

# Activation of Na<sup>+</sup> and K<sup>+</sup> Pumping Modes of (Na,K)-ATPase by an Oscillating Electric Field\*

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Serpseru and Tsong (Serpseru, E. H., and Tsong, T. Y. (1983) *J. Membr. Biol.* 74, 191–201; (1984) *J. Biol. Chem.* 259, 7155–7162) reported activation of a K<sup>+</sup> pumping mode of (Na,K)-ATPase by an oscillating electric field (20 V/cm, 1.0 kHz). Their attempts to activate Na<sup>+</sup> pumping at the same frequency were unsuccessful. We report here activation of a Na<sup>+</sup> pumping mode with an oscillating electric field of the same strength as used previously (20 V/cm) but at a much higher frequency (1.0 MHz). At 3.5 °C and the optimal amplitude and frequency, the field-induced, ouabain-sensitive (0.2 mM ouabain incubated for 30 min) Rb<sup>+</sup> influx ranged between 10 and 20 amol/red blood cell/h, and the corresponding Na<sup>+</sup> efflux ranged between 15 and 30 amol/red blood cell/h, varying with the source of the erythrocytes. No Rb<sup>+</sup> efflux nor Na<sup>+</sup> influx was stimulated by the applied field in the frequency range 1 Hz to 10 MHz. These results indicate that only those transport modes that require ATP splitting under the physiological condition were affected by the applied electric fields, although the field-stimulated Rb<sup>+</sup> influx and Na<sup>+</sup> efflux did not depend on the cellular ATP concentration in the range 5 to 800 μM. Computer simulation of a four-state enzyme electroconformationally coupled to an alternating electric field (Tsong, T. Y., and Astumian, R. D. (1986) *Bioelectrochem. Bioenerg.* 15, 457–476; Tsong, T. Y. (1990) *Annu. Rev. Biophys. Biophys. Chem.* 19, 83–106) reproduced the main features of the above results.

(Na,K)-ATPase plays an important role in the regulation of cellular Na<sup>+</sup> and K<sup>+</sup> concentrations and is one of the key enzymes responsible for maintaining the osmotic balance of cells and transmission of action potentials (1–6). The human erythrocyte maintains its intracellular sodium and potassium ion composition relatively constant even against large gradients of these ions across the membrane because of the activity of this enzyme. The exchange of Na<sup>+</sup> and K<sup>+</sup> through the red cell membrane has several pathways, but, at physiological concentrations and membrane potential, only the extrusion of Na<sup>+</sup> and influx of K<sup>+</sup> catalyzed by (Na,K)-ATPase represent active transport (7–11). The energy required is provided by the hydrolysis of ATP. Under a variety of conditions, it has been found that for each ATP consumed 3 Na<sup>+</sup> are extruded from the cytoplasm concomitantly with the

accumulation of 2 K<sup>+</sup> (12, 13). Because of this unbalanced charge translocation the net reaction catalyzed by the enzyme should be electrogenic. The catalytic cycle of the enzyme is specifically inhibited by ouabain (14–16).

Data from different laboratories indicate that varying the activity of the (Na,K)-ATPase can change the membrane potential of cells or vesicles (17, 18, 29). However, various uncoupled transport modes at rates very much smaller than the maximal velocity have been demonstrated by Karlisch and Stein (19).

Previously it was reported that the K<sup>+</sup> pumping mode of the (Na,K)-ATPase of human erythrocytes was influenced by external electric fields. An oscillating electric field of 20 V/cm at 1.0 kHz activated the K<sup>+</sup> pumping mode without consumption of ATP (20, 21). No activity of a Na<sup>+</sup> pumping mode was stimulated by similar applied fields. The soundness of some of these results, however, was in question because of the large standard deviation. Since most membrane integral proteins of cells are constantly exposed to electric fields of 20 to 500 kV/cm (reflecting a transmembrane electric potential, Δψ, of 10 to 250 mV), either from the cellular metabolism or from external sources, it was essential to investigate further the electrical responses of the (Na,K)-ATPase. We report here the activation of the Na<sup>+</sup> pumping mode and the determination of the electric parameters for stimulation of (Na,K)-ATPase.

## EXPERIMENTAL PROCEDURES

**Materials**—<sup>22</sup>Na and <sup>86</sup>Rb were obtained from Amersham Corp. Ouabain and other chemicals were from Sigma and were of the highest quality available. Vanadate was from Aldrich. Liquiscint was supplied by Yellow Springs Instruments. Fresh blood samples were obtained from healthy young adults by venipuncture.

**Experimental Set-up and Electric Stimulation**—The device for the voltage stimulation has been described (20). Basically, it consists of a cylindrical Plexiglas chamber with adjustable volume of 150-μl capacity. The chamber is connected to a Heath Zenith SG-1271 Function Generator which can generate an alternating (a.c.) field of various wave forms between 1 Hz and 1.0 MHz. For an a.c. frequency higher than 1.0 MHz, a Wavetek 20 MHz AM/FM/PM Generator Model 148A was used. This instrument can generate an a.c. field of various wave forms between 0.01 and 20 MHz. The voltage and frequency are monitored by the differential amplifier unit (7A22) of a Tektronix 7704A oscilloscope. At the two sides of the chamber are platinum electrodes supported by brass holders. The brass holders are hollow and cold water can circulate through to control the

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<sup>1</sup> The abbreviations used are: Δψ, transmembrane electrical potential; RBC, red blood cell, in this case, of human; a.c., alternating electric field; Δψ<sub>m</sub>, maximal transmembrane electrical potential; S, an erythrocyte sample stimulated with an electric field; NS, a control erythrocyte sample not stimulated by an electric field; OS, an erythrocyte sample treated with ouabain and stimulated with an electric field in the presence of ouabain; ONS, a control erythrocyte sample treated with ouabain but without stimulation by an electric field; Ω, ohm.

temperature of the cell suspension. In order to suppress the ATP hydrolysis-linked transport activity, experiments were done at 2 °C. Within the range of electric fields used, temperature elevation due to currents was less than 1.5 °C. Control samples were kept at 3.5 °C for correction.

In all experiments freshly drawn human blood, in the presence of heparin, was centrifuged for 10 min at 4 °C and the buffy coat and plasma were discarded. Red cells were then washed at 1000 × *g* three times with 5 volumes of a cold medium containing 150 mM NaCl, 5 mM KCl, and 27 mM sucrose in 10 mM Tris/HCl buffer at pH 7.4 for 5 min each time. The Na<sup>+</sup> and the K<sup>+</sup> contents of an erythrocyte sample were determined with a Corning Model 450 flame photometer. When loading of Rb<sup>+</sup> or Na<sup>+</sup> was required, radioactive tracer of the ion was added to the incubation medium. The concentration determined by the flame photometry and the radioactivity of the ion were then used to calculate ion fluxes.

**Ion Influx Experiments**—The washed cells were incubated in an isotonic solution containing 12.5 mM NaCl, 1 mM MgCl<sub>2</sub>, 243 mM sucrose in Tris/HCl buffer at pH 7.4 for Rb<sup>+</sup> influx, or containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, and 27 mM sucrose in 10 mM Tris/HCl at pH 7.4 for Na<sup>+</sup> influx, with or without incubation in 0.2 mM ouabain for 30 min, at room temperature. After incubation, stock solutions were added to give final ion concentrations of 12.5 mM RbCl, 2.5 mM NaCl, 243 mM sucrose, 1 mM MgCl<sub>2</sub>, and 10 mM Tris at pH 7.4 at a hematocrit of 10–15% with 20–25 cpm/pmol of <sup>86</sup>Rb for Rb influx, or 150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 27 mM sucrose, and 10 mM Tris at pH 7.4 at a hematocrit of 10–15% with 8–12 cpm/pmol of <sup>22</sup>Na for Na<sup>+</sup> influx. 20-μl aliquots were taken at zero time; 150 μl of suspension was placed into the chamber, which was stimulated at the desired voltage and frequency at the indicated temperature. Another part of suspension was kept under the same condition except that there was no voltage stimulation. In some experiments control samples were also kept in the electric stimulation chamber (in contact with the platinum electrodes) without subjecting them to electric stimulation. This exposure to the platinum electrodes did not have detectable effects. At the end of stimulation, two 30-μl aliquots for each kind of sample, stimulated and nonstimulated, were withdrawn and the cells were washed three times with 0.5 ml of the same ice-cold medium without radioactive tracer. Then the washed cells were dissolved in 1 N NaOH, bleached with H<sub>2</sub>O<sub>2</sub> containing 10% ascorbic acid, and neutralized with 1 N HCl. 0.3 ml of suspension was mixed with 5 ml of Liquescent and counted in a Packard scintillation counter for <sup>86</sup>Rb influx. The washed cells were counted directly in a Packard Auto-gamma Scintillation Spectrometer 5266 for <sup>22</sup>Na influx. The hematocrit of each sample was determined and was usually 10–15%.

The results are expressed as attomoles/red blood cell (RBC)/h or ions/pump/s (attomole = 10<sup>-18</sup> mol), by assuming 93 μm<sup>3</sup> cell volume, 200 (Na,K)-ATPase molecules per RBC, and also that 50% of the packed cell volume was intracellular space. The quantity of each type of ion transported per cell in 1 h was always less than 1% of the quantity of that type of ion present inside the cell at the beginning of the experiment. Changes in extracellular concentrations were negligible.

**Ion Efflux Experiments**—Before ion efflux experiments were performed, radioactive tracer was loaded into the cells. Ion loading was done by passive diffusion. The washed cells were suspended in 10 mM NaCl, 15 mM KCl, 1 mM MgCl<sub>2</sub>, 200 mM sucrose in 10 mM Tris/HCl buffer at pH 7.4 at a hematocrit of 30–40% with 8–12 cpm/pmol of <sup>22</sup>Na<sup>+</sup> for Na<sup>+</sup> efflux, or 140 mM RbCl, 10 mM NaCl, 1 mM MgCl<sub>2</sub>, and 10 mM Tris/HCl at pH 7.4 at a hematocrit of 30–40% with 20–25 cpm/pmol of <sup>86</sup>Rb for Rb<sup>+</sup> efflux, for overnight at 4 °C. After loading, cells were washed three times with cold isotonic buffer of the same composition as mentioned above for each kind of influx without a radioactive tracer. A second wash was done immediately after the first but a third wash was done 1 h after the second wash. This procedure eliminated nonspecific binding of <sup>22</sup>Na<sup>+</sup> to the membrane and improved the accuracy of the experiments. Then the cells were suspended to a hematocrit of 8–12% in the stimulating medium, which contained 150 mM NaCl, 5 mM KCl, 2 mM MgCl<sub>2</sub>, 27 mM sucrose in 10 mM Tris/HCl with or without 0.2 mM ouabain at pH 7.4 for Na<sup>+</sup> efflux, or 2.5 mM NaCl, 12.5 mM RbCl, 2 mM MgCl<sub>2</sub>, and 243 mM sucrose in 10 mM Tris/HCl at pH 7.4 with or without 0.2 mM ouabain at a hematocrit of 8–12% for Rb<sup>+</sup> efflux. The cell suspension was incubated for 30 min at room temperature before the stimulation. 20-μl aliquots were withdrawn before 150 μl of suspension was placed into the chamber. <sup>86</sup>Rb<sup>+</sup> or <sup>22</sup>Na<sup>+</sup> efflux was stimulated by an a.c. electric field of 20 V/cm at 1.0 kHz for the K<sup>+</sup> pumping mode

or at 1.0 MHz for the Na<sup>+</sup> pumping mode for 60 min at 3 °C. The controlled sample was kept at 3.5 °C under the same conditions except for voltage stimulation. Two 30-μl aliquots taken from both the stimulated and the nonstimulated samples at the end of stimulation were washed with 0.5 ml of cold nonradioactive medium. 0.3 ml from the supernatant of the first wash was taken and counted directly for Na<sup>+</sup> efflux. For Rb<sup>+</sup> efflux 0.3 ml of the supernatant was mixed with 5 ml of Liquescent and counted as mentioned under "Ion Influx Experiments." Hematocrits were checked for each sample.

**Depletion of Cytoplasmic ATP**—Fresh red blood cells were suspended, at 8% hematocrit, in an isotonic solution of 10 mM NaCl, 25 mM sodium arsenate, 200 mM sucrose, 10 mM Tris/HCl at pH 7.4 and were incubated at 37 °C for 1 h. Thereafter, the cells were washed three times in a solution of 10 mM NaCl, 250 mM sucrose, 10 mM Tris/HCl, pH 7.4. To determine the ATP content, 300 μl of distilled water was added to a 100-μl red cell suspension to hemolyze the red cells. 50 μl of 50% trichloroacetic acid was then added. The ghosts were removed by a high speed centrifugation, and the supernatant was adjusted to pH 7.4 with NaOH before the determination of ATP concentration.

The two methods listed were used. In general they gave consistent results.

**1) Luciferin/Luciferase Assay for ATP Content**—The method of Streher (22) was used to prepare luciferin/luciferase assay solution. Briefly, 50 mg of vacuum-dried firefly lanterns (from Sigma) was extracted, with grinding, at 0 °C with 5 ml of 0.1 M sodium arsenate, pH 7.4, for 2–5 min. The suspension was filtered into a test tube kept in an ice bath, and 50 mg of magnesium sulfate was then added and thoroughly mixed. After the magnesium sulfate addition, the suspension was transferred into another tube, which was wrapped with Saran Film. 100 μl of the freshly prepared luciferin/luciferase mixture was added to 800 μl of 0.1 M Tris/HCl buffer, pH 7.4, in a cuvette, and the luminescence was determined with a LKB Wallac 1250 Luminometer. A standard curve was made with known concentrations of ATP.

**2) Fluorometric Assay for ATP Content**—D-Glucose, ATP, and NADP<sup>+</sup> are converted to ADP, NADPH, and 6-phosphogluconate in the presence of glucose-6-phosphate dehydrogenase and hexokinase. The increase in the fluorescence of NADPH can be used to assay ATP concentration, as described by Williamson and Corkey (23). A cuvette was filled with 2 ml of assay buffer containing 50 mM triethanolamine-HCl, 10 mM MgCl<sub>2</sub>, 5 mM EDTA, at pH 7.4. To this 10 μl (1 M) of glucose, 10 μl (10 mg/ml) of NADP<sup>+</sup> and 5 μl (0.2 mg/ml) of glucose-6-phosphate dehydrogenase were added and mixed thoroughly. After 1–2 min, 5 μl (2 mg/ml) of hexokinase was added. The fluorescence level was recorded. 10 μl of an ATP standard or unknown sample was then added, and the fluorescence increase was measured. The ATP concentration of a sample was read from a calibration curve obtained with ATP standards. The excitation wavelength was 307 nm and the emission wavelength was 452 nm, using an Aminco-Bowman spectrofluorometer.

## RESULTS

**Ouabain-sensitive, Alternating Electric Field-stimulated Rb<sup>+</sup> Influx and Na<sup>+</sup> Efflux**—When red cells in an isotonic suspension were exposed to an oscillating electric field, depending on frequency there was an increased Rb<sup>+</sup> influx or Na<sup>+</sup> efflux with respect to a control sample which was kept at the same temperature without electric stimulation. Table I gives the results of such experiments, using an a.c. field of 20 V/cm at two frequencies: 1.0 kHz and 1.0 MHz. These two frequencies were chosen to make a direct comparison because they were optimum for activating the Rb<sup>+</sup> (K<sup>+</sup>) pumping mode and the Na<sup>+</sup> pumping mode, respectively. The samples were kept at 2 °C. When the electric field was applied, the temperature of the suspension was only slightly elevated, but in no case did the sample temperature at its steady state reach higher than 3.5 °C. Thus, control samples were kept at 3.5 °C. In Table I, Rb<sup>+</sup> influx of a.c. stimulated samples was 23.5 amol/RBC/h compared with that of samples pretreated with 0.2 mM ouabain, which was 11.1 amol/RBC/h (OS). This value was identical to that of the control samples including the nonstimulated (NS) and the ouabain-treated-nonstimulated samples (ONS). The increase of Rb<sup>+</sup> influx was also inhibited by 0.1

TABLE I  
Electric field-stimulated pump transport of Rb<sup>+</sup> and Na<sup>+</sup> at 3.5 °C

For measurements of ion concentration by flame photometry and ion movement using radioactive tracers see "Experimental Procedures." Each value is the mean of 3–5 measurements. Standard deviation is given in parentheses. 1 amol = 1 attomole =  $1 \times 10^{-18}$  mol. 1 amol/RBC/h = 0.0108 mmol/liter cells/h. Values varied for erythrocyte samples from different individuals. Data given in this table were obtained from blood samples of a single individual. With samples from different individuals, Rb<sup>+</sup> influx values were in the range 10–20 amol/RBC/h and Na<sup>+</sup> efflux values were in the range 15–30 amol/RBC/h.

	Cellular ion conc			Medium ion conc				Measured ion movement						
	Na	K	Rb	Na	K	Rb	Mg	NS	S	ONS	OS	NS-ONS	S-OS	S-NS
	<i>mM</i>			<i>mM</i>				<i>amol/RBC/h</i>						
20 V/cm a.c., 1.0 kHz														
Rb influx	6	75	27	2.5	0	12.5	2	13.0 (0.3)	23.5 (1.2)	10.1 (0.6)	11.1 (0.15)	2.9 (0.6)	12.4 (1.2)	10.5 (1.2)
Rb efflux	6	65	15	2.5	0	12.5	2	42.1 (1.7)	43.4 (1.1)	41.7 (1.5)	41.6 (1.5)	0.4 (1.7)	1.8 (1.5)	1.3 (1.7)
Na influx <sup>a</sup>	6	75	0	150	5	0	2	3.2 ( $<0.1$ )	3.54 (0.2)	4.0 (0.1)	6.2 (0.3)	−0.8 (0.1)	−2.7 (0.2)	0.4 (0.2)
Na efflux <sup>a</sup>	6	75	0	150	5	0	2	4.3 (2.0)	6.2 (0.6)	1.7 (0.1)	1.9 (0.8)	−1.9 (2.0)	4.3 (0.6)	1.9 (2.0)
20 V/cm a.c., 1.0 MHz														
Rb influx	6	75	27	2.5	0	12.5	2	10.6 (3.8)	10.4 (3.5)	8.8 (1.8)	8.9 (1.6)	2.1 (3.8)	1.5 (3.5)	−0.5 (3.5)
Rb efflux	6	65	15	2.5	0	12.5	2	38.3 (2.0)	37.7 (1.0)	40.4 (0.3)	39.5 (1.1)	−2.1 (2.0)	−1.8 (1.0)	−0.6 (2.0)
Na influx <sup>a</sup>	6	75	0	150	5	0	2	6.1 (0.6)	6.9 (0.9)	6.6 (0.3)	6.9 ( $<0.1$ )	−0.5 (0.6)	0.0 (0.9)	0.8 (0.9)
Na efflux <sup>a</sup>	6	75	0	150	5	0	2	4.0 (2.7)	20.8 (3.2)	2.0 (0.1)	5.3 (1.8)	2.0 (2.7)	15.5 (3.2)	16.8 (3.2)

<sup>a</sup> In Na<sup>+</sup> influx and efflux experiments, Rb<sup>+</sup> was not added because our intention was to demonstrate the active pumping of Na<sup>+</sup>. K<sup>+</sup> was present on both sides of the membrane.

mM vanadate (data not shown). In contrast, neither Rb<sup>+</sup> efflux, Na<sup>+</sup> influx, nor Na<sup>+</sup> efflux was stimulated by a 1.0-kHz a.c. field. In the Rb<sup>+</sup> influx experiments, the red cells were preloaded with 27 mM Rb<sup>+</sup> (cytoplasmic K<sup>+</sup> 75 mM) and the external medium contained only 12.5 mM Rb<sup>+</sup>. Yet the applied a.c. field stimulated the influx rather than the efflux of Rb<sup>+</sup>, and this stimulated activity was inhibited by an inhibitor of the (Na,K)-ATPase or ouabain (Table I).

Stimulation of Na<sup>+</sup> efflux required a much higher a.c. frequency, namely 1.0 MHz. For the experiments reported in Table I, Na<sup>+</sup> efflux under the influence of such a field was significantly increased to 20.8 amol/RBC/h from 5.3 amol/RBC/h in ouabain-treated samples. There was no increase of Na<sup>+</sup> influx, Rb<sup>+</sup> influx, or Rb<sup>+</sup> efflux. Again, this a.c. stimulated activity was blocked by pretreating samples with 0.1 mM vanadate. The Rb<sup>+</sup> influx values of inhibitor-treated samples are, within experimental uncertainty, identical to those of NS and ONS samples. The results confirm that the applied a.c. field activated the (Na,K)-ATPase of the red cells, and only transport in the direction normally driven by splitting ATP was affected.

Varying with the individual from whom the red cells were taken, the a.c. stimulated activity under the optimal conditions was in the range 10–20 amol/RBC/h for Rb<sup>+</sup> influx and 15–30 amol/RBC/h for Na<sup>+</sup> efflux. However, the ratio of Rb<sup>+</sup>/Na<sup>+</sup> = 2:3 was not strictly maintained for red cells from single individuals. For example, in one case the net stimulated activity using optimal fields was 12 amol/RBC/h for the Rb<sup>+</sup> flux and was 31 amol/RBC/h for the Na<sup>+</sup> flux (Figs. 1 and 2).

**Optimal Electric Field Strength for Stimulation of (Na,K)-ATPase**—Previous studies of Serspersu and Tsong (20, 21) have shown that there was an optimum field strength of 20 V/cm (peak-to-peak) when a 1.0-kHz a.c. field was used for stimulating the Rb<sup>+</sup> (K<sup>+</sup>) pumping activity of the enzyme. Our result confirms their observation (Fig. 1B). The same

behavior was found for the Na<sup>+</sup> pumping mode. Data shown in Fig. 1A were obtained using a.c. fields of 1.0 MHz at different field strengths. Maximum stimulation of Na<sup>+</sup> efflux occurred at an electric field of 20 V/cm, at 3.5 °C. In this set of experiments, the rate of Na<sup>+</sup> efflux in the stimulated sample was 53.5 amol/RBC/h. In the control samples, including NS, OS, and ONS, the passive Na<sup>+</sup> effluxes of these samples were approximately 24.1 amol/RBC/h. The net voltage-stimulated Na<sup>+</sup> efflux was approximately 30 amol/RBC/h. Above or below the electric field strength of 20 V/cm, the stimulated, ouabain-sensitive Na<sup>+</sup> efflux was reduced.

**Frequency Dependence**—Another rather intriguing observation of Serspersu and Tsong (20) was that there was also an optimum frequency for the a.c. activation of the Rb<sup>+</sup> (K<sup>+</sup>) pumping mode. Their results showed that when 20 V/cm a.c. fields were used, the optimum frequency for Rb<sup>+</sup> influx was 1.0 kHz. However, a theoretical analysis by Blank (24) based on a surface compartmental model predicted that 200 Hz would be the optimum. His calculations made use of values of Rb<sup>+</sup> permeability available in the literature. The results of Serspersu and Tsong (20, 21) do not include data points at this frequency. In order to check whether 200 Hz is the real maximum, we have repeated the experiment to include more data points. The result shown in Fig. 2B confirms the observation of Serspersu and Tsong (20) that the optimum frequency for activating the Rb<sup>+</sup> pump using a 20 V/cm a.c. field is approximately 1.0 kHz. Data in Fig. 2B suggest that the frequency dependence of the Rb<sup>+</sup> pump is not symmetrical, and there may be another peak around 50 kHz.

A more striking result of our experiment is the finding of an optimum frequency for activating the Na<sup>+</sup> pumping mode at 1.0 MHz. For several years we have been unable to activate the Na<sup>+</sup> pumping mode because our search was limited to frequency range 1 to 10 kHz. The result of an experiment is shown in Fig. 2A. At 10 kHz, the Na<sup>+</sup> pumping mode activity



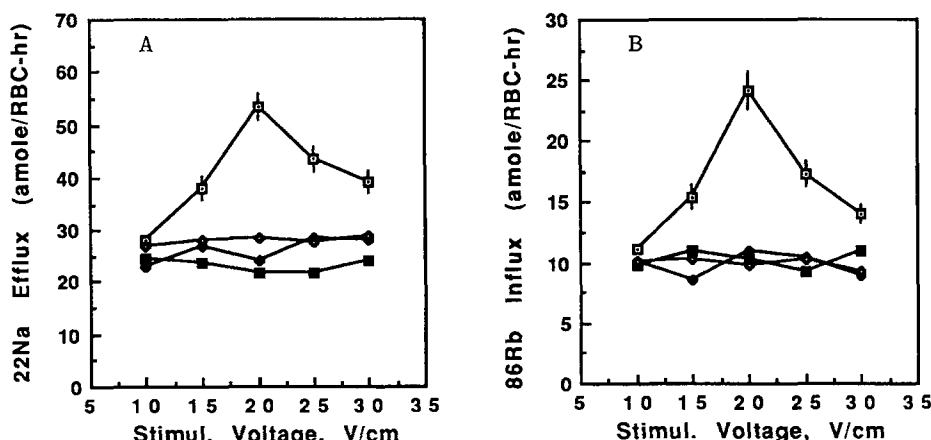
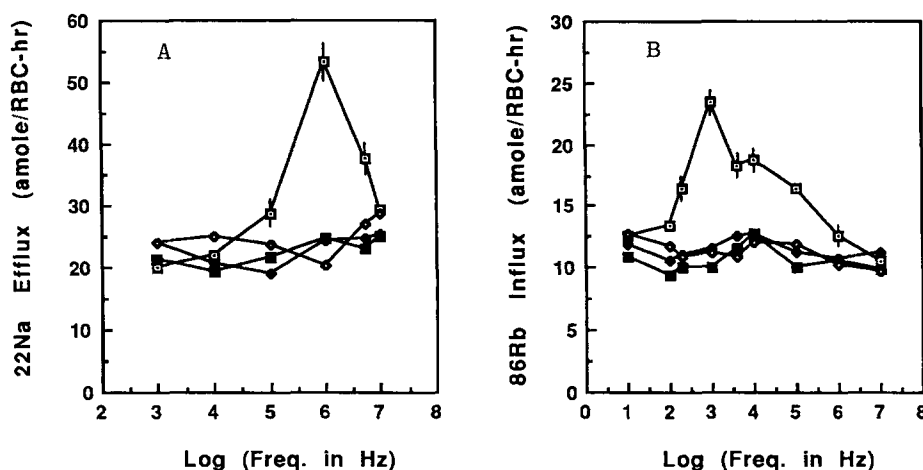


FIG. 1. Amplitude dependence of electric field-stimulated  $\text{Na}^+$  pumping and  $\text{Rb}^+$  pumping. A,  $\text{Na}^+$  efflux. Red blood cells were treated with an a.c. electric field at 1.0 MHz, with different field strengths for 60 min at 3 °C. 20- $\mu\text{l}$  aliquots at the beginning and 30- $\mu\text{l}$  aliquots at the end of stimulation were drawn for radioactivity assay of  $^{22}\text{Na}^+$  content. Two other aliquots from each sample were also drawn for determining hematocrit index. Symbols:  $\blacklozenge$ , NS;  $\square$ , S;  $\blacksquare$ , ONS; and  $\diamond$ , OS. 1 amol/RBC/h = 0.0108 mmol/liter of cells/h. Each point is a mean of 3–6 determinations. Error bars are given for data points with standard deviations larger than the symbols. B,  $\text{Rb}^+$  influx. Experimental procedures and symbols used are the same as those in A, except that ionic compositions of the cytoplasmic and external media were different. These compositions are given in Table I and under "Experimental Procedures."

FIG. 2. Frequency dependence of the  $\text{Rb}^+$  pumping and  $\text{Na}^+$  pumping activities using the optimal a.c. field of 20 V/cm. A,  $\text{Na}^+$  efflux stimulated by an a.c. field of 20 V/cm was measured at different frequencies. Symbols:  $\blacklozenge$ , NS;  $\square$ , S;  $\blacksquare$ , ONS; and  $\diamond$ , OS denote samples as described in Fig. 1A. Other details are the same as Fig. 1. B,  $\text{Rb}^+$  influx stimulated by an a.c. field of 20 V/cm was measured for NS ( $\blacklozenge$ ), S ( $\square$ ), ONS ( $\blacksquare$ ), and OS ( $\diamond$ ) samples, as was described by Serpersu and Tsong (20, 21). More data points were obtained to ensure that the optimum frequency indeed occurred at 1 kHz. A shoulder is seen around 10 kHz.



was hardly discernible (Fig. 2A), whereas at 1.0 MHz, the maximum net stimulated  $\text{Na}^+$  efflux was 29.3 amol/RBC/h. No  $\text{Rb}^+$  influx,  $\text{Rb}^+$  efflux, nor  $\text{Na}^+$  influx was detected under these experimental conditions.

**Lack of Dependence on Cytoplasmic ATP**—At 3.5 °C, there is little ATP hydrolysis activity (3). It would appear that the observed effect could not be attributed to voltage stimulation of ATP hydrolysis. To ascertain that this indeed was the case, we have used ATP-depleted red cells to perform the electric stimulation experiment. The result on the  $\text{Na}^+$  pumping mode is shown in Table II to be compared with samples in which ATP was at a normal level. For the fresh red blood cells, the ATP level varied between 600 and 800  $\mu\text{M}$ , and the net stimulated activity (S – OS) was 19.8 amol/RBC/h. In ATP-depleted samples ( $[\text{ATP}] < 15 \mu\text{M}$ ), the value was 15.1 amol/RBC/h. The slight difference is probably due to experimental variation rather than to a reduced level of stimulated activity. We have found that it is difficult to reduce the ATP level of red blood cells below 10  $\mu\text{M}$  without severely degrading the state of the red cells. It remains uncertain whether or not the 10  $\mu\text{M}$  of ATP is essential for the activation of the enzyme by the electric field. In Table II, data obtained at 26 °C are also shown. At this temperature, the ouabain-sensitive activity

was 8.0 amol/RBC/h (NS – ONS) in the ATP-containing cells and 2.8 amol/RBC/h in the ATP-depleted cells. The a.c. stimulated  $\text{Na}^+$  efflux was 29.6 amol/RBC/h (S – OS) for the ATP-containing cells and 27.9 amol/RBC/h for the ATP-depleted cells. Serpersu and Tsong (20, 21) reported that the electric field-stimulated  $\text{Rb}^+$  or  $\text{K}^+$  pumping activity was not sensitive to cellular ATP level between 10  $\mu\text{M}$  and 1 mM.

**Efficiency of Electric Fields with Different Waveforms**—Our previous analysis based on the concept of "Enforced Enzyme Oscillations by Electroconformational Coupling" (see below) predicted that an electric field of square waveform would have a higher efficiency than the sinusoidal waveform for driving an uphill transport reaction (25, 26). To test this prediction we have performed the electric field activation of  $\text{Na}^+$  pumping mode using a square waveform of 20 V/cm. With a square waveform electric field (20 V/cm at 1.0 MHz), the net stimulated activity was 32.2 amol/RBC/h. In this case, the sinusoidal a.c. field gave only 29.3 amol/RBC/h. The square waveform appears to be approximately 10% more efficient (25).

**Other Properties**—In order to examine whether or not the erythrocytes were damaged after exposure to the electric field, we have compared the extent of hemolysis of the stimulated samples (20 V/cm at different frequency) with that of non-

TABLE II

Effect of cytoplasmic ATP on voltage activation of Na<sup>+</sup> pumping mode

Na<sup>+</sup> efflux was measured. The cytoplasmic concentration of Na<sup>+</sup> was 6 mM and of K<sup>+</sup> was 75 mM. The medium ion concentration was 150 mM for Na<sup>+</sup>, 5 mM for K<sup>+</sup>, and 2 mM for Mg<sup>2+</sup>. Data are from blood samples of one individual.

Sample	Temp	[ATP]	NS	S	ONS	OS	NS-ONS	S-ONS	S-NS	S-OS
	°C	μM								
Fresh RBC	3.5	600-800	18.5 (2.2)	36.5 (1.2)	15.7 (2.1)	16.7 (1.8)	2.8 (2.1)	20.8 (2.1)	18.0 (2.2)	19.8 (1.8)
	26	600-800	24.4 (2.8)	44.8 (1.7)	16.4 (2.8)	15.2 (2.3)	8.0 (2.8)	28.5 (2.8)	20.5 (2.8)	29.6 (2.8)
	3.5	5-15	14.4 (0.2)	30.4 (3.0)	13.3 (2.8)	15.4 (1.3)	1.2 (2.8)	17.2 (3.0)	16.0 (3.0)	15.1 (3.0)
ATP-depleted RBC	26	5-15	20.2 (2.2)	43.3 (3.0)	17.4 (1.6)	15.4 (2.8)	2.8 (2.2)	25.9 (3.0)	23.1 (3.0)	27.9 (3.0)

stimulated samples. The extent of red cell hemolysis, measured by hemoglobin release, was similar for the electric field-stimulated samples and control samples and in all cases was below 1%.

It was reported that vanadate can inhibit the activity of (Na,K)-ATPase through the block of the phosphorylation cycle (27, 28). Erythrocytes were incubated with 0.1 mM vanadate for 30 min at room temperature and then exposed to voltage stimulation of 20 V/cm at 1.0 MHz, at 3.5 °C, as described above. The field-stimulated Na<sup>+</sup> efflux was  $0.47 \pm 1.8$  amol/RBC/h (three determinations). Likewise, with a 1-kHz field, the stimulated Rb<sup>+</sup> influx was  $1.5 \pm 0.91$  amol/RBC/h (three determinations). Thus, vanadate completely inhibited the a.c. stimulated Na<sup>+</sup> and Rb<sup>+</sup> pumping activities. The results of Serpersu and Tsong (21) show that vanadate only partially inhibited the electric field-induced Rb<sup>+</sup> influx. The discrepancy is attributed to the insufficient reaction time with vanadate in the previous publication.

## DISCUSSION

**Electric Field-induced Transmembrane Potential**—Many authors have derived mathematical expressions, starting from the Maxwell relation, to calculate transmembrane potential induced by an electric field (see *e.g.* Refs. 26, 29 and references cited therein). Let us consider a simplified case in which a spherical shell, with an inner radius of  $a$  and an outer radius of  $b$ , is exposed to an electric field  $E$ . If the conductivities of the internal medium and the external medium are equal and much higher than that of the shell material and if the thickness of the shell is much smaller than  $a$  or  $b$ , then the maximal transmembrane potential generated by an electric field of  $E$ ,  $\Delta\psi_m$ , is,<sup>2</sup>

$$\Delta\psi_m = 1.5 a E \quad (1)$$

Equation 1 has been shown experimentally to be applicable to cells (30, 31) and lipid vesicles (32, 33). The shape of a human erythrocyte is biconcave and is far from spherical. Miller and Henriquez (34) have applied the three-dimensional finite element solution with the boundary conditions of Neumann and Rosenheck (29) to compute  $\Delta\psi_m$  for an erythrocyte and found that  $\Delta\psi_m$  induced across an erythrocyte membrane is 22% less than that calculated for a sphere. For a field of 20 V/cm (peak-to-peak),  $\Delta\psi_m$  across an erythrocyte membrane would be 10 mV. The maximal electric field across the lipid bilayer is thus 10 mV/5 nm or 20 kV/cm.

In our experiments oscillating electric fields were used. When the cycle time of an a.c. field is comparable with the

$RC_m$  constant ( $R$  being the resistance of the suspending medium and  $C_m$  being the capacitance of an erythrocyte), the magnitude of the transmembrane potential induced by the electric field is reduced and can no longer be calculated according to Equation 1. In such a case, the Schwann Equation should apply (35).

$$\Delta\psi_m = 1.5 a E / [1 + (\omega \tau)^2]^{1/2} \quad (2)$$

where  $\omega$  is the angular velocity of the a.c. field and,

$$\tau = a C_m (r_i + r_e / 2) \quad (3)$$

$r_i$  and  $r_e$  being the specific resistance of the cytoplasmic fluid and the external medium, respectively. We have used  $a = 5$  μm,  $C_m = 1$  microfarad/cm<sup>2</sup>, and  $r_i = r_e = 100$  Ωcm to obtain a  $\tau$  of 0.075 μs. The value of  $\omega \tau$  is 0.47 for a 1 MHz field and 4.7 for a 10 MHz field. The optimal transmembrane electric field for Rb<sup>+</sup> pumping was 20 kV/cm and for Na<sup>+</sup> pumping was 18 kV/cm after this correction. Within experimental uncertainty, the optimal field strengths for both pumping modes are identical.

**Features of the Electric Activation**—The present study shows that both Na<sup>+</sup> and K<sup>+</sup> pumping modes of the (Na,K)-ATPase can respond to electric fields and that the two modes can function independently of each other: when a 1.0 kHz a.c. field was used K<sup>+</sup> pumping mode was activated without the activity of the Na<sup>+</sup> pumping mode, and when a 1.0 MHz a.c. field was used the Na<sup>+</sup> pumping mode was activated without the activity of the K<sup>+</sup> pumping mode. Although the uncoupled Na<sup>+</sup> transport mode has been known to occur under certain conditions (6), *in vivo* the two modes are generally coupled.

Another surprising observation is the wide separation of the optimum frequencies for the two pumping modes. Although the optimum field strength for the two modes is identical, *i.e.* at 20 V/cm, the optimum frequency differs by 3 decades. Presumably, the optimum amplitude reflects the extent of the electrically induced conformational change or the energy factor and the optimum frequencies reflect the relaxation process or the kinetic factor (26, 36, 37). Analysis presented later supports this contention. The faster response of the Na<sup>+</sup> mode than that of the K<sup>+</sup> mode may reflect a difference in the basic design of the two cation binding sites.

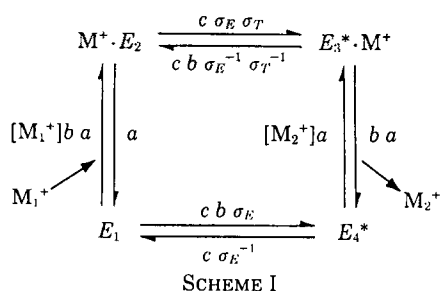
The next question we asked is why there is such a wide discrepancy between the measured rate of ion translocation and the optimum frequency. At 3.5 °C, the rate of Rb<sup>+</sup> transport is 15–25 ions/s/pump whereas the optimum frequency is 1.0 kHz, nearly 50-fold greater than one would expect. Likewise, the rate of the Na<sup>+</sup> transport is only 15–30 ions/s/pump whereas the optimum frequency is 1 MHz, nearly 3000-fold greater than one would expect. The number of ions pumped per cycle/red cell is approximately 0.003 for Na<sup>+</sup> and 2.1 for

<sup>2</sup> A typographic error is found in the paper of Teissie and Tsong (32). In the equation of Neumann and Rosenheck (29) shown in page 1550, the two 9s in the denominators should read 9 λ.

Rb<sup>+</sup> when we assume that each red cell contains 200 molecules of (Na,K)-ATPase. Because an applied field-induced transmembrane potential is not uniformly distributed, not every enzyme molecule is expected to be activated. By an integration of the transmembrane potential over a spherical surface one would predict that the fraction of enzyme that can be activated is one-third, or approximately 70 molecules. The observed values of 0.003 and 2.1 are much smaller than 70. However, these results can be reproduced by kinetic analysis of the four-state membrane transport model discussed below. The analysis indicates that each cycle of stimulation translocates less than a stoichiometric quantity of ligand.

Whether any of the stimulated transports was a net transport against the electrochemical potential defined by the Nernst equation remains to be investigated. It is, however, clear that of the four transport modes studied, only the two that require splitting of ATP under the physiological conditions, *i.e.* Na<sup>+</sup> efflux and Rb<sup>+</sup> (or K<sup>+</sup>) influx, were stimulated by the a.c. fields. The contention that the applied a.c. field only stimulated one-for-one ion exchange is not supported by the experimental data. As shown in Tables I and II, there was no stimulation of back fluxes of either Rb<sup>+</sup> or Na<sup>+</sup>; and, since the optimum frequency for activating the two pumps was different by 3 decades, the applied a.c. field did not stimulate a one-to-one exchange of the two ions.

**Enforced Enzyme Oscillations by Electroconformational Coupling**—To understand how an enzyme can be driven by an oscillating electric field, we have previously proposed that the conformation of a membrane integral enzyme with net charges or rich in helix dipoles will undergo a conformational transition under the influence of an electric field (35–37). An electric field favors enzyme states with higher molar electric moments than states with lower molar electric moments. An oscillating electric field can cause such an enzyme to oscillate among its various conformational states; and, if this enforced conformational oscillation is coupled to ligand binding reactions, the enzyme can efficiently utilize the electric energy for pumping a substrate or an ion against its concentration gradient (35–38). We have used this concept to interpret the results of Figs. 1 and 2 using the four-state model shown below.



In the scheme, *E* represents the enzyme and M<sup>+</sup> represents either Rb<sup>+</sup> or Na<sup>+</sup>. The numerical subscripts refer to four states of the enzyme *E*. The rate coefficients are given in terms of three parameters, *a*, *b*, and *c*, which are characteristic of the enzyme and independent of the electric field. The *c* is a scaling factor which sets the time scale for the conformational transition steps, the *a* sets the time scale for the association/dissociation steps, and *b* is the zero field equilibrium constant between the states M<sup>+</sup>·*E*<sub>2</sub> and *E*<sub>3</sub>\*·M<sup>+</sup> and between the states *E*<sub>1</sub> and *E*<sub>4</sub>\*. *b* is also the association constant of M<sub>1</sub><sup>+</sup> and 1/*b* is the association constant of M<sub>2</sub><sup>+</sup>. The electric field dependence of the conformational transitions is given by  $\sigma_E = \exp(x\alpha)$ . The *x* is the effective number of charges moved across the membrane in the conformational

transition of the protein and is known as the displacement charge. The field dependence due to the electrogenicity of the transport is given by  $\sigma_T = \exp(z_S\alpha)$ . The *z<sub>s</sub>* is the charge on the substrate. In both cases, the  $\alpha$  is the potential difference between the two surfaces of the membrane multiplied by *e*/(2*kT*). Here, *e* is the charge of an electron, *k* is the Boltzmann constant, and *T* is the Kelvin temperature. The factor 2 was introduced in order to divide the effect of the electric field equally between the forward and the backward rate constants. Notice that the field dependencies are apportioned equally between the forward and reverse transitions as the simplest case. Flux is clockwise provided that *b* > 1; it is counterclockwise if *b* < 1. Also for simplicity, we have selected the parameters to display symmetry on both sides of the membrane when the field is 0. In general, the kinetic behavior of the enzyme in Scheme I can be simulated by writing down the four differential equations, one for each enzyme state, and solving the system numerically.

$$\frac{d[E_1]}{dt} = -(b a [M_1]^+ + c b \sigma_E) [E_1] + a [M^+ E_2] + c \sigma_E^{-1} [E_4^*] \quad (4)$$

$$\frac{d[M^+ E_2]}{dt} = -(c \sigma_E \sigma_T + a) [M^+ E_2] + c b \sigma_E^{-1} \sigma_T^{-1} [E_3^* M^+] + b a [M_1^+ E_1] \quad (5)$$

$$\frac{d[E_3^* M^+]}{dt} = -(b a + c b \sigma_E^{-1} \sigma_T^{-1}) [E_3^* M^+] + c \sigma_E \sigma_T [M^+ E_2] + a [M_2^+ E_4^*] \quad (6)$$

$$\frac{d[E_4^*]}{dt} = -(a [M_2^+] + c \sigma_E^{-1}) [E_4^*] + c b \sigma_E [E_1] + b a [E_3^* M^+] \quad (7)$$

Rather than solving nonlinear differential equations and calculating the change in concentration of M<sub>1</sub><sup>+</sup> and M<sub>2</sub><sup>+</sup> as a function of time, as was done by Tsong and Astumian (26, 36), it is convenient to keep the concentrations of M<sub>1</sub><sup>+</sup> and M<sub>2</sub><sup>+</sup> constant and to evaluate the distribution of the enzyme in each state as a function of time by solving Equations 4–7. These distributions can then be used to calculate the instantaneous flux through each transition. At steady state where each of the time derivatives in Equations 4–7 is 0 and the magnitudes of the fluxes through each transition are equal, the flux around the circle is clockwise when [M<sub>1</sub><sup>+</sup>] > [M<sub>2</sub><sup>+</sup>], counterclockwise when [M<sub>2</sub><sup>+</sup>] > [M<sub>1</sub><sup>+</sup>], and 0 when [M<sub>1</sub><sup>+</sup>] = [M<sub>2</sub><sup>+</sup>]. The protein behaves as a facilitated diffusion transport system, or a Michaelis-Menten type of enzyme embedded in a cell membrane (39). This is true regardless of the value of the steady state membrane potential  $\psi_0$ . When an a.c. field  $\psi = \psi_0 + \psi_1 \cos \omega t$  is applied, the situation becomes quite different. When [M<sub>2</sub><sup>+</sup>] = [M<sub>1</sub><sup>+</sup>], the a.c. field causes the enzyme state probabilities to oscillate (Fig. 3A), and also causes net clockwise flux to occur (Fig. 3B). The stimulated cyclic flux of the substrate for Scheme I is dependent on the frequency of the oscillating field. In Fig. 4 two sets of kinetic parameters were used to stimulate the a.c. induced Na<sup>+</sup> and Rb<sup>+</sup> pumping by the (Na,K)-ATPase. The main factor determining the frequency optimum is the time scale for the association/dissociation reaction.

In these simulations, we have taken into account a few experimental observations. First, to comply with the results of Bahinsky *et al.* (40) that transport of two K<sup>+</sup> is non-electrogenic, we assumed that the *E*<sub>1</sub> to *E*<sub>4</sub>\* transition involves a compensatory movement of two negative charges. Second, we assumed that transport of 2 Rb<sup>+</sup> occurred in concert, *i.e.* in a single step (41). Likewise, transport of 3 Na<sup>+</sup> also occurred in a single step. Third, the utilization of the four-state scheme required that binding of ligand subsequent to the association of the first ligand is fully cooperative. And finally, no account

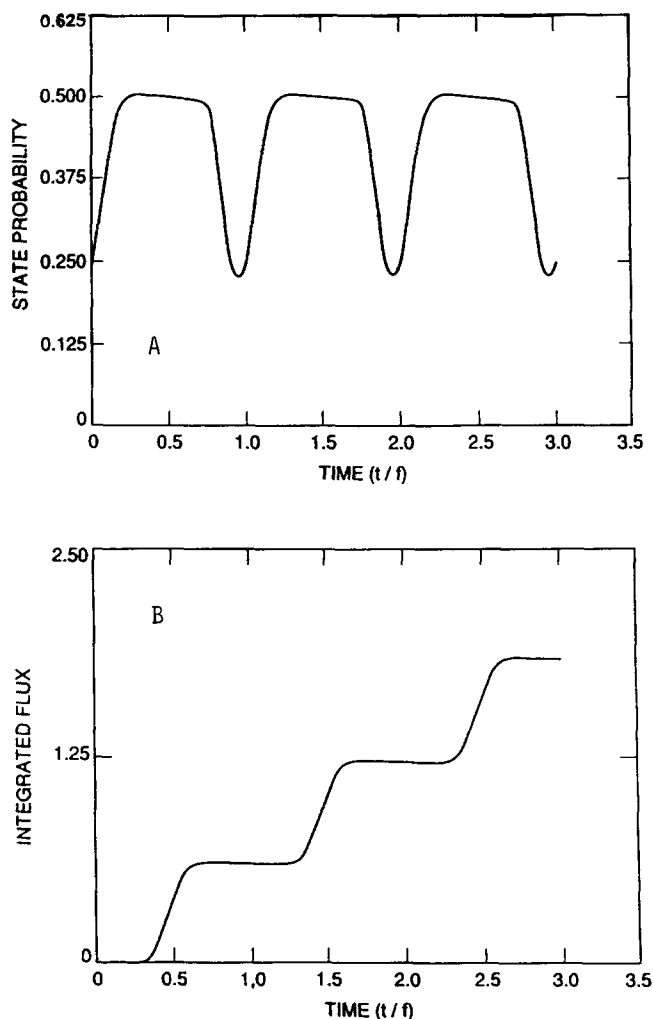


FIG. 3. Enforced electroconformational oscillations of enzyme states. Computer analysis was done to demonstrate an a.c. induced conformational oscillation and the subsequent clockwise pumping of substrate, using the kinetic Scheme I shown in the text with these parameter values:  $b = 10$ ,  $a = 1000 \text{ s}^{-1}$ ,  $c = 1000 \text{ s}^{-1}$ ,  $z_s = 0$ , and  $x\psi = -1$ . See text for the meaning of the parameters. A, state probability (minimum of 0 and maximum of 1) of  $M^+E_2$  as a function of the a.c. cycle. B, net clockwise flux of  $M^+$  from  $M_1^+$  to  $M_2^+$  induced by the a.c. field. Integrated flux is given as a function of the a.c. cycle.

of the effect of phosphorylation of the enzyme is made. We propose that the interaction that allows free energy coupling between ATP hydrolysis and ion pumping may well be coulombic. The phosphorylation is known to play a role in linking the two functions of  $K^+$  and  $Na^+$  pumping *in vivo* (1–6, 42). Amplitude optimum can also be explained by considering the effect of induced dipoles. This term depends on the square of an electric field and always has a positive sign (36). At some field intensity the induced dipole term exceeds the permanent dipole term, and the net effect is to lock the enzyme into certain states. The efficiency of field stimulation decreases beyond that point.

**Conclusion**—The opening/closing of many channel proteins is known to depend on the transmembrane electric field (43–45). However, electroactivation of enzymes has not been studied in detail. Besides the (Na,K)-ATPase, mitochondrial, chloroplast, and thermophilic bacterial ATPases have been shown previously to utilize energy from applied electric fields for synthesizing ATP (46–49). The concept of the electroconformational coupling has also been used to interpret these results (36). One should recognize that if an enzyme can

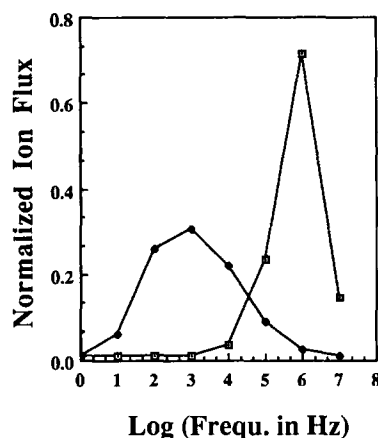


FIG. 4. Computer simulation of the frequency dependence of the  $Na^+$  and the  $Rb^+$  pumpings based on the model of the electroconformational coupling. Scheme I, shown in the text, was used to simulate the frequency dependence of the electric field induced net  $Na^+$  efflux (Fig. 2A) (□) and net  $Rb^+$  influx (Fig. 2B) (◆), based on the concept of electroconformational coupling. Relative rate of ion flux is plotted against logarithm of the frequency. The bias factor,  $b$ , which measures the relative affinity of  $E_1$  and  $E_4^*$  for  $M^+$ , was 500. The flank factor,  $a$ , which measures the relative rate (no unit), was 1 for  $Rb^+$  influx and 1000 for  $Na^+$  efflux, and  $c$  was 1 (also relative rate) for both  $Rb^+$  and  $Na^+$  pumping modes. The charge displacement of protein,  $x$ , was  $-2$  for both pumping modes.  $z_s$  was 2 for  $Rb^+$  and 3 for  $Na^+$  pumping modes and  $\psi = 20 \cos \omega t$ , in V/cm, where  $\omega = 2\pi f$ ,  $f$  being frequency of the a.c. field. See text for details.

capture energy from an applied electric field, it should also respond to an endogenous field of similar intensity and waveform. Thus, studies of the electric activation of these enzymes should contribute to our understanding of the action of these enzymes, *in vivo*. The concept of enzyme oscillators has also been considered for (Na,K)-ATPase by Post (50).

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