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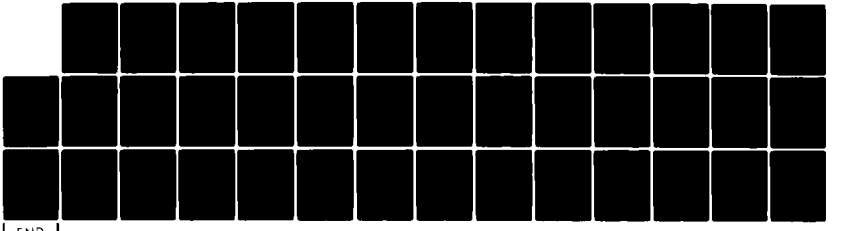

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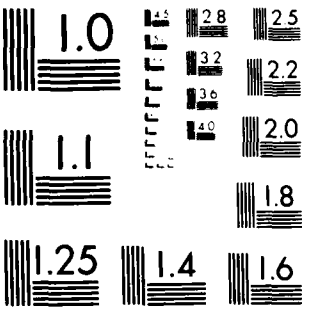

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DO ISOLATED MEMBRANES AND PURIFIED VESICLES PUMP SODIUM? - A CRITICAL REVIEW AND REINTERPRETATION.

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Acknowledgements. G.N.L. is supported by NIH grants 2-R01-CA16301-03 and 2-R01-GM11422-13 and by Office of Naval Research Contract N0014-71-C-0178. W.N. is supported by the Veterans Administration Medical Research Service and by Office of Naval Research Contract N00014-76-C-1166.

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I. BACKGROUND - THE MEMBRANE-PUMP THEORY.

Since the discovery that cells are the basic units of life, there have been two different general viewpoints about the physical state of the living cell - the membrane theory, and the bulk-phase theories. The fundamental assumption of the membrane theory is that the interior of the cell and of its organelles is essentially a dilute aqueous solution in which ions are dissolved much as they are in a Ringers solution; hence, the discontinuity of the cell from its environment depends upon a structural barrier, the plasma membrane. The fundamental assumption of the bulk-phase theories is that the physical state of the water and solutes within the cell is not that of a dilute aqueous solution; the discontinuity between the cell and its environment reflects a general property of the entire cell substance.

Several observations led, in the 1930's, to the ascendance of the membrane theory, and simultaneously, to the near-extinction of the bulk-phase, or as it was then called, the "colloid" concept (1,2). These included a) the approximate equality of total concentration of ions, expressed as millimoles per liter of cell water, with that of the external incubation medium, and b) the swelling and shrinking of cells as if they were behaving as true osmometers, when placed into a medium containing poorly penetrating solutes.

The old, and most fundamental, observation, that cells maintain markedly asymmetric ion concentration gradients between themselves and the external medium, was then explained by a combination of membrane impermeability (as to Na^+, keeping cell Na^+ low), and a Donnan equilibrium (as for K^+, keeping cell K^+ high), in the form of the theory of Boyle and Conway (3).

This relatively settled state of affairs did not last long, and was thrown into turmoil by the observation, of Heppel (4) and of Steinbach (5), that Na^+ does in fact readily enter and leave the cell. With both absolute membrane impermeability and a Donnan equilibrium refuted there was only one way left that cells could maintain an asymmetric concentration of Na^+ between two dilute aqueous solutions - it had to be pumped out continually to make up for the steady leaking of Na^+ down its apparent electrochemical gradient, into the cell (6,7). Pumping requires an exogenous source of free energy, and the process by which the stable asymmetric low concentration of cell Na^+ was maintained was called active transport.

This modified version of the membrane theory gained support from the discovery of what was thought to be the "pump" itself - the Na,K-ATPase, first isolated by Skou in 1957 (8). By the mid 1960's a large body of evidence suggested that the Na,K-ATPase is in fact the Na^+ pump, with hydrolysis of the "high energy" phosphate bond of ATP being the exogenous source of free energy needed to perform active transport. Thus, the kinetics of activation of the ATPase, the stoichiometric relations to K^+ and Na^+, the effect of inhibitors such as ouabain, and other features of the Na,K-ATPase are similar in studies of the extracted enzyme and of ion transport in cells (9,10).

However, as strong as the evidence linking the Na,K-ATPase with the pump may be, it remains circumstantial. Why is this so? There are two sets of reasons. First, an entirely different theory could predict a similar linkage between the two phenomena, and indeed such a theory has long been proposed (11-13). Second, the evidence is not as strong or as consistent as would seem. We will develop the argument in four parts.

1. The Changing Definition of Active Transport.

The original definition of active transport, and the reason for postulating the Na^+ pump in the first place, had to do with the observation that cells maintain an asymmetric ion concentration difference that is stable in time and that deviates from a Donnan equilibrium. Having decided, however, that the Na,K-ATPase is the pump, one did not feel constrained to rigorously study the steady state distribution of ions itself, which required long term incubations, sterile conditions, absence of cell deterioration, and ability to reach a new steady-state level within a reasonable time after exposure to an altered incubation medium. Instead, one relied upon short term ion flux experiments, and auxiliary features of the pump, such as ouabain-inhibition, ATP hydrolysis, or ATP-induced increase in ion flux, then became the major criteria for identifying, and defining, active transport. At the same time, the definition of active transport lost its relationship to the fundamental assumption upon which the membrane-pump theory was based, that of dilute solution theory. Thus, Best and Taylor's textbook of physiology states (p. 1-23) (14) "an active transport process is defined, not by demonstrating that flux is thermodynamically uphill, but only by demonstrating that flux is coupled to metabolism". In response to this, Hazlewood (15) has succinctly pointed out that "if such a change in the definition is indeed accepted, then it is not clear what fundamental assumptions are basic to the definition. If it is a definition by statement, it may be impossible to test". In other words, it may have wandered beyond the reach of scientific method.

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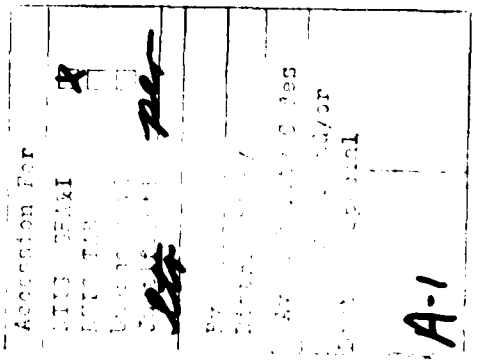

2. The Red Cell Ghost and the Perfused Squid Axon - Incompleteness of the "Proof" of Active Transport.

During the 1960's and early 1970's, a large amount of kinetic data of Na^+ and K^+ transport and of the Na,K-ATPase accumulated, and a wide variety of models were proposed to describe the workings of what was thought to be a coupled Na^+-K^+ pump. In order to see how the pump behaved in response to variations in cell ion contents, one had to find ways to overcome some of the obstacles mentioned in section 1. Techniques were devised to manipulate cell ion concentrations, such as the "resealing" of red blood cell ghosts (16), the study of genetically variable sheep red cells (17), the treatment of red cells with PCMBS to make them leaky (18,19), and the removal of the gel-like interior of the squid axon and direct perfusion of it with various solutions (20,21). Indeed, the red cell ghost and the perfused squid axon, with their ability to show increase in flux of Na^+ or of K^+ in the presence of ATP, were accepted as even more definitive proof of the membrane-pump concept. As we shall see, however, in Section IV, these preparations are not able to perform true active transport, and actually provide evidence against the membrane-pump concept.

3. Mechanism of the Pump - The Inadequacy of Kinetic Studies.

The techniques mentioned above permitted development of the flux-activation studies, in which initial influx or efflux was determined as a function of external or cell ion concentration. These studies helped to narrow down the number of plausible mechanisms of action of the pump (22,23). However, the extent to which any kinetic study is able to distinguish between various possible transport models, is limited. This

conclusion has been reached by a number of reviewers within the membrane-pump community itself.

In his review of the Na,K-ATPase in 1975, Skou (24) concluded "There are so many unsolved problems, ... that it is not possible to set up a kinetic model for the transport of the cations based on experimental evidence. It has not yet been possible to define and to isolate a pure system. Nor has it been possible to relate the intermediary steps in the reaction to molecular events and thereby to get the information necessary to formulate a mechanistic model for the transport". Similarly, Hoffman (25) recently said "It should be recognized that the molecular mechanism of translocation and its relation to transphosphorylation is still obscure even in the light of evidence that reversible transformation of different forms of EP (ATPase enzyme-phosphate) might be associated with particular types of cation exchange". In discussing the flux activation studies, Garrahan and Garay (22) said "But, however useful these studies may be, they are hampered by the fact that almost always more than one kinetic scheme will fit the experimental results. The distinction between alternative models lies beyond the realm of kinetics and probably will become possible only when procedures to measure the binding of cations to purified ATPase preparations become available".

Glynn (23), in a recent review of the Na^+ pump, said "If the great mass of work that has been done had led to the general acceptance, even provisionally and even in outline, of a hypothesis accounting for the working of the pump, we could have described that hypothesis and then considered the evidence for it. Unfortunately, no such hypothesis exists."

4. The Search for Purified Pumps.

The direction to be taken in order to improve this state of affairs was indicated by the following statement by Hokin (26): "On the assumption that the Na,K-ATPase is the molecular machine for Na^+ and K^+ transport, several models have been proposed to explain its mechanism..... The type of evidence that has led to the formulation of these models has been of an indirect nature. Obviously, a more direct approach to the elucidation of the molecular mechanism of Na^+ and K^+ transport would be to completely purify the Na,K-ATPase in its biologically active form and to perform physical and biological studies on the purified molecule". Thus, the realization of the limitations of kinetic studies in whole cells then led to the search for simpler systems. The simpler models have taken two forms - the phospholipid bilayers (27), and the vesicles. The bilayers have been useful for studying membrane permeability (28), but have not yielded a fully-functioning "pump" (29). Indeed, in regard to the isolation of ionophores from ATPases and other ion "transport" systems, P. Mueller who, together with Rudin and others had initiated the great surge of interest in lipid bilayers (27), has recently said (30), "A lot of us have spent a wasted ten years or so trying to get these various materials into bilayers or something that is planar barrier or something that can be recognized as having physiological significance..."

In 1974 and 1975, purified Na,K-ATPase-phospholipid vesicles were prepared, and appeared to actively pump Na^+ and K^+ and to establish an asymmetric concentration gradient of these ions. These studies have been widely quoted as having proven, once and for all, the presence of the Na^+ pump (26,31). In Section V, we critically review this claim and in Section VI offer an alternative interpretation. Our discussion will

revolve around four major interrelated questions: 1) Is there sufficient energy available to operate the Na^+ pump in the intact cell? 2) Do ion gradients make ATP by forcing the pump to run backwards? 3) Do natural membrane preparations, such as the red cell ghost and the perfused squid giant axon actually pump ions? 4) Do reconstituted purified phospholipid-ATPase "vesicles" pump Na^+?

II. IS THERE SUFFICIENT ENERGY TO OPERATE THE Na^+ PUMP IN THE INTACT CELL?

In 1962, Ling (11) found an energy need of the apparent pumping-out of Na^+ by frog muscle that exceeded by 15–30 times the energy available in the form of ATP. The problem of the energy requirement of the Na^+ pump, as well as that of just some of the other 20 or so pumps that must be postulated (e.g., calcium, magnesium), has been discussed in detail elsewhere (13,15,32). Similar problems have been noted in bacteria (33) and in smooth muscle (34).

Three sets of ad hoc postulations have been made to account for this dilemma.

1. Ussing (35) postulated that much of the measured efflux of Na^+ was simply exchange diffusion (exchange of Na^+ for Na^+) occurring via the pump carrier, so that the net energy-requiring efflux was much less than assumed. However, if Na^+ for Na^+ exchange diffusion is to serve the purpose of reducing the energy need, then the rate of Na^+ efflux must show a strict dependence upon Na^+ concentration in the external medium, and in the absence of external Na^+ there should be no Na^+ efflux via the exchange mechanism. This does not obtain in the frog muscle, nor does exchange diffusion make any significant contribution to Na^+ efflux in nerve, red cell, or smooth muscle (36-40).

2. A second ad hoc postulation designed to reduce the energy need of Na^+ efflux was the localization of Na^+ in the sarcoplasmic reticulum of muscle (41). This has been disproven experimentally (37); furthermore, recent electron microprobe analyses failed to show significant Na^+ sequestration in the sarcoplasmic reticulum, but indicated rather even distribution of Na^+ throughout the cell (42).

3. The third explanation for the apparent energy deficit for pumping Na^+ out is that given by Glynn (43). He proposes that metabolic energy is required only for net outward movement, and that the inward leaking of Na^+ down its electrochemical gradient is coupled to outward movement of Na^+ against the electrochemical gradient. Part of the rationale for this postulation is the apparent ability of artificially imposed steep concentration gradients of ions to drive a net synthesis of ATP by making the pump run backwards. We discuss this concept, and the data appearing to support it, in Section III. The statement that "metabolic energy is required only for the net outward movement" (43) means that, under normal physiological conditions when the level of cell Na^+ is constant, the energy dissipated by the leakage of Na^+ molecules down their concentration gradient is harnessed by the pump in order to move an equal number of molecules out. This concept is a violation of the fundamental laws of thermodynamics. For indeed, what Glynn has proposed is a perpetual motion machine; it is similar to the demon first imagined by James Clerk Maxwell - the character which, by operating a frictionless shutter between two closed compartments, was capable of sorting out nitrogen and oxygen into separate contiguous compartments from a mixture of both without energy expenditure! Such a demon has yet to be found in the real world and it is doubtful that cell membranes are an exception to this rule.

In any case, there already exists evidence against this concept, for if energy is harnessed from Na^+ leaking inward, then this leaking must be coupled to the outward pump. Yet ouabain, a specific poison of the Na,K-ATPase and of the Na^+ "pump", has been found by Horowicz and Gerber (44) not to affect the inward Na^+ flux into muscle.

III. DO ION GRADIENTS MAKE ATP BY FORCING THE PUMP TO RUN BACKWARDS?

In 1966 Jagendorf and Uribe (45) described the formation of ATP by chloroplasts (in the dark), upon transfer from a medium of pH 3.8 to one of pH 8.0. They concluded that the hydrogen ion gradient provided the energy source for the formation of ATP, in accord with the chemiosmotic hypothesis of Mitchell (46). Many similar and related observations have been made in mitochondria (47) sub-mitochondrial particles (48) and bacteria (49,50). These cells or organelles are generally felt to make ATP by dissipating a proton gradient through the backwards running ATPase proton pump.

Garrahan and Glynn (51), and later Glynn and Lew (52) found that red cells in high-Na^+, K^+-free medium also could make ATP and Cockrell et al (53) found synthesis of ATP associated with K^+ efflux in mitochondria. It was presumed that ATP formation was coupled to the net efflux of K^+ down its electrochemical gradient backwards through the pump.

Makinose and Hasselbach (54) showed ATP synthesis stoichiometrically related to calcium efflux in calcium-loaded sarcoplasmic reticulum vesicles. Others (55-57) have confirmed this observation, and all concluded that the imposed calcium gradient allowed reversal of the inwardly-directed calcium pump.

There are, however, a variety of experimental findings which provide serious doubts that it is an electrochemical gradient of the ions per se that allows the formation of ATP. Only two need be cited here.

1. Taniguchi and Post (58) studied the guinea pig kidney-derived Na,K-ATPase vesicles; they concluded "it seems likely that binding of Na^+ was sufficient for synthesis of ATP and that a subsequent step releasing Na^+ (from the opposite face of the membrane) was not necessary".

2. Knowles and Racker (59) studied purified calcium-ATPase from rabbit muscle, and concluded "we have observed ATP generation fr· · ADP and P_i without the formation of an ion gradient across a membrane. propose that the energy derived from ion-protein interaction drives the mation of ATP".

Others have drawn similar conclusions (60,61,62,63). That ion gradients per se are not essential for the formation of ATP is not surprising, for, when one looks at the other side of the coin and questions the mechanism by which ATP performs its "energetic" role in maintaining the resting state of the cell, one finds increasing evidence that it is not the hydrolysis of the so-called high energy phosphate bond that provides energy, but rather the adsorption of ATP onto key enzymes and other proteins. A detailed discussion of this evidence has been given elsewhere (13).

IV. DO RED BLOOD CELL GHOSTS AND PERFUSED SQUID AXONS ACTUALLY PUMP IONS?

The perfused squid giant axon can maintain a normal resting potential and ability to conduct action potentials; it contains functioning Na,K-ATPase; and it is able to increase efflux of Na^+ by addition of ATP. The red cell ghost contains normally-functioning Na,K-ATPase, maintains its "sidedness" with regard to K^+ and Na^+ activation and ouabain inhibition

and maintains the proper stoichiometric relations between ion transport and the ATPase. With preservation of these features, one would think that these preparations would be able to demonstrate the ultimate function of active transport - a net movement of ions against an electrochemical gradient, with the establishment of a stable asymmetric ion concentration difference between two dilute aqueous solutions. In fact, they are not able to do so.

1. The perfused squid giant axon (64), in which most of the axoplasm was removed, had a component of Na^+ efflux that was sensitive to K^+ and ouabain in the presence of ATP. However, ATP did not induce a net Na^+ efflux against an electrochemical gradient.

2. The dialyzed squid giant axon (65,66) is frequently cited as proof of active Na^+ transport. In this preparation, a capillary tube was inserted longitudinally through the axoplasm, and most of the axoplasm remained intact within the cell. This, unlike the perfused axon, maintained a relatively normal baseline Na^+ efflux and K^+ influx. ATP induced an increase in Na^+ efflux, an increase in K^+ influx, but also an increase in Na^+ influx. However, the net movements were in the wrong direction: the dialyzed axon showed a net gain of Na^+ and a net loss of K^+ during the course of the experiment. Hence, this preparation also does not "prove" active transport by a membrane pump.

3. Red blood cell ghosts are often cited as proving active transport. Freedman's careful review in 1973 made it quite clear that the direct demonstration of a net movement of Na^+ or K^+ against an electrochemical gradient had not yet been shown (67). Freedman (68) recently found that red cell ghosts prepared by the Bodemann-Passow technique can indeed re-accumulate K^+, and extrude Na^+, to a small but significant

degree. However, Ling and Balter (69) have shown that the ghosts used by Freedman contain considerable cytoplasm, and Ling and Tucker (70) have shown that intact, non-leaky "white" ghosts, which are freed of the bulk of their cytoplasm but do contain the Na,K-ATPase, are not able to accumulate K^+ or extrude Na^+. Again, these data, taken together, support the bulk phase adsorption concept more than they do the membrane-pump theory.

The fact that these natural membrane preparations, which are intact and retain the Na,K-ATPase with the proper orientation and stoichiometry, are not able to actively pump K^+ and Na^+ in the proper direction and to establish and maintain a steady-state asymmetric ion concentration difference between two dilute aqueous solutions, itself provides a disproof of the membrane-pump concept. This shortcoming should raise one's suspicions about the ability of synthetic vesicles and cell fragments to pump ions.

V. DO RECONSTITUTED PURIFIED PHOSPHOLIPID-ATPase VESICLES PUMP Na^+?

1. The General Nature of Vesicles.

The use of vesicles to study "transport" of solutes was pioneered in the early 1960's by the extensive studies of Kaback and associates in bacterial systems (71) and by Hasselbach, Martonosi, and their associates in sarcoplasmic reticulum (72,73). Over the past few years hundreds of papers have appeared describing properties of vesicles prepared from a wide variety of biological sources. Many of these preparations are fragments of cells or organelles; apparently, they are assumed to be surrounded by a single bilayer membrane, although some may be multilamellar. Our aim in this review is to determine whether reconstituted vesicles do or do not "pump" Na^+ (and K^+). From this standpoint, a vesicle can be

conceived as a proper model of the living cell, in terms of the membrane-pump theory, only if it is hollow inside and contains the "pump" within the surface membrane. Hence, knowledge of the structure and water content of the vesicle preparation is of paramount importance.

Many studies, regretably, do not report water content or give data from which it could be calculated. Kaback reports 63% water in his bacterial vesicles (74), Hirata et al report less than 50% in their bacterial vesicles (75), MacDonald and Lanyi report 67% in their Halobacterium vesicles (76) and McKinley and Meissner report something less than 75% in their sarcoplasmic reticulum vesicles (77).

Clearly, these preparations are not simply vesicles; they contain as much or more solid matter (phospholipid and protein) than the original cells from which they were derived. This may be underscored by noting a discrepancy between their water content and their size. If they are simply vesicles enclosed by a bilayer membrane that is 50-100 Å wide (assuming no water in the membrane), and if they contain 70% water, then they cannot be larger than 0.04-0.08 μ in diameter. Since the membrane itself would contain water, the maximum size of the vesicles would be still smaller. Yet, the bacterial vesicles (74,76), the sarcoplasmic reticulum vesicles (78,79), and the synaptosomes (80), all vary in diameter from 0.1 to 1.0 microns. Indeed, if one looks back upon the electron microscope pictures of these preparations (for example Kaback, reference 71, figure 2), one may find solid bodies as well as apparently-hollow "vesicles". Again, from the standpoint of "proving" the existence of a membrane-pump, vesicles are meaningful only if they contain free water within a hollow interior, and contain a pump only within the surface membrane. Thus, although these preparations (cell fragment vesicles) are

useful in isolating specific enzymatic functions, they are of no advantage over the original whole cells in differentiating between the alternative theories of solute accumulation - the membrane-pump concept or the bulk-phase adsorption concept, because they do not satisfy the basic requirement of a model of hollow membrane-enclosed sacs.

In the remainder of our discussion, we deal only with the vesicles that are claimed to prove the presence of Na^+ or K^+ pumping energized by ATP.

2. H^+,K^+-ATPase Vesicular Fragments.

Sachs, Schackman, et al (81,82) claimed H^+ and Rb^+ pumping by the K^+-ATPase in vesicular fragments of gastric mucosa. H^+ uptake was assessed by measuring pH of the incubating medium, and Rb^+ release was assessed in vesicles separated from the radiolabelled incubation medium by Millepore filtration and four ice-cold choline chloride washes. The results were described as nanomoles ion per mg protein. The water contents of the vesicles, which were given as a "volume" of 2 µl/mg, presumably mg of protein, would be at most 66%.

The main observation was that ATP induced H^+ uptake and Rb^+ release. The authors went to some length to demonstrate that both Rb^+ or H^+ were in the free state in the membrane-enclosed sac of free water. In figure 5 of the first paper (81), they showed a linear relation between H^+ uptake (in presence of ATP) or Rb^+ uptake (in absence of ATP), and the reciprocal of the osmolarity (π) of the medium, in which osmolarity was varied by adding mannitol. Ion contents were expressed as nanomoles/ mg protein. The extrapolation of the apparent linear relation to zero Rb^+ or H^+ uptake at infinite osmolarity ($1/\pi = 0$) led them to conclude that "binding" must have been minimal.

However, the amount of water in the vesicles at various osmolarities was not determined but only assumed to follow the behavior of a perfect osmometer. The validity of this assumption is doubtful (see 83,84,85).

A far more serious error in their evaluation of the significance of their findings reveals itself from a quantitative assessment of the buffering capacity of the vesicles. As we shall make clear in the next paragraph, there is compelling reason to believe that the bulk of the H^+ that had entered the vesicles was not in the free state as the authors claimed.

As given by the original authors, the protein content of these vesicles was approximately one third or 33.3% of the weight of the vesicles. The average amino acid residue weight in many proteins studied is 112 (ref. 11, p. 48). One liter of vesicles therefore contains a total of 333/112 or 3.0 moles of amino acid residues in their proteins. The average molar percentage of glutamic and aspartic acid residues in many proteins is at least 10% (ref. 11, p. 47 and ref. 86). Therefore one liter of vesicles must contain no less than 3.0 x 0.1 = 0.3 moles of β- and γ-carboxyl groups.

From Fig. 6 of the data of Sachs et al (81) the addition of ATP caused the release of about 100 nmoles of Rb^+ per mg of proteins; this converts to $100 \times 10^{-9} \times 333 \times 10^{3} = 3.33 \times 10^{-2}$ moles/liter of vesicle. An equalmolar amount of H^+ must have entered the vesicles in exchange.

By taking into account the total concentration of the β- and γ-carboxyl groups present in the vesicle, the total molarity of H^+ that has entered the vesicles, and the well-known pK value of the β- and γ-carboxyl group of about 4.0 (87), one can easily determine the percentage of this H^+ that would remain free inside the vesicle. This

turns out to be 0.05%. In other words 99.95% of the H^+ that has entered the cell, must exist in the vesicles associated with the β- and γ-carboxyl groups of the vesicle proteins.

This conclusion makes it all but impossible to interpret the data in terms of the membrane-pump model, the basic tenet of which is that the bulk of cell ions exist in the cell water in a state that is like that of a dilute aqueous solution.

On the other hand, the data can be readily interpreted in terms of the association-induction hypothesis. One tentative view is as follows: According to the association-induction hypothesis the interaction of ATP with cellular proteins may affect the protein-water-ion system by changing the electronic distribution in the protein. As a result the electron-density of the β- and γ-carboxyl group, expressed as the c-value, increases (11). Such an increase of c-value is equivalent to a rise of the pK value of these groups; in consequence the preference for H^+ by these sites, relative to that of Rb^+, increases. This preference in turn leads to a movement of H^+ into the vesicles replacing the adsorbed Rb^+.

3. Purified Na,K-ATPase - Reconstituted Vesicles.

The purification of Na,(K)-ATPase was accomplished in several laboratories around 1971 (88-91), and the phospholipid requirement for optimal functioning of the Na,K-ATPase had been defined (92,93). One of the polypeptide fractions of the Na,K-ATPase was shown to enhance Na^+ permeability of a bilayer membrane (94), but the bilayer preparations did not demonstrate active transport per se (29).

In 1974, Goldin and Tong (95), and Hilden, Rhee and Hokin (96), reported reconstitution of purified renal medulla and rectal gland Na,K-

ATPase into phospholipid vesicles, and claimed their ability to demonstrate the establishment of a sustained Na^+ gradient under the influence of ATP. Subsequently, coupled Na^+ and K^+ (or rubidium) transport were described in vesicles made with enzyme from brain (97), rectal gland (98,99), electric organ (100), and renal medulla (101,102). Additional studies, in sarcoplasmic reticulum fragments (77,103), or vesicles, dealt only with efflux of K^+ or Na^+ from pre-loaded vesicles, and did not attempt to demonstrate active transport *per se*.

Of the eight studies of reconstituted Na,K-ATPase, that of Racker and Fisher (100) reported Na^+ uptake as amount of Na^+ per milligram of vesicle protein, and did not attempt to define vesicle volume or water content. That of Anner *et al* (101) expressed results as "mM", but did not indicate at all just how water content was measured. The remaining six studies (95-99,102) did deal with the question of vesicle volume, and appeared to show active transport of Na^+ by our criterion - the establishment of a stable, asymmetric concentration difference between two dilute aqueous solutions. We will review these studies in detail.

a. Techniques of Preparation and Separation of Na,K-ATPase Vesicles.

Figure 1 gives a schematic flow diagram of the basic technique used by Goldin and Tong (95) and by Hilden, Rhee and Hokin (96). The technique can be divided into three stages.

Stage 1. Vesicle preparation. Vesicles were made by the method of Racker (104). Cholate-solubilized phospholipid (lecithin, alone or with phosphotidylserine), and purified Na,K-ATPase were mixed and then subjected to prolonged dialysis to remove the cholate and allow vesicle formation.

Stage 2. Isotope loading. The vesicle suspension was equilibrated in a buffer containing radioactive isotope (e.g., ^{22}Na). In some cases isotopes were incorporated into vesicles as they were formed (Stage 1).

Stage 3. Separation of the isotope-loaded vesicles from isotope in the surrounding incubation mixture. This was done by passage through a sephadex column. The vesicles with the labeled Na^+ appeared first in the effluent (fraction 3 of Fig. 1) to be followed by effluent containing no radioactivity (fraction 4) and eventually by the great peak of free ^{22}Na (fraction 5).

b. Measurement of Volume (and Water Content).

The technique described for demonstrating active Na^+ and K^+ transport involved no direct measurement of the volume of the vesicles collected in fraction 3 of Figure 1. Instead, an indirect measurement of the volume of the vesicles (called "trapped volume") was made using labeled inulin or labeled D-glucose as a trapped volume probe. These probes were incorporated into the vesicles either during the dialysis step (Stage 1) or after it (Stage 2). Since addition of ATP to the incubation mixture during loading with radioactively labeled glucose produced no change in the amount of labeled glucose collected with the vesicle fraction, it was assumed that ATP did not change the size of the trapped volume of the vesicles. This constancy of trapped volume is one of the basic assumptions upon which the interpretation of the data rests. The second basic assumption is that there does not occur a differential leakage of ions out of the vesicles during passage through the sephadex column.

c. Major Findings of the Studies.

The major findings of these papers were that addition of ATP to the buffer at the stage of isotope loading, produced a large gain in the total amount of ^{22}Na recovered in the vesicle fraction collected from the column (fraction 3). This gain of vesicle Na^+ was interpreted as due to the activity of the Na,K-ATPase, which, making use of energy from hydrolysis of ATP, pumped Na^+ into the vesicles, either alone or in exchange for K^+. That Na^+ was not pumped out of the vesicles as one would expect if the vesicles resemble living cells, was interpreted as being due either to the fact that the vesicle membranes happened to be turned inside out, or to the fact that only about half the pumps were oriented in such a way as to be accessible to the externally-applied ATP (102). Membrane vesicles with the normal orientation have not been reported.

d. The Fundamental Discrepancies.

i. Inequality of "trapped volume" measured by different probes.

As mentioned above, the two basic assumptions made in interpreting the data were 1) the constancy of the trapped volume and 2) the negligible loss of labeled ions during the passage of the vesicles through the column. It follows that in the absence of ATP, the trapped volume for Na^+ and for glucose, as a percentage of the total radioactivity in the incubation medium, should be constant and equal to the trapped volume determined by inulin.

In Table I we show all the available trapped volume data from the original papers of Goldin and Tong (95) and of Hilden, Rhee and Hokin (96), determined with inulin, with glucose and with labeled Na^+. Two facts stand out. First, the values, in the absence of ATP, are widely

different, contrary to expectations from the authors' assumptions. Second, without exception, the trapped volumes for Na^+ are much smaller than the trapped volumes determined with inulin or glucose. This inconstancy of the trapped volume suggests that much of the labeled Na^+ has been lost to the sephadex column and that the presence of ATP only reduced this rate of loss.

ii. Estimated leakage of Na^+ into the sephadex column.

The marked discrepancy in trapped volumes determined by different probes, in the absence of ATP, alone provides compelling evidence for differential leakage or exchange of ^{22}Na. Indeed, from the data given in Goldin and Tong's original paper for the time course of ^{22}Na accumulation in absence of ATP (figures 5, 7 and 8 of reference 95), the half-time of exchange of labelled Na^+ was 5-10 minutes. The normal elution time of the vesicles was given by Hilden and associates as 5-7 minutes; the elution time of Goldin and Tong's experiments must have been longer, almost 35 minutes. The times for passage through the column, plus the rapid rates of labeled ion exchange, again suggest that a large amount of the trapped ^{22}Na can be expected to be lost during the passage through the column.

There is, however, apparently conflicting data regarding the exchange diffusion of ^{22}Na, and in Goldin's later paper (102), the half-time of exchange was 2-3 hours (figure 7). The reason for this conflict was not discussed. We will provide the explanation in a moment.

In addition, it should be pointed out that Hilden, Rhee and Hokin did present evidence which convinced them that loss of radioactive Na^+ during elution of the vesicles was insignificant. They loaded vesicles with Na^+ in the presence of ATP, and separated the Na^+-loaded vesicles from the ^{22}Na in the external solution by passage through the column.

They then incubated the vesicles (25°C), in the now-nonradioactive buffer collected with the vesicles, for various lengths of time and passed each of these samples through the column once more. By comparing the counts collected with the vesicles the second time with the counts collected after the first elution, the loss of radioactivity during the incubation and the second passage through the column was calculated. From these data it was concluded that "under the standard conditions (5-7 minutes for elution of vesicles) a maximum of 15-30% of the ^{22}Na may have been lost from the vesicles". Furthermore, vesicles reincubated in the absence of ATP seemed to lose even less ^{22}Na.

How can one reconcile these data with the marked discrepancy in trapped volumes determined by different probes? The underlying assumption of Hilden et al's estimates was that the efflux of ^{22}Na follows first-order kinetics with a single rate constant. This must not be the case, and there must be a fast fraction of ^{22}Na exchange or efflux. If, as we surmise, ATP is slowing down the overall ^{22}Na exchange-efflux into the Sephadex column, then it might unmask the fast fraction. And, indeed, plots of the time courses of ^{22}Na "entry" into the vesicles (especially figure 5 of 96 and figure 9 of 102), suggest that somewhere around 20% of the labeled Na^+ in the vesicles exchanged with a half-time of approximately 30 minutes; the bulk of labeled Na^+ (80%) exchanged at a much faster rate with a half-time of only about 5 minutes. By taking this factor into account one can see that there must have been a much greater loss of ^{22}Na from the vesicles during the first elution. In Hilden et al's testing for leakage, the first passage through the column had already removed the bulk of the fast exchanging fraction. During the subsequent incubation and second passage through the column much less was lost since they were then

dealing only with the slowly exchanging fraction of ^{22}Na left in the vesicles.

It is now evident that the apparent conflict between Goldin and Tong's original time courses for ^{22}Na exchange in the absence of ATP, which showed half-times of around 5 minutes, and Goldin's later one which showed a half-time of 2-3 hours and which led him to conclude that no leakage into the column occurred, is simply due to the fact that in the original study, samples were taken over 5 to 60 minutes, while in the later study none were taken before 30 minutes. That is to say, in figure 7 of the later study (102), Goldin is showing the <u>slow</u> fraction of Na^+ exchange, while in curve (a) of figure 5 of the original (95) he is showing primarily the <u>fast</u> fraction. Thus, in figure 9 of the later paper, with data points all within 30 minutes, ^{22}Na exchange again shows a rapid component occurring within 5 minutes, and then a very slowly-rising one therafter.

One then raises the question, where does the slowly exchanging fraction come from? We suggest that it represents Na^+ associated with the anionic groups of the phospholipid or protein of the vesicles. The vesicles prepared by both groups contain rather huge amounts of phospholipids. Indeed, the ratio of phospholipid to water in the vesicle is about 1 to 1 (e.g., given as 0.9 ml of buffer per gram of phospholipid by Goldin and Tong). At this concentration, there are about 500 mmoles of phosphate groups per liter of vesicles. That a substantial portion of ^{22}Na may be associated with these anionic sites, in addition to that which may be adsorbed to the enzyme, is to be expected (see Section VI).

<u>iii. Osmotic imbalance</u>.

In each of their first papers, Goldin and Tong (95) as well as

Hilden, Rhee and Hokin (96) concluded that in the presence of ATP, the ATPase pumped Na^+, as chloride, into the vesicles so that the Na^+ concentration reached about 3 times that of the control, without a change in the volume of the vesicles.

The vesicles formed without ATP must be in osmotic equilibrium with the incubation medium; i.e., the osmotic activity inside and outside of the vesicles must be equal. If indeed pumping in the presence of ATP had led to a three-fold increase in Na^+ concentration, while not altering the concentration of the other major cation present, K^+, then the osmotic activity would have risen from about 300 milliosmolar to about 380 milliosmolar. The vesicles should have swollen by some 26%. Yet the addition of ATP did not change the trapped volume as determined by glucose beyond a trivial 0.04% (96).

Again, we think it more reasonable to assume that ATP did not cause a three-fold increase of Na^+ (and chloride) accumulation; it only slowed down the leakage or exchange of the labeled Na^+ into the sephadex column.

iv. Inconsistancy in trapped volume determination by dual-labeling with ^{42}K and ^{22}Na.

In their second paper, Hilden and Hokin (98) did not report glucose, inulin, or other trapped volumes, but appear to have shown an equal trapped volume for Na^+ and K^+, as seen in the "typical" experiment shown in their Fig. 1. This is also indicated in the typical experiment shown in Table VIII, although it is not clear whether or not this is the same experiment as shown in Fig. 1. In their ~~1976~~ paper, however, Hilden and Hokin (99) found that the trapped volumes for Na^+ without ATP were, in percentage of total labeled Na^+, 0.22 without ouabain and 0.14 with ouabain. The corresponding trapped volumes for K^+ were, in percentage of

total labeled K^+ in the same samples, 1.29 without ouabain and 0.21 with ouabain. These data are summarized in our Table II. This marked difference in the trapped volumes for K^+ and Na^+, in the absence of ATP, shows once more that the basic assumption upon which the authors base their interpretation is incorrect. These differences also must be due to different exchange rates of labeled Na^+ and K^+ during their passage through the sephadex column.

Additional data given in the same paper (99) is equally contradictory. The trapped volume for K^+ in the absence of ATP was 1.29% in twice substituted enzyme vesicles; with ouabain and still without ATP, the trapped volume dropped to 0.21%. There was a loss of 80-85% of the isotope content as a result of the application of ouabain to vesicles containing no ATP (this also occurred, although to a lesser extent, in the experiment of Racker and Fisher (100). In contrast, the drop in K^+ content in response to ATP (without ouabain) was only from 1.29% to 0.80% a loss of only 33%.

Comparing these two sets of values one may, following the same logic used by Hilden and Hokin, make the following claim: ouabain (without ATP) can energize K^+ pumping better than ATP (without ouabain) can! Once again, we feel that these discrepancies may all be artifact due to differential leakage of ions into the sephadex columns.

v. Summary.

In summary, these studies do not prove that the Na,K-ATPase pumps Na^+ or K^+. The labeled ion collected in the vesicle fraction from the sephadex column does not represent the labeled ion concentration in the vesicles before their passage through the column. The two assumptions upon which the authors base their interpretation of the data, that the trapped volume is constant and that there is negligible leakage of ions into the sephadex

column, are not valid. Leakage, or exchange of labeled isotope with nonlabeled ion in the buffer of the sephadex column, are the major events underlying their observations, and ATP simply causes less leakage of Na^+, not net gain of Na^+.

VI. AN ALTERNATIVE INTERPRETATION OF THE VESICLE DATA.

Figure 2 reproduces an old diagram illustrating, according to the association-induction hypothesis (12), the consequences of the adsorption of ATP onto a protein. These are, first, an allosteric alteration in the ionic preference of the beta- and gamma-carboxyl groups of the protein, and second, the multilayer polarization of water by the polypeptide backbone. Figure 3 incorporates this diagram into the vesicle model. Indeed, this picture is in essence what Ling has suggested as the molecular mechanism for selective ionic permeability of the cell surface since 1953 (105,106). The relative roles of polarized water, and of membrane lipid, in determining permeability, have been recently reviewed (107).

In this model, the passage of Na^+ could occur via the water layers polarized by the surface proteins and phospholipids, or via association with, followed by dissociation from, the fixed anionic sites at the surface. Without ATP the water molecules are much closer to a state of normal water with relatively high permeability for solutes such as Na^+ and K^+; as a result more of the labeled Na^+ introduced into the vesicles exchanges with nonlabeled Na^+ in the Sephadex column, after the vesicles have separated from their incubation medium but before they have reached the collection tube. Inclusion of ATP in the incubation mixture during the isotope loading stage causes a conformational change of the ATPase protein, allowing at least a part of the polypeptide chains to become extended.

The exposed backbone NH, CO groups then polarize deep layers of water, reducing its solubility for Na^+ (q_{Na}) as well as reducing the diffusion coefficient of Na^+ in this water (D_{Na}). Since permeability is a product of q_{Na} and D_{Na}, there is a reduced rate of exchange of the labeled Na^+ trapped in the vesicle with nonlabeled ions in the column. Hence, vesicles treated with ATP retain more labeled Na^+ during their passage through the Sephadex column. On the other hand, an increased preference of carboxyl groups for K^+, in response to ATP, would enhance the exchange of labeled K^+ by the adsorption-desorption route; hence, the decrease in the amount of labeled K^+ in the vesicle fraction. Direct evidence for the cooperative binding of K^+ to the Na,K-ATPase, as predicted by the association-induction hypothesis, was recently reported by Matsui and associates (108); indeed, the data, as expressed by Scatchard analysis, exactly parallels that seen in whole cells (109).

Although our interpretation of their data differs profoundly from that of the original authors, we feel equally strong in our appreciation of the significance of the experimental work itself. Indeed, they have succeeded in showing the extent to which the most fundamental properties of the protein-polarized water system can be captured in a purified <u>in vitro</u> preparation, and can be controlled by interaction with phospholipids and with ATP. The data do not, however, provide proof of the existence of the Na^+ pump, and the data are more simply, and more completely, explained by the association-induction hypothesis.

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Table I

Na,K-ATPase Vesicle "Trapped Volume" Data

Probe	-ATP	+ATP	Reference
Inulin	5%	-	96, Fig. 1
Glucose	0.66%	0.70%	96, Table IV
	1.8%	-	95
Sodium	0.3-0.4%	1.5-2%	96, Tables I & IV
	1-2%	-	96, Fig. 4
	-	5%	96, Fig. 5
	2.5%	5%	96, Fig. 10
	0.3-0.4%	0.6-0.9%	95, Fig. 5,7,8

Table II

Trapped Volumes (% isotope content) of Doubly-Labelled Na,K-ATPase Vesicles

(From tables II and III of Hilden and Hokin, 99).

Probe	Ouabain	-ATP	+ATP
^{22}Na	No	0.22%	1.35%
^{42}K	No	1.29%	0.86%
^{22}Na	Yes	0.14%	0.15%
^{42}K	Yes	0.21%	0.20%

Legends

Figure 1 (a) Preparation of Reconstituted Na,K-ATPase Vesicles. Phospholipid (e.g., Lecithin) is solubilized in cholate, and vesicles are formed during prolonged dialysis.

(b) Separation of Vesicles Through Sephadex Column. See text for details.

Figure 2 Schematic representation of a portion of a protein molecule undergoing cooperative transformation in response to the cardinal adsorbent, ATP. Adsorbed water molecules are perceived as existing in a state of polarized multilayers, but are schematically depicted as one layer. Modified from Figure 25, Reference 12.

Figure 3 Incorporation of the concept of Figure 2 into the vesicle model.

FIGURE 1 a

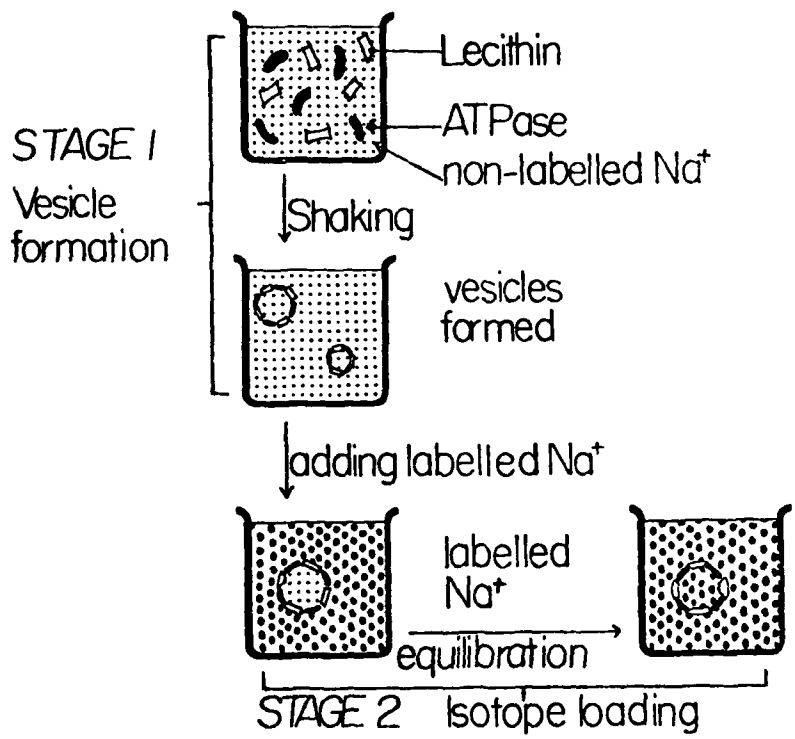
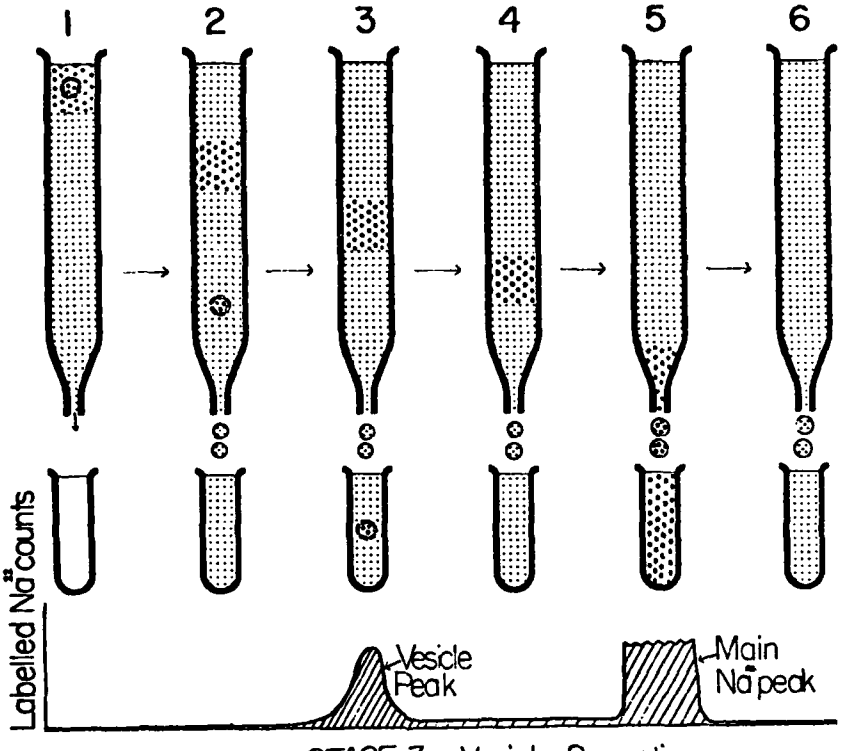

Lecithin
ATPase
non-labelled Na+
STAGE I
Vesicle formation
Shaking
vesicles formed
adding labelled Na+
labelled Na+
equilibration
STAGE 2 Isotope loading

FIGURE 1 b



STAGE 3 - Vesicle Separation

FIGURE 2

ADSORBED H_2O
POLYPEPTIDE CHAIN
ANIONIC SIDE CHAIN
ATP
B
FREE H_2O
H_2O
A

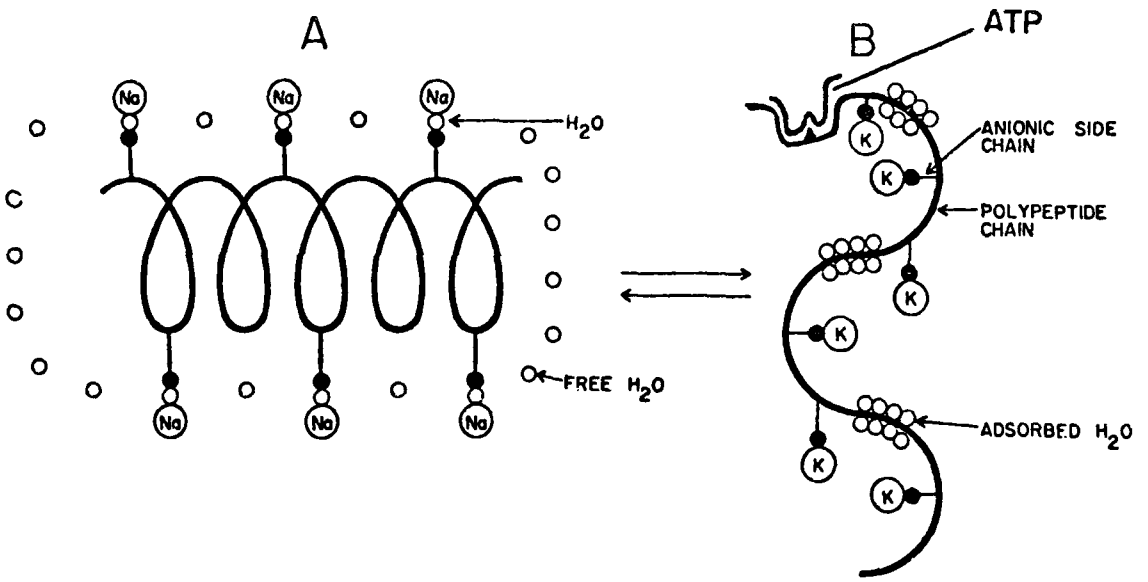
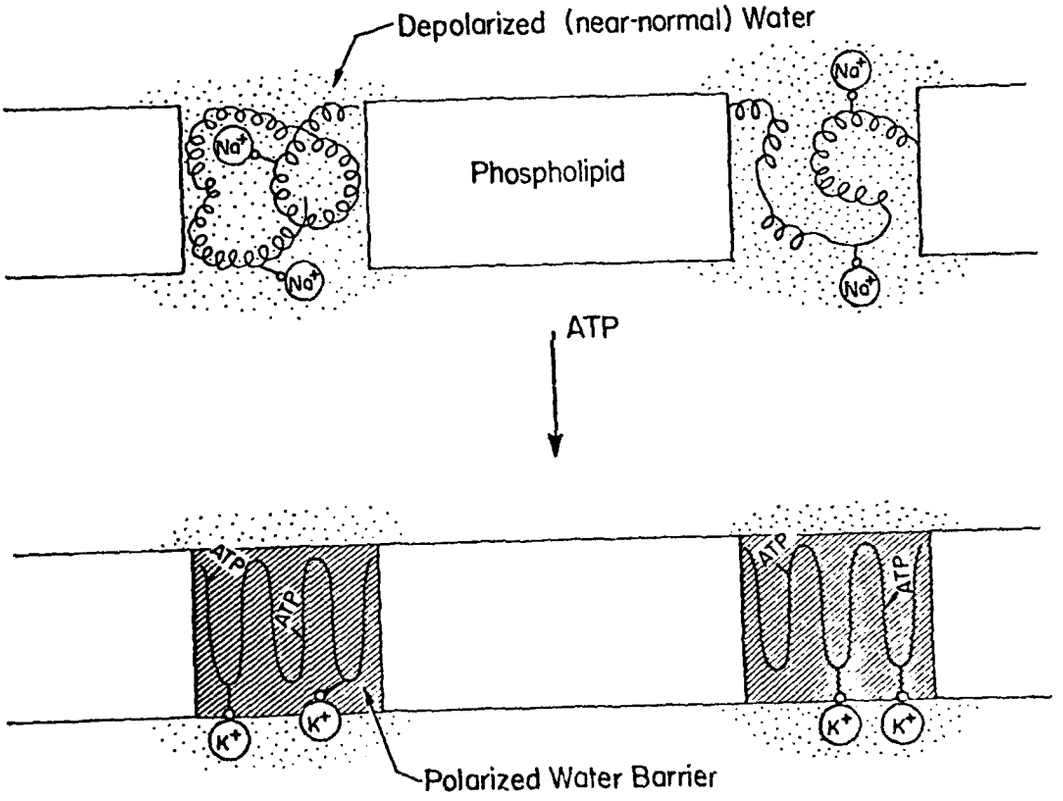
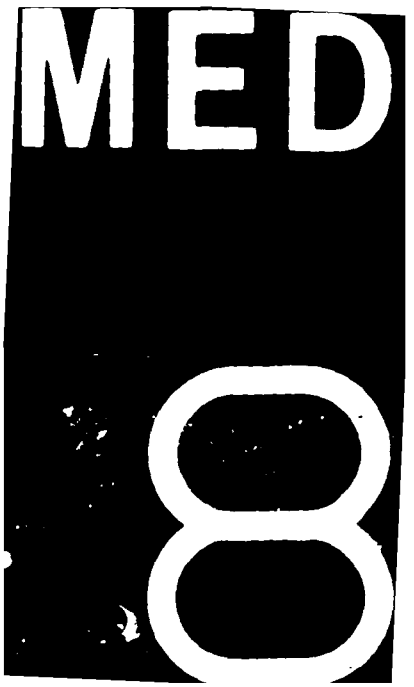

A
B
ATP
Na
H_2O
FREE H_2O
K
ANIONIC SIDE CHAIN
POLYPEPTIDE CHAIN
ADSORBED H_2O

FIGURE 2

FIGURE 3


Depolarized (near-normal) Water
Phospholipid
Na+
Na+
Na+
Na+
ATP
ATP
ATP
ATP
ATP
K+
K+
K+
K+
Polarized Water Barrier


MED
8