Cation Transport and Structure of the Red-Cell Plasma Membrane

By Joseph F. Hoffman, Ph.D.

This paper recounts recent work concerning the Na and K permeability of red blood cells. The discussion deals principally with results derived from isolated red-cell membranes, or ghosts. Using defined isolation techniques, a system of ghosts is described in which Na is actively pumped. Like intact cells, the pump requires extracellular K and energy derived from the metabolism. The source of energy for the pump has been assessed by incorporating normally impermeable substrates into the ghost interior at the time of hemolysis. The specific and direct substrate of the pump has been identified as ATP. Further studies are described that correlate the activity of the Na pump with an ATPase. This ATPase, like the pump, requires both Na and K for activity and is inhibited by cardiac glycosides. The evidence points toward the involvement of this ATPase as an intermediate component of the pump reaction.

This paper is concerned with two aspects of the red cell. The first is the molecular organization of the membrane; the second, its permeability to cations. In addition, an attempt will be made to evaluate the possible relationship between structure and function, at least for certain features of the membrane. Much of the information to be presented derives principally from studies on red-cell ghosts. For the purpose of this paper, the term ghost will be used to denote an isolated plasma membrane. Ghosts can be derived from red cells by hemolysis with hypotonic solutions. It should be recognized that the ghost at hemolysis does not break, but, after hemolysis, the ghost recovers its impermeability to hemoglobin and can, with proper treatment, regain its normal permeability to cations. It will be anticipated that the properties possessed by any particular type of ghost will be dependent upon its manner of isolation. In representing an isolated system, ghosts offer the advantage of considerable simplification in the study of many problems, as well as greater latitude in experimental manipulation.

Morphological Features of the Plasma Membrane

Although it is not my purpose to review the various evidences concerning the intimate structure of the plasma membrane, I would like to consider briefly its molecular morphology. Our present understanding of the architecture of the red-cell membrane has been obtained primarily from studies on ghosts, by microscopic observation and by chemical analysis.

The ghost has been found by electron microscopy to consist of two components: plaques and fibers. The plaques are short cylinders, about 40 Å in thickness and on the average 250 Å in diameter, and appear to be packed in an hexagonal array over the entire surface. The fibers lie underneath the plaques and measure about 20 Å in diameter and vary in length from 100 to 400 Å. The total thickness of the dried membrane is estimated by several methods to be 60 to 70 Å. The surface ultrastructure of the ghost is pictured as being composed of plaques and fibers which are held together by the ether-extractable lipid. If the lipid is radially oriented and arranged as a bimolecular leaflet, that is, plaque—lipid—lipid—fiber, then the results are consistent with the paucimolecular theory of Danielli, Davson, and Harvey. On the other hand,
since ether is known to remove only a portion of the lipid, the remaining lipid could be contained in the plaques but oriented tangentially, consistent with the pore hypothesis of the type proposed by Parpart and Ballentine. On this basis, these two, rather different theories might be reconciled.

It is known that the membrane is composed primarily of lipid and protein. The original work of Parpart and Dziemian showed that in mammalian red cells all of the lipid was contained in the ghost. They also found that while the total lipid content of the ghosts of a number of species was not particularly different, the ratio of the amount of lipid to protein varied significantly. In recent years this work has been considerably extended particularly with respect to the chemistry of the lipids as provided by the development of chromatographic techniques. Thus, Dawson et al. and de Gier and Van Deenen (as well as others) have analyzed the ghosts of different species of red cells and have found differences not only in the various classes of lipids represented but also differences in the fatty-acid content within the various types of lipid present in the different ghosts.

**Lipid Composition and Permeability**

With this as background, let us now consider certain relationships between lipid composition and cation permeability. We have recently had occasion to measure the cation permeability of two altered types of red cells, each possessing patterns of lipid composition different from normal. The first type studied were red cells from essential fatty-acid deficient rats. Watson found that these rats were different from normal controls in that they were almost completely devoid of linoleic acid, linolenic acid, and arachidonic acid but contained instead mainly eicosatrienoic acid. This is to say that the total number of double bonds is decreased but the average chain length of the fatty acids present is slightly increased. The altered fatty acids are found primarily in the phospholipids and comprise about 40 per cent of the fatty acids present. The total lipid content of the deficient cell is the same as the normal control. In addition, the deficient cell appears normal in shape and size. Red cells were also studied from two patients with acanthocytosis. These red cells characteristically have a thorny or spiny appearance (hence, the name acanthocyte). Acanthocytes were found by Ways, Reed, and Hanahan to contain normal amounts of total phospholipid, but the lecithin fraction was decreased to about half the normal value, being compensated by an increase in the sphingomyelin fraction. In addition, the acanthocytes, like the rat cells, were deficient in linoleic acid. Thus, these two types of cells present rather specific but not identical alterations in their lipid constituents. However, the permeability to Na and K of each type of abnormal cell was found to be the same as its normal control. This is based on measurements of influx and outflux of both Na and K under a number of different conditions. These results indicate that these lipids (at least the altered ones) are either not involved in the various pathways of normal cation transport or, if they are, that these types of alteration are not important for the transport processes.

**General Considerations of Cation Composition of Red Cells**

The concentrations of Na and K in the red cells of various species of mammals can be divided into three distinct classes. The red cells of most mammals (such as man, rabbit, rat) have intracellular concentrations high in K and low in Na. But the carnivores (represented by the dog or cat) are just the reverse: their red cells are high in Na and low in K. The third class, as you might expect, is variable. This occurs in the order Artiodactyla (represented by the pig, ox, or sheep) and contains species whose red cells are either high in K or high in Na. Of particular interest in this connection is the sheep. Two types of sheep have been found; the red cells of one type, like man, are high K while the other type, like the dog, is low K. These two types of sheep have been shown by Evans to represent a single gene difference, the low K type being inherited as a Mendelian
dominant. We will have reason to discuss sheep again but for the moment consider the relation between cation content and lipid composition. Although the lipid data are by no means extensive enough to evaluate this relationship in depth, there does not appear to be any correlation between Na or K content and lipid composition. This is so whether the comparison is made on the basis of the various types of lipid present or with the fatty-acid composition. (The two types of sheep, by the way, have been found by Vaughan and Oliveira18 to possess the same lipid composition.) This lack of correlation is also true when comparison is made between the lipid composition and the Na and K permeability of the red cells of various species. It might, perhaps, be anticipated that in the absence of a correlation between lipid composition and Na and K content, that there would also be no correlation with cation permeability. But this, of course, depends upon the relationship between the cation content of a cell and its permeability.

Before we take up the relation between permeability and cation content, it will be helpful to define a few terms. This can be done with the cell as diagrammed in figure 1. The cell contains Na and K as well as anions. Now there are three conceptual pathways19 which can be used to characterize the movements of cations across membranes. The first is by passive diffusion represented by the term leak. Passive diffusion is downhill in the sense that the ions move in the direction of their electrochemical gradients. Exchange diffusion is a special type of diffusion and can be observed only with radioactive tracers. Active transport is represented by the term pump. Active transport is defined as the movement of an ion uphill, that is, against its electrochemical potential gradient.19 Movement of ions by the mechanism of active transport requires the expenditure of energy. Thus, the pump involves specifically the exchange of K outside for Na inside; exchange diffusion involves the exchange of Na for Na or K for K; the leak can be thought of as the combined movement of a cation with an anion. Net movements of ions can only occur by the leak or by the pump pathways.

Let us examine how the permeability characteristics of a membrane control the cellular concentrations of Na and K (or how the cation content of a cell reflects the operation of certain membrane parameters). For this we need to consider only the relationship between the pump and the leak, for these are the only pathways by which changes in Na and K content can occur. The intracellular cation composition can be controlled by considering that the pump operates to compensate for the changes brought about by the diffusion leaks. Tosteson and I20 have developed a quantitative formulation of these relationships that also includes the idea of a coupled 1 to 1 Na-K pump. That is, that the pump flux of Na is tightly linked to the pump flux of K, an idea for which there is a considerable amount of evidence.21 (An example of this is discussed later.) From the equations developed it is possible either to predict (a) from known concentrations of Na and K on the two sides of the membrane, the membrane permeabilities needed to maintain constant the internal concentrations of cations or, alternatively, (b) from known membrane permeabilities, the equilibrium intracellular Na and K concentration (for a given extracellular condition). The pertinent membrane parameters turn out to be, for any given cell, the ratio of the two
leaks, Na to K; the ratio of the permeabilities, pump to leak; and the coupling ratio of the pump. We were able to test these relationships by measurements of the various membrane parameters on the two types of sheep mentioned earlier, that is, the high-K and low-K types. Although the predicted membrane parameters can be expected to be quite dissimilar for the two cell types, the results obtained for each type proved to be in satisfactory agreement with prediction. By way of explanation, consider the following example: Assume that a cell, sitting in plasma, has a certain leak permeability to Na and another, but higher leak permeability to K. Assume also that they cannot change, that their ratio is fixed. Now, add to this system the operation of a 1 to 1 coupled Na-K pump. It should be clear that the faster the pump turns over, the higher will be the intracellular concentration of K. This is the situation in the sheep, although somewhat simplified, since the various ratios of parameters are different for the two types of sheep. Since red cells are in osmotic equilibrium with their environment, and since the volume of a red cell is directly dependent on its cation content, control of the intracellular cation composition, as set out in this formulation, also offers a mechanism for the regulation of cell volume. At the same time, this presents a physiological role for the pump. Obviously, any alteration in the membrane parameters, such as inhibition of the pump or the application of hemolytic agents, will lead ultimately to the cell’s demise.

\textbf{Mg}^{++}, \textit{An Internal Membrane Cohesive}

I would like now to discuss evidence indicating that the red-cell membrane contains an internal cohesive (or cement) which acts to control monovalent cation permeability. Let us first consider an assay system for K$^+$ retention by ghosts. Hemolysis: 1 vol. cells + 10 vol. H$_2$O (+ test substance) Reversal: Addition of KCl + NaCl (K/Na = 5/1) to make medium 0.17 M Incubation: 40 minutes at 37°C. Washing: 0.17 M NaCl (pH = 7.4) Analysis: (K$^+$) content of ghosts

In essence what is being done here is to first make ghosts, fill them with K, and then to see how well they hold on to this K, comparing different hemolysis conditions only. In this instance, cells are hemolyzed in 10 volumes of water containing various substances. The term reversal refers to the addition of sufficient salt to make the 10 volumes of water isotonic (relative to plasma). During reversal, salt enters the ghost. Since the salt is mainly K, the ghosts are now high K. The ghosts are stabilized by incubation at 37°C and then washed with a K-free medium. After three washes, the K content of the ghosts is determined. A high K content can be taken as an indication that the ghosts have become relatively impermeable to K (that is, not very leaky). In contrast, a low K content indicates that the K was lost by washing and that the ghosts are quite permeable to K. Thus, the K content of ghosts is inversely related to its permeability. Figure 2 shows the effects of

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{K retention of ghosts after hemolysis in Mg or Ca solutions. The final K concentration of the ghosts, (K)$_0$, is expressed in mM K/liter of ghosts.}
\end{figure}
adding small amounts of Mg++ or Ca++ at the time of hemolysis. It is apparent that Mg++ is without effect on the K content of ghosts but that Ca++ has marked and deleterious effect in that it produces a considerable increase in permeability of the ghost to K.

Figure 3 shows the effect of adding adenosinetriphosphate (ATP) to the initial hemolytic solution, along with Mg++ or Ca++.

![Figure 3](image)

**Figure 3**

*K* retention of ghosts after hemolysis in 2 mM Na₂ATP+ divalent cation.

Although the effect of ATP is presented here, similar results have been obtained with ethylenediamine tetraacetic acid (EDTAA), the point being that it is the chelation property that is of interest. It is seen that ATP in the absence of divalent cations makes the ghosts permeable to K and that the addition of either Mg++ or Ca++ can prevent or at least protect against this action. It is evident that as the binding capacity of the ATP becomes saturated or neutralized, the ghosts are high K. As the concentration of the divalent cation is increased beyond this point, the effects are similar to those presented in figure 2. That is, ghosts in the presence of Mg++ remain high K, but Ca++ makes the ghosts leaky to K. In fact, the Ca++ curve is simply shifted to the right, depending upon the amount of ATP (or EDTAA) that was present at hemolysis.

Another point can be made. The agents which prevent K+ retention act only during hemolysis, since addition after hemolysis does not result in any membrane damage. In addition, the injury once produced cannot be repaired after hemolysis by adding the alternate member of the complex. These results indicate that a divalent cation, presumably Mg++, stabilizes the internal molecular arrangement of the membrane. This divalent cation appears to be necessary for normal monovalent cation permeability. Only while hemolysis is occurring can the cohesive be removed by chelation or replaced by Ca++. Either way the ghost is made permeable to cations.

**Na Permeability of Ghosts**

Now, in the space that remains, I want to consider three aspects of one subject, and this is active Na transport. Since we will be dealing with ghost systems, we will first compare the transport properties of ghosts with that of intact cells, paying particular attention to the pump. We will then consider the energy source for the pump and finally its correlation with an enzyme.

It is perhaps instructive to mention the rationale behind these studies on ghost systems. It is because intact cells are known to be impermeable to all of the phosphorylated metabolic intermediates that ghosts are used. As Straub showed, these types of compounds can be easily incorporated into ghosts at the time of hemolysis. In addition, metabolic manipulation of intact cells is rather limited, since there are always possible interrelated reactions occurring under any one set of conditions, but in ghosts study of single reactions is possible by elimination of interference, either by dilution or removal of substrate, while at the same time creating a cytoplasm of one's own choice. And, lastly, we are studying the properties of the cell membrane in isolation.
For this work ghosts were prepared as outlined in figure 4. As before, cells are hemolyzed with 10 volumes of water, containing now a trace amount of Na$^{24}$. The resultant ghosts, thus labeled with Na$^{24}$, are subsequently washed with MgCl$_2$ to remove as much of the excess Na$^{24}$ as possible. Following this, the ghosts are suspended in 30 volumes of an isotonic medium, and the loss of Na$^{24}$ is measured over the course of several hours. (It should be mentioned that these ghosts contain approximately only 9 per cent of their original hemoglobin and enzymic capacity.)

Figure 5 shows the rate of loss of Na$^{24}$ from ghosts suspended in different media. Inosine serves here as an energy source. It enters the Embden-Meyerhof pathway via the hexosemonophosphate shunt and as a result stimulates glycolysis.$^{26, 27}$ Since the membrane is permeable to inosine, it is added only to the incubation medium. Strophanthidin, a cardiac aglycone, has been shown by Schatzmann$^{28}$ to have the specific action of inhibiting active transport without affecting metabolism. With this in mind, strophanthidin is used here simply as a tool to assess the presence, as well as the activity, of the pump. There are two types of media: a Na-containing medium and a Na-free medium in which the Na has been replaced with Mg$^{2+}$. In both types of media, 5 mM K is present. The difference between curves A and C is due to the action of strophanthidin and, thus, represents the activity of the pump, the active transport of Na. The difference between curves A and B is due to the presence of Na and, thus, represents Na exchange diffusion. This applies also to curves C and D. Curve D represents the leak alone, since in the presence of strophanthidin the pump is inhibited and there is no Na exchange diffusion in a Na-free medium. Thus, ghosts behave as intact cells, in that all three types of transport can be demonstrated.

Figure 6 shows the activation of the pump by extracellular K in reconstituted ghosts. The outward rate constant, $\omega_{K_{Na}}$, expresses the fraction of the intracellular Na that is lost or exchanged per hour and, in this sense,
is a measure of the Na outflux. The four curves represent the same four conditions present in figure 5. It is seen that the outflux of Na increases as the K in the medium increases, ultimately reaching saturation. This is so in the presence or absence of Na exchange diffusion. This K activation of the pump can be inhibited by the addition of strophanthidin. This type of activation of the Na outflux by extracellular K was first observed in muscle by Steinbach and in red cells by Harris and Maizels. It is the basis of the idea of a coupled pump—that is, that for the pump to operate, there is an obligatory exchange of K for Na, that the pump outflux of Na is dependent upon extracellular K.

Thus, there is an equivalence between strophanthidin and the removal of K, in that either one results in the inhibition of the pump. Both approaches have been used in the ghost work below in assessing the activity of the pump. It is of interest to also mention a word concerning the mode of action of strophanthidin. Glynn has shown with red cells that strophanthidin and K compete for the pump site. This competition can also be demonstrated on ghosts. While still other features will be illustrated, the characteristics of the reconstituted ghost system, as seen in figures 5 and 6, clearly demonstrate the similarity between ghosts and intact cells with regard to the activity of the pump.

**ATP, The Substrate of the Pump**

Table 1 shows the effects of different substrates on the activation of the pump in two types of ghost systems, incubated in a Mg-K medium. Fresh refers to ghosts prepared from fresh blood, that is, immediately after withdrawal from a donor. The results presented in figures 5 and 6 were obtained using fresh ghosts. Depleted refers to ghosts prepared from washed red cells that had been depleted of their endogenous metabolites by incubation at 37°C for 18 to 24 hours in the absence of substrate. It has been found that cells so treated have lost essentially all of their ATP, adenosinediphosphate (ADP), hexosediphosphate (HDP), and diphosphoglyceric acid (DPG), and so forth. Now, the point of table 1 rests on the differential effect obtained on depleted ghosts with inosine and adenosine. In fresh ghosts, the activation of the pump by substrate is shown by the increase in the per cent Na released upon the addition of inosine or adenosine to the control ghosts. Thus, either nucleoside will serve as substrate, with inosine and adenosine being equally effective. In depleted ghosts this is simply not so. It is only with adenosine that the pump can be activated and not with inosine.

This differential action is significant because it provides the clue to the identity of the specific energy source or substrate for the pump. This can be argued as follows. Consider only the depleted-ghost system. While there is no lactate formed by the depleted control ghosts, the addition of inosine or adenosine results in a considerable production of lactic acid, the rate of lactate production being the same from either inosine or adenosine. Not only is glycolysis stimulated, thus indicating a turnover of high-energy phosphate, but it can also be shown that both inosine and adenosine, equally well, lead to the partial replenishment of all of the phosphorylated glycolytic intermediates. Preliminary experiments have shown that inosine-triphosphate (ITP) is synthesized from inosine and ATP from adenosine. Since the difference brought about by these two substrates cannot be related to the intermediates, the difference between these two conditions must, then, be due to the different nucleotides formed. This appears to eliminate the participation of compounds other than ATP or ADP.

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**Table 1**

*The Influence of Different Substrates on the Outflux of Na from Fresh and Depleted Ghosts*

<table>
<thead>
<tr>
<th>Compound in final medium</th>
<th>% Na released in 90 minutes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Fresh</td>
</tr>
<tr>
<td>Control</td>
<td>22.0</td>
</tr>
<tr>
<td>Strophanthidin</td>
<td>20.5</td>
</tr>
<tr>
<td>Inosine</td>
<td>71.7</td>
</tr>
<tr>
<td>Adenosine</td>
<td>73.8</td>
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</table>
in the pump reaction. This can be tested in ghost systems, and this is what follows.

Figure 7 (A and B) illustrates the effect of incorporated ATP and ADP on the pump outflux of Na in reconstituted depleted ghosts. These compounds are incorporated at hemolysis as the neutral Mg complex. Consider the ATP ghosts. It is apparent in figure 7A that the Na outflux from ghosts containing ATP can be inhibited by the addition of strophanthidin: thus, ATP by itself stimulates the pump. This activation is not particularly affected by the addition of inosine or adenosine. It should be mentioned that ATP added to the medium has no effect on the outflux of Na for either the case shown in this figure or to control ghosts. It will be noted in figure 7B that ADP will also activate the pump. Without going into the details of the evidence, it is very probable that this result with ADP is actually due to the formation of ATP by the enzyme, adenylic kinase, known to be present in this system. Thus, for our purposes, ATP in both instances is responsible for the activity of the pump.

The specificity of the incorporated nucleotide with regard to the activation of the pump is shown in table 2. Here, comparison is made of the relative effectiveness of four different nucleotides: ATP, ITP, guanosinetriphosphate (GTP), and uridinetriphosphate (UTP). Again, the strophanthidin sensitivity indicates the activity of the pump. It is apparent that of these compounds, only ATP will run the pump.

Figure 8 shows the effect on Na outflux of incorporated phosphoenolpyruvate (PEP). In figure 8C it can be seen that the pump is hardly affected, that it is not activated by PEP alone. However, if a trace amount of ADP is incorporated along with PEP, then as shown in figure 8D, the pump is stimulated. It can be shown that the incorporated trace amount of ADP, by itself, is insufficient to run the pump. Rather, the function of ADP is that it acts as the phosphate acceptor in the reaction brought about by the enzyme, pyruvate kinase. Thus, in the conversion of PEP to pyruvate acid, ADP is phosphorylated to become ATP. It is the ATP that drives the

<table>
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<tr>
<th>Incorporated substrate</th>
<th>% Na released in 80 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>50.6 + Stroph. 22.8</td>
</tr>
<tr>
<td>ITP</td>
<td>27.6 + Stroph. 22.2</td>
</tr>
<tr>
<td>GTP</td>
<td>25.9 + Stroph. 25.5</td>
</tr>
<tr>
<td>UTP</td>
<td>33.4 + Stroph. 33.2</td>
</tr>
<tr>
<td>Control</td>
<td>29.1 + Stroph. 24.2</td>
</tr>
</tbody>
</table>

Figure 7

The effect of incorporated ATP (A) and ADP (B) on the Na outflux from depleted ghosts. ATP and ADP were incorporated into the ghosts at hemolysis. The term, alone, means that no other substrate is present.

Figure 8

The effect of incorporated phosphoenol pyruvate (PEP) and other substrates on the outflux of Na from depleted ghosts. See text for discussion.
pump, and, if we can anticipate a little, it is the pump, by reacting with ATP, that re-
generates the ADP, thus allowing it to re-
cycle. The increased stimulation brought about by inosine or adenosine raises these
previously depleted ghosts to the fresh ghost
level. The conclusion to be drawn from these,
as well as other experiments (involving for
the most part inhibitors), is that any reaction
that will generate ATP will drive the pump.
But more important, ATP is shown to be the
direct and immediate energy source of the
pump; the link between metabolism and the
transport process is ATP.

While the results presented in figure 9
emphasize these conclusions, they also show
a somewhat different approach, and this is
competition between the pump and the hexo-
kine reaction for ATP. Although this ex-
periment is here demonstrated on ghosts, it
was first carried out by Dunham on intact
cells. One of the properties of the previously
described ghost system is that glucose is not
able to be utilized. The reason for this, as
found by Lionetti and his colleagues, is that
the enzyme, hexokinase, is denatured by hemol-
ysis. This, however, can be overcome by in-
corporating (in this instance, yeast) hexo-
kine along with the ATP at hemolysis. The
result of this type of an experiment is pre-
sented in figure 9. It is evident, that, as
before, the pump is activated by ATP alone.
The addition of glucose to this system results
in the complete inhibition of the pump. This
same inhibition of the pump can be brought
about by glucose in the presence of iodoacetic
acid (IAA). These results can be explained
on the following basis: For the conversion
of glucose to glucose-6-PO₄ by hexokinase, ATP
is required and is degraded to ADP. Since
glucose is present in considerable quantity,
this reaction is constantly taking place. Now
the pump also uses ATP but in this instance
is inactive. Apparently, the hexokinase reac-
tion preferentially utilizes all of the ATP.
All the ATP formed from ADP by the adenyl-
kinase would similarly be swallowed up
by the phosphorylation of glucose. So much
for the pump and its substrate, ATP.

The Correlation of the Pump with ATPase
If the pump uses ATP, then there must be
an ATPase. If this is so, then it might be
expected that such an ATPase would display
many of the properties known to be charac-
teristic of the pump. Such an enzyme which
requires Na and K for activation has been
found—it was discovered by Skou in 1957 in
crab nerve. It was found in the red cell
by Post et al., as well as by Dunham and
Glynn, and was shown to be attached to the
membrane. I would like, now, to describe
some of the properties of this ATPase and
then to consider the correlation between this
ATPase and the pump.

While the red-cell ATPase can be prepared
in a variety of ways, all yielding, at least
qualitatively, the same results, there are two
features common to all preparations that I
want to mention. First, the enzyme has not
as yet been solubilized; it is always studied
in association with the ghost membrane.
Second, the ATPase is studied on ghosts com-
pletely permeable to ATP, Mg, Na, and K.
This distinction is important since we will be
comparing the properties of the enzyme, on
the one hand, with the characteristics of the
pump, on the other. It should be understood
that these studies are carried out on two sep-
rate types of ghost systems.
Table 3 shows the basic properties of the enzyme. The first line shows the total enzymatic activity of the ghosts, obtained by incubation with MgATP + Na + K. If we add to this strophanthidin, then the activity is partially reduced as seen in the second line. The difference between these is given on the third line. For convenience only, we will call the residual activity Apyrase, the difference component, the ATPase. It is the ATPase that concerns us. The ATPase displays two features which make it of rather unusual interest. The first, as noted earlier, is that it requires for activation both Na and K. The second is that the activation due to Na and K can be inhibited by strophanthidin. It can also be shown that the activation of this ATPase by K in the presence of Na corresponds reasonably well to the activation of the pump outflux of Na with extracellular K. Conversely, the Na activation of the enzyme in the presence of K yields a similar correspondence. In addition to these relationships, the competition between K and cardiac glycosides, mentioned before in connection with the pump, can also, as Dunham and Glynn found, be demonstrated on the ATPase. One further point, and this is with respect to the spatial localization of the enzyme. Ryan and I were able to show that the Apyrase is located on the outside of the membrane, the ATPase on the inside surface.

We were also interested in the substrate specificity of the ATPase. This is presented in table 4. It is apparent, as with the pump, that the ATPase is specific for ATP, that ITP, UTP, and GTP are inactive. In addition, it is of interest that ADP is also inactive. In other experiments, it was found, in this regard, that the addition of ADP inhibits the rate of breakdown of ATP.

In figures 10 and 11, the effect of Ca is examined first on the enzyme, then on the pump. Figure 10 shows how the enzymatic activity of ghosts is affected by divalent cations. The incubation medium contains Na, K, and ATP. The results, as shown in the curve marked No Mg ++ ± strophanthidin, indicate that there is no appreciable breakdown of ATP in the absence of Mg ++. However, when Mg ++ is added, there is a marked decrease in the activity. This effect is shown by the curve marked Mg ++ ± strophanthidin. The addition of Ca ++ is shown by the curve marked Mg ++ ± strophanthidin ± Ca ++. The results indicate that Ca ++ inhibits the activity of the ATPase. The results also show that the Ca ++ effect is dependent on the presence of strophanthidin. The Ca ++ concentration in the incubation mixture is given on the abscissa. See text for discussion.
sence of Mg++; that is, the enzymatic activity is not affected by Ca++. However, if Mg++ is added, then, as shown by the two uppermost curves, the addition of Ca results in a marked stimulation of the enzymatic activity. The top curve represents the total activity, that is, the composite of ATPase + Apyrase. The second curve represents the activity of the Apyrase alone. This Apyrase curve shows that, although the Apyrase is Mg++ requiring, it is Ca++ activated. Now, the difference between the top two curves represents the ATPase activity, and this is plotted in the lower portion of figure 10, with an expanded ordinate. The point to be made is that the ATPase (in contrast to the Apyrase) is inhibited at all concentrations by Ca++.

Figure 11 shows the effect of Ca++ on the pump flux of Na in reconstituted ghosts. These ghosts were prepared by incorporating, at hemolysis, ATP complexed with different amounts of Ca++. For reasons given before, the binding capacity of the ATP has to be saturated; therefore, Ca++ replaced Mg++, as shown on the abscissa of figure 11. As indicated by the upper curve, the total Na outward flux diminishes as the Ca++ concentration is increased. The middle curve represents the diffusion leakage of Na and shows, as anticipated by the previous discussion, that this is increased by Ca++. The difference between these curves reflects the activity of the pump, and this is presented in the lower portion of the figure. The important point here is that the pump is inhibited by intracellular Ca++, in a manner similar to that seen for the ATPase. It should be mentioned that neither the pump nor the leak is in any way affected by the addition of Ca++ to the medium, up to twice the normal plasma levels. Thus, the action of Ca++ on the membrane is asymmetrical. Since the pump is inhibited by Ca++, it would be to the physiological advantage of the cell to exclude Ca++. Perhaps the fact that Ca++ is virtually absent from the intact red cell is, in this sense, significant.

The main characteristics common to both the pump and the ATPase may be summarized as follows:

- Activation by Na +K
- Substrate specificity
- pH sensitivity
- Inhibition by: Ca++, F−, Cu++, strophantidin
- Unaltered by: IAA, NaN3, AsO4, DNP, CN−, 1−

The similarity in the properties of the pump compared to the ATPase is striking and strongly points toward the conclusion that the ATPase is an intimate component of the pump. Unfortunately, limitations in space prevent presentation of all of the experimental data upon which this correlation is based. However, it is important to mention that while there are differences between intact cells and reconstituted ghosts in the pump activity, as affected by pH and certain of the metabolic inhibitors, it can be shown that these differences arise as a result of effects on ATP synthesis rather than as differences in the pump itself. This is to say that there has been no

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**Figure 11**

The inhibition of the Na outflux from ghosts by incorporated Ca. \(^{40}\ ^{6}KNa\) is the outward rate constant of Na expressed in units of reciprocal hours. See text for discussion.
instance where the effect of any agent has been found to have an action different on the pump than on the enzyme.

An additional approach to the correlation between the pump and the ATPase has emerged from an analysis of the relationship between the pump flux of a cell and its ATPase activity. Tosteson et al., 41 in studying the two types of sheep red cells referred to before, found that the magnitude of both the pump flux and the ATPase activity was approximately four times greater in the high-K than in the low-K type. This type of result has been extended by Bonting et al., 42 to include a variety of tissues where the pump and ATPase activity paralleled each other over a range in absolute magnitude of about 50,000-fold. The relative constancy in the ratio of pump to ATPase points toward the conclusion that variations in the pump capacity of different cells reflect differences in pump number rather than turnover number.

I would like to close with a brief consideration of the molecular device in the membrane responsible for active transport. Many different kinds of models have been suggested, but, as far as I am aware, no evidence exists to experimentally discriminate between one type of model or another. 43 My own bias, at the present time, is toward the concept of a rotator or gate-type mechanism in which the ATPase itself is considered to be the pump. This, of course, is based upon the correlation between the pump and the ATPase. Although the actual process may prove to be different, this formulation has the useful virtue of at least organizing the relevant features of the transport reaction. A description of the operation of a rotator mechanism has been previously presented. 43

References

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