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PREPARATION AND PROPERTIES OF THE ($Na^+ + K^+$)-ATPase OF PLASMA MEMBRANES FROM EHRLICH ASCITES CELLS

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SUMMARY

A new rapid method was devised to isolate plasma membranes from Ehrlich ascites tumor cells. 35% of the total ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) is recovered in the membrane fractions with less than 0.3% contamination with nuclei or mitochondria. The ($Na^+ + K^+$)-ATPase is unlike that of most systems in its low molecular activity (2000 min^{-1} at 37 °C) and high sensitivity to K^+ inhibition, which seems to be due to a K^+-induced increase in the dissociation constant for ATP at low (0.1 mM) potassium concentrations. The dissociation constant for ATP was found to be 0.45 μM which is near that reported for other systems.

The number of ATP binding sites was found to be 47 pmoles/mg membrane protein from binding studies and 43 pmoles/mg membrane protein from phosphorylation studies.

The data obtained support the view that the Na^+-activated ATPase is part of the ($Na^+ + K^+$)-ATPase.

INTRODUCTION

As part of a study of transport at the subcellular level with Ehrlich ascites cell membranes, we have devised a method to obtain high yields of plasma membranes in a short space of time. This method makes use of a cell-rupturing procedure which minimizes contamination by other cellular constituents, unlike most methods for plasma membrane preparation which yield a mixed microsomal fraction. The method can be adapted to prepare from 10 to 200 mg membrane protein in a single preparation. Although Ehrlich ascites cells have been shown to possess a ($Na^+ + K^+$)-activated ATPase, (EC 3.6.1.3)[1–3] the details of the partial reactions have not been studied for this system. Since Ehrlich cells, unlike erythrocytes and other mammalian cells, can be rapidly depleted of K^+ and filled with Na^+, the responsiveness of the ($Na^+ + K^+$)-ATPase to Na^+ and/or K^+ in this tissue may be different from that of other tissues.

The present communication describes our procedure for isolating plasma membranes and the properties of the ($Na^+ + K^+$)-ATPase of this system.

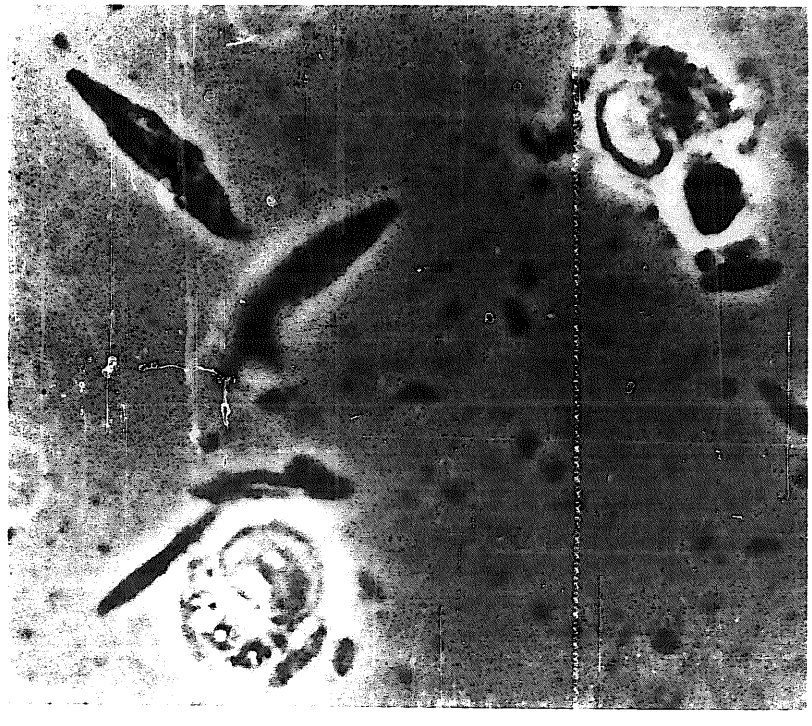
EGTA, ethyleneglycol-bis(β-aminoethyl ether)- *N,N'*-tetraacetic acid.

METHODS AND MATERIALS

Isolation of plasma membranes from Ehrlich ascites cells

Ehrlich ascites cells, maintained as described previously, were used[4]. The cells were washed 4 times in the cold with isotonic saline to remove red cells and soluble contaminants. Then the cells were pelleted and resuspended at room temperature in 10 times their volume with 1.0 mM $ZnCl_2$ and allowed to stand at room temperature for 10 min. This step is a modification of the procedure of Warren *et al.*[5] for isolating plasma membranes from L cells and it appears to prevent the action of liberated digestive enzymes and to "stabilize" the membrane, *i.e.* prevent its disintegration on further manipulation.

The cells at this stage are swollen, but intact as seen at 200-fold magnification under phase contrast. The cytoplasmic material appears aggregated around the nucleus as noted by Warren *et al.*[5] for L cells. After 10 min, the cells were chilled in crushed ice and all subsequent procedures were done on ice or at 4 °C. After 10 min on ice, the cells were lysed by forcing the suspension out of a 30-ml plastic syringe fitted with an 8-cm flat-tipped needle (internal diameter = 1 mm), the needle being pressed firmly against the bottom of the tube. Lysis was monitored using a phase contrast microscope, and care was taken to prevent foaming. The lysate was then layered over four 200-ml sucrose gradients (25–40%(w/w) sucrose containing 0.5 mM $ZnCl_2$). The gradients were then centrifuged in a PR-I International Centrifuge at $1500 \times g$ for 45 min at 4 °C. With this procedure, the majority of nuclei, still intact, were pelleted and the lower one third of the gradient contained the plasma membranes. The top two-thirds of the sucrose gradient were discarded. The plasma membrane fraction was collected in two parts, that portion directly above the nuclei being collected separately from the bulk of the lower third of the gradient since the former portion was contaminated with nuclei. Both the nuclei-free and nuclei-contaminated



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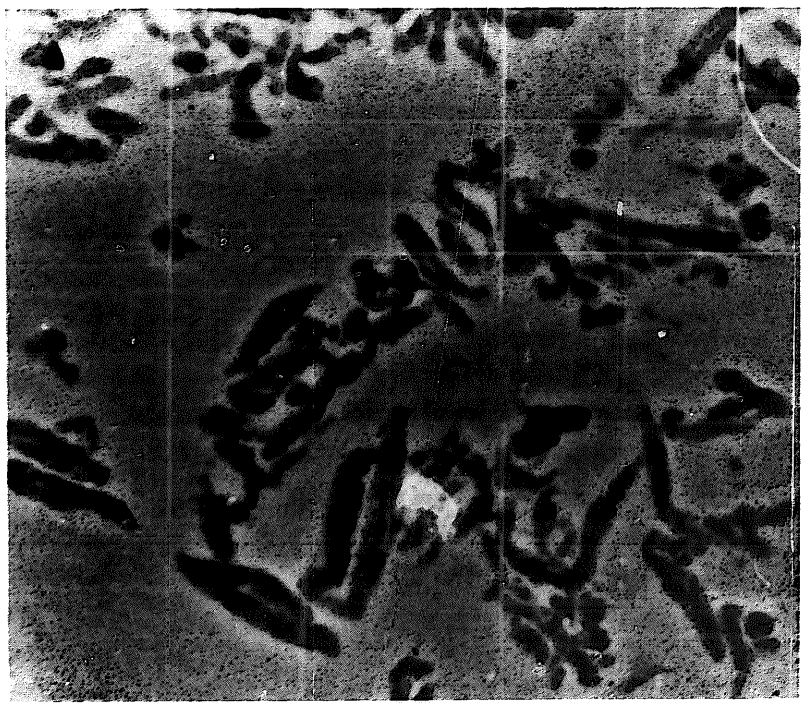

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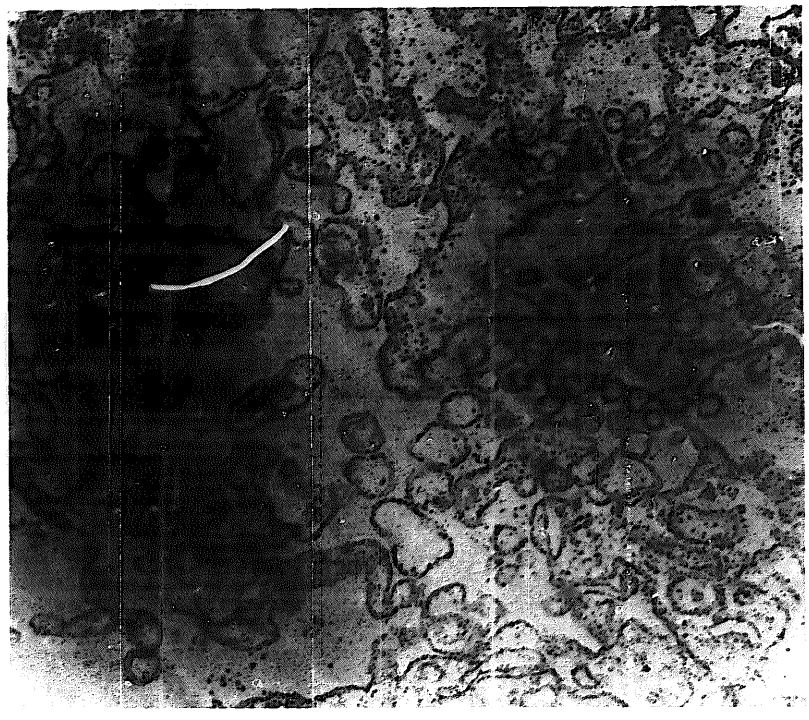

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Fig. 1. Microscopic examination of Ehrlich ascites cell plasma membranes. A shows the cell lysate and B the final membrane suspension in 15% dimethylsulfoxide. Both are at 192-fold magnification under phase contrast. Kodak tri-X pan was used and exposed for 0.5 s. The large elongated membranous objects are the plasma membranes. C is an electron micrograph of the membrane preparation at 17000× magnification prepared by Dr Sarah Gibbs of the Biology Department at McGill University. The stains used were 4% uranyl acetate in 40% ethanol for 25 min at 40 °C followed by Reynolds lead nitrate. The black dots are artifacts of the fixation procedure[17].

fractions were made 1 mM with respect to EDTA at pH 7.5 using Na^+ EDTA (Tris at this stage had adverse effects on the membranes). The EDTA treatment restored both the Mg^{2+}-ATPase and the $(Na^+ + K^+ + Mg^{2+})$-ATPase activities (Fig. 2). Both nuclei-contaminated and nuclei-free fractions were then centrifuged in a refrigerated Sorvall RC-2B at 12000×*g* for 30 min. The nuclei-contaminated fraction was resuspended in 15%(v/v) dimethylsulfoxide and subjected to several cycles of differential centrifugation to remove the contaminating nuclei, then pooled with the nuclei-free fraction and recentrifuged in a refrigerated Sorvall centrifuge at 3000×*g* for 10

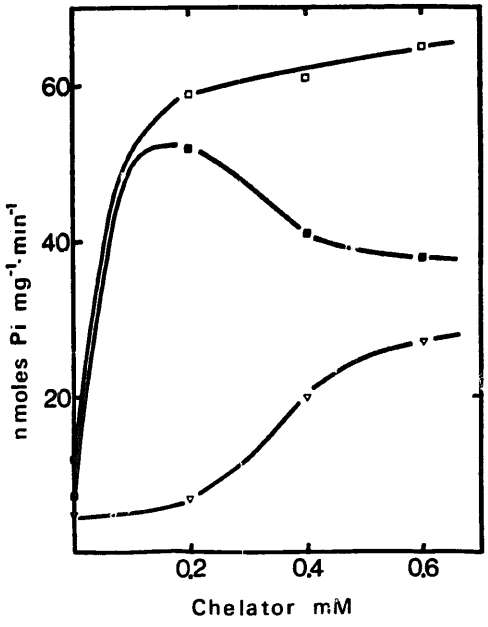

Fig. 2. The effect of Zn^{2+} removal on the Mg^{2+} and $(Na^+ + K^+)$-ATPase activities. Both EDTA and EGTA were used as chelators. The final reagent concentrations were 20 mM Tris–HCl, pH 7.5, 250 μM ATP (Tris salt), 250 μM $MgCl_2$ for (■). In addition (□) had 80 mM NaCl and 17 mM KCl. (△) = (□) − (■). The preparation was used directly as it came out of the sucrose gradient. 10-min incubations were done at 37 °C.

min. This final pellet was resuspended in 15%(v/v) dimethylsulfoxide to give a protein concentration of 1.0–2.0 mg/ml. The membranes are stored frozen at −20 °C. A yield of 15–20 mg membrane protein is obtained from approximately 12 ml of packed cells. Recently this procedure has been scaled up seven fold by using an RC-3 centrifuge for the sucrose gradient and a Polytron homogenizer (Kinematica, Lucerne, Switzerland) to rupture the cells.

Chemical analyses

Protein was measured by the method of Lowry *et al.*[6], RNA by the orcinol method[7] and by the absorbance at 260 nm[8], sialic acid by the thiobarbituric acid method[9], and DNA by the diphenylamine method[10].

Enzyme assays

ATPase activity was measured either by following the release of ^{32}P from γ-^{32}P-labelled ATP prepared according to Post *et al.*[11] as modified by Blostein[12] or by measuring the inorganic phosphate released using the phosphomolybdate method[13].

p-Nitrophenylphosphatase activity was measured by following the release of *p*-nitrophenol at 400 nm. NADH or succinate–cytochrome *c* reductase activities were measured by following the increase of absorption at 550 nm[14].

[^{32}P]ATP-binding studies

Prior to use, the membrane preparation was washed free of the storage medium and resuspended in 2.5 mM [^{14}C]citrate (Tris salt, pH 7.5). All manipulations were carried out at 0 °C. [^{14}C]Citrate was used to measure the trapped fluid associated with the pellet to correct for non-specific trapping of a highly negatively charged ion. To 0.5 ml of the membrane suspension was added a solution containing 10 mM EDTA (Tris salt) pH 7.5, 0.3 mM GTP (Tris salt) pH 7.5 and [γ-^{32}P]ATP ranging from 0.15 to 10 μM ATP in one series and 1.0 to 100 μM in second series which also contained 10 mM K^+. The final volume was 1.0 ml. The resulting mixture was centrifuged at 13000 × *g* in a fixed angle Sorvall centrifuge at 4 °C for 3 min. The supernatant was decanted into a precooled tube. The walls of the tube containing the pellet were dried with lint-free lens paper and the pellet was extracted with 1.0 ml of ice-cold 5% trichloroacetic acid and centrifuged at 3000 × *g* for 1 min. Duplicate samples of the pellet extract were removed and counted using Bray's solvent. 50 μl of the original supernatant, with sufficient trichloroacetic acid added to make it comparable to the pellet sample, were counted. To correct for contamination with $^{32}P_i$, a second aliquot of the sample, with carrier P_i added to minimize losses of $^{32}P_i$, was treated with charcoal to remove ATP. Sufficient trichloroacetic acid was added to make this solution comparable to the pellet extract. ATP hydrolysis was minimal in these experiments (maximum 4% of total ATP added) since GTP suppressed the Mg^{2+}-ATPase without inhibiting the $(Na^+ + K^+)$-ATPase. The absence of $(Na^+ + K^+)$-activated GTPase is known[2].

Membrane phosphorylation

Membranes were incubated with the ATP concentration given in the text using [γ-^{32}P]ATP in a medium containing 40 mM Tris–HCl buffer and the specified Mg^{2+}, Na^+ and K^+ concentrations, in a final volume of 0.5 ml. Incubations were initiated by the addition of ATP to yield a final concentration of 250, 25 or 2.5 μM. Chase experiments were done by incubating parallel tubes for 30 s followed by addition of [^{31}P]ATP in excess (500 μM final at the two lower ATP concentrations and 25 mM at the highest) to one of the tubes and incubating it for a further 30 s. The reactions were stopped with 5.0 ml of ice-cold 5% trichloroacetic acid containing 2.5 mM ATP and 5 mM NaH_2PO_4. Each sample was filtered at 4 °C using a millipore filter (0.6 μ pore). The filtrate was collected into a 15-ml centrifuge tube containing 0.5 g charcoal to adsorb the ATP. The filter was washed twice with 1 ml of the cold trichloroacetic acid, ATP, phosphate mixture (see above) and these washings were pooled with the first filtrate. The filter was washed a further four times (eight times for the high ATP concentration) with 1-ml aliquots of the wash mixture and these washings were discarded. The filters were then placed on a planchet, dried, and counted in a Nuclear Chicago gas-flow planchet counter. On some occasions liquid scintillation counting was used. The charcoal-treated filtrate was also counted. This procedure permitted us to measure membrane phosphorylation and ATPase activity on the same sample. Protein determinations were done on the initial membrane suspension.

Materials

Neuraminidase, ATP, NADH, cytochrome *c*, NaCl, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA), tris(hydroxymethyl)aminomethane (Tris), GTP (Tris salt), rotenone, *p*-nitrophenyl phosphate and oligomycin were purchased from Sigma Chemical Co., St. Louis, Mo. Ouabain was obtained from Nutritional Biochemical Co., Cleveland, Ohio, and antimycin A from Schwartz-Mann, Orangeburg, N.Y. Other reagents were obtained from Fisher Chemical Co., Montreal, Que. All radioactive materials were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Characteristics of the membrane fraction

The use of Zn^{2+} during the isolation procedure for plasma membranes raises the possibility that alterations in the membrane structure or irreversible inactivation of membrane proteins may occur. That activities present in the plasma membrane are restored after the removal of Zn^{2+} is shown by two lines of evidence. First, intact cells treated with Zn^{2+} and then washed free of Zn^{2+} have esssentially the same amino acid transport activity as untreated cells. For example, glycine at 2 mM was accumulated 10.8-fold in untreated cells and 9.5-fold in Zn^{2+}-treated cells. Na^+ removal abolished transport in both cases. Second, both the Mg^{2+}- and (Mg^{2+} + Na^+ + K^+)-ATPase are largely inactive in membrane preparations containing Zn^{2+}, but activity is restored on addition of EDTA or EGTA (Fig. 2). As will be shown, these preparations have a high (Na^+ + K^+)-activated, ouabain-sensitive ATPase relative to the Mg^{2+} activity. Other reports have also appeared in the literature where Zn^{2+} was used in the isolation of plasma membranes from L cells[15] and chick embryo fibroblasts[16] which yielded preparations whose ATPase activity was restored on removing the Zn^{2+}.

The membranes can be stored in 15% dimethylsulfoxide at −20 °C for at least a month without detectable change in the (Na^+ + K^+)-ATPase activity or in protein labelling activity.

The following criteria were used to assess the composition and purity of the plasma membrane fraction of the Ehrlich ascites cells.

Light and electron microscopy. The characteristic appearance of the plasma membrane fraction under phase contrast and in the electron microscope is shown in Fig. 1. The preparation appears to be relatively free from other cellular components.

No mitochondria were detected although an occasional nucleus was found. Particles scattered in the electron micrographs are probably artifacts of fixation (Fig. 6 of ref. 17).

Oxygen utilization. That the preparation is relatively free from mitochondria is further supported by the fact that measurements of oxygen uptake with the Clark oxygen electrode showed that the preparation has only 2% of the respiratory activity found in the lysate and that this residual O_2 utilization is insensitive to antimycin A and rotenone, but is completely inhibited by CN^-. The oxygen uitlization does not lead to H_2O_2 formation since the addition of catalase has no effect.

Distribution of chemical constituents. The distribution of several cellular components in the fraction designated as the plasma membrane is given in Table I. About 35% of the total (Na^+ + K^+)-ATPase acitivity found in the lysate is recovered

TABLE I

CHEMICAL CHARACTERIZATION OF THE PLASMA MEMBRANE PREPARATION

The $(Na^+ + K^+)$-ATPase and the Mg^{2+}-ATPase were measured at 250 μM ATP and 50 μM $MgCl_2$. The incubation was for 10 min at 37 °C. The $(Na^+ + K^+)$-ATPase is the increment in ATPase on addition of 75 mM Na^+ and 25 mM K^+ to the reaction medium. A freshly prepared lysate was used for all assays except the protein assay. Respiration was measured with a Clark oxygen electrode.

	$(Na^+ + K^+)$-ATPase activity (nmoles/mg protein/min)	*Mg^{2+}-ATPase activity (nmoles/mg protein/min)*	*DNA (μg/mg protein)*	*RNA (μg/mg protein)*	*Sialic acid (nmoles/mg protein)*	*NADH–cytochrome c reductase activity (nmoles/mg protein/min)*	*Protein (%)*	*Antimycin A inhibited respiration (nmoles O_2· mg protein^{-1} h^{-1})*	*Rotenone-inhibited respiration (nmoles O_2· mg protein^{-1} h^{-1})*
Lysate	2.1	8.0	66	72*	10.9	6.5**	100	101	37
Upper band	6.2	33.8	0.6	—	—	—	9.4	—	—
Pellet	1.6	8.5	100	—	—	—	88	—	—
Plasma membr. (final suspension)	46.9	23.2	10±2. (4)	75* 57***	20±2 (5)	8.2**	2.5±0.3 (3)	ND†††	ND(<0.1)
% of total activity in PMF†	38††	5.3††	0.30	2.6	5.0	3.2	2.5	ND	ND(<0.3)

* By absorption at 260 nm (average of 2 different preparations).
** Average of 2 determinations.
*** By orcinol method (average of 2 different preparations).
† Plasma membrane fraction.
†† Results of a typical experiment. The average values are 35 ± 4 (3) and 5 ± 1 (3).
††† ND: Not Detectable.
Rotenone was used at 1.1 μg/ml and Antimycin A at 0.6 μg/ml.

in the plasma membrane fraction. Only 5% of the Mg^{2+}-ATPase is recovered, a finding consistent with the observation that ATPase activity is contained in other membranes, such as mitochondria. 0.3% of the total DNA (deoxyribose content) is recovered in the plasma membrane fraction. Although some of this is due to contaminating nuclei or nuclear debris, the possibility that some small fraction of the DNA is a natural constituent of plasma membranes cannot at this time be excluded. The RNA content of the plasma membranes is very similar to that obtained by Juliano *et al.*[18] whose work supports the idea that RNA is a normal constituent of the plasma membrane. The low sialic acid content of Zn^{2+}-treated membranes was also observed by Glick *et al.*[19]. Although Wallach and his associates[1,2] have also isolated and purified a plasma membrane preparation from Ehrlich ascites cells it is impossible to compare our yields to theirs since they do not indicate what fraction of the total (Na^+ + K^+)-ATPase (nor indeed of any of the markers) is recovered in the membrane fraction. Comparing the Mg^{2+}-ATPase and the (Mg^{2+} + Na^+ + K^+)-ATPase, Wallach and Ullrey[2] showed that the activity of the (Na^+ + K^+)-ATPase is about the same as the activity of the Mg^{2+}-ATPase, that is addition of Na^+ + K^+ doubles the rate of ATP hydrolysis. In our preparation the addition of monovalent cations nearly trebles the ATPase, that is the monovalent cation activated activity is twice as large as the Mg^{2+}-ATPase (Table II). In agreement with Wallach and Ullrey[2], we also find that the (Na^+ + K^+)-ATPase activity is greatest at pH 8.5. The specific activity of our preparation is 70% of theirs when assayed under conditions of incubation similar to theirs.

TABLE II

CHARACTERISTICS OF THE (Na^+ + K^+)-ACTIVATED ATPase AND THE Mg^{2+}-ATPase

The Mg^{2+} concentration is 150 μM for 250 μM ATP and 50 μM for 25.0 and 2.5 μM ATP. 0.5 mM EGTA is present. The Na^+-ATPase is the increment of ATPase observed on addition of 75 mM Na^+. The "chasable" Na^+-phosphorylation is that ^{32}P-label which is lost after 30 s incubation with non-radioactive ATP (see Methods).

ATP concentration:	*250*	*250*	*25*	*2.5*
temp. (°C):	*37*	*20*	*20*	*20*
(Na^+ + K^+)-ATPase (nmoles/mg per min)	62 ± 4 (9)	20 ± 1 (5)	8.4, 7.2	*
Na^+-ATPase (nmoles/mg per min)	**	**	2.3, 2.8	1.9 ± 0.3 (13)
Mg^{2+}-ATPase (nmoles/mg per min)	27 ± 6 (10)	9 ± 1 (4)	7.9, 2.8	3.6 ± 0.5 (13)
"Chasable" component of Na^+-dependent phosphorylation (pmoles/mg protein)	***	43 ± 2 (3)	34	27 ± 3 (13)

* At this ATP concentration, K^+ inhibits ATPase at all concentrations examined.

** No reliable determination because of high Mg^{2+}-ATPase.

*** Values obtained were difficult to interpret because of the apparent activation of other alkali metal cation-controlled systems.

The $(Na^+ + K^+)$-ATPase

To study the properties of the $(Na^+ + K^+)$-activated ATPase of this membrane preparation the following activities were assayed. (1) The $(Na^+ -- K^+)$-stimulated ATPase. (2) The Na^+-stimulated ATPase. (3) The incorporation of γ-^{32}P from ATP into trichloroacetic acid-precipitated membrane material. (4) The exchangeability of the protein-bound ^{32}P.

TABLE III

Mg^{2+} CONCENTRATION, MEMBRANE PHOSPHORYLATION AND ATPase ACTIVITY

Membranes (270 mg) were incubated for 1 min at 37 °C in a medium containing 250 μM ATP (Tris salt) 40 mM Tris–HCl, pH 7.5; 0.5 mM EGTA (Tris salt, pH 7.5) in a final volume of 0.5 ml

Membrane phosphorylation (pmoles/mg)				*ATPase activity (μmoles P_i per minute per mg protein)*			
Mg (μM)	—	$+Na^+$	$+(Na^+ + K^+)$	—	$+Na^+$	$+(Na + K^+)$	*Δ = $Na^+ + K^+$ + ATPase
0	20		18				
50	44	92	76	27.4	21.0	62.5	35.1
100	100	160	148	34.6	30.1	81.3	46.7
150	153	234	227	37.2	31.2	85.0	47.8

*Δ = values in Column 7 *minus* values in Column 5. Na^+ was present at a concentration of 75 mM and K^+ at 25 mM.

Our first objective was to obtain the experimental conditions which would optimize the monovalent cation ATPase relative to the Mg^{2+} baseline. The results in Table III show that with 250 μM ATP as substrate, a concentration of 0.1 mM Mg^{2+} is nearly optimal for both the $(Na^+ + K^+)$-ATPase and the Mg^{2+}-ATPase. At concentrations between 0.05 and 0.15 mM Mg^{2+} there is a constant relationship between the Mg^{2+}-ATPase and the $(Na^+ + K^+)$-ATPase.

The effect of Mg^{2+} concentration on the phosphorylation of the membrane proteins shows that both the Mg^{2+}-dependent and the $(Mg^{2+} + Na^+)$-dependent phosphorylations increase with Mg^{2+} concentration albeit not to the same extent.

At 250 μM ATP, both Na^+ and K^+ are required to stimulate ATP hydrolysis. The results in Fig. 3 show the effects of K^+ on the hydrolysis of ATP and on labelling of membrane material. It may be seen that with 5 mM K^+, hydrolysis of ATP is optimal as also shown by Wallach and Ullrey[2]. The decrease in protein labelling is also nearly at its lowest with 5 mM K^+. That the increased rate of hydrolysis is related to the decreased level of phosphorylated protein is suggested by the almost reciprocal relationship of the two curves (Fig. 3).

It has been proposed by Blostein[12] that with red cell ghosts the use of very low ATP concentrations permits one to measure the Na^+-dependent component of the $(Mg^{2+} + Na^+ + K^+)$-ATPase and, since the level of membrane phosphorylation by Mg^{2+} alone is greatly reduced at low ATP, a better measurement of the Na^+-stimulated membrane phosphorylation is also obtained. The data in Figs 3 and 4 show that at 2.5 μM ATP, Na^+ stimulates both the phosphorylation of membrane

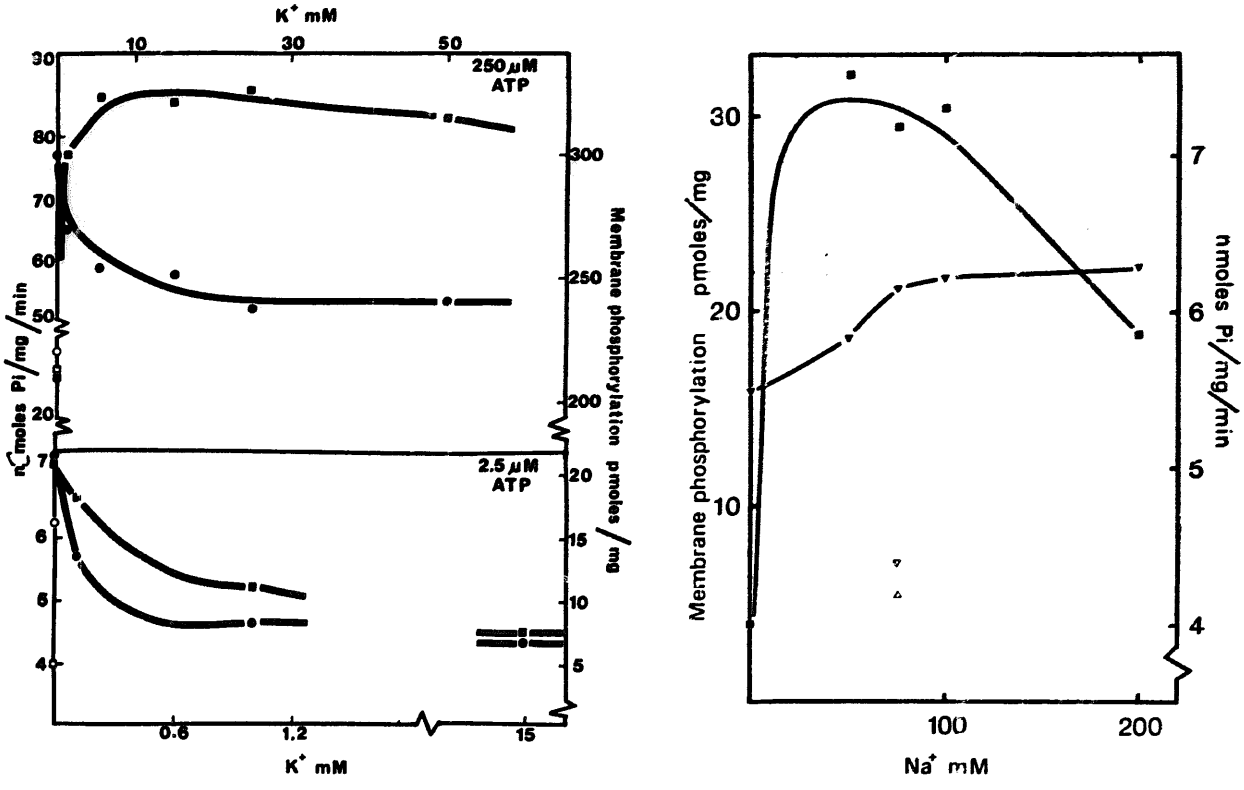

Fig. 3. Response to K^+ concentration at 2.5 and 250 μM ATP. The medium contained 40 mM Tris–HCl, pH 7.5, 75 mM Na^+, and 0.5 mM EGTA (Tris salt, pH 7.5). The Mg^{2+} concentration was 50 μM with 2.5 μM ATP and 150 μM with 250 μM ATP. Incubation was for 30 s at 21 °C with 2.5 μM ATP and for 1 min at 37 °C with 250 μM ATP. 92 mg membrane protein in a volume of 0.5 ml was used with the low ATP concentration and 220 mg with the higher ATP concentration. The ATPase activity (□, ■) and the membrane phosphorylation (○, ●) are indicated, the open symbols representing activity in absence of Na^+.

Fig. 4. Response to Na^+ concentration at 2.5 μM ATP. The medium contained 40 mM Tris–HCl, pH 7.5, 50 μM Mg^{2+}, 500 μM EGTA (Tris salt, pH 7.5) and 92 mg membrane protein in a volume of 0.5 ml. Incubations were for 30 s at 21 °C. The ATPase activity (■) and the membrane phosphorylation (▲, ▽, △) are indicated. The hollow triangles represent the levels of phosphorylation in the presence of 15 mM KCl (△) and that after 10 min incubation (△) without K^+.

protein and the release of P_i. At this ATP concentration, K^+, even at 0.1 mM, decreases both the hydrolysis of ATP and the labelling of the protein. It is also evident in Fig. 3 that K^+ decreases protein labelling to a greater extent than the ATPase activity. The fact that both ATPase and labelling of protein are reduced suggests that K^+ decreases the rate of phosphorylation of protein in addition to stimulating the rate of dephosphorylation. In several systems examined K^+ inhibits ATPase activity at low ATP concentrations[20–22]. However, this system appears to be particularly sensitive to K^+ since 0.1 mM K^+ reduces ATPase activity in presence of 75 mM Na^+. Even with red cell membranes from LK sheep cells which are known to be sensitive to inhibition by K^+ (ref. 21), 3 mM K^+ is required for 30% inhibition at 0.2 μM ATP. K^+ concentrations of 0.1 mM are not inhibitory[21].

The possibility was examined that the inhibition of ATPase by K^+ was due to an increased K_{diss} for ATP. Recently Hegyvary and Post[23], and Norby and Jensen[24] showed that the dissociation constant for ATP in purified ATPase preparations from

kidney and brain microsomes respectivley increased when the K^+ concentration was increased.

The Scatchard plot in Fig. 5 shows that in Ehrlich ascites cell membranes, K^+ increases the K_{diss} for ATP from 0.45 to 2.7 μM. Although our method for determining K_{diss} for ATP is different from that described by Norby and Jensen[24] or Hegyvary and Post[23], the K_{diss} for ATP in absence of K^+ is similar to the latter values (compare 0.45 μM to 0.12 μM and 0.3 μM, the values quoted by Norby and Jensen[24],

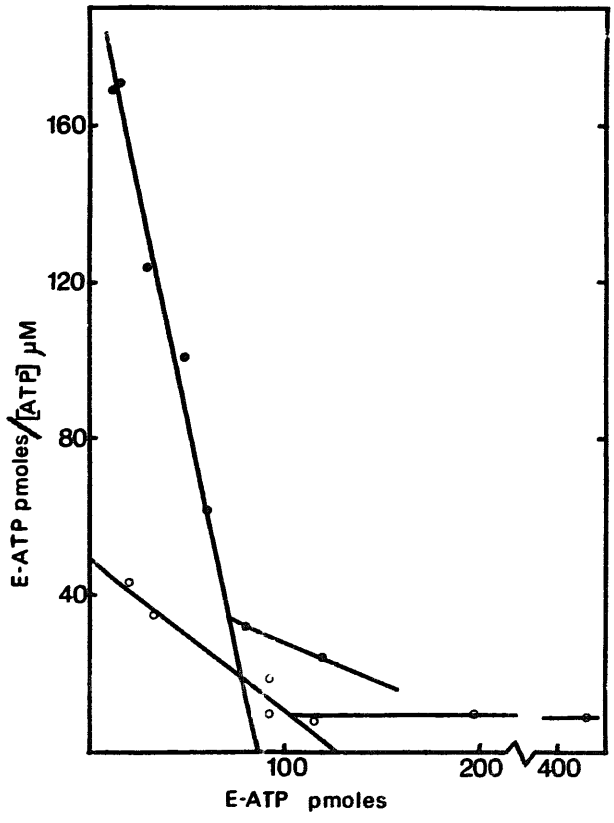

Fig. 5. Scatchard plot for ATP binding. See Methods for experimental detail. In the absence of K^+ (●) the K_{diss} = 0.45 μM and in its presence (○) the K_{diss} increased to 2.7 μM. The intercept on the ordinate in the absence of K^+ (●) indicates that 47 pmoles ATP are bound per mg protein.

and Hegyvary and Post[23], respectively). We find, as did Hegyvary and Post[23], that K^+ alters not only the K_{diss} but also the apparent number of ATP binding sites. While the significance of this change is unclear, the qualitative effect of K^+ on ATP binding is striking, indicating that at low ATP, K^+ is likely to reduce membrane phosphorylation and hence ATPase by diminishing the association of the enzyme with ATP. It is perhaps significant that a Na^+-stimulated, K^+-inhibited ATPase activity is observed at those ATP concentrations which are below the K_{diss} for ATP in presence of K^+. When the ATP concentration is well above the K_{diss} in presence of K^+ (25 μM, or 10 times the estimated K_{diss} value) K^+ stimulates ATPase activity.

In absence of K^+, high Na^+ (> 150 mM) inhibits the ATPase activity (an observation also seen by Wallach and Ullrey[2]), but does not decrease the protein phosphorylation suggesting that Na^+ may have an affinity, albeit small, for the K^+-dependent hydrolytic sites.

Properties of the phosphoprotein intermediate

The demonstration that the $(Na^+ + K^+)$-ATPase in our system can be divided into phosphorylating and dephosphorylating steps led us to examine whether the phosphorylated intermediate in this system behaves kinetically in a manner consistent with its role as a rapidly turning-over intermediate and in line with the rate of turnover observed in other systems[21,25–27,30]. The data in Fig. 6 show that Na^+ increases the level of phosphorylated component which can be "chased out" by addition of excess unlabelled ATP. Without addition of ATP, the level of the ^{32}P intermediate decreases with time of incubation as a function of the remaining [^{32}P]ATP concentration. The data in Fig. 6 also show that the labile component in absence of Na^+ is small. The Na^+-stimulated incorporation is hydroxylamine-sensitive. (Results not shown.)

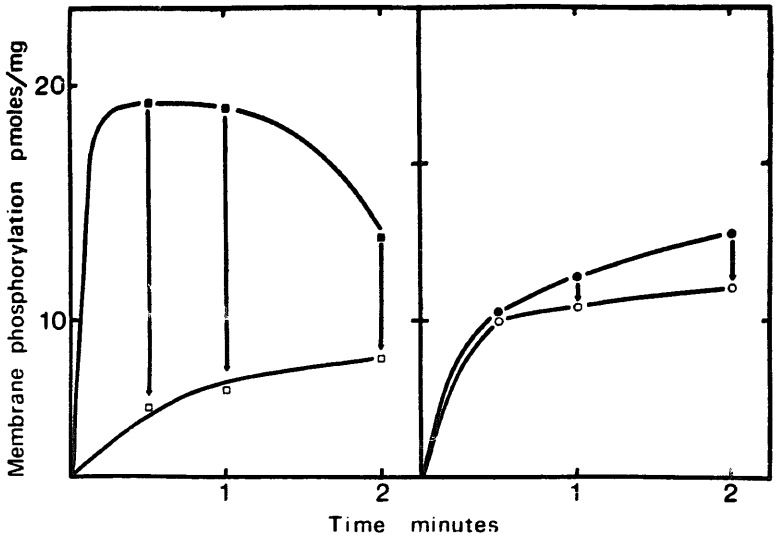

Fig. 6. Time course of membrane phosphorylation at 2.5 μM ATP. The medium contained 40 mM Tris–HCl, pH 7.5, 50 μM Mg^{2+}, 500 μM EGTA (Tris salt, pH 7.5) and 92 mg membrane protein in a 0.5-ml volume. Incubations were done at 20 °C. The values in the absence (●, ○) and in the presence (■, □) of 75 mM Na^+ are shown. The hollow symbols represent the level of phosphorylation remaining after a 30-s chase with [^{31}P]ATP (see Methods and Materials for details.) The arrows span the regions of chasable phosphorylation.

In addition to increasing the labile phosphorylation, Na^+ decreases the "stable" phosphorylation (*i.e.* the labelling not decreased by addition of non-radioactive ATP). This decrease in "stable" phosphorylation in presence of Na^+ is not due to competition for, or depletion of, ATP since the stable labelling is reduced in presence of Na^+ and oligomycin where the ATP concentration remains high (see Fig. 8). The significance of the stable labelling and the action of Na^+ thereon are unknown. However, the effect of Na^+ is observed consistently and requires that a correction be made for the "true" Na^+-stimulated formation of "chasable" intermediate. Skou has also observed that Na^+ decreases the "stable" component of the phosphorylation[28].

The data in Fig. 7 show the inhibition of ATPase activity by ouabain. It may be noted that at 2.5 μM ATP, both ouabain and K^+ decrease the ATPase acitivity and that with 5.0 mM K^+, the residual ATPase is not further inhibited by ouabain. Under the present experimental conditions, incubation with ouabain does not cause a major decrease in the level of the phosphorylated intermediate, in presence or absence

of K^+ (Fig. 7). (Indeed it may be noted that with 2.5 μM ATP and 0.1 mM K^+, the addition of ouabain increases the level of the phosphorylated intermediate while ATPase activity is further reduced.) The rate of chase of ^{32}P intermediate, however, is decreased with ouabain. For example in the absence of ouabain, complete loss of ^{32}P label from the protein is observed within 30 s (the first sample taken) after the addition of excess non-radioactive ATP at 20 °C. In contrast, in presence of ouabain, a similar loss of radioactivity from ^{32}P-labelled intermediate requires 10 min incubation in presence of excess non-radioactive ATP. A similar effect was observed by Sen *et al.*[29]. These data suggest that the inhibition by ouabain of the $(Na^+ + K^+)$-ATPase is caused by a decreased rate of turnover of the intermediate.

If oligomycin is used instead of ouabain, there is no detectable change in the rate of loss of ^{32}P label from the protein in presence of excess ATP (complete loss of radioactivity within 30 s after addition of ATP). However, oligomycin does increase

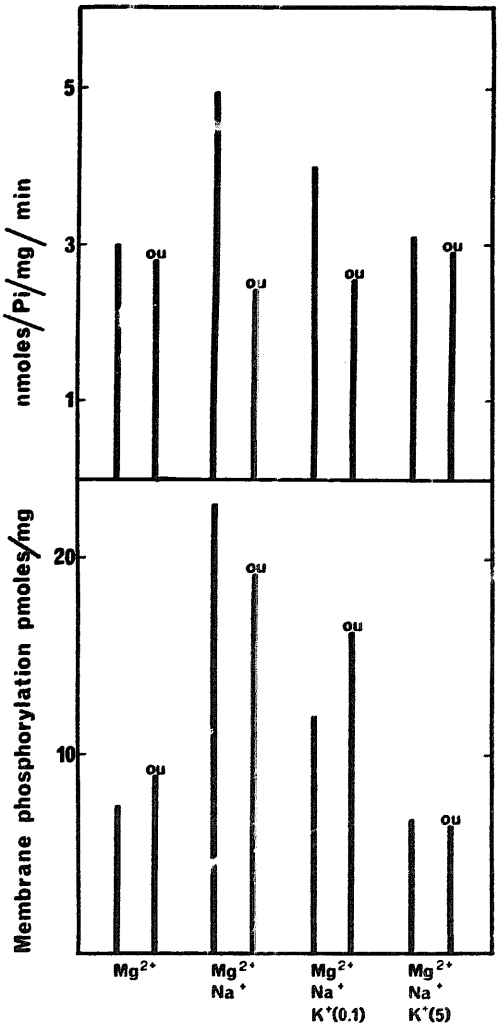

Fig. 7. The effect of 1 mM ouabain on the ATPase activity and membrane phosphorylation at 2.5 μM ATP. The medium contained 40 mM Tris–HCl, pH 7.5, 50 μM Mg^{2+}, 500 μM EGTA (Tris salt, pH 7.5) and 92 mg membrane protein in 0.5 ml. Incubations were done at 20 °C for 30 s. The bars distinguished by the letters OU show the activity in the presence of ouabain while the other bar of the pair is the activity in the absence of ouabain. Where the presence of Na^+ and K^+ are indicated the concentrations were 75 mM for Na^+ and 0.1 and 5.0 mM for K^+.

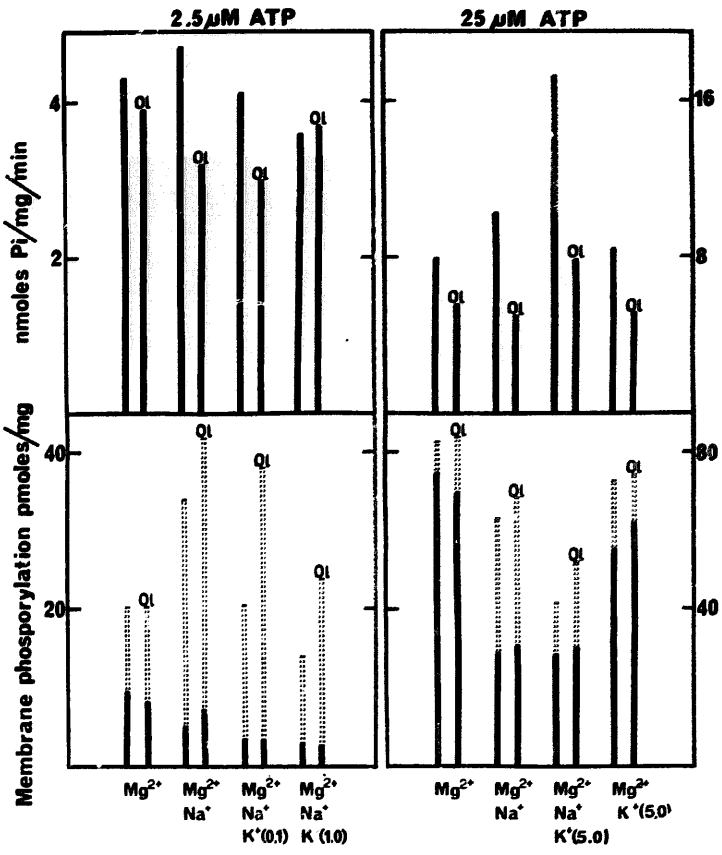

Fig. 8. The effect of 20 μg/ml oligomycin on the ATPase activity and the degree of membrane phosphorylation at 25 μM ATP and 2.5. μM ATP. The medium contained 40 mM Tris–HCl, pH 7.5, 50 μM Mg^{2+}, 500 μM EGTA (Tris salt, pH 7.5). The bars distinguished by the letters OL show the activity in the presence of oligomycin while the other bar of the pair is the activity without oligomycin. The dotted portion is the fraction of the phosphorylation which can be reduced by incubation for 30 s with excess non-radioactive ATP (see Methods and Materials). Where the presence of Na^+ is indicated, the concentration was 75 mM. The K^+ concentrations (mM) are those shown in the figure. 80 mg membrane protein were used per tube with 2.5 μM ATP and 185 mg membrane protein with 25 μM ATP, in a volume of 0.5 ml. Incubation was at 20 °C for 30 s.

the level of the phosphorylated intermediate in presence of 0.1 mM K^+ in a manner similar to that seen with ouabain (Fig. 8).

The contrasting results obtained with ouabain and oligomycin on the turnover of the intermediate, that is on the rate at which the intermediate breaks down and is rephosphorylated by ATP, are consistent with the notion that the intermediate exists in two forms, E_1P and E_2P[30,31]. The sequence of events in the hydrolysis of ATP is believed to be $E + ATP \rightleftarrows E_1P \rightarrow E_2P \rightarrow E + P_i$. Oligomycin has been shown to act at the site of conversion of E_1P to E_2P whereas ouabain inhibits the hydrolysis of E_2P. An exchange with ATP is possible only at the level of E_1P[30,31]. In agreement with this mechanism it would be anticipated that ouabain but not oligomycin would reduce the rate of chase in presence of ATP while net hydrolysis of ATP would be reduced by both inhibitors.

The data in Fig. 9 show that like most other systems examined, this preparation has a K^+-activated, *p*-nitrophenylphosphatase activity which is inhibited by Na^+ (ref. 32).

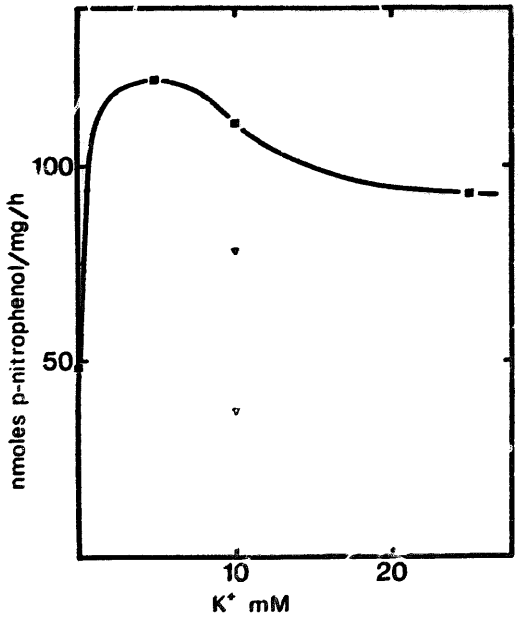

Fig. 9. *p*-Nitrophenylphosphatase activity as a function of the K^+ concentration (■).An initial concentration of 250 μM *p*-nitrophenylphosphate was used along with 150 μM Mg^{2+}, 40 mM Tris–HCl, pH 7.5, 500 μM EGTA (Tris salt, pH 7.5). (■) Control, no Na^+; (▼) contains 25 mM Na^+ and (▽) contains 75 mM Na^+. Incubations were performed at 37 °C for 30 min.

DISCUSSION

Single cell preparations offer an opportunity to separate plasma membranes as relatively large pieces, thereby minimizing the contamination with other sub-cellular components such as the endoplasmic reticulum. The relatively simple procedure that we have devised permits us to separate appreciable quantities of plasma membranes from Ehrlich ascites cells in a short space of time. 140 mg membrane protein can be prepared within 6 h by a single operator (M.C.) The membrane fraction can be stored for as long as one month with no significant loss of the $(Na^+ + K^+)$-activated ATPase. We have regularly observed that the Mg^{2+}-ATPase activity changes more (decreases) with storage than does the $(Na^+ + K^+)$-ATPase. Earlier methods for isolating plasma membranes from Ehrlich ascites cells, described by Wallach[1,2] and Schon and Menke[3] required more time to prepare without offering preparations which had higher $(Na^+ + K^+)$-ATPase activity per mg protein. The present procedure gives a 20-fold enrichment of the $(Na^+ + K^+)$-ATPase with a 35% yield of the total $(Na^+ + K^+)$-ATPase. Earlier investigators[1–3] failed to report the yield of $(Na^+ + K^+)$-ATPase activity or the enrichment based on the original lysate, making comparisons impossible.

That our preparation is minimally contaminated by whole mitochondria and nuclear material may be seen by (a) the visual appearance of the preparation under the electron microscope, and light microscope, (b) the low content of DNA, and (c) the low content of respiratory markers of mitochondria. The question of contamination with endoplasmic reticulum is more difficult to assess since our preparation does contain about 2.5% of the total RNA and 3.2% of the NADH–cytochrome *c* reductase. Since several groups[18,33–35] have now concluded that RNA is a normal constituent of the plasma membrane, we cannot dismiss the presence of RNA as due to contamination with endoplasmic reticulum. Although NADH–cytochrome *c* reductase has been used

as a marker of the endoplasmic reticulum it is by no means certain that it is absent from the plasma membrane and in fact it has been shown in red cell membranes[36]. Our present data does not permit us to come to any firm conclusions about this marker except to point out that if it is a maker for endoplasmic reticulum or the exterior mitochondrial membrane, the contamination is small, being 3.2% of the total activity in the cell.

The present study is, to the best of our knowledge, the first to examine the characteristics of the phosphorylated intermediate of the $(Na^+ + K^+)$-ATPase in these cells. Many of the aspects of the enzyme are similar to those observed in other systems. However the turnover number of the ATPase at 20 and 37 °C is not as great as that observed in other tissues[21,25-27,30], 500 per min and 2000 per min respectively, based on the optimal $(Na^+ + K^+)$-ATPase at pH 8.5 and 45 phosphorylation sites per mg membrane protein. From ATP binding studies (Fig. 5), 47 pmoles ATP are bound per mg membrane protein. This correlates very well with phosphorylation data (Table II) which show that 43 ± 2 pmoles ^{32}P per mg membrane protein are incorporated in presence of Na^+. Assuming 1 mg membrane protein per 10^8 cells*, there are approximately $2 \cdot 10^5$–$3 \cdot 10^5$ phosphorylation sites per cell which is well inside the range reported in the literature[27]. At high ATP concentrations (250 μM), one observes the usual $(Na^+ + K^+)$-stimulation of ATPase acitivity, an increase by Na^+ and a decrease by K^+ of the amount of phosphoprotein labelled from [γ-^{32}P]ATP. Nearly maximal stimulation of ATP hydrolysis (and decrease in phosphoprotein label) are obtained in presence of 5 mM K^+ with 50% stimulation at 0.5 mM K^+, a value close to that reported for a number of other systems[37].

The recent demonstrations that K^+ decreases ATP binding to the membrane preparations (refs 23, 24, and this paper) help to resolve the question whether the Na^+-ATPase is a different enzyme from the $(Na^+ + K^+)$-ATPase[38]. Our data and those reported by other investigators[23,24,39] on ATP binding are consistent with a dual role for K^+, inhibiting Na^+-ATPase at concentrations of ATP near or less than the dissociation constant of the enzyme and stimulating Na^+-ATPase at concentrations of ATP in excess of the dissociation constant. Further, it may be deduced from our data that K^+ inhibits phosphorylation of the labile intermediate and that the Na^+-stimulated ATP-ase is a component of the $(Na^+ + K^+)$-ATPase. Thus at low ATP levels, Na^+ increases ATP-ase activity, an increase which is almost completely abolished by either ouabain or oligomycin. K^+, added at 0.1 mM, decreases the level of the labile intermediate in presence or absence of ouabain. Since there is little ATPase activity in presence of ouabain or oligomycin, the action of K^+ on the intermediate must be due to a reduction of phosphorylation of the intermediate and not due to an increased rate of turnover of the intermediate. If the intermediate whose level is decreased by low K^+ concentrations is unrelated to the $(Na^+ + K^+)$-ATPase, the addition of specific inhibitors of the ATPase, ouabain (or oligomycin) should not alter the level of this intermediate. The data, however, show (Figs 7+8) that with low K^+ the level of the intermediate is higher in presence of ouabain or oligomycin than in their absence. Such a result could be due to a fortuitous occurrence or to the fact

* The assumption of 1 mg membrane protein per 10^8 cells is based on a count of $4 \cdot 10^8$ cells per ml of packed cells, a 35% yield of membrane protein, and the recovery of 18 mg protein from 12 ml of packed cells.

that K^+ inhibits the formation of the labile intermediate of the $(Na^+ + K^+)$-ATPase and the inhibition of formation is overcome by the agents, ouabain or oligomycin. It is unlikely that the results obtained are fortuitous and that we are dealing with two intermediates, one whose level is decreased by K^+ and the other whose level is increased by ouabain (or oligomycin) since (1) in absence of K^+, ouabain (or oligomycin) has little or no effect on the steady state level of the intermediate (Figs 7+8), and (2) if K^+ is elevated to 1 or 5 mM, little or no increase in the steady state level of the intermediate is observed on addition of ouabain or oligomycin.

Our results are consistent with the interpretation that K^+ decreases the labelling of the intermediate at low ATP levels and that ouabain and oligomycin overcome these effects of K^+, either by antagonizing the action of K^+ on phosphorylation or decreasing still further the hydrolysis of the intermediate so that its steady state level is elevated despite a decreased rate of formation. At higher K^+ concentrations the inhibition of phosphorylation is so great that it cannot be overcome by ouabain or oligomycin. Siegel and Goodwin[40] have also provided evidence for the identity of the Na^+ and the $(Na^+ + K^+)$-ATPase.

Thus Ehrlich cell membranes possess a $(Na^+ + K^+)$-ATPase whose properties are similar to those observed in other systems except that the enzyme is particularly sensitive to inhibition by K^+. This sensitivity does not appear to be due to a decreased affinity of the enzyme for ATP, but rather an increased affinity of the enzyme for K^+.

The high inhibitory activity of K^+ in this system even in presence of excess Na^+ leads one to speculate that at low ATP levels, Na^+ pumping in these cells will cease despite an increase in cell Na^+ and a lowering of the cell K^+.

The low turnover number of the $(Na^+ + K^+)$-ATPase also correlates with the relatively high Na^+ content of these cells[41].

After this paper was submitted we learned of the work of Forte *et al.*[42] on the preparation of a plasma membrane fraction from Ehrlich ascites cells. Their method uses Ca^{2+} probably instead of Zn^{2+} to prevent rupture of nuclei. Whilst the preparations seem similar in many regards such as degree of contamination with DNA and enrichment of the $(Na^+ + K^+)$-ATPase, the yield of $(Na^+ + K^+)$-ATPase is low, about 1.5% of the total $(Na^+ + K^+)$-ATPase, whereas with our procedure 35% of the total $(Na^+ + K^+)$-ATPase is recovered and our procedure requires less time and is adaptable to handling of large volumes and hence larger quantities of membranes may be prepared.

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