydrophilic. The signal produced by a salt of a lipophylic anion, sodium dodecyl sulfate, was opposite in direction. This latter change is probably due to the association of the lipophylic anion with the lipid membrane creating a new, perhaps more intense, summation dipole at the water-lipid interface, oriented with the positive side to the experimental chamber and negative side to the membrane. Analogously, ATP and other hydrophilic organic polyanions could associate with the membrane in such a way as to create a change in the summation dipole in the direction opposite from the change due to sodium dodecyl sulfate. This orientation would require

one or more associated cations to point toward or to enter the predominately cation permeable membrane, while the ATP remains on the water side of the dipole with its tripolyphosphate moiety still associated with its cations. This association of polyphosphate with the phosphate of the phospholipid by way of bridging counterions, an anion-cation-anion sandwich, would resemble the association of organic phosphates in organic solution or in aqueous sols. Such an association would explain why a second challenge with ATP did not produce a second response, and why the electrogenic response is nearly the same with 0.5 mM as with 50 mM Na_2ATP. The association would be strong enough to overcome a 40 mV bias imposed on the membrane in either direction as in Fig. 1*b*, even with a low impedance external shunt.

If ion association produced the electrical change, then the OCV would be a linear function of the quantity of associated ATP, or of the bound cardiolipin sites, just as emf across a fixed capacitor is a linear function of the charge stored. Then the OCV would follow the mass action equation of ion association instead of the Nernst equation. In Fig. 2*b* the highest OCV obtainable (corrected for the Nernst contribution) is assumed to relate full binding of cardiolipin sites, and the lower four points are replotted according to the mass action equation, log [ATP]= log [bound cardiolipin/free cardiolipin]$-$log K (assoc). The result is a straight line with the zero intercept at log [ATP]$=-\log K_a=-3.7$. This gives a K_a of 5×10^3 which is appreciably lower than 5×10^6 found for the binding of ATP to Na-K-ATPase (Hegyvary & Post, 1971). But the binding of ATP to the membrane is not nucleotide specific. Considering that ATP must have at least one more binding site to bind to ATPase to confer nucleotide specificity, e.g., the 6 amino group (Hegyvary & Post, 1971), this association constant is reasonable.

In Fig. 1*b*, the "ATP current" through a low impedance external circuit peaked at 1.8×10^{-9} A and returned to baseline 12 min later. The area subtended is about 6.5×10^{-7} coulombs which corresponds to 6.7×10^{-12} moles or 4×10^{12} cations oriented toward or entering the membrane. Considering the entire 1-mm diameter hole, or 7.9×10^{13} Å^2, that would be one Na^+ per 20 Å^2, should the model be appropriate. With pure cardiolipin in decane the ATP current was frequently larger, equivalent to about one Na^+ per 15 Å^2. If the lipid at the water interface is entirely cardiolipin with an estimated surface area of 70 Å^2 per molecule (Fleisher & Rouser, 1965), then there would be 4.7 Na^+ per phospholipid in the model proposed here. Unless more than one ATP molecule is associated with one phospholipid molecule in the model, then more

than one Na^+ must be oriented per molecule of ATP associated. The alignment of several Na^+ per ATP would be attractive as part of a model for the transfer of Na^+ across cell membrane where there are about three Na^+ transported per ATP hydrolyzed. Moreover these counterions would be inserted into a milieu which is essentially lipid and which would be favorable for maximal transfer to the cation of the energy of hydrolysis of the polyphosphate (Hyman, 1966).

Cardiolipin is thought to occur predominantly, if not entirely, in the mitochondrial membrane. Orientation of ATP or ADP within the mitochondria on the cardiolipin of the mitochondrial membrane, perhaps on cardiolipin as a component of the ATPase of that membrane, would generate a potential or charge separation.

Whether cardiolipin is a true component of the ATPase of the cell wall or whether its presence in this preparation is due to contamination of the microsomes with mitochondria will be discussed below. Either way, enough phosphatidyl serine was present to entertain the possibility that association of ATP with this acidic phospholipid has a role in electrogenesis at the cell membrane. If cardiolipin were present in membrane ATPase it would not only offer greater electrogenesis than phosphatidyl serine but it could explain the effects of phospholipase A. This enzyme abolishes the electrogenic response of intact microsome fragments containing ATPase which are embedded in an inert supporting membrane (Jain *et al.*, 1972), and it also abolishes ATP binding to Na-K-ATPase (Hegyvary & Post, 1971). Cardiolipin is rapidly hydrolyzed by phospholipase A (Van Deenan, 1966).

The belief that cardiolipin exists only in mitochondria or perhaps also in lysosomes (Korn, 1966; Fleischer & Rouser, 1965) is largely based on its absence in the carefully studied microsomes of liver cells (Fleisher & Rouser, 1965; Dallner, Siekevitz & Palade, 1965; Strickland & Benson, 1960), and in the easily prepared membranes of erythrocytes (Dodge & Phillips, 1967). However significant quantities of cardiolipin have been found by presumably adequate methods in microsomes of pig heart (Marinetti, Erbland & Stotz, 1958) and beef heart (Fleischer & Rouser, 1965). The demonstration of cardiolipin in this solubilized ATPase derived from microsomes of canine renal outer medulla, raises the question of whether or not this candidate for an electrogenic scheme may be present in the microsomes of some tissues, perhaps those tissues which are more active in Na^+ and K^+ transport.

Identification of individual phospholipids in subcellular fractions has been hampered by contamination of the preparation of one subcellular

element by another, and by the inadequacy of earlier chromatographic separations. A preparation of microsomes found free of mitochondria by electron photomicrographs may still have significant cytochrome oxidase activity, suggesting that it is contaminated by fragments of mitochondria. These fragments would contain cardiolipin. Chromatographic separations prior to Marinetti in 1958 were either cumbersome or inadequate, and according to Fleischer and Rouser (1965) even Marinetti's paper chromatograms may fail to detect cardiolipin. More recent two dimensional thin layer chromatographic techniques are adequate to separate and identify cardiolipin. Although the preparation of ATPase in this work was not controlled by electron microscopy and by assaying cytochrome oxidase, the cardiolipin found is likely to be from microsomes because it would require almost 25% contamination by mitochondria to make the cardiolipin content 5% of the phospholipids. Further work in this laboratory has demonstrated that cardiolipin is present in microsomes of the outer renal medulla prepared with precautions against mitochondrial contamination and present in the Na-K-ATPase derived from these microsomes.

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