

# Membrane Compartmentalized ATP and Its Preferential Use by the Na,K-ATPase of Human Red Cell Ghosts

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**ABSTRACT** This paper describes work which begins to define the molecular organization in the region of the membrane that comprises the functional domain of the Na:K pump. The membrane-bound phosphoglycerate kinase (PGK) and Na,K-ATPase appear to be directly linked via a compartmentalized form of ATP. Evidence for the membrane pool of ATP is based on the labeling characteristics of the phosphoproteins by [ $\gamma$ - $^{32}\text{P}$ ]ATP of ghosts incubated under various conditions. Preincubation of ghosts in the presence of ATP at 37°C, but not at 0°C, completely obscures the formation of the Na-phosphoprotein in ghosts washed and subsequently incubated in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP. In contrast to the Na component, the Mg component of phosphorylation is only slightly altered by preincubation with ATP. ATPase activity measured as  $^{32}\text{P}_i$  liberated during the subsequent incubation at 0°C, reflects completely the differential effects of preincubation with ATP on  $^{32}\text{P}$  incorporation into phosphoprotein. ATP placed within the pool by preincubation can be removed by operating the Na,K-ATPase or the PGK reaction in the reverse direction by use of exogenous substrates. Alternatively, the membrane pool of ATP can be formed also from exogenous substrates by running the PGK reaction in the forward direction. These results, while providing direct support for a membrane compartment of ATP, also indicate the location of this compartment in relation to the PGK and the Na,K-ATPase. In addition, these results also imply that the Mg and Na components are different enzymatic entities since substrate ATP can be derived from separate sources.

## INTRODUCTION

In the course of experiments on the solubilization of human red cell ghost Na,K-ATPase, we noted that preincubation of membranes with nonradioactive ATP (followed by thorough washing) resulted in dissociation of the pattern of phosphorylation of the ghosts after exposure to gamma-labeled ATP by use of the procedure defined by Blostein (1968). Thus, while the Mg-dependent phosphorylation was unaffected by the preincubation, the Na-dependent fraction had disappeared. This result led us to postulate that the preincubation with ATP resulted in the filling of a membrane ATP pool which was closely related to the Na,K-ATPase but not to the Mg-ATPase. Preliminary accounts of this work have

been presented previously (Proverbio and Hoffman, 1972; Hoffman, 1973; Hoffman and Proverbio, 1974).

#### MATERIALS AND METHODS

##### *Preparation of Red Cell Ghosts*

Hemoglobin-free ghosts were prepared by the method of Hoffman and Ryan (see Heinz and Hoffman, 1965) from unwashed packed cells (20,000 g) and were stored at  $-20^{\circ}\text{C}$  in a solution which contained 15.3 mM NaCl, 1.7 mM Tris, and 0.1 mM EDTA (pH 7.4 at  $23^{\circ}\text{C}$ ).<sup>1</sup> To ready the ghosts for use, the frozen ghosts were thawed at room temperature and were then washed three times at  $4^{\circ}\text{C}$  with 20 vol of 17 mM Tris (pH 7.5 at  $4^{\circ}\text{C}$ ) by centrifugation at 15,000 g. After washing, the ghosts were concentrated (about 15 mg protein/ml) before being either preincubated (and/or) labeled as indicated below.

##### *Preincubation of Ghosts*

Preincubation refers to different treatments to which the ghosts were subjected before labeling with or exposure to either  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  or  $[8\text{-}^3\text{H}]\text{ATP}$ . Thus, ghosts prepared as described above were suspended in about 20 vol of an ice-cold solution which contained 40 mM either NaCl or choline Cl, 2 mM  $\text{MgCl}_2$ , 0.25 mM EDTA, and 10 mM Tris (pH 7.5 at  $0^{\circ}\text{C}$ ). In some experiments the solution also contained 20 mM KCl. The ghosts were then washed three times, at  $0^{\circ}\text{C}$ , by repeated centrifugation at 15,000 g for 5 min. The ghosts were then suspended in 10 vol of the same solution used in their washing but to which were added various substances, such as ATP, which are specified in connection with the relevant experiments, and the pH of the solution was adjusted according to the temperature of use. The ghost suspension was then placed in a reciprocating shaker bath and incubated for various periods (usually 30 min) and at different temperatures (usually  $0^{\circ}\text{C}$  and  $37^{\circ}\text{C}$ ). Upon the completion of the incubation period the ghosts were again washed four times at  $0^{\circ}\text{C}$  with 17 mM Tris (pH 7.5) as described before. At the end of the fourth wash the concentrated ghosts, which assumed different volumes depending on the treatment, were diluted about threefold relative to the control (no treatment) with 17 mM Tris (pH 7.5) and placed at  $0^{\circ}\text{C}$  ready for the next part of the experiment which involved incubation in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  or  $[8\text{-}^3\text{H}]\text{ATP}$ . It should be noted that even though the degree of packing of the ghosts varied depending upon the type of treatment, the ghosts were porous in the sense that under all circumstances they remained permeable to all added substrates; that is, no resealing of the ghosts occurred as a result of any pretreatment. Just why the relative density of the ghosts varied under the different conditions is not known.

It should be emphasized that the stated pH of the various media used throughout this paper refers to the pH ( $\pm 0.1$  pH unit) of that solution at the indicated temperature ( $\pm 2^{\circ}\text{C}$ ).

<sup>1</sup> *Abbreviations used in this paper:* ATP, ADP, AMP, adenosine tri-, di- and monophosphate; ATPase, adenosine triphosphatase; CTP, cytidine triphosphate; dATP, 2'-Deoxy-ATP; 1,3 DPG, 1,3-diphosphoglycerate; EDTA, ethylene diamine tetraacetic acid; GAP, triose-P, glyceraldehyde-3-phosphate; GAPD, glyceraldehyde phosphate dehydrogenase; GTP, guanosine triphosphate; ITP, inosine triphosphate; NAD<sup>+</sup>, NADH, oxidized and reduced forms of nicotinamide-adenine dinucleotide; PCA, perchloric acid; 3-PGA, 3-phosphoglycerate; PGK, phosphoglycerate kinase; P<sub>i</sub>, inorganic phosphate; SDS, Na dodecyl sulfate; TCA, trichloroacetic acid, UTP, uridine triphosphate. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., except for PCA, which was purchased from Allied Chemical Co., Morristown, N. J., TCA, from Merck and Co., Rahway, N. J., and CTP, from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

*Labeling of Ghosts with  $^{32}\text{P}$  or  $^3\text{H}$* 

In general the procedure outlined by Blostein (1968) was followed throughout. 0.35 ml of the ghost suspension (about 3–5 mg protein/ml) was incubated in Corex glass tubes (15 ml) in a final volume of 0.50 ml in a standard medium which contained in final concentration, unless otherwise stated, 2  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (approximately 10 Ci/mM and less than 2% hydrolyzed, kindly supplied by Dr. Paul Greengard), 12  $\mu\text{M}$   $\text{MgCl}_2$ , 10 mM Tris (pH 7.5). In addition the medium also contained either 50 mM NaCl or 50 mM choline Cl. The concentration of KCl when present was 10 mM. It should be understood that the 2  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP was comprised of 2  $\mu\text{M}$  Tris-ATP plus approximately  $10^6$  cpm [ $\gamma$ - $^{32}\text{P}$ ]ATP per 0.50 ml final suspension volume. In some experiments [ $8$ - $^3\text{H}$ ]ATP (approximately 5 Ci/mM, obtained from New England Nuclear, Boston, Mass.) was used in place of [ $\gamma$ - $^{32}\text{P}$ ]ATP in equivalent amounts. The purity of the [ $8$ - $^3\text{H}$ ]ATP was verified by thin layer chromatography with the method indicated below.

The labeling was carried out over a 15-s period which was started (zero time) by adding the ghost suspension (kept at 0°C) to the standard medium (also at 0°C) which contained either [ $\gamma$ - $^{32}\text{P}$ ]ATP or [ $8$ - $^3\text{H}$ ]ATP and either NaCl, choline Cl, or KCl. The reaction was stopped by the addition, with stirring, of 4.5 ml of an ice-cold solution which contained 5% trichloroacetic acid (TCA) + 0.1 mM  $\text{Na}_2\text{ATP}$  + 1.0 mM  $\text{H}_3\text{PO}_4$ . The TCA-insoluble fraction (denatured ghosts) was then washed four times with the same solution with an RC-3 Sorvall refrigerated centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.) (4,400 *g* for 5 min). After removal of the supernate the washed ghosts were solubilized with 1.5 ml NCS (Nuclear Chicago Solubilizer, Amersham/Searle Corp., Arlington Heights, Ill.) and quantitatively transferred to a counting vial by pipette with the use, as diluent, of the counting solution (toluene containing appropriate amounts of PPO and POPOP) to a total volume of 12 ml. The vial was then counted by liquid scintillation (Nuclear Chicago). Total protein was determined on samples of the original suspension of ghosts by the method of Lowry et al. (1951) with crystalline bovine serum albumin (Miles Laboratories, Inc.) used as standard. Controls were also run in order to evaluate the effects of different medium ingredients on the protein determination.

In some experiments involving the use of [ $8$ - $^3\text{H}$ ]ATP the labeling procedure was altered so that the relative specific activity of the [ $8$ - $^3\text{H}$ ]ATP as well as the temperature and length of incubation was different from that stated above. The detailed circumstances in which these altered conditions were used are given in the text in connection with the relevant experiments.

*ATPase Activity*

ATP hydrolysis was determined by using the charcoal method previously described (Heinz and Hoffman, 1965). The incubation conditions and medium used for measuring ATPase activity were identical to those used for the  $^{32}\text{P}$  labeling studies except that the incubation time at 0°C was, as suggested by Blostein (1968), extended to 20 min. Thus, ghosts were incubated with 2  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP + 12  $\mu\text{M}$   $\text{MgCl}_2$  + 10 mM Tris (pH 7.5). In addition, either 50 mM choline Cl or NaCl was included in the incubation medium together with 10 mM KCl when desired in order to estimate, respectively, Mg-ATPase, Na-ATPase, and Na,K-ATPase. ATPase activity was stopped by adding to 0.5 ml of reaction mixture 4.5 ml chilled 5% TCA.  $^{32}\text{P}$  in cpm was determined on the supernate, after centrifugation for 10 min at 3,600 *g*, before and after the addition (with subsequent centrifugation) of 1.2 ml ( $1/4$  teaspoon) activated charcoal (Merck). Since charcoal removes unhydrolyzed [ $\gamma$ - $^{32}\text{P}$ ]ATP, the ATPase activity was measured by the rate of  $^{32}\text{P}_i$  appearance (Crane and Lipmann, 1953). All samples were counted in Bray's solution (Bray, 1960) by liquid scintillation with a counting efficiency of approximately 95%.

Fluorometric methods were used for the enzymatic determination of ATP (Bergmeyer, 1965, p. 551) and ADP and AMP (Bergmeyer, 1965, p. 573) under the specific conditions as described in the references. Thin layer chromatography was also used for separating ATP, ADP, and AMP according to the method of Pataki (1967) that utilizes cellulose "chromogram" sheets obtained from Eastman Kodak (Rochester, N. Y.) (6065 cellulose with fluorescent indicator).

The forward and back reactions of the triose phosphate dehydrogenase-phosphoglycerate kinase system were carried out according to the methods of Bücher (1955) employing the conditions specified in the appropriate legends given in Results.

All experiments were carried out at least twice with duplicate samples analyzed.

## RESULTS

### *Membrane Phosphorylation and ATPase Activity*

Table I shows that the ghost system used in the present study exhibits character-

TABLE I  
ATPase ACTIVITY AND  $^{32}\text{P}$  INCORPORATION DURING INCUBATION WITH  
 $2 \mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  AT  $0^\circ\text{C}$

Incubation conditions	ATPase (pmol/mg protein $\times$ h)	$^{32}\text{P}$ Incorporation (pmol/mg protein)			
		at 15 s	at 30 s		
			Control	+EDTA at 15 s	+ATP at 15 s
Mg	181	0.67	0.68	0.37	0.23
Mg+Na	301	1.21	1.25	0.67	0.40
Mg+Na+K	291	0.71	—	—	—

Ghost preparation and incubation conditions carried out as specified in Materials and Methods. ATPase estimated by  $^{32}\text{P}$  liberation after 20 min of incubation.  $^{32}\text{P}$  uptake measured on separate samples after 15 and 30 s of incubation, stopping the reactions by the addition of TCA. 0.05-ml additions were made at the end of the initial 15-s incubation period to give a final concentration of either 10 mM Tris (control), 10 mM EDTA, or 150  $\mu\text{M}$  nonradioactive Tris-ATP + 150  $\mu\text{M}$   $\text{MgCl}_2$  (pH 7.5). Incubation was then continued for another 15 s before analysis for incorporated  $^{32}\text{P}$ .

istics the same as those previously established by Blostein (1968, 1970). Thus, Na acts to increase the rate of hydrolysis of ATP as well as to increase the amount of  $^{32}\text{P}$  that can be incorporated into the membranes after 15 s of incubation with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . The addition of K inhibits the Na-stimulated increment in incorporated  $^{32}\text{P}$  even though K has no effect on the activity of the ATPase obtained in the presence of Na. The effect of K on decreasing the amount of the Na-stimulated component of  $^{32}\text{P}$  incorporation is presumably due to K catalyzing the rate of dephosphorylation (Post et al., 1969). The inability of K to increase further the ATPase activity above that obtained with Na alone is probably related to the low concentration of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  used in its assay (Blostein, 1968) and is considered in a separate publication.<sup>2</sup>

It is convenient for subsequent reference to call the Na-stimulated component of  $^{32}\text{P}$  incorporation the *Na component* in order to distinguish this component from the *Mg component* or the amount of  $^{32}\text{P}$  incorporated in the presence of

<sup>2</sup> Proverbio, F., and J. F. Hoffman. Manuscript in preparation.

Mg alone. The size of the Na component is taken as the difference between the amount of labeling obtained with (Na + Mg) and with (Mg) alone. In an analogous way the total ATPase can be divided into a *Na-ATPase* component and a *Mg-ATPase* component, depending upon the presence or absence of Na in the incubation medium.

The lability of the  $^{32}\text{P}$  incorporated under the various circumstances becomes apparent upon the addition of an excess of nonradioactive ATP or of EDTA to the reaction mixture after 15 s of incubation. As shown in Table I the levels of  $^{32}\text{P}$  contained within the ghosts decrease, in contrast to the controls, during a subsequent 15-s period of incubation when either nonradioactive ATP or EDTA is added in the presence or absence of Na. Thus, subsequent incubation with nonradioactive ATP indicates the turnover of the incorporated  $^{32}\text{P}$  while incubation with EDTA results in dephosphorylating the system, presumably by tying up Mg required for the phosphorylation reaction (Blostein, 1968).

*Effect of Preincubation on the Subsequent Incorporation of  $^{32}\text{P}$*

The basis for the present paper begins with the observation that the formation of the Na-stimulated component of  $^{32}\text{P}$  incorporation can be completely inhibited by preincubation of the ghosts at 37°C in the presence of nonradioactive ATP. This is shown in Table II. Thus ghosts were preincubated under the various conditions shown for 30 min at either 0°C or 37°C. Subsequently, ghosts were thoroughly washed (see Materials and Methods) to remove all bulk solution ATP before incubation with [ $\gamma$ - $^{32}\text{P}$ ]ATP under standard conditions in order to measure the level of  $^{32}\text{P}$  incorporation into the Mg component compared with the Na component. It is apparent that the Na component was absent when nonradioactive ATP was present in the preincubation medium and when the preincubation was carried out at 37°C.

While there are a number of alternative explanations for this observed effect, the remainder of this paper is concerned with demonstrating that the reason that  $^{32}\text{P}$  incorporation into the Na component is inhibited is the presence of a membrane pool of ATP. ATP can gain access to this membrane compartment (pool) only during incubation at 37°C and not during incubation at 0°C. Once in the membrane pool ATP can be utilized at 0°C and affects predominantly the Na component and not the Mg component of phosphorylation. This means that the Na component would use preferentially the membrane pool of nonradioactive ATP while the Mg component would derive its ATP primarily from the bulk solution [ $\gamma$ - $^{32}\text{P}$ ]ATP. Therefore, even though the Na component is undergoing phosphorylation, it is not being labeled with  $^{32}\text{P}$  because it cannot use, at 0°C, bulk [ $\gamma$ - $^{32}\text{P}$ ]ATP. The Mg component becomes labeled with  $^{32}\text{P}$  since it draws mainly on bulk ATP. It is possible that the failure to get  $^{32}\text{P}$  into the Na component at 0°C could be due to the mixing of bulk [ $\gamma$ - $^{32}\text{P}$ ]ATP with the nonradioactive ATP in the membrane pool, thereby reducing its specific activity to such an extent that no  $^{32}\text{P}$  could appear to be incorporated into the membrane protein. But since this alternative requires that the Na component can be labeled only via the membrane pool this possibility can be eliminated on the basis, as shown in Table II, that the Na component can be labeled at 0°C without going

through the membrane pool, since the membrane pool route is open and accessible only at 37°C. (Alternatively, if nonradioactive ATP bound during preincubation were released during the labeling incubation with [ $\gamma$ - $^{32}$ P]ATP, it would change the specific activity of the bulk [ $\gamma$ - $^{32}$ P]ATP and would therefore affect the Mg-component the same as the Na-component). Thus it is necessary to suppose that the Na component, at 0°C, can use *either* bulk *or* membrane pool-located ATP, but if ATP is present in the membrane pool, previously loaded by preincubation at 37°C, then the preference for the pool source overrides and therefore masks any  $^{32}$ P incorporation from the bulk solution.

TABLE II  
EFFECT OF PREINCUBATION IN THE PRESENCE OF Mg, Na AND ATP ON  
THE SUBSEQUENT INCORPORATION OF  $^{32}$ P FROM [ $\gamma$ - $^{32}$ P]ATP

Preincubation condition		$^{32}$ P Incorporation (pmol/mg protein)		
Medium	°C	Incubation media		
		Mg	Na + Mg	$\Delta$
No preincubation		1.01 $\pm$ 0.02	1.76 $\pm$ 0.16	0.75
Mg	0	0.67 $\pm$ 0.07	1.29 $\pm$ 0.10	0.62
Mg+ATP	0	0.44 $\pm$ 0.06	1.01 $\pm$ 0.16	0.57
Mg+Na	0	0.68 $\pm$ 0.04	1.20 $\pm$ 0.14	0.52
Mg+Na+ATP	0	0.47 $\pm$ 0.02	0.94 $\pm$ 0.13	0.47
Mg	37	0.43 $\pm$ 0.04	0.98 $\pm$ 0.13	0.55
Mg+ATP	37	0.34 $\pm$ 0.06	0.38 $\pm$ 0.08	0.04
Mg+Na	37	0.56 $\pm$ 0.06	1.03 $\pm$ 0.14	0.47
Mg+Na+ATP	37	0.36 $\pm$ 0.08	0.38 $\pm$ 0.07	0.02

Preincubation was carried out for 30 min at either 0°C or 37°C in a medium which contained 10 mM Tris (pH 7.5) + 0.25 mM EDTA and when indicated with 2 mM MgCl<sub>2</sub>, 1.5 mM Tris ATP, and 40 mM NaCl. When Na was absent, 40 mM choline Cl was added instead. After preincubation, the ghosts were thoroughly washed at 0°C with 17 mM Tris (pH 7.5) before incubation with [ $\gamma$ - $^{32}$ P]ATP to study  $^{32}$ P incorporation in the presence of Mg or Na + Mg as described in the legend to Table I and in Materials and Methods. The difference ( $\Delta$ ) between the columns headed Na + Mg and Mg measures the  $^{32}$ P incorporated into the Na component. The average values are given  $\pm$  SEM where  $n = 19$ .

Since the conditions of preincubation determine the subsequent characteristics of  $^{32}$ P incorporation it was of interest to study the effects of certain variations in these conditions. For instance, as shown in Table II, the presence of Na during preincubation did not alter the preincubation effect provided Mg and ATP were present. However, Table III shows that Mg was required since preincubation of the ghosts in the presence of ATP alone did not prevent or alter the rate of incorporation of  $^{32}$ P into the Na component.

As already indicated in Table II the temperature of preincubation has a marked effect on the incorporation of  $^{32}$ P into the Na component. Table IV shows the effects of changing the temperature of preincubation from 0°C to 37°C. It is apparent again that preincubation with ATP suppresses the subsequent incorporation of  $^{32}$ P into the Na component and that this effect occurs only at 37°C. Perhaps effects of preincubation on  $^{32}$ P incorporation could be

demonstrated at lower temperatures by extending the time of preincubation and/or increasing the concentration of MgATP, but this has not been evaluated. If the idea is correct that a membrane pool of ATP is established by preincubation it is obscure why access to it has the temperature dependence noted in Table IV.

To test the specificity of the nucleotide requirement for the effect of preincubation on incorporation of  $^{32}\text{P}$  into the Na component, ghosts were preincubated

TABLE III  
THE EFFECT OF Mg ON THE PREINCUBATION EFFECT OF ATP IN ALTERING THE SUBSEQUENT INCORPORATION OF  $^{32}\text{P}$  INTO THE Na COMPONENT

Preincubation condition		$^{32}\text{P}$ Incorporation (pmol/mg protein)		
Medium	°C	Incubation media		
		Mg	Na+Mg	$\Delta$
ATP+Mg	37	0.58	0.64	0.06
ATP	37	0.46	0.78	0.32

Preincubation conditions, except for the variations noted were carried out as described in the legend to Table II and in Materials and Methods.

TABLE IV  
THE EFFECT OF THE TEMPERATURE OF PREINCUBATION ON THE SUBSEQUENT INCORPORATION OF  $^{32}\text{P}$  FROM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  INTO THE Na COMPONENT ( $\Delta$ )

Preincubation temperature °C	$^{32}\text{P}$ Incorporation (pmol/mg protein)		
	Incubation media		
	Mg	Na+Mg	$\Delta$
0	0.68±0.06	1.19±0.05	0.51
7	0.58±0.04	1.00±0.08	0.42
17	0.56±0.01	1.03±0.03	0.47
27	0.53±0.02	0.95±0.01	0.42
37	0.58±0.07	0.64±0.05	0.06

Preincubation carried out for 30 min in the presence of Mg + ATP as described in legend to Table II and in Materials and Methods. The average values are given  $\pm$  SEM where  $n = 6$ .

in the presence of different nucleotide triphosphates or some of their breakdown products such as ADP, nucleosides, purines, and  $\text{P}_i$ . As shown in Fig. 1, the incorporation of  $^{32}\text{P}$  into the Na component is affected only when the preincubation is carried out with ATP. This is so despite considerable variation in the total amount of  $^{32}\text{P}$  incorporated into the two different components. This type of specificity parallels completely the ATP specificity of the Na:K pump (or the Na,K-ATPase) previously found for human red cell ghosts (Hoffman, 1962). The result with ADP is also consistent with the fact that the low level of adenylate kinase activity that has been found to be present in the type of ghost preparation used in this study (Parker and Hoffman, 1967) is inadequate in generating sufficient ATP to affect incorporation of  $^{32}\text{P}$  into the Na component (cf. Fig. 4).

*Effect of Preincubation on the Subsequent Hydrolysis of ATP*

Since, as discussed in relation to Table I, there is turnover of the phosphorylated intermediate of  $^{32}\text{P}$  into the Na component, it was of interest to see if there was a comparable effect of preincubation on the subsequent utilization of ATP as assayed with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . ATPase activity can be studied under the same experimental circumstances as the incorporation of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  by increasing the time of incubation at  $0^\circ\text{C}$  from 15 s to 20 min and by measuring the

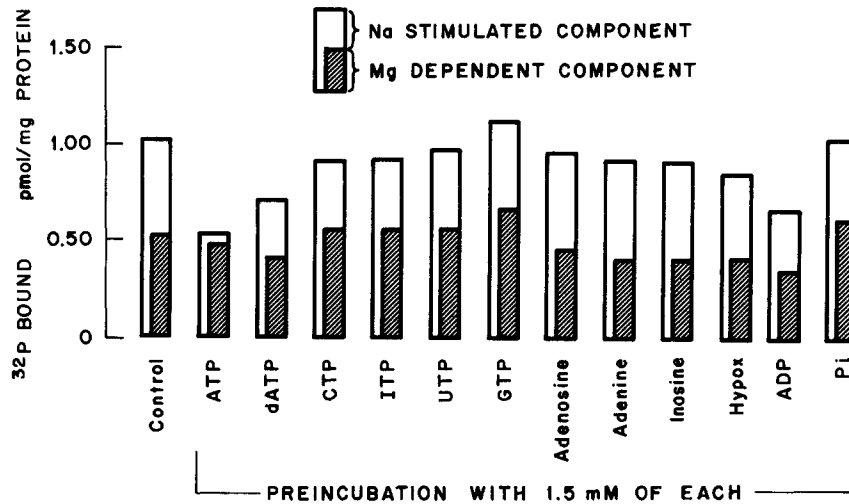


FIGURE 1. The effect of preincubation in the presence of different substances on the subsequent incorporation of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Preincubation was carried out for 30 min at  $37^\circ\text{C}$  in a medium containing 1.5 mM of each of the substances indicated together with 2 mM  $\text{MgCl}_2$ , 40 mM choline Cl, 0.25 mM EDTA, and 10 mM Tris (pH 7.5). Sodium salts of the nucleotides and  $\text{H}_3\text{PO}_4$  ( $\text{P}_i$ ) were used. Hypox stands for hypoxanthine. Preincubations and labeling with  $^{32}\text{P}$  were carried out as described in Materials and Methods. The total height of each bar represents the  $^{32}\text{P}$  bound in the presence of Na + Mg. The shaded portion of each bar represents the  $^{32}\text{P}$  incorporated in the presence of Mg alone (Mg-stimulated component) while the difference represents the  $^{32}\text{P}$  incorporated into the Na component as indicated in the inset. The variability shown in the amounts of  $^{32}\text{P}$  incorporated into the two components is due in part to the fact that this figure represents a composite of several different experiments.

appearance of  $^{32}\text{P}_i$  in the medium (Blostein, 1968). The results as shown in Table V on ATPase activity parallel completely the results presented in Table II on  $^{32}\text{P}$  incorporation. Thus, only the activity of the Na-ATPase is suppressed by preincubation with ATP and this effect, like incorporation of  $^{32}\text{P}$  into the Na component, can be obtained only when the preincubation is carried out at  $37^\circ\text{C}$ . The fact that preincubation at  $37^\circ\text{C}$  reduced the Na-ATPase activity by about 40% did not alter the differential effects of the different preincubation conditions.

*Time-Course of Membrane Phosphorylation*

The time-course of  $^{32}\text{P}$  incorporation into ghosts was studied after preincubation under different conditions in order to compare the pattern of  $^{32}\text{P}$  incorporation



into the two different phosphoprotein components. (The time period chosen was the same [20 min] as used in the ATPase assay.) Fig. 2 shows the time-course for  $^{32}\text{P}$  incorporation from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  into the Na and Mg components when the preincubation was carried out at  $0^\circ\text{C}$  in the presence of different concentrations of ATP or when there was no preincubation at all. As before (Table II), the Na component was not affected by the conditions of preincubation but there was significant incorporation of  $^{32}\text{P}$  into the Mg component which increased with time of incubation and which was inhibited by ATP in rough proportion to its concentration in the preincubation medium. Fig. 3 shows again that in using control conditions of preincubation at  $37^\circ\text{C}$  (see Table II) the incorporation of  $^{32}\text{P}$  into the Na-component is unaffected by preincubation in the presence of Mg

TABLE V  
THE EFFECT OF PREINCUBATION IN THE PRESENCE OF Mg, Na AND ATP  
ON SUBSEQUENT HYDROLYSIS OF ATP

Preincubation condition		ATP Hydrolysis (pmol/mg protein $\times$ h)		
Medium	$^\circ\text{C}$	Incubation media		
		Mg	Na+Mg	$\Delta$
No preincubation		200 $\pm$ 8	290 $\pm$ 12	90
Mg	0	232 $\pm$ 3	314 $\pm$ 2	82
Mg+ATP	0	182 $\pm$ 1	258 $\pm$ 2	76
Mg+Na+ATP	0	184 $\pm$ 1	280 $\pm$ 2	96
Mg	37	182 $\pm$ 3	228 $\pm$ 2	46
Mg+ATP	37	174 $\pm$ 1	172 $\pm$ 5	-2
Mg+Na	37	200 $\pm$ 6	248 $\pm$ 2	48
Mg+Na+ATP	37	188 $\pm$ 2	188 $\pm$ 4	0

Preincubation and ATPase activity carried out as described in Materials and Methods. Ghosts were thoroughly washed after preincubation and before ATPase assay. ATPase activity was measured as  $^{32}\text{P}$  liberated from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  after incubation for 20 min at  $0^\circ\text{C}$ . The difference ( $\Delta$ ) between columns headed Na + Mg and Mg measures the ATPase activity associated with the Na-ATPase. The average values are given  $\pm$ SEM where  $n = 6$ .

or Mg + Na but that incorporation of  $^{32}\text{P}$  into the Mg component increases very similarly to the rate observed when the preincubation was carried out at  $0^\circ\text{C}$  in the absence of ATP. The results presented in Fig. 4 show that when preincubation was carried out at  $37^\circ\text{C}$  in the presence of different concentrations of ATP, ATP decreased incorporation of  $^{32}\text{P}$  into both the Mg component and the Na component. The effects of preincubation with ATP on the Mg component are similar whether the ghosts are preincubated at  $0^\circ\text{C}$  or at  $37^\circ\text{C}$  (compare Fig. 2). Thus while it is not known what factors are responsible for the time-dependent increase in  $^{32}\text{P}$  incorporation into the Mg component (perhaps due to nonspecific binding of  $^{32}\text{P}_i$  liberated during the incubation), the observed effects seem to be independent of the temperature of preincubation. This contrasts sharply with the preincubation temperature and ATP-dependent effects associated with incorporation of  $^{32}\text{P}$  into the Na component, in addition to the fact that labeling of the Na component is evidently saturated by 60-s incubation. It is also of

interest, as shown in Fig. 4, that the suppression of  $^{32}\text{P}$  incorporation into the Na component is dependent upon the concentration of ATP present during preincubation. This indicates that the amount of ATP in the membrane pool can be varied by controlling the preincubation conditions, and we shall consider below

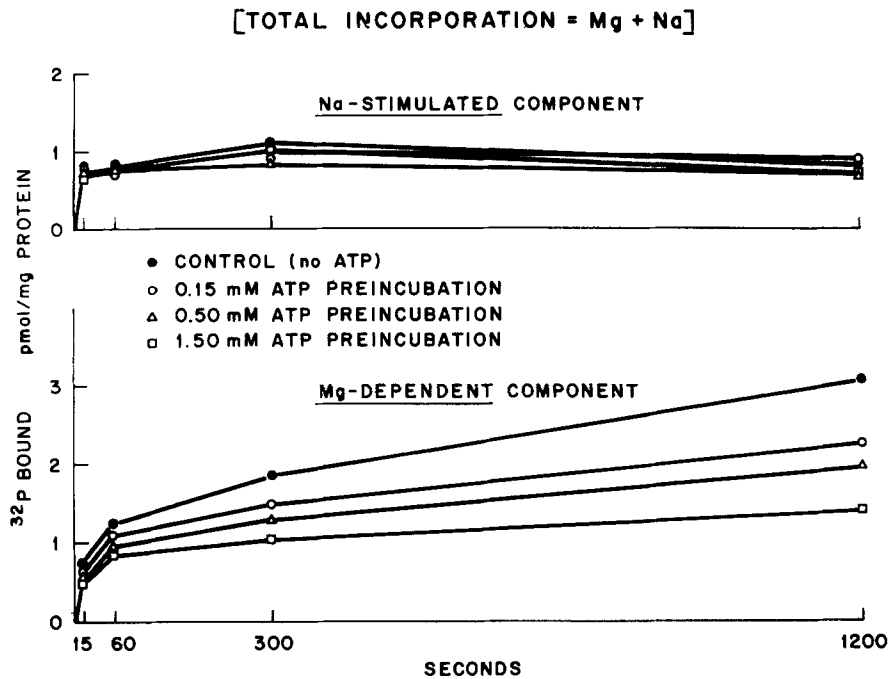


FIGURE 2. Time course of  $^{32}\text{P}$  incorporation from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  at  $0^\circ\text{C}$  into ghosts preincubated for 30 min at  $0^\circ\text{C}$  with different concentrations of ATP. Preincubation and  $^{32}\text{P}$  incorporation were carried out as described in Materials and Methods except that the concentration of ATP in the preincubation medium was varied as indicated (zero, ●; 0.15 mM ATP, ○; 0.50 mM ATP, △; and 1.5 mM ATP, □) with the concentration of  $\text{MgCl}_2$  held constant at 2 mM and that  $^{32}\text{P}$  incorporation was accessed at 15, 60, 300, and 1,200 s. As before, the ghosts were thoroughly washed after preincubation before the beginning of the incubation to measure  $^{32}\text{P}$  incorporation. The upper and lower panels separate the incorporation due to the Na component from the Mg component, respectively, recognizing that the Na component is obtained from the difference between the incorporation obtained in the presence of Na + Mg and that obtained with Mg alone.

the effects of this type of manipulation on incorporation of  $^{32}\text{P}$  into the Na component.

#### *Uptake and Lability of $[8\text{-}^3\text{H}]\text{ATP}$ Binding to Ghosts*

It was of interest to estimate the fraction of  $^{32}\text{P}$  taken up by ghosts which could be due to bound nucleotide rather than incorporation into membrane phosphoprotein. To estimate this fraction, ghosts were incubated with  $2\ \mu\text{M}$   $[8\text{-}^3\text{H}]\text{ATP}$  and

the rate of incorporation of  $^3\text{H}$  was compared directly with the rate of incorporation of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  into ghosts incubated under identical conditions. The results are shown in Table VI. While the incorporation of  $^{32}\text{P}$  followed the same pattern as found previously (see Table I and Fig. 2), the incorporation of

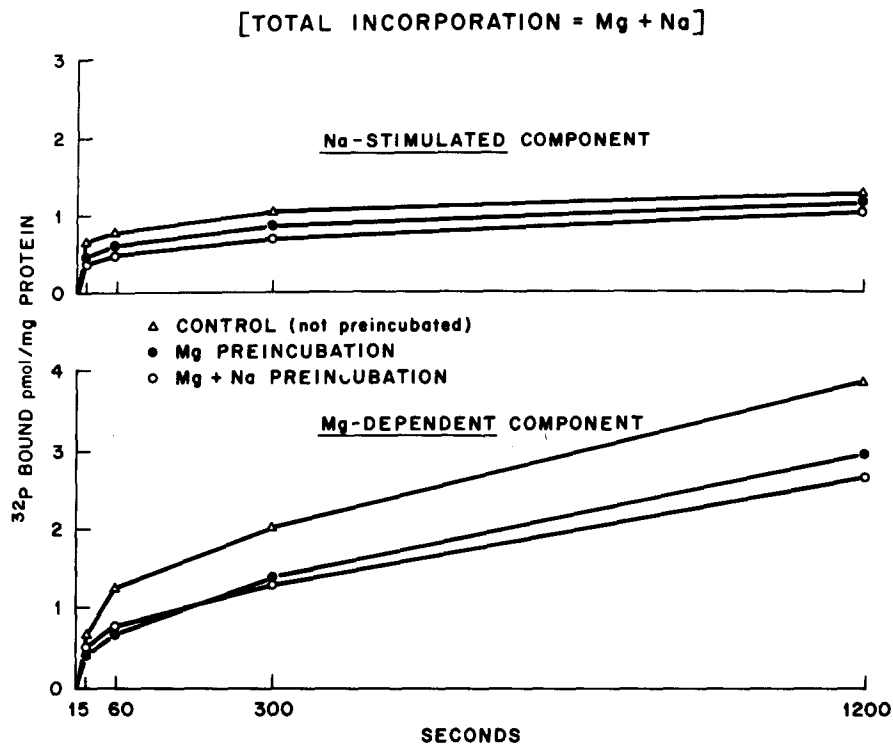


FIGURE 3. Time course of  $^{32}\text{P}$  incorporation from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  during incubation at  $0^\circ\text{C}$  into ghosts preincubated for 30 min at  $37^\circ\text{C}$  in the presence of either Mg or Na + Mg. Preincubation and  $^{32}\text{P}$  incorporation carried out as described in Materials and Methods except that the incorporation of  $^{32}\text{P}$  into ghosts was measured at 15, 60, 300, and 1,200 s as indicated. The concentration of Mg in the preincubation was 2 mM and contained either 40 mM choline Cl (Mg alone, ●) or 40 mM NaCl (Na + Mg, ○). As before the ghosts were thoroughly washed after preincubation before the beginning of the incubation to measure  $^{32}\text{P}$  incorporation. The distinction between the upper and lower panels is the same as described in the legend to Fig. 2.

$[\text{8-}^3\text{H}]\text{ATP}$  was found to reach a maximum level at 15 s of incubation. Furthermore, the amount of incorporated  $^3\text{H}$  during this time was not affected by the presence or absence of Na or Na + K in the medium. That the bound nucleotide (taken as  $^3\text{H}$  content) is labile can be demonstrated by carrying out an experiment comparable in design to that presented in Table I but using  $[\text{8-}^3\text{H}]\text{ATP}$  instead of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . As shown in Table VII the addition of either EDTA or nonradioactive ATP decreases, by about 50% in 45 s, the amount of  $^3\text{H}$  associ-

ated with the ghosts, presumably by complexation with Mg or by altering the specific activity of the bound nucleotide, respectively (as discussed in connection with the results presented in Table I). The lability of the bound nucleotide is also not affected by Na. Since the quantity of  $^3\text{H}$  bound is small (approximately 10% of the  $^{32}\text{P}$  that can be incorporated into ghosts in the presence of Na + Mg),

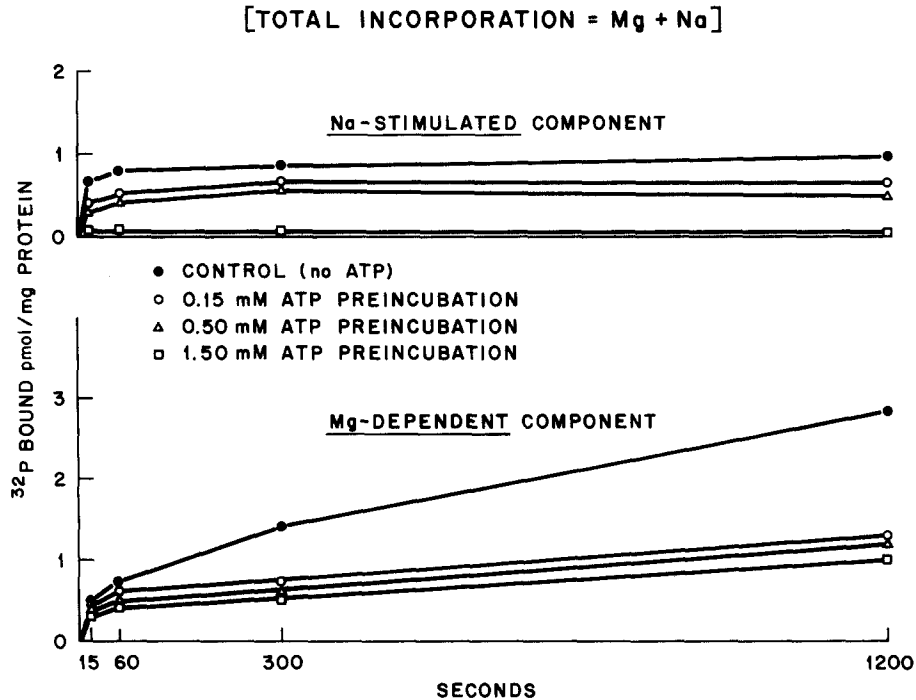


FIGURE 4. Time course of  $^{32}\text{P}$  incorporation from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  during incubation at  $0^\circ\text{C}$  into ghosts preincubated for 30 min at  $37^\circ\text{C}$  with different concentrations of ATP. Preincubation and  $^{32}\text{P}$  incorporation were carried out as described in Materials and Methods except that the concentration of ATP was varied as indicated (zero,  $\circ$ ; 0.15 mM ATP,  $\circ$ ; 0.50 mM ATP,  $\triangle$ ; and 1.5 mM ATP,  $\square$ ) with the concentration of  $\text{MgCl}_2$  held constant at 2 mM and that  $^{32}\text{P}$  incorporation was assessed at 15, 60, 300, and 1,200 s. As before, the ghosts were thoroughly washed after preincubation before the beginning of the incubation to measure  $^{32}\text{P}$  incorporation. The upper and lower panels have the same meaning as described in the legend of Fig. 2.

these findings imply that the major component of the bound  $^{32}\text{P}$  represents formation of phosphoprotein from the terminal phosphate of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , consistent with previous conclusions (Blostein, 1968; Knauf et al., 1974). In addition, if the bound  $^3\text{H}$  is assumed to represent bound nucleotide, the quantity present after TCA treatment is much too small to represent ATP in the membrane pool since only a single molecule of nucleotide would be bound per 10 or so glycoside binding sites (pumps).

*Unloading the Membrane Pool of ATP*

To see if the effect of preincubation with ATP could be reversed, preincubated ghosts were pretreated in different ways before we measured in the usual way the incorporation of  $^{32}\text{P}$  into the Na component from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . The results of

TABLE VI  
COMPARISONS OF TIME COURSE AND EXTENT OF  $^{32}\text{P}$  INCORPORATION FROM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  WITH  $[8\text{-}^3\text{H}]\text{ATP}$  BINDING TO GHOSTS UNDER THE SAME CONDITIONS

Seconds incubated	$^{32}\text{P}$ and $^3\text{H}$ content (pmol/mg protein)					
	Incubation media					
	Mg		Mg+Na		Mg+Na+K	
	$^{32}\text{P}$	$^3\text{H}$	$^{32}\text{P}$	$^3\text{H}$	$^{32}\text{P}$	$^3\text{H}$
15	0.54	0.11	1.05	0.11	0.51	0.11
60	0.80	0.13	1.50	0.11	0.77	0.12
300	1.32	0.14	2.33	0.13	1.36	0.13
1,200	2.54	0.13	3.44	0.14	2.52	0.13

Ghosts not preincubated. Ghosts incubated as described in Materials and Methods with either  $2\ \mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  or  $[8\text{-}^3\text{H}]\text{ATP} + 12\ \mu\text{M}$   $\text{MgCl}_2 + 10\ \text{mM}$  Tris (pH 7.5) together with either  $50\ \text{mM}$  NaCl or  $50\ \text{mM}$  choline Cl. When present,  $20\ \text{mM}$  KCl was added to the incubation medium. At the indicated times, the reaction was stopped by the addition of TCA and the ghosts processed for  $^{32}\text{P}$  and  $^3\text{H}$  content. Comparison is made of the uptake of  $^{32}\text{P}$  and  $^3\text{H}$  into ghosts when either Mg, Mg + Na or Mg + Na + K are present in the incubation medium.

TABLE VII  
INCORPORATION AND TURNOVER OF  $[8\text{-}^3\text{H}]\text{ATP}$  IN GHOSTS INCUBATED AT  $0^\circ\text{C}$  WITH  $2\ \mu\text{M}$   $[8\text{-}^3\text{H}]\text{ATP}$  AS DESCRIBED IN MATERIALS AND METHODS

Incubation conditions	$[8\text{-}^3\text{H}]\text{ATP}$ uptake (pmol/mg protein)			
	at 15 s	at 60 s		
		Control	+EDTA at 15 s	+ATP at 15 s
Mg	$0.09 \pm 0.002$	$0.09 \pm 0.002$	$0.05 \pm 0.001$	$0.04 \pm 0.005$
Mg+Na	$0.10 \pm 0.004$	$0.10 \pm 0.003$	$0.06 \pm 0.001$	$0.05 \pm 0.005$

The incubation medium also contained  $12\ \mu\text{M}$   $\text{MgCl}_2$ ,  $10\ \text{mM}$  Tris (pH 7.5) together with either  $50\ \text{mM}$  choline Cl (Mg medium) or  $50\ \text{mM}$  NaCl (Mg + Na medium).  $[8\text{-}^3\text{H}]\text{ATP}$  content was measured on separate samples after 15 and 60 s of incubation using TCA to stop the reactions. 0.05-ml additions were made at the end of the initial 15-s incubation period to give a final concentration of either  $10\ \text{mM}$  Tris (control),  $10\ \text{mM}$  EDTA, or  $150\ \mu\text{M}$  nonradioactive Tris ATP +  $150\ \mu\text{M}$   $\text{MgCl}_2$ . Incubation was then continued for another 45 s before analysis for bound  $^3\text{H}$ . The average values are given  $\pm$  SEM where  $n = 4$ .

experiments in which the membrane pool of ATP was depleted by reincubation at  $37^\circ\text{C}$  are shown in Tables VIII and IX. The general plan of this type of experiment is first to load the membrane pool with ATP, followed by thorough washing of the ghosts to remove bulk ATP, as before. Second, the ghosts are then resuspended in media of varying composition and reincubated at  $37^\circ\text{C}$ . The

TABLE VIII  
DEPLETION OF THE MEMBRANE POOL OF ITS ATP BY INCUBATION AT  
37°C

First preincubation 30 min, 37°C Medium	Second preincubation 90 min, 37°C Medium	<sup>32</sup> P incorporation (pmol/mg protein)		
		Incubation media		
		Mg	Na+Mg	Δ
Mg	Not preincubated	0.65	1.20	0.55
Mg+ATP	Not preincubated	0.62	0.65	0.03
Mg	Tris 17 mM	0.50	0.90	0.40
Mg+ATP	Tris 17 mM	0.62	1.00	0.38

Ghosts were preincubated twice before the subsequent incorporation of <sup>32</sup>P from [ $\gamma$ -<sup>32</sup>P]ATP was determined. The first preincubation was carried out in the usual way (see legend, Table II) by incubating the ghosts at 37°C for 30 min in the presence of 2 mM MgCl<sub>2</sub> ± 1.5 mM Tris ATP. The ghosts were then thoroughly washed at 0°C as before with 17 mM Tris (pH 7.5) before being preincubated a second time. The second preincubation of ghosts was carried out at 37°C for 90 min with 17 mM Tris (pH 7.5) as the suspension medium. The control ghost suspensions (not preincubated) were handled in the same way but kept at 0°C during the second preincubation. At the end of the second preincubation, the ghosts were again washed with 17 mM Tris and then incubated with [ $\gamma$ -<sup>32</sup>P]ATP to estimate <sup>32</sup>P incorporation in the presence of Mg or Mg + Na, as described in the legend to Table I and in Materials and Methods. The difference ( $\Delta$ ) between the columns headed Na + Mg measures the <sup>32</sup>P incorporated into the Na component.

TABLE IX  
ACCELERATING THE RATE OF DEPLETION OF THE MEMBRANE POOL OF  
ITS ATP BY INCUBATION WITH Na + K

First preincubation 30 min, 37°C Medium	Second preincubation 15 min, 37°C Medium	<sup>32</sup> P incorporation (pmol/mg protein)		
		Incubation media		
		Mg	Na + Mg	Δ
Mg+ATP	Not preincubated	0.63±0.04	0.64±0.08	0.01
Mg+ATP	Mg	0.65±0.02	0.67±0.03	0.02
Mg+ATP	Mg+Na	0.57±0.03	0.62±0.05	0.05
Mg+ATP	Mg+Na+K	0.69±0.04	1.00±0.06	0.31

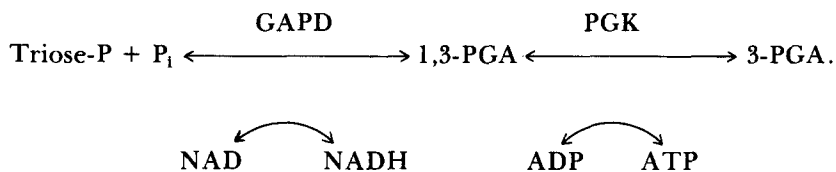
Ghosts were preincubated twice before the subsequent incorporation of <sup>32</sup>P from [ $\gamma$ -<sup>32</sup>P]ATP was determined. The first preincubation was carried out in the usual way (see legend, Table II) by incubating the ghosts at 37°C for 30 min in the presence of 2 mM MgCl<sub>2</sub> + 1.5 mM Tris ATP, as described in Materials and Methods. The ghosts were then thoroughly washed at 0°C with 17 mM Tris (pH 7.5) before they were preincubated a second time. The second preincubation of ghosts was carried out at 37°C for 15 min with the ghosts suspended in a medium which contained 10 mM Tris (pH 7.5) + 0.25 mM EDTA + 2 mM MgCl<sub>2</sub> + 40 mM NaCl and 10 mM KCl as indicated. When Na was absent, 40 mM choline Cl was added instead. The control ghosts (not preincubated) were suspended in 17 mM Tris (pH 7.5) and kept at 0°C during the second preincubation. At the end of the second preincubation, the ghosts were again washed with 17 mM Tris and then incubated with [ $\gamma$ -<sup>32</sup>P]ATP to estimate <sup>32</sup>P incorporation in the presence of Mg and Mg + Na as already described (see Materials and Methods or legend, Table I). The difference ( $\Delta$ ) between the columns headed Na + Mg and Mg measures the <sup>32</sup>P incorporated into the Na-component. The average values are given ± SEM where  $n = 4$ .

ghosts are again washed and the presence or absence of the Na component of phosphorylation is determined. Thus, the reappearance of the Na component indicates the effectiveness of treatment during the second incubation in reversing the effect of the pretreatment with ATP. Table VIII presents evidence that

the membrane pool of ATP can be eliminated when the ghosts are subjected to a second preincubation for 90 min. Depletion of the membrane pool of ATP by this means is a relatively slow process which appears to occur during the 90-min incubation in a nonlinear fashion since there is no apparent loss of ATP from the pool after 15, 30, or 60 min in the presence of Tris (data not shown). Results which indicate that the rate of removal of ATP from the membrane pool can be accelerated are presented in Table IX, in which it is shown that the combined presence of Na + K leads to unloading the membrane pool of its ATP within 15 min. (15 min of incubation with Na + K was used since we found that some pool ATP was still present at 10 min.) Presumably, the membrane pool would disappear at a faster rate when the second incubation was carried out in the presence of Na + Mg than with Mg alone but we have no information on this point. These results, while indicating the lability of the membrane pool of ATP, also point to the activity of the Na,K-ATPase and presumably the Na-ATPase in accelerating the rate of pool depletion and provide further evidence that the pool of ATP is located within the membrane in the region of the Na:K pump.

*PGK Activity and the Membrane Pool of ATP.*

This section is concerned with showing that the activity of the glyceraldehyde phosphate dehydrogenase (GAPD) and phosphoglycerate kinase (PGK) can be used to regulate the amount of ATP present in the membrane pool. It is known from previous work (Ronquist and Ågren, 1966; Schrier, 1966; Eckel et al., 1966; Parker and Hoffman, 1967) that GAPD and PGK activities are present in hemoglobin-free ghosts of human red cells and that interactions between the Na:K pump and the GAPD-PGK sequence have also been demonstrated (Eckel et al., 1966; Parker and Hoffman, 1967). That the GAPD-PGK system is also operative in the ghosts as used in the present experiments is shown by the results presented in Figs. 5 and 6 in which the reversible flow of substrates through the coupled system occurs as summarized in the equation:



The results presented in Figs. 5 and 6 clearly show that ghosts by themselves can catalyze the forward and backward reactions, provided the ghosts are incubated with the appropriate substrates and cofactors necessary for running the sequence involving the two enzymes, PGK and GAPD. That the forward reaction is accelerated by the addition of ghosts but not when exogenous PGK is also added indicates that in this situation membrane-bound GAPD presumably is rate limiting. Addition of exogenous GAPD to ghost accelerates the reduction of NAD, implying that in this circumstance PGK may be rate limiting since the rate is fastest when only exogenous PGK + GAPD are present without ghosts. Although more experiments involving the direct assay of substrates and products would be necessary to establish the rate-limiting features of this reaction sequence, these results provide further evidence that PGK and GAPD are

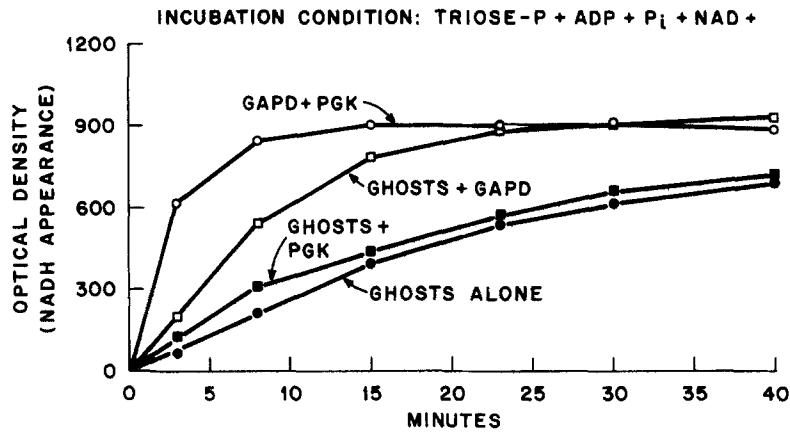


FIGURE 5. Capacity of ghosts to carry out the PGK reaction in the forward direction. NAD reduction (or NADH formation) was measured as an increase in optical density at 366 nm. Reactions were performed at 23°C in a cuvette and tracked spectrophotometrically over the indicated times. The reaction medium contained in final concentration (mM): Triose-P (0.83), NAD (0.415), ADP (0.25), Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (50.0), MgSO<sub>4</sub> (5.0), and glycine (132). Where indicated, the reaction medium also contained 0.005 ml (10 mg/ml) GAPD; 0.010 ml (10 mg/ml) PGK, and 0.010 ml (10 mg/ml) ghosts. Final volume was 3 ml, pH 6.9. The reaction was begun (zero time) by adding the ghosts to the medium containing the various constituents indicated.

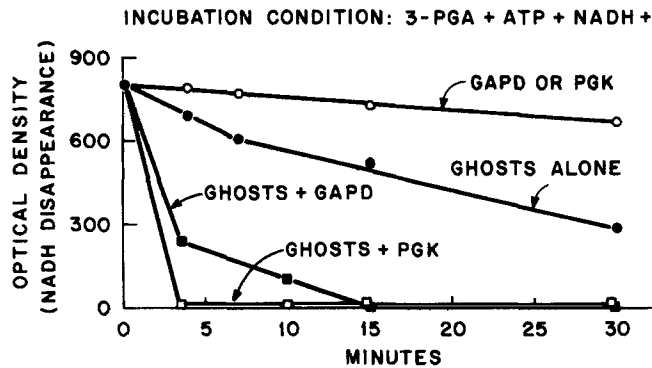


FIGURE 6. Capacity of ghosts to carry out the phosphoglycerate kinase (PGK) reaction in the backward direction. NADH oxidation was measured as a decrease in optical density at 366 nm. Reactions were carried out at 23°C in a cuvette and followed spectrophotometrically over the indicated times. The reaction medium contained in final concentration (mM): 3-PGA (5.0), ATP (0.30), MgSO<sub>4</sub> (5.0), NADH (0.25), NaHCO<sub>3</sub> (17.5), cysteine (20.0), and glycine (106.5). Where indicated, the reaction medium also contained 0.005 ml (10 mg/ml) glyceraldehyde phosphate dehydrogenase (GAPD); 0.010 ml (10 mg/ml) PGK, and 0.010 ml (10 mg/ml) ghosts. Final volume was 3 ml, pH 7.0. The reaction was begun (zero time) by adding the ghosts to the medium containing the various constituents indicated.

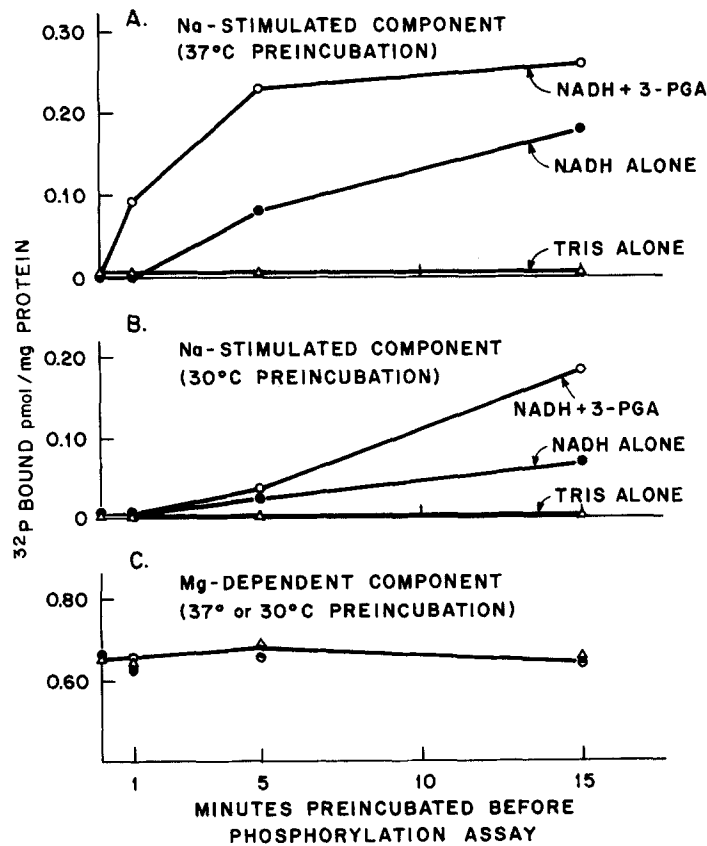


present as component parts in the fabric of the membrane. This conclusion is further substantiated by results presented in Fig. 6 in which it is shown that ghosts per se contain the capacity to catalyze the backward reaction when provided again with the appropriate substrates and cofactors. This is apparent from the fact that NADH oxidation is accelerated by the addition of ghosts and that the rate of oxidation can be further accelerated by the exogenous addition of either GAPD or PGK. This latter result is also consistent with the previous interpretation concerning the rate-limiting features as determined in the present assay system.

Fig. 7 presents results concerned with utilizing the membrane-bound GAPD-PGK system running in the backward direction, to remove ATP previously placed in the membrane pool by the preincubation procedure. In the sense that the purpose here is to unload in another way the membrane pool of its ATP, these studies extend the approach taken in the previous section. Thus, ghosts were preincubated with ATP in order to load the membrane pool with ATP. After thorough washing, the ghosts were then reincubated for various lengths of time (at either 37°C or 30°C) in the presence of selected substrates required to run the PGK reaction backward. If running the PGK reaction backward resulted in the removal of ATP from its membrane pool, this should be detectable by the ability to incorporate  $^{32}\text{P}$  into the Na component upon incubation with [ $\gamma$ - $^{32}\text{P}$ ]ATP. On this basis then, the results shown in Fig. 7 (graphs A and B) demonstrate that ATP can be removed from its membrane pool by the operation of the PGK-GAPD reaction sequence. (Note that the Mg component of phosphorylation [Fig. 7, graph C] does not appear to be affected by any of these maneuvers involving the PGK-GAPD system.) It should be noted that the increased rate of reappearance of the Na component obtained in the presence of NADH alone is due to the medium used since separate experiments show that approximately the same rate is obtained with the same medium but without NADH. The reason for the difference in rate with the medium along compared to Tris is not known. Nevertheless, the fact that the rate of reappearance of the Na component is accelerated (at either temperature) by the addition of only the combination 3-PGA + NADH indicates that the PGK-GAPD system resident in the membrane operates on ATP present in the pool. These results imply then that the PGK-GAPD system is linked to the Na-ATPase (and, therefore, presumably the Na:K pump) through a common pool of ATP. On a comparative basis, the ATP pool can be emptied faster at 37°C by running the PGK-GAPD system backward than by incubation in the presence of Na + K (Table IX).

Just as it was possible to remove ATP from its membrane pool by running the PGK-GAPD system backwards, it should also be possible to place ATP within the pool by operating this reaction sequence in the forward direction. This is what has been done in the experiments summarized in Table X. In these experiments ghosts were first preincubated under various conditions which included exposure to those substrates required to run the GAPD-PGK reaction sequence forward. The ghosts were then washed, as previously described, before measuring the incorporation of  $^{32}\text{P}$  into the Na component from [ $\gamma$ - $^{32}\text{P}$ ]ATP. The failure to incorporate  $^{32}\text{P}$  into the Na component is taken to represent the

synthesis of ATP by the GAPD-PGK system and its deposition directly into the membrane pool. As shown in Table X, preincubation with the complete medium prevents the subsequent incorporation of  $^{32}\text{P}$  into the Na component. On the other hand, incorporation of  $^{32}\text{P}$  into the Na component is normal when the preincubation is carried out in the absence of triose-P. This result not only represents a control for the incubation carried out with the complete medium but also indicates that the amount of ADP present as substrate is insufficient to alter incorporation into the Na component even if it were converted to ATP, say by an adenylate kinase. This may be important since ATP added exogenously is still effective in this circumstance in masking  $^{32}\text{P}$  incorporation into the Na component. On the other hand, if the Na component is to be blocked, it is necessary to preincubate with concentrations of ATP greater than 0.5 mM (see Fig. 4). Since ATP generated by the forward reaction requires only the addition of substrates (Triose-P,  $\text{P}_i$ , NAD, and ADP) and not enzymes (GAPD and PGK) and since this newly made ATP can act to block the appearance of  $^{32}\text{P}$  into the Na component, these results, as discussed previously with regard to the backward reaction, can be used to specify the organization of the membrane in the region of the pump in terms of an energy-yielding reaction (PGK) and an energy-

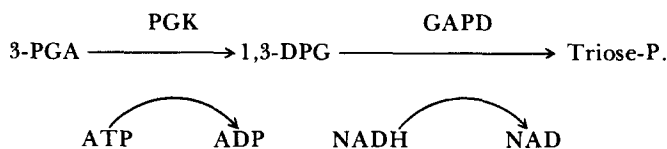


utilizing reaction (Na,K-ATPase). Thus, ATP generated by the PGK reaction would be made available for preferential use by the Na:K pump. The membrane compartmentation of ADP as substrate for the PGK has been previously suggested on other grounds (Parker and Hoffman, 1967) and is entirely consistent with the thesis developed here.

*The Size of the Membrane Pool of ATP*

One approach for estimating the size of the membrane pool would be, after filling the pool with labeled ATP, to measure the amount of the label released after exposing the ghosts to conditions known to deplete the pool of its ATP. This type of approach would be workable if the amount of nonspecific binding of ATP to the ghosts (which is appreciable [Heinz and Hoffman, 1965]) could be minimized, otherwise the membrane pool would be obscured. After a few preliminary attempts it became clear that this experiment was feasible if one used the following design. As described in Table XI, the ghosts were incubated a total of four times. (The first three incubations are preparatory since it is only during the fourth that depletion of the pool is measured.) The first incubation

FIGURE 7. (*opposite*) Removal of ATP from the membrane pool by running the PGK reaction in the backward direction:



First, the membrane pool was loaded by preincubation of ghosts with 2 mM MgCl + 1.5 mM Na<sub>2</sub>ATP for 30 min at 37°C as described in Materials and Methods (see legend, Table II). Next, the ghosts, after washing, were reincubated a second time (at either 37°C or 30°C), in the presence of various substrates in order to run the PGK reaction backward: 0.5 ml (5 mg/ml) ghosts were suspended, in 5.0 ml final volume, in either 17 mM Tris Cl (pH 7.5) or in a medium which contained in final concentration (mM): MgSO<sub>4</sub> (5.0), NaHCO<sub>3</sub> (17.5), cysteine (20.0), glycine (106.5) and, as indicated, either 3-PGA (5.0) and/or NADH (0.25), with a final pH 7.0. (It is important to note that analysis showed that the NADH and 3-PGA solutions used as well as the second preincubation medium was K-free, i.e. less than 0.05 mM.) The second preincubation was begun (zero time) by adding the ghosts to the medium containing the various constituents indicated. Samples were withdrawn from the second preincubation at 1, 5, and 15 min and immediately centrifuged (0°C, 12,000 g for 5 min) and the ghosts washed four times at 0°C with 17 mM Tris Cl (pH 7.5). The ghosts were then incubated at 0°C for 15 s as previously described, with [ $\gamma$ -<sup>32</sup>P]ATP to study <sup>32</sup>P incorporation in the presence of Mg or Na + Mg. The difference between the amount of <sup>32</sup>P incorporated in the presence of Na + Mg and Mg alone represents the <sup>32</sup>P bound by the Na component of the membrane (upper panels, A and B). The amount of <sup>32</sup>P incorporated in the presence of Mg alone represents the <sup>32</sup>P bound by the Mg component of the membrane (lower panel, C). In this experiment each analysis was carried out in duplicate and another experiment of the same type yielded completely comparable results.

was carried out in the presence of either nonradioactive ATP or UTP to try to minimize the subsequent binding of radioactive ATP to nonspecific sites. To label the membrane pool, ghosts were exposed in both types of experiments to [<sup>3</sup>H]ATP only during the second incubation. Presumably, [<sup>3</sup>H]ATP can exchange with any nonradioactive ATP that could be contained within the membrane pool as a result of the first incubation since there is essentially no difference between the <sup>3</sup>H content of ATP- and UTP-exposed ghosts. (As shown

TABLE X  
LOADING THE MEMBRANE POOL WITH ATP GENERATED BY RUNNING  
THE PGK REACTION IN THE FORWARD DIRECTION

Preincubation condition	<sup>32</sup> P incorporation (pmol/mg protein)		
	Incubation media		
	Mg	Na+Mg	Δ
Not preincubated	0.78	1.05	0.27
Complete medium	0.68	0.71	0.03
Complete medium minus triose-P	0.67	0.87	0.20
Complete medium minus triose-P + 1.5 mM Na <sub>2</sub> ATP	0.64	0.66	0.02

Ghosts were preincubated for 45 min at 37°C in the presence of various substrates in order to run the PGK reaction forward. 0.5 ml (5 mg/ml) ghosts were suspended, in 5.0 ml final volume, in what is referred to as the complete medium which contained in final concentration (mM): Triose-P (0.83), NAD (0.415), ADP (0.25), Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (50.0), MgSO<sub>4</sub> (5.0), and glycine (132) with final pH 6.9. Two types of controls were carried out, as indicated, in which Triose-P was left out of the complete medium in one instance and ATP was added in its place in the other. Ghosts which were not preincubated were kept at 0°C in 17 mM Tris Cl (pH 7.5) until ready for use in the <sup>32</sup>P incorporation portion of the experiment. At the end of the preincubation period the ghosts were washed, as described before, with 17 mM Tris Cl (pH 7.5) at 0°C (5 min, 12,000 g). The ghosts were then incubated at 0°C for 15 s, as again already described, with [ $\gamma$ -<sup>32</sup>P]ATP to study <sup>32</sup>P incorporation in the presence of Mg or Na + Mg. The difference ( $\Delta$ ) between the amount of <sup>32</sup>P incorporated in the presence of Na + Mg and Mg alone represents the <sup>32</sup>P incorporated into the Na component. In this experiment duplicate samples were prepared and analyzed in duplicate. An additional experiment of the same type gave completely comparable results.

in Fig. 1, UTP cannot substitute for ATP in the pool since preincubation with UTP does not alter the subsequent incorporation of <sup>32</sup>P into the Na component.) The third incubation was carried out in order to reduce the amount of <sup>3</sup>H bound nonspecifically to the ghosts, as found by Heinz and Hoffman (1965). The change in the <sup>3</sup>H content that results from the fourth incubation of the ghosts in the presence of Mg + Na compared to Mg + Na + K is taken as representing the amount of ATP (as [<sup>3</sup>H]ATP) that was originally incorporated into the membrane pool. It should be noted that the total time of incubation of ghosts at 37°C, after they have been loaded with [<sup>3</sup>H]ATP, that is the sum of the third and

fourth incubations, is 30 min. As discussed before (Tables VIII and IX), this length of time is not sufficient to deplete the membrane pool of its ATP when ghosts are incubated either in Mg alone or with Mg + Na. On the other hand, 15 min is sufficient, as shown in Table IX, to unload all of the ATP from the membrane pool when the incubation is carried out with Mg + Na + K. The

TABLE XI  
RELEASE OF [<sup>3</sup>H]ATP FROM PREINCUBATED GHOSTS

Incubation after which ghosts analyzed	<sup>3</sup> H content (nM/mg protein) first incubation			
	Experiment A		Experiment B	
	ATP	UTP	ATP	ATP+ouabain
2nd incubation ([ <sup>3</sup> H]ATP)	2.07±0.04	1.78±0.05	—	—
3rd incubation (Tris)	1.18±0.02	1.03±0.01	—	—
4th incubation with:				
Mg	0.81±0.02	0.70±0.01	0.80±0.01	0.62±0.01
Mg+Na	0.71±0.01*	0.59±0.01‡	0.71±0.03‡	0.62±0.02
Mg+Na+K	0.64±0.01*	0.51±0.02‡	0.63±0.01‡	0.60±0.01

The ghosts used in this experiment were incubated a total of four separate times with different conditions for each incubation. After each incubation the ghosts were washed, at 0°C, four times as previously described with 17 mM Tris Cl (pH 7.5) to ready them for the next incubation but after the fourth incubation, the washed ghosts were concentrated by centrifugation and samples removed for determinations of <sup>3</sup>H and protein content. Samples for <sup>3</sup>H determination were first dissolved in 1.5 ml NCS and then counted after adding 12 ml Toluene counting solution (see Materials and Methods).

The first incubation of the ghosts was carried out at 37°C for 30 min in a medium which contained 10 mM Tris (pH 7.5) + 0.25 mM EDTA + 2 mM MgCl<sub>2</sub> and either nonradioactive 1.5 mM Na<sub>2</sub>ATP or 1.5 mM Na<sub>2</sub>UTP (Exp. A). In Exp. B, the conditions were the same except that the first incubation was carried out with 1.5 mM Na<sub>2</sub>ATP in the presence and absence of 1 × 10<sup>-3</sup> M ouabain.

The second incubation was carried out at 37°C for 20 min with the ghosts suspended in a similar medium but which contained 10 mM Tris Cl (pH 7.5) + 0.25 EDTA + 2 mM MgCl<sub>2</sub> + 1.5 mM Na<sub>2</sub>ATP + sufficient [<sup>3</sup>H]ATP (uniformly labeled) to give a specific activity of about 2 × 10<sup>12</sup> dpm/mol ATP.

The third incubation was carried out at 37°C for 15 min with the ghosts suspended in a medium which only contained 17 mM Tris Cl (pH 7.5).

The fourth incubation was carried out at 37°C for 15 min with the ghosts suspended in a medium which contained 10 mM Tris Cl (pH 7.5) + 0.25 mM EDTA + 2 mM MgCl<sub>2</sub> together with 40 mM NaCl and 10 mM KCl as indicated. When Na was absent, 40 mM choline Cl was added instead. The average value for two separate experiments of each type is given ± SEM where *n* = 7 for the different conditions in both Exp. A and B. The differences between the values marked \* and ‡ are statistically significant to the level that *P* < 0.01 for those marked \* and *P* < 0.05 for those marked ‡. Two other experiments where the first incubation was carried out in the presence of ATP gave results completely comparable to those presented here.

difference in the content of ghosts (Exp. A) incubated with Mg + Na + K and Mg + Na is 0.07 nM/mg protein or 0.08 nM/mg protein for ghosts initially incubated with ATP and UTP, respectively. While the same amount (0.07 nM/mg protein) of ATP is lost in Exp. B as in Exp. A upon incubation in the presence of Mg + Na + K compared to Mg + Na, it appears that the presence of ouabain prevented [<sup>3</sup>H]ATP from entering the pool during the second incubation since the membrane content of [<sup>3</sup>H]ATP in the presence of ouabain was the

same as in its absence after incubation with Mg + Na + K. (This effect of ouabain is presented without explanation.) Taking 75 pM ATP/mg protein as an average value, and assuming 0.6 pg protein/ghost, means that there would be approximately 27,000 molecules of ATP per ghost associated with the membrane pool. If the number of Na:K pumps is assumed to be equivalent to the number of glycoside binding sites (Ingram, 1970), then there would be about 100 molecules of pool ATP associated with each pump.

#### DISCUSSION

The main conclusions to be drawn from the studies presented in this paper are, first, that ATP can be compartmentalized within the human red cell membrane, and second, that this compartmentalized form of ATP is preferentially used by the Na:K pump, implying that the Na-stimulated phosphoprotein component of the membrane may be a separate system from the Mg component. These conclusions stem from the findings that when hemoglobin-free frozen-thawed ghosts are preincubated with nonradioactive ATP, the formation of the phosphoprotein associated with Na stimulation (Table II) as well as the Na-stimulated ATPase (Table V) activity are not seen. To obtain these effects of preincubation it was shown that Mg was required (Table III), that a preincubation temperature of 37°C (or at least above 27°C) was necessary (Table IV), that the preincubation effect was specific for ATP (Fig. 1), and that the extent of the preincubation effect was dependent on the ATP concentration present during preincubation (Fig. 4). If it is assumed that preincubation with ATP is tantamount to filling a membrane compartment (pool) with ATP then the results can be summarized by the model presented in Fig. 8. For this discussion it should be kept in mind that formation of phosphoproteins and ATPase activities are assessed at 0°C as previously described.

As depicted in this model (Fig. 8) there are two sources of ATP. These are pool and bulk ATP. When the pool is empty, normal  $^{32}\text{P}$  labeling of the Na component as well as the Mg component is obtained from bulk  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ; in addition, the Na-ATPase and the Mg-ATPase activities are both accurately reflected by the rate of liberation of  $^{32}\text{P}_i$  in the medium. On the other hand, when ATP (nonradioactive) is contained within the membrane pool, then the apparent utilization of bulk  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  by the Na-sensitive system is changed. In this situation the Mg-sensitive system is seen to behave essentially as it did previously but there is now no flow of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  through the Na-sensitive components. Presumably, the Na-sensitive system is still active but since it now preferentially uses pool ATP which is nonradioactive rather than bulk ATP, the flow of  $^{32}\text{P}_i$ , as seen before, disappears. This model then provides an explanation for the preincubation effects of ATP as described in this paper.

There are at least two other ways of interpreting the effects of preincubation with ATP in the subsequent pattern of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  utilization by the membrane components. These are that preincubation could result either in the formation of an inhibitor or in inducing vesiculation of the membrane. In either case, specificity would be required of the inhibitor or of the type of vesicle formed in order to account for the selectivity of the effects on the Na-sensitive system

compared to the Mg-sensitive system. While neither of these alternatives can be ruled out, there is also no evidence in its favor. Thus, the breakdown products which occur during preincubation with ATP can be entirely accounted for by analyzing the products present in the supernate after preincubation either enzymatically or by thin layer chromatography. All of the products from this

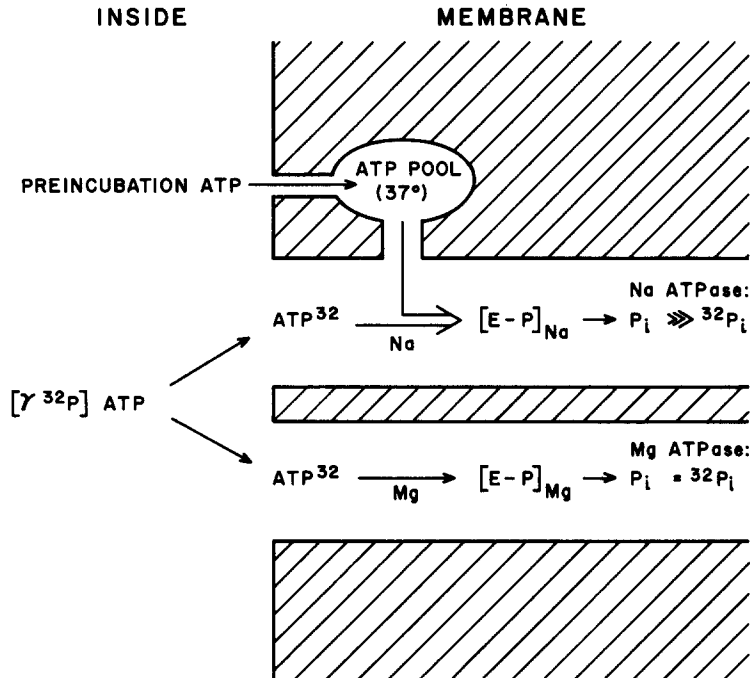


FIGURE 8. Model of membrane containing a compartmentalized ATP. ATP can be placed in this compartment by preincubation or by the operation of the membrane-bound GAPD-PGK system. ATP present in this pool is used in preference to bulk ATP (denoted as [ $\gamma$ - $^{32}P$ ]ATP) for the formation of the phosphoprotein ( $[E-P]_{Na}$ ) and therefore for the formation of  $P_i$  liberated by the Na-ATPase. In contrast, only bulk ATP is used by the Mg component for the formation of its phosphorylation ( $[E-P]_{Mg}$ ) and the production of  $^{32}P_i$  from [ $\gamma$ - $^{32}P$ ]ATP. See text for further discussion.

preincubation (ADP, adenine, hypoxanthine, and  $P_i$ ) were tested separately (Fig. 1) and found to be ineffective in altering the subsequent incorporation of  $^{32}P$  into the Na component. With regard to vesiculation, the conditions of preincubation are not compatible with the known circumstances (Penniston and Green, 1968; Steck et al., 1970; Katsumata and Asai, 1972) under which vesiculation appears to occur. In addition, the appearance of preincubated ghosts used in the present work under phase and interference light microscopy is the same as the population of control ghosts, whether preincubated or not; although ghost shapes vary, there is no evidence of vesiculation observable at this level of resolution.

Perhaps the most convincing evidence in favor of the concept of a membrane pool for ATP is the fact that the contents of the pool can be influenced by the activity of the GAPD-PGK system. Both of these enzymes are known, from other work (Schrier, 1966, 1967; Ronquist and Ågren, 1966; Parker and Hoffman, 1967; Nilsson and Ronquist, 1969; Tillmann et al., 1975), to be contained in the human red cell membrane and the data presented in Figs. 5 and 6 confirm this under the conditions used in the present experiments. Thus when the appropriate substrates are added to ghosts for running the GAPD-PGK system in either the forward or the backward direction, which presumably results in either the synthesis or the removal, respectively, of ATP within the membrane, the ability subsequently to incorporate  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  into the Na component is likewise either inhibited (Table X) or regenerated (Fig. 7). It should be pointed out in connection with the running of the GAPD-PGK system in the forward direction that the possibly exists that the ATP generated from the ADP added as substrate (0.25 mM) enters the membrane pool from an exogenous source (since the incubation is carried out at 37°C) rather than being placed there enzymatically. This possibility is unlikely in view of the fact that the concentration of ATP that could be generated (measured to be less than 0.1 mM) is insufficient to fill the pool and prevent incorporation of  $^{32}\text{P}$  into the Na component (see Fig. 4). In fact it was with just this consideration in mind that the specific conditions for testing the forward reaction were adopted.

To the extent that the Na-phosphoprotein and its associated ATPase activity as seen in red cell ghosts represent intermediate activities of the Na,K-ATPase and therefore the Na:K pump (see Blostein, 1968, 1970; see footnote 2), it is evident on the basis of the previously discussed results that the ATP-generating and ATP-consuming reactions in the membrane have in common the membrane pool of ATP. This same conclusion was reached on rather different grounds by Parker and Hoffman (1967) and confirmed by Okonkwo et al. (1975) in studies concerned with defining circumstances under which the membrane-bound PGK system was rate controlling in terms of the activity of the Na:K pump and the rate of lactate production.<sup>3</sup> Therefore, as presented in Fig. 9, it would appear that it is possible to specify in general terms the organization of the membrane in the region of the Na:K pump in the sense that, within this microdomain, ADP and ATP are compartmentalized in juxtaposition to the GAPD-PGK reaction sequence and the Na:K pump apparatus. Another conclusion can also be drawn from the fact that the Na component of the membrane draws preferentially on pool ATP while the Mg component uses bulk ATP. As depicted in Fig. 8, these results imply that these two enzyme systems are separate entities. While this conclusion is based on kinetic evidence as presented in this paper, a separate paper demonstrates on a physical basis that these two membrane components

<sup>3</sup> Chillar and Beutler (1976) correctly point out that one type of experiment used by Parker and Hoffman (1967) to consider that PGK is rate limiting for glycolysis is open, in the light of recent advances, to another interpretation. It is unfortunate that in the attempt of Chillar and Beutler (1976) to criticize the hypothesis, that membrane PGK is closely associated with the Na:K pump, they ignore the more relevant and substantive results presented in that paper (Parker and Hoffman, 1967) as well as the work of others (Okonkwo, et al., 1975; Sachs, 1972; Segel, et al., 1975, and Tillman, et al., 1975).



can be separated from each other<sup>4</sup> by density gradient centrifugation after solubilization with SDS by use of a modification of the procedure described by Dunham and Hoffman (1970).

Another point to be discussed has to do with the size estimate of the number of molecules of ATP associated with the membrane pool as presented in Table XI. It should be recalled that this measurement is based on the idea that the addition of K accelerated the rate of depletion of the membrane pool of its ATP in such a manner that the combination K + Na + Mg was effective in 15 min compared to

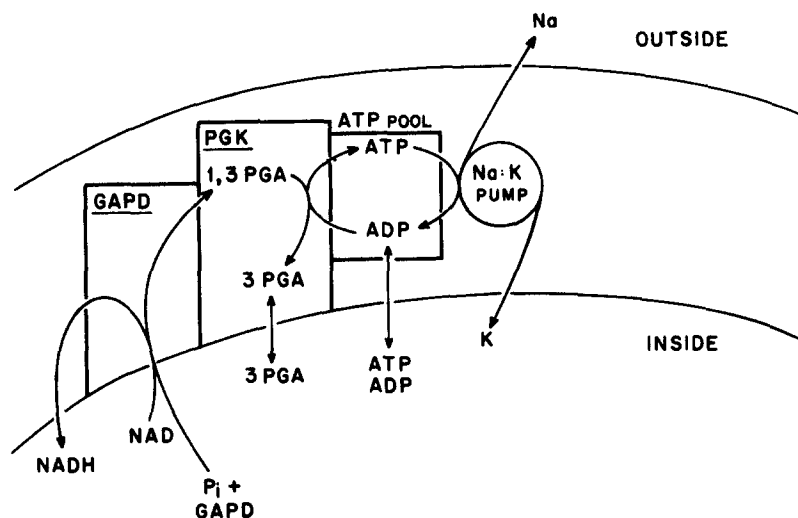


FIGURE 9. Model representation of the intrinsic organization of the membrane in the region of the Na:K pump. The enzymes, glyceraldehyde phosphate dehydrogenase (GAPD) and phosphoglycerate kinase (PGK), are depicted as being locked in sequence and connected to the Na:K pump via the membrane pool of ATP. Substrate molecules present on the inside (cytoplasm) have access to the membrane bound enzymes under the various circumstances described in the text but it is the ATP present within the membrane pool that is preferentially used by the Na:K pump. This model of enzyme arrangement is similar to that previously proposed by Schrier (1966).

incubation with only Na + Mg (Table IX). It was suggested that this effect of K together with Na was to increase the rate of ATP utilization by activation of the Na,K-ATPase. This could be so but it is necessary to know the concentration of ATP involved as substrate before this accelerating role of K can be interpreted in this way. This is because, as shown by Blostein (1968) as well as others (Czerwinski et al., 1967; Neufeld and Levy, 1969; Mårdh and Zetterquist, 1972), at low levels of ATP, K (with Na) fails to activate the Na,K-ATPase, presumably because K decreases the affinity of the system for ATP (Post et al., 1969; Hegyvary and Post, 1971; Robinson, 1967). Thus, in the present experiments it is not possible to know whether the effect of K is mediated by the Na,K-ATPase or

<sup>4</sup> Proverbio, F., and J. F. Hoffman. Manuscript in preparation.

by some other mechanism. On the other hand, if K does in fact act through the Na,K-ATPase, then it would imply that the concentration of ATP in the pool is somewhat larger than  $5 \mu\text{M}$  (see Blostein, 1968, and Mardh and Zetterquist, 1972). On this basis it is of interest to calculate the number of molecules of ATP in the membrane pool. If the turnover number of the Na,K-ATPase at  $37^\circ\text{C}$  is taken as 6,000 ions per ouabain binding site per minute (Hoffman and Ingram, 1969) and if 3 Na ions are transported per ATP utilized (Sen and Post, 1964), then 2,000 molecules of ATP would be consumed per site per minute. If the concentration of ATP in the pool is taken as  $5 \mu\text{M}$  and the Na,K-ATPase is assumed to have approximately 2% of the activity at  $37^\circ\text{C}$  at this substrate level as it has at 1 mM ATP, and if it takes 15 min to deplete the pool, then each pool would contain approximately 600 molecules of ATP. In view of the various assumptions made this value can be considered to compare favorably with the estimate made from the results presented in Table XI. Perhaps it will be possible to develop a more direct and accurate estimate of the size of the membrane pool of ATP.

A final comment should be made with regard to the possibility that a membrane pool of ATP associated with the Na:K pump might be present and functional in the intact red blood cell. Indirect evidence that this might be the case has already been inferred from the presence of the relevant enzyme systems in the membrane (Schrier, 1963, 1966) and from analysis of the inhibitory effects of ouabain on lactate production in intact cells (Whittam and Ager, 1965) as extended to ghost systems (Parker and Hoffman, 1967; Okonkwo et al., 1975). Evidence of a different sort, though still indirect, can be seen in the work reported by Feig et al. (1972), Segel et al. (1975), and Sachs (1972) where cation transport was studied in intact cells which had been glucose depleted. This treatment yielded cells which still contained a high concentration of ATP (generated from 2,3 DPG) but were depleted of triose-phosphate as well as all proximal substrates. That the transport of Na and K in these altered cells was markedly reduced but not eliminated under circumstances where an ample concentration of ATP was present to drive the pump implies that the operation of the membrane-associated PGK may be required for either efficient or maximum activity of the pump. Obviously, a more detailed and systematic analysis of the interrelationship is needed and it would also be interesting to study in this regard PGK-deficient red cells (Kraus et al., 1968, and Segel et al., 1975).

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