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# The Link between Metabolism and Active Transport of Sodium in Human Red Cell Ghosts*

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**Summary.** Reconstituted human red blood cell ghosts have been used to assay various metabolic intermediates for their role in driving the Na:K pump. ATP was identified as the primary substrate of the pump. The main evidence was based on 1) the finding that the only requirement for activation of the pump was the presence of ATP, whether incorporated directly or generated by an ATP-yielding reaction; 2) the discriminating effects of various metabolic inhibitors; 3) the competition between the pump and the hexokinase reaction for ATP; and 4) the difference in effects of adenosine and inosine in activating the pump in energy-depleted ghosts. ADP was found to affect the Na:K pump due to the presence of an adenylate kinase and perhaps because of an effect on the phosphoryl potential. The sidedness of action of the cardiotonic steroid, strophanthidin, was evaluated and found to inhibit the Na:K pump only from the outside of the membrane. Inhibition of the pump by strophanthidin was also found to spare ATP in reconstituted ghosts provided the nonspecific phosphatase activity was suppressed.

The active transport of cations across the membrane of red blood cells is known to be driven by energy derived from glycolysis (Danowski, 1941; Harris, 1941). Since this initial work the characteristics of the transport process itself have been further defined as well as its dependence on metabolism (*see* Maizels, 1954; Tosteson, 1955; Glynn, 1957*a*); in addition, the various metabolic pathways and enzymatic capacities of red cells have become known in greater detail (*see* Prankerd, 1956; Altman, 1959). This information necessarily forms the basis for the present investigation.

This paper is concerned with the demonstration that ATP itself provides the immediate source of energy for the transport mechanism in red cells. Much work has been done toward the establishment of this relationship (Gardos, 1954; Gerlach, 1956; Dunham, 1957*a*, *b*; Whittam, 1958). The most direct evidence is that of Dunham (1957*a*, *b*) and Whittam (1958) in which the rate of utilization of ATP was correlated with the influx of K under a variety of experimental conditions. These studies (as well as Gardos, 1956; Pragay, 1957) clearly indicate that the overall reaction for transport was dependent upon phosphate bond energy. However, the problem remained to evaluate whether this utilization of ATP was direct or that intermediate steps were possibly involved between the dephosphorylation of ATP and the pump; that is, the determination of the specific chemical reaction coupled to the transport mechanism.

It has been possible to approach the solution of this problem, as described below, by the use of ghost systems. With ghosts, in attaining a greatly simplified and controlled metabolism, it is possible to assay particular substrates for their role in initiating and sustaining the activity of the transport mechanism. Thus, the advantage offered by ghosts lies in the fact that compounds to which the cells are normally impermeable can be incorporated inside the ghosts at the time of hemolysis (Straub, 1953). Immediately subsequent to this incorporation a portion of the ghosts sponta-

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* **Dedication.** In dedicating this article to Professor Walther Wilbrandt, I would like to acknowledge my indebtedness to him for I have been much influenced by many of his stimulating and substantive contributions over the years. The last time I spoke with Professor Wilbrandt was during his visit to Yale in March, 1979. As we were discussing then some of the data contained in this paper, Professor Wilbrandt expressed surprise that we were reviewing an almost completed manuscript, albeit written years before. He urged me to publish it. The paper up to the Discussion section is presented as it was written in 1965, but the discussion has been shortened and updated. While the circumstance is sad for its appearance in this volume, I hope that the paper represents an appropriate contribution to Professor Wilbrandt's memory.

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neously reconstitutes and provides a system in which the flux of cations can be measured (Hoffman, 1962*a*). The previous use of ghost systems in this regard as well as more recent studies will be considered in the Discussion. Although the experiments below show that the activity of the pump, resident in the membrane, operates on intracellularly produced ATP it should be recognized that the mechanism by which this energy is converted into osmotic work is at present not known.

Brief accounts of most of the work reported in this paper have been presented previously (*cf.* Hoffman, 1960*a*, *b*, 1961, 1962*b*).

## Materials and Methods

### *Compounds, Solutions and Abbreviations*

The sodium salts of the following chromatographically pure compounds were used: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; UTP, uridine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; IDP, inosine 5′-diphosphate; IMP, inosine-5′monophosphate. Each of these nucleotides was obtained from several commercial sources and used without further purification. PEP (phosphoenolpyruvate) was obtained from Calbiochem-Behring Corp. as the tricyclohexylammonium salt. The sodium salts of phosphocreatine (CrP) and ATPPPP (adenosine 5′-tetraphosphate) as well as thiamine pyrophosphate chloride (ThPP) were obtained from Sigma Chemical Co.; the sodium salts of CTP (cytidine 5′-triphosphate) and deoxyATP as well as deoxyadenosine, deoxyinosine and the magnesium salt of PRPP (5-phosphoylribose-1-pyrophosphate) were obtained from Pabst Laboratories.

$AsO_4$ is sodium arsenate; IAA, sodium iodoacetate; $NaN_3$, sodium azide, and NaF, sodium fluoride.

Hexokinase used in the experiments relative to Fig. 3 was obtained from Nutritional Biochemicals Corporation (yeast, 28,000 KM units/gram at 30 °C); to Table 7, from Sigma Chemical Co. (yeast, Type IV).

Adenosine, inosine and strophanthidin were obtained from a number of commercial sources. Disodium ethylenediaminetetracetate (EDTA) was obtained from Fisher Scientific Co.; acetylcholine chloride (ACh), from Merck and Co. and physiostigmine salicylate, from Burroughs Wellcome and Co.

Uniformly labeled $[^{32}P]Na_2ATP$ with a specific activity approximately 1 millicurie/gram was obtained from Schwarz Bioresearch, Inc.

Concentrations are expressed in millimoles per liter. Abbreviations like $MgCl_2$-Tris stand for a 9 to 1 mixture of osmotically equivalent solutions; for example, 24 mM $MgCl_2$-Tris contains 9 parts of 24 mM $MgCl_2 + 1$ part 34 mM Tris (hydroxymethyl) aminomethane; similarly, 17 mM $KCl-PO_4$ contains 9 parts of 17 mM $KCl + 1$ part 11 mM $K_2HPO_4/KH_2PO_4$ mixture.

### *Collection and Handling of Blood*

Blood, from healthy young adults, was drawn into a polyethylene bag containing heparin dissolved in a small volume of isotonic buffered saline. Substitution of sodium citrate (*cf.* Hoffman, 1962*a*) for heparin did not influence the results. After collection the blood was centrifuged at *ca.* 15,000 × *g* for 5 min and the plasma and buffy coat discarded. The packed cells were treated as follows:

*Fresh ghosts.* Fresh ghosts refers to the ghosts prepared immediately from the packed cells as obtained above. The total lapse of time between the withdrawal of the blood to the completion of the last ghost washing was always less than 4 hours.

*Depleted Ghosts.* The packed cells as obtained above were suspended in an equal volume of a solution containing 153 mM KCl, 9.4 mM $Na_2HPO_4$ and 1.6 mM $NaH_2PO_4$ (pH = 7.4) plus chloromycetin (2 mg/100 ml) to prevent bacterial contamination. This suspension was incubated at 37 °C, with constant but gentle shaking for 18 to 24 hr. Afterwards, the mixture was centrifuged as before and the supernatant discarded. The remaining "depleted" packed cells were either studied directly or used to prepare *depleted ghosts.*

### *Preparation of Ghosts*

The following procedure, slightly modified from Hoffman (1962*a*), was used for the routine preparation of $^{24}Na$-labeled ghosts. One volume of packed cells (C), either fresh or depleted, was hemolyzed in 10 volumes of a cold (2–4 °C) hypotonic solution (H), the composition of which is described below. In all cases the hemolytic ratio, C/H = 1/10, was used unless otherwise noted. After hemolysis the mixture was left in the cold for 20 min before centrifugation at approximately 18,000 × *g* for 5 min. The ghosts were then washed 4 times using for each wash 10 volumes of solution kept at 2–4 °C. The washing solution used for most experiments was 24 mM $MgCl_2$-Tris. The concentration of $MgCl_2$ in this solution can be varied without affecting the results but must have an osmotic pressure greater than that of the hemolyzate in order to minimize the occurrence of any rehemolysis of the ghosts during washing. The $^{24}Na$-labeled ghosts, concentrated from the fourth wash, were added directly to the incubation medium as given below.

### *Composition of the Hemolysis Solution*

$^{24}Na$ was added, in trace amount, to all hemolysis solutions. Control ghosts (no added substrate) were prepared by hemolysis in 1 mM $MgCl_2$. For the preparation of ghosts with incorporated substrate the hemolysis solution contained, unless noted otherwise, 2 mM substrate + 2 mM $MgCl_2$ when the substrate was ATP, ADP, UTP, GTP, CTP, deoxy ATP, PRPP, ITP, IDP, or IMP or 2 mM substrate + 1 mM $MgCl_2$ when the substrate was AMP, PEP, CrP, ThPP, or ACh. The experiments in which ADP (trace) was added refer to either 0.1 mM ADP + 1 mM $MgCl_2$ or 0.1 mM ADP + 2 mM PEP (or ITP) + 2 mM $MgCl_2$. Solutions, when necessary, were neutralized by the addition of either Tris, HCl or NaOH. Other compounds added to the hemolysis solutions as well as certain alterations in the above conditions are presented in relation to the relevant experiments. It was shown using the methods given by Hoffman (1958) that complete hemolysis occurred (no intact cells remained) in all of the hemolysis conditions used in this work.

Further, considering ATP as the prototype for incorporated substrate, it has been found that, after hemolysis, ATP is distributed to equal concentration between ghosts and their hemolyzate (i.e., the same as the distribution of $^{24}Na$) and that the ghosts retain, like $^{24}Na$, ATP upon subsequent washing.

It is to be understood that compounds referred to as *incorporated substrates* were presented to the ghosts only at the time hemolysis.

### Measurement of Na Outflux

The method used is essentially the same as the one described by Hoffman (1962*a*): one volume of $^{24}$Na-labeled ghosts was dispensed into different flasks each containing approximately 30 volumes of medium (*see below* for composition) and put in a Dubnoff shaker at 37 °C. Aliquots of 5 ml removed at various times over a 2 to 3 hr period were centrifuged immediately at 20,000 × *g* for 5 min at 4 °C. The zero time sample was taken from each flask after mixing but prior to the 37 °C incubation. Samples of 3 ml of each ghost-free supernatant (S) were pipetted and counted. In addition, 3 ml of each suspension mixture (M) were also counted. The percent $^{24}$Na released from the ghosts for each time, *t*, was calculated from relation:

$$\frac{(R)_S^t}{(R)_M} \times 100,$$

where $(R)$ equals the counts per minute. For the reasons given previously (Hoffman, 1962*a*) the value of $(R)_S^{t=0}$ is subtracted from $(R)_M$ and all other $(R)_S^{t=t}$ taken from each flask before calculating the percent released. The conversion of the percent $^{24}$Na released per unit time to the actual outflux of Na (in mM/liter ghosts × hr) is given in Hoffman (1962*a*). Since, in some experiments, the rate of release of $^{24}$Na from ghosts deviates slightly from a single exponential (but with no significant alteration of the results), the data are expressed in units of percent $^{24}$Na released per unit time as a relative measure of the outflux. All experiments have been performed at least twice and the results are reproducible to the extent that the same qualitative effects are readily observable for each type of experiment performed, but the variation inherent in the different ghost preparations excludes comparison on a quantitative basis.

The incubation medium contained 108 mM $MgCl_2$-Tris + 17 mM KCl-$PO_4$ (pH = 7.4). Any alterations in the composition of this medium (by additions or substitutions) are indicated in connection with the relevant experiments. Substrates to which the membrane is normally permeable, such as adenosine, adenine or inosine, were added only to the final incubation medium at a concentration of 3 mM.

All analytical procedures used are identical to the ones previously described (Hoffman, 1962a). Lactic acid was measured chemically by the method of Barker and Summerson (1941) and enzymatically using lactic acid dehydrogenase and DPN as supplied by C.F. Boehringer and Soehne, GMBH, Mannheim, Germany. Inorganic phosphate ($P_i$) was estimated by the method of Berenblum and Chain (1938). This method is based on the formation of an inorganic phosphomolybdate complex soluble in butyl alcohol. $^{32}P_i$ was estimated by counting appropriate aliquots of the butyl alcohol extract using either an end-window GM tube and planchets or a liquid scintillation counter and Bray's (1960) solution.

### Operational Definition of Active Transport

The fraction of the total Na outflux that can be inhibited by strophanthidin is taken to represent the activity of the Na pump. However, it should be emphasized that it was independently established for many of the experimental conditions discussed here that the strophanthidin-sensitive component of the Na outflux was essentially equivalent to the inhibition produced by the removal of K from the medium (*cf.* Harris & Maizels, 1951; Harris, 1954; Glynn, 1957*b*). Further justification for the use of this definition in ghost systems is considered by Hoffman (1962*a*). Thus, for the purposes of this paper, an estimate of the Na pump for each condition can be taken as the difference between the percent $^{24}$Na released when strophanthidin is absent compared to when it is present.

## Results

### Differential Stimulation of Na Outflux

These experiments are concerned with the activation of the Na pump in fresh and depleted ghosts by substrates added to the final incubation medium. Table 1A shows the effect of various substances on the

**Table 1.** Effect of various substrates on the rate of loss of $^{24}$Na from fresh and depleted ghosts

| Exp. | Substrate incorporated | Compound added to incubation medium[b] | Percent $^{24}$Na released in 90 min | |
|---|---|---|---|---|
| | | | Fresh ghosts | Depleted ghosts |
| A | Control[a] | Alone[c] | 22.0 | 19.3 |
| | | Strophanthidin ($1 \times 10^{-4}$ M) | 20.5 | 16.4 |
| | | Inosine (3 mM) | 71.7 | 17.3 |
| | | Adenosine (3 mM) | 73.8 | 35.5 |
| | | Glucose (7 mM) | 23.2 | – |
| B | ATP | Alone | 64.0 | 61.4 |
| | | Strophanthidin ($1 \times 10^{-4}$ M) | 13.5 | 19.1 |
| | | Inosine (3 mM) | 72.1 | 67.1 |
| | | Adenosine (3 mM) | – | 71.7 |
| | | Glucose (7 mM) | 69.9 | – |
| C | ADP | Alone | 50.4 | 47.6 |
| | | Strophanthidin ($1 \times 10^{-4}$ M) | 12.3 | 21.5 |
| | | Inosine (3 mM) | 54.3 | 50.7 |
| D | PEP | Alone | 54.9* | 23.9 |
| | | Strophanthidin ($1 \times 10^{-4}$ M) | 39.1* | 19.0 |
| | | Inosine (3 mM) | 77.0* | 36.8 |
| | | Adenosine (3 mM) | – | 43.0 |

[a] Control means that no substrates were incorporated at hemolysis.

[b] The incubation medium contained 108 mM $MgCl_2$-Tris + 17 mM KCl-$PO_4$ (pH 7.4) as described in Materials and Methods.

[c] The term, alone, indicates no addition to final medium.

* Medium contains Na in place of Mg.

rate of loss of $^{24}$Na from fresh and depleted ghosts without any incorporated substrate. It is apparent that in fresh ghosts the nucleosides, inosine and adenosine, are equally effective in stimulating active Na transport. But these two nucleosides are not at all comparable in this regard when presented to depleted ghosts. In depleted ghosts, adenosine leads to activation of the Na pump under circumstances where inosine is without effect (a similar experiment is recorded in Tables 3 and 5). The significance of this result will be considered below. It should be noted that the slight inhibition in the loss of $^{24}$Na seen upon the addition of strophanthidin presumably represents an effect on a downhill flux. This component is always small and presumably can be neglected with regard to the main concern of this paper. The remaining strophanthidin-insensitive component of the outflux is not appreciably altered whether or not substrate has been added to the medium.

This difference in the response of depleted ghosts to inosine and adenosine can also be demonstrated on depleted intact cells (Table 2). The red cells used in this experiment were first preincubated at 37 °C for 18 hr in the absence of substrate. The cells were then labeled with $^{24}$Na during the next and final 4 hr of the preincubation period. After preincubation the cells were washed with and suspended in a solution containing 112 mM MgCl$_2$, 15 mM KCl and 17 mM Tris buffer (pH = 7.4). The rate of appearance of $^{24}$Na was measured for 90 min using the identical methods as described for ghosts; Na outflux was calculated from the equation: $^{o}M_{\mathrm{Na}} = {}^{o}k_{\mathrm{Na}}\ (\mathrm{Na})_c$, where $^{o}M_{\mathrm{Na}}$ is the Na outflux in mM Na/liter cells × hr; $^{o}k_{\mathrm{Na}}$ is the outward rate constant in hr$^{-1}$ and (Na)$_c$ is the cellular concentration of Na in mM Na/liter cells (*cf.* Hoffman, 1962*a*). The results show that the Na pump is activated with adenosine but not with inosine during the 90-min flux period. The magnitude of the pump flux obtained under these conditions is essentially the same as the value obtained in fresh cells in the presence of Na (*cf.* Glynn, 1957*b*). It should be stated that the conditions necessary for the demonstration of this differential stimulation with the nucleosides have been found to vary for different bloods primarily in the required length of preincubation.

**Table 2.** Effect of inosine and adenosine on the outflux of Na ($^{o}M_{\mathrm{Na}}$) and lactate production in depleted intact red blood cells in mM Na/liter cells × hr[a]

| Compound added to incubation medium | Concentration (mM/liter) | $^{o}M_{\mathrm{Na}}$ (mM/liter cells·hr) | Lactate production |
|---|---|---|---|
| Control | – | 0.20 | 0.00 |
| Strophanthidin | 0.05 | 0.21 | 0.00 |
| Inosine | 5.0 | 0.27 | 6.56 |
| Adenosine | 5.0 | 1.50 | 5.52 |

[a] The concentration of Na in cells was 8.5 mM Na/liter cells.

The significance of this differential activation of the pump depends upon the metabolic state of depleted cells. Depletion of the cellular energy reserves, brought about by the preincubation procedure, results in the loss of practically all ATP, ADP, HDP and 2,3-DPG (Dunham, 1957a, b); Whittam, 1958). The addition of nucleoside stimulates glycolysis, the production of lactate by inosine or adenosine being comparable both in depleted cells (Table 2) and in depleted ghosts (*see later*). While it is known (Dische, 1951) that nucleosides enter the Embden-Meyerhof pathway from the pentose shunt, the present results indicate that not only is there a turnover of high-energy phosphate, but also imply that these nucleosides provide for the replenishment of the normal glycolytic intermediates similar to that observed in the type of ghosts studied by Lionetti et al., (1961) and in cold-stored intact cells by Gabrio, Finch and Huennekens (1956). The difference between the action of these two nucleosides with regard to the pump, then, is probably due to the different nucleotides formed rather than related to any of the various intermediates. (We have observed [*unpublished experiments*] in depleted ghosts a net synthesis of ITP given inosine or ATP, given adenosine.) This would appear to eliminate the participation of compounds other than the nucleotides, ATP and ADP, in the transport reaction. These considerations provide the basis for the concept that ATP is the direct substrate of the pump. Evidence in support of this concept is developed in the remainder of this paper.

### *Effect of Incorporated ATP, ADP and PEP*

In the experiments described above, the activation of the Na pump is presumably brought about by the ATP generated from the glycolytic utilization of nucleoside. A more direct approach is presented in Table 1, B, C and D and Figs. 1 and 2 in which the action of specific compounds, incorporated into ghosts at the time of hemolysis is assessed. As before a comparison is made between fresh and depleted ghosts as well as the effect of various compounds added to the final incubation medium for each hemolysis condition.

Table 1, B and C, shows that incorporated ATP or ADP will provide for the restitution of the Na pump. The activation produced by either ATP or ADP is essentially the same in fresh and depleted ghosts and in both, the addition of nucleoside causes

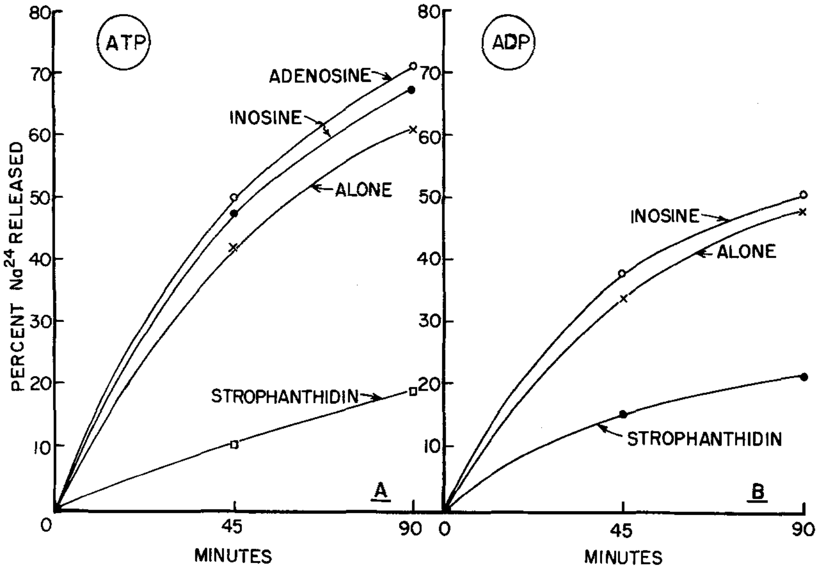

**Fig. 1.** Rate of release of $^{24}Na$ from depleted ghosts containing incorporated ATP (left, *A*) or ADP (right, *B*). The data presented in this Figure are from the same two relevant experiments presented in Table 1. The incubation conditions are given in the legend of Table 1. (Taken from Hoffman, 1962*b*)

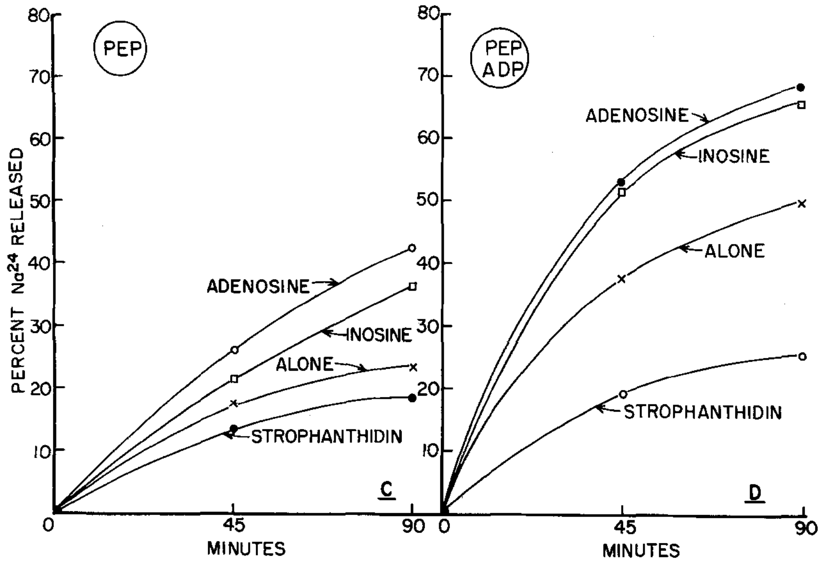

**Fig. 2.** Rate of release of $^{24}Na$ from depleted ghosts containing incorporated PEP (left, *C*) or PEP+ADP (trace) as presented in the right side (*D*) of the Figure. The data given for PEP are from the same experiment as presented in Table 1. The incubation conditions for both sets of incorporated substrates are the same as described in the legend of Table 1. (Taken from Hoffman, 1962 *b*)

only a small enhancement of the outflux. The time-course of the loss of $^{24}Na$ from the depleted ghosts presented in Table 1, B and C, is shown in Fig. 1, *A* and *B*, respectively. It should be noted that ATP is more effective than ADP in stimulating the active transport of Na. The fact that both of these nucleotides are effective may reflect the activity of an adenylic kinase known to be present in red cells (Overgaard-Hansen, 1957). Although the experimental distinction between ATP and ADP with regard to their primacy in the pump reaction will be considered later, these results are consistent with the idea that ATP is the immediate substrate of the pump.

If the conclusion is correct that the pump will use only ATP then it should be possible to activate the pump through a reaction that would generate ATP. Such a reaction is represented by the conversion of PEP to pyruvic acid which utilizes ADP as phosphate acceptor to yield ATP. This reaction can be run in either fresh or depleted ghosts and the results are presented in Table 1D and Fig. 2. The assay on fresh ghosts was performed in a medium containing 153 mM NaCl-$PO_4$ rather than the standard 108 mM $MgCl_2$-Tris (as used for the depleted ghosts) and therefore, the relative rate of loss of Na is increased (by about one-fifth) due presumably to the presence of Na exchange diffusion. Even so, it is clear the PEP incorporated into fresh ghosts will result in the activation of the Na pump. In depleted ghosts it can be seen that PEP is inactive in this regard although a small stimulatory effect is obtained from the addition of nucleosides. The time-course of the loss of

$^{24}$Na from the depleted ghosts in Table 1 D is given in Fig. 2 *C*.

The failure of incorporated PEP to stimulate the pump in depleted ghosts is evidently due to the absence of ADP in the depleted ghost system, thus preventing the reaction, PEP to pyruvic acid, from taking place. This situation can be corrected by incorporating a "trace" amount of ADP along with PEP into the depleted ghosts at the time of hemolysis. The result of this type of an experiment is given in Fig. 2 *D*. It is apparent that PEP + ADP (trace) leads to activation of the Na pump. The addition of nucleosides increases the pump activity even further, equivalent to the activation observed in depleted ghosts containing incorporated ATP (*compare* Fig. 1 *A*). Evidence that the incorporated "trace" amount of ADP is insufficient, in itself, to activate the pump is given in Table 5.

### *Concentration Dependence of the Pump on Incorporated ATP*

Table 3 shows the effect on the Na outflux of three different concentrations of ATP incorporated into ghosts. The results of two comparable experiments are presented: depleted ghosts were used in one and fresh ghosts were used in the other (the latter represented by the values given in brackets). The concentrations of ATP as shown refer to the concentration of ATP present in the hemolysis solution at the time of hemolysis. It is apparent that the pump (taken as the alone-strophanthidin difference component) is partially activated by 0.2 mM ATP and is essentially operating maximally with 0.6 mM ATP. It should be noted that both inosine and adenosine are capable of stimulating the pump to operate at a level exceeding that obtainable with incorporated ATP. This effect is presumably dependent upon the intracellular concentration of ADP. It will be shown in a subsequent section (*see* Table 8) that the magnitude of the pump flux is evidently sensitive to the ratio, ATP/ADP. As this ratio decreases the pump flux also decreases. The addition of nucleoside to this system could result in decreasing the concentration of ADP or increasing the concentration of ATP, or both. Thus, the metabolism of the nucleoside would tend to maximize the ATP/ADP ratio and may account for the difference in the pump rate under the two sets of conditions. It should be mentioned that ADP can be formed from ATP by the enzymatic activity of the pump, by adenylate kinase and by the activity of nonspecific phosphatases present within the ghost system (*see* Table 10). These three activities would contribute, in the absence of nucleoside, to a production of ADP at a level proportional to the amount of incorporated ATP. The fact that adenosine is more effective than inosine in stimulating the pump at the lower concentrations of ATP would presumably be related to this availability of ADP since, as inferred in the previous discussion, the pump requires ATP.

Table 3 is also of interest in another connection. This has to do with the apparent Michaelis constant ($K_{1/2}$) of the pump for ATP. Because of insufficient accuracy these data can only provide, at best, an order of magnitude estimate of the apparent $K_{1/2}$ which, from the data presented in Table 3 (using the 30- as well as the 60-min flux points), was found to range from 0.3 to 2.6 mM incorporated ATP. However, the relationship of the incorporated ATP to the actual concentration of intracellular ATP present in the ghosts at the time of assay is unknown. The extent of the breakdown of incorporated ATP during the preparation of the ghosts is presumably small since the procedure is carried out in the cold (*see* discussion related to Table 12). Another consideration has to do with the relative cell volume that the ghosts have at the time of assay, at least the cell volume of those ghosts that are doing the pumping (that is, releasing $^{24}$Na). As discussed before (Hoffman, 1962*a*) these ghosts must shrink in the assay medium to perhaps one-tenth the volume they possess after they have hemolyzed in the hemolysis solution thereby increasing the concentration of ATP. These unknowns limit even more so the usefulness of the range of $K_{1/2}$ values noted above.

**Table 3.** Effect of different concentrations of incorporated ATP on the outflux of $^{24}$Na from fresh and depleted ghosts[a]

| Incorporated substrate | ATP (mM) | Percent $^{24}$Na released in 60 min | | | |
|---|---|---|---|---|---|
| | | Alone | Stroph. | Inosine | Adenosine |
| Control | 0 | 12.4 | 12.7 | 17.3 | 33.6 |
| | | (15.7) | (10.7) | (–) | (55.9) |
| ATP | 0.2 | 23.1 | 15.9 | 48.8 | 72.4 |
| | | (39.5) | (13.9) | (–) | (73.7) |
| | 0.6 | 47.8 | 13.4 | 60.2 | 73.9 |
| | | (52.7) | (13.3) | (–) | (76.5) |
| | 2.0 | 50.6 | 13.3 | 68.9 | – |
| | | (48.6) | (10.3) | (–) | |

[a] Two experiments are presented: The results obtained using depleted ghosts are unbracketed while the brackets refer to the results obtained using fresh ghosts. The hemolysis solution contained 1.2, 1.6 or 2.0 mM $MgCl_2$ added to 0.2, 0.6 or 2.0 mM ATP, respectively. The control refers to no added substrate but hemolyzed in 1 mM $MgCl_2$. Alone indicates no addition to the final medium. The concentration of strophanthidin (stroph.) is $1 \times 10^{-4}$ M; inosine, 3 mM— adenosine, 3 mM.

## *Specificity of the Incorporated Nucleotide for Activation of the Na Pump*

Having established that the Na pump would run on incorporated ATP it was of interest to evaluate the specificity of the nucleotide required for activation. This acquired additional interest because of the differential action of the nucleosides, inosine and adenosine, already described, with the inference that ITP generated from inosine was inactive. Table 4 shows the effect different nucleotides, incorporated into depleted and fresh ghosts, had on the activation of the Na outflux. With regard to depleted ghosts, it is clear that of the compounds tested only ATP results in any marked stimulation of the pump. It should, however, be noted that there may be a small and reproducible (*see* Table 6) strophanthidin-sensitive component of Na outflux observable with ITP. The magnitude of the pump activation obtained with ATP and ITP appears to parallel the activity obtained when these compounds are used as substrates for the Na, K-ATPase associated with the membrane (*cf.* Hoffman, 1962*b*). Even so, the results presented in Table 4 demonstrate the marked preference the pump has for ATP.

In contrast to the results obtained using depleted ghosts, GTP, CTP and deoxyATP, when incorporated into fresh ghosts, are able to produce a significant stimulation of the Na outflux as also shown in Table 4. This differential action is particularly impressive in the case of deoxyATP. It cannot, however, be assumed that any of these nucleotides serve as direct substrates for the pump. Rather, it is more likely that these results point toward the activity of a nucleoside diphosphokinase (working in conjunction with an adenine ribonucleotide) that presumably is present in the more metabolically competent fresh ghost system (*cf.* Berg & Joklik, 1954; Sacktor & Cochran, 1957). With regard to deoxyATP, the explanation may be more complicated than this since it has been found (data not shown) that deoxyadenosine, but not deoxyinosine, will stimulate the Na pump in control-depleted ghosts similar to the stimulation produced by adenosine (but not by inosine) as presented in Table 1. Since, in preliminary experiments, it was found that lactate was formed from deoxyadenosine (in amounts similar to that produced from adenosine as seen in Table 7) and that the presence of incorporated deoxyATP inhibits this lactate formation, it is not clear from the above results whether the oxidation of the ribosyl moiety occurs before or after the formation of the nucleotide in the synthesis of ATP.

**Table 4.** Effect of different nucleotides incorporated into depleted and fresh ghosts on the outflux of Na

| Types of ghosts | Substrate incorporated | Percent $^{24}$Na released in 80 min | |
|---|---|---|---|
| | | Alone[b] | Strophanthidin[c] |
| Depleted | ATP | 50.6 | 22.8 |
| | ITP | 26.7 | 21.1 |
| | GTP | 25.9 | 25.5 |
| | UTP | 33.4 | 33.2 |
| | CTP | 26.4 | 18.5 |
| | DeoxyATP | 28.7 | 21.9 |
| | Control[a] | 29.1 | 24.2 |
| Fresh | ATP | 56.7 | 15.9 |
| | ITP | 24.1 | 17.6 |
| | GTP | 30.8 | 18.7 |
| | CTP | 33.4 | 20.4 |
| | DeoxyATP | 53.1 | 20.6 |
| | Control | 22.0 | 19.1 |

[a] Control refers to no substrate added at hemolysis. Control ghosts were hemolyzed in the presence of 1 mM $MgCl_2$. For the nucleotides, the hemolysis solution contained in each instance was 2 mM $MgCl_2$ + 2 mM nucleotide.

[b] Alone means that no addition was made to the final incubation medium.

[c] Strophanthidin concentration is $1 \times 10^{-4}$ M.

## *Effect of Various Substances of the Active Transport of Na*

Table 5 extends the list of incorporated substrates which have been assayed for their possible involvement in the pump reaction. It should be understood that each row represents a single experiment (except for the separate pairs of experiments in which ACh and ITP were used) and since each experiment was carried out on different preparations of ghosts comparison with each other is only valid on a qualitative basis. All experiments were performed using depleted ghosts. It is apparent that ADP and ATP are the only incorporated substrates which will activate the Na pump. It was to be expected that AMP and IMP would be inactive in this regard but the fact that CrP, ThPP, PRPP and ATPPPP are also inactive is a further indication of the pump specificity for the high energy phosphate of ATP. The results obtained with ACh corroborate previous evidence (*cf.* Parpart & Hoffman, 1952) that ACh is without effect on the pump. ACh was ineffective when incorporated into ghosts and tested alone or when incubated in the presence of added ACh. These results were unaltered by the addition of $1.3 \times 10^{-5}$ M physostigmine. It should also be mentioned that ACh was without effect on the active transport of Na in ghosts containing incorporated ATP.

**Table 5.** Effect of various incorporated compounds on the outflux of Na in depleted ghosts

| Incorporated substrate | Percent $^{24}$Na released in 90 min | | | |
|---|---|---|---|---|
| | Alone$^{b}$ | Stroph. (1 × $10^{-4}$ M) | Inosine (3 mM) | Adenosine (3 mM) |
| Control$^{a}$ | 29.0 | 26.1 | 31.2 | 52.3 |
| AMP | 24.9 | 20.1 | 22.2 | – |
| IMP | 34.4 | 36.5 | 34.6 | 44.9 |
| IDP | 27.8 | 25.4 | 22.3 | 31.2 |
| CrP | 23.7 | 21.0 | – | – |
| ThPP | 22.4 | 20.3 | – | 49.0 |
| PRPP | 27.1 | 22.7 | 45.4 | 55.7 |
| ATPPPP | 29.9 | 28.0 | 40.2 | 38.7 |
| ACh | 21.7 | 20.3 | – | – |
| | 22.1$^{c}$ | 20.5$^{c}$ | – | – |
| ADP (trace) | 28.9 | 22.9 | 22.5 | 59.6 |
| ITP | 27.6 | 22.2 | 26.3 | 26.7 |
| ITP+ADP (trace) | 23.1 | 18.6 | – | 32.2 |

[a] Control refers to ghosts prepared in the absence of any substrate added to the hemolysis solution but in the presence of 1 mM $MgCl_2$. All other hemolyses carried out in 2 mM concentrations + 2 mM $MgCl_2$ except in the instance of ADP (trace) as described in Materials and Methods.

[b] Alone means that no addition was made to the final incubation medium.

[c] Superscript c indicates that 2 mM ACh was added to the final incubation medium.

It is of interest that not only is the ADP (trace) inactive by itself but that the pump is not activated upon the addition of inosine. Evidently the difference between fresh and depleted ghosts is, in this respect, more than can be accounted for by the presence or absence of ADP (*see* Discussion).

In the experiments with ITP, portions of the same preparation of depleted cells were used to incorporate ITP and ITP+ADP (trace). As pointed out before (Table 4) the pump is essentially inactive in ghosts containing ITP. However, it should be noted that the action of adenosine is effectively blocked under these conditions but not in ghosts with incorporated IMP, IDP or ITP+ADP (trace). The mechanism underlying this inhibition is not at present known.

### *Effect of Metabolic Inhibitors on the Activity of the Na Pump*

**Table 6.** Effect of inhibitors on the active transport of Na in depleted ghosts containing incorporated ATP or ADP$^{a}$

| Exp. | Incorporated substrate | Percent $^{24}$Na released in 90 min | | | |
|---|---|---|---|---|---|
| | | Alone | Stroph. | Inosine | Adenosine |
| 1 | ADP + $NaN_3$ | 32.3 | 17.9 | – | – |
| | | 37.1$^{a}$ | 16.5$^{a}$ | – | – |
| | ATP + $NaN_3$ | 44.5 | 14.3 | – | – |
| | | 47.0$^{a}$ | 15.9$^{a}$ | – | – |
| 2 | Control | 29.0 | 25.1 | 31.2 | 52.3 |
| | ADP + ($NaN_3$) | 47.3 | 22.0 | – | – |
| | | 52.6$^{a}$ | 22.7$^{a}$ | – | – |
| | ATP + ($NaN_3$) | 55.3 | 20.7 | – | – |
| | | 59.9$^{a}$ | 21.2$^{a}$ | – | – |
| 3 | ATP + (IAA) | 62.0 | 28.7 | 70.2 | – |
| | | 72.2$^{b}$ | 27.8$^{b}$ | – | – |
| 4 | ATP + $AsO_4$ | 39.6$^{c}$ | 22.6$^{c}$ | 38.0$^{c}$ | – |
| 5$^{e}$ | ATP | 46.1 | 26.3 | 59.0 | 58.8 |
| | ATP + ($AsO_4$) | 48.2$^{c}$ | 29.6$^{c}$ | 50.5$^{c}$ | 46.0$^{c}$ |
| | ATP + ($F^-$) | 24.2$^{d}$ | 22.3$^{d}$ | 21.5$^{d}$ | 23.1$^{d}$ |

[a] The inhibitors used in experiments 1 and 4 were present at hemolysis as well as in the washing solutions. The inhibitors enclosed in brackets (experiments 2, 3 and 5) were added only to the final incubation medium. The superscript letters refer to the following additions to the final incubation medium: *a*, 5 mM $NaN_3$; *b*, 5 mM NaF; *c*, 5 mM $Na_2AsO_4$; *d*, 5 mM NaF; *e*, medium contains Na in place of Mg and the values indicate the percent $^{24}$Na released in 60 min. Other additions to the final incubation medium are Stroph. (strophanthidin) at $1 \times 10^{-4}$ M; inosine, 3 mM and adenosine, 3 mM.

The effects of metabolic inhibitors as tested on ghosts are to be compared with their known action on transport and metabolism as observed in intact red cells. Thus, fluoride was shown by Harris (1941), Danowski (1941) and Maizels (1951) to inhibit both active cation transport and glycolysis in human red cells. Similarly, Maizels (1951) and Love, Cronvich and Burch (1955) showed that IAA also acts to inhibit both active transport and glycolysis. In contrast, $NaN_3$ has no effect on either cation transport or glycolysis in human red cells (Maizels, 1951). Arsenate is known to stimulate glycolysis but inhibits active transport, presumably by blocking the esterfication of inorganic phosphate (*cf.* Hewitt & Nicholas, 1963), and thus the formation of ATP. It is also of interest to note that $AsO_4$ prevents the utilization of nucleosides such as adenosine (Prankerd & Altman, 1954).

In contrast to intact cells, it is possible in ghosts to test directly the effect an inhibitor has on the pump as distinct from its effect on metabolism by using, as indicated in Table 6, depleted ghosts containing incorporated ATP. In this type of ghost preparation the pump is active but there is no detectable glycolysis (*see later*). In the two separate sets of experiments (the ghosts in each set were made from the same preparation of depleted cells) presented in Table 6 in which $NaN_3$ was used, the preparative procedure was altered only to the extent that in experiment 1 but not in experiment 2 ghosts were hemolyzed and washed in the presence of 5 mM $NaN_3$. This procedural change was made in order to maximally expose the ghosts to $NaN_3$ and to circumvent any possible permeability barrier for $NaN_3$. It can be seen that $NaN_3$ was without effect on the outflux of Na from ghosts containing ADP or ATP whether or not $NaN_3$ was present throughout the preparative procedure or added only in the final incubation medium. Table 6 (as well as Fig. 3) also shows that the Na pump is not inhibited by IAA. The slight stimulation of Na outflux seen with IAA (as well as with $NaN_3$) may be due to the effect of added Na in stimulating Na exchange diffusion. It is also apparent that $AsO_4$ had no influence on the Na pump. The effect of $AsO_4$ was tested in two ways. In experiment 4 the ghosts were prepared, as in experiment 1 with $NaN_3$, by hemolysis, in and washing with, solutions containing 5 mM $Na_2AsO_4$. The $AsO_4$ results given in experiment 5 were obtained using ghosts prepared in the standard manner to which $AsO_4$ was present only in the final incubation medium. Experiment 5 represents the results from one experiment using a single preparation of ghosts suspended in the different solutions as indicated. In this instance, by comparing the effect of $AsO_4$ with its control (i.e., ATP without added $AsO_4$), it can be seen that the Na pump is unaltered but the stimulation produced by the addition of nucleosides is completely blocked by the presence of $AsO_4$. All of the above results on ghosts indicate, as anticipated from the intact cell studies, that the inhibitors $NaN_3$, IAA and $AsO_4$ act only indirectly on the pump by preventing the glycolytic synthesis of ATP. Given ATP, these inhibitors are without effect.

In contrast to the above discussed inhibitors, $F^-$ was found to inhibit the active transport of Na. This inhibition represents a direct action of $F^-$ on the pump, as distinct from its known effect on glycolysis, since it was observed in ghosts containing incorporated ATP. The assay, as given in Table 6, was carried out in a Na medium to obviate any possible interference by $MgCl_2$. This effect of $F^-$ is consistent with the result that $F^-$ inhibits the Mg-dependent Na, K-ATPase as reported by Hoffman and Ryan (1960, *cf.* Hoffman, 1961). Kirschner (1964) has also presented evidence for this dual action of $F^-$ in intact pig red cells.

### *Competition between the Active Transport System and the Hexokinase Reaction*

It was shown in Table 1 that glucose added to control fresh ghosts was inactive in the sense that it failed to stimulate the Na pump. The reason for this, as shown by Lionetti et al. (1956) is that the enzyme, hexokinase, is inactive or absent in ghosts (apparently lost or denatured during the hemolytic process) and, thus, prevents the glycolytic use of glucose. It is possible, however, to circumvent this deficiency by introducing new hexokinase into the interior of the ghost at the time of hemolysis. The results of an experiment in which depleted cells were hemolyzed in the presence of a solution containing hexokinase and ATP is illustrated in Fig. 3. It is apparent that, as previously shown, the pump is activated by ATP alone and is inhibited by strophanthidin. This also indicates that these ghosts, under these conditions, are not influenced by the presence of incorporated hexokinase. However, the addition of glucose to this system results in the complete inhibition of the pump and that this inhibition by glucose occurs in the presence of IAA. These results can be explained on the basis that there is a competition between the pump and the hexokinase reaction for the available ATP. The hexokinase requires ATP to convert glucose to glucose-6-$PO_4$ and since glucose is present in excess this reaction dominates the ATP utilization, thus resulting in the inhibition of the pump. Any ATP generated by glyco-

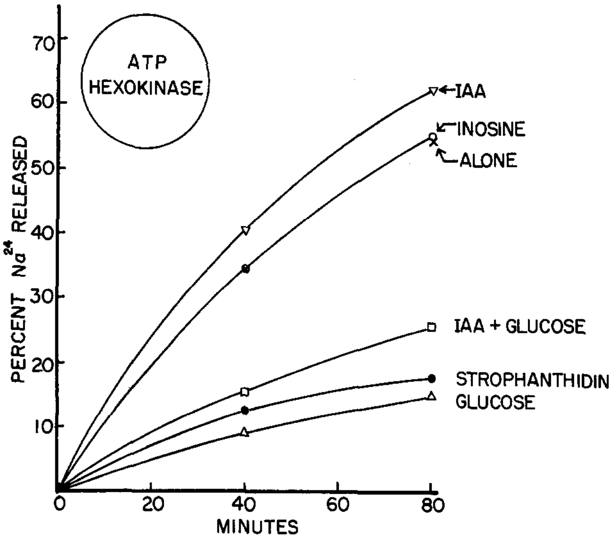

**Fig. 3.** Effect of glucose on the rate of release of $^{24}Na$ from depleted ghosts containing incorporated ATP+hexokinase. The composition of the hemolysis solution was 2 mM $Na_2ATP$+2 mM $MgCl_2$+400 µg/ml hexokinase. Alone means that no addition was made to the final incubation medium. The concentration of NaIAA was 1 mM; inosine, 3 mM; glucose, 11 mM; strophanthidin, $1 \times 10^{-4}$ M. *See text* for discussion. (Taken from Hoffman, 1962*b*)

lysis or by the action of adenylate kinase would similarly be preferentially used in the phosphorylation of glucose. This same type of competitive inhibition of the pump by the addition of glucose has been previkously demonstrated on intact cells by Dunham (1957*b*) under appropriately set optimum conditions. However, in intact cells, glucose will produce only partial inhibition of the pump. Presumably, the difference between ghosts and intact cells in this regard reflects the difference in the relative concentration of the two competing ATP utilizing systems.

As an extension of the above type of experiment, it was of interest to test the effect of incorporating different amounts of hexokinase on the action of glucose with regard to the pump as well as on lactic acid production. These data are shown in Table 7 and represent the results obtained using a single preparation of fresh cells for the preparation of the ghosts. Two different amounts of hexokinase were incorporated and the concentrations given in the Table refer to the concentration present in the hemolyzing solution. Lactate production was linear with time and was measured at hourly intervals for a period of 3 hr. With regard to control ghosts, it is apparent that glucose, as before, has no effect on the pump and has only a minimal effect on glycolysis. Adenosine on the other hand, stimulates the pump and results in a considerable production of lactic acid. This situation is markedly altered, however, in ghosts containing the low concentration (2 µg/ml) of incorporated hexokinase. In contrast to control ghosts, glucose is now utilized, as evidenced by the increased lactate production, and results in a concomitant activation of the Na pump. It should be stated that in other experiments in which this low concentration of hexokinase was used the incorporation of ATP does not change the over-all pattern of results. This is to say that although the addition of ATP to this system will in itself activate the pump, the effect of glucose is unchanged to the extent that the same glycolytic rate is maintained and that glucose actually results in a slight stimulation in the Na pump. However, as shown in the bottom portion of Table 7, this is not at all the case when ATP was used in conjunction with the high concentration (200 µg/ml) of incorporated hexokinase. In this instance, it can be seen that the addition of glucose inhibits (as in Fig. 3) the pump activity even though considerable glycolysis occurs. This emphasizes the previous conclusion regarding the competitive effects these two reactions have for the generated ATP. It should be noted that the lactate produced from glucose appears as a dependent function on the amount of incorporated hexokinase. The fact that the metabolism of adenosine is unaffected by the presence or absence of hexokinase or ATP reflects the difference in pathways used by these two substrates.

**Table 7.** Competition between the active transport system and the hexokinase reaction[a]

| Substances incorporated | Additions to incubation medium | Percent $^{24}$Na released in 60 min | Lactate production (mM/liter ghosts · hr) |
|---|---|---|---|
| Control | alone | 30.4 | 0.11 |
| | glucose | 30.4 | 0.17 |
| | glucose + strophanthidin | 25.8 | 0.16 |
| | adenosine | 68.1 | 2.83 |
| Hexokinase (2 µg/ml) | alone | 32.2 | 0.06 |
| | glucose | 59.9 | 1.50 |
| | glucose + strophanthidin | 23.8 | 1.70 |
| | adenosine | 67.9 | 2.76 |
| ATP + hexokinase (200 µg/ml) | alone | 45.4 | 0.18 |
| | glucose | 12.6 | 2.46 |
| | glucose + strophanthidin | 10.3 | 2.52 |
| | adenosine | 50.9 | 2.60 |

[a] Hexokinase incorporated into fresh ghosts at two different concentrations (values refer to the hexokinase concentration in the hemolysis solution). Alone means that no addition was made to the final incubation medium. Concentration of strophanthidin is $1 \times 10^{-4}$ M, glucose, 11 mM; adenosine, 4 mM.

The reason for presenting in Table 7 the results from an experiment using fresh rather than depleted ghosts stems from the fact that glucose has no effect on depleted ghosts containing incorporated hexokinase. This deficiency is corrected by incorporating ATP along with the hexokinase for glucose can then be metabolized. Since the intent was to demonstrate the activation of the pump using glucose alone, the effect is pronounced in fresh ghosts compared to the small enhancement seen with glucose added to depleted ghosts already stimulated with incorporated ATP.

It remains to emphasize the difference between fresh and depleted ghosts with regard to the control levels of lactate production. While there is sometimes a minimal but real lactate production measureable in control fresh ghosts, as shown in Table 7, no lactate production has ever been detectable in comparable preparations of control-depleted ghosts incubated for as long as 6 hr at a hematocrit of approximately 30%. It can further be stated that there is also no observable lactate produced by depleted ghosts containing incorporated ATP using these same incubation conditions.

Addition of adenosine or inosine to control-depleted ghosts will result, as with fresh ghosts, in the production of lactate.

### *Distinction between ATP and ADP as the Substrate for the Pump Reaction*

It was considered in the previous discussion that the stimulation of the pump by ADP was, in fact, indirect and due to ATP since ATP could be formed by the activity of the enzyme, adenylate kinase (2 ADP $\rightleftharpoons$ ATP+AMP). This supposition was based on the presumed presence of the enzyme and the observation (Tables 1 and 6, Fig. 1) that the stimulation of the pump by ADP was always less than that obtainable with comparable amounts of ATP. In an attempt to dissociate the effects of ADP from ATP and to define further the discrimination of the pump in this regard two types of experiments were performed. The first type has to do with the effects of incorporating different combinations of substrates used in the adenylate kinase reaction; the second deals with decreasing the concentration, and hence the activity, of the adenylate kinase by dilution of the cellular contents.

Table 8 shows the results of the first type of experiment in which various adenine nucleotides were incorporated into depleted ghosts. The depleted ghosts used in this experiment were derived from the same preparation of depleted cells. It is apparent that the strophanthidin-sensitive flux varies and is dependent upon the particular type or combination of nucleotides that were incorporated at hemolysis. The results obtained using ATP or ADP are consistent with the data previously presented (Tables 1 and 6 and Fig. 1) in showing activation of the pump in contrast to AMP (*cf.* Table 5) which is essentially negative in this regard. It should also be mentioned that the pump, as shown in Table 3, is already nearly maximally activated by the incorporation of 0.6 mM ATP under this same set of conditions. This concentration of ATP, by the way, is approximately the same as that calculated from the known equilibrium constant of the adenylate kinase reaction in this situation.

It would be expected from the known properties of the red cell adenylate kinase (Kashket & Denstedt, 1958; Tatibana, Nakao & Yoshikawa, 1958; Cerletti & Bucci, 1960) that the equilibrium concentrations of ATP, ADP and AMP would be the same independent of the direction the equilibrium is approached. This is to say that the effects of incorporating 2 mM ADP should be the equivalent of incorporating 1 mM ATP+1 mM AMP. It is apparent from the data in Table 8 that this is not the case, that ATP+AMP is more effective than ADP alone. While the involvement of the adenylate kinase is not excluded by these data, the following explanation is consistent with all of the results presented in Table 8. If it is assumed that ATP is the preferred substrate of the pump, then these results represent the effects of two different processes going at the same time. One is the activity of the adenylate kinase, the other is represented by product (ADP) inhibition of the pump reaction. Thus, the fact that ADP alone is active is indicative of adenylate kinase activity. The fact that ATP alone is more effective than ATP+ADP (i.e., direct inhibition by ADP) or than ATP+AMP (i.e., indirect inhibition by ADP which has been generated by the adenylate kinase) are illustrative of product inhibition.

The above explanation is further substantiated by studies carried out by Hoffman & Ryan (*cf.* Hoffman, 1962*b*) on the Na,K-ATPase of hemoglobin-free human red cell ghost membranes. It was found that the Na,K-ATPase is specific for ATP and that ADP is inactive in the sense that it is not broken down by this enzyme. On the other hand, the addition of ADP to the enzyme preparation inhibits the utilization of ATP. Although this inhibition could be demonstrated as a decrease in the total Na,K-ATPase activity (comparing ATP alone to ATP+ADP) it was best observed using [$^{32}$P]ATP in the presence and absence of nonradioactive ADP, where differences between liberated P$_i$ and $^{32}$P$_i$ can be used to measure the relative utilization of ADP and ATP. In this circumstance it is possible to show not only the inhibition of the Na,K-ATPase by ADP but that in the presence of ADP essentially all enzyme activity is derived from the specific utilization of [$^{32}$P]ATP as

**Table 8.** Effect of incorporating different adenine nucleotides on the outflux of Na in depleted ghosts[a]

| Incorporated substrates (mM) | Percent $^{24}$Na released in 60 min | | $\Delta$ Stroph. |
|---|---|---|---|
| | alone (*a*) | Stroph. (*b*) | (*a*–*b*) |
| ATP (2.0) | 52.8 | 26.0 | 26.8 |
| ADP (2.0) | 37.8 | 25.5 | 12.3 |
| AMP (2.0) | 30.5 | 25.0 | 5.5 |
| ATP (1.0)<br>+AMP (1.0) | 45.3 | 26.0 | 19.7 |
| ATP (1.0)<br>+ADP (1.0) | 40.7 | 24.7 | 16.0 |
| ADP (1.0)<br>+AMP (1.0) | 33.7 | 22.3 | 11.4 |

[a] Hemolysis solutions contained 2 mM total nucleotide + 2 mM $MgCl_2$. Alone means that no addition was made to the final incubation medium. Stroph. (strophanthidin) was added at a concentration of $1 \times 10^{-4}$ M. The delta ($\Delta$) stroph. column refers to the difference between the values in columns *a* and *b*.

substrate. If these considerations are applicable to the ghost preparation as used in Table 8 they imply that in the absence of adenylate kinase in the ghost interior, ADP alone would be inactive as an incorporated substrate.

A possible mechanism for the product inhibition referred to before is inherent in the formulation by Lipmann (1960) of the concept of a phosphoryl potential in describing the bond energy obtainable in the high-energy phosphates. At constant pH and concentration of inorganic phosphate, the free energy of the bond is an explicit function of the ratio, ATP/ADP, the higher the ratio the greater the free energy. If this is so, then in the experiments reported above, the inhibition of the pump brought about by ADP can be expressed as a decrease in the phosphoryl potential. This approach emphasizes the energy requirements necessary to run the pump and its control by the ratio, ATP/ADP.

If the adenylate kinase is in any way responsible for the pump activity seen with incorporated ADP it might be possible to reduce its effectiveness by lowering its intracellular concentration. This is approachable, at least in principle, on the basis that the adenylate kinase is a soluble enzyme which comes out of the cell upon hemolysis (Kashket & Denstedt, 1958; Tatibana et al. 1958; Cerletti & Bucci, 1960). If the adenylate kinase diffuses or is partitioned to equilibrium during the hemolytic process, then the relative concentration of enzyme remaining in the ghost after hemolysis can be easily controlled by prior adjustment of the ratio of volume of solution used to hemolyze a unit volume of cells. Since hemoglobin is known to diffuse to equilibrium (i.e., for any given hemolytic ratio, the concentration of hemoglobin in ghosts, at their hemolytic volume, is the same as the concentration of hemoglobin in the hemolysate) this technique of altering the hemolytic ratio was used to make ghosts containing different concentrations of hemoglobin (Hoffman, 1958). If the assumption is made that adenylate kinase becomes distributed at hemolysis to the same extent as hemoglobin, then the hemolytic ratio, as determined by the fractional content of hemoglobin remaining in ghosts relative to their original (intact cell) hemoglobin content, is a measure of the relative dilution of the cellular concentration of adenylate kinase.

**Table 9.** Outflux of Na from fresh ghosts containing either incorporated ATP or ADP as a function of the hemolytic ratio[a]

| Hemolytic ratio | Percent $^{24}$Na released in 120 min | | | | Δ Stroph. | |
|---|---|---|---|---|---|---|
| | ATP ghosts | | ADP ghosts | | Δ ATP | Δ ADP |
| | Alone (*a*) | Stroph. (*b*) | Alone (*c*) | Stroph. (*d*) | (*a–b*) | (*c–d*) |
| 1/9 | 57.3 | 21.1 | 42.2 | 13.9 | 35.4 | 28.3 |
| 1/27 | 57.8 | 23.3 | – | – | 34.5 | – |
| 1/45 | 54.7 | 30.1 | 41.0 | 18.0 | 24.6 | 23.0 |
| 1/85 | 60.1 | 31.0 | 41.1 | 21.3 | 29.1 | 18.8 |
| 1/1500 | 59.5 | 31.0 | 45.1 | 29.3 | 28.5 | 15.8 |

[a] The different hemolytic ratios represent the different dilutions of the cellular hemoglobin that occurred at hemolysis using various volumes of hemolyzing solution. The hemolytic ratio was varied and measured as described in the text. The ghosts whose ratios varied from 1/9 to 1/85 were prepared by a single hemolysis. The bottom row represents ghosts that were made by two successive hemolyses as described in the text. Alone means that no addition was made to the final incubation medium. The concentration of strophanthidin (stroph.) is $1 \times 10^{-4}$ M. Delta (Δ) ATP or ADP refer to the differences in the values of columns (*a–b*) and (*c–d*), respectively.

The result of an experiment designed to test the above approach on the relationship between ADP and adenylate kinase activity with regard to the pump is presented in Table 9. The four top rows represent the results from one set of experiments using fresh ghosts made from aliquots of a single preparation of intact cells. The bottom row refers to a different experiment which will be discussed separately. With reference only to the upper portion of the Table, the outflux of Na was measured in ghosts containing either ATP or ADP incorporated at different hemolytic ratios. The hemolytic ratio was estimated in the following way. Samples of known volume of the hemolysis mixtures as well as a known volume of the original packed intact cells, were diluted and their optical density determined spectrophotometrically (540 nm). The hemolytic ratio was calculated, after correction for dilution, from the ratio of the optical densities, hemolysate to intact cells, for each of the four conditions. The concentration of hemoglobin was also determined on equal volumes of ghosts (separated by centrifugation) and hemolysates. The fact that the optical densities of these samples were the same and identical to the optical density of an equivalent portion of the hemolysis mixture indicates that hemoglobin was distributed to equilibrium and that, necessarily, 100% hemolysis took place in each condition (Hoffman, 1958). The hemolytic ratio, determined in this manner, measures the actual dilution the hemoglobin undergoes at the time of hemolysis, that is, at the hemolytic volume. Since the hemolytic ratio, for each condition, as measured on ATP ghosts and ADP ghosts were almost the same, they are listed under a single heading. The ATP and ADP ghosts as presented in Table 9 were prepared under conditions slightly modified from those described in Materials and Methods. The only change introduced was that each hemolyzing solution contained 6 mM NaCl in addition to its other constituents. This modification was found necessary in order to provide spontaneously reconstituted ghosts at the lower hemolytic ratios. In an attempt to minimize variations different portions of the same ATP or ADP solution were used to hemolyze cells to obtain the various hemolytic ratios indicated.

It is apparent in Table 9 that there is a decrease in the strophanthidin-sensitive or pump component of Na outflux as the cell contents are diluted over this 10-fold range (1/9 to 1/85). This applies to both the ATP- and ADP-containing ghosts although the reduction obtained with ADP ghosts is proportionately greater than that obtained with ATP ghosts. However, the primary change seen in the pump flux of Na, in both types of ghosts, results from an increase in the strophanthidin-insensitive flux. Presumably, this increase in the strophanthidin-insensitive component represents an increase in the leak permeability of the ghosts since the concentration of strophanthidin as used here was shown to produce maximum inhibition. While this consideration implies that the differences are real in the relative effects of dilution on the pump flux of Na in both kinds of ghosts, it cannot be said the decrease in the pump seen with ADP, compared to ATP, is due specifically to a decrease in the concentration of adenylate kinase. Although it is evident that changes in the hemolytic ratio have little influence on the ability of ATP to run the pump, the fact is that ADP is still active even after its incorporation into ghosts whose contents have been diluted 1,500-fold.

This result is illustrated in the last row of Table 9. These ghosts are of special interest in that they were prepared using two successive hemolytic steps in which ATP or ADP (along with $^{24}$Na) was incorporated only during the second hemolysis. This was accomplished by means of the following procedure. For the first hemolysis, one volume of cells was completely hemolyzed in 51 volumes of solution containing 25 mM NaCl + 1 mM $MgCl_2$. The resultant ghosts were harvested by centrifugation and then rehemolyzed (i.e. the second hemolysis) in the ratio one volume of ghosts to 46 volumes of solution containing 6 mM NaCl + 2 mM $Na_2ATP$ or (2 mM $Na_2ADP$) + 2 mM $MgCl_2$. The tonicity of the solution used in the first hemolysis is sufficiently low to obtain 100 percent hemolysis but it is higher than the tonicity of the solution used for the second hemolysis (*cf.* Hoffman, 1958). Thus, the ghosts derived from the first hemolysis are rehemolyzed when placed in the hypotonic media containing the nucleotides. The hemolytic ratio that obtains in the first hemolysis is 1/32 based on the fact that the cells swell to their hemolytic volume (approximately 1.6 times their initial or original volume) prior to hemolysis. The hemolytic ratio at the second hemolysis is taken as the actual ratio, 1/46, since the ghosts from the first hemolysis are essentially at their hemolytic volume. Thus, the overall hemolytic ratio is the product of the two separate hemolytic steps or approximately 1/1,500. These twice-hemolyzed ghosts are white to very light pink and contain, after isolation from the second hemolytic step, 12.6 mM Na/liter ghosts and less than 0.2 mM K/liter ghosts. Ghosts so prepared were washed as previously described and their Na outflux measured in the usual manner. Given the fact that twice-hemolyzed ghosts can be reconstituted with regard to their ability to pump, their advantage over a single hemolysis procedure in obtaining large cellular dilutions is obvious.

It is evident in Table 9 that the pump in ATP-containing ghosts prepared at 1/1,500 is the same as that seen at the higher hemolytic ratios. This is in contrast to the ghosts containing ADP since the pump level in these ghosts has continued to decrease. Even so, ADP is still quite active in these ghosts. If the assumption is made that the pump is specific for ATP (from the argument presented earlier on the interaction of ADP and ATP on the Na, K-ATPase) and that any activity still seen with ADP reflects the adenylate kinase, then it would appear that the adenylate kinase was simply not diluted out using these hemolytic procedures. This would mean that the adenylate kinase was trapped within the ghosts in the sense that it was not diluted out as was hemoglobin or that the activity of the remaining enzyme was sufficient to account for the observed effects of ADP. This last possibility appears quite likely in view of the very high adenylate kinase activity exhibited by red cells (*cf.* Altman, 1959). However, regardless of the inferential evidence, further work is still needed (perhaps with the use of a specific inhibitor of the adenylate kinase or by studying the effects of incorporated adenylate kinase) to separate a direct effect of ADP on the pump from its indirect activity via the adenylate kinase.

## *Breakdown of ATP in Ghosts*

It has been previously shown in intact cells (Dunham, 1957*a*, *b*; Whittam, 1958) that the inhibition of the pump by cardiotonic steroids (e.g. strophanthidin) results in a decreased rate of breakdown or sparing effect on intracellular ATP. In the first attempts to demonstrate a similar relationship in reconstituted ghosts it was observed that instead of any effect of sparing on incorporated ATP, the rate of ATP utilization was unaltered when the pump was inhibited with strophanthidin. Since it is known that ghosts possess considerable nonspecific phosphatase activity that is unmasked upon hemolysis (*cf.* Clarkson & Maizels, 1952), it was thought that this increased phosphatase activity might be responsible for the failure to find any effect the pump might have on ATP breakdown. If the nonspecific phosphatase activity could be differentially reduced or eliminated without any effect on the pump, then it might be possible to demonstrate a sparing effect by strophanthidin on the rate of utilization of ATP. That this was, in fact, possible is shown by the experiments described below.

For clarity of presentation we will consider first the procedure used to modify the nonspecific phosphatase activity; second, the effect this procedure has on the pump capacity of the ghosts and finally, the change in the rate of breakdown of incorporated ATP that occurs upon the inhibition of the pump. While only the results from single experiments of the three types are reported it should be understood that each is typical of several, yielding comparable results.

The results concerned with the inhibition of the nonspecific phosphatase are presented in Table 10. In this experiment the total ATPase activity expressed as µM inorganic phosphate ($P_i$) liberated/ml ghost mixture × hr, was determined at zero, 40 and 80 min using the four incubation media with the indicated composition. For each case the amount of $P_i$ liberated increased linearly with time. The ghost mixture used in these measurements was prepared as follows. Ghosts were prepared in the standard manner as described in Materials and Methods. Thus, fresh cells were hemolyzed with 10 volumes of 1 mM $MgCl_2$, centrifuged and washed 4 times with 17 mM $MgCl_2$-Tris. These washed ghosts were concentrated by centrifugation at 15,000 × *g*, then frozen at −20 °C and thawed at room temperature. The freeze-thawing procedure was repeated 3 times after which 100 percent rehemolysis was shown to have occurred. One ml of this ghost mixture (containing now permeable ghosts plus the hemolysate that was trapped at the initial preparative hemolytic step) was incubated with 4 ml of each of the media as given in Table 10. This particular type of ghost mixture was chosen for assay in order to approximate as closely as possible the ATPase activity of the closed, reconstituted ghost system as studied before with regard to the pump. The upper two rows show that the ATPase activity is large and not inhibited by strophanthidin. In contrast, the lower two rows show that the incubation conditions are such as to reduce the total ATPase activity and that the remaining ATPase activity is now inhibited by strophanthidin. Evidently the addition of equimolar amounts of Mg and EDTA with concomitant preservation of the Mg/ATP ratio as used in the upper two rows, results in the inhibition of the nonspecific phosphatase and allows the strophanthidin-sensitive component to be observed. Thus, these results indicate that by the incorporation of EDTA it may be possible to demonstrate a sparing effect on ATP utilization by strophanthidin. However, before this is considered it is necessary to evaluate the effect incorporated EDTA has on the active transport of Na in reconstituted ghosts.

**Table 10.** Effect of EDTA on the ATPase activity of a ghost mixture[a]

| Medium contents in mM/liter | | | | | | µM $P_i$ liberated/ ml ghost mixture × hr |
|---|---|---|---|---|---|---|
| NaCl | KCl | $Na_2ATP$ | $MgCl_2$ | $Na_2EDTA$ | Stroph. | |
| 40 | 20 | 2.0 | 1.0 | 0 | 0 | 4,87 |
| 40 | 20 | 2.0 | 1.0 | 0 | 0.2 | 5.27 |
| 40 | 20 | 2.0 | 4.0 | 3.0 | 0 | 1.66 |
| 40 | 20 | 2.0 | 4.0 | 3.0 | 0.2 | 0.79 |

[a] The ghost mixture was prepared, as described in the text, by rehemolyzing a reconstituted ghost preparation. The ATPase of this mixture was determined in each of the four medium compositions indicated in the Table. Each medium also contained 10 mM Tris buffer and the assay was carried out at 37 °C at pH 7.4.

**Table 11.** Effect of incorporated EDTA on the outflux of Na in fresh ghosts containing ATP[a]

| Hemolysis condition | Percent $^{24}$Na released in 90 min | |
|---|---|---|
| | Control | Strophanthidin ($1 \times 10^{-4}$ M) |
| ATP | 53.7 | 23.7 |
| ATP+EDTA | 61.0 | 24.4 |

[a] *See text* for discussion.

The effect of incorporated EDTA on the Na pump is shown in Table 11. The ghosts in this experiment were made using different portions of the same preparation of fresh cells. The hemolysis solution for the control ATP ghosts contained 1 mM $Na_2ATP$ + 1 mM $MgCl_2$. For the ATP+EDTA ghosts, the hemolysis solution contained 1 mM $Na_2$ ATP + 4 mM $MgCl_2$ + 3 mM $Na_2$ EDTA. The washing procedure and the flux assay were performed as previously described with the exception that the tonicity of the wash solution was increased to 34 mM $MgCl_2$-Tris in order to prevent any rehemolysis from occurring. It is apparent that the activity of the pump in ghosts containing EDTA is not only comparable to but somewhat increased over the control level. If the nonspecific phosphatase activity is in fact decreased by EDTA, then

**Table 12.** Rate of breakdown of incorporated [$^{32}$P]ATP in fresh reconstituted ghosts and the effect of incorporated EDTA[a]

| Hemolysis condition | Time in miutes | Liberated $^{32}P_i$ in cpm | | Percent inhibition |
|---|---|---|---|---|
| | | Control | Stroph. ($1 \times 10^{-4}$ M) | |
| [$^{32}$P]ATP | 0 | 0 | 0 | – |
| | 40 | 1,230 | 1,432 | −18.8 |
| | 80 | 2,003 | 1,972 | + 1.5 |
| | 140 | 3,474 | 3,028 | +11.5 |
| [$^{32}$P]ATP +EDTA | 0 | 0 | 0 | – |
| | 40 | 537 | 438 | +18.4 |
| | 80 | 772 | 449 | +41.8 |
| | 140 | 851 | 641 | +24.7 |

[a] The rate of liberation of $^{32}P_i$ was measured for 140 min. *See text* for details and discussion.

the enhanced activity of the pump seen in these ghosts may be the result, *pari passu,* of the preservation of a relatively higher level of ATP. In any event, the results presented in Tables 10 and 11 document the feasibility of the approach used to study directly the utilization of ATP by the pump.

Table 12 shows the measurements concerned with the ATP utilization by reconstituted ghosts. The ghosts used in these experiments were prepared in the same manner as that described for Table 11 but substituting uniformly labeled [$^{32}$P]ATP for nonradioactive ATP. In addition, the assay was carried out at a hematocrit of 20 percent rather than the usual 3 percent. [$^{32}$P]ATP was used in order to facilitate the $P_i$ analyses and to distinguish its appearance from any other origin of intracellular $P_i$. The values given in the Table have been normalized for the purpose of comparison to the same initial total counts/unit sample of ghosts and show only the relative changes that occur upon incubation. It should also be stated that approximately half of the incorporated ATP was broken down in 140 min in the upper control [$^{32}$P]ATP ghosts. It is clear from the data presented in Table 12 that the effects observed closely parallel the results as presented in Table 10. Thus, the total ATPase activity is reduced by the incorporation of EDTA and that under this circumstance, strophanthidin is seen to decrease significantly the rate of breakdown of incorporated ATP. While it would be of considerable interest to evaluate the stoichiometric relationship between the ATP spared by the inhibition of the pump to the active transport of Na the several uncertainties inherent in the measurements (even when performed at the same time on the same ghosts) preclude the usefulness of such an estimation. This will be elaborated in the discussion.

### *Asymmetrical Action of Strophanthidin*

Since many of the results and conclusions presented in this paper depend upon the use of strophanthidin, it is of interest to consider in this section the action of strophanthidin itself, particularly with regard to its locus of action on the membrane. Thus, it appeared possible with reconstituted ghosts to evaluate directly whether the inhibition produced by strophanthidin results from its interaction with the membrane from the inside or from the outside, or both. In view of the finding by Solomon, Gill and Gold (1956) and Glynn (1957*b*) that external K can reduce or prevent the inhibition of the pump by cardiac glycosides (or aglycones), and the fact that the pump flux of Na is coupled to external K, it could be anticipated that strophanthidin would be found to interact with the membrane from the outside; i.e., the K side of the pump. Caldwell and Keynes (1959) in comparing the effects of ouabain when injected directly into the interior of the squid axon with its effects when applied externally, found that Na outflux was inhibited only under the latter circumstances. This would appear to eliminate the possibility that strophanthidin could act from the inside. However, as pointed out by Caldwell and Keynes (1959), it is possible that the high intracellular K may act to prevent the inhibition by ouabain and since glycosides are known to be strongly bound by proteins (Hatcher & Eggleston, 1919), it is also possible that the injected ouabain was bound by the axoplasm to such an extent that its concentration at the membrane was too low be effective. This latter difficulty can be minimized by the use of aglycones rather than glycosides, since aglycones are known (*cf.* Straub, *1931*) to be much less firmly bound to proteins or membranes (they can be easily washed off) than glycosides. This is the reason that strophanthidin was used in the present experiments. The results show that although strophanthidin can be trapped inside ghosts at hemolysis, it has no effect on the pump unless it is present in the external medium (Table 13).

Part *A* of Table 13 is concerned with the effect of incorporated strophanthidin on the outflux of Na. The data given in Part *B* provides an evaluation of whether or not the ghosts used in Part *A* did in fact contain an inhibiting concentration of strophanthidin. The test and control ghosts used in Part *A* were made from fresh cells and prepared for assay in the standard manner as previously described. The only preparative difference between the test and control ghosts lies in the hemolysis condition; i.e. in the presence or absence of strophanthidin. The control ghosts were made by hemolysis in 9 volumes of solution containing 0.56 M ethanol + 1 mM $MgCl_2$. The test ghosts were

**Table 13.** Part *A*. Effect of incorporated strophanthidin on the outflux of Na from fresh ghosts (*See text* for discussion)

| Type ghosts | Hemolysis condition for preparation of ghosts | Percent $^{24}$Na released in 130 min | | |
|---|---|---|---|---|
| | | Alone[a] | Inosine + added strophanthidin[b] | Inosine[c] |
| Control | alcohol | 26.4 | 21.2 | 61.7 |
| Test | strophanthidin + alcohol | 23.6 | 19.8 | 58.7 |

[a] Alone means no additions to the final incubation medium.

[b] The concentration of strophanthidin added in the final incubation medium is $1 \times 10^{-4}$ M.

[c] Concentration for inosine is 3 mM.

Part *B*. Effect of extracts of ghosts used in Part *A* on the outflux of Na from a new control preparation of fresh ghosts[a]

| Additions to incubation medium | Percent $^{24}$Na released in 120 min from control ghosts | Percent inhibition |
|---|---|---|
| Inosine | 70.1 (66.4) | – |
| Inosine + added strophanthidin | 32.1 (17.9) | 100 |
| Inosine + extract of test ghosts | 58.7 (48.0) | 30.0 |
| Inosine + extract of control ghosts | 67.0 (61.8) | 8.1 |

[a] The concentration of inosine added in the final incubation medium is 4 mM and strophanthidin, $1 \times 10^{-4}$ M. The results of a second separate experiment are given in brackets. *See text* for details and discussion.

made by hemolysis in a solution having the same composition as the control but containing in addition $1.5 \times 10^{-3}$ M strophanthidin. This is the only exposure to strophanthidin the test ghosts received other than in the subsequent addition of strophanthidin to the incubation medium as part of the assay of $^{24}$Na outflux. It is apparent in Part *A* (Table 13) that there is no difference in the pump flux of Na between the control and test ghosts. This same result has been observed in three separate experiments. This is tantamount to saying that strophanthidin has no effect on the pump from the inside of the membrane provided the test ghosts did contain incorporated strophanthidin.

The demonstration that the test ghosts did contain sufficient strophanthidin to inhibit significantly the Na pump is given in Part *B* (Table 13). Extracts of the control and test ghosts were prepared in the following manner. A portion of the packed ghosts of each kind used in Part *A* were frozen and stored at −20 °C for two weeks (in order to allow decay of the $^{24}$Na). The packed ghosts were then thawed at room temperature and diluted with 20 volumes of incubation medium. This mixture was then completely rehemolyzed by blending in a Waring blender for one minute at 0–2 °C. The ghosts were then removed from the mixture by centrifugation at approximately 25,000 × *g* for 10 min. The supernatant resulting from this procedure represents the extract referred to in Part *B*. The assay for the outflux of $^{24}$Na was carried out using a new preparation of fresh ghosts made from blood obtained from the same individual as used in Part *A* (in order to avoid any immunological complications). It can be seen that the extract of test ghosts results in a significant inhibition of the Na pump in contrast to a slight inhibition seen with the control extract. (The results of a second completely separate experiment, carried out using similar extracts of Part *A* ghosts, are given in parantheses in Part *B*. In addition, similar results for the two types of extracts are also obtained when the assay is carried out using depleted ghosts containing incorporated ATP.) Since the extracts used in Part *B* were diluted prior to assay, the original ghosts (Part *A*) presumably contained approximately 20 times the concentration of strophanthidin as represented by the degree of inhibition in Part *B*. This is to say that the results as given in Part *B* indicate that the ghosts as assayed in Part *A* did contain an inhibitory concentration of strophanthidin and therefore support the conclusion that strophanthidin interacts with the membrane only from the outside.

The actual concentration of strophanthidin contained in the test ghosts of Part *A* was estimated by interpolation of the inhibitory effect of adding different concentrations of strophanthidin to the assay medium containing the control ghost extract as assayed in Part *B*. The concentration of added strophanthidin which produces the same inhibition as obtained with the test ghost extract was approximately $1.2 \times 10^{-6}$ M. This becomes $2.4 \times 10^{-5}$ M after correction for the dilution of the original ghost extract. It will be recalled that the strophanthidin concentration added to the hemolysis medium was $1.5 \times 10.^{-3}$ M. Therefore, the reconstituted ghosts retained at least one percent of the strophanthidin to which they were exposed and since only 10 to 20 percent of the ghosts become reconstituted (*cf.* Hoffman, 1962) the retention factor would be increased to 10 percent. While it is possible that the reconstituted ghosts lost strophanthidin during their preparation, there is a separate problem in knowing the actual concentration of strophanthidin contained in the hemolysis medium, per se. This is due to the limited water solubility of strophanthidin, estimated as being approximately $3 \times 10^{-5}$ M by Dr. S. Hajdu (*personal communication*).

Presumably the actual concentration of strophanthidin contained in the test ghosts lies between these two limits but must remain unknown in the absence of further work.

As an independent check with regard to the presence or absence of strophanthidin, the test ghost extract was subjected to a sensitive and specific bioassay utilizing the staircase phenomenon of the frog heart (Hajdu, 1957). It was found (the assay was kindly performed by Dr. S. Hajdu) that the test ghost extract contained an amount of cardiotonic steroid equivalent to $2 \times 10^{-6}$ M in good agreement with the estimate ($1.2 \times 10^{-6}$ M) given before. In contrast, the bioassay was completely negative with regard to the control ghost extract indicating that this extract was devoid of any cardiac active material.

## Discussion

The main thrust of this paper has been the determination in red blood cells of the primary source of energy for the Na:K pump. The conclusion to be drawn is that the metabolic link between the cytoplasm and the membrane transport process is ATP. This conclusion is essentially based on a) the results obtained with different incorporated substrates in which the activation of the pump was shown to have a specific dependence on ATP, whether incorporated directly or generated from a specific ATP-yielding reaction; b) the discriminating effects of various metabolic inhibitors; c) the characteristics of the competition between the pump and the hexokinase reaction for ATP; and d) the possible differential activation of the pump in depleted systems by different nucleosides, in addition to various correlative measurements attending the above types of studies. Since this evidence, taken as a whole, appears to eliminate the possible involvement of any soluble high-energy phosphorylated intermediate between ATP and the membrane complex, it follows that ATP is the direct substrate of the pump. More recently, extensive work has been carried out on the fate of the terminal phosphate of ATP within the pump and establishing the set of reactions associated with the events of transphosphorylation coupled to the translocation of Na and K (*see* Glynn & Karlish, 1975; Cavieres, 1977; Post, 1979; Skou and Nørby, 1979; Hobbs & Albers, 1980).

The early work carried out by Teorell (1952) and by Szekely, Mányai and Straub (1952) showed that ghosts of human red blood cells could be reconstituted with regard to their osmotic properties and relative cation permeability. Straub (1953) then showed that ghosts could be made to accumulate K at the expense of intracellular ATP but it was Gardos (1954) who by adding ATP to the hemolyzing medium was able to increase the intracellular concentration of ATP. Gardos (1954) found that K accumulation was increased, although not in stoichiometric amounts, by increasing intracellular ATP. One reason for mentioning this initial work is because of its historical importance. Another reason for mentioning it is that while the conclusion is correct (as supported, for example, by the results presented in the present paper) that the pump runs on intracellular ATP, the basis for this conclusion is perhaps more inferential than substantive. This is because the hemolysis conditions as employed by Gardos (1954) were such that (a) not all the cells hemolzyed (we found, for instance, in repeating these experiments that the proportion of intact cells present varied in proportion to the ATP concentration in the medium, from about 10% intact cells at low ATP (approximately 12 mM) to as much as 30% at high ATP (about 36 mM)); (b) that ATP entered intact cells as well as ghosts and that lactate was produced, at almost a third of the normal rate, by the reconstituted system. We found, in fact, that by using the conditions of Gardos (1954), namely, a hemolytic ratio of one volume cells to two volumes medium containing 10 mM ATP at pH 2.3 at 0–2. °C, but increasing the tonicity of the medium to isotonic by adding NaCl, that the ATP content of the cells could be increased as much as threefold without any appreciable hemolysis occurring. Thus, these observations raise a reasonable doubt concerning the extent to which the accumulated K can be assigned to the ghosts in the system and to the primacy of ATP in being the direct substrate of the pump.

It was pointed out in connection with the results presented in Tables 3 and 12 that because of various uncertainties (such as ghost volume, heterogeneity of the population, scatter of data) that it was not possible to estimate either the $K_{1/2}$ of the pump for intracellular ATP or the relationship between the number of Na ions pumped to the ATP utilized. There are now several measures of the pump stoichiometry in red cells (Sen & Post, 1964; Whittam & Ager, 1965; Garrahan & Glynn, 1967) indicating that the ratio is 3 Na/ATP. In addition, recent and more accurate estimates of the $K_{1/2}$ of the red cell pump for ATP are in agreement and range from about 200 to 400 mM (Post, Sen & Rosenthal, 1965; Karlish & Glynn, 1974; Kennedy, Lunn & Hoffman, 1980). With regard to the results presented in Table 8 we have since investigated in a systematic way the effects of controlled variations in the ratio ATP/ADP on pump function (Kennedy et al., 1980). It was found that decreasing the ratio ATP/ADP, at constant ATP, decreased the pump flux of Na, indicating the relative importance of the phosphoryl potential as already discussed.

Another aspect of the results which should be discussed is the significance of the differences in the effects of adenosine and inosine on activating the Na pump. One consequence of the difference is that the redox theory as proposed by Conway (1957) would not seem applicable to the Na:K pump in red cells since, as shown in Table 2, lactate is briskly produced from both inosine and adenosine under circumstances where the pump is activated only by the metabolism of adenosine. Adenosine and inosine also seem to have different effects depending upon whether fresh or depleted ghosts were used (Tables 1 and 5). Thus, where both nucleosides appear comparable in fresh ghosts, their effects in depleted ghosts, as already alluded to, are different. The basis for this difference would appear to be complex since as shown in Table 1, inosine fails to stimulate fresh or depleted ghosts loaded with ADP but not when either type is loaded with PEP or PEP+ADP (Fig. 1); it is also evident in Table 5 that, in depleted ghosts inosine is inactive in the presence of ADP as well as in the presence of the inosine nucleotides, these latter nucleotides also suppressing activation by adenosine. As suggested earlier, one difference between fresh and depleted ghosts could be the activity of a nucleotide diphosphokinase (Parks & Agarwal, 1973), another unknown in these various experiments is the purity of the substrates incorporated. None of the substances were purified prior to use and the possibility that trace amounts of nucleotides or other types of metabolizable substrates are present cannot, of course, be ruled out. It may be, for instance, that a combination of the presence of a nucleoside diphosphokinase together with trace contaminates might be responsible for the relative stimulation of the uncoupled Na efflux (Karlish & Glynn, 1974) with nucleotides which in the present work appear unable to activate the Na:K pump. Perhaps the most important difference between inosine and adenosine in activating the Na:K pump in depleted ghosts lies in the concentrations of the nucleosides used (3 mM) in the present work. Askari and Rao (1968) found that when the concentration of either inosine or adenosine was $10^{-4}$ M, inosine was just as effective as adenosine in stimulating the Na:K pump. But when the nucleoside concentration was increased to $10^{-3}$ M and above, the differences already noted were observed such that adenosine continued to activate under circumstances where inosine did not. The metabolic basis for this difference in effectiveness is not at present known, but it may be that the activation of the pump by ATP may be inhibited by the presence of significant concentrations of their hypoxanthine analogs.

One final comment should be made relative to the sidedness of action of strophanthidin as analyzed in Table 13. The conclusion that cardiotonic steroids only act on the outside (the K-activating side) of the Na:K pump in human red cells has been dramatically confirmed by Perronne and Blostein (1973) by analysis of the effects of ouabain on right-side out versus inside-out vesicles.

All of the work reported in this paper was carried out while the author was a member of the Laboratory of Kidney and Electrolyte Metabolism, Section of Membrane Physiology, National Heart, Lung and Blood Institute, Bethesda, Maryland.

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