BBA 75631

STUDIES ON THE PARTIAL REACTIONS CATALYZED BY THE $(Na^+ + K^+)$-ACTIVATED ATPase

III. RELATION OF K^+-DEPENDENT *p*-NITROPHENYLPHOSPHATASE TO Na^+ TRANSPORT IN RED CELL GHOSTS

A. ASKARI AND S. N. RAO

Department of Pharmacology, Cornell University Medical College New York, N.Y. (U.S.A.)

(Received December 7th, 1970)

SUMMARY

1. When resealed ghosts labeled with $^{22}Na^+$ are suspended in a Na^+-free medium, the addition of K^+ and *p*-nitrophenylphosphate to the medium initiates a ouabain-sensitive efflux of Na^+. If this efflux is to occur in a Na^+-containing medium, the presence of ATP in the medium is also needed. The object of this work was the further study of these phenomena.

2. Na^+ efflux was not stimulated if *p*-nitrophenylphosphate were placed inside the ghosts; and when ghosts or intact cells were incubated in a solution containing *p*-nitrophenylphosphate no evidence for the rapid uptake of the substrate was obtained.

3. Under optimal conditions the stimulation of Na^+ efflux by *p*-nitrophenylphosphate was greater than the stimulation of efflux by adenosine.

4. When ghosts were suspended in a Na^+-free medium containing *p*-nitrophenylphosphate, the addition of any of the alkali cations, except Na^+, to the medium stimulated Na^+ efflux. The order of effectiveness of the cations was: $K^+ > Rb^+ > Cs^+ = Li^+$.

5. The inhibitory effects of external Na^+ were greater at lower K^+ concentrations. Kinetic studies showed, however, that the interaction between Na^+ and K^+ was not of the simple competitive type.

6. The inhibitory effect of external Na^+ was reversed by the addition of certain nucleotides (ATP, CTP, ADP) to the medium. ATP was the most effective. ITP, CDP, and AMP were ineffective.

7. Oligomycin did not affect the stimulation of Na^+ efflux into a Na^+-free medium. In a Na^+-containing medium the lower ranges of tested concentrations of oligomycin stimulated the efflux. This ATP-like effect of oligomycin disappeared as the concentration of the antibiotic was raised. These higher concentrations could also block the effects of ATP in a Na^+-containing medium.

8. Stimulation of Na^+ efflux under all of the above various conditions could be blocked by ouabain or P_i added to the medium.

9. In any experiment where Na^+ efflux was stimulated in the presence of *p*-nitrophenylphosphate, there was an increase in the production of *p*-nitrophenol.

10. The data are consistent with the hypothesis that the K^+-dependent phosphatase "segment" of the $(Na^+ + K^+)$-activated ATPase is on the outside surface of the membrane, and that the phosphatase is the primary translocator of Na^+ and K^+.

INTRODUCTION

In human red cells the $(Na^+ + K^+)$-activated ATPase complex (ATP phosphohydrolase, EC 3.6.1.3) of the membrane utilizes ATP that approaches the complex from the matrix side of the membrane to catalyze the coupled movements of Na^+ and K^+ across the membrane[1-5]. Several different molecular mechanisms for these ion translocations have been proposed, and the common feature of all is the postulate that ion movements are intimately related to the turnover of the phosphoenzyme intermediate within the membrane[6]. On the assumption that the hydrolysis of *p*-nitrophenylphosphate by the enzyme complex is equivalent to the breakdown of the phosphorylated intermediate, we studied the effects of *p*-nitrophenylphosphate on Na^+ transport in resealed red cell ghosts and found that if this substrate approaches the medium side of the membrane it mimics some of the actions of ATP on Na^+ transport[7]. Here we present the results of the continuation of these studies. The question of the site of action of *p*-nitrophenylphosphate is studied in more detail; the Na^+ efflux that is stimulated by *p*-nitrophenylphosphate is compared with the metabolically energized efflux; optimal conditions for the stimulation of efflux by *p*-nitrophenylphosphate are characterized; and the effects of ouabain and oligomycin, two different modifiers of the enzyme complex, on the stimulation of Na^+ efflux by *p*-nitrophenylphosphate are investigated. A preliminary account of portions of this work has been presented[8].

MATERIALS AND METHODS

Several types of resealed ghosts labeled with $^{22}Na^+$ were used in these studies. The following steps were common to the procedures used for all preparations. (1) Hemolysis of intact red cells. (2) Labeling with $^{22}Na^+$. (3) Washing with a buffered $MgCl_2$ solution. (4) "Reconstitution" and suspension in the final incubation medium. Unless otherwise stated the experiments were performed with ghosts which are deficient in substrates and have low internal Na^+ concentrations. These were prepared by hemolysis of cells in 10 vol. of water. The procedure for the preparation of these ghosts, and their composition and properties, have been described elsewhere[9]. Ghosts similar to the above, but with higher internal Na^+ concentrations (those used in experiments of Fig. 8) were prepared by hemolyzing the cells in solutions containing varying amounts of NaCl. The compositions of the various hemolyzing and wash solutions were the same as those described by HOFFMAN[10]. The concentration of Na^+ within these ghosts was determined immediately after hemolysis. Ghosts filled with ATP or *p*-nitrophenylphosphate were prepared, according to the method described before[11], by hemolysis of the cells either in a solution containing 2 mM ATP, or in one which contained 4 mM *p*-nitrophenylphosphate.

Time course of the release of $^{22}Na^+$ from the ghosts was determined as previously described[9,11]. In experiments where *p*-nitrophenylphosphate was used, in addition to

the measurement of radioactivity, at each time interval the amount of *p*-nitrophenol in the mixture was also determined[12]. For the majority of the experiments the data are presented as changes in the rate constant of Na^+ efflux. This constant was calculated from the experimental data on the time course of release of $^{22}Na^+$ as described before[11]. Thus each point representing a rate constant in the various figures of this paper is in fact the slope of a line such as those presented in Fig. 1. It should be emphasized that such lines for each experimental condition were obtained by actual measurements of the amount of $^{22}Na^+$ released from the ghosts at a minimum of three different time intervals.

Hemoglobin-free ghosts were prepared by the method of PARKER AND HOFFMAN[13]. After the final wash the membranes were frozen and thawed twice, before they were used for the determination of enzyme activity.

Nucleotides, *p*-nitrophenylphosphate, adenosine, ouabain, and oligomycin were obtained from Sigma Chemical Co. (St. Louis, Mo.)

RESULTS

Na^+ efflux in ghosts that are filled with ATP or p-nitrophenylphosphate

Using a procedure similar to the one employed for the preparation of ATP-filled ghosts, we obtained $^{22}Na^+$-labeled ghosts which retained a considerable amount of *p*-nitrophenylphosphate after repeated washings. Efflux of Na^+ from these ghosts and the disappearance of *p*-nitrophenylphosphate were measured and compared with Na^+ efflux and ATP breakdown in ATP-filled ghosts. The data presented in Table I clearly show that in ghosts filled with *p*-nitrophenylphosphate Na^+ efflux is not significantly stimulated by the addition of K^+ to the medium. It is also apparent that *p*-nitrophenylphosphate is indeed hydrolyzed in the ghosts, but that the hydrolysis is not affected by the presence of K^+.

Permeability of the red cell membrane to p-nitrophenylphosphate

Retention of *p*-nitrophenylphosphate by the ghosts during the preparation and

TABLE I

EFFECTS OF INTERNAL ATP AND *p*-NITROPHENYLPHOSPHATE ON Na^+ EFFLUX

Ghosts labeled with $^{22}Na^+$ and filled either with ATP or *p*-nitrophenylphosphate were prepared as described in MATERIALS AND METHODS. Portions of each preparation were incubated at 37° for 1 h in 25 vol. of the following solutions: (1) 160 mM choline chloride–10 mM Tris·HCl (pH 7.4). (2) 154 mM choline chloride–10 mM Tris·HCl (pH 7.4)–6 mM KCl. At the beginning and the end of the incubation the levels of $^{22}Na^+$, P_i, and the "easily hydrolyzable phosphate" of the ghosts were measured as described before[11]. Levels of *p*-nitrophenylphosphate were also determined[12,33]. Prior to the incubation the ATP-filled ghosts contained 0.9 μmoles/ml P_i, and 2.41 μmoles/ml "easily hydrolyzable phosphate". Ghosts filled with *p*-nitrophenylphosphate contained 1.2 μmoles/ml P_i and 0.90 μmole/ml *p*-nitrophenylphosphate.

	p-Nitrophenylphosphate-filled ghosts incubated		*ATP-filled ghosts incubated*	
	Without K^+	*With K^+*	*Without K^+*	*With K^+*
% of $^{22}Na^+$ released in 1 h	18	17	15	35
% decrease in ATP or *p*-nitrophenylphosphate content	90	90	35	49

washing of the ghosts suggested that the ghost membrane is relatively impermeable to this compound. This conclusion seemed to be in contrast to that reached by GARRAHAN *et al.*[14] in their studies on the hydrolysis of *p*-nitrophenylphosphate by the intact red cells. Therefore, we incubated washed intact cells and resealed ghosts in solutions containing the compound, and measured the amounts of *p*-nitrophenylphosphate and *p*-nitrophenol in the media, the cells, and the ghosts. The results which are summarized in Table II do not indicate a rapid uptake of the organic phosphate by either the intact cells or the ghosts. It is evident, however, that a considerable quantity of *p*-nitrophenol accumulates within the cells or the resealed ghosts.

TABLE II

UPTAKES OF *p*-NITROPHENYLPHOSPHATE AND *p*-NITROPHENOL BY INTACT RED CELLS AND GHOSTS

Packed red cells were obtained from fresh human blood by centrifugation and several washings in isotonic saline. Ghosts were prepared as described in MATERIALS AND METHODS but without the use of $^{22}Na^+$ label. In each experiment 5 ml of cells or ghosts were added to 20 ml of a solution containing 145 mM KCl, 20 mM Tris·HCl (pH 7.4), and *p*-nitrophenylphosphate. In experiments with intact cells the concentration of this substrate was 5 mM, and in those with ghosts the concentration was 2 mM. Immediately after the addition of cells or ghosts to the medium, an aliquot of the suspension was withdrawn, immersed in ice, mixed with trace amounts of $^{22}Na^+$, and centrifuged in cold at 20 000 × *g* for 5 min. The supernatant was carefully separated from the pellet. An aliquot of the supernatant, and the pellet were mixed with equal volumes of 8 % $HClO_4$, cooled, and centrifuged. *p*-Nitrophenylphosphate and *p*-nitrophenol contents of the acid extracts were determined[12,33]. Radioactivities of aliquots of extracts were also measured. From these counts the amount of medium that was trapped in each pellet was calculated[34]. The measured values of *p*-nitrophenylphosphate and *p*-nitrophenol of the ghosts and the cells were corrected for the trapped medium. The same procedure was repeated after the ghosts or the cell suspensions were incubated at 37° for the indicated periods.

	p-Nitrophenylphosphate (μmoles)			*p-Nitrophenol (μmoles)*		
	0 min	*30 min*	*60 min*	*0 min*	*30 min*	*60 min*
Expt. 1:						
Medium	100	61		0.8	30.8	
Cells	0	1.1		0	8.5	
Expt. 2:						
Medium	39		28.5	0.3		8.5
Ghosts	0		0.6	0		2.2

Comparison between the stimulation of Na^+ efflux by p-nitrophenylphosphate and the metabolically energized Na^+ efflux

When washed ghosts are suspended in a Na^+-free medium the simultaneous addition of *p*-nitrophenylphosphate and K^+ to the medium stimulates the efflux of Na^+ from the ghosts[7]. It was of interest to compare the magnitude of this stimulation with that which is obtained when energy is supplied to the membrane through substrate metabolism. The ghosts can use the ribose of either inosine or adenosine for lactate production. Fig. 1. shows the effects of *p*-nitrophenylphosphate and adenosine on the time course of the release of $^{22}Na^+$ from the ghosts in three different preparations. The data show that in each preparation stimulation of efflux by *p*-nitrophenylphosphate is greater than that obtained with adenosine. It should be pointed out that the adenosine and the K^+ concentrations used in the above experiments are the maximum effective concentrations for the metabolic stimulation of the pump in these

ghosts[9]. That the *p*-nitrophenylphosphate and the K^+ concentrations used in experiments of Fig. 1 are also optimal is evident from the data of the following section.

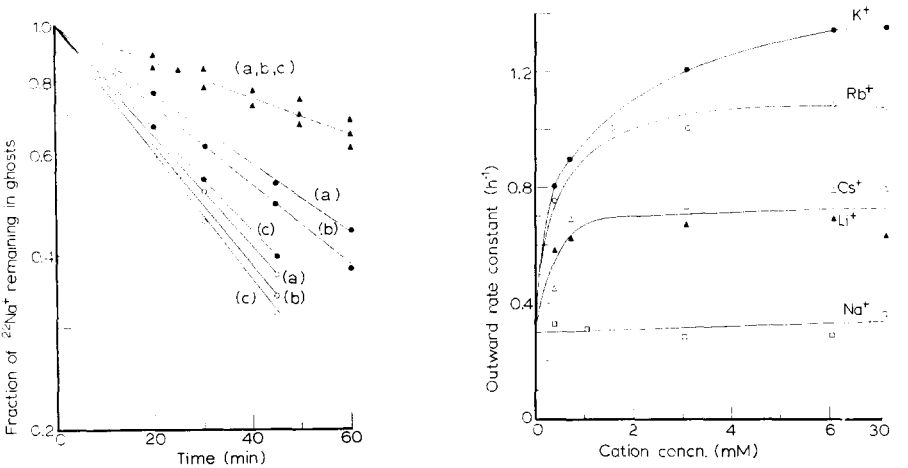

Fig. 1. Comparison between the effects of adenosine and *p*-nitrophenylphosphate on Na^+ efflux. Three different ghost preparations (a, b, and c) were used. Portions of each preparation were incubated in 30 vol. of the following media: (1) ▲, 154 mM choline chloride, 6 mM KCl, 10 mM Tris·HCl (pH 7.4). (2) ●, medium 1 containing 1 mM adenosine. (3) ○, medium 1 containing 1 mM *p*-nitrophenylphosphate.

Fig. 2. Effects of various alkali cations on the stimulation of Na^+ efflux by *p*-nitrophenylphosphate. The control medium contained 160 mM choline chloride, 10 mM Tris·HCl (pH 7.4), and 1 mM *p*-nitrophenylphosphate. The various cations were added as chloride salts. Whenever these salts were added, equivalent concentrations of choline chloride were omitted from the control medium. ●, K^+; ○, Rb^+; ▲, Li^+; △, Cs^+; □, Na^+.

Optimal conditions for the stimulation of Na^+ efflux into a Na^+ free medium by p-nitrophenylphosphate

We showed before that the presence of K^+ in the medium is necessary for the stimulation of Na^+ efflux by *p*-nitrophenylphosphate, and the concomitant increase in the hydrolysis of this compound[7]. Fig. 2 shows the effects of varying concentrations of several alkali cations on Na^+ efflux into an isotonic choline chloride solution containing *p*-nitrophenylphosphate. It is evident that the addition of Na^+ to the medium has no significant effect on the rate of release of $^{22}Na^+$ from the ghosts. All other alkali cations stimulate the efflux. The order of effectiveness being $K^+ > Rb^+ > Cs^+, Li^+$.

The effects of varying concentrations of *p*-nitrophenylphosphate on Na^+ efflux in the presence of a fixed and maximal effective concentration of K^+ were also studied. Half-maximal stimulation was obtained at about 0.3 mM substrate, and maximal effect was observed in the range of 0.9–5 mM substrate.

In none of the above experiments Mg^{2+} is included in the incubation solutions. In view of the known requirements of K^+-dependent *p*-nitrophenylphosphatase for Mg^{2+} (see refs. 14, 15), the question of the necessity of the addition of Mg^{2+} may arise. We point out that the $^{22}Na^+$-labeled ghosts are washed and suspended for storage in a medium containing $MgCl_2$. It may be estimated that under our standard experimental condition, the Mg^{2+} that is added to the incubation medium with the ghosts is sufficient to make the final Mg^{2+} concentration about 0.5 mM. Addition of more Mg^{2+}, up

to 10 mM, has no effect on the magnitude of efflux. Further increase in Mg^{2+} concentration causes a gradual inhibition of the substrate hydrolysis and the related efflux of Na^+. This is in accord with the known inhibitory effects of high Mg^{+2} concentrations on the K^+-dependent phosphatase of the partially purified enzyme preparations[15].

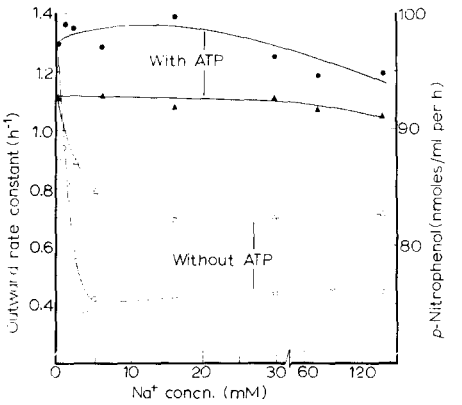

Fig. 3. Inhibitory effects of varying concentrations of external Na^+ on the K^+-dependent *p*-nitrophenylphosphatase (△, ▲) and the associated Na^+ efflux (○, ●). Incubation media: (1) ○, △, 154 mM choline chloride, 6 mM KCl, 10 mM Tris·HCl (pH 7.4), 1 mM *p*-nitrophenylphosphate, and the indicated concentrations of NaCl replacing equivalent concentrations of choline chloride. (2) ●, ▲, Medium 1 containing 0.5 mM ATP.

In several experiments similar to those of Figs. 1 and 2 replacement of choline chloride with tetramethylammonium chloride did not affect the results.

Effect of external Na^+ on the stimulation of Na^+ efflux by p-nitrophenylphosphate

We have shown[7] that if ghosts are suspended in an isotonic NaCl solution, the addition of *p*-nitrophenylphosphate and K^+ to the medium does not cause the stimulation of Na^+ efflux. This inhibitory effect of external Na^+ on Na^+ efflux was studied in more detail. The data of Fig. 3 show that as little as 3 mM Na^+ in the external medium is sufficient to block completely the stimulated efflux of Na^+ and hydrolysis of the substrate. To see if anything about the nature of the inhibitory effect of external Na^+ could be learned the effects of varying concentrations of K^+ on the efflux in the presence of several fixed concentrations of external Na^+ were determined. The data plotted in Fig. 4 indicate that Na^+ is not a simple competitive inhibitor of the K^+ activation.

Effects of nucleoside phosphates on the stimulation of Na^+ efflux by p-nitrophenylphosphate

The data of Fig. 3 show that the addition of 0.5 mM ATP to the medium overcomes the inhibitory effects of all tested concentrations of external Na^+ on Na^+ efflux. Effects of varying concentrations of K^+ on Na^+ efflux into various Na^+-containing media in the presence of ATP were determined. The results which are included in Fig. 4 clearly show that ATP overcomes the inhibitory effects of Na^+ at all K^+ concentrations. Fig. 5 shows the effects of varying concentrations of ATP on a fixed

inhibitory concentration of Na^+. Also included in Fig. 5 are data indicating that ATP, within the range of tested concentrations, has no significant effect on the stimulation of Na^+ efflux into a Na^+-free medium by *p*-nitrophenylphosphate. In Table III the effects of several other nucleoside phosphates are compared with that of ATP. At the tested concentrations only ADP and CTP could overcome the inhibitory effect of external Na^+ on Na^+ efflux. It is evident, however, that neither is as effective as ATP.

Effects of oligomycin on the K^+-dependent p-nitrophenylphosphatase and the stimulation of Na^+ efflux by p-nitrophenylphosphate

The rationale for the use of ATP in the above experiments to overcome the inhibitory effects of external Na^+ was the finding that the K^+-dependent *p*-nitrophenylphosphatase of a partially purified (Na^+ + K^+)-activated ATPase from brain is further activated by the simultaneous presence of Na^+ and ATP (refs. 16–18). Recent studies of our laboratory on the effects of oligomycin on the brain enzyme showed that (a) oligomycin does not inhibit the K^+-dependent *p*-nitrophenylphosphatase[18]; (b) the antibiotic prevents the ATP-stimulation of the K^+-dependent activity[18,19]; and (c) under proper conditions oligomycin, like ATP, can further activate the K^+-dependent *p*-nitrophenylphosphatase[19,20]. In view of these findings we decided to study the effects of oligomycin on the K^+-dependent *p*-nitrophenylphosphatase and the related Na^+ movements of the functionally intact membrane. We were particularly interested to see if oligomycin could replace ATP in experiments such as those of Fig. 5. Before initiating such studies, however, we prepared fragmented hemoglobin-free ghosts, and studied the properties of the K^+-dependent *p*-nitrophenylphosphatase in this relatively pure membrane preparation. The kinetic parameters for K^+-activa-

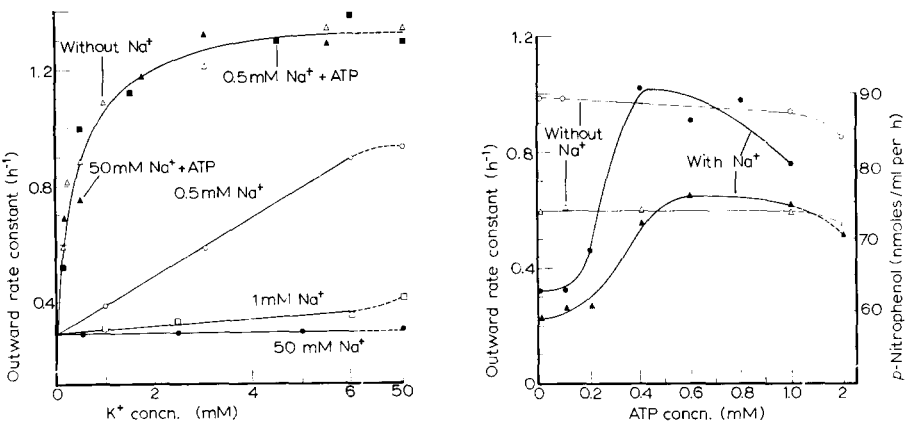

Fig. 4. Effects of varying concentrations of K^+ on Na^+ efflux in ghosts which were suspended in: △, control medium (160 mM choline chloride, 10 mM Tris·HCl (pH 7.4), 1 mM *p*-nitrophenylphosphate, and the indicated concentrations of KCl replacing equivalent concentrations of choline chloride); ○, control medium with 0.5 mM NaCl; □, control medium with 1 mM NaCl; ●, control medium with 50 mM NaCl replacing 50 mM choline chloride; ■, control medium with 0.5 mM NaCl + 0.5 mM ATP; ▲, control medium with 50 mM NaCl + 0.5 mM ATP.

Fig. 5. Effects of varying concentrations of ATP on Na^+ efflux (○, ●) and *p*-nitrophenol production (△, ▲). Incubation media: ○, △, 154 mM choline chloride, 6 mM KCl, 10 mM Tris·HCl (pH 7.4), and 1 mM *p*-nitrophenylphosphate; ●, ▲, similar to the above medium but with 154 mM NaCl replacing choline chloride.

tion, Na^+ inhibition of the K^+-dependent activity, and ATP activation in the presence of Na^+ and K^+ were very much the same as reported by others who have used similar preparations of red cell membranes[14,21,22]. The multiple effects of oligomycin on the red cell enzyme have not been reported before and are shown in Fig. 6. The results are qualitatively the same as those obtained with the brain enzyme[19]. The main difference being that the activating effect of oligomycin on the *p*-nitrophenylphosphatase of the hemoglobin-free membranes is obtained within a much wider range of oligomycin concentrations than that observed with the brain enzyme. Having confirmed the similar effects of oligomycin in the above two preparations, we then tested its effects on the resealed ghosts. The data presented in Fig. 7 show that (a) oligomycin does not inhibit the stimulation of Na^+ efflux into the Na^+-free medium by *p*-nitrophenyl-

TABLE III

REVERSAL OF THE INHIBITORY EFFECTS OF EXTERNAL Na^+ BY VARIOUS NUCLEOSIDE PHOSPHATES

Ghosts were incubated in 30 volumes of a solution containing 154 mM NaCl, 6 mM KCl, 1 mM *p*-nitrophenylphosphate, the indicated amounts of nucleotides, and 10 mM Tris·HCl (pH 7.4).

Added nucleotide	*% of $^{22}Na^+$ released in 1 h*
None	30
0.4 mM ATP	68
0.4 mM CTP	54
0.4 mM ITP	33
1 mM ITP	28
0.4 mM ADP	38
1 mM ADP	50
1 mM CDP	30
1 mM AMP	36

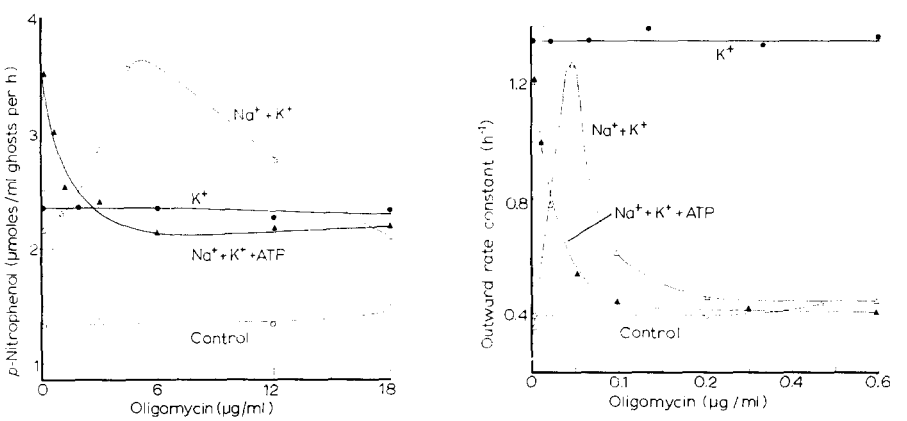

Fig. 6. Effects of varying concentrations of oligomycin on the *p*-nitrophenylphosphatase activity of fragmented membranes of red cells. All reaction mixtures contained 4 mM substrate and 4 mM Mg^{2+}. ○, without K^+, Na^+ and ATP; ●, 0.5 mM K^+; △, 0.5 mM K^+ + 10 mM Na^+; ▲, 0.5 mM K^+ + 10 mM Na^+ + 0.1 mM ATP.

Fig. 7. Effects of varying concentrations of oligomycin on Na^+ efflux in ghosts that were suspended in: ○, control medium (160 mM choline chloride, 10 mM Tris·HCl (pH 7.4), and 1 mM *p*-nitrophenylphosphate); ●, control medium with 6 mM KCl; △, control medium with 6 mM KCl + 6 mM NaCl; ▲, control medium with 6 mM KCl + 6 mM NaCl + 0.5 mM ATP.

phosphate; (b) in a Na^+-containing medium where *p*-nitrophenylphosphate alone is not effective the addition of either ATP or the lower ranges of tested oligomycin concentrations stimulates Na^+ efflux; and (c) when activating concentration of ATP and oligomycin are added together the effects are not additive, and in fact each seems to antagonize the other.

Effects of cardiac glycosides

Experiments were done to determine the effect of several concentrations of ouabain on Na^+ efflux from the ghosts under the following conditions: (1) When efflux into a Na^+-free medium was stimulated by the addition of *p*-nitrophenylphosphate. (2) When activation of efflux into a Na^+-containing medium was obtained by the addition of *p*-nitrophenylphosphate and ATP. (3) When efflux into a Na^+-containing medium was stimulated by *p*-nitrophenylphosphate and oligomycin. In all cases the stimulation of Na^+ efflux could be blocked completely at about 10^{-5} M ouabain.

Effect of internal Na^+ on the stimulation of Na^+ efflux by p-nitrophenylphosphate

Ghosts with varying internal Na^+ concentrations were prepared and the effects of *p*-nitrophenylphosphate on Na^+ efflux into a Na^+-free medium were determined. A fixed concentration of K^+ was added to the media in all cases. In Fig. 8 Na^+ efflux is plotted as a function of apparent internal Na^+ concentration. (As described in MATERIALS AND METHODS this is the concentration of Na^+ in ghosts at the time of hemolysis. It is not the "true" internal Na^+ concentration. To obtain this the measured values should be multiplied by a volume correction factor[10].) Na^+ efflux is the product of the apparent internal Na^+ concentration and the outward rate constant. It is evident from the results that the magnitude of the efflux that is dependent on *p*-nitrophenylphosphate is constant within the tested range of internal Na^+ concentrations. Clearly internal Na^+ has no inhibitory effect on the efflux. Whether the increasing Na^+ concentration has an activating effect on the substrate-dependent efflux can not be determined from the data of Fig. 8. With the standard methods that we use,

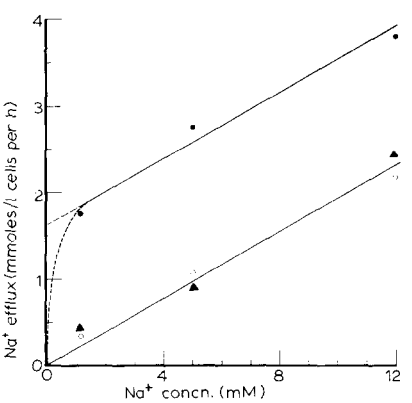

Fig. 8. Effects of varying internal Na^+ concentrations on Na^+ efflux. Incubation media: ○, control medium (154 mM choline chloride, 10 mM Tris·HCl (pH 7.4), and 6 mM KCl); ●, control medium with 1 mM *p*-nitrophenylphosphate(▲, control medium with 1 mM *p*-nitrophenylphosphate and 3 mM NaCl.

ghosts with internal Na^+ concentrations lower than about 1 mM are not convenient to prepare. It is conceivable, therefore, that there is an activating effect of internal Na^+ on Na^+ efflux, and that the maximum activation has been reached at this Na^+ concentration. Also included in Fig. 8 are the data showing that even in ghosts with the highest internal Na^+ concentration, the efflux that is dependent on *p*-nitrophenylphosphate is inhibited by the presence of a small concentration of Na^+ in the medium.

Effects of orthophosphate on the stimulation Na^+ efflux by p-nitrophenylphosphate

In the course of our early studies the stimulation of Na^+ efflux by *p*-nitrophenylphosphate could not be obtained consistently. We were able to overcome this problem by using either fresh solutions of the substrate or stock solutions which were frozen rather than kept at 4°. These observations led us to suspect the possible inhibitory effects of low concentrations of orthophosphate. The effects of varying concentrations of this ion, added as potassium phosphate buffer (pH 7.4), were examined on Na^+ efflux into (a) Na^+-free medium with added *p*-nitrophenylphosphate; and (b) Na^+-containing medium with added ATP and *p*-nitrophenylphosphate. Under both conditions the same inhibitory effect of orthophosphate was observed. Half-maximal inhibition of efflux was obtained at 0.2 mM orthophosphate, and at concentrations of 0.8 mM or higher the stimulation of Na^+ efflux by *p*-nitrophenylphosphate was maximally inhibited.

Correlation between the stimulation of Na^+ efflux by p-nitrophenylphosphate and the production of p-nitrophenol

In some of the above experiments (Figs. 3 and 5) the data obtained from the measurements of *p*-nitrophenol are presented along with th efflux data. It is evident that whenever Na^+ efflux is stimulated or inhibited there is a corresponding increase or decrease in *p*-nitrophenol production. Such measurements were routinely made in all of the experiments described here, and the same correlation between the magnitude of efflux and the rate of breakdown of the substrate was observed in all.

DISCUSSION

The interpretation of the major finding of this and a previous paper[7], namely the stimulation of Na^+ efflux by *p*-nitrophenylphosphate, would become easier if one could decide on which side of the membrane the K^+-dependent hydrolysis of the substrate is occurring. The fact that *p*-nitrophenylphosphate which is added to the medium is hydrolyzed by the intact red cells and ghosts, can be interpreted to mean that either the substrate first penetrates the membrane and is then broken down within the cell, or that the substrate can be acted upon by enzymes that are located on the outside of the cell membrane. The accumulation of the product of the reaction, *p*-nitrophenol, within the cells or ghosts is also of little value in determining the site of hydrolysis of the substrate. *p*-Nitrophenol is a weak acid (pK, 7.15), and at pH values used in our experiments one would expect it to pass through the membrane easily regardless of the side at which it is formed. On the other hand the ability of ghosts to trap *p*-nitrophenylphosphate in spite of repeated washings during the preparative procedure, and the absence of significant accumulation of the compound within the cells or ghosts indicate that the penetration of this phosphate ester through

the membrane is slow, if any, and similar to that of ATP and other organic phosphates[23]. On the basis of this conclusion, and considering that K^+-dependent hydrolysis of *p*-nitrophenylphosphate can be detected when the compound is placed in the medium and not when it is trapped in the ghosts, it seems that the K^+-dependent hydrolysis occurs on the medium side of the ghost membrane. Further support for this is provided by the observation that the effects of all the tested modifiers of the K^+-dependent phosphatase activity (K^+, Na^+, ATP, ouabain, orthophosphate and oligomycin) are also obtained by the addition of these agents to the medium. Considering the known limited permeability of the membrane to some of these modifiers, and the low effective concentrations of all, we feel that in the absence of compelling evidence to the contrary, the simplest assumption is to place the K^+-dependent *p*-nitrophenylphosphatase with its active site and the proposed modifying sites[17–19] on the medium side of the membrane.

An interesting outcome of these studies is the finding that there are subtle differences between the properties of the *p*-nitrophenylphosphatase of the functionally intact ghosts and those of the enzyme activity of the fragmented membranes of the red cells. The K^+-dependent activity of the intact ghosts is inhibited by Na^+ as expected from studies on the more purified enzyme. But both the K^+ concentration causing half-maximal activation and the Na^+ concentration causing half-maximal inhibition are significantly lower in the case of the activity of the intact ghost. (compare Figs. 2 and 3 with the data of ref. 14). The nature of inhibition by Na^+ also seems to be somewhat different. As evident from our data, the interaction between Na^+ and K^+ is clearly not of the simple competitive type. On the other hand data with a more purified preparation show apparent competition between the cations[11]. Another point of difference is the response of the enzyme to the activating concentrations of oligomycin and ATP. With the partially purified enzyme from brain, the activation by oligomycin or ATP in the presence of Na^+ is accompanied by the lowering of the apparent K_m for K^+ to a level that is below the K_m that is obtained in the absence of Na^+ + ATP or Na^+ + oligomycin[19]. GARRAHAN *et al.*[21] have made similar observations in relation to the activating effects of ATP on the enzyme activity of the fragmented red cell membranes, and our data show similar activating effects of ATP and oligomycin in hemoglobin-free ghosts. In all of these preparations the enzyme activity at a fixed K^+ concentration can be greater in the presence of Na^+ and a modifier than in their absence. On the other hand in the functionally intact ghosts ATP or oligomycin can only overcome the inhibitory effects of Na^+. The *p*-nitrophenylphosphatase activity, and the concomitant Na^+ efflux, in the presence of the modifiers never becomes greater than that obtained in the presence of K^+ alone. The reason for these discrepancies is not known. Perhaps the subtle differences are due to the "more organized" nature of the enzyme in the resealed ghost preparations. It is interesting to note that PRIESTLAND AND WHITTAM[24] have shown that the kinetics of the interactions of Na^+ and K^+ with the (Na^+ + K^+)-activated ATPase in resealed ghosts are also different from those in the fragmented red cell membranes.

The extensive data of this paper which show a strict correlation between the activity of the K^+-dependent *p*-nitrophenylphosphatase and Na^+ efflux, give further support to one of the conclusions of our initial studies. We had suggested[7] that the K^+-dependent breakdown of *p*-nitrophenylphosphate is sufficient for the stimulation of Na^+ efflux. This conclusion was strictly justified in the relatively simple case of

efflux into the Na^+-free medium. In the Na^+-containing medium, the necessity of the presence of a nucleoside phosphate in the medium created additional complications. Although it has been shown repeatedly that ATP can be used for ion movements only when it is on the matrix side of the membrane, it might have been suspected that in the presence of *p*-nitrophenylphosphate it is the ATP that is being utilized, albeit in an unusual way, to stimulate Na^+ efflux. The studies with oligomycin rule out this possibility. Obviously, as long as the inhibitory effect of Na^+ on the K^+-dependent *p*-nitrophenylphosphatase is overcome (either by ATP or by oligomycin) the stimulation of Na^+ efflux is obtained. Therefore, we now feel more confident in assuming that under the experimental conditions described in this paper a phosphatase that can be approached by its substrate on the medium side of the membrane is the translocator for Na^+.

The major point that is to be considered now is whether the Na^+ efflux mediated by the above translocator phosphatase is related to the physiological processes of the coupled Na^+, K^+-pump. Although certain similarities are immediately obvious it is important to examine these in some detail. In the following sections the properties of Na^+ efflux that is stimulated by *p*-nitrophenylphosphate are compared with those of the Na^+ efflux energized by ATP that is either placed inside the ghosts or generated by substrate metabolism.

1. As the limited data presented in this paper (Fig. 1) indicate, in each of three preparations where direct comparisons were made, the magnitude of Na^+ efflux that is stimulated by *p*-nitrophenylphosphate is slightly greater than that of the metabolically stimulated efflux. From the results of experiments that were done for other purposes in our laboratory during the past few years, we have also compared the stimulation of efflux by *p*-nitrophenylphosphate in about 50 different preparations with the efflux that was stimulated by adenosine or inosine in over 100 similar ghost preparations. Although precise calculations were not done, it seemed clear to us that the stimulation by *p*-nitrophenylphosphate was always equal to or greater than the stimulation by metabolism.

2. In both cases Na^+ efflux is stimulated by the presence of K^+ (or a "not-sodium ion"[25]) in the medium. In the absence of external Na^+ the curve of activation of efflux as a function of K^+ concentration is hyperbolic in shape in both cases, and the concentration of K^+ at which the efflux is half-maximally stimulated is also the same (Fig. 2 and ref. 24). It is appropriate to point out here that at the moment we do not have sufficient data to show directly that the stimulation of Na^+ efflux by *p*-nitrophenylphosphate is accompanied by a simultaneous K^+ influx. But in view of the requirement of the efflux for external K^+ it is not unreasonable to assume that this efflux, like the physiological Na^+ efflux, represents an exchange process.

3. In both cases Na^+ efflux is inhibited by external Na^+. The quantitative aspects of Na^+ inhibition are, however, different. The stimulation by *p*-nitrophenylphosphate can be completely blocked by external Na^+, and this inhibition may be either completely or partially reversed by ATP added to the medium. In intact cells where the pump is driven by metabolism, complete inhibition of Na^+ efflux by external Na^+ is never observed. Only when low and suboptimal concentration of K^+ are used the inhibitory effects of external Na^+ becomes evident[24,26].

4. A corollary of the above different effects of external Na^+ is that if Na^+ efflux that is dependent on *p*-nitrophenylphosphate is to occur in a medium containing

physiological concentrations of Na^+, the presence of ATP in the medium is required. There is no such requirement when Na^+ efflux is being stimulated by ATP that is placed or generated on the matrix side of the membrane. This difference, however, may be more apparent than real. It is generally assumed, as we did in a previous paper[7], that in ATP-filled ghosts or intact cells ATP is located only within the cytoplasm. But the ATP-filled ghosts are prepared under conditions where the entire cell membrane (inside and outside) is exposed to high concentrations of ATP, and it is possible that even after several washings some ATP may remain bound to the outside surface of the membrane. Similarly, in the intact red cell it is not unreasonable to imagine that the external membrane surface may be exposed to ATP that is synthesized within the membrane. The possibility of ATP synthesis in association with, or within, the membrane phase of erythrocytes has been suggested repeatedly[27–29] and has become even more attractive since the findings of RONQUIST[23,30] which indicate that portions of triosephosphate dehydrogenase and phosphoglycerate kinase of the intact red cell are on the cell surface, and that these enzymes can accept substrates from the medium and release some ATP into the medium.

5. As we have already indicated our present data are not conclusive as to whether the efflux that is observed in the presence of *p*-nitrophenylphosphate is activated by internal Na^+. It is interesting to note, however, that in experiments very similar to those of Fig. 8, but in which Na^+ efflux was stimulated by substrate metabolism, evidence for the dependence of active efflux on internal Na^+ concentration was clearly obtained[10]. This means that either the Na^+ efflux that is stimulated by *p*-nitrophenylphosphate is independent of internal Na^+ concentration, or that this efflux is more sensitive to increasing internal Na^+ concentrations than the metabolically stimulated Na^+ efflux. The question of whether the response of the phosphatase-mediated Na^+ efflux to internal sodium is drastically different from that of the physiological Na^+ efflux must remain open.

6. The major difference between the Na^+ efflux that is stimulated by *p*-nitrophenylphosphate and the ATP-driven efflux for which no obvious explanation comes to mind is the different sensitivities of the two processes to external orthophosphate. The first process is inhibited by small concentrations of orthophosphate, but as far as we know there is no inhibition of active Na^+ efflux by external orthophosphate either in the intact cells or ATP-filled ghosts.

On the whole the above comparison indicates to us that the Na^+ efflux which is activated by *p*-nitrophenylphosphate is not merely a curious artifact, and that it indeed represents the operation of the classical coupled Na^+,K^+-pump. Therefore, we feel that any mechanism for the physiological manner of the operation of the pump through the (Na^+ + K^+)-activated ATPase must be consistent with the observations described here. It should be emphasized, however, that at the moment the present findings may be incorporated into several equally plausible models. The reason for this being that recent studies with the partially purified enzyme indicate that the relationship between the K^+-dependent *p*-nitrophenylphosphatase and the (Na^+ + K^+)-activated ATPase may be much more complicated than originally suspected[18,19,31,32]. It is not appropriate to discuss the various possible models here, but the essential features of the working hypothesis that we prefer to test may be summarized as follows:

1. Both Na^+ and K^+ translocations are associated with the K^+-dependent phosphatase "segment" of the ($Na^+ + K^+$)-activated ATPase complex.

2. The primary role of the ATP-protein transphosphorylation "segment" is the production of a substrate for the translocator phosphatase.

3. It is the substrate production step that is controlled by the internal Na^+ concentrations.

ACKNOWLEDGEMENTS

This investigation was supported by U.S. Public Health Service Grant HE-10884 from the National Heart Institute, and by a grant-in-aid from the New York Heart Association.

REFERENCES

1 G. GARDOS, *Acta Physiol. Hung.*, 6 (1954) 191.
2 J. F. HOFFMAN, *Federation Proc.*, 19 (1960) 127.
3 R. WHITTAM, *Nature*, 196 (1962) 134.
4 P. G. GARRAHAN AND I. M. GLYNN, *J. Physiol.*, 192 (1967) 217.
5 A. SEN AND R. L. POST, *J. Biol. Chem.*, 239 (1964) 345.
6 P. MITCHELL, *Advan. Enzymol.*, 29 (1967) 33.
7 A. ASKARI AND S. N. RAO, *Biochem. Biophys. Res. Commun.*, 36 (1969) 631.
8 A. ASKARI AND S. N. RAO, *Abstr., 14th Meeting Biophys. Soc., Baltimore, Md., 1970*, 12a.
9 A. ASKARI AND S. N. RAO, *J. Pharmacol. Exptl. Therap.*, 163 (1968) 407.
10 J. F. HOFFMAN, *J. Gen. Physiol.*, 45 (1962) 837.
11 A. ASKARI AND S. N. RAO, *J. Pharmacol. Exptl. Therap.*, 172 (1970) 211.
12 O. A. BESSEY, O. H. LOWRY AND M. J. BROCK, *J. Biol. Chem.*, 164 (1946) 321.
13 J. C. PARKER AND J. F. HOFFMAN, *J. Gen. Physiol.*, 50 (1967) 893.
14 P. J. GARRAHAN, M. I. POUCHAN AND A. F. REGA, *J. Physiol.*, 202 (1969) 305.
15 B. J. R. PITTS AND A. ASKARI, *Biochim. Biophys. Acta*, 227 (1971) 453.
16 H. YOSHIDA, T. NAGAI, T. OHASHI AND Y. NAKAGAWA, *Biochim. Biophys. Acta*, 171 (1969) 178.
17 A. ASKARI AND D. KOYAL, *Biochem. Biophys. Res. Commun.*, 32 (1968) 227.
18 D. KOYAL, S. N. RAO AND A. ASKARI, *Biochim. Biophys. Acta*, 225 (1971) 11.
19 A. ASKARI AND D. KOYAL, *Biochim. Biophys. Acta*, 225 (1971) 20.
20 A. ASKARI, *Biochim. Biophys. Acta*, 191 (1969) 198.
21 P. G. GARRAHAN, M. I. POUCHAN AND A. F. REGA, *J. Membrane Biol.*, 3 (1970) 26.
22 J. D. JUDAH, K. AHMED AND A. E. M. MCLEAN, *Biochim. Biophys. Acta*, 73 (1963) 527.
23 G. RONQUIST, *Acta Physiol. Scand.*, 74 (1968) 594.
24 R. N. PRIESTLAND AND R. WHITTAM, *Biochem. J.*, 109 (1968) 369.
25 R. L. POST, S. KUME, T. TOBIN, B. ORCUTT AND A. K. SEN, *J. Gen. Physiol.*, 54 (1969) 306s.
26 P. J. GARRAHAN AND I. M. GLYNN, *J. Physiol.*, 192 (1967) 175.
27 D. E. GREEN, E. MURER, H. O. HULTON, S. H. RICHARDSON, B. SALMON, G. P. BRIERLY AND H. BAUM, *Arch. Biochem. Biophys.*, 112 (1965) 635.
28 G. RONQUIST, *Acta Chem. Scand.*, 21 (1967) 1484.
29 S. L. SCHRIER, *Biochim. Biophys. Acta*, 135 (1967) 591.
30 G. RONQUIST, *Acta Physiol. Scand.*, 76 (1969) 312.
31 C. HEGYVARY AND R. L. POST, *Abstr., 14th Meeting Biophys. Soc., Baltimore, Md., 1970*, 11a.
32 J. D. ROBINSON, *Arch. Biochem. Biophys.*, 139 (1970) 164.
33 O. A. BESSEY AND R. H. LOVE, *J. Biol. Chem.*, 196 (1952) 175.
34 J. FUNDER AND J. O. WIETH, *Acta Physiol. Scand.*, 71 (1967) 105.