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Intracellular Na^+, K^+ and Cl^- activities in Ehrlich ascites tumor cells

W. David Dawson and Thomas C. Smith *

Department of Physiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284 (U.S.A.)

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Ehrlich ascites tumor cell membrane potential (V_m) and intracellular Na^+, K^+ and Cl^- activities were measured under steady-state conditions in normal saline medium (Na^+ = 154, K^+ = 6, Cl^- = 150 mequiv./l). Membrane potential was estimated to be -23.3 ± 0.8 mV using glass microelectrodes. Intracellular ion activities were estimated with similar glass electrodes rendered ion-selective by incorporation of ion-specific ionophores. Measurements of V_m and ion-activity differences were made in the same populations of cells. Under these conditions the intracellular Na^+, K^+ and Cl^- activities are 4.6 ± 0.5; 68.3 ± 8.0; and 43.6 ± 2.1 mequiv./l, respectively. The apparent activity coefficients for Na^+ and K^+ are 0.18 ± 0.02 and 0.41 ± 0.05 respectively. These are significantly lower than the activity coefficients expected for the ions in physiological salt solutions (0.71 and 0.73, respectively). The activity coefficient for intracellular Cl^- (0.67 ± 0.03), however, is close to that of the medium (0.73), and the transmembrane electrochemical potential difference for Cl^- is not different from zero. The results establish that the energy available from the Na^+ electrochemical gradient is much greater than previously estimated from chemical measurements.

Introduction

Ehrlich ascites tumor cells have been useful in the study of unidirectional and net ion fluxes, ion movements during volume regulation, pH regulation, and co-transport of inorganic (e.g., $H_2PO_4^-$, H^+) and organic (e.g., amino acids) substrates. Complete evaluation of the results of these studies has been impossible, however, since quantitation of the energy gradients affecting these processes requires knowledge of the intracellular ion activities. These measurements have yet to be accomplished in the Ehrlich cell.

It has been postulated that a large fraction of the total cell Na^+ might be sequestered in the nucleus [1], thus reducing the cytoplasmic Na^+ activity. This possibility is based on experiments in which the nuclei of disrupted Ehrlich cells were centrifuged through a nonpolar solvent phase and their contents then analyzed for Na^+, K^+ and Cl^-. Approximately 90% of the total cell Na^+ was associated with the nuclear compartment. A different technique for isolating the nuclear compartment led to the conclusion that approximately 61% of the cell Na^+ was not associated with the cytoplasm [2]. However, the difficulty of isolating undamaged nuclei has prevented verification of these results as reliable estimates of intracellular ion compartmentation. In addition, binding of cations to polyanionic proteins and nucleic acids may also play an important role in regulation of the intracellular ion activity.

Previously estimates of free Na^+, K^+ and Cl^- concentrations in the cytoplasm have depended on measurements of these ions in cell extracts by means of flame photometry, atomic absorption spectrometry or, in the case of Cl^-, amperometric measurements. The methods do not account for

* To whom correspondence should be addressed.

possible intracellular compartmentation. The development of ion-specific resins [3,4] which can be incorporated into glass microelectrodes has made possible more direct measurements of intracellular ion activities. Estimates from a number of tissues using such ion-specific microelectrodes [5,6] show considerable compartmentation of intracellular ions, especially Na^+ and K^+. These observations have emphasized the need for corrections of the estimates of the ion electrochemical energy gradients.

We have previously measured membrane potentials in Ehrlich cells using glass microelectrodes filled with potassium acetate [7]. These potentials are stable (approximately -23.0 mV) and are sensitive to extracellular K^+, Ca^{2+} and valinomycin [2,7,8]. Using slightly modified techniques, we have constructued ion-specific microelectrodes for Na^+, K^+ and Cl^- [5]. These electrodes have been calibrated and used to determine the intracellular ionic activities in these cells.

Materials and Methods

Cell suspensions

Experiments were performed with Ehrlich ascites tumor cells (Lettré strain; hyperdiploid) which were maintained in Ha/ICR male mice by weekly transplantation. Tumor-bearing animals with growths between 10 and 12 days were used. Cells were removed from unanaesthetized animals by peritoneal aspiration and washed free of ascitic fluid. The wash and incubation medium was saline buffered to pH 7.4 with 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid). The ionic composition was: $[Na^+] = 154$ mM; $[K^+] = 6$ mM; $[Cl^-] = 150$ mM; $[Ca^+] = 2$ mM; and $[Mg^{2+}] = 0.2$ mM. Cell suspensions were incubated in 50 or 500 ml Erlenmeyer flasks under an air atmosphere at 21–24°C on a gyrorotary shaker set for 48 oscillations per min. In general, the cell suspensions were incubated in flasks large enough to insure a shallow, well aerated system.

Intracellular ion contents

The methods used to determine intracellular Na^+, K^+ and Cl^- contents by flame photometry (Na^+ and K^+) and amperometric titration (Cl^-) have been previously described [2]. Briefly, measurements of cellular ion contents were made on 1% perchloric acid extracts of cell pellets from either 1.0 or 0.2 ml aliquots of cell suspension. The Cl^- contents of the perchloric acid extract and incubation medium were analyzed with a Buchler-Cotlove auto-titrator, while Na^+ and K^+ contents were determined with a Beckman Klina flame photometer using Li^+ as an internal standard. All estimations of intracellular ion contents were corrected for trapped extracellular fluid using freshly dialyzed [^{3}H]methoxyinulin (ICN) as an extracellular space marker. Cellular water content was determined from wet and dry weights of the pellets [2].

Microelectrodes

Glass microelectrodes were prepared from Na^+ borosilicate microfilament capillaries (A-M Microsystems). The capillaries were pulled on a horizontal micropipette puller (Industrial Science Assoc.), yielding two tapered glass capillaries with tip diameters less than 0.2 microns. The barrels of these capillaries were then placed (tip-up) in a solution of 300 mM potassium acetate. The tip was filled by the wick action of the microfilament. The remainder of the electrode was completely filled with potassium acetate using a syringe and 30 gauge needle. Electrodes may be used immediately for potential measurements as previously described [8], or stored for several weeks in the potassium acetate solution.

Ion-selective microelectrodes

Capillaries identical to those used for conventional microelectrodes (described above) were used for the fabrication of ion-specific microelectrodes. Capillaries were pulled as before and then placed tip-up in a solution of 0.01–0.025% Dow-Corning 1107 silicone in acetone. The electrode tips filled within 10 s by capillarity. The electrodes were then removed from the solution and placed in a clean, dry 10 ml beaker and cured in an oven overnight at 100–120°C. After cooling and microscopic inspection these electrodes were placed tip-up in the desired ligand and once again allowed to fill by capillarity. Although the electrode tips fill within 30 min to 2 h depending on the ligand (Cl^--selective is the fastest, Na^+-selective is the slowest), a higher percentage of usuable electrodes

was obtained if they were allowed to fill overnight. After the electrode tips were filled with ion-selective resin, the electrodes were back-filled with either 1.0 M NaCl (Na^+- and Cl^--selective electrodes) or 300 mM potassium acetate (K^+-selective electrodes) and stored in these solutions overnight. With this method, eight to ten electrodes of each ion specificity could be made per day with 20–60% of these being usable for impalement.

Ion-selective ligands

Cl-selective ligand (Corning No. 477913) was obtained from Corning Medical, Medfield, MA. K^+-selective ligand (Corning No. 477317) was also obtained from Corning Medical and LIX-110 K^+-selective ligand was obtained from WPI, New Haven, CT. Both K^+-selective ligands gave similar results. Na^+-selective ligand was fabricated in our laboratory according to previously published methods [9]. Specifically, the unopened shipping vial containing 50 mg of Na^+ ligand I (Fluka, Switzerland) was centrifuged for 10 min at low speed. The vial was then opened and 177 μl 3-nitro-*o*-xylene (Aldrich Chemical Co., Milwaukee, Wisconsin) plus 2.5 mg sodium tetraphenylborate (Fluka) was added to Ligand I. This mixture represents a 2-fold greater concentration of the ligand than previously described [9]. Since the sodium tetraphenylborate dissolves slowly, the ligand was not used for at least 24 h after its preparation. All ligands were kept dry and in the dark at room temperature (approx. 22°C). There was no change in the performance characteristics of the ligands during the period of their use.

Measurement of the membrane potential

The membrane potential of Ehrlich ascites tumor cells was determined as previously described [7,8] under the same conditions used to evaluate the intracellular activities of Na^+, K^+ and Cl^-. Cell suspension, diluted 60 : 1 with saline medium, was added to the four quadrants of an X-plate. The cells were allowed to settle and attach to the bottom of the plate. This occurred in about 5 min. Attachment of the cells was facilitated by the presence of Ca^{2+}, but did not require special treatment of the X-plates. In addition, the presence of Ca^{2+} in the medium has been shown to alter cation permeabilities and to increase the membrane potential from approximately -10 mV (without Ca^{2+}) to -23 mV [8]. The effect of Ca^{2+} is maximal at 2.0 mM, the concentration used for all experiments in this study.

The X-plates containing diluted cell suspensions were placed on a modified microscope stage and observed with 100X magnification. The potential difference between cell cytoplasm and medium was recorded as previously described [8]. The potential recorded was considered a valid measurement of the membrane potential if three criteria were met:

(1) the potential changed abruptly upon insertion of the electrode;

(2) the potential was stable for at least 30 s;

(3) upon withdrawal of the electrode the potential returned to its pre-impalement value.

Approx. 60% of the attempts at cell impalement yielded responses which satisfied these criteria. When they were not fulfilled the measurement was rejected.

The cell membrane resistance (R_m) was also estimated during an impalement. Typically, total circuit resistance increased by about 20 MΩ following impalement. During stable membrane potential recordings, the membrane resistance could be measured several times. An unchanging resistance was taken as further evidence for the absence of electrical shunting (leakage) around the electrode.

Estimation of intracellular ion activities

The potential of a perfectly selective ion specific electrode may be written as:

$$V = V_o + S \log a_i \quad (1)$$

where V_o (mV) is a constant and S (mV) is the change in the output of the electrode that corresponds to a 10-fold change in ion activity, a_i. If the electrode is not perfectly ion-selective, but has finite responsiveness to other ions in a mixed solution, the potential output is a more complex function of the solution and electrode characteristics [10]. For example, if the electrode is responsive to two monovalent ions, i and j, in a mixture and responds to 10-fold changes in either i or j with the same potential change, S, then the output is given by:

$$V = V_o + S \log(a_i + k_{ij} a_j) \quad (2)$$

where k_{ij} is the selectivity coefficient of the elec-

trode for j with respect to i. The characteristics of the electrode (S and k_{ij}) can be estimated from separate calibrations in solutions containing purely i or j, or by recalibrating in solutions of known, fixed activities of interfering ions. The cation-specific electrodes used for these studies were highly selective (Na^+-selective: $k_{Na:K}$ between 0.02 and 0.03; K^+-selective: $k_{K:Na}$ between 0.016 and 0.02) and yielded calibration responses in pure solutions with S in the range 55–60 mV, compared to a value of 58.0 mV expected for an ideal electrode. The Cl^--selective electrodes gave calibration responses with S values somewhat less than the ideal (range 48–55 mV), even though the calibrating solutions were nominally free of interfering anions. The basis for the deviation from ideality was not explored. Under the incubation conditions used for these studies (dilute cell suspension containing Cl^- as the only anion), the major intracellular anion that might significantly interfere with determination of cellular Cl^- activity is phosphate. However, addition of 10 mM total phosphate to the calibration solutions was without measureable effect on the electrode output, suggesting minimal interference. Selectivity toward other anions was not tested.

Even though ion selective electrodes may exhibit nearly ideal behavior, their use to estimate intracellular activity is complicated by two factors. First, the intracellular output includes the membrane potential, V_m. If reliable estimates of V_m can be obtained, the total change in electrode output can be corrected for its contribution. However, for an ion in electrochemical equilibrium a special problem arises, since the electrode response to altered ion activity upon impalement may be exactly matched by the contribution of V_m [5]. Thus, a perfectly satisfactory impalement may give no electrical signature, obviating application of our normal criteria for validating measurements. Resolution of this problem is considered later with respect to estimates of intracellular Cl^- activity. Second, when advancing the electrode into the cell not only does the activity of the ion of interest change, but also the activity of the interfering ions. The total change in electrode potential (ΔV_t) is given by:

$$\Delta V_t = S \log\{[a_i^c + k_{ij}a_j^c]/[a_i^m + k_{ij}a_j^m]\} + V_m \qquad (3)$$

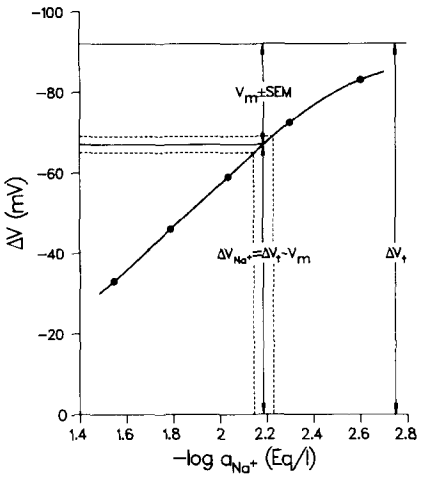

Fig. 1. Na^+-selective electrode calibration and use for intracellular ion activity estimates. For calibration, the electrodes were first immersed in the saline bathing solution and the electrode output was taken as zero. Electrode output was then determined in test solutions of known a_{Na^+} (2.0 to 21.0 mequiv./l) in the presence of 60 mequiv./l K^+ to mimic the intracellular a_{K^+}. The calibration curve is shown. Insertion of the electrode into a cell yields a potential change (ΔV_t) reflecting both the intracellular a_{Na^+} and V_m. Correction of the response for the contribution from V_m permits the a_{Na^+} corresponding to ΔV_{Na^+} to be read directly from the calibration curve.

where the superscript c and m indicate cellular and medium solutions, respectively. If S differs for the ions, or k_{ij} is a function of activity, then the use of the calibration characteristics determined from calibration curves in solutions of fixed contents is inappropriate for the calculation of intracellular ion activities [5]. This proved to be the case in the present studies for the use of Na^+-selective electrodes. Even though $k_{Na:K}$ ranged between 0.02 and 0.03, the electrode response to a_{Na} (2–20.9 mequiv./l) in the presence of $a_K = 60$ mequiv./l (to mimic intracellular activity) was not linear. This non-linearity may have reflected either altered responsiveness to Na^+ as opposed to K^+ (difference in S) or dependence of $k_{Na:K}$ on a_{K^+}.

Consequently, for measurement of intracellular ion activities we have adopted the method of mixed solutions [5]. A typical calibration curve for a Na^+ selective electrode is shown in Fig. 1. Each

electrode was calibrated by measuring its response to varying ionic activities in the presence of possible interfering ions. The concentrations of possible interfering ions were selected to mimic those estimated for the cell cytoplasm. Prior to calibration, each electrode potential was set to 0 millivolts (mV) when immersed in the normal saline medium. In this way deviations from an ideal response would be automatically included in the calibration curve for the electrode [5]. Na^+-selective electrodes were calibrated in solutions containing 60 mequiv./l KCl and Na^+ activities from 2.0 to 21.0 mequiv./l. K^+- and Cl^--selective electrodes were calibrated in solutions with activities of 13–110 mequiv./l in the presence of 20 mequiv./l NaCl. Each electrode calibration was repeated at least five times and electrodes with deviations greater than 1 mV were not used.

The ionic activities of the calibration solutions were calculated as described by Fujimoto and Kubota [11]. The activity coefficients (γ_{ion}) for the ions in the incubation medium were 0.73 for K^+ and Cl^- and 0.71 for Na^+.

Results

Membrane potential measurements

In the present study the membrane potential of Ehrlich ascites tumor cells has been measured using microelectrodes filled with 300 mM potassium acetate. We have previously demonstrated that the use of potassium acetate instead of KCl as a filling solution yields measurements of improved stability. We have attributed the improvement to elimination of leakage of the filling solution into the cell [7]. Tip potentials of these electrodes range from 0 to 10 mV, and tip resistances are about 30–40 MΩ. A typical potential recording is shown in Fig. 2a. Stable membrane potentials of steady-state Ehrlich cells have been recorded for as long as 15 min. During this time neither changes in membrane integrity nor cell morphology are apparent from visual inspection or electrical characteristics. The average membrane potential of these cells is -23.3 ± 0.8 mV (S.E.; $n = 66$). This value is in agreement with those from previous electrophysiological studies [2,7,8] and with estimates based on the Cl^- equilibrium distribution [8,12].

Intracellular ion activities in steady-state Ehrlich cells

Representative recordings of impalements with K^+- and Na^+-selective microelectrodes are shown in Fig. 2b and 2c. The criteria used for accepting ion activity measurements were: (1) an initial sharp deflection upon impalement to a new, relatively stable potential; and (2) following withdrawal of the electrode, a return of the potential to its pre-impalement value. Based on these criteria, an ion-selective electrode would be calibrated, used for several impalements (6–12), and then recalibrated to insure that no changes in the electrical characteristics had occurred.

Upon impalement of a cell with a ion-selective electrode, an immediate potential change (ΔV_t) is recorded. Two factors contribute to the magnitude of this change: (1) the ion activity difference between medium and cell (ΔV_{ion}); and, (2) the membrane potential difference (V_m). To isolate the change in response due to the difference in ion activity, V_m must be measured and subtracted from the total response, ΔV_t. The potential change resulting from the ion activity difference (ΔV_{ion}) is

$$\Delta V_{ion} = \Delta V_t - V_m \qquad (4)$$

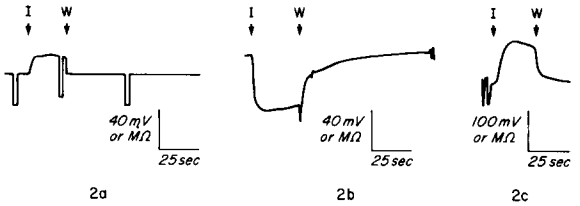

Fig. 2. (a) Reproduction of an oscilloscopic record of the membrane potential ($V_m = -22.4$ mV) of an Ehrlich ascites tumor cell. The measured resistance (R_m) increases by 10 MΩ during impalement. The total time course of this impalement if 20 s. (b) Reproduction of an oscilloscopic record of the potential change measured with a K^+-selective microelectrode. The maximum potential change is 50 mV and is of opposite polarity to V_m. Since the K^+-selective electrode measures both the ion activity difference and the membrane potential difference, V_m must be added to the recorded change to obtain the potential change (V_{ion}) reflecting the K^+ activity difference. The time course of this measurement is 30 s. (c) Typical oscilloscopic record of the potential change found with use of a Na^+-selective microelectrode. The maximum potential change here is 100 mV and is of same polarity as V_m. Thus, V_m is subtracted from the Na^+-selective electrode measurements to obtain the potential change (V_{ion}) due to the Na^+ activity difference. The time course of this measurement is 30 s.

The small size of Ehrlich tumor cells (diameter ≈ 12 μm) renders it difficult to reliably measure ΔV_t and V_m in the same cell, since this would require impalement with separate electrodes. Thus, the average V_m of the population of cells was measured and used to correct ΔV_t. Typical results obtained using this method are shown in Fig. 1 for a Na^+-selective electrode. After ΔV_t is measured in 6–12 cells, a mean is calculated. This value is then corrected for the contribution of the membrane potential (V_m). The intracellular activity that corresponds to a_{Na^+} can then be read directly from the axis representing the ion activity.

The impalement of cells with the cation-selective electrodes yielded large changes in the electrode output (Na^+-electrode: average $\Delta V_t = -92.0 \pm 2.0$ mV (S.E.; $n = 25$); K^+-electrode: average $\Delta V_t = 36.0 \pm 1.0$ mV (S.E.; $n = 32$)). Consequently, the criteria for establishing the validity of the measurements could be clearly applied. Approx. 60% of our attempted impalements using these electrodes gave potential responses consistent with these criteria. In the case of Cl^--selective electrodes, however, the success of attempted impalements could not be so clearly judged. A considerably larger percentage of the attempts, compared to cation-selective or V_m measurements, resulted in no significant change in electrode output. As has been previously noted [5], a perfectly selective, ideal electrode would show no response for an ion in electrochemical equilibrium. However, since we have no criterion for validating the impalements in the absence of electrical signals, we did not include any null responses for estimating a_{Cl^-}. The average ΔV_t from those impalements which resulted in measureable changes was -2.9 ± 0.6 mV (S.E.; $n = 16$). It is notable that these were obtained with electrodes showing S values less than that expected for ideal electrodes. Only these estimates were used to evaluate a_{Cl^-}.

Table I gives the results from the measurements of ion contents and ion activities in steady-state cells. The intracellular K^+ activity is 68.3 ± 8.0 mequiv./l, while that for Na^+ is 4.6 ± 0.5 mequiv./l. Coupled with the measurements of total ion contents, these values yield apparent activity coefficients (γ_{app}) of 0.41 ± 0.05 and 0.18 ± 0.02 for K^+ and Na^+, respectively, in the intracellular compartment. These estimates are similar to those made in other cells [9,13,14] and reflect cation compartmentation and/or binding to macromolecules. Neither Na^+ nor K^+ is in electrochemical equilibrium. The Cl^- activity is 43.6 ± 2.1 mequiv./l. Compared to the chemically measured Cl^- (65.3 mequiv./l), this yields an apparent activity coefficient of 0.67 ± 0.03, close to that in the physiological medium (0.73). Thus, under the steady-state conditions there is little indication for compartmentation. Furthermore, application of the Nernst equation to the transmembrane Cl^- distribution yields an equilibrium potential of -23.4 mV. This is not different from the membrane potential and is consistent with our finding that attempts to impale the cells with the Cl^--selective electrodes frequently resulted in no change in the electrode output.

TABLE I

ACTIVITIES, AVERAGE CONCENTRATIONS, AND APPARENT ACTIVITY COEFFICIENTS FOR INTRACELLULAR Na^+, K^+ AND Cl^- IN EHRLICH ASCITES TUMOR CELLS

Ehrlich ascites tumor cells were incubated in physiological saline medium at 21–24°C to establish steady-state cellular ion contents. The intracellular ion activities were measured using ion-selective microelectrodes. The average intracellular concentrations of the ions were determined by spectrophotometry (for Na^+ and K^+) and amperometrically (for Cl^-). The apparent activity coefficients (γ_{ion}^{app}) were calculated as the ratio of activity to concentration. Data represent mean ± S.E. for activity and average concentration determinations. Numbers of observation are given in parentheses.

Ion	Intracellular concentration (mequiv./l)	Intracellular activity (mequiv./l)	γ_{ion}^{app}
Na^+	25.3 ± 0.5	4.6 ± 0.5 (25)	0.18 ± 0.02
K^+	165.1 ± 1.2	68.3 ± 8.0 (32)	0.41 ± 0.05
Cl^-	65.3 ± 0.4	43.6 ± 2.1 (16)	0.67 ± 0.03

Discussion

Ion-selective microelectrodes have been used to estimate the intracellular ion activities in a number of cell systems [5,6,14]. In this study similar ion-selective electrodes have been employed to evaluate the intracellular activities of Na^+, K^+ and Cl^- in Ehrlich ascites tumor cells. Steady-state

cells incubated in normal saline have intracellular activities of 4.6 ± 0.5 and 68.3 ± 8.0 mequiv./l, respectively, for Na^+ and K^+. Comparison of these values to estimates based on chemical measurements ($[Na^+] = 25.3$ mequiv./l cell water; $[K^+] = 165.1$ mequiv./l cell water) yields apparent activity coefficients of 0.18 ± 0.02 and 0.41 ± 0.05 for Na^+ and K^+, respectively. Activity coefficients less than unity indicate cellular compartmentation, although our results do not permit identification of the compartments (e.g., sequestration by organelles or binding to macromolecules). Similar findings have been reported in epithelial cells [5,6] and in cardiac muscle [13].

Ehrlich cells incubated in normal saline have a $[Cl^-]$ of 65.3 ± 0.4 mequiv./l cell water when measured amperometrically. Impalement of Ehrlich tumor cells with Cl^--sensitive electrodes, gives an estimation of 43.6 ± 2.1 mequiv./l for cell Cl^- activity (apparent activity coefficient = 0.67 ± 0.03). Substitution into the Nernst equation (medium Cl^- activity is 109.5 mequiv./l) predicts an equilibrium potential of -23.4 mV, which is not different from the membrane potential (-23.3 mV) measured with potassium acetate-filled microelectrodes. The conclusion that Cl^- is in electrochemical equilibrium is consistent with our current understanding of Cl^- transport in steady-state Ehrlich ascites cells. Of the three mechanisms for Cl^- movement which have been characterized (i.e., conductive, anion exchange and cation-dependent co-transport [15]), only the cation-dependent co-transport of Cl^- with Na^+ and K^+ envisions coupling to energy sources which might establish non-equilibrium conditions. However, it is well established that inhibition of the co-transport by furosemide is without effect on the Cl^- distribution in steady-state cells; only unidirectional Cl^- fluxes are altered (cf. Ref. 15). It seems apparent that under these conditions Cl^- is not maintained out of electrochemical equilibrium.

Taken together, the results indicate that 59% of the intracellular K^+ and 82% of the intracellular Na^+ do not contribute to the free cation pools. On the other hand, these results also show that γ_{Cl}^{app} is not different from that in the medium and Cl^- is in electrochemical equilibrium. Consequently, at least under steady-state conditions, chemical estimates of the Cl^- distribution accurately predict the membrane potential.

The demonstration of apparent Na^+ and K^+ compartmentation in these cells has important implications concerning processes which depend on their electrochemical potential gradients [1,2]. Two examples serve to emphasize the consequences. First, estimates of V_m which use indirect methods, such as the accumulation of fluorescent dyes or lipophilic ions, generally give values greater than those obtained by direct electrophysiological techniques [7,16,17]. This difference can be appreciated by comparing the V_m obtained in the present study (-23.3 ± 0.8 mV) to the estimates achieved using carbocyanine dyes (about -60 mV) [16,17]. As we have previously pointed out, part of this difference undoubtedly reflects altered cellular metabolism in the presence of the dyes [20]. Our finding that intracellular K^+ shows significant compartmentation offers an additional explanation for the discrepancy. The indirect methods rely on knowledge of the K^+ equilibrium potential for calibration, using chemical measures of cellular K^+ for its calculation. Clearly this procedure will lead to a serious over-estimation of the calibrating potential. While the experimental conditions in the present studies are not fully comparable to those using the indirect potential probes, we can achieve a rough evaluation of the magnitude of the over-estimation using the data in Table I. Regardless of the $[K^+]$ in the medium, calculation of the K^+ equilibrium potential using the chemical measurement for cellular K^+ (165.1 mequiv./l) will over-estimate the true value (based on $a_{K^+} = 68.3$ mequiv./l) by 22.6 mV. The effects of this mis-calibration on the derived value for V_m under physiological conditions need to be more thoroughly considered in future studies which rely on K^+ equilibrium potentials. Second, the Na^+-gradient hypothesis for amino acid transport in these cells envisions that the energy source is the Na^+ electrochemical potential gradient [1,18]. However, all attempts to demonstrate that the Na^+ gradient contains sufficient energy have failed. It is of interest that previous studies have suggested that if the Na^+ gradient is the energy source, then γ_{Na}^{app} would need to be about 0.40 [19]. In light of our finding that approximately 82% of intracellular Na^+ is not electrochemically active, the energy balance needs to be reexamined.

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