Membrane-bound ATP Fuels the Na/K Pump

Studies on Membrane-bound Glycolytic Enzymes on Inside-Out Vesicles from Human Red Cell Membranes

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ABSTRACT ATP stimulates Na transport into inside-out vesicles (IOVs) made from human red cell membranes; strophanthidin inhibits the ATP-stimulated transport. The substrates for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) (glycolytic enzymes bound to the cytoplasmic surface of the red cell membrane) also stimulate Na transport into IOVs without added ATP. The elution of GAPDH from the membranes prevents the stimulation by the substrates, but not by exogenous ATP. Hexokinase plus glucose (agents that promote breakdown of ATP) prevent stimulation of Na transport by exogenous ATP but not by the substrates for GAPDH and PGK. [^{32}P]orthophosphate is incorporated into a membrane-bound organic phosphate compound shown chromatographically to be ATP. The level of membrane-bound ATP is decreased when Na is added, and this decrease is inhibited by strophanthidin. When further synthesis of [^{32}P]ATP is blocked by the addition of unlabeled orthophosphate, all of the membrane-bound [^{32}P]ATP is dissipated by the addition of Na. From these observations it was concluded that membrane-bound glycolytic enzymes synthesize ATP and deposit it in a membrane-associated compartment from which it is used by the Na/K pump.

INTRODUCTION

The immediate energy source for the active transport of Na and K is provided by the hydrolysis of ATP. The mature human red blood cell synthesizes ATP exclusively through glycolysis. It has been suggested that in these cells two enzymes of the glycolytic pathway, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), are associated with the Na/K pump (Schrier et al., 1966; Parker and Hoffman, 1967). With orthophosphate and NAD as substrates, GAPDH converts glyceraldehyde-3-phosphate (G3P) to 1,3-diphosphoglycerate (1,3-DPG). The subsequent enzyme in the pathway, PGK, catalyzes the transfer of a phosphate group from 1,3-DPG

to ADP, forming ATP. It has also been suggested that the ATP synthesized by membrane-associated GAPDH and PGK is compartmentalized in a "membrane pool," which is used preferentially by the Na/K pump (Hoffman and Proverbio, 1974; Okonkwo et al., 1975; Proverbio and Hoffman, 1977). Indirect evidence for the existence of a pump-associated ATP pool was provided by the inhibition of active transport in cells high in ATP, and depleted of triose phosphates (Sachs, 1972; Segel et al., 1975), and the inhibition of the use of ^{32}P-labeled ATP by Na,Mg-ATPase after pretreatment with nonradioactive ATP (Proverbio and Hoffman, 1977). The suggestion of a membrane pool of ATP, however, has met with criticism. Chillar and Beutler (1976) and De and Kirtley (1977), using permeable ghosts (red cell membranes obtained by hypotonic lysis), were unable to find a coupling between PGK and the Na/K pump. Chillar and Beutler suggested that the Na/K pump readily provides ADP for and uses ATP from glycolytic enzymes other than PGK, whereas De and Kirtley could not find an effect of ouabain on the production of 3-phosphoglycerate, the product of PGK. Beutler et al. (1978) later suggested that only one pool of ATP existed in the intact cell. They found, using [^{32}P]orthophosphate to label the PGK-derived ATP, that essentially all the ^{32}P-labeled ATP was available for sugar phosphorylation by cytoplasmic hexokinase, suggesting the presence of a single pool of ATP, which constituted the "soluble" ATP in the cytoplasm.

To investigate the mechanism by which ATP is supplied to the Na/K pump, and particularly to assess the roles of glycolytic enzymes and ATP bound to or otherwise associated with the membrane, we have used inside-out vesicles (IOVs) prepared from red blood cells. IOVs provide an experimental system free from some of the difficulties of intact cells and resealed ghosts. Unlike intact cells and resealed ghosts, IOVs afford direct access to the cytoplasmic membrane surface. Furthermore, the large extravesicular volume of the IOVs precludes the changes in concentrations of substrates at the cytoplasmic surface, which can occur rapidly in cells and ghosts. With this system, we found that the substrates for GAPDH and PGK support active Na transport through synthesis by the membrane-bound enzymes of ATP, and that the ATP also remains bound to the membrane.

MATERIALS AND METHODS

Blood from healthy human donors was drawn into heparinized syringes. The red cells were washed three times at 4°C by centrifugation, aspiration, and resuspension in an isotonic solution containing choline-Cl (150 mM) and Tris-HCl (10 mM), pH 7.4 (290 mosmol/liter).

Preparation of IOVs

IOVs were prepared by a modification of the method of Steck (1974). Unless otherwise noted, all solutions were ice-cold and were made using glass-distilled water containing Tris-EGTA (0.1 mM), pH 7.0. Red cells washed in isotonic choline-Cl were lysed in 40 vol of 5 mM Tris-HCl, pH 8.0. After 10 min on ice, the suspension of ghosts was centrifuged (30,000 *g*) and the clear red supernate was removed by aspiration. The ghosts were then suspended in 10 vol of Tris-EGTA (0.1 mM), pH 7.0. After another

10 min on ice, 20 vol of 5 mM Tris-HCl, pH 8.0, was added and the suspension was centrifuged. Suspension and centrifugation were repeated once more and the pellet was resuspended in 25 vol of 0.5 mM Tris-HCl, pH 8.0. The suspension was allowed to sit on ice for 1 h. The suspension was then centrifuged and the pellet was homogenized by passing it five times through a 1-in, 27-gauge hypodermic needle on a 5-ml syringe. The homogenate (2 ml) was layered on top of 3 ml of a dextran T-70 solution (4.46% [wt/vol] in 0.5 mM Tris-HCl, pH 8.0) in a polycarbonate tube (11 × 120 mm) and centrifuged for 40 min at 30,000 *g*. The vesicles at the interface were removed and washed by centrifugation, successively, in solutions of 10, 20, and 40 mM Tris-glycylglycine; all three solutions also contained $MgCl_2$ (0.1 mM), pH 7.4. The osmolarity of the 40-mM solution was 52 mosmol/liter, as determined using a model 3L osmometer (Advanced Instruments, Inc., Needham Heights, Mass.). The vesicles, at a membrane protein concentration of ~0.1 mg/ml, were incubated at 4°C overnight in the desired "loading" solution, typically containing KCl (5 mM), $MgCl_2$ (1.0 mM), choline-Cl (20 mM), and glycylglycine (2.5 mM), pH 7.4, and the solution was equiosmolar with the vesicles (52 mosmol/liter). Before use, the vesicles were centrifuged and resuspended in the loading solution to give a final membrane protein concentration of ~1 mg/ml.

Preparations of the vesicles, negatively contrasted, were examined by transmission electron microscopy. The preparations contained vesicles of diameters ranging from 0.42 to 1.67 μm (mean diameter, 0.80 μm, $n = 55$). No larger vesicles or membrane fragments were seen.

IOVs depleted of their bound GAPDH were prepared by a procedure similar to that of Kant and Steck (1973). Washed ghosts were incubated on ice for 20 min in 20 vol of 150 mM NaCl and 5 mM Tris-HCl, pH 8.0. The ghosts were then washed by centrifugation and aspiration twice in 20 vol of the same solution and resuspended in 20 vol of 5 mM Tris-HCl, pH 8.0. The suspension was centrifuged and the pellet was suspended in 20 vol of 0.5 mM Tris-HCl, pH 8.0. The pellet was collected again and resuspended in 25 vol of the same solution. The suspension was allowed to sit on ice for 1 h. The suspension was centrifuged and the pellet was homogenized and purified on a layer of dextran as described above.

To separate and determine the presence of the various membrane proteins, the IOVs were analyzed by electrophoresis in 5% polyacrylamide disk gels with 0.2% sodium dodecyl sulfate (SDS) as described by Fairbanks et al. (1971). Gels were stained with Coomassie Blue and scanned at 550 nm with a Beckman model 24 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Bands were identified and designated according to the scheme of Steck (1972).

"Sidedness" of Vesicles

The percent of vesicles that were inside out was determined from acetylcholinesterase activity as described by Steck (1974). Acetylcholinesterase is confined to the outer surface of the red cell membrane (Firkin et al., 1963). The assay uses 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to follow the appearance of free thio groups during the hydrolysis of acetylthiocholine. Vesicles in buffer with and without Triton X-100 are added to a solution containing DTNB and acetylthiocholine-Cl. The reaction is followed with a recording spectrophotometer; an increase in absorbance at 412 nm corresponds to acetylcholinesterase activity. The percent of vesicles that are inside out is given by $[(1 - A_{in})/A_{tr}] \times 100$, where A_{in} is the acetylcholinesterase activity of the intact vesicles and A_{tr} is the activity of the vesicles disrupted with Triton X-100. Generally, 80% of each vesicle preparation was inside out.

Measurement of Na Influxes in Vesicles

The method was a modification of that of Blostein (1979). The vesicles were added to a final protein concentration of 0.1 mg/ml to flux media containing ^{22}Na (5 μCi/ml). The flux media were isosmotic with the loading solution; their compositions are given with the results of each experiment. In some experiments, valinomycin was included at 1 μM. At desired times, 0.20–0.25-ml aliquots of the suspension were removed and placed directly onto 1.2-μm Millipore RA 25-mm filters (Millipore Corp., Bedford, Mass.). The filters were washed with 15 ml of ice-cold nonradioactive medium, after which the funnel (Millipore XX10-025-14) was removed and the edges of the filters were washed with 1.5–2 ml of wash solution. Intravesicular ^{22}Na associated with the filter was measured with an autogamma crystal well scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Fluxes are expressed as nanomoles of Na per milligram membrane protein per minute.

The protein on filters was determined using a procedure provided by the Millipore Corporation. Protein on the filters was stained with Ponceau-S in a solution of trichloroacetic acid (TCA) (0.125% [wt/vol] Ponceau-S, 1.9% [wt/vol] TCA). The filters were then washed three times in 5 ml of 5% (vol/vol) acetic acid. Stained protein was eluted from the filters with 1 ml of 1 M NaOH. The filters were removed and the solutions were acidified with 2 ml of 1 M HCl. The protein concentration was calculated from absorbance at 520 nm and standard curves were prepared using known amounts of bovine serum albumin on filters.

Membrane-associated ATP

The procedure was to incubate vesicles in media with [^{32}P]orthophosphate, and then wash and extract them by a method that separates inorganic and organic phosphates (Garrahan and Glynn, 1967). The radioactivity of the organic phosphate was then determined, and the identity of the ^{32}P-labeled organic phosphate was determined by chromatography.

Specifically, IOVs were incubated, with and without ADP, in a medium containing the appropriate substrates (4 mM NAD, 2 mM G3P, and 0.5 mM [^{32}P]orthophosphate). At various times, a 0.2-ml aliquot was removed and placed onto a Millipore filter. To eliminate nonspecific binding to filters, the filters had been prewashed with 2 ml of choline-Cl solution (52 mosmol/liter) containing 250 μM P_i and 10 μM ATP. The filters were then washed with 20 ml of the same solution. The filters were placed into glass test tubes and 5 ml of ice-cold 0.1% Triton X-100 containing 10 μM ATP was added. The solutions containing the filters were vortexed for 40 s. The filters were removed and 1 ml of 5% ammonium molybdate in 8 M H_2SO_4 was added. Ice-cold isobutanol (6 ml) was added and the solution was vortexed for 20 s. The solution was centrifuged briefly (200 *g*) and the isobutanol layer, along with the lipid at the interface, was removed by aspiration. To remove all ^{32}P-labeled orthophosphate from the aqueous layer, 2 μmol of unlabeled carrier orthophosphate was added and the isobutanol extraction was repeated. This procedure was repeated once more, followed by a fourth isobutanol extraction without the addition of carrier P_i. Radioactivities of aliquots of the aqueous layer were determined in a liquid scintillation counter (Beckman LS-255; Beckman Instruments, Inc.) by Cerenkov scattering, giving the amount of labeled organic phosphate present.

Chromatographic separation of labeled products was carried out on PEI-cellulose layers in 1 M HCOOH and 1 M LiCl as described by Randerath and Randerath (1967). This technique enables a clear separation of ATP and ADP (R_f: ATP, 0.33; ADP, 0.70).

Abbreviations

The following abbreviations will be used in this paper: 1,3-DPG, 1,3-diphosphoglycerate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EGTA, ethyleneglycol-bis-*N,N′*-tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde-3-phosphate; IOVs, inside-out vesicles; NAD, nicotinamide adenine dinucleotide; P_i, orthophosphate; PGK, phosphoglycerate kinase; 3PG, 3-phosphoglycerate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

Sources of Materials

Highly purified choline-Cl was obtained from the Chemical Division of Syntex Agri-Business Inc., (Springfield, Mo.) and was not purified further. [^{32}P]Orthophosphoric acid, γ-^{32}P-ATP, and ^{22}NaCl were obtained from New England Nuclear (Boston, Mass.). Dextran T-70 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Acetylthiocholine-Cl, ammonium persulfate, ADP, ATP, DTNB, EGTA, G3P, hexokinase, NAD, Ponceau-S, SDS, TCA, Triton X-100, and valinomycin were from Sigma Chemical Co., (St. Louis, Mo.). Acrylamide and *N,N′*-methylene-bisacrylamide were from Bio-Rad Laboratories (Richmond, Calif.).

RESULTS

Na Transport into IOVs: Stimulation by ATP

Preliminary experiments were performed to characterize the transport of Na into IOVs with K at the intravesicular (extracellular) membrane surface. As shown in Fig. 1, ATP stimulates Na influx through the Na/K pump (we show below that the ATP-stimulated flux is inhibited by strophanthidin). The active Na transport is enhanced in vesicles treated with the K ionophore valinomycin. This is because intravesicular K remains constant in valinomycin-treated vesicles and is therefore not rate-limiting, whereas in the absence of valinomycin, the vesicles are rapidly depleted of K because of their high surface:volume ratio, resulting in a decrease in Na influx.

In Fig. 1, the rate of the ATP-dependent Na uptake was ~5.4 nmol Na/mg membrane protein·min. Below we present additional measurements of ATP-stimulated fluxes ranging from 4.8 to 8.8 nmol/mg·min, with a mean of 6.5 (n = 6; Figs. 1–3; Tables I, III, and IV; the values from Table II are not included because the protein concentrations in these experiments were not measured directly). Using 0.6 pg membrane protein/red cell ghost (Proverbio and Hoffman, 1977), 1.15×10^{13} red cells/liter of cells (Dunham and Hoffman, 1978), and correcting for the fraction of vesicles that are inside out (0.8), an ATP-stimulated uptake of 6.5 nmol/mg·min is equivalent to an efflux of Na in intact cells of 3.3 mmol/liter cells·h. This flux is greater than the ATP-stimulated fluxes of 0.7 mmol/liter cells·h measured in IOVs by Blostein and Chu (1977) and 0.28–0.36 mmol/liter cells·h reported by Sze and Solomon (1979). However, our rate of ATP-dependent Na uptake is still considerably less than 8 mmol/liter cells·h, the maximum active flux that can be obtained in intact cells (Mercer and Dunham, 1981 [in press]; J. R. Sachs, personal communication). At the concentrations of Na and K at the two surfaces of the IOV membranes, the maximum rate of transport might be expected. There is no apparent explanation for the failure to obtain it.

Effects of Ouabain and Strophanthidin on Na Transport

The results of an experiment designed to confirm that the ATP-stimulated Na transport is mediated by the Na/K pump are shown in Table I. IOVs were incubated, with and without ATP, in Na media containing ouabain or

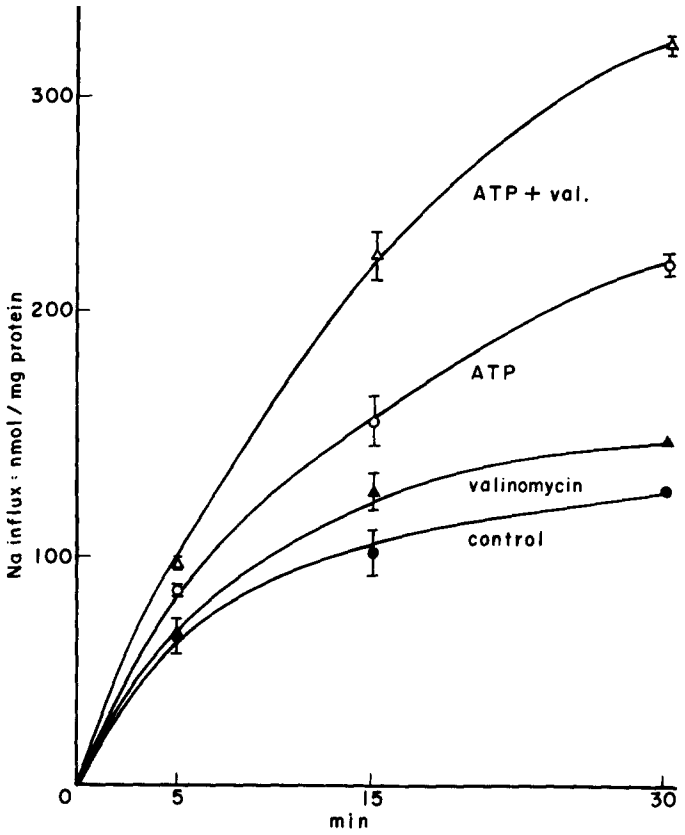

FIGURE 1. Time-course of Na uptake into IOVs in the presence of ATP and valinomycin. ^{22}Na uptake was measured by adding vesicles loaded with KCl (5 mM), $MgCl_2$ (1.0 mM), choline-Cl (20 mM), and Tris-glycylglycine (2.5 mM), pH 7.4, to flux media containing NaCl (20 mM), KCl (5 mM), $MgCl_2$ (1.0 mM), Tris-glycylglycine (2.5 mM), ATP (1 mM); and valinomycin (1 μM) as indicated. At the times indicated, 0.2-ml aliquots of the suspension were removed and placed directly onto a 1.2-μm Millipore RA 25-mm filter. The filters were washed with 15 ml of ice-cold nonradioactive medium and the intravesicular ^{22}Na associated with the filter was measured with an autogamma counter. ●, control; ○, 1 mM ATP present; ▲, 1 μM valinomycin present; △, 1 mM ATP and 1 μM valinomycin present. Each point represents the mean of three determinations ± SEM. (Error bars are omitted when smaller than the symbols.)

strophanthidin. In IOVs, extravesicular (cytoplasmic) ouabain should have no effect on active Na influx because ouabain is impermeant and the ouabain-binding site, on the external surface of the pump, is in the interior of the vesicle. However, strophanthidin, an aglycone inhibitor of the pump to which

the membranes are permeable, should inhibit active transport in the vesicles. Table I shows that extravesicular ouabain had little effect on active Na transport, and that strophanthidin completely inhibited it. The lack of effect of ouabain on active transport in IOVs (in confirmation of the results of Perrone and Blostein [1973]) confirms that the vesicles are sealed. More importantly, the inhibition of the ATP-stimulated Na transport by strophanthidin supports the view that the ATP-stimulated transport in IOVs is mediated by the Na/K pump.

Effects of the Substrates of GAPDH and PGK on Na Transport

The effects of P_i, ADP, and ADP plus P_i on active Na transport into IOVs are shown in Fig. 2. IOVs were incubated, with or without ATP, in Na media containing ADP and P_i as indicated. Neither ADP (0.5 mM) nor P_i (0.5 mM)

TABLE I
EFFECTS OF OUABAIN AND STROPHANTHIDIN ON ATP-PROMOTED Na TRANSPORT IN IOVs

	Na influx		
	Control	Ouabain	Strophanthidin
	nmol/mg protein·min		
Control	14.5±0.1	14.8±0.3	12.8±0.2
+ ATP	23.3±0.2	22.7±0.5	13.3±0.1
Δ (ATP-stimulated flux)	8.8±0.2	7.9±0.6	0.5±0.2

Conditions and procedures were the same as those described for Fig. 1 except that the vesicles contained 5 mM NaCl and 15 mM choline-Cl (KCl, $MgCl_2$, and Tris-glycylglycine were as before). IOVs were incubated at 25°C with ouabain (10 μM) or strophanthidin (10 μM) for 5 min. The vesicles were then added to flux media containing valinomycin (1 μM). Some aliquots of flux medium contained, in addition, ATP (1 mM), ouabain (10 μM), or strophanthidin (10 μM), as indicated. After 15 min, the vesicles were filtered and washed, and intravesicular ^{22}Na was determined. Shown are total influxes without (first line) and with ATP (second line) and the total minus the control influx (third line: ATP-stimulated flux). Fluxes are means ± standard errors of the mean or ± standard errors of differences (ATP-stimulated fluxes) ($n = 3$).

had much effect in the absence of ATP. However P_i, ADP, and ADP plus P_i, in the presence of ATP, inhibited the ATP-activated Na transport slightly. The inhibition of active transport by cytoplasmic P_i and ADP is not unexpected. Inhibition by P_i was shown in intact cells by Garay and Garrahan (1975), who suggested that it was brought about by decreasing the rate of dephosphorylation of the pump (i.e., product inhibition). Competitive inhibition of the Na/K pump by ADP was demonstrated by Hexum et al. (1970) in measurements on Na,K-ATPase activity of fragmented membranes from rat brain (ADP might also act as a product inhibitor, though probably with low affinity).

The modification of nucleotide concentrations by adenylate kinase, particularly the formation of ATP from ADP, ought not to be a problem with

IOVs; Cavieres and Glynn (1979) showed in experiments on resealed ghosts that adenylate kinase does not remain bound to the membranes. In any case, ADP did not stimulate transport (Fig. 2).

ADP and P_i are two of the substrates of GAPDH and PGK. Table II shows the results of three experiments in which all of the substrates were tested (except, of course, glyceraldehyde-1,3-diphosphate, which is made by GAPDH). IOVs were added to a medium containing NAD, ADP, and G3P (all of the substrates except P_i). (NAD was added at a relatively high concentration, 4 mM, to overcome the NADase activity known to exist in red cells [Alivisatos et al., 1956]. The concentrations of G3P and NAD were well above their apparent K_m's for GAPDH from human red cells; the concentration of P_i was near its K_m of 0.8 mM [Oguchi, 1970].) These vesicles, without

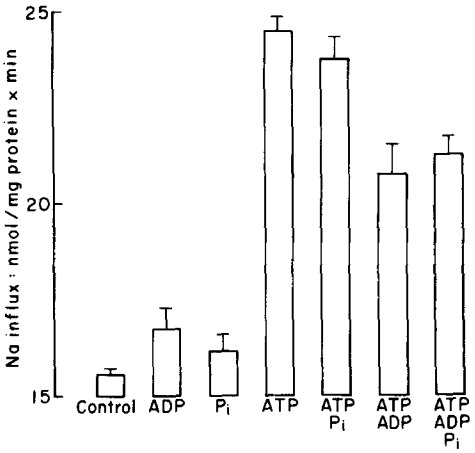

FIGURE 2. Effects of P_i, ADP, and P_i plus ADP on ATP-stimulated Na influx into IOVs. Conditions and procedures were the same as those described for Fig. 1. Concentrations of ligands employed were: ADP, 0.5 mM; P_i, 0.5 mM; ATP, 1 mM. To emphasize the relative heights of the *bars*, the origin of the ordinate was set at 15 nmol Na/mg·min. Shown are means ± SEM ($n = 4$).

P_i, are designated preparation A in Table II. The addition of ATP stimulated the Na flux by ~64% (preparation B compared with A). The addition of P_i also stimulated the flux (24%; preparation C), but not as much as ATP did. Preparation C is, of course, the condition with all of the substrates of GAPDH and PGK, but no added ATP. The stimulation was highly significant ($P < 0.01$, $n = 6$, randomization test for matched pairs; Siegel, 1956). Addition of ATP and P_i together (preparation D) stimulated Na transport to the same extent as ATP alone did.

Fig. 3 shows the results of an experiment in which we tested the effects of other combinations of the substrates. Na transport was stimulated only when all of the substrates required for ATP synthesis were present.

Elution of GAPDH

If GAPDH is involved in supplying ATP to the Na/K pump, then elution of GAPDH from the membranes will abolish activation of the pump by the substrates of GAPDH and PGK. From experiments based on this rationale, further evidence demonstrating the importance of membrane-associated GAPDH in supplying ATP to the pump is provided in Fig. 4 and Table III. Fig. 4 shows scans of SDS polyacrylamide gels after electrophoresis of membrane proteins from control IOVs and IOVs with GAPDH (band 6 as designated by Steck [1972]) removed (see Materials and Methods). Although the substrates for GAPDH and PGK stimulated Na transport in IOVs containing GAPDH, they had no effect on Na transport in GAPDH-depleted

TABLE II

INFLUENCE OF THE SUBSTRATES FOR GAPDH AND PGK ON Na INFLUX INTO IOVs

Preparation	Experiment 1	2	3
	nmol/mg protein·min		
A) Control (ADP, G3P, NAD)	10.0±0.5	18.9±0.6	20.2±0.3
B) + ATP (percent stimulation)	15.3±0.3 (53)	31.7±1.7 (67)	34.8±0.9 (72)
C) + P_i (percent stimulation)	13.4 ± 1.0 (34)	21.5±0.3 (14)	25.4±0.7 (25)
D) + ATP + P_i (percent stimulation)	—	33.6±1.0 (78)	33.5±1.3 (66)

Vesicles were prepared the same way as those described in Fig. 1. The flux medium contained NaCl (20 mM), KCl (5 mM), valinomycin (1 μM), buffer, and $MgCl_2$ as before, and the substrates ADP (1 mM), G3P (2 mM), and NAD (4 mM). Preparation A (control) had no further constituents. Preparations B–D had ATP (0.5 mM) and/or P_i (0.5 mM) as indicated. Shown in parentheses are the stimulations (as percents) by these additions. Results of three separate experiments are shown. Fluxes are expressed as nmol Na/mg protein·min (assuming 0.1 mg protein/ml; individual protein determinations were not carried out in this experiment). Values are means ± standard errors of the mean ($n = 3$).

vesicles (Table III). ATP stimulated the transport of Na into GAPDH-depleted vesicles, showing that the pump itself was unaffected by the removal of GAPDH.

Compartmentalization of ATP: Effects of Hexokinase

The ATP synthesized by PGK on the IOVs seems likely to be compartmentalized in some way because ATP released into the extravesicular medium by the IOVs would probably be too dilute to fuel the pump. Compartmentalized ATP should be inaccessible to agents in the medium that would degrade it; an effective way to degrade ATP is by the phosphorylation of glucose catalyzed by hexokinase. Table IV shows the results of two experiments in which the

presence of compartmentalized ATP, synthesized by GAPDH and PGK, was tested according to this rationale. Vesicles were added to media containing NAD, ADP, and G3P, with hexokinase or hexokinase plus glucose, and with or without P_i. (Hexokinase alone had a slight inhibitory effect on passive,

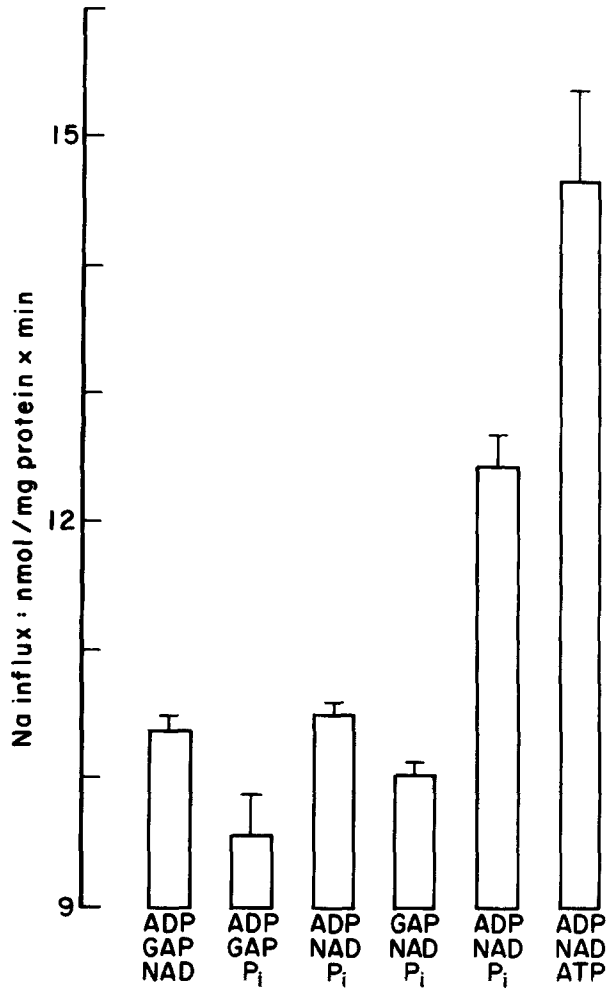

FIGURE 3. Effects on Na influx in IOVs of various combinations of substrates of GAPDH and PGK. In one aliquot, ATP was also included as indicated. Vesicles were prepared as described in Table II. Concentrations of ligands were: ADP, 1 mM; G3P, 2 mM; NAD, 4 mM; P_i, 0.5 mM; ATP, 1 mM. Valinomycin was present at 1 μM. The origin of the ordinate was set arbitrarily at 9 nmol/mg·min. Shown are means ± SEM ($n = 4$).

ATP-independent, Na transport in the vesicles. Therefore, all aliquots contained hexokinase, and hydrolysis of ATP was activated by addition of glucose. We have no explanation for this intriguing effect of hexokinase.)

Summarized at the bottom of Table IV, from the means of fluxes from the

two experiments, are data for the stimulation of influx by P_i and by ATP both when no glucose was added (therefore there was no breakdown of ATP by hexokinase) and when glucose was added (activating breakdown of ATP). Na transport promoted by ATP from PGK (activated by addition of P_i) was not inhibited when exogenous ATP would have been degraded (the stimulation by glucose was probably not significant). On the other hand, hexokinase plus glucose prevented promotion of Na transport by exogenous ATP (hexokinase alone did not prevent it). These results confirm that membrane-bound

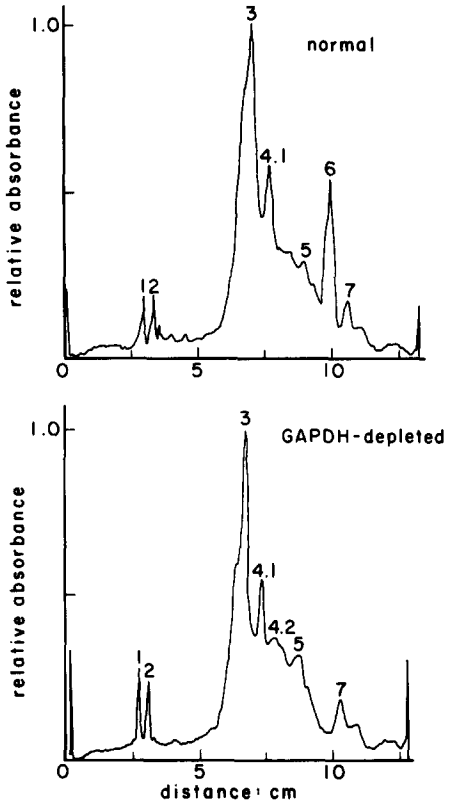

FIGURE 4. Scans of gels after electrophoresis of proteins from normal IOVs (*top*) and GAPDH-depleted IOVs (*bottom*). 5% polyacrylamide disk gels in 0.2% SDS (method of Fairbanks et al. [1971]) were stained with Coomassie Blue and scanned at 550 nm. The origins are at the left. Bands are identified according to Steck (1972); band 6 is GAPDH.

PGK supplies ATP directly to the Na/K pump without accessibility of the ATP to the extravesicular medium. These results also provide direct evidence that this ATP is compartmentalized at the membrane.

Incorporation of [^{32}P]Orthophosphate into Membrane-bound Organic Phosphate

We attempted to verify the existence of the membrane pool of ATP by labeling it with [^{32}P]orthophosphate. The results of these experiments are

TABLE III

Na INFLUX INTO IOVs DEPLETED OF GAPDH AND INTO NORMAL IOVs IN THE PRESENCE OF THE SUBSTRATES FOR GAPDH AND PGK

	Control			GAPDH-depleted		
	Control	P_i	ATP	Control	P_i	ATP
	nmol/mg protein·min					
Total	9.3±0.2	11.7±0.1	14.2±0.2	14.4±0.2	14.1±0.2	19.3±0.2
Δ	—	2.4±0.2	4.9±0.2	—	−0.3±0.2	4.9±0.2

Conditions and procedures were identical to those in Table II. Na uptake was determined in the presence of GAPDH-PGK substrates, using IOVs (control) and GAPDH-depleted IOVs, and prepared as described in Materials and Methods (Fig. 4 demonstrates the depletion). The vesicles were suspended in a medium containing ADP (1 mM), G3P (2 mM), NAD (4 mM), and valinomycin (1 μM). Control media had no additional constituents. P_i (1 mM) or ATP (1 mM) were added to other preparations as indicated. Fluxes are means ± standard errors of the mean (n = 3). Δ indicates stimulation by P_i or ATP as incremental flux over control.

TABLE IV

EFFECT OF HEXOKINASE AND GLUCOSE ON THE STIMULATION OF Na TRANSPORT IN IOVs BY ATP AND BY THE SUBSTRATES FOR GAPDH AND PGK

	Na influx					
Experiment	Control	Glucose	P_i	P_i + glucose	ATP	ATP + glucose
	nmol/mg protein·min					
1	7.5±0.1	6.6±0.3	8.8±0.5	10.5±1.3	12.3±1.9	7.5±0.6
2	4.9±0.1	6.4±0.5	7.6±0.7	8.7±0.2	10.2±0.7	5.5±0.6
Mean	6.2	6.5	8.2	9.6	11.2	6.5

Summary of stimulation

	No glucose (hexokinase inactive)	With glucose (hexokinase active)
	nmol/Na/mg·min	
By P_i	2.0	3.1
By ATP	5.0	0.0

Conditions and procedures were similar to those described in Fig. 1. IOVs were added to flux media containing hexokinase (3 U/ml), valinomycin (1 μM), ADP (1 mM), G3P (2 mM), and NAD (4 mM). Control preparations had no additional constituents. Other preparations contained, in addition: glucose (2.5 mM), P_i (0.5 mM), and/or ATP (1 mM), as indicated. Fluxes are means ± standard errors of the mean (n = 3). Results from two separate experiments are shown; the summary of stimulation is based upon the means.

shown in Table V. IOVs loaded with choline-Cl (K-free) were incubated in media containing [^{32}P]orthophosphate, NAD, and G3P, with or without ADP. After 45 min, the vesicles were washed in nonradioactive medium and the organic phosphate was extracted. As seen in Table V, the vesicles incubated

with all of the substrates necessary for ATP production incorporated roughly twice as much P_i into organic phosphate as did vesicles to which ADP was not added. Although the errors in several of the experiments were large (and the stimulation of incorporation by ADP was of questionable significance in two of the experiments), there was an indication of stimulation by ADP of incorporation of P_i into membrane-bound organic phosphate in all four of the experiments carried out. Parker and Hoffman (1967) proposed that there might be a membrane-bound pool of ADP. Although unsupported by direct evidence, such a pool would explain the incorporation of P_i observed without added ADP (Table V). This possibility was not tested. Another possible explanation for the incorporation of ^{32}P in the absence of added ADP is that it represents the synthesis of 1,3-DPG. In the model we will develop below to explain our results, it is implicitly assumed that there will be a membrane-

TABLE V

INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO MEMBRANE-BOUND ORGANIC PHOSPHATE IN IOVs INCUBATED WITH THE SUBSTRATES OF GAPDH AND PGK

	Phosphate incorporated		
Experiment	Control	ADP	Δ
		nmol/mg protein	
1	0.63±0.23	1.19±0.15	0.56
2	0.37±0.09	1.06±0.51	0.69
3	0.16±0.06	0.41±0.25	0.25
4	1.04±0.06	1.58±0.31	0.54

IOVs containing 20 mM choline-Cl, 1 mM $MgCl_2$, and 2.5 mM Tris-glycylglycine, pH 7.4, were suspended in a medium of the same composition, which also contained G3P (2 mM), NAD (4 mM), and [^{32}P]orthophosphate (0.5 mM, 250 μCi/ml). This suspension is designated "control." ADP (1.0 mM) was added to some aliquots to initiate the activity of PGK. The vesicles were incubated for 45 min and then washed, after which the organic phosphate was extracted and counted for radioactivity as described in Materials and Methods. Δ indicates stimulation by ADP of phosphate incorporation over control. Values are means ± standard errors of the mean ($n = 4$).

associated pool of 1,3-DPG as well as of ATP, but we have no direct relevant results on 1,3-DPG.

Fig. 5 shows the rate of incorporation of phosphate into membrane-bound organic phosphate. It was complete, or nearly so, in 2 min, and therefore the rate was too rapid to measure accurately by our method.

Demonstration That Membrane-bound Organic Phosphate Is ATP

Fig. 6 shows chromatographic evidence that the organic phosphate synthesized by the IOVs is ATP. ^{32}P-labeled organic phosphate extracted from the vesicles was chromatographed, along with standards (both P_i and ATP labeled with ^{32}P) on PEI-cellulose plates. The major peak of radioactivity of the organic phosphate extracted from the membranes corresponded to the ATP standard.

A standard of ADP was not run; however, this technique enables a clear separation of ATP and ADP (Randerath and Randerath, 1967).

Use of the Membrane-bound ATP by the Na/K Pump

The ATP associated with the membrane of IOVs incubated with the substrates for GAPDH and PGK, if accessible to the Na/K pump, should be used when the pump is operating. Fig. 7 shows the results of an experiment in which IOVs, loaded with 5mM KCl, were incubated in Na-free medium containing [^{32}P]orthophosphate and the other necessary substrates. After sufficient time to incorporate [^{32}P]orthophosphate into ATP to a constant level (45 min), the

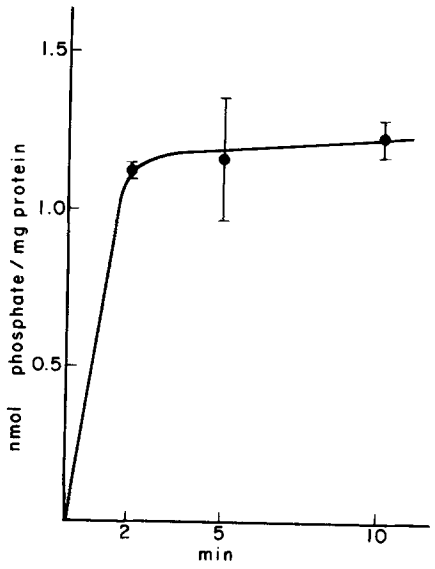

FIGURE 5. Time-course of incorporation of [^{32}P]orthophosphate into organic phosphate bound to IOVs. Vesicles containing choline-Cl (20 mM), $MgCl_2$ (1 mM), and Tris-glycylglycine (2.5 mM), pH 7.4, were suspended in a medium of the same composition that also contained G3P (2 mM), NAD (4 mM), ADP (1 mM), and [^{32}P]orthophosphate (0.5 mM; 200 μCi/ml). The vesicles were incubated for the times indicated, and then were washed and extracted for ^{32}P-labeled organic phosphate as described in Materials and Methods.

vesicles were placed in media containing Na, with or without strophanthidin. The addition of Na caused the loss of ATP from the vesicles, presumably by initiating the transport of ions by the pump and the concomitant hydrolysis of ATP. This decrease in bound ATP caused by Na was blocked by the addition of strophanthidin, confirming that the decrease was mediated by the pump and that the bound organic phosphate is ATP. In the experiment in Fig. 7, bound ATP approached a constant level 30 min after the addition of Na, which should represent the balance achieved when both synthesis (by PGK) and use (by the pump) are taking place. Fig. 8 shows a similar experiment, except that an excess of unlabeled P_i (10 mM) was added along

with the Na, preventing further synthesis of [^{32}P]ATP after the pump was activated. The strophanthidin-inhibitable decline in membrane-bound ATP was much more rapid than in Fig. 7 (where synthesis of [^{32}P]ATP continued). The amount of membrane-bound ATP fell in 5 min to ≤10% of the level at time zero in IOVs that were synthesizing [^{32}P]ATP but not using it. This

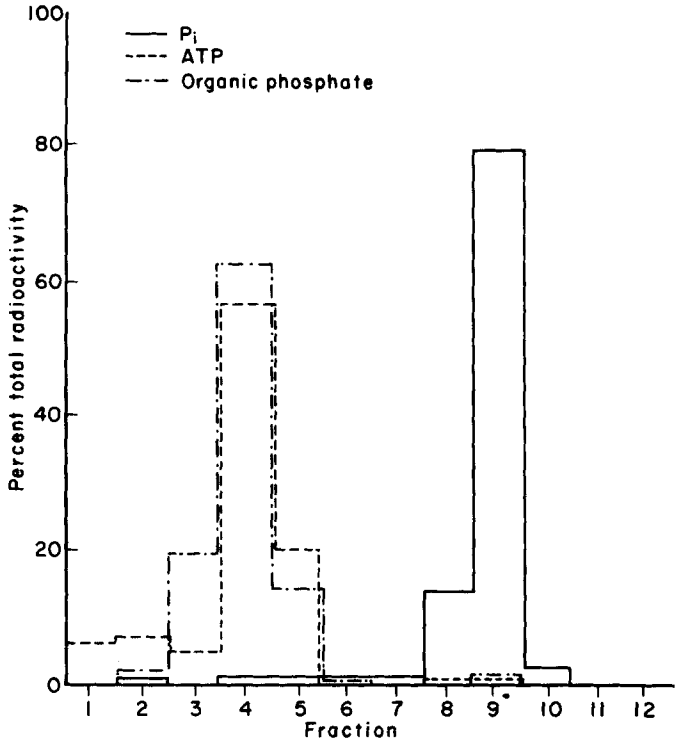

FIGURE 6. Chromatography of ^{32}P-labeled organic phosphate extracted from IOVs. Vesicles were prepared the same as those in the experiment in Fig. 5. The incubation medium, including [^{32}P]orthophosphate, was also the same as for Fig. 5. After incubation for 45 min, the vesicles were washed and extracted for organic phosphate. This was spotted on PEI-cellulose plates, along with the standards [^{32}P]orthophosphate and [^{32}P]ATP. After the solvent front moved 12 cm, the plate was dried and cut into 12 sections. The radioactivity of each was determined by Cerenkov scattering in a liquid scintillation counter. Similar results were obtained in two other experiments on separate preparations of vesicles.

observation confirms that at least most of the ATP synthesized at the membranes and bound to them is available to fuel the pump.

DISCUSSION

The main conclusions to be drawn from this study are that the glycolytic enzymes GAPDH and PGK, bound to the red cell membrane, synthesize ATP

and deposit it in a membrane-associated compartment, and that this membrane-bound ATP is used by the Na/K pump. The major points of evidence were: (*a*) active Na transport into inside-out vesicles was promoted by the addition of the substrates for GAPDH and PGK, and without added ATP; (*b*) the GAPDH-PGK complex synthesizes ATP, which remains associated with the membrane; (*c*) this bound ATP fuels the pump because its level is reduced by the addition of Na, and this reduction is inhibited by strophanthidin; and (*d*) the membrane-bound ATP is compartmentalized in the sense that it is inaccessible to degradation by hexokinase.

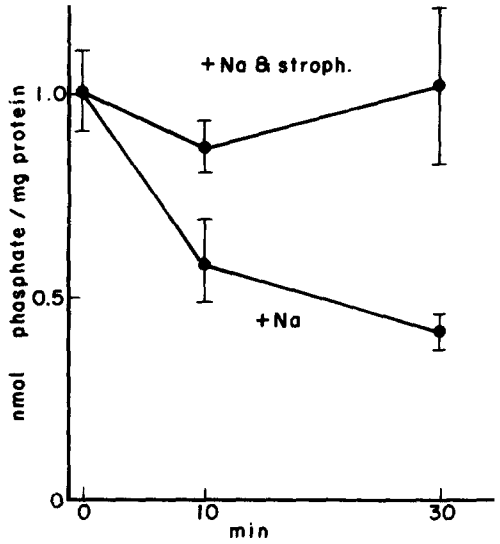

FIGURE 7. Use of membrane-bound ATP by the Na/K pump of IOVs, which were simultaneously transporting Na and synthesizing [^{32}P]ATP. Vesicles were prepared the same way as described for Fig. 5. They were incubated in a medium containing KCl (5 mM), choline-Cl (20 mM), $MgCl_2$ (1 mM), Tris-glycylglycine (2.5 mM), pH 7.4, ADP (1 mM), G3P (2 mM), NAD (4 mM), and [^{32}P]orthophosphate (0.5 mM, 250 μCi/ml). After 45 min, the vesicles were suspended in a similar medium, except that the concentrations of ADP, G3P, NAD, P_i, and choline were reduced by half, Na was added to a final concentration of 10 mM, and valinomycin was added to 1 μM. To some aliquots strophanthidin was also added (1 μM) as indicated. At various times, samples of vesicles were removed, washed, and extracted for organic phosphate as described in Materials and Methods.

The functional relationship between the three enzymes in the multienzyme complex is represented diagrammatically in Fig. 9. ATP is in parentheses to indicate that we think it is compartmentalized. 1,3-DPG is probably also compartmentalized, but we have no evidence that it is. GAPDH and PGK are, of course, peripheral proteins and Na,K-ATPase is an integral protein; the structural relationship between the proteins is not known. Much if not all

of the membrane-associated GAPDH is bound to the anion-exchange protein of band 3 (Kliman and Steck, 1980). It may be that a small fraction of these GAPDH molecules has a functional relationship with the Na/K pumps, although it has been suggested that GAPDH bound to band 3 protein is

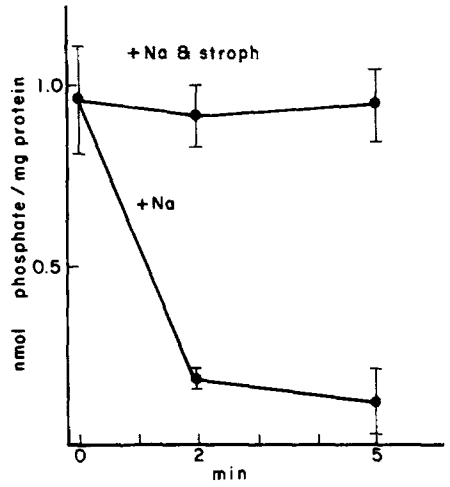

FIGURE 8. Use of membrane-bound ATP by the Na/K pump of IOVs in which [^{32}P]ATP synthesis was stopped at the same time the pump was activated. The experiment was carried out the same way as the one in Fig. 7, except that when the flux was initiated by Na, the orthophosphate concentration was increased to 10 mM, thereby stopping the synthesis of [^{32}P]ATP from [^{32}P] orthophosphate.

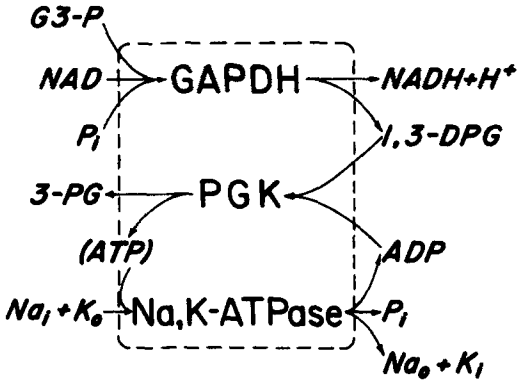

FIGURE 9. Functional relationship between two glycolytic enzymes and the Na/K pump. ATP is in parentheses to indicate compartmentalization. Other ligands, such as 1,3-DPG and ADP, may also be compartmentalized.

reversibly inhibited (I.-H. Tsai, S. N. P. Murthy, and T. L. Steck, personal communication). Alternatively, a small subpopulation of GAPDH has separate binding sites on or near the pumps. It is not known where PGK molecules are bound. It is also not known if other glycolytic enzymes are associated with

the enzyme system. Finally, there may be proteins other than glycolytic enzymes that serve as a part of the ATP compartment.

Similar schemes were proposed several years ago for functional relationships between the glycolytic enzymes and the Na/K pump (Parker and Hoffman, 1967; Proverbio and Hoffman, 1977; Schrier et al., 1975; Segel et al., 1975). More recently, such a multienzyme system was proposed on the basis of measurements of ^{31}P nuclear magnetic resonances (NMR) of 2,3-DPG and G3P in red cells and bound to IOVs and on an effect of ouabain on the NMR (Fossel and Solomon, 1977 and 1979; Solomon, 1978). The shift in NMR caused by ouabain is consistent with the binding of a small subpopulation of GAPDH to sites associated with the pumps such as we proposed above (and separate from band 3 protein); otherwise, a complex interaction must be proposed between band 3 and the pump.

From the measurements of the amount of organic phosphate synthesized by PGK and bound to the membrane, it ought to be possible to estimate the number of ATP molecules, bound or otherwise compartmentalized, associated with each pump. In so doing, it is necessary to accept that the synthesized, bound organic phosphate is ATP and that it is associated with Na/K pumps. Figs. 6–8 support these conclusions. Assuming 0.6 pg of protein/red cell membrane (Proverbio and Hoffman, 1977) and 250–500 pump sites/cell (see Table I of Joiner and Lauf [1978]), one can calculate from the data in Table V that there are 350–700 molecules of ATP associated with each pump. This range of values is in remarkable agreement with Proverbio and Hoffman (1977), whose estimate, based on indirect evidence, was 100–600 molecules of ATP per pump.

There are several important uncertainties associated with our calculation of the number of ATP molecules per pump. First, the data in Table V were obtained under conditions where the pump was not running, and Fig. 7 suggests that the steady state level of bound ATP is two- to threefold lower when the pump is running than when it is not. Second, the method for production of the IOVs undoubtedly elutes glycolytic enzymes from the membranes, and some of these enzymes may come off the pumps (although the pumps or associated molecules may be stronger binding sites than band 3). This would account for the lower activation of the pump by the substrates of GAPDH and PGK than by exogenous ATP (Table II), although this argument raises the question of the accessibility to the pump, either directly or indirectly by the way of the bound compartment, of bulk phase ATP. Elution of the glycolytic enzymes from the pump would reduce the synthesis of ATP available for incorporation into the membrane-associated compartment. Furthermore, these enzymes or other peripheral proteins may comprise a part of the compartment or the boundaries of it, whatever the mechanism of retention of ATP. This second consideration could mean that the calculated number of ATP molecules per pump is underestimated by two- to threefold.

A third uncertainty stems from elution of peripheral proteins in general in the preparation of the IOVs. If a substantial amount of protein is eluted, then 0.6 pg of protein per membrane cannot be used to calculate ATP molecules

per cell or per pump. A comparison of the electrophoretic patterns of the proteins of IOVs (Fig. 4) and of extracts of otherwise untreated ghosts (Sauberman et al., 1979) shows that there is a much higher proportion of band 3 (the major integral protein) in IOVs relative to bands 1 and 2 (the major peripheral proteins) than in the ghosts. Therefore, much of the peripheral protein has been lost from the IOVs. The magnitude of this reduction in total membrane protein, which leads to an overestimate in the number of ATP molecules per pump, is of the order of 1.5–2-fold (peripheral proteins are ~55% of total membrane protein of ghosts; Fairbanks [1980]).

The nature of the association of ATP with the membrane is obscure. The ATP molecules could be electrostatically bound to membrane constituents, integral and/or peripheral. Alternatively, the ATP molecules could be in solution in an occluded volume in or adjacent to the membrane, perhaps enclosed in part by GAPDH and PGK molecules or other peripheral proteins. If the ATP is assumed to be compartmentalized in solution, some arbitrary limits of the dimensions of the ATP compartment can be estimated. If the concentration of ATP in the compartment or "pool" is taken, simply for the purpose of illustration, to be 10 mM, then a compartment containing 350 molecules would occupy a volume of ~60,000 nm^3. This is two orders of magnitude greater than the volume occupied by an enzyme molecule such as GAPDH or PGK (~140 $nm^3/10^5$ mol wt, $\rho = 1.2$). Various assumptions about the geometry of the association of the ATP compartments with the membrane can give estimates of up to 10% of the total membrane surface required to accommodate all of the ATP. Although the uncertainties associated with this estimate are legion (several are discussed above), it can lead to several general conclusions. First, given the probable volume occupied by the ATP, it is unlikely that the bound ATP is situated *within* the membrane (cf. Proverbio and Hoffman [1977]); more likely it is between the integral part of the membrane and the peripheral proteins. Second, for the same reason, more than one molecule each of GAPDH and PGK are probably necessary to provide the boundaries of the ATP compartment associated with each pump (De and Kirtley [1977] showed that 230 molecules of PGK are bound per red cell membrane, astonishingly close to some of the lower estimates of the number of Na/K pumps per cell; Joiner and Luf [1978]). Furthermore, these considerations regarding the size of ATP compartments could apply in a general sense if ATP were bound rather than contained in solution in an occluded space. Further speculation is not warranted.

Arguments against the coupling of membrane-bound glycolytic enzymes to the Na/K pump have been advanced by De and Kirtley (1977) and Beutler et al. (1978). De and Kirtley failed to detect coupling between the activities of PGK and the pump; Beutler et al. suggested that PGK-derived ATP was not compartmentalized in the membrane because it was available for phosphorylation of sugar catalyzed by hexokinase. However, the arguments based on these observations suffer from the fact that most of the PGK in red cells cannot be associated with the pumps.

Although there is in red cells synthesis of ATP by enzymes that are not

coupled to Na/K pumps, and the pumps may be able to use this ATP (Hoffman, 1962), there is evidence from intact cells of the importance of the membrane-associated enzymes in fueling the pump. Sachs (1972) and Segel et al. (1975) found that cells depleted of triose phosphates but not of ATP had a reduced rate of activity of the Na/K pump. More recently, we found that reducing the concentration of P_i in intact red cells to <0.5 mmol/liter caused inhibition of the pump (Mercer and Dunham, 1981 [in press]).

Coupling of glycolysis to the Na/K pump by way of membrane-bound glycolytic enzymes may occur in other types of cells as well. For example, Paul et al. (1979) found evidence in vascular smooth muscle that glycolysis appears to be coupled to transport of Na and K, whereas oxidative metabolism is coupled to the energy requirements of contraction. In what may be related observations, evidence has been presented for binding of creatine phosphokinase to the surface membrane of myocardial cells and for functional coupling of this enzyme to the Na/K pump (Saks et al., 1977; Grosse et al., 1980).

In general, there are two types of advantages that can accrue from organized multienzyme systems such as the one we are proposing (GAPDH, PGK, and Na,K-ATPase; see Fig. 9): efficiency and regulation (Welch, 1977). Increased efficiency may result simply from proximal juxtaposition of the enzymes; or the physical association of the enzymes (and other aspects of their resultant local environment) may alter their catalytic properties in an advantageous manner, enhancing efficiency. Secondly, the associated enzyme system may bestow opportunities for regulation not possible with the separate proteins; the opportunities include compartmentalization of and local control of concentrations of substrates, products, and other regulator ligands. For a detailed discussion of the functional significance of organized multienzyme systems, see Welch (1977).

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