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Electrogenic and Electroneutral Transport Modes of Renal Na/K ATPase Reconstituted into Proteoliposomes

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Summary. This paper describes measurements of electrical potentials generated by renal Na/K-ATPase reconstituted into proteoliposomes, utilizing the anionic dye, oxonol VI. Calibration of absorption changes with imposed diffusion potentials allows estimation of absolute values of electrogenic potentials.

ATP-dependent Na_{cyt}/K_{exc} exchange in K-loaded vesicles generates large potentials, up to 250 mV. By comparing initial rates or steady-state potentials with ATP-dependent ^{22}Na fluxes in different conditions, it is possible to infer whether coupling ratios are constant or variable. For concentrations of Na_{cyt} (2–50 mM) and ATP (1–1000 μM) and pH's (6.5–8.5), the classical $3Na_{cyt}/2K_{exc}$ coupling ratio is maintained. However, at low Na_{cyt} concentrations (<0.8 mM), the coupling ratio is apparently less than $3Na_{cyt}/2K_{exc}$.

ATP-dependent $Na_{cyt}/congener_{exc}$ exchange in vesicles loaded with Rb, Cs, Li and Na is electrogenic. In this mode congeners, including Na_{exc}, act as K_{exc} surrogates in an electrogenic $3Na_{cyt}/2congener_{exc}$ exchange. (ATP+Pi)-dependent K_{cyt}/K_{exc} exchange in K-loaded vesicles is electroneutral.

ATP-dependent "uncoupled" Na flux into Na- and K-free vesicles is electroneutral at pH 6.5–7.0 but becomes progressively electrogenic as the pH is raised to 8.5. The ^{22}Na flux shows no anion specificity. We propose that "uncoupled" Na flux is an electroneutral $3Na_{cyt}/3H_{exc}$ exchange at pH 6.5–7.0 but at higher pH's the coupling ratio changes progressively, reaching 3Na/no ions at pH 8.5. Slow passive pump-mediated net K uptake into Na- and K-free vesicles is electroneutral, and may also involve K_{cyt}/H_{exc} exchange.

We propose the general hypothesis that coupling ratios are fixed when cation transport sites are saturated, but at low concentrations of transported cations, e.g., Na_{cyt} in Na/K exchange and H_{exc} in "uncoupled" Na flux, coupling ratios may change.

Key Words Na/K-pump · electrogenicity · reconstituted vesicles · variable coupling

Introduction

It is now well accepted that the Na/K pump in physiological conditions is electrogenic (Thomas, 1972; Gadsby, 1984; Glynn, 1984). This is the consequence of the unequal stoichiometry of 3Na ions extruded for 2K ions taken up per cycle. In red cells, the "classical" 3:2 stoichiometry was inferred on the basis of flux and ATP hydrolysis measurements (Post & Jolly, 1957). Electrogenicity has been demonstrated directly using a potential-sensitive carbocyanine dye (Hoffman, Kaplan & Callahan, 1979). In other cells such as nerve, muscle and oocytes, the most convincing studies have used a combination of Na flux and current measurements to show that the pump-mediated current equals about $\frac{1}{3}$ of the active Na flux (Lederer, 1980; Thomas, 1984; De Weer, Rakowski & Gadsby, 1987; Eisner, Valdeolmillos & Wray, 1987; De Weer, Gadsby & Rakowski, 1988). In the excitable cells, reports of different coupling ratios, have appeared from time to time, but when interfering fluxes unrelated to the pump are taken into account, the coupling ratio is considered in hindsight to be 3Na per 2K. Thus the consensus of opinion is that the coupling ratio is 3Na:2K per cycle and is fixed (*see* De Weer et al., 1988). However, studies on electrogenicity using whole cells are restricted by the low potentials generated by the pump (typically 1–10 mV), due to the low resistance of the membranes (nerve, muscle) or by the low density of the pumps (red cells).

In view of these limitations, we decided to look at the electrogenic properties of the pump in phospholipid vesicles reconstituted with pig kidney Na/K-ATPase (Karlish & Pick, 1981). These vesicles show a number of advantages for such studies including a very low passive permeability to cations, control of sidedness of ligand effects, the possibility of probing the cytoplasmic surface of inside-oriented pumps and the simplicity of interpretation of effects due to the presence of only the one transport system. It was expected that active Na/K exchange would generate large electrogenic potentials in the vesicles and thereby allow a more detailed examination of Na:K coupling ratios than is possible with whole cells. The transport properties of the vesicle system have now been described in detail. These include a coupling ratio of 3Na/2K for active Na/K exchange, abnormal flux modes characterized previously in red cells, including two types of

Na/Na exchange, K/K exchange, "uncoupled" Na flux, and pump reversal (for reviews *see* Karlish & Stein, 1985; Karlish, 1988*b*) and additional modes such as slow passive K fluxes in the absence of other pump ligands (Karlish & Stein, 1982*b*).

An interesting finding with red cells is that the so-called "uncoupled" Na flux into a Na- and K-free medium, in which ATP is hydrolyzed and 2 or 3 Na ions are extruded per cycle (Glynn & Karlish, 1976), is electroneutral (Dissing & Hoffman, 1983). Observation of a ouabain-sensitive sulphate efflux from red cells treated with DIDS the inhibitor of the anion exchange system, led Dissing and Hoffman (1983) to propose that, in Na- and K-free media, the pump mediates an electroneutral cotransport of Na with anions (presumably 3Na:3Cl). It was clearly of interest to look at electrogenicity in the reconstituted vesicles when the pumps are carrying out "uncoupled" Na and other abnormal flux modes.

The tool chosen for the present experiments was the anionic potential-sensitive dye oxonol VI (*see* Bashford & Smith, 1978; Waggoner, 1985). Oxonol dyes have been used extensively to look at electrical potentials in liposomes (Bashford et al., 1979), sub-mitochondrial particles (Smith & Chance, 1979), chloroplasts (Schuurmans, Casey & Kraayenhof, 1978; Admon, Shahak & Avron, 1982) and phospholipid vesicles reconstituted with cation pumps including, in this laboratory, the F_1-F_0 ATPase (Navarro & Essig, 1984; Shahak, Admon & Avron, 1982). This dye is thought to diffuse into vesicles in response to inside-positive potentials and undergo a shift in absorption spectrum upon binding to the interior membrane (Bashford et al., 1979). Measurement of absorption changes using a dual wavelength spectrophotometer provides sensitive detection of the expected potentials.

This paper describes first the use of the oxonol dye to measure electrogenic potentials generated by Na/K exchange and then examines the potentials generated by the pump in abnormal flux modes, exploiting the high sensitivity of the measurement. Preliminary reports of this work have appeared (Goldshleger et al., 1987*b*; Karlish, 1988*a*). See also Apell and Bersch (1987) for another recent study of electrogenic potentials using oxonol VI. Earlier work of Dixon and Hokin (1980) documented electrogenicity in reconstituted vesicles.

Materials and Methods

Preparation of Na/K-ATPase and Reconstituted Vesicles

Na/K-ATPase was prepared from fresh pig kidney red outer medulla by the rapid procedure described by Jorgensen (1974). Protein by the method of Lowry and ATPase activity were determined as in Jorgensen (1974). Specific activities were in the range 13–20 U/mg protein. Before use, the enzyme was dialyzed at 4°C against 1000 volumes of a solution containing histidine, 25 mM, pH 7.0; EDTA(Tris), 1 mM.

The enzyme was reconstituted into phospholipid vesicles essentially as described (Karlish & Pick, 1981; Karlish & Stein, 1985; Karlish, 1988*b*). For preparation of the phospholipid a suspension of soybean lecithin (~60 mg/ml) in Tris · HCl, 500 mM; histidine, 25 mM, pH 7.0 was sonicated and dialyzed for 4 hr at room temperature against 200–300 volumes of histidine, 25 mM, pH 7.0, subjected to a cycle of freeze-thaw-sonication, and then dialyzed overnight at 4°C against 500 volumes of histidine, 25 mM, pH 7.0; EDTA, 1 mM. Small dilutions of the phospholipid during these procedures were taken into account by comparing absorption (light scattering) of the suspensions at 420 nm as an approximate measure of concentration. The room temperature dialysis was an effective way of removing an unexpected endogenous K-conductance (*see* Results) and contaminating K.

The reconstitution procedure was, in brief, as follows: dialyzed enzyme was mixed with recrystallized cholate(Tris) (at 6 mg cholate per mg protein), and added to a suspension of the treated phospholipid (at 40 mg phospholipid per mg protein) containing the salts to be incorporated within the vesicles. The suspension was frozen rapidly in liquid nitrogen, allowed to thaw slowly at room temperature and was sonicated briefly with a Bransonic 12 sonicator until it was partially cleared. Unless stated otherwise vesicles contained histidine, 25 mM, pH 7.0; EDTA(Tris), 1 mM, and the chloride salt of K,Na,Rb,Cs,NH4, choline or Tris, at 300 mM, as described in the figure legends. After reconstitution, vesicles were centrifuged twice at low speed (1200 rpm, or $\approx 900 \times g$ for 4 min) in an International centrifuge, on short columns of Sephadex G-50 equilibrated with ice-cold Tris · HCl, 300 mM; histidine, 25 mM, pH 7.0 (*see also* Penefsky, 1977).

Measurement of Potentials

Measurements were made using an Aminco DW-2a dual wavelength spectrophotometer, with wavelength settings of 628–590 nm and a slit width of 2 nm. Absorption changes were usually monitored on a recorder. Spectra were recorded in the split beam mode and the data were stored and analyzed using an Apple II computer interfaced with the spectrophotometer. The sample cell was thermostated and fitted with a magnetic stirrer. A stock solution in dry ethanol of oxonol VI (1–2 mM) was prepared, divided into aliquots, and stored at −20°C. A fresh aliquot of the dye was used for each experiment.

For each measurement of a potential, vesicles (20–150 μl containing ~0.5–3.75 mg lipid) were suspended in the appropriate reaction medium (total volume 2 ml), undergoing continuous stirring in the sample cell at 20°C, and a small volume 2–5 μl of the oxonol VI was added to give final concentrations 0.2–2 μM dye. The absorption was monitored and when the signal had become stable following equilibration of the dye with the lipid (1–5 min), a small volume of solution, containing ATP(Tris) or another ligand to initiate the reaction of interest, was added, and the time course of the absorption change was recorded. The initial rate and steady-state level of the absorption change (e.g., as in Fig. 2) were measured and either used as such for comparison with other conditions or were converted to absolute units of voltage as described below.

Membrane resistance was estimated as follows (*see* Apell & Bersch, 1987). When the oxonol signal had reached a steady level (*see* Fig. 2), KCl, 5 mM and then vanadate(Tris), 0.5 mM were added to rapidly block the pump, and the exponential dissi-

pation of the signal was recorded. The rate constants $k(\text{sec}^{-1})$ of the exponential decay curves were obtained graphically, and the resistance R_m ($\Omega \cdot \text{cm}^2$) was calculated as $-1/(k \cdot C_m)$, where C_m ($\sim 1\ \mu\text{F} \cdot \text{cm}^{-2}$) is the membrane capacitance.

To convert absorption changes to units of voltage (mV) the former were calibrated with imposed diffusion potentials, using pH gradients plus a protonophore FCCP or Li gradients plus a specific Li ionophore AS701 (Shanzer, Samuel & Korenstein, 1983). By the first method FCCP, 5 μM was first added to the suspension of vesicles with dye. Then aliquots of 1 M sulfuric acid (3–20 μl) were added and transient absorption changes were observed with a rise time within the mixing time of the system (about 1 s). Control samples without vesicles were also run to take into account effects of the acidity on the absorption of the dye itself. The difference in peak height of the signal with and without vesicles was taken to be a measure of the voltage change. The pH's of the suspensions before and after addition of the acid were measured. This method was useful for pH's above 5.5; at lower pH's the corrections for effects of pH on the dye's absorption were too large and became unreliable. As an alternative method, vesicles were suspended in media containing LiCl, 1–50 mM plus Tris · HCl to a total concentration of 300 mM, histidine, 25 mM, pH 7.0, and then AS701, 10 μM, was added and the peak height of the absorption change was recorded. With concentrations of ionophore up to 20-fold higher the height of the signal was the same, but it was maintained for a shorter time, due presumably to a more rapid dissipation of the Li gradient. This method was used with K- or Tris-loaded vesicles, but not with Na-loaded vesicles, for the AS701 has significant conductance to Na (Shanzer et al., 1983).

Measurement of Isotope Fluxes

Active and passive fluxes were measured at room temperature 20–24°C (*see* Karlish & Pick, 1981; Karlish & Stein, 1985; Karlish, 1988*b*), in the specific experimental conditions given in the figure legends. In brief, vesicles were warmed to room temperature and 40–100 μl aliquots were mixed in duplicate with volumes of the appropriate reaction mixtures containing ^{22}Na, with or without ATP(Tris) to activate, or ^{86}Rb, with or without vanadate(Tris) to inhibit, the pump-mediated fluxes. After periods of time within the linear phase of the different reactions, isotope uptake was stopped either by transfer of the suspensions to ice-cold columns of Dowex-50 X8 50–100 mesh (Tris-form) or by addition first of 100 μl of an ice-cold solution containing NaCl, 300 mM; histidine, 25 mM, pH 7.0, and then transfer to the Dowex columns. The vesicles with trapped isotope were immediately eluted into counting vials with 1 ml of an ice-cold solution of sucrose, 300 mM. Total isotope and the column background were measured in each experiment. ^{22}Na was measured by scintillation counting and ^{86}Rb by its Cerenkov radiation. ATP-activated or vanadate-inhibited fluxes were estimated from the difference in isotope uptake with or without the ATP or vanadate, respectively. Initial rates of fluxes were calculated as nmol of Na or Rb uptake/10 μl vesicles/min. In accordance with our previous practice we refer to cations bound to cytoplasmic or extracellular sites on the pump by the subscripts cyt or exc, respectively.

Calculations

Linear and nonlinear regression analysis was performed using an IBM-compatible personal computer and the program Enzfitter (Elsevier-BIOSOFT).

Materials

Oxonol VI was obtained from Molecular Probes. ^{22}Na and ^{86}Rb were obtained from Amersham International. Dowex 50 X8 50–100 mesh and choline chloride were from Merck. Before use, the Dowex was converted to the Tris form, and the choline chloride was recrystallized from hot ethanol. The Li ionophore AS701, was a gift from Dr. A. Shanzer of the Weizmann Institute. Sephadex G-50 (fine) was obtained from Pharmacia. Vanadate-free ATP and ADP were from Boehringer. Creatine phosphate, creatine phosphokinase and soyabean phospholipid were from Sigma. ATP, ADP, creatine phosphate and ortho vanadate were converted to the Tris salts by passage over columns of Dowex-50 (Tris). All other materials were of analytical grade.

Abbreviations

DIDS,4′,4-diisithiocyano-2,2′-stilbene disulfonic acid; AS701, N,N′-diheptyl-N,N′-diethylether, 5,5-dimethyl-3,7-dioxanone; and FCCP, *p*-trifluoromethyoxycarbonyl cyanide phenylhydrazone.

Results

When ATP is added to a medium containing Na plus Mg and vesicles loaded with K, it is expected that the ATP-dependent Na/K exchange will generate a potential positive inside, (Fig. 1, top left), causing the anionic oxonol (Fig. 1, top right) to accumulate within the vesicle. Figure 1 (bottom) shows a typical absorption spectra of the dye before and after addition of ATP to the medium containing vesicles, the spectrum with ATP being recorded in the steady-state of the absorption change (*see* Fig. 2). The red shift in the absorption spectrum with a minimum at 590 nm and maximum at 628 nm in the difference spectrum is typical for oxonol when accumulated in vesicles (Bashford et al., 1979). All subsequent measurements were made using the wavelengths 628 minus 590 nm, i.e., the pair giving the maximal signal change.

Figure 2 shows a typical time course of the oxonol signal when ATP is added to the reaction medium. To verify the presumed mechanism, a number of control experiments were performed. For example, induction of the signal required the simultaneous presence of Na plus Mg and then ATP. It did not occur in reconstituted vesicles preincubated with K plus vanadate, or in vesicles reconstituted with enzyme pretreated with ouabain (but addition of ouabain to reconstituted vesicles did not block the response). No signal was observed in plain lipid vesicles. These observations confirm that we are dealing with a phenomenon sustained by active Na/K-pumps in an inside-out orientation.

As discussed below, the initial change in voltage reflects the rate of charging of the membrane capacitance, while the steady-state level of the volt-

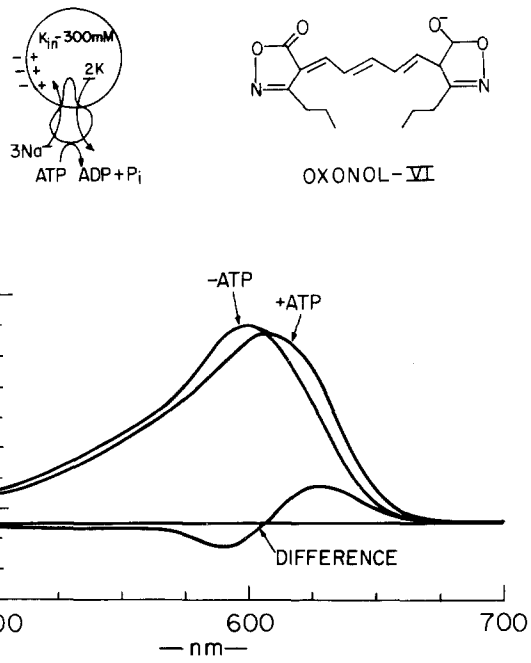

Fig. 1. Absorption spectra of oxonol VI in vesicles reconstituted with renal Na/K-ATPase. Vesicles were loaded with KCl, 300 mM and suspended in a medium containing Tris · HCl, 300 mM; histidine, 25 mM, pH 7.0. Vesicles (50 μl) were suspended in 2 ml of a medium containing Tris · HCl, 250 mM; NaCl, 50 mM; $MgCl_2$, 3 mM; histidine, 25 mM, pH 7.0, and oxonol VI, 2 μM. The absorption spectrum was recorded between 500 and 700 nm at 2 nm per sec. ATP(Tris), 3 mM, was then added and after 30 sec the spectrum was recorded again

Table 1. Calculated resistances of reconstituted vesicles

Internal ion	pH	Resistance $\Omega \cdot cm^2 \cdot 10^7$
K	7.0	6.14 ± 0.54 (n = 4)
	8.5	4.72 ± 0.3 (n = 3)
Tris	8.5	4.86 ± 1.22 (n = 3)
Na	7.0	2.86 ± 0.17 (n = 4)

K-, Na- and Tris · HCl-loaded vesicles were prepared and suspended in the standard Tris · HCl/histidine solution. The resistance of K vesicles was measured under the conditions of Fig. 2 with pH of the medium adjusted to 7.0 or 8.5, that of the Na vesicles under conditions of Fig. 9, and that of the Tris · HCl vesicles under conditions of Fig. 10, with the pH 8.5 medium. In each case KCl, 5 mM was added in the steady state and then vanadate, 0.5 mM was added. In the case of the Tris vesicles, addition of KCl caused a small rise in the absorption; vanadate was added only when this was complete. From the exponential traces of absorption decrease, plots were made of log (amplitude$_{t=0}$–amplitude$_{t=t}$) versus time, and rate constants were calculated by linear regression.

age is achieved when the inward pump current equals the outward passive leakage current via the membrane resistance. In the absence of additions other than ATP the steady-state signal was maintained for a certain period and then declined slowly, as the level of K in the vesicles fell.

The resistance of the vesicle membrane was estimated from the time constant ($R_m \cdot C_m$) of passive dissipation of the voltage, when the pump current was turned off suddenly in the steady-state by adding K ions and then vanadate (Fig. 2). Assuming C_m equals 1 $\mu F/cm^2$, R_m could be estimated. Table 1 collects together measurements from vesicles containing K, Tris or Na ions. The resistances so estimated are high, the highest value found, $6.14 \cdot 10^7$ $\Omega \cdot cm^2$ (in K-containing vesicles at pH 7.0) being consistent with that expected for the soybean lipid membrane. The calculated resistance in the experiments of Apell and Bersch (1987) was five-to-10-fold higher than found here, due probably to the use of a lower temperature (11°C) and pure lipids. The resistance could be reduced deliberately by adding ionophores, such as valinomycin or FCCP. When these were added prior to ATP, no signal was seen and when added in the steady state the signal collapsed immediately.

For quantitation of the size of potentials, and comparison of oxonol absorption changes in different conditions or vesicles, it was essential to ensure that the responses were in the linear range with respect to lipid and dye concentration. Figure 3 shows a titration of the absorption change at a fixed dye concentration (2 μM) and varying volumes of vesicles (lipid ~25 mg/ml). The amplitude is saturated at approximately 50 μl of vesicle per cuvette (0.4 mg/ml), indicating that all the dye has been accumulated. Any attempt to estimate the size of the potential would obviously underestimate it in this condition. With a fixed amount of lipid (0.3 mg/ml) the amplitude of the response was proportional to dye concentration up to about 1.0 μM, increased further up to 2 μM while slightly less than linear, and actually decreased at 10 μM (*not shown*). The lack of linearity and even inhibition at increasing dye concentrations could be due to short circuiting of the potential by the lipid-soluble dye itself. In experiments where small potentials were to be measured (*see* Figs. 6, 9 and 10) the sensitivity of the measurement was increased by raising the volume of vesicles and concentration of dye, while maintaining the ratio of lipid to dye.

The calibrations in Fig. 4 were performed using ion specific ionophores, AS701 (Li) and FCCP (H) and inwardly oriented gradients of Li or H, respectively. Transient diffusion potentials were thus imposed across the reconstituted vesicle membranes and the peak dye response was recorded as de-

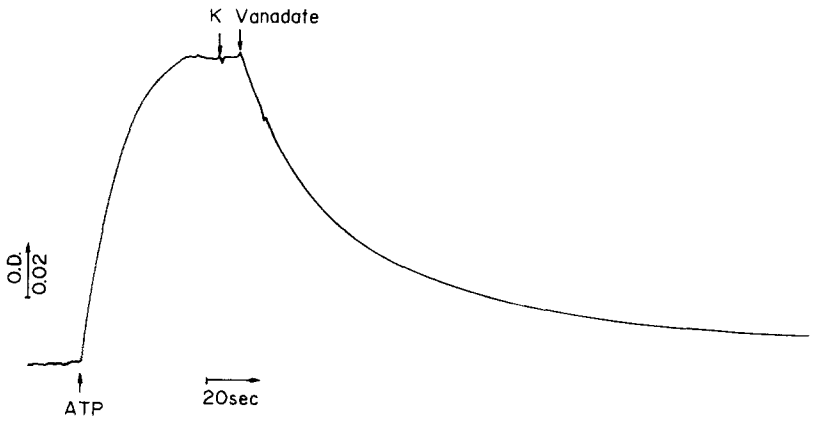

Fig. 2. Time-course of oxonol absorption change accompanying active Na/K exchange. Vesicles (30 μl) prepared as in Fig. 1 were suspended in the same medium as in Fig. 1. ATP(Tris), 500 μM was added, followed by KCl, 5 mM and vanadate(Tris), 2 mM, in the steady state, as indicated. Full scale absorption was 0.2 optical density units

scribed in Methods. The upper and lower pair of lines represent calibrations with FCCP and AS701 with two different lipid plus dye combinations used in the experiments in Table 2 (*see below*). The identical responses observed by the two procedures, at the same lipid-to-dye ratios, support the assumption that the peak height of the absorption change upon changing pH_{out} or Li_{out} is a reliable measure of the voltage change, and furthermore that the H or Li gradients do not dissipate appreciably during the measurement. The oxonol absorption is linearly related to the voltage and for each pH or Li_{out}, the change in absorption, ΔA, is proportional to the change in the Nernst potential, $\Delta V = -R \cdot T/F \cdot \ln X_{in}/X_{out}$, (where X refers to H or Li, respectively) with a slope of 58 mV per decade of concentration at 20°C. Notice also that calibrations were the same for KCl- or Tris · HCl-loaded vesicles. Appreciable oxonol signals were observed upon addition of acid even in the absence of FCCP, suggesting that the soybean lipid is selectively permeable to protons even without the added protonophore.

Table 2 presents values of electrogenic potentials for different types of vesicles and conditions. The maximal potentials generated by active Na/K exchange were very large, 209–281 mV as we have reported (Goldshleger et al., 1987*b*; Karlish, 1988*a*). Apell and Bersch (1987) have reported potentials of a similar size. Since these estimates are based on smaller calibrating potentials (0–~120 mV, *see* Fig. 4) there might seem to be uncertainty that the oxonol signal amplitude is proportional to voltage at the very high values. However, experiments showing proportionality of the signal amplitude and the pump-mediated Na flux over a wide range of values (*see* for example Table 3) suggest that in fact the potential and signal amplitude are linearly related even at high potentials. The large size of the electrogenic potentials must be the consequence of the very high resistance of the vesicle membrane (Table 1), relative to the pump current. The high sensitivity of the measurements permits detection of small potentials (Table 2).

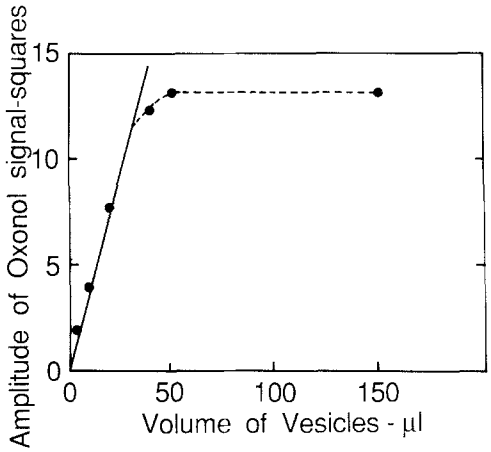

Fig. 3. Oxonol absorption change at different vesicle to dye ratios. Vesicles were prepared and suspended in the same reaction medium as in Fig. 1. The amplitude of the ATP-induced oxonol signal was measured with 5–150 μl vesicles and a fixed oxonol VI concentration of 2 μM

ATP-Dependent Na/K Exchange

In this section we describe experiments to measure initial rates and steady-state amplitudes of oxonol signals compared with the rates of active ATP-dependent ^{22}Na uptake into K-loaded vesicles. The initial rate of the voltage change should be proportional to the pump current, or active sodium flux multiplied by $\frac{1}{3}$ assuming the classical 3Na : 2K coupling ratio. The steady-state voltage should be proportional to the pump current at that voltage (*see* Discussion). Comparison of oxonol signals with active fluxes in different conditions, therefore, provides evidence as to the constancy or otherwise of

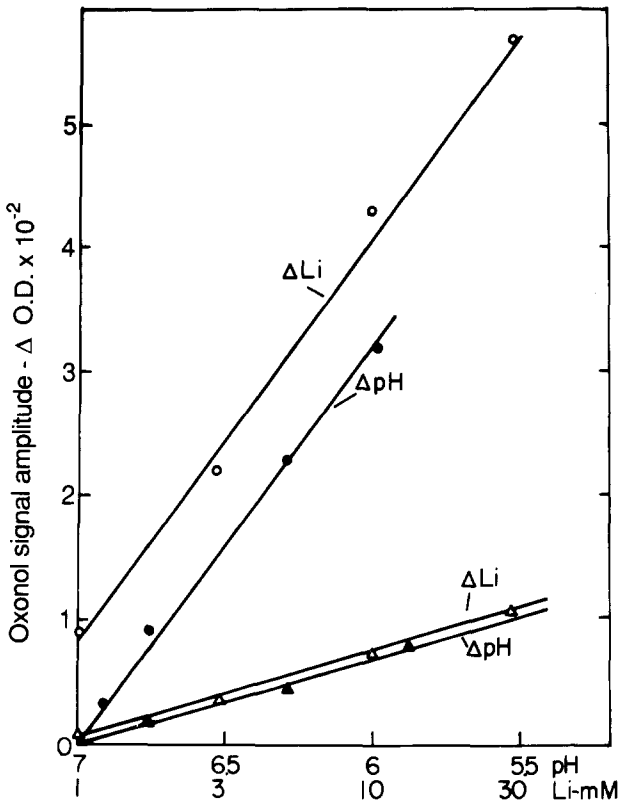

Fig. 4. Calibration of oxonol absorption changes with diffusion potentials. Vesicles were prepared to contain KCl, 300 mM or Tris · HCl, 300 mM. KCl-loaded vesicles were suspended in the reaction medium of Fig. 1 at (*i*) 150 μl vesicles and 2 μM dye, ▲ or (*ii*) 30 μl vesicles and 0.25 μM dye, ●. FCCP, 5 μM was added and then the appropriate aliquot of acid to each sample. The Tris · HCl-loaded vesicles were suspended in reaction media containing Tris · HCl + LiCl at a total concentration of 300 mM; histidine, 25 mM, pH 7.0 at (*iii*) 150 μl vesicles and 2 μM dye, △, or (*iv*) 30 μl vesicles and 0.25 μM dye, ○. AS701, 10 μM was then added to each sample

the coupling ratio, provided that the membrane resistance is constant or known.

Figure 5 presents an experiment on the ATP dependence of the initial rate of the oxonol signal and active ^{22}Na flux. Control experiments showed that oxonol VI, up to 11 μM, did not affect the rate of Na/K-exchange. The data were fitted with the following values of K_m in μM: oxonol signal rate, 34.0 ± 6.7; ^{22}Na flux, 30.93 ± 6.7. The curves are indistinguishable and the line drawn in the figure is the best fit to all the points (K_m 33.14 μM). Clearly the coupling ratio is maintained constant over the entire range of ATP concentrations. A point of interest is that at the saturating ATP concentration the rate of active ^{22}Na uptake is linear for at least 1 min (*not shown*). Since the electrogenic potential is fully developed at 1 min (Fig. 2), the rate of transport cannot be significantly retarded by the electrogenic potential in our conditions.

Table 2. Absolute values of electrogenic potentials

Date	Na/K exchange pH 7.0	Uncoupled Na flux 7.0 (mV)	Uncoupled Na flux 8.5 (mV)	Na/Na exchange 7.0
9-6-87	209.0	—	—	3.45
10-6-87	281.0	1.2	4.3	—
24 6-87	255.0	0.73	3.1	4.7
Average	248.0 ± 21	0.96	3.7	4.08

The experimental traces from which these potentials have been calculated are as follows: K vesicles; oxonol absorption changes were from experiments exactly like that in Fig. 2, except that the oxonol concentration was 0.25 μM and the calibration lines were those in Fig. 4 using 30 μl vesicles and 0.25 μM dye. Na and Tris vesicles: oxonol absorption changes were those from the experiments of Figs. 9 (left) and 10, at pH 7.0 and 8.5, respectively, and similar ones, and the calibration lines were those in Fig. 4, using 150 μl vesicles and 2 μM dye, and another similar one.

The high sensitivity of the absorption measurements is demonstrated by the traces in Fig. 6 of signals accompanying Na/K-exchange at very low ATP concentrations 0.1, 0.25, and 0.5 μM, respectively. Here it was necessary to include the ATP regenerating system to maintain constant the concentrations of ATP. These concentrations of ATP sustain Na/K-exchange at about 0.4, 1.0 and 2.0% of the maximal rate, the signals corresponding to about 1.5, 3.0 and 6.0 mV, respectively. The rate and amplitude are proportional to the ATP concentration as expected for an ATP concentration far below the K_m. The traces in Fig. 6 serve as useful positive controls for comparison later with small signal sizes expected in conditions where low rates of Na/K exchange are expected (*see* Fig. 7 below) or in abnormal flux modes with low rates.

Figure 7, shows an experiment comparing the initial rate of the oxonol response and the initial rate of ATP-dependent ^{22}Na uptake over the sodium concentration range of 0.2–50 mM. The data were fitted to the Hill equation to obtain values of V_{max}, $K_{0.5}$, and n_H, and then replotted as the ratio v/V_{max} versus Na concentration. The data for the initial rates of the oxonol response above about 2 mM Na and the ^{22}Na flux are superimposable. The line drawn is the theoretical line for $K_{0.5}$ 16.6 mM and n_H 1.48 using all the points above 2 mM Na from both measurements. An unexpected finding was that the linear relation between the rate of the flux and oxonol signal is not maintained at low Na concentrations. As seen from the inset of Fig. 7 with expanded scales, oxonol signals were not detected below 0.8 mM Na. The active ^{22}Na flux at 0.8 mM Na, about 0.8% of the maximal rate, is of a size that

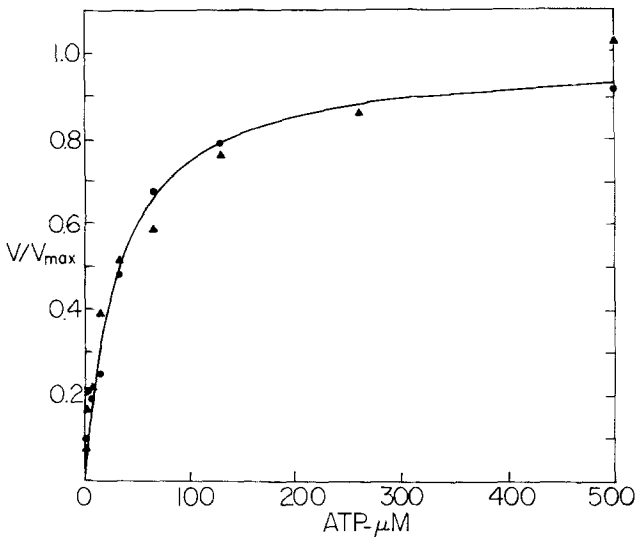

Fig. 5. ATP-dependence of Na/K exchange and the oxonol absorption change. K-loaded vesicles were prepared as in Fig. 1. For measurement of oxonol signals, 30 μl of vesicles were suspended in the reaction medium of Fig. 1, containing oxonol VI, 2 μM and in addition phosphocreatinine(Tris), 1 mM, and creatinine phosphokinase 10 U/ml. ATP(Tris) 1–500 μM was added to initiate the absorption change. For measurement of the ATP-dependent ^{22}Na uptake, 50 μl of vesicles were mixed with 50 μl of a reaction medium such that the same final concentrations of all constituents were as in the oxonol measurements, ^{22}Na ~2 · 10^6 cpm per sample, and ATP 2–500 μM, or zero. After 30-sec incubation at room temperature, 100 μl of ice-cold NaCl, 300 mM was added and the suspension was removed immediately to the cold Dowex columns. The extrapolated maximal rate of active flux was 30.8 nmol Na/10 μl vesicles/min. ▲ ^{22}Na flux; ● oxonol signal

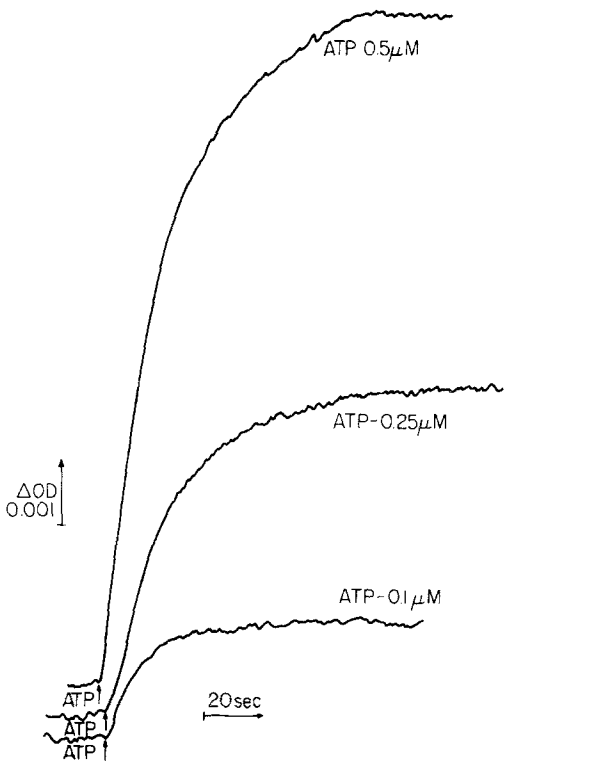

Fig. 6. Oxonol absorption changes at very low ATP concentrations. K-loaded vesicles were prepared as in Fig. 1 and 150 μl were suspended with oxonol VI, 2 μM in a reaction medium similar to that of Fig. 1, but containing NaCl, 30 mM; creatine phosphate, 0.5 mM; creatine phosphokinase, 10 U/ml. ATP was added to give final concentrations of 0.125–0.5 μM. Full scale absorption was 0.01 optical density units

should have produced a readily detectable signal. Notice the size of signals in Fig. 6, recorded when the rate was reduced to 0.4–1.0% of maximal by reducing the ATP concentration, but with a saturating Na concentration.

Figure 8 presents the initial rates of oxonol signal and ^{22}Na flux as a function of pH. The curves are essentially superimposable except at pH 9.0 where the oxonol signal almost disappeared. At pH 9.0 the resistance of the membrane increased precipitously, due probably to the reduction in ionic strength of the medium as the Tris becomes largely deprotonated (*not shown*). It is probably this which reduces the signal amplitude.

ATP-Dependent Na/Congener Exchange

In the experiment in Table 3, rates of ATP-dependent ^{22}Na uptake, oxonol signal amplitude and membrane resistance were measured for six sets of vesicles containing K,Rb,Cs,NH_4,Li or Na ions. With the exception of the NH_4-loaded vesicles (*see* Discussion), there is a remarkably good correlation between the potential and the rate of flux. The result suggests strongly that the classical 3 : 2 coupling ratio holds for the congener ions. Notice that in the Na-loaded vesicles the resistance appears to be significantly lower than in other vesicles, a result seen also in Table 1. In Na-loaded vesicles the flux being measured is an ATP-dependent Na/Na exchange, described for red cells (Blostein, 1983*b*) and reconstituted vesicles (Forgac & Chin, 1982; Cornelius & Skou, 1985). The actual potential generated by Na/Na exchange (4.08 mV, *see* Table 2) is only about 1.6% of that generated by Na/K exchange (248 mV), while the flux rate is close to 3% of maximal Na/K exchange in these vesicles (Table 3, and *see* Karlish & Stein, 1985). When the higher conductance of Na-loaded vesicles is taken into account the ratios of fluxes and voltages for Na/Na compared to Na/K exchange are similar.

Figure 9 shows the result of an experiment in which the oxonol responses of Na-loaded vesicles were compared in a medium to which ATP or ATP plus ADP was added. The signal with ATP alone is

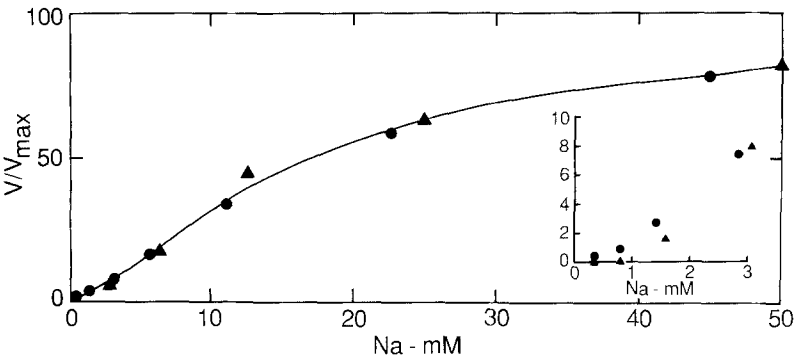

Fig. 7. Na_{cyt} dependence of Na/K exchange and oxonol absorption changes. K-loaded vesicles were prepared as in Fig. 1. For oxonol signal measurements 30 μl of vesicles were suspended in a reaction medium similar to that in Fig. 1, but containing NaCl, 0.78–50 mM plus Tris · HCl to a total concentration of 300 mM. The reaction was initiated by addition of ATP, 500 μM. The point in the figure at 0.35 mM is taken from another similar experiment. For measurement of the ATP-dependent ^{22}Na uptake, 40 μl of vesicles were mixed with 40 μl of a reaction medium such that the final concentrations of Na were 0.35–45.3 mM (plus ^{22}Na, ~ $6 \cdot 10^5$ cpm per sample); ATP, 500 μM or zero, and those of other constituents were as in the oxonol measurements. After incubation for 1 min at room temperature, the samples were removed to Dowex columns. ● ^{22}Na flux; ▲ oxonol signal

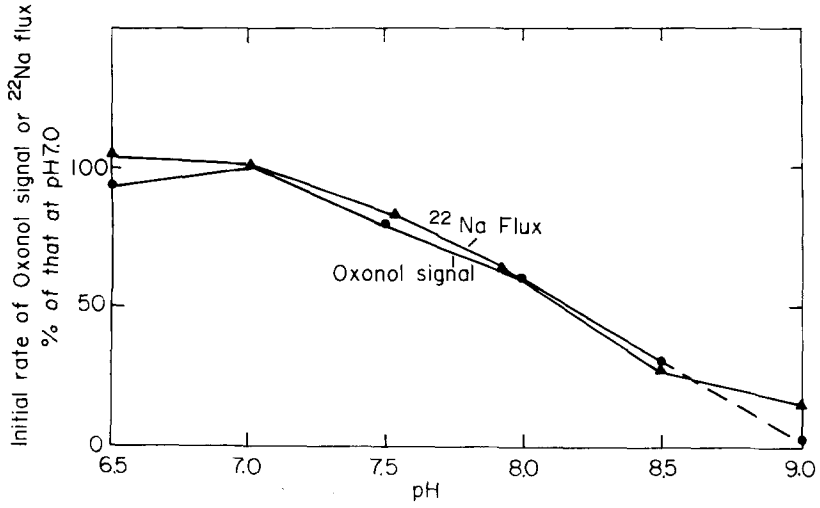

Fig. 8. pH dependence of Na/K exchange and oxonol absorption changes. K-loaded vesicles were prepared as in Fig. 1. For measurement of the oxonol signals 30 μl vesicles were suspended in a reaction medium similar to that in Fig. 1, except that NaCl was at 30 mM and the pH's were adjusted to 6.5–9.0 as indicated. The reaction was initiated by addition of ATP, 500 μM. For measurement of the ATP-dependent ^{22}Na uptake 30 μl of vesicles were mixed with 100 μl of a reaction medium such that the final pH's of the medium were 6.5–9.0; NaCl, 30 mM (plus ^{22}Na $1.05 \cdot 10^6$ cpm per sample); ATP, 500 μM or zero and concentrations of other constituents were as in the oxonol measurements. After 1-min incubation at room temperature the suspension was removed to Dowex columns

Table 3. Cation selectivity at the extracellular surface for active Na flux and the electrogenic potential

Ion	Na flux		Oxonol signal amplitude		Calculated resistance
	Rate nmol/10 μl/min	Ratio X/K	Units	Ratio X/K	$\Omega \cdot cm^2 \cdot 10^7$
K	2.12	1	11.75	1	2.53
Rb	1.98	0.93	11.14	0.95	2.95
Cs	1.97	0.93	9.5	0.81	2.31
NH_4	0.83	0.39	0	0	—
Li	0.74	0.35	4.77	0.41	1.87
Na	0.06	0.028	0.27	0.023	0.72

K-, Rb-, Cs-, NH_4-, Li and Na-loaded vesicles were prepared and suspended in the standard Tris · HCl/histidine medium. The ATP-dependent oxonol absorption changes and membrane resistances were measured exactly as in Fig. 2. The ATP-dependent ^{22}Na uptakes were measured exactly as in Fig. 5, using ATP, 3 mM.

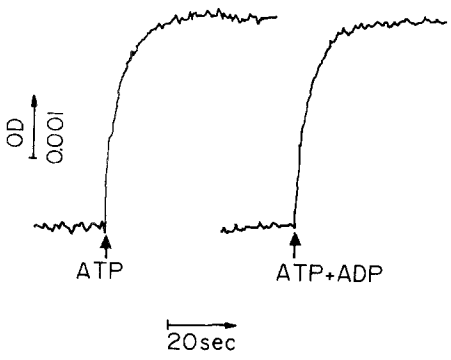

Fig. 9. Oxonol absorption changes accompanying Na/Na exchange. Na-loaded vesicles were prepared and suspended in the standard Tris · HCl; histidine medium as described in Materials and Methods. Vesicles (150 μl) were suspended in the reaction medium of Fig. 1, containing oxonol, 2 μM. The reaction was initiated by addition of ATP, 300 μM (left) or ATP, 300 μM plus ADP, 30 μM (right). Full scale absorption change was 0.01 optical density units

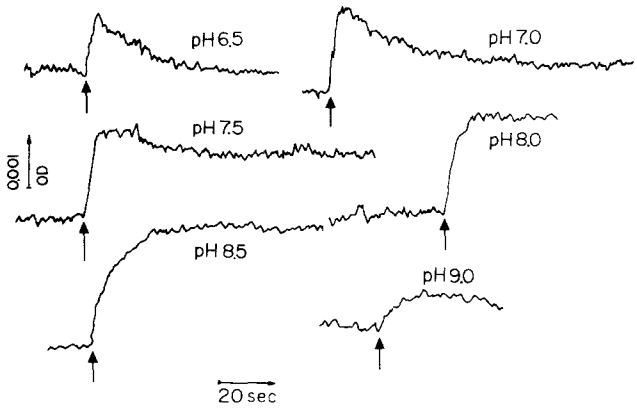

Fig. 10. Oxonol absorption changes accompanying "uncoupled" Na flux. Tris · HCl-loaded vesicles were prepared and suspended in the standard medium as described in Materials and Methods. Vesicles (150 μl) were suspended in reaction media similar to that in Fig. 1, containing oxonol, 2 μM, with the pH's adjusted to 6.5–9.0 as indicated. The reaction was initiated by addition of ATP, 30 μM. Full scale absorption change was 0.01 optical density units

that generated by the electrogenic Na/Na exchange just discussed. In the presence of ADP and ATP the pump should be carrying out the classical electroneutral 3Na/3Na exchange described by Garrahan and Glynn (1967*c*). In our conditions, addition of ADP together with ATP accelerated the ^{22}Na flux by 30–50% (*not shown*, and *see* Fig. 3 of Karlish & Stein, 1985). The oxonol signals in the two conditions were identical (*see* Discussion).

"Uncoupled" Na Flux

In our system, the equivalent of the "uncoupled" Na efflux from red cells is an ATP-dependent Na uptake into vesicles devoid of both K and Na. Previously we reported the existence of such a flux, with a rate about 1% of that of Na/K exchange (*see* Karlish & Stein, 1985). If the uncoupled flux were completely electrogenic (i.e., moving 3 positive charges per cycle) we might have expected to see a oxonol signal about as great as that generated by Na/K exchange at 0.5 μM ATP as in Fig. 6.

The experiment in Fig. 10 depicts the oxonol signal at different pH's. This and many similar experiments produced quite a different answer from that predicted above. It is consistent with Dissing and Hoffman's (1983) finding in red cells, that at a pH near neutral the uncoupled flux is electroneutral, but shows in addition that at higher pH this flux becomes electrogenic. At pH 6.5 a small transient response was observed but no steady-state difference was detectable. The transient response was almost certainly the result of the presence of traces of contaminating K ions. The vesicles suspensions contain up to 20 μM K, detectable by flame photometry, and deriving from the lipid. The K is trapped within the vesicles and should be pumped out upon addition of ATP, producing a transient voltage. In support of this explanation, it was found that if the transient was elicited by a low concentration of ATP (1 μM) and sufficient time elapsed to allow hydrolysis of the ATP (5 min), a second addition of ATP produced no further response. Figure 10 shows that as the pH of the medium was raised a distinct steady-state potential was superimposed on the transient. At pH 7.0 this was very small but was just detectable, while it became prominent at pH 8.5. At pH 8.5 the signal returned to baseline if a low concentration of ATP was used and sufficient time for its complete hydrolysis had elapsed. A second addition of ATP elicited a second signal, of the same amplitude (*not shown*), consistent with this being a real phenomenon associated with "uncoupled" flux at pH 8.5. Measurement of the vesicle conductance at pH 7.0 and 8.5 showed that this was a little higher at the higher pH (Table 1). As mentioned above the conductance at pH 9.0 in the Tris media is much higher than at pH 8.5 and this probably explains the precipitous fall in the amplitude at this pH.

The oxonol signal amplitude was then compared in parallel experiments with the maximal rate of uncoupled flux at the different pH's, *see* Fig. 11. The flux rate falls while the potential rises. Thus it appears a change of the coupling ratio must occur. The oxonol response in Fig. 11 was measured at 50

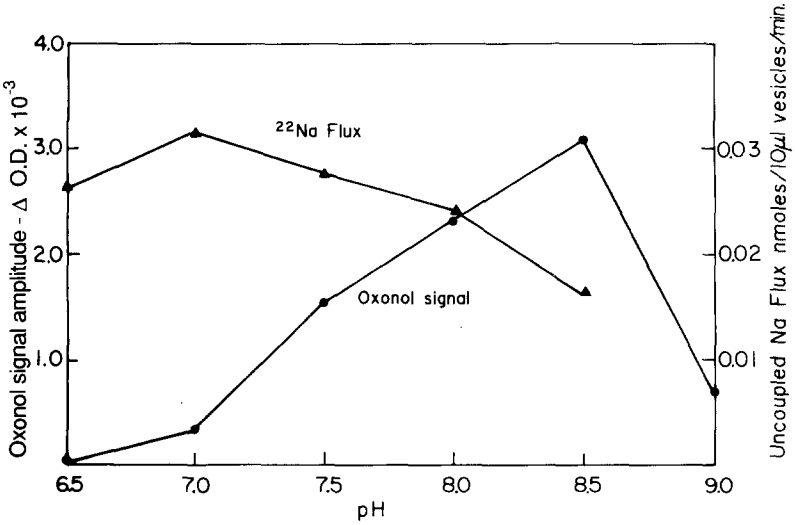

Fig. 11. pH dependence of "uncoupled" Na flux and oxonol absorption changes. The oxonol signal amplitudes are the average of two experiments, that in Fig. 10 and another essentially identical experiment. For measurement of ATP-dependent ^{22}Na uptake, 30 μl of vesicles were mixed with 100 μl of a reaction medium such that the final concentrations of the constituents were: NaCl, 2.4 mM (plus ^{22}Na, 1.48 · 10^6 cpm per sample); Tris · HCl, 300 mM; histidine, 25 mM, pH's 6.5–8.5 as indicated; $MgCl_2$, 3 mM; with ATP, 90 μM or without ATP. After 5-min incubation at room temperature the suspensions were transferred to the Dowex columns. Prior control experiments showed that the reaction was linear in this time range and was saturated at the NaCl concentration used (*see* Karlish & Stein, 1985)

mM Na so as to compare it with the oxonol response in K-loaded vesicles, as in Fig. 8. The "uncoupled" Na flux in Fig. 11 could not be measured at 50 mM but was measured at 2.4 mM Na which too is saturating (Karlish & Stein, 1985). Calibration of the oxonol signals in Tris- and K-loaded vesicles gave the absolute values of the potential generated by uncoupled flux at pH 7.0 and 8.5 recorded in Table 2, i.e., 0.96 and 3.7 mV. The comparable values of voltage generated by Na/K exchange were 248 and 55 mV, respectively (the latter is calculated from Fig. 8 and similar experiments).

In experiments with Tris-loaded vesicles, a possible concern is that entry of Na into the vesicles either by leakage or via the pump raises the intravesicular Na and converts the "uncoupled" flux to the Na/Na exchange. This can be excluded for reasons given in the Discussion.

It has been suggested by Dissing and Hoffman (1983) that electroneutrality of the "uncoupled" Na flux in red cells reflects a 3Na/3Cl cotransport. If this were the case for kidney enzyme, one might expect to observe some specificity for the anion at the putative cytoplasmic anion site. Vesicles were therefore prepared to contain, and were suspended in media containing, Tris salts of various anions, and the ATP-dependent ^{22}Na uptake was measured. As seen in Table 4 we could detect no preference of this flux for chloride compared to the other monovalent anions including gluconate and even aspartate.

We propose in Fig. 13, the electroneutral "uncoupled" Na flux is in reality a $3Na_{cyt}/3H_{exc}$ exchange while electrogenic "uncoupled" flux is a net movement of 3Na ions per cycle.

Passive K Fluxes

K-loaded vesicles suspended in a K-containing medium sustain a K/K exchange. This exchange occurs at a very low rate in the absence of ATP and P_i (~1% of max. Na/K exchange) (Karlish & Stein, 1982*b*) at a somewhat higher rate in the presence of ATP or P_i (2–4% of max. Na/K exchange) (Karlish & Stein, 1982*a*), and at a maximal rate in the presence of both ATP and P_i (15% of max. Na/K exchange) (Karlish, Lieb & Stein, 1982). No oxonol responses were detected when ATP or P_i or both ligands were added to K-loaded vesicles suspended in a medium containing K, so as to activate K/K exchange. This suggested, not unexpectedly, that this exchange is electroneutral.

A more interesting experiment was to look for responses when K(Rb) is added to vesicles devoid of K or Na. As shown previously, in these conditions, a very slow vanadate-inhibited net K(Rb) uptake on inside-out oriented pumps is detected (rate about 0.5% of max. Na/K exchange) (Karlish & Stein, 1982*b*). If this flux were electrogenic it should be possible to detect it. An important precaution was the use of lipid treated as described in Methods

Table 4. Lack of anion specificity of "uncoupled" Na uptake and vanadate-inhibited net Rb uptake

Anion	Uncoupled Na uptake nmol per min	Vanadate inhibited Rb uptake per 10 μl vesicle
Chloride	0.033	0.0065
Nitrate	0.031	0.0078
Formate	0.021	0.0099
Gluconate	0.046	—
Aspartate	0.029	0.0098

For the ^{22}Na flux experiment, vesicles were prepared to contain 300 mM Tris-chloride, -nitrate, -formate, -gluconate, and -aspartate, respectively, plus histidine, 25 mM, and were suspended in the different solutions of 300 mM Tris-anion/histidine. The flux was measured by mixing 50 μl vesicles with 50 μl of a reaction medium such that the final composition was Tris-anion, 300 mM; Na-anion, 2.14 mM (plus ^{22}Na $1.95 \cdot 10^6$ cpm per sample); Mg-anion, 3 mM with ATP, 0.66 mM or without ATP. After 5-min incubation at room temperature the suspensions were transferred to Dowex columns. For the ^{86}Rb experiment, vesicles were prepared to contain 100 mM Tris-chloride, -nitrate, -formate, and -aspartate, respectively, plus histidine, 25 mM, pH 7.0, and were suspended in the different solutions of 100 mM Tris-anion/histidine. The flux was measured by mixing 50 μl of vesicles with 50 μl of a reaction medium such that the final composition was Tris-anion, 100 mM; RbCl, 2.71 mM (plus ^{86}Rb $2.93 \cdot 10^6$ cpm per sample); without or with vanadate(Tris), 0.25 mM plus $MgCl_2$, 1 mM. After 5-min incubation at room temperature suspensions were transferred to Dowex columns. Note that all samples contained about 5 mM chloride deriving from the RbCl and solution of ^{86}Rb. Solutions of Tris-, or Na-, or Mg-anion were prepared by neutralizing Tris base, NaOH, or $MgOH_2$, respectively, with solutions of hydrochloric, nitric, formic, gluconic or aspartic acids, respectively. Prior to use the gluconic acid solution was decolorized with active charcoal and filtered.

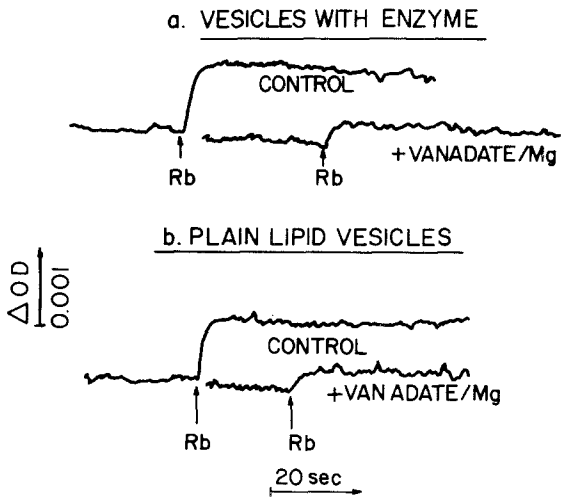

Fig. 12. Absence of oxonol absorption changes accompanying net passive Rb uptake. Plain lipid vesicles or vesicles reconstituted with enzyme, loaded with choline chloride, 100 mM; histidine, 25 mM, pH 7.0, were prepared and suspended in the choline/histidine solution. Vesicles (150 μl) were suspended in the choline/histidine solution with oxonol, 2 μM. RbCl, 2.5 mM, or vanadate, 0.5 mM, plus Mg, 1 mM, were added as indicated. Control experiments showed that the rate of net vanadate-inhibited Rb uptake was maximal when vesicles were prepared in this way rather than in the standard way used in this paper. Full scale absorption changes was 0.01 optical density units

to remove the endogenous K conductance. If the untreated lipid was used, addition of K induced an appreciable oxonol response. The traces in Fig. 12 show that addition of Rb to reconstituted vesicles made with the treated lipid induced a small response, and this was reduced in the presence of Mg plus vanadate. However, these were not pump-mediated events since they were seen also when plain lipid vesicles were used. We conclude that this mode of the pump does not generate a potential positive inside. This flux also shows no specificity towards chloride compared to very different anions (*see* Table 4).

Discussion

The voltage changes monitored by the oxonol signals can be analyzed simply assuming the existence of a battery (the pump) in parallel with the passive resistance and capacitance of the surrounding membrane (*see* Apell & Bersch, 1987). The pump-induced potential can be described formally as a charging of the capacitor.

$$I = A \cdot C_m \cdot dV/dT. \quad (1)$$

I is the total membrane current across A the area of membrane in question, C_m is the capacitance per unit area and dV/dT is the rate of change of potential.

$$I = I_p + I_l. \quad (2)$$

I_p is the pump current and I_l is the passive leakage current through the membrane resistance. For Na/K exchange one can write:

$$I_p = (n - m)/n \cdot J_{Na} \cdot F. \quad (3)$$

n and m are the stoichiometries of Na and K transport per cycle, respectively (assumed to equal 3 and 2, respectively); J_{Na} is the active Na flux in moles per unit time in the area of interest and F is Faraday's constant.

$$I_l = A \cdot V/R_m. \quad (4)$$

V is the membrane potential and R_m is the membrane resistance ($\Omega \cdot cm^2$).

Initially, upon switching on the pump battery by addition of ATP, the potential is zero and $I_l = 0$. In this state:

$$I = I_p. \tag{5}$$

Combining Eqs. (1), (3) and (5) we have:

$$dV/dT = (n - m)/n \cdot J_{Na} \cdot F/A \cdot C_m. \tag{6}$$

Hence the initial rate of change of the voltage should be proportional to the active Na flux.

In the steady-state the voltage becomes constant when the pump current equals the leakage current via the membrane resistance.

$$I_p\# = I_l. \tag{7}$$

$I_p\#$ is the pump current in the steady state. Strictly speaking $I_p\#$ is not equal to the initial pump current I_p, since the pump is working against a potential. In practice the difference may be small or negligible (*see below*).

Combining Eqs. (3), (4) and (7) we have:

$$V = (n - m)/n \cdot J_{Na}\# \cdot F \cdot R_m/A. \tag{8}$$

The steady-state potential should be proportional to the steady-state active transport rate multiplied by the resistance.

When the battery is switched off in the steady state by addition of vanadate (Fig. 2), the pump current is zero and the voltage dissipates via the membrane resistance. In this condition:

$$I = I_l. \tag{9}$$

From the definitions above one can write:

$$dV/dT = -V/R_m \cdot C_m. \tag{10}$$

The time constant of the exponential relaxation is $R_m \cdot C_m$. C_m is assumed to equal 1 μF/cm^2 and so R_m can be estimated (Table 1).

The discussion below tests these concepts utilizing the voltage and flux data for Na/K exchange. We then compare electrogenic potentials and fluxes for Na/K exchange, assuming a coupling ratio of 3Na for 2K (Karlish & Pick, 1981), in different conditions and in abnormal pump modes, in order to establish the coupling ratios.

Na/K and Na/Congener Exchange

The identical dependence of the initial rates of the oxonol signal and active Na flux on Na, ATP and protons over wide concentration ranges (Figs. 5–7) shows that the rate of generation of the potential is exactly proportional to the pump current, as predicted by Eq. (6). As seen from Eq. (8) the steady-state voltage should depend on the steady-state current and also resistance R_m; the latter was not measured in early experiments. When variations in R_m were taken into account the amplitude of the oxonol responses was usually proportional to the initial rate of ^{22}Na flux. A good example of this is seen in the experiment of Table 3 for the series of counter ions K, Rb, Cs, Li and Na. The result is consistent with Eq. (8) and shows that, in our conditions, the steady-state flux $J_{Na\#}$ is not significantly different from the initial flux J_{Na}, (as inferred also from linearity of the rate of Na/K exchange over the rise time of the potential). We conclude that it is reasonable to use the analysis above to compare voltages and fluxes for modes of the pump with known or unknown stoichiometries, respectively.

One strong conclusion from the results in Figs. 5–7 is that the coupling ratio, 3Na for 2K, must be fixed over the wide concentration ranges of Na, ATP and protons. Furthermore for the series K, Rb, Cs, Li, and Na a coupling ratio of 3Na/2congener ions also holds. Thus these vesicle experiments confirm the classical 3 : 2 coupling ratio from whole cell studies and of course extend the range of conditions in which it appears to be true.

Nevertheless the surprising observation (Fig. 7) that the oxonol signal disappeared at low Na_{cyt} concentrations (<0.8 mM) which should still have sustained a clearly detectable signal, seems to indicate a reduction to less than $3Na_{cyt}$ per $2K_{exc}$. A reduced Na/K coupling ratio at low Na_{cyt} does not contradict evidence for fixed coupling in whole cells (De Weer et al., 1988) for it is not possible to study electrogenicity in whole cells at these low Na_{cyt} levels in the absence of K ions. Indication for an altered coupling ratio at low Na_{cyt} comes from experiments using inside-out vesicles from red cells. Blostein (1983*a*) has reported a reduction in Na/Rb coupling ratio from 1.22 at 1.8 mM Na to 0.44 at 0.18 mM Na. Protons can be transported by the Na/K pump in the absence of cytoplasmic Na at a low enough pH (5.5–6.2) (Hara & Nakao, 1986; Polvani & Blostein, 1988). One might, therefore, suppose that replacement of sodium by protons could account for the reduction in the Na/ATP coupling ratio. Nevertheless, utilizing passive proton fluxes as indirect monitors of electrogenic potentials, Polvani and Blostein (1989) have obtained evidence very recently which too is consistent with a true reduction in coupling ratio at low Na_{cyt}. Therefore, although it is usually assumed that the pump can function only when the full complement of 3Na are bound, it now seems possible that at very low Na concentrations, transport can occur at a reduced rate with less than 3Na ions bound.

An unexpected result in Table 3 was that no potential was detected in vesicles prepared with NH_4Cl. A reasonable explanation is the following. Dilution of NH_4-loaded vesicles into the reaction medium should produce an outwardly directed gradient of NH_4 ions and hence also of protons due to free equilibration of NH_3 across the lipid membrane. (Dilution of NH_4-loaded cells is a well-known method to induce cytoplasmic acidity.) Since as mentioned above the vesicles appear selectivity permeable to protons even in the absence of added protonophores, the outwardly directed proton gradient could simply short-circuit an electrogenic potential produced by $3Na/2NH_4$ exchange.

Na/Na Exchange

Na/Na exchange observed in the presence of ATP only is similar to that described for red cells in which Na acts as a poor K surrogate (Blostein, 1983*b*). This flux has been demonstrated in reconstituted vesicles (Forgac & Chin, 1982) and a coupling ratio of about $3Na_{cyt}/2Na_{exc}$ per ATP hydrolyzed has been measured (Cornelius & Skou, 1985). The measurements of voltage and fluxes in Tables 2 and 3 show that the potential generated by this flux is about 1.6% of that generated by Na/K exchange while the rate of flux is close to 3% of Na/K exchange. Although initially this was taken to indicate that only some of the Na/Na exchange cycles could be electrogenic (Goldshleger et al., 1987*b*; Karlish, 1988*a*), when the lower resistance of Na-loaded vesicles is taken into account the coupling ratio is inferred to be $3Na_{cyt}/2Na_{exc}$ in our conditions also.

It was expected that addition of ADP to vesicles carrying out ATP-activated Na/Na exchange would reduce the size of the potential by accelerating electroneutral ATP-plus ADP-dependent 3:3 Na/Na exchange (Garrahan & Glynn, 1967*b*) at the expense of the electrogenic 3:2 exchange (Fig. 9). This was not found, although ADP at the concentration used in the experiment accelerates the ^{22}Na flux by about 50% (*see* Karlish & Stein, 1985). One explanation of the paradox could be that the rate of the step accelerated by ADP (E_1P—E_1) is much slower than the rate of phosphorylation (E_1—E_1P) or the conformational change (E_1P—E_2P). Thus electroneutral $3Na_{cyt}/3Na_{exc}$ exchange could be activated by ADP, without significantly diminishing electrogenic $3Na_{cyt}/2Na_{exc}$ exchange. The concentration of ADP used in the vesicle experiment, 30 μM, is low compared to concentrations required to activate Na/Na exchange in red cells (Glynn & Hoffman, 1971). In the vesicles, however, 30 μM ADP was optimal for activating Na/Na exchange in the presence of 300 μM ATP, while higher concentrations of ADP were inhibitory (*unpublished results*).

"Uncoupled" Na Flux

There are three reasons for excluding the possibility that "uncoupled" flux into the vesicles reflects Na/Na exchange. (*i*) At pH 6.5 the "uncoupled" flux is electroneutral while the Na/Na exchange is electrogenic. If in reality the "uncoupled" flux were all $3Na_{cyt}/2Na_{exc}$ exchange, a potential of 1–1.5 mV predicted for the observed Na flux would have been easily detectable. (*ii*) The potential generated by "uncoupled" flux at pH 8.5; 3.7 mV is about the same as that generated by Na/Na exchange at pH 7.0 (*see* Table 2). However in Na-loaded vesicles there is a sharp pH-dependent fall in the potential accompanying Na/Na exchange with a value at pH 8.5 of only about 0.5 mV, i.e., close to the detection limit (*unpublished*) (*iii*) Control experiments have shown that it is necessary to load vesicles with at least 100 mM Na to activate Na/Na exchange fully (*unpublished*). Within the 20 sec or so necessary to generate the oxonol response at pH 8.5, Fig. 11, net Na entry by passive leakage is negligible (*see* Karlish & Pick, 1981). The turnover rate of uncoupled Na flux via the pump is about 0.07 sec^{-1} at pH 8.5; thus in 20 sec, two turnovers could have occurred and 6 Na ions could have entered via the pump. Assuming an absolutely minimal radius of 100 Å for freeze-thaw-sonicated vesicles, the internal concentration of Na after one turnover could have risen no higher than 1 mM.

A comparison of the ratios of voltages and fluxes generated by "uncoupled" Na flux and Na/K exchange at the different pH's allows one to deduce the coupling ratio of the "uncoupled" flux in the pH range of 6.5–8.5.

Based on Eq. (8) we can write:

$$V_{Unc}/V_{Na/K} = (f \cdot J_{Unc})/(0.33 \cdot J_{Na/K}) \cdot (R_{mT}/R_{mK}). \quad (11)$$

The resistances in Tris (R_{mT}) and K (R_{mK}) vesicles are equal at pH 8.5 (*see* Table 2) and we assume this to be true at other pH's. The symbol f is the fraction of the uncoupled Na flux which carries charge and can vary from 0 to 1. For Na/K exchange, 0.333 is the fraction of the Na flux carrying charge. The value of $(V_{Unc}/V_{Na/K})/(J_{Unc}/J_{Na/K})$ can therefore vary between 0 and 3.

The voltages (in mV) generated by "uncoupled" Na flux and Na/K exchange were, respectively, 0 and 228 at pH 6.5, 0.96 and 248 at pH 7.0, and 3.7 and 55 at pH 8.5 (*see also* Table 2). The maximal rates (in nmol/10 μl vesicles/min) of the uncoupled flux and Na/K exchange were, respectively, 0.025 and 3.35 at pH 6.5 (calculated from Figs. 11 and 8), 0.0275 ± 0.00035 and 3.26 ± 0.51 (n = 7) at pH 7.0, and 0.0168 ± 0.0012 and 0.71 ± 0.13 (n = 3) at pH 8.5. Thus the values of $V_{Unc}/V_{Na/K}$

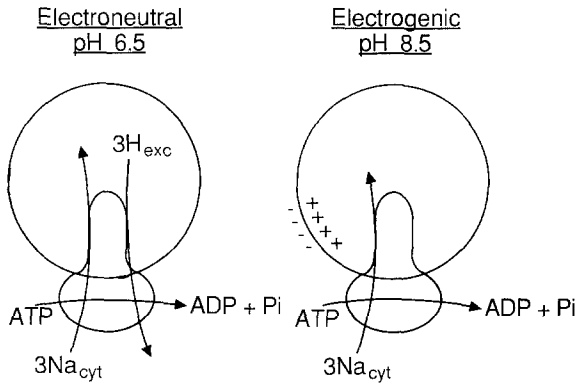

Fig. 13. Proposed ATP-dependent electroneutral Na/H exchange or electrogenic net Na flux in Na- and K-free vesicles

at pH's 6.5, 7.0 and 8.5 were 0, 0.38 and 6.8%, respectively, while the values of $J_{Unc}/J_{Na/K}$ at pH's 6.5, 7.0 and 8.5 were 0.7%, 0.89 ± 0.089% and 2.37 ± 0.16%, respectively. The quotients $(V_{Unc}/V_{Na/K})/(J_{Unc}/J_{Na/K})$ at pH's 6.5, 7.0 and 8.5 are therefore, 0, 0.42 and 2.86, respectively. Thus the "uncoupled" Na flux is electroneutral at pH 6.5. At pH 7.0 the ratio has risen a little but is still too small for the flux to be considered electrogenic. At pH 8.5 the "uncoupled" flux appears to be fully electrogenic, 3Na being transported without being exchanged for other ions.

Figure 13 (left) represents the proposed electroneutral $3Na_{cyt}/3H_{exc}$ exchange mode of the "uncoupled" flux. The change from electroneutral behavior at pH 6.5 to electrogenic behavior at pH 8.5 may be explained economically by supposing that the pump can cycle even when the three protons are not bound, due to the reduction in their concentration, Fig. 13 (right). At the intermediate pH's one might have coupling ratios of 3Na/2H or 3Na/1H or different proportions of the cycles 3Na/3H or 3Na/no ions. A pump-mediated ATP-dependent Na/H exchange in inside-out vesicles from red cells has been demonstrated recently (Polvani & Blostein, 1988). This exchange was observed at pH 6.2 but not at pH 6.8. A proton movement accompanying "uncoupled" Na uptake into reconstituted vesicles has also been reported (Forgac & Chin, 1982). This was interpreted as a passive flux driven by an electrogenic potential, but no evidence for a potential was provided.

In view of the lack of specificity of the "uncoupled" flux for anions (Table 4) the suggestion of electroneutral $3Na_{cyt}/3Cl_{cyt}$ cotransport (Dissing & Hoffman, 1983) is clearly not supported. Recently, transient charge movements associated with the first cycle of ATP-dependent Na movements have been detected on renal Na/K ATPase adsorbed to lipid bilayers (Fendler et al., 1985; Borlinghaus, Apell & Läuger, 1987). This finding also argues against $3Na_{cyt}/3Cl_{cyt}$ cotransport, for such a mode of transport would not carry charge even in the first cycle.

The proposal for a Na/anion cotransport for red cells was made following observation of a ouabain-inhibited sulphate efflux into a medium devoid of both Na and K (Dissing & Hoffman, 1983). The cells were pretreated with DIDS the inhibitor of band 3 the anion exchanger (Cabantchik, Knauf & Rothstein, 1978). A fundamental requirement for the hypothesis of pump-mediated Na/anion cotransport is that no anion fluxes occur via band 3. If this condition does not hold a ouabain-inhibited anion flux might be explained by the parallel actions of a ouabain-inhibited electroneutral Na/H exchange and anion/hydroxyl or anion/bicarbonate exchange via band 3. Considering the enormous excess of band 3 molecules, about 10^6 (Cabantchik et al., 1978), over pumps, about 250 per cell (Erdmann & Hasse, 1975), and the higher maximal turnover rate of band 3, about 10^4 sec^{-1} (Brahm, 1977), compared with about 150 sec^{-1} (Jorgensen, 1974) for the pump, it seems conceivable that even if say 99% of band 3 molecules had been poisoned with DIDS, a sufficient anion/hydroxyl or anion/bicarbonate flux might have remained to facilitate the parallel pathways proposed above.

Passive K Fluxes

The lack of oxonol signal in vesicles carrying out a K/K exchange was not surprising. K/K exchange is thought to involve the occluded form $E_2(2K)$ in which 2K ions are occluded per pump molecule (Forbush, 1987; Shani, Goldshleger & Karlish, 1987).

The lack of an oxonol signal in the slow passive net K flux mode and the lack of anion specificity for this flux (Fig. 12 and Table 4) lead us to propose that for this mode also, in the absence of Na or K at the extracellular surface, protons may be bound and transported in exchange for the K at the cytoplasmic surface.

Cation Binding Sites

The suggestion of an ATP-dependent Na/H exchange in the absence of Na and K at the extracellular surface parallels the recent finding that the pump sustains an ATP-dependent H/K exchange in the absence of Na at the cytoplasmic surface (Hara & Nakao, 1986; Polvani & Blostein, 1988). If our interpretation is correct, the voltage versus pH curve

in Fig. 11 is equivalent to a titration of the groups involved in the range pH 6.5–8.5. The pK of these groups is about 7.5, a value which has an interesting implication. Based on effects of voltage on the pump (Goldshleger et al., 1987*a*) and inactivation of cation occlusion by DCCD (Shani-Sekler et al., 1988), we have proposed that, two of the three cation binding sites contain carboxyl groups, and 2K and 2Na bind to the same two residues which are in a nonaqueous environment. The pK of carboxyl groups in water would normally be 4–5. Carboxyls in a nonaqueous environment might well have raised pKs of 7.5.

By using complexes of H_3O, Na or K complexes with crown ethers as models of cation sites, Boyer (1988) has argued recently that in transport systems which can bind and transport either Na or protons, it is likely that the protons are transported as hydronium ions. Protons can substitute for Na ions at the cytoplasmic surface and for K ions at the extracellular surface of the Na/K pump, and may, therefore, be bound and transported as hydronium ions. The corollary of the hypothesis as regards the alkali metal ions would be that these must be wholly or largely dehydrated in order to fit into the ion binding cavities and coordinate with surrounding oxygen or other electron donating atoms in specific complexes.

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