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# Potassium-Activated Phosphatase from Human Red Blood Cells

## The Asymmetrical Effects of $K^+$, $Na^+$, $Mg^{++}$ and Adenosine Triphosphate

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*Summary.* The cell membrane $K^+$-activated phosphatase activity was measured in reconstituted ghosts of human red cells having different ionic contents and incubated in solutions of varying ionic composition. When $K^+$-free ghosts are suspended in $K^+$-rich media, full activation of the phosphatase is obtained. Conversely, very little ouabain-sensitive activity is detected in $K^+$-rich ghosts suspended in $K^+$-free media. These results, together with the fact that $Na^+$ competitively inhibits the effects of $K^+$ only when present externally, show that the $K^+$ site of the membrane phosphatase is located at the outer surface of the cell membrane. The $Mg^{++}$ requirements for $K^+$ activation of the membrane phosphatase are fulfilled by internal $Mg^{++}$. Addition of intracellular $Na^+$ to ATP-containing ghosts raises the apparent affinity of the enzyme for $K^+$, suggesting that the sites where ATP and $Na^+$ produce this effect are located at the inner surface of the cell membrane. The asymmetrical features of the membrane phosphatase are those expected from the proposed role of this enzyme in the $Na^+-K^+$-ATPase system.

It is now widely accepted that the unequal distribution of $Na^+$ and $K^+$ between living cells and their surroundings is maintained by an energy-dependent translocation of $Na^+$ and $K^+$ across the cell membrane, the energy coming from the hydrolysis of adenosine triphosphate (ATP) at the inner surface of the cell membrane. One of the possible approaches to get insight into the mechanisms involved in this process is to try to separate the overall ATP hydrolysis by the cation transport system ($Na^+-K^+$-ATPase) into a sequence of partial reactions. During the last few years, work in this field has provided evidence suggesting that the hydrolysis of

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ATP by the $Na^+ - K^+$-ATPase system of cell membranes proceeds in at least two steps: (1) a $(Mg^{++} + Na^+)$-dependent step involving the transference of phosphate from ATP to a protein of the enzyme system; and (2) a $K^+$-activated release of inorganic phosphate ($P_i$) from the phosphorylated protein (Albers, Fahn & Koval, 1963; Whittan, Wheeler & Blake, 1964; Post, Sen & Rosenthal, 1965; Gibbs, Roddy & Titus, 1965; Ahmed & Judah, 1965; Hokin, Sastry, Galsworthy & Yoda, 1965; Nagano, Kanazawa, Mizuno, Tashima, Nakao & Nakao, 1965; Rodnight, Hems & Lavin, 1966; Blostein, 1968). The sequence of reactions can be written as follows:

$$\text{ATP} + \text{enzyme} \xrightleftharpoons{Mg^{++}\ Na^+} \text{enzyme} - P_i + \text{ADP} \qquad (1)$$

$$\text{enzyme} - P_i + H_2O \xrightarrow{K^+} \text{enzyme} + P_i. \qquad (2)$$

It has been suggested that the $K^+$-activated and ouabain-sensitive phosphatase activity found in most cell membranes is the expression of the ability of the $Na^+ - K^+$-ATPase system to hydrolyze other phosphate esters apart from the phosphorylated intermediate (Judah, Ahmed & McLean, 1962; Bader & Sen, 1966).

One of the most prominent features of the $Na^+ - K^+$-ATPase system lies in the fact that $K^+$ is only effective as an activator at the external surface of the cell membrane, whereas $Na^+$ and ATP are only effective at the internal surface of the cell membrane (Glynn, 1962; Whittam, 1962), $P_i$ being released within the cell (Schatzmann, 1964; Marchesi & Palade, 1967). If, as mentioned above, the $K^+$-activated phosphatase were the part of the system responsible for the last step in the $Na^+ - K^+$-dependent hydrolysis of ATP, it can be predicted that $K^+$, $Na^+$ and ATP should affect the phosphatase with the same asymmetrical requirements as those of the $Na^+ - K^+$-ATPase, and that the phosphatase should release $P_i$ within the cell. In the first paper of this series (Garrahan, Pouchan & Rega, 1969) in which the kinetic properties of the $K^+$-activated phosphatase from red cell membranes were described, it was also shown that phosphatase substrates are not hydrolyzed by the membrane phosphatase unless they have access to the internal surface of the cell membrane, suggesting that $P_i$ is released within the cell.

In this paper we report experiments in which membrane phosphatase activity was measured in resealed ghosts of human red cells to determine at which surface of the membrane $K^+$, $Na^+$, $Mg^{++}$ and ATP are effective. The results show that the membrane phosphatase has asymmetrical requirements for $K^+$, $Na^+$, $Mg^{++}$ and ATP which are similar to those of the $Na^+ - K^+$-ATPase system.

A preliminary account of some of the experiments reported here has already been published (Rega, Pouchan & Garrahan, 1969).

## Materials and Methods

### *Preparation of Resealed Ghosts*

The method described here is based on that of Lepke and Passow (1968). Human blood from hematologically normal adults was used, either freshly drawn or as outdated blood-bank blood. Coagulation was prevented with acid-citrate-dextrose solution. The blood was centrifuged at $1{,}750 \times g$ for 10 min, and the plasma and buffy coat were removed by aspiration. The remaining cells were washed three times with about 5 volumes of 150 mM ice-cold choline chloride solution. After the last wash, the cells were spun down for 15 min at $10{,}000 \times g$. A known volume of washed and packed cells was squirted into 300 volumes of a stirred ice-cold lysing solution containing 5 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.2 at 20 °C). In the experiments in which the effect of internal ATP was tested, 0.75 mM Tris-salt of the nucleotide was also present in the lysing solution. The hemolyzate was kept at 0 °C for 10 min. Low temperature and standing was found to be necessary to allow the intracellular soluble phosphatase normally present in red cells to equilibrate with the lysing solution (*cf.* Garrahan *et al.*, 1969). After this step, the hemolyzate was stirred and sufficient concentrated salt solution was added to restore the tonicity to about 320 ideal mosm. The salt solution consisted of a mixture of different salts in proportions chosen to give the desired internal composition of the resealed ghosts. The ghost suspension was centrifuged at 5 °C for 15 min at $17{,}000 \times g$, and most of the supernatant was discarded. The ghosts were resuspended in the remaining supernatant (about 30 ml) and incubated at 37 °C for 40 min. At the end of this period, the ghosts were spun down and washed three times with an ice-cold isotonic solution of either choline chloride or Tris–HCl (pH 7.8 at 37 °C). After they had been washed, the ghosts were suspended in more of the wash solution ready for use.

### *Estimation of the Volume of Resealed Ghosts*

A portion of the washed ghosts packed by centrifugation at $17{,}000 \times g$ for 15 min was mixed with about 10 volumes of wash solution containing an adequate amount of a membrane-free hemolyzate in distilled water, freed of $Na^+$ and $K^+$ by treatment with Amberlite IR-120 resin (Tris-form). The suspension was stirred and spun down for 15 min at $17{,}000 \times g$. A known volume of the packed ghosts was hemolyzed in distilled water, and its $Na^+$ or $K^+$ concentration measured by flame photometry. The extracellular hemoglobin in the packed ghosts was measured in a ghost-free supernatant obtained by centrifuging a mixture of the packed ghosts with a known volume of hemoglobin-free wash solution. Hemoglobin was estimated by its absorbance at 541 nm.

The net volume of the ghosts in the pellet was then calculated from the distribution volume of hemoglobin in the pellet, according to the following equation:

$$V_g = V_t(1 - \mathrm{Hb}_p/\mathrm{Hb}_w) \qquad (3)$$

where $V_g$ is the ghost volume, $V_t$ the volume of the pellet, $\mathrm{Hb}_p$ the extracellular hemoglobin content per liter of pellet, and $\mathrm{Hb}_w$ the hemoglobin concentration per liter of wash solution. This procedure seems to be justified since it is known that after hemolysis hemoglobin does not penetrate into unsealed ghosts (Hoffman, 1958), and there is

no evidence of hemoglobin binding to the outer surface of the red cell membrane. A lower estimate of the volume of resealed ghosts was obtained on the assumption that their cation concentration is equal to that of the solution in which they were sealed, according to the following equation:

$$V_s = V_g \frac{C_g}{C_e} \tag{4}$$

where $V_s$ is the volume of resealed ghosts, $C_g$ the concentration of $Na^+$ or $K^+$ per liter of ghosts, and $C_e$ the concentration of $Na^+$ or $K^+$ in the solution in which the ghosts were sealed.

### *Measurement of the Phosphatase Activity*

Phosphatase activity was measured estimating the release of p-nitrophenol (p-NP) from p-nitrophenylphosphate (p-NPP) by the ghosts as previously described (Garrahan *et al.*, 1969). In all experiments, the amount of ghosts present was that which gave a hematocrit of 5 % calculated on the original volume of cells. The composition of the incubation media varied in the different experiments but the total osmolarity was kept at 320 ideal mosm, the pH at 7.8 (at 37 °C) and the p-NPP (Tris-salt) concentration at 6.3 mM. All inculations were carried out at 37 °C for a 20- to 30-min period.

### *Sources of Materials*

The Tris salt of p-NPP was prepared from Sigma 104 phosphatase substrate (p-nitrophenylphosphate disodium tetrahydrate) as previously described (Garrahan *et al.*, 1969), ATP, also from Sigma, was obtained as the crystalline disodium salt and converted to its Tris salt by running small volumes (about 2 ml) of distilled water containing 0.25 mmole of ATP disodium salt through a column containing about 5 g Amberlite IR-120 (H) resin (British Drug Houses Ltd.), followed by 30 ml of distilled water. The total effluent was adjusted to pH 7.8 with Tris base. Ouabain (Strophantin-G, octahydrate) was obtained from Sigma. All other salts and reagents were analytical reagent grade. Solutions were prepared in twice glass-distilled water.

## Results

### *On the System Used*

The interpretation of the results to be presented in this paper needs to take into account the following two properties of our experimental system. (1) It is generally accepted that when resealed ghosts are prepared, only a fraction of the total population regains its low permeability to cations after sealing, the remaining ghosts exchanging their internal cations freely with those in the bathing solutions (Hoffman, 1962). If the composition of the resealed ghosts is supposed to be equal to the composition of the medium in which the ghosts were sealed, the amount of resealed ghosts in a preparation can be calculated from the distribution volume of trapped solutes. (2) The active site of the membrane phosphatase is only accessible through the internal surface of the cell membrane (Garrahan *et al.*, 1969). Therefore, one

way to avoid the contribution of the unsealed ghosts to the phosphatase activity of the preparation would be to trap into the resealed ghosts a non-penetrating substrate such as acetyl phosphate (Garrahan *et al.*, 1969). We found in practice that, because of its high instability in solution, acetyl phosphate is practically lost during the incubation period necessary for sealing. p-NPP, a more stable substrate, was therefore chosen. However, p-NPP passes very quickly through the red cell membrane (Garrahan *et al.*, 1969) and for this reason has to be added in the final incubation media. Under these conditions, the substrate will be hydrolyzed by both the resealed and the unsealed ghosts. Thus, when asymmetric effects of cations placed externally are studied, the activity of the unsealed ghosts must be taken into account.

### *Location of the $K^+$ Site*

*Effects of extracellular $K^+$.* Table 1 compares the $K^+$-dependent phosphatase activity of $K^+$-free resealed ghosts in a medium containing 50 mM $K^+$ with the activity of the same ghosts after disruption of their membrane, which allows $K^+$ to have access to both sides of the membrane. It is clear that abolition of the permeability barrier does not significantly change the $K^+$-dependent activity. Estimation of the volume of resealed ghosts shows that at least 50% of the total population of ghosts recovered its low permeability to cations. Hence, had $K^+$ been needed internally, the $K^+$-dependent phosphatase activity of disrupted membranes should have been about 100% higher than the activity in intact ghosts. Moreover, assuming a rate of penetration of 10 mmoles $K^+$/liter cells per hr (Hoffman, Tosteson & Whittam, 1960), the average internal $K^+$ concentration in the initially

Table 1. *Effect of $K^+$ on $K^+$-dependent p-NPP hydrolysis in resealed ghosts and in disrupted ghosts*[a]

| Experiment | $K^+$-dependent p-NPP hydrolysis (mmole p-NP/liter original cells/hr) | | Volume of ghosts occupied by resealed ghosts (%) |
|---|---|---|---|
| | Resealed ghosts | Disrupted ghosts | |
| 1 | 0.342 | 0.368 | 53 |
| 2 | 0.342 | 0.323 | 51 |
| 3 | 0.369 | 0.395 | 58 |

[a] The solution in which the ghosts were sealed was composed of: 110 mM NaCl, 10 mM $MgCl_2$, and 40 mM Tris–HCl (pH 7.8). The $K^+$-dependent p-NPP hydrolysis is the difference between the activities in a medium containing 50 mM KCl, 90 mM choline chloride, 10 mM $MgCl_2$, and 20 mM Tris–HCl (pH 7.8) and the activities in a medium in which all the KCl was replaced by NaCl. Disrupted ghosts were prepared by freezing and thawing the incubation mixture.

$K^+$-free ghosts during the experiment would have been 2.5 mM. Even disregarding the competitive effect of internal $Na^+$, 2.5 mM $K^+$ is three times lower than the $K^+$ concentration needed for half-maximal activation of the phosphatase (Garrahan *et al.*, 1969). These results, showing that full activation of the $K^+$-dependent phosphatase can be accomplished by external $K^+$, support the view that the $K^+$ site of the enzyme is located on the outer surface of the cell membrane. To confirm this view, the effect of intracellular $K^+$ was also tested.

*Effects of Intracellular $K^+$.* Table 2 shows the ouabain-sensitive phosphatase activity of ghosts containing 50 mM $K^+$ in the presence and absence of external $K^+$. It is clear that the ouabain-sensitive phosphatase activity of $K^+$-rich ghosts is largely abolished when they are transferred from a $K^+$-containing medium to a nominally $K^+$-free medium. In two of the three experiments, a residual ouabain-sensitive p-NPP hydrolysis in the absence of external $K^+$ was detected. If internal $K^+$ were effective as activator, from the measured values of activity in the 50 mM $K^+$ medium and the amount of resealed ghosts, it can be calculated that the activity in the 0 mM $K^+$ medium would have been at least two to three times larger than the measured activity.

*Effects of Extracellular $Na^+$.* As $Na^+$ is known to compete for the $K^+$ site of the membrane phosphatase (Garrahan *et al.*, 1969), extracellular location of the $K^+$ site implies that inhibitory effects of $Na^+$ should only be exerted at the outer surface of the membrane.

The results given in Table 1 showing that, in going from ghosts whose inner surface is exposed to 110 mM $Na^+$ to disrupted membranes exposed

Table 2. *Ouabain-sensitive p-NPP hydrolysis in $K^+$-rich ghosts*[a]

| Experiment | Ouabain-sensitive p-NPP hydrolysis (mmole p-NP/liter original cells/hr) | | Volume of ghosts occupied by resealed ghosts (%) |
|---|---|---|---|
| | 0 mM $K^+$ medium | 100 mM $K^+$ medium | |
| 1 | 0.002 | 0.125 | 49 |
| 2 | 0.072 | 0.330 | 45 |
| 3 | 0.053 | 0.310 | 45 |

[a] The solution in which the ghosts were sealed was composed of: 50 mM KCl, 10 mM $MgCl_2$, 10 mM NaCl, and 85 mM Tris–HCl (pH 7.8). The 0 mM $K^+$ medium contained: 130 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.8). The 100 mM $K^+$ medium was similar to the 0 mM $K^+$ medium except that 100 mM KCl replaced an equivalent amount of NaCl. The ouabain-sensitive p-NPP hydrolysis is the difference between the rates in the absence and in the presence of $10^{-3}$ M ouabain.

Table 3. *Effect of external $Na^+$ on $K^+$-dependent p-NPP hydrolysis in resealed ghosts*[a]

| Experiment | $Na^+$ concn. in the solution in which the ghosts were sealed (mM) | $K^+$-dependent p-NPP hydrolysis (mmole p-NP/liter original cells/hr) | | Inhibition by $Na^+$ (%) |
|---|---|---|---|---|
| | | 0 mM $Na^+$ medium | 40 mM $Na^+$ medium | |
| 1 | 60 | 0.432 | 0.288 | 33 |
| 2 | 60 | 0.243 | 0.198 | 19 |
| 3 | 60 | 0.350 | 0.278 | 20 |
| 4 | 60 | 0.180 | 0.144 | 20 |
| | 0 | 0.180 | 0.144 | 20 |

[a] The solution in which the ghosts were sealed was composed of: 60 mM NaCl, 40 mM choline chloride, 10 mM $MgCl_2$, and 40 mM Tris–HCl (pH 7.8). When $Na^+$ was absent, in the solution in which the ghosts were sealed, all NaCl was replaced by KCl. The 0 mM $Na^+$ medium contained: 20 mM KCl, 80 mM choline chloride, 10 mM $MgCl_2$, and 40 mM Tris–HCl (pH 7.8). In the 40 mM $Na^+$ medium, 40 mM NaCl replaced an equivalent amount of choline chloride. The $K^+$-dependent p-NPP hydrolysis is the difference between the activities in the above mentioned media and the activities in media in which all the KCl was replaced by choline chloride.

to about 2.5 mM $Na^+$, there is no appreciable change in $K^+$-dependent phosphatase activity, suggest that internal $Na^+$ is ineffective as an inhibitor. Experiments 1–3 in Table 3 show that addition of external $Na^+$ leads to partial inhibition of $K^+$-dependent phosphatase activity of $Na^+$-rich ghosts. Although these results together with those in Table 1 suggest that $Na^+$ is only effective as an inhibitor at the external surface of the cell membrane, they do not discard the possibility that the simultaneous presence of $Na^+$ at both sides of the membrane is required for the inhibitory effects to be expressed. To test this point, the effect of external $Na^+$ on the $K^+$-dependent phosphatase activity of $Na^+$-rich and $Na^+$-free resealed ghosts prepared from the same batch of red cells was compared (Table 3, Exp. 4). It is clear that neither control activities nor the effect of extracellular $Na^+$ is altered by the addition of $Na^+$ inside the ghosts.

### *Location of the $Mg^{++}$ Site*

Activation by $K^+$ of the hydrolysis of p-NPP by the red cell membrane phosphatase is absolutely dependent on the presence of $Mg^{++}$. As reversal of hemolysis is dependent on internal $Mg^{++}$ (Hoffman, 1962), only the effect of external $Mg^{++}$ could be tested. The effects of external $Mg^{++}$ on

Table 4. *Effect of external $Mg^{++}$ on $K^+$-dependent p-NPP hydrolysis in resealed ghosts containing $Mg^{++}$* [a]

| Experiment | $K^+$-dependent p-NPP hydrolysis (mmole p-NP/liter original cells/hr) | | Volume of ghosts occupied by resealed ghosts (%) |
|---|---|---|---|
| | 0 mM $Mg^{++}$ medium | 5 mM $Mg^{++}$ medium | |
| 1 | 0.135 | 0.288 | 63 |
| 2 | 0.171 | 0.333 | 49 |

[a] The solution in which the ghosts were sealed was composed of: 15 mM NaCl, 5 mM $MgCl_2$, 90 mM choline chloride, and 40 mM Tris–HCl (pH 7.8). The 0 mM $Mg^{++}$ medium contained: 15 mM NaCl, 50 mM KCl, 50 mM choline chloride, 40 mM Tris–HCl (pH 7.8), and 0.25 mM sodium ethylenediaminetetraacetate (EDTA). In the 5 mM $Mg^{++}$ medium, 5 mM $MgCl_2$ replaced an equivalent amount of choline chloride. The $K^+$-dependent p-NPP hydrolysis is the difference between the activities in the above mentioned media and the activities in a media in which all the KCl was replaced by choline chloride.

the $K^+$-dependent phosphatase activity of $Mg^{++}$-containing ghosts are shown in Table 4. The persistence of $K^+$-dependent phosphatase activity when $Mg^{++}$ is absent from the incubation medium makes it clear that extracellular $Mg^{++}$ is not essential for this activity to be expressed. Judging by the number of ghosts that recovered their low cation permeability, the approximately twofold increase in activity upon addition of $Mg^{++}$ to the external medium can be entirely accounted for by penetration of $Mg^{++}$ into the unsealed ghosts. These results suggest, therefore, that the $Mg^{++}$ site of the phosphatase is located at the inner surface of the cell membrane.

### *Effects of ATP and $Na^+$*

We have shown elsewhere (Rega, Garrahan & Pouchan, 1968) that, in the presence of ATP, adequate amounts of $Na^+$ lower the concentration of $K^+$, giving half-maximal activation of the membrane phosphatase. This effect has tentatively been related to the participation of the $K^+$-activated phosphatase in the overall $Na^+ - K^+$-ATPase reaction. If this were true, from the known asymmetric requirements of the $Na^+ - K^+$-ATPase system, the ATP-dependent effects of $Na^+$ should require both ATP and $Na^+$ at the inner surface of the cell membrane. This prediction was tested measuring $K^+$-dependent phosphatase activity in ghosts containing either ATP or ATP + $Na^+$, suspended in media with either 5 or 30 mM $K^+$. Results in Table 5 show that addition of intracellular $Na^+$ raises the $K^+$-

Table 5. *$K^+$-dependent p-NPP hydrolysis in resealed ghosts containing either ATP or ATP + Na*

| Experiment | $K^+$-dependent p-NPP hydrolysis (mmole p-NP/liter original cells/hr) | | | | $K^+$-dependent p-NPP hydrolysi in the 5 mM $K^+$ mediu / $K^+$-dependent p-NPP hydrolysi in the 30 mM $K^+$ medi | |
|---|---|---|---|---|---|---|
| | 5 mM $K^+$ medium | | 30 mM $K^+$ medium | | | |
| | Ghosts containing ATP | Ghosts containing ATP + $Na^+$ | Ghosts containing ATP | Ghosts containing ATP + $Na^+$ | Ghosts containing ATP | Ghosts containing ATP + $Na^+$ |
| 1 | 0.049 | 0.148 | 0.284 | 0.369 | 0.17 | 0.40 |
| 2 | 0.018 | 0.072 | 0.135 | 0.144 | 0.13 | 0.50 |
| 3 | 0.072 | 0.180 | 0.459 | 0.360 | 0.16 | 0.50 |
| 4 | 0.009 | 0.063 | 0.225 | 0.207 | 0.04 | 0.30 |

[a] For each experiment, the ATP and the ATP + $Na^+$ ghosts were obtained from a sin hemolyzate. The ATP-ghosts were resealed in a solution containing: 0.75 mM ATP, 110 r choline chloride, 5 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.8). In the ATP + $Na^+$ gho 20 mM NaCl replaced an equivalent amount of choline chloride. About half the ATP initia present must have been hydrolyzed during the incubation necessary for sealing (*see* Dunhan Glynn, 1961).

The 30 mM $K^+$ medium contained: 30 mM KCl, 80 mM choline chloride, 10 mM MgC and 40 mM Tris-HCl (pH 7.8). In the 5 mM $K^+$ medium, 25 mM KCl was replaced by equivalent amount of choline chloride. The $K^+$-dependent p-NPP hydrolysis is the differe between the activities in the above mentioned media and the activities in a medium which the KCl was replaced by choline chloride.

dependent phosphatase activity in the 5 mM $K^+$ medium. It is known that $Na^+$ does not change the maximum rate of $K^+$-dependent phosphatase activity in the presence of ATP (Rega *et al.*, 1968). Hence, the differences in activities between the two kind of ghosts in the 5 mM $K^+$ medium must be ascribed to an increase in apparent affinity for $K^+$ induced by ATP + $Na^+$ from the internal surface of the membrane, provided that in each of the experiments in Table 5 the total number of ghosts was the same. This, however, does not seem to be the case judging by the differences in activities between the two kinds of ghosts in the 30 mM $K^+$ medium (Table 5). Effects owing to differences in ghost recovery are canceled if, instead of comparing the absolute activites, the ratio of $K^+$-dependent phosphatase activity in the 5 mM $K^+$ medium/$K^+$-dependent phosphatase activity in the 30 mM $K^+$ medium in the two kinds of ghosts is compared. Results in Table 5 show that in all experiments there is a large increase of this ratio upon addition of internal $Na^+$, lending strong support to the idea that the observed effects of internal $Na^+$, in the presence of ATP, are due to an increase in the apparent affinity of the phosphatase for $K^+$.

## Discussion

The main conclusion to be drawn from the experiments reported in this paper is that the red cell membrane $K^+$-activated phosphatase has a definite orientation within the membrane structure. As a result of this, the specific sites of the enzyme are accessible through only one of the surfaces of the membrane, lending spatial asymmetry to its requirements for substrate and cofactors. The actual asymmetric requirements of the phosphatase for its substrate (Garrahan *et al.*, 1969), $K^+$, $Na^+$ and ATP, seem to be the expected from the proposed role of the phosphatase in the dephosphorylation step of the $Na^+ - K^+$-ATPase reaction.

Our results show that the membrane phosphatase activity is stimulated by external $K^+$. This assertion is based on the facts that the $K^+$-dependent activity when $K^+$ is only present externally equals the activity when $K^+$ has access to both sides of the membrane, and that the ouabain-sensitive activity of $K^+$-containing ghosts in $K^+$-free media is much smaller than the expected had the $K^+$-site been located internally. The residual ouabain-sensitive phosphatase activity observed in the absence of external $K^+$ can only in part be accounted for by leak of $K^+$ from the ghosts, if the effective concentration of $K^+$ is assumed to be the concentration measured in the bulk of the medium. If $K^+$ ions accumulated at the surface of the cells, the effective concentration might be much higher. Ouabain-sensitive ATPase activity not accounted for by $K^+$ concentration in the bulk of the medium has also been observed in $K^+$-containing ghosts suspended in $K^+$-free media (Garrahan & Glynn, 1967). Further evidence for the external location of the $K^+$ site comes from the fact that $Na^+$ is effective as an inhibitor only when present in the external media.

It is known that the $K^+$-site of the $Na^+ - K^+$-ATPase system is located externally (Glynn, 1962; Whittam, 1962), and that combination of $K^+$ with this site is competitively antagonized by external and not by internal $Na^+$ (Whittam & Ager, 1964). Similar asymmetric features are therefore shared by the $K^+$ site of the phosphatase and the $K^+$ site of the ATPase. This fact supports rather strongly the proposed physiological role of the $K^+$-activated phosphatase, since, if this enzyme were responsible for the dephosphorylation step in the overall $Na^+ - K^+$-ATPase reaction, the $K^+$ sites of both the phosphatase and the ATPase should be the same and hence have the same location in the membrane.

The active site of the $Na^+ - K^+$-ATPase system and its specific site for $Na^+$ are located at the inner surface of the cell membrane (Glynn, 1962; Whittam, 1962). In this paper we have shown that the increase in apparent

affinity for $K^+$ of the red cell membrane phosphatase induced by the simultaneous presence of $Na^+$ and ATP (Rega *et al.*, 1968) is also evident in sealed ghosts when both $Na^+$ and ATP are only present internally. Internal location of the ATP and the $Na^+$ sites of the red cell membrane phosphatase, together with the fact that the ATP-dependent effects of $Na^+$ are specific for ATP and blocked by oligomycin (Garrahan, Pouchan & Rega, 1970), provide indirect evidence that the sites for ATP and $Na^+$ of the phosphatase may be the same as those of the ATPase system.

Although the requirement of the membrane phosphatase for internal $Mg^{++}$ seems at first in agreement with a similar requirement of the ATPase, they are quite different in nature. In fact, the role of $Mg^{++}$ in the $Na^+ - K^+$-ATPase reactions seems to be restricted to the formation of the ATP – Mg complex that is the effective substrate for the system, $Mg^{++}$ not being required for the $K^+$-activated hydrolysis of the phosphorylated intermediate (Gibbs *et al.*, 1965). On the other hand, the $K^+$-activated hydrolysis of artificial substrates by the membrane phosphatase is absolutely dependent on the presence of $Mg^{++}$, which seems to combine with a site that is independent of and does not interact with the substrate and $K^+$-sites (Garrahan *et al.*, 1969). For these reasons, the exact physiological meaning of the internal location of the $Mg^{++}$ site of the phosphatase remains obscure.

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