J. Membrane Biol. 20, 75–97 (1975)

Cation Permeability and Ouabain-Insensitive Cation Flux in the Ehrlich Ascites Tumor Cell

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Received 17 June 1974; revised 23 September 1974

Summary. The components of Na and K flux across the plasma membrane have been investigated in the Ehrlich ascites tumor cell. At intracellular K levels of approximately 100 mM, unidirectional K influx is composed of a ouabain-sensitive component, a ouabain-insensitive, nondiffusional component and a diffusional component. Unidirectional K efflux is composed of an external K-dependent component and a diffusional component. Upon reduction of intracellular K to approximately 50 mM, the external K-dependent component becomes maximal and diminishes upon further reduction of intracellular K. Unidirectional Na efflux is composed of a ouabain-sensitive component, a diffusional component and a saturable, external Na-dependent, ouabain-insensitive component. Unidirectional Na influx may be accounted for by a diffusional component, based on estimates of membrane permeability to Na, membrane potential and Na distribution. This would suggest that the ouabain-insensitive, external Na-dependent Na efflux is not Na–Na exchange.

The origin of the cell membrane potential has not been previously established in the Ehrlich ascites cell. From the diffusional components of Na and K flux determined in these experiments, the membrane permeabilities to Na and K have been estimated. These permeabilities, in conjunction with the Na and K distributions across the plasma membrane, predict a cell membrane potential of −18 mV (inside negative). Passive Cl distributions in these cells predict a cell membrane potential of −21 mV, which is in agreement with previous microelectrode measurements and dibenzyldimethylammonium distributions. The results are therefore consistent with the conclusion that the magnitude and polarity of the cell membrane potential in the Ehrlich ascites cell is dictated primarily by Na and K.

The Ehrlich ascites tumor cell maintains asymmetric distributions of Na and K across the plasma membrane, internal Na being low relative to external Na and internal K being high relative to external K. Upon exposure to the inhibitor of the Na, K-activated ATPase, ouabain, these gradients move toward equilibrium, indicating the presence of an active, coupled pump mechanism for Na and K (Hempling, 1958; Maizels, Remington & Truscoe,

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1958; Levinson, 1967). These observations establish that the movements of Na and K across the ascites cell membrane are composed of at least two components, passive fluxes which tend to move the cations towards electrochemical equilibrium and active fluxes which oppose these passive movements and maintain the asymmetric distributions.

Investigations on various other cells have established that Na and K movements across the plasma membrane are composed of other than ouabain-sensitive cation transport and passive cation flux. The presence of electrically silent, one for one exchange mechanisms for Na and/or K has been indicated in red blood cells (Hoffman & Kregenow, 1966), frog muscle (Ussing, 1949; Keynes & Swan, 1959), liver cells (Claret & Mazet, 1972), mouse eggs (Powers & Tupper, 1974) and Girardi cells (Lamb & McCall, 1972). Furthermore, in the human red blood cell, active Na extrusion has been established which is distinct from the ouabain-sensitive component of Na transport (Sachs, 1971). In the Ehrlich ascites cell, Hempling (1972) has observed that the transition from nonsteady-state to steady-state conditions is characterized by a large increase in the unidirectional fluxes of K coupled with a net gain of K. Inhibition of the active Na – K pump blocked the net gain of K but a large portion of the unidirectional K fluxes remained. This suggests the presence of a K – K exchange mechanism, based on the presence of large unidirectional fluxes which do not contribute to net K movements. It has also been observed in the Ehrlich ascites cell that the magnitude of the unidirectional Na influx is dependent on internal Na concentration (Aull & Hempling, 1963). This has been taken to suggest the presence of a Na – Na exchange mechanism.

The ionic basis of the cell membrane potential has not been previously established in the Ehrlich ascites cell. The distributions of Na and K and the membrane permeability to these cations has been shown to account for the magnitude and polarity of the cell membrane potential in numerous cells, both excitable and nonexcitable. Values for Na and K permeability may be determined, knowing the magnitude of the respective diffusional fluxes. Based on previous observations in the Ehrlich ascites cell, as well as other types of cells, we reasoned that the ouabain-insensitive fluxes of Na and K might well be made up of components other than diffusional fluxes. Knowledge of these components would be necessary prior to the determination of membrane permeabilities.

In this study we present evidence for the presence of Na and K fluxes in the Ehrlich ascites cell which do not fall in the category of active fluxes mediated by the ouabain-sensitive Na – K pump nor in the category of diffusional fluxes. In the case of K, the evidence suggests that a component

of K efflux exists which is dependent on the presence of external K. The magnitude of this component is also sensitive to intracellular K levels, in that it diminishes at levels of intracellular K above or below 50 to 60 mM. In the case of Na, a large component of Na efflux dependent on the presence of extracellular Na exists. It is conceivable that this represents an exchange mechanism. However, from membrane permeability to Na, the diffusional component of Na influx has been estimated and it can account for the entire unidirectional influx of Na, as determined from measurement of total Na efflux and the assumption of steady-state. Under these circumstances, the Na-dependent Na efflux would appear to represent a net Na efflux and not a Na – Na exchange. By exclusion of these fluxes and the ouabain-sensitive fluxes from the total unidirectional fluxes of Na and K, we have made estimates of the magnitudes of the respective diffusional fluxes. We observe that these diffusional fluxes predict permeabilities to Na and K which are able to closely predict the magnitude and polarity of the cell membrane potential in the Ehrlich cell. Therefore, these studies provide evidence for the physiological basis of the membrane electrical properties of this cell and they indicate the existence of components of Na and K flux which have not previously been investigated. The functional significance of these fluxes is presently unknown, but their relationship to nutrient transport will be of interest.

Materials and Methods

Ehrlich Lettre (ELD) cells were grown in female Ha/ICR mice and maintained by 12- to 14-day transfers. The line was a gift of Dr. T. Hauschka, Roswell Memorial Park. Ten- to 12-day growths were used for the experiments described here. Cells were aspirated from the peritoneal cavity, washed in Krebs-Ringer's solution (mM: 147 NaCl, 6 KCl, 1 $MgSO_4$, 0.1 $CaCl_2$, 46 Tris-OH, pH 7.4 with H_2PO_4) twice by centrifugation and incubated at 26 to 28 °C in a reciprocal shaker. Prior to each experiment, the cells were preincubated 30 to 45 min to obtain steady-state conditions. When handled in this manner, the cells contain, at the termination of the incubation, 117 ± 12 mM internal K and 57 ± 12 mM internal Na (SEM, $n = 7$ separate cell populations). All subsequent values are given as the standard error of the mean where n is the number of separate measurements.

Unidirectional influx of K was measured from the kinetics of ^{42}K or ^{86}Rb uptake. ^{86}Rb serves as an analog of K uptake in these cells (e.g., *see* Fig. 7), an observation consistent with numerous cell types. At zero time a trace amount of ^{42}K or ^{86}Rb was added to the suspension medium. At intervals, aliquots of cells were removed and rapidly pelleted (45 sec) in a microcentrifuge. Samples of the supernatant were removed for measurement of the external specific activity (cpm/mole). The pellet was then quickly rinsed with cold isotonic choline chloride (200 mM choline chloride, 10 mM Tris-Cl, pH 7.4) and lysed in distilled water. The lysate was then transferred to a counting vial and isotope content was measured by detection of Cerenkov radiation in a liquid scintilla-

tion counter. Subsequent to counting, the same samples were then analyzed for internal Na and K content with an internal standard flame photometer. Aliquots were also removed during the course of the experiments for cell water measurements. Cells were pelleted, the tube blotted dry and weighed. The pellets were dried overnight and weighed again. Cell water was determined from wet weight minus dry weight after appropriate corrections for trapped extracellular water, as determined from ^{14}C-inulin space corrections. Typically, in cells treated in this manner, extracellular space represented 10.2± 1.4% (n=20) of total pellet water. Based on the extracellular space, appropriate corrections were applied to the isotope uptake and flame photometric measurements. All values for ion concentration are given based on cell water content.

Unidirectional Na and K efflux were determined from the kinetics of ^{24}Na and ^{42}K (or ^{86}Rb) loss. Cells were preloaded with tracer by incubation in Krebs-Ringer's containing trace amounts of the appropriate isotope. The cells were then pelleted, washed twice in Krebs-Ringer's solution or the appropriate modification of this solution and resuspended. At intervals, samples were removed and the cells were pelleted in a microcentrifuge. The supernatant was removed and the pellet was rinsed in cold isosmotic choline chloride. The pellet was then lysed in distilled H_2O and the lysate was analyzed for radioactivity and Na and K content as described above. Pellet water was determined as described above.

Cell membrane potentials were determined from the steady-state distribution of ^{36}Cl. Cells were incubated under various conditions with trace amounts of ^{36}Cl. Influx of the isotope was measured as described above for K. At isotope equilibrium internal specific activity equals external specific activity and exchangeable internal Cl may be determined. Chloride has been shown to distribute passively in the Ehrlich ascites cell (e.g., Simonsen & Nielsen, 1971; Aull, 1972) such that its distribution reflects the cell membrane potential, i.e.,

$$V_m = V_{Cl} = \frac{RT}{F} \ln \frac{Cl_i}{Cl_o} \tag{1}$$

where V_m is the cell membrane potential and V_{Cl} is the chloride equilibrium potential. Cl_i and Cl_o are the internal and external chloride activities, respectively. It has recently been demonstrated that the distribution of another membrane potential probe(dibenzyldimethylammonium) gives results similar to those based on ^{36}Cl distribution (Cespedes & Christensen, 1974).

Cell numbers and cell diameters were determined under the various experimental conditions in a cell-counting chamber under phase-contrast microscopy.

The unidirectional efflux and the unidirectional influx were determined from the following considerations (Sheppard, 1962). In a closed, two-compartment system the rate coefficients (k_i and k_o) for the unidirectional influx and efflux are, respectively:

$$k_i = \frac{J_i}{C_o V_o} \tag{2}$$

$$k_o = \frac{J_o}{C_i V_i}. \tag{3}$$

J_i and J_o are the influx and efflux, respectively, in moles/time. C_i and C_o are the inside and outside concentrations and V_i and V_o the volumes. The unidirectional efflux rate coefficient (k_o) of Na or K was evaluated directly from the slope of plots of ln (R_t/R_{t_0}) versus time where R_t equals the count inside at time t and R_{t_0} equals the count inside at time zero (e.g., Figs. 2 and 4).

Unidirectional K influx was derived from the following considerations. At steady state $J=J_i=J_o$. Therefore, from Eqs. (2) and (3):

$$k_i+k_o=J\frac{1}{C_oV_o}+\frac{1}{C_iV_i}. \tag{4}$$

$K=k_i+k_o$ and may be evaluated from the slope of plots of $\ln[1-R_t/R_{t_\infty}]$ versus t (e.g., Fig. 7).

From K and the known volumes and concentrations the unidirectional influx, J_i, may be calculated in moles time^{-1} by rearranging Eq. (4):

$$J_i=K\frac{C_oV_o\cdot C_iV_i}{C_oV_o+C_iV_i}. \tag{5}$$

Dividing through by the appropriate area term yields the flux in moles cm^{-2} time^{-1}.

Results

The Components of Na Flux

Aull and Hempling (1963) have reported that the unidirectional efflux of Na in the Ehrlich ascites cell is composed of a rapidly exchanging component and a slow component. Under our experimental conditions we do not observe the presence of fast and slow components, but rather a single exchangeable component (e.g., Fig. 2). Knowledge of the presence of two exchangeable components is important in analysis of unidirectional fluxes and in assessing the contribution of the components to cell electrical properties. We therefore undertook analysis of Na efflux using experimental conditions described by Aull and Hempling (1963) to assure ourselves that the absence of a fast component was not a result of our experimental design. Two such effluxes are illustrated in Fig. 1. As is shown, we again only observe one exchangeable component. The rate constant for this component is approximately 13×10^{-4} sec^{-1}. The two components demonstrated by Aull and Hempling (1963) have rate coefficients of exchange of 10×10^{-4} sec^{-1} and 58×10^{-4} sec^{-1} (given as 3.5 and 20.8 hr^{-1}). The former slow component has a rate of exchange in reasonable agreement with our single component. The rapid component does not appear under our normal experimental procedures, as outlined in Materials and Methods and it does not appear in our cells under experimental procedures as described by Aull and Hempling (1963). Therefore, we conclude that in our cell strain the unidirectional Na efflux derives from a single kinetic compartment. One basic difference in the cells used in the two studies is their Na content. Based on a mean diameter of 16 μm, which is consistent for both studies, the internal Na content for the cells used in the experiments of Aull and

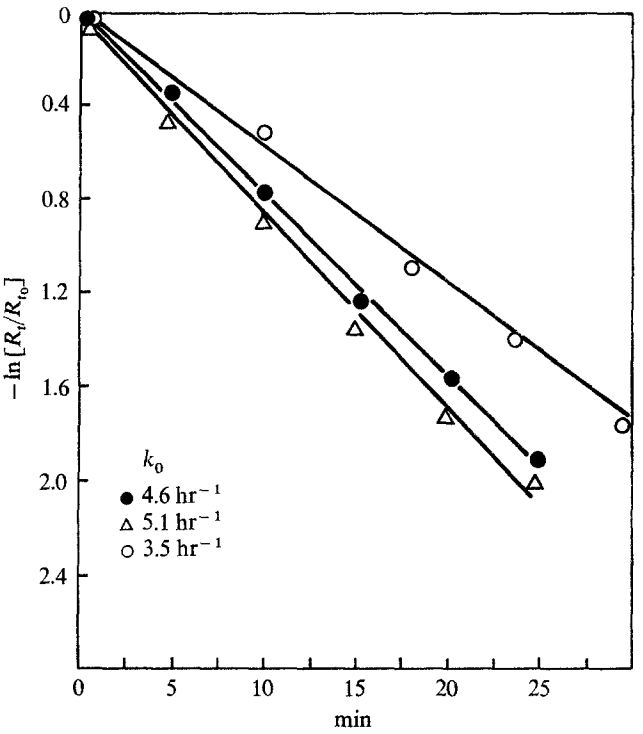

Fig. 1. Unidirectional Na efflux in the Ehrlich ascites tumor cell. The Figure illustrates two experiments carried out on separate cell populations according to procedures described by Aull and Hempling (1963); i.e. cells preloaded with ^{24}Na were washed twice with cold, tris-buffered isosmotic choline chloride and resuspended in Krebs-Ringer's solution. At intervals, aliquots were removed, the cells were pelleted and the supernatant removed. The pellet was washed twice in cold choline chloride and assayed for ^{24}Na and Na content (*see* Materials and Methods). A single kinetic compartment with a unidirectional efflux rate coefficient of approximately 4.8 hr^{-1} is indicated in these cells. Internal Na was monitored over the duration of the efflux experiments and in the two experiments it was 59 ± 5 mM (n = 6) and 59 ± 3 mM (n = 7). The open circles represent the slow component observed by Aull and Hempling (1963, Fig. 2) replotted here for comparison

Hempling (1963) is approximately 20 mM (given as 0.41 μmoles/10^7 cells) versus approximately 60 mM in the cells used in the present studies. Furthermore, it is quite likely that other distinctions exist in the two cell lines, since they represent populations carried under different conditions for several years.

The presence of 1 mM ouabain reduces the unidirectional Na efflux by $45 \pm 6\%$ (n = 4); e.g., Fig. 2*A*. The remaining ouabain-insensitive component of the efflux exchanges with a rate constant of $5.3 \pm 0.4 \times 10^{-4}$ sec^{-1} (n = 8) and represents a flux of 9.8 ± 1.4 pmoles cm^{-2} sec^{-1} (n = 7) (Table 1).

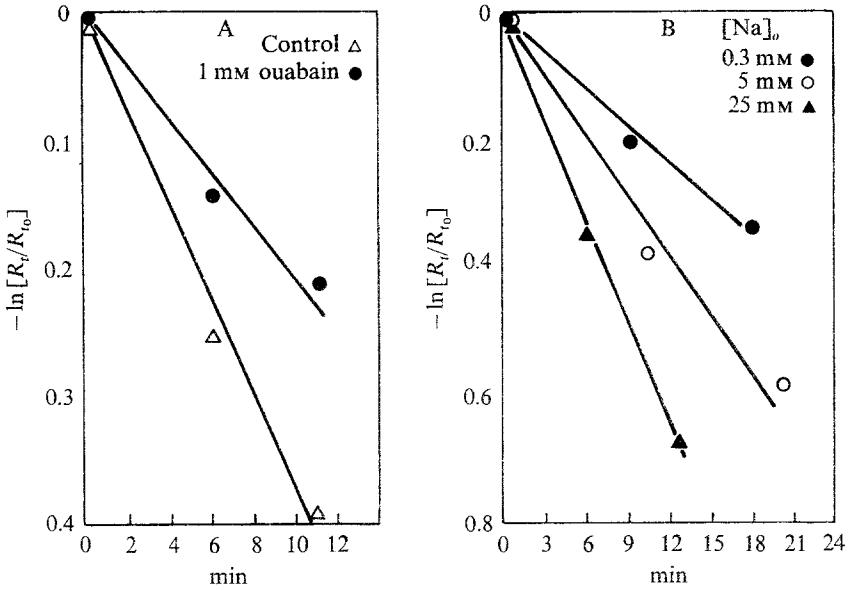

Fig. 2. (*A*) The effect of 1 mM ouabain on the unidirectional efflux of Na. In this particular experiment the control cells contained an internal Na of 37 mM and the ouabain-treated cells an internal Na of 46 mM at the termination of the efflux. Unidirectional Na efflux in control cells was 6.8 pmoles cm^{-2} sec^{-1} versus 4.8 pmoles cm^{-2} sec^{-1} in the ouabain-treated cells. (*B*) Typical experiment illustrating the effect of increased extracellular Na on Na efflux in ouabain-inhibited cells. The Figure illustrates the efflux of Na in three cell aliquots obtained from the same population of cells. At zero time, the cells were resuspended in Na-free, 5 mM Na or 25 mM Na medium (choline substitutes for Na). External Na, as determined from samples of the medium, was 0.3 mM upon resuspension of cells in Na-free medium. Internal Na was measured in the same samples in which ^{24}Na was determined (*see* Materials and Methods). The maximum change in intracellular Na was 12% over the duration of the efflux experiment in the three conditions. Intracellular Na under the three conditions, as averaged from zero time to the termination of the efflux, was: $[Na]_o$ 0.3 mM, $[Na]_i$ 8 mM; $[Na]_o$ 4 mM, $[Na]_i$ 11 mM; $[Na]_o$ 25 mM, $[Na]_i$ 24 mM. The unidirectional fluxes for this particular experiment are 0.8, 1.7 and 6.1 pmoles cm^{-2} sec^{-1} in 0.3, 5 and 25 mM external Na, respectively

Table 1. Ouabain-insensitive Na efflux in the Ehrlich ascites tumor cell (observed versus predicted)[a]

$[Na]_i$ (mM)	k_o (10^{-4} sec^{-1})	Na efflux[b] (pmoles cm^{-2} sec^{-1})	Na efflux[c] (pmoles cm^{-2} sec^{-1})
64±5 n=7	5.3±0.4 n=8	9.8±1.4 n=7	10.6

[a] All values are ± SEM. n is the number of separate experiments.
[b] Na efflux as measured from ^{24}Na loss (*see* Materials and Methods).
[c] Na efflux as predicted from a saturable Na-dependent efflux plus a unidirectional passive efflux at 147 mM $[Na]_o$ (*see text* and Fig. 3).

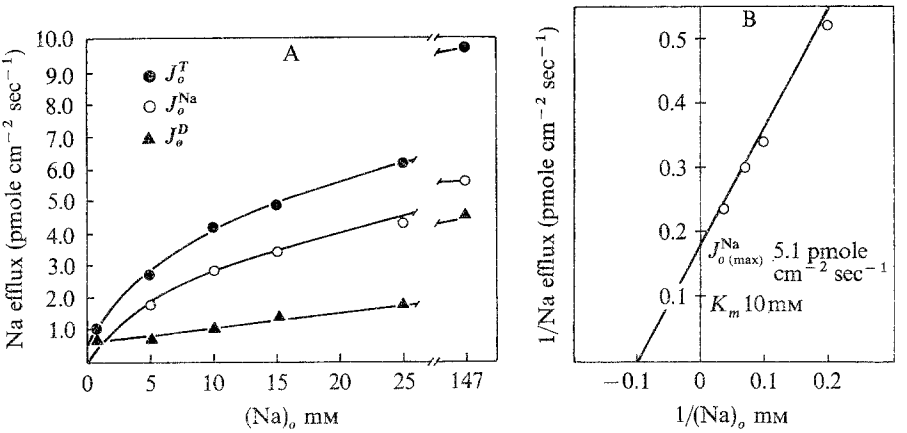

Fig. 3. (*A*) The components of ouabain-insensitive, unidirectional Na efflux at varying external Na concentrations in the Ehrlich ascites tumor cell. Total ouabain-insensitive Na efflux (J_o^T) at varying external Na was obtained from experiments such as those illustrated in Fig. 2*B*. The diffusional component of the Na efflux (J_o^D) was determined as described in Results. The difference between these two components represents the efflux of Na dependent on external Na (J_o^{Na}). (*B*) Reciprocal plot of the external Na-dependent component of Na efflux illustrated in (*A*) over the range of 5 mM to 25 mM external Na. The plot yields a saturation value ($J_{o\,(max)}^{Na}$) of 5.1 pmoles cm^{-2} sec^{-1} and an apparent K_m for external Na of 10 mM

This ouabain-insensitive Na efflux can be shown to be composed of at least two components, an external Na-dependent Na efflux and an external Na-independent Na efflux. The Na-dependent component is demonstrated by the observation that a reduction of external Na results in a reduction of Na efflux which cannot be accounted for by predicted changes in a diffusional efflux. Furthermore, the Na-dependent component can be shown to be a saturable component. The following experiments have led to these conclusions.

Cells preloaded with ^{24}Na were washed and resuspended in Krebs-Ringer's solution containing 1 mM ouabain and varying amounts of Na (choline substitutes for Na). Upon exposure to reduced external Na, the cells gradually lose internal Na. To determine the magnitude of the unidirectional flux it is desirable that a constant internal Na concentration be maintained over the duration of the flux experiment. Therefore, conditions were chosen such that the fluxes were measured over intervals in which little or no change in internal Na concentration occurred. This was achieved by monitoring the washout of ^{24}Na over intervals of 10 to 20 min upon exposure to medium containing reduced extracellular Na. Typical results of such experiments are illustrated in Figs. 2*B* and 3*A*. Cells washed in

Na-free, 1 mM ouabain medium and resuspended in this medium have a unidirectional Na efflux of approximately 0.8 pmole cm^{-2} sec^{-1}. Under these conditions the active Na efflux and the externally Na-dependent Na efflux are abolished. It should be noted that external Na under these conditions is not zero but of the order of 10^{-4} M. However, this is well below the K_m of the Na-dependent process *(see below)*, indicating that the Na-dependent Na efflux is virtually absent. It is assumed that this ouabain-insensitive, external Na-independent efflux represents the diffusional efflux of Na ($J^D_{o(Na)}$), that is the efflux of Na whose magnitude is dependent on the internal Na concentration, the cell membrane potential and the membrane permeability to Na. From this unidirectional efflux, in conjunction with the cell membrane potential (V) and the internal Na concentration (Na_i), the cell membrane permeability to Na (P_{Na}) may be determined (Hodgkin & Katz, 1949):

$$P_{Na}=\frac{J^D_{o\ (Na)}}{(Na)_i}\frac{1-e^{-VF/RT}}{VF/RT}. \tag{6}$$

Cell membrane potential under these conditions is -26 ± 5 mV (n = 10), as determined from ^{36}Cl distribution; internal Na is 12 ± 3 mM (n = 12); and the value for $J^D_{o(Na)}$ is 0.8 pmole cm^{-2} sec^{-1} (Fig. 3*A*). This yields a value for P_{Na} of $1.1\pm0.3\times10^{-7}$ cm sec^{-1}. From Eq. (6), knowing P_{Na}, the diffusional efflux of Na can be determined at varying membrane potentials and internal Na concentrations. The total unidirectional Na efflux ($J^T_{o(Na)}$) at increasing Na concentrations is illustrated in Fig. 3*A*. The diffusional component of the efflux, as determined from Eq. (6) knowing appropriate cell membrane potential and internal Na at the varying external Na concentrations, is also plotted. The difference between total unidirectional Na efflux and diffusional Na efflux represents the Na-dependent component of the efflux ($J^{Na}_{o(Na)}$). A reciprocal plot of this component (Fig. 3*B*) indicates an apparent K_m for the Na-dependent efflux of 10 mM external Na and its magnitude at saturation to be approximately 5.7 pmoles cm^{-2} sec^{-1}.

If the assumption is correct that the component of Na efflux in the presence of ouabain and the absence of Na represents the diffusional efflux of Na, then the following prediction can be made and tested. Upon saturation of the Na-dependent component of Na efflux in the presence of ouabain, the unidirectional efflux of Na should increase only as the diffusional component increases. At normal external Na (147 mM) the Na-dependent component is saturated. Therefore, the total unidirectional efflux of Na is the sum of the Na-dependent component at saturation (5.7 moles cm^{-2} sec^{-1}) plus the diffusional component (4.9 pmoles cm^{-2} sec^{-1}), as determined

from our value for P_{Na}. This yields a predicted value for the total unidirectional, ouabain-insensitive, Na efflux of 10.6 pmoles cm^{-2} sec^{-1} at normal external Na. In seven experiments (Table 1) the ouabain-insensitive, unidirectional Na efflux has been measured to be 9.8 ± 1.4 pmoles cm^{-2} sec^{-1}. The values are in good agreement and support the hypothesis that the diffusional component for Na efflux is represented by the external Na-independent, ouabain-insensitive component.

The presence of Na-dependent Na efflux has been demonstrated previously in other cells and in certain cases it has been defined as electrically silent, one for one exchange diffusion, as originally envisioned by Ussing (1949). It is also conceivable that such a Na-dependent flux may represent net Na efflux, as opposed to the absence of a net flux in true exchange diffusion. Such a possibility would be analogous to the external Na-dependent, ouabain-insensitive net Na efflux observed in the human red blood cell (Sachs, 1971). Our data suggest that the Na-dependent Na efflux observed in the present experiments is not a Na – Na exchange mechanism but rather a net Na efflux. This conclusion is based on the following observations. Under physiological conditions the cells maintain a constant internal Na of approximately 60 mM for indefinite periods. Therefore, they are in steady-state such that total unidirectional Na influx ($J^T_{i(Na)}$) equals total unidirectional Na efflux. Total unidirectional efflux equals the diffusional component (4.9 pmoles cm^{-2} sec^{-1}) plus the Na-dependent component (5.7 pmoles cm^{-2} sec^{-1}) plus the ouabain-sensitive component (8.4 pmoles cm^{-2} sec^{-1}), yielding a value of 19.0 pmoles cm^{-2} sec^{-1}. Therefore, total unidirectional Na influx equals 19.0 pmoles cm^{-2} sec^{-1}. The diffusional component of this influx ($J^D_{i(Na)}$) may be determined, knowing P_{Na} (1.1×10^{-7} cm sec^{-1}), external Na concentration (147 mM) and cell membrane potential (V, -21 ± 4 mV, n = 6) as determined from ^{36}Cl distribution; i.e.,

$$J^D_{i(Na)} = P_{Na} \cdot (Na)_o \frac{VF/RT}{1 - e^{-VF/RT}}. \tag{7}$$

This yields a value of 23.3 pmoles cm^{-2} sec^{-1}. Thus, within experimental error, the unidirectional influx of Na in these cells can be accounted for entirely by a diffusional Na influx. This suggests that the Na-dependent component of the Na efflux is not an exchange mechanism. If it were, nonexchange Na influx would then be nearly double nonexchange Na efflux, leading to Na accumulation in the cells. This does not occur.

If the unidirectional Na influx is composed entirely of a diffusional influx, measurements of ^{24}Na uptake would be expected to yield values

equivalent to the magnitude of the diffusional component. Attempts at such measurements have been unsuccessful. Extracellular Na is high relative to intracellular Na. Therefore, the extracellular space corrections are large. The results have been highly variable in our hands and we have been unable to determine the unidirectional Na influx accurately. To our knowledge, such measurements have not been made by others. In previous studies values for Na influx have been obtained as the difference between measured efflux and net flux (e.g., Aull & Hempling, 1963), as we have done in the present study.

The Components of K Flux

The unidirectional efflux of K has been measured through the loss of ^{42}K or ^{86}Rb from cells preloaded with the appropriate tracer. Removal of external K results in an approximately 75% reduction in the unidirectional K efflux rate coefficient (Fig. 4). This observation is consistent with the presence of an externally K-dependent K efflux. Cells exposed to K-free solutions gradually lose intracellular K. Furthermore, the removal of external K alters cell membrane potential. Therefore, these variables must be accounted for to evaluate the presence of a K-dependent K efflux. The following experimental design was chosen.

Aliquots of cells preloaded with tracer were washed three times in K-free Krebs-Ringer's solution (Na substitutes for K). The cells were then resuspended in normal (6 mM K) or K-free solutions. These solutions contained 1 mM ouabain to prevent reaccumulation of intracellular K in the 6 mM K medium. In the absence of ouabain, the cells in 6 mM K Krebs-Ringer's rapidly reaccumulate K such that their intracellular K is not equivalent to that of the cells in K-free medium. The presence of ouabain maintains equivalent levels of intracellular K. The presence or absence of ouabain does not affect the unidirectional K efflux, as determined in experiments in K-free solutions (Fig. 4). Changes in cell membrane potential were significant upon removal of external K. ^{36}Cl distributions in the presence or absence of external K indicate a hyperpolarization of cell membrane potential in K-free medium to -31 ± 3 mV ($n = 4$) as compared to -14 ± 4 mV ($n = 4$) in 6 mM K, 1 mM ouabain medium. A typical ^{36}Cl distribution experiment is illustrated in Fig. 5. This hyperpolarization in cell membrane potential will contribute to the reduction in unidirectional K efflux as seen in K-free medium, since the increased intracellular negativity will slow the diffusional component of the efflux. We assume that the unidirectional K efflux in K-free medium represents the diffusional component ($J^D_{o(K)}$). From this component, the cell membrane potential and intra-

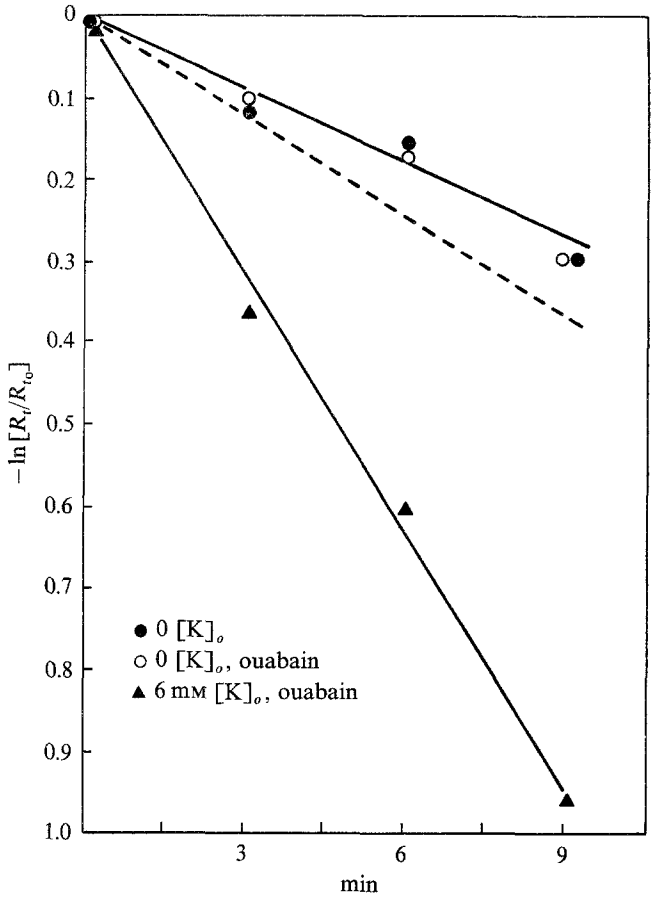

Fig. 4. The unidirectional efflux of K in K-free versus 6 mM K Krebs-Ringer's in the Ehrlich ascites tumor cell. Typical experiment illustrating the effect of K removal and the presence or absence of ouabain on the unidirectional K efflux. In all conditions intracellular K did not change significantly from the initiation of the efflux to its termination, as measured from the same samples in which ^{42}K was determined (*see* Materials and Methods). In this particular experiment in zero K, zero K ouabain and 6 mM K ouabain, medium intracellular K was, respectively, 45 ± 4 mM, 46 ± 2 mM and 38 ± 1 mM ($n = 4$). In 6 mM K medium the unidirectional efflux is 19.8 pmoles cm^{-2} sec^{-1}. Removal of external K reduces it to 6.7 pmoles cm^{-2} sec^{-1}. The presence of ouabain does not significantly alter this value. The dashed line has a slope corrected for the effect of the membrane potential hyperpolarization (resulting from K removal) on the diffusional component of the K efflux (*see* Results)

cellular K concentration, the membrane permeability to K (P_K) may be determined. The relationship is equivalent to that for P_{Na} [Eq. (6)]. The appropriate values are as follows: $J^D_{o(K)}$ equals 3.2 ± 0.5 pmoles cm^{-2} sec^{-1} ($n = 4$); K_i equals 25 ± 3 mM ($n = 4$). This yields a value for P_K of $2.5 \pm 0.5 \times 10^{-7}$ cm sec^{-1}. Knowing P_K, the diffusional component of the efflux

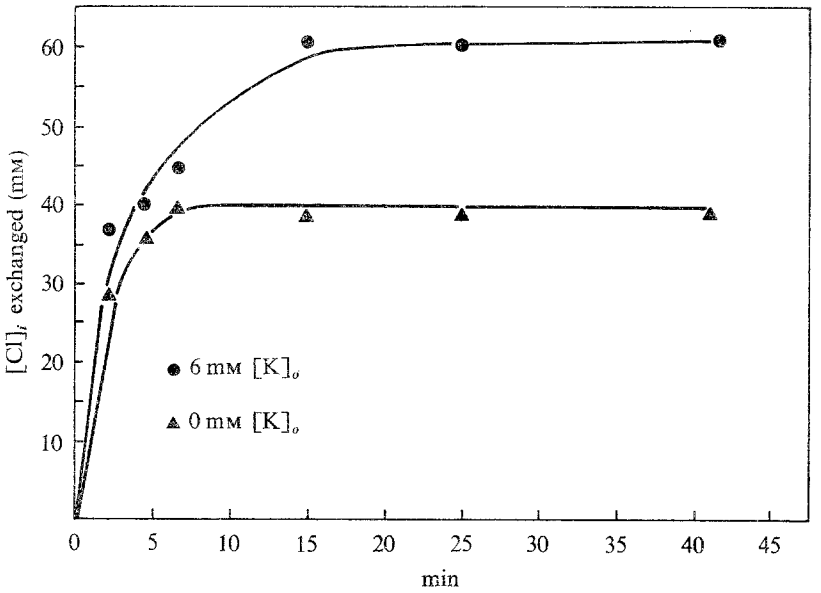

Fig. 5. The influx and steady-state distributions of ^{36}Cl in cells incubated in the presence or absence of extracellular K. ^{36}Cl uptake was determined as described in Materials and Methods. Cl distributes passively in these cells and the steady-state distribution of Cl indicates a membrane potential of -24 mV in the 6 mM K medium versus -35 mV upon K removal. The hyperpolarization is consistent with the increased outward driving force on K ion

at varying membrane potentials may be determined (Hodgkin & Katz, 1949):

$$J_o^D = P_K(K)_i \frac{VF/RT}{1-e^{-VF/RT}}. \quad (8)$$

The diffusional component of K efflux was determined at a cell membrane potential equivalent to that of cells in the presence of 6 mM external K, 1 mM ouabain (i.e., -14 mV). As illustrated in Fig. 4, the unidirectional efflux rate coefficient for the diffusional component, when corrected for the membrane potential change, is still approximately 60% less than that observed in the presence of K. This suggests the presence of a large, non-diffusional, external K-dependent K efflux. The experiment illustrated in Fig. 4 is typical of 12 such experiments. Subsequent experiments indicate that the external K-dependent K efflux is not a saturable component with respect to intracellular K but rather, it diminishes with increasing intracellular K. Varying intracellular K concentrations were obtained by incubating cells for periods of time in K-free medium prior to resuspension in

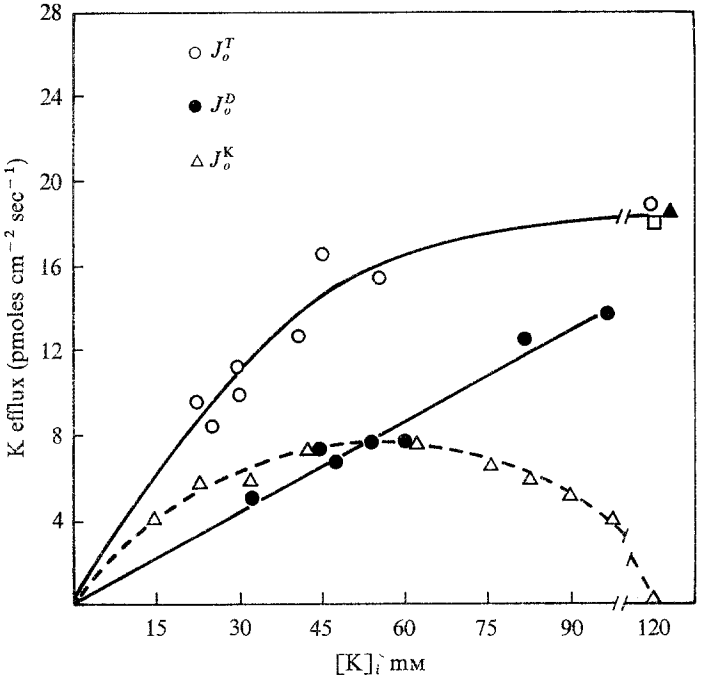

Fig. 6. The components of unidirectional K efflux under conditions of increasing intracellular K in the Ehrlich ascites tumor cell. The diffusional component (J_o^D) was measured in the absence of external K, as in Fig. 4. The different internal K concentrations were obtained by incubation for varying times in K-free medium. Ouabain at 1 mM is present in all experiments up to 94 mM intracellular K. It is absent in the experiments in which intracellular K is 120 to 124 mM, since in its presence these levels of K are rapidly lost. The square represents the diffusional component of unidirectional K efflux at 120 mM intracellular K, as determined from the values of P_K and cell membrane potential. It cannot be measured directly since removal of external K results in reduction of intracellular K below normal levels in a brief period of time. The filled triangle represents the mean value for the unidirectional influx of K, as determined from K uptake experiments (18.3 ± 2.9 pmoles cm^{-2} sec^{-1} (n = 12), e.g. *see* Fig. 7). The values for influx and efflux are in good agreement, indicating the presence of steady-state conditions in normal Krebs-Ringer's. The open triangles represent the unidirectional efflux of K dependent on the presence of external K (J_o^K) at varying internal K. They are obtained from the difference between J_o^T and J_o^D over the range of intracellular K

6 mM or K-free solutions. The unidirectional efflux was then measured in the presence or absence of external K. Correction for membrane potential hyperpolarization in K-free medium was made from ^{36}Cl distributions and the total and diffusional components were determined. As illustrated in Fig. 6, total unidirectional K efflux ($J_{o(K)}^T$) increases with increasing intracellular K, as does the diffusional component. It should be noted that the near linear increase in the diffusional K efflux with increasing internal K

content is not expected. As seen in Eq. (8), it is a function of not only the concentration of K but also membrane potential. However, the changes in membrane potential over the range of 30 to 60 mM internal K are slight and therefore the increase in intracellular K predominates the increase in diffusional efflux. At levels of intracellular K above 60 mM, Eq. (8) would predict a somewhat steeper rise in the diffusional component than that measured experimentally; i.e., a diffusional efflux of approximately 16 pmoles cm^{-2} sec^{-1} versus a measured value of 13 pmoles cm^{-2} sec^{-1} at 82 mM intracellular K. The K-dependent component is defined as the difference between the total K efflux and the diffusional efflux. It does not saturate but rather, it achieves a maximum at approximately 50 mM internal K and diminishes at increasing intracellular K concentrations. The discrepency between the magnitude of the diffusional component, as measured versus calculated from Eq. (8), would indicate that the K-dependent component may diminish somewhat more rapidly at elevated intracellular K levels. Our present data do not allow us to determine whether the externally K-dependent K efflux observed represents an exchange mechanism for K or a net K efflux dependent on extracellular K. Data of Hempling (1972) would suggest that it may represent a K – K exchange mechanism (*see* Discussion).

Unidirectional K influx has been investigated from the kinetics of ^{42}K or ^{86}Rb uptake. In 12 experiments the total unidirectional K influx was 18.3 ± 2.9 pmoles cm^{-2} sec^{-1}. This value is close to total unidirectional efflux (Fig. 6), indicating steady-state conditions. As has been previously determined (Maizels *et al.*, 1958; Levinson, 1967), a large component of K influx is sensitive to ouabain (e.g. Fig. 7). In our hands this pump-mediated flux ($J^P_{i(K)}$) represents approximately 52% of the total unidirectional K influx. Knowing membrane permeability to K, external K concentration and cell membrane potential, the diffusional component of K influx ($J^D_{i(K)}$) can be determined under physiological conditions from a relationship equivalent to that in Eq. (7). This yields a value of 2.3 pmoles cm^{-2} sec^{-1}. The measured total unidirectional influx of K ($J^T_{i(K)}$) was 18.3 ± 2.9 pmoles cm^{-2} sec^{-1} ($n = 12$). The ouabain-sensitive component plus the diffusional component account for 65%. The remainder of the K influx is, therefore, ouabain-insensitive and nondiffusional. Intracellular K in these cells was 101 ± 8 mM. As seen from Fig. 6, at 101 mM K approximately 4 pmoles cm^{-2} sec^{-1} of K efflux are dependent on external K. If this represents a one for one K exchange, then approximately 22% of the influx of K is accounted for by this exchange component. This plus the 65% comprised of the ouabain-sensitive flux and the diffusional flux accounts for 87% of

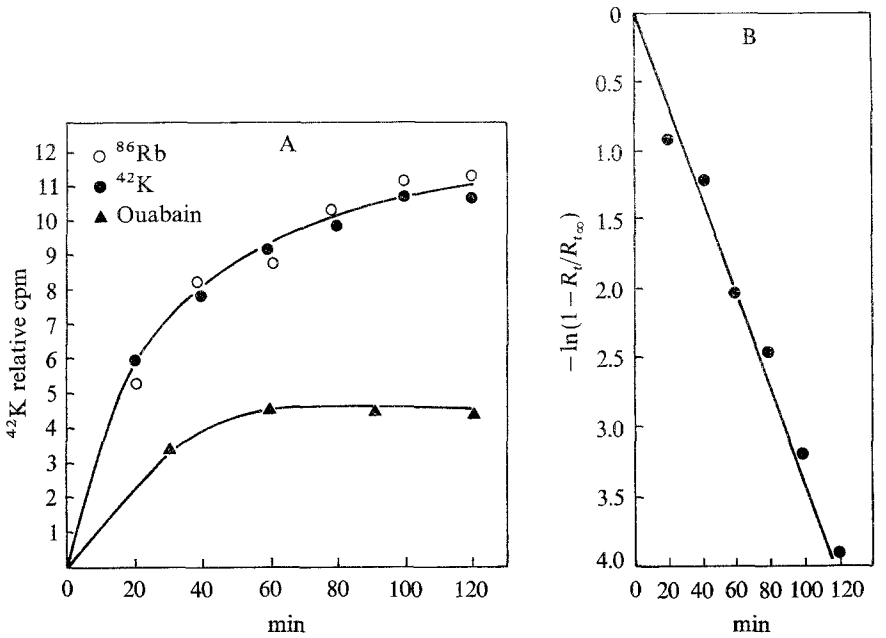

Fig. 7. K influx in the Ehrlich ascites tumor cell. Cells were incubated in medium containing trace amounts of ^{86}Rb or ^{42}K and isotope uptake was determined as described in Materials and Methods. (*A*) Comparison between the uptake of ^{86}Rb and ^{42}K and the effect of 1 mM ouabain on K influx. (*B*) Plot to obtain the unidirectional K influx. The slope of the line represents the rate constant K (the sum of the unidirectional potassium influx plus efflux rate coefficients (k_i+k_o). The value for $R_{t\infty}$ was obtained from computer plots yielding a linear relationship for $-\ln(1-R_t/R_{t\infty})$ versus time. From k_i+k_o, unidirectional K influx is obtained [Eq. (5), text]. The experiments illustrated here are typical of 12 such experiments measuring total unidirectional K influx (18.3 ± 2.9 pmoles cm^{-2} sec^{-1}) and four such experiments measuring ouabain-sensitive unidirectional K influx (9.5 ± 1.9 pmoles cm^{-2} sec^{-1})

the total unidirectional K influx. The remaining 13% may be the result of compounded experimental error, or represent in whole or in part a component of K influx as of yet unidentified.

Discussion

The Relationship of the Diffusional Fluxes of Na and K to Cell Membrane Potential and Conductance

In numerous cells, both excitable and nonexcitable, the magnitude and polarity of the cell membrane potential has been shown to result from the membrane permeability to, and the distributions of, Na and K (e.g., *see*

Williams, 1970). Microelectrode studies indicate the magnitude of the Ehrlich ascites cell membrane potential to be -20 to -30 mV, inside negative (Johnstone, 1959; Lassen, Nielsen, Pape & Simonsen, 1971). Passive distribution of Cl and dibenzyldimethylammonium also indicate membrane potential values of -20 to -30 mV (e.g., Simonsen & Nielsen, 1971; Cespedes & Christensen, 1974). The origin of the cell membrane potential in the Ehrlich ascites cell has not been previously established. In this study we have made estimates of the magnitude of the membrane permeability to Na and K, based on the diffusional fluxes of these cations. From these permeabilities it is possible to establish the magnitude and polarity of a cell membrane potential which would arise if due exclusively to the distributions of, and membrane permeabilities to Na and K (Hodgkin & Katz, 1949);

$$V=\frac{RT}{F}\ln\frac{P_{\mathrm{K}}(\mathrm{K})_o+P_{\mathrm{Na}}(\mathrm{Na})_o}{P_{\mathrm{K}}(\mathrm{K})_i+P_{\mathrm{Na}}(\mathrm{Na})_i}. \tag{9}$$

The use of this relationship assumes the membrane is in steady-state, ionic mobility is constant throughout the membrane and the potential field throughout the membrane is constant. Furthermore, the contribution, if any, of electrogenic pumping to the membrane potential is assumed to be insignificant. For the cells used in this study P_{K} equals approximately 2.5×10^{-7} cm sec^{-1}. In normal Krebs-Ringer's $(\mathrm{Na})_o$ is 147 mM and $(\mathrm{K})_o$ is 6 mM. In seven separate cell populations $(\mathrm{Na})_i$ equaled 57 ± 12 mM and $(\mathrm{K})_i$ equaled 117 ± 14 mM. Using Eq. (9), these yield a value for cell membrane potential of -18 mV. In normal Krebs-Ringer's, Cl distributions in these cells indicate a membrane potential of -21 ± 4 mV ($n=6$). The results are therefore consistent with the conclusion that cell membrane potential in the Ehrlich ascites cell is dictated primarily by the distributions of and permeabilities to Na and K. This is further supported by the following observations. In a cell in which the membrane potential is a function of Na and K, an increase in extracellular K would be expected to depolarize the membrane potential. Microelectrode measurements by Lassen *et al.* (1971) on Ehrlich ascites cells suggest this to be true in that elevation of external K to 155 mM depolarized cell membrane potential from approximately -26 mV to -7 mV. Conversely, a reduction in extracellular K would be expected to hyperpolarize the cell membrane potential. Our studies on ^{36}Cl distribution confirm this. Removal of external K leads to hyperpolarization from approximately -24 mV to -35 mV (e.g., Fig. 5). An increased membrane conductance to K ion would be expected to hyperpolarize cells as a result of the increased contribution of K to the cell membrane potential.

Cespedes and Christensen (1974) have observed that in valinomycin-treated Ehrlich ascites cells the membrane potential hyperpolarizes to approximately -35 mV as compared to -20 mV on control cells. This is in agreement with the increased K conductance induced by valinomycin. It should also be noted that the membrane potentials in the study of Cespedes and Christensen (1974) were determined from the distribution ratio of dibenzylmethylammonium and these values are in good agreement with those determined from ^{36}Cl distribution.

In a recent study by Pietrzyk and Heinz (1974), evidence has been presented for nuclear sequestration of Na in the Ehrlich ascites cell. In normal Krebs-Ringer's they estimate that cytoplasmic Na concentration is approximately 17% of overall Na concentration. Reduction of extracellular Na to values below 30 mM results in the disappearance of nuclear sequestration of Na. K distribution appears to be uniform. In the present study, values for P_{Na} were determined in Na-free medium. Based on the observations of Pietrzyk and Heinz (1974), overall Na concentration would reflect cytoplasmic Na under these conditions. Therefore, the magnitude of P_{Na} would be unaffected. However, their data would suggest that cytoplasmic Na at normal extracellular Na would be reduced to approximately 10 mM versus 57 mM based on total Na content. Insertion of this value into Eq. (9) would yield a somewhat lower membrane potential value (-14 mV versus -18 mV).

Knowing the appropriate permeabilities or fluxes, the membrane conductances to Na, K and Cl may be calculated from the following equations (Hodgkin & Katz, 1949):

$$G_K = P_K \frac{F^2}{RT} \frac{V_m}{V_K - V_m} \frac{(K)_o - (K)_i e^{-V_m F/RT}}{1 - e^{-V_m F/RT}} \tag{10}$$

$$G_{Na} = P_{Na} \frac{F^2}{RT} \frac{V_m}{V_{Na} - V_m} \frac{(Na)_o - (Na)_i e^{-V_m F/RT}}{1 - e^{-V_m F/RT}}. \tag{11}$$

Since chloride is in electrochemical equilibrium:

$$G_{Cl} = \frac{F^2}{RT} J^D_{o(Cl)}. \tag{12}$$

V_K and V_{Na} are the equilibrium potentials for K and Na (-77 mV and $+25$ mV, respectively) and $J^D_{o(Cl)}$ is the diffusional Cl efflux (7.0×10^{-11} mole cm^{-2} sec^{-1}, *see below*). All other values are as previously given.

Applied to the present results, G_K equals 74 μmho cm^{-2} and G_{Na} equals 3 μmho cm^{-2}. From experiments such as those of Fig. 5, unidirectional Cl influx has been estimated. This yields a value of 7.0×10^{-11} mole $cm^{-2} sec^{-1}$, which is in good agreement with previous observations (Aull, 1967; Lassen *et al.*, 1971). Since unidirectional Cl influx equals unidirectional efflux, Eq. (12) may be applied and G_{Cl} equals 260 μmho cm^{-2}. This assumes all Cl flux is diffusional. The presence of a mediated Cl flux would reduce the actual diffusional flux and in turn yield a reduced conductance. However, it is likely that the bulk of membrane conductance is due to Cl conductance, since a large portion of the flux would have to be nondiffusional to reduce G_{Cl} to values equivalent to G_K or G_{Na}. Furthermore, Hempling and Kromphardt (1965) have observed that Cl efflux is independent of external Cl, suggesting the absence of Cl – Cl exchange. If the bulk of ionic current is carried by Na, K and Cl, then total membrane conductance is approximately 340 μmho cm^{-2}. This yields a specific membrane resistance of approximately 3000 Ω cm^2. Microelectrode measurements by Lassen *et al.* (1971) yield a specific membrane resistance of 70 Ω cm^2. Based on their estimates of chloride resistance (4000 Ω cm^2 versus 3800 Ω cm^2 in the present study) they have concluded that their microelectrode measurements are dominated by a large leakage current around the microelectrode. Our data would also indicate this to be true.

The Components of Na and K Flux

Fig. 8 is a schematic illustration of the components of Na and K flux under physiological conditions, as indicated by the present experiments. Previous observations of Aull and Hempling (1963) on the Ehrlich ascites cell are pertinent to our present results. They have observed the following. An increase in intracellular Na achieved by cooling of the cells in turn results in an increase in the unidirectional Na influx, as measured from the difference between efflux and net flux. These observations were made at external Na concentrations of 138 to 172 mM. When external Na was dropped to 18 mM a large reduction in unidirectional Na influx occurred. In certain of these experiments the cells were near steady-state, which indicates that the reduction in extracellular Na also reduces unidirectional Na efflux. The change is greater than could be accounted for by an alteration in diffusional Na efflux. This observation is consistent with the present experiments in which we observe a component of Na efflux which is reduced at lowered external Na. Aull and Hempling (1963) suggest, based on the increase in Na influx with increasing internal Na concentration, the presence

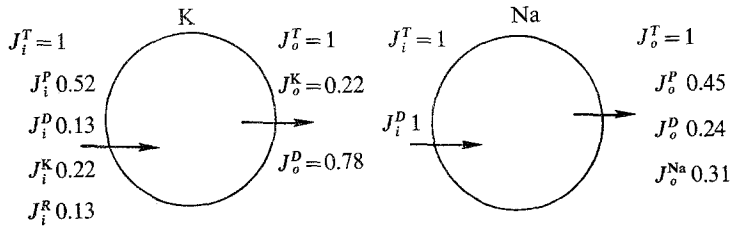

Fig. 8. Schematic illustration of the components of unidirectional Na and K influx and efflux, as suggested by the present experiments. In normal Krebs-Ringer's the cells maintain constant internal Na and K concentrations, indicating steady-state conditions. Therefore total unidirectional efflux equals influx ($J_i^T = J_o^T$). These are arbitrarily given values of one. For Na it equals approximately 19 pmoles cm^{-2} sec^{-1} and for K it equals approximately 18 pmoles cm^{-2} sec^{-1}. The components of the unidirectional fluxes are given as the appropriate fraction of the total flux. The Figure represents conditions of approximately 100 mM internal K, 70 mM internal Na. The superscripts refer to: *T*, the total flux; *P*, the ouabain sensitive pump components; *D*, the diffusional components; Na, the external Na-dependent component; K, the external K-dependent component, and *R*, the residual component. *See text* for further details

of a Na – Na exchange process. It is conceivable that the externally Na-dependent Na efflux which we observe represents one and the same system with an external as well as internal dependence on Na concentration. Our data suggest that, under physiological conditions, the externally Na-dependent Na efflux is not acting in a one for one exchange mode but rather as a net Na efflux. This is based on the calculation of the diffusional Na influx of these cells and the observation that this influx can account for the total influx obtained from the steady-state assumption. However, it should be emphasized that the magnitude of the diffusional component of Na influx is obtained from the value of P_{Na}, the membrane potential and the extracellular Na concentration. Based on the experimental error associated with determination of P_{Na} and membrane potential, a portion of Na influx could be nondiffusional. This, in conjunction with the presence of an external Na-dependent Na efflux, leaves open the possibility of a Na – Na exchange component. A dual function of the component would not be unique since the ability of exchange mechanisms to revert to net unidirectional fluxes has been observed. For example, Sachs (1971) has demonstrated in the human blood cell that a ouabain-insensitive, furosemide-sensitive, externally Na-dependent Na efflux acts as a Na – Na exchange mechanism at elevated extracellular Na but results in a net extrusion of Na at reduced extracellular Na. Garrahan and Glynn (1967) have demonstrated in the red blood cell that increases in external potassium result in the conversion of a Na – Na exchange mechanism to a Na – K exchange mechanism. It has also been

observed in Girardi cells that ouabain inhibits Na – K exchange and simultaneously increases Na – Na exchange, an observation consistent with the conversion of a pump to an exchange mechanism (Lamb & McCall, 1972).

As to our observations on K flux, previous experiments of Hempling (1958, 1972) are also pertinent. We have observed the presence of an external K-dependent K efflux which is maximal at approximately 50 mM intracellular K (Fig. 6). Upon further elevation of intracellular K, this component diminishes. Hempling (1958, 1972) observes the following. Cells maintained at 0 to 4 °C gain Na and lose K. Upon transition to elevated temperatures there is a large increase in the unidirectional K fluxes and coupled to them a net gain in potassium. Upon inhibition of the active Na – K pump, the net gain of K is eliminated but a large portion of the unidirectional K fluxes remain. Hempling (1972) has suggested these remaining K fluxes represent a K – K exchange mechanism since they do not contribute to net K flux. His experiments were run under conditions in which intracellular K levels were of the order of 30 to 50 mM. It is at this level of intracellular K that our present experiments indicate the presence of a large external K-dependent component of K efflux, which could conceivably represent a K – K exchange mechanism. The functional significance of the disappearance of this external K-dependent K efflux at elevated intracellular K is not clear. However, a variability in the magnitude of K – K exchange mechanisms has been previously reported. For example, the magnitude of K – K exchange is dependent on intracellular phosphate levels in the red blood cell. At reduced intracellular phosphate it is diminished whereas at elevated internal phosphate it is high (Glynn, Lew & Luthi, 1970). It is quite reasonable to assume that elevation of intracellular K in the Ehrlich ascites cell is accompanied by changes in other cellular components, among them factors which could alter the degree of the external K-dependent K efflux. Hempling (1972) has suggested that at low temperatures, which correlate with the reduced intracellular K, a large portion of the K flux is by an exchange mechanism, whereas at normal intracellular K it is primarily inward pump-mediated and outward diffusional. This is based on the observation that an initial lowering of temperature from 25 °C does not decrease K influx and efflux to the same extent but rather results in K loss. If the K-dependent K efflux observed in the present experiments represents an exchange mechanism, then our observations would support the concept of its reduction at normal temperatures, which correlate with normal intracellular K and its enhancement at lowered temperatures, which correlate with reduced intracellular K.

The present data also indicate that the external K-dependent K efflux observed at reduced intracellular K is ouabain-insensitive. Both ouabain-

sensitive and ouabain-insensitive exchange mechanisms have been observed. In Girardi cells Na – Na exchange components exist which are ouabain-sensitive and insensitive (Lamb & McCall, 1972). In human red blood cells, the phosphate dependent K – K exchange is ouabain-sensitive (Glynn *et al.*, 1970). However, in goat red blood cells, this component of K – K exchange is ouabain-insensitive (Dunham & Bleier, 1973).

In previous studies, inhibitors such as ethacrynic acid and furosemide have been used to identify the presence of ouabain-insensitive, nondiffusional components of Na and K flux (e.g., Hoffman & Kregenow, 1966; Sachs, 1971). In the present study we present evidence for the presence of such components in the Ehrlich ascites cell. We have subsequently observed that there exist components of Na and K flux in this cell that are sensitive to furosemide and these components are distinct from the ouabain-sensitive components. Furthermore, the magnitude of these furosemide-sensitive components is in reasonable agreement with the magnitude of the Na-dependent Na efflux and the K-dependent K efflux identified in the present study, suggesting they may be one and the same. These experiments will be presented in detail elsewhere.

The helpful comments of Mr. Douglas Powers and Dr. Philip Dunham are greatly appreciated. This work was supported by grants from the American Cancer Society and the Syracuse University Educational Improvement Fund.

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