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Intracellular Compartmentation of Na^+, K^+ and Cl^- in the Ehrlich Ascites Tumor Cell: Correlation with the Membrane Potential

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Summary. The intracellular distribution of Na^+, K^+, Cl^- and water has been studied in the Ehrlich ascites tumor cell. Comparison of the ion and water contents of whole cells with those of cells exposed to La^{3+} and mechanical stress indicated that La^{3+} treatment results in selective damage to the cell membrane and permits evaluation of cytoplasmic and nuclear ion concentrations. The results show that Na^+ is sequestered within the nucleus, while K^+ and Cl^- are more highly concentrated in the cell cytoplasm. Reduction of the $[Na^+]$ of the incubation medium by replacement with K^+ results in reduced cytoplasmic $[Na^+]$, increased $[Cl^-]$ and no change in $[K^+]$. Nuclear concentrations of these ions are virtually insensitive to the cation composition of the medium. Concomitant measurements of the membrane potential were made. The potential in control cells was -13.7 mV. Reduction of $[Na^+]$ in the medium caused significant depolarization. The measured potential is describable by the Cl^- equilibrium potential and can be accounted for in terms of cation distributions and permeabilities. The energetic implications of the intracellular compartmentation of ions are discussed.

Ehrlich ascites tumor cells actively extrude Na^+ and accumulate K^+, and as a consequence maintain these ions away from their electrochemical equilibria (Hempling, 1958; Maizels, Remington & Truscoe, 1958). The cells also avidly accumulate certain amino acids, achieving intracellular levels at steady state manyfold higher than that in the extracellular medium (Christensen & Riggs, 1952; Schultz & Curran, 1970). Such an accumulation of solute against its electrochemical gradient requires an input of energy. The sensitivity of amino acid transport to the $[Na^+]$ in the medium (Christensen & Riggs, 1952; Christensen, Riggs, Fischer & Palatine, 1952; Kromphardt, Grobecker, Ring & Heinz, 1963), as well as concomitant changes in Na^+ and K^+ movements (Riggs, Walker & Christensen, 1958), have led to the postulation of the "ion-gradient hypothesis" of amino acid transport.

According to the "ion-gradient hypothesis" the energy for amino acid transport is derived directly from the electrochemical gradients of Na^+ and K^+, which in turn are maintained by the active cation transport system (Riggs, Walker & Christensen, 1958; Eddy, Mulcahy & Thomson, 1967). Indeed, Eddy (1968*a, b*), by investigating the role of ion gradients in metabolically inhibited cells, demonstrated that cation gradients can be used to energize, at least partially, amino acid accumulation. However, the continued uptake of amino acids under conditions of reversed cation gradients (Shafer & Jacquez, 1967; Jacquez & Shafer, 1969; Shafer & Heinz, 1971) and the correlation of amino acid transport with cellular ATP content (Potashner & Johnstone, 1971; Johnstone, 1974) question the importance of the cation gradients as sources of energy for amino acid transport.

The resolution of how much energy, if any, cation gradients contribute to drive amino acid movements requires quantitation of the energy available from the gradients. Such a quantitation in turn requires: (a) accurate measurement of the ion concentrations within the intracellular transport compartment; and (b) precise determination of the membrane potential. There is still lack of agreement among investigators concerning both of these parameters.

With respect to intracellular ion concentrations, Pietrzyk and Heinz (1974) have pointed out that all of the evidence indicating insufficient cation energies to account for amino acid transport is based on the assumption that no intracellular compartmentation of ions occurs. These authors have studied the possibility of partition of intracellular ions between nuclear and cytoplasmic compartments. Using lyophilized, fractionated Ehrlich cells, they found that the major part of Na^+ and Cl^- was closely associated with DNA, indicating appreciable sequestration in the nucleus. Consequently, the assumption of a single cellular compartment would lead to an overestimation of the $[Na^+]$ in the cytoplasm. Unfortunately, lyophilized, fractionated cells may not show ionic partitioning which is representative of viable, metabolically active cells. No successful direct determination of nuclear or cytoplasmic ion contents has been previously reported.

The value of the membrane potential under any of the experimental conditions used is similarly uncertain. In general, three different methods have been applied to estimate the membrane potential of the Ehrlich ascites tumor cell. Direct measurements of the membrane potential using intracellular microelectrodes have yielded values of -20 to -40 mV (Johnstone, 1959; Lassen, Nielsen, Pape & Simonsen, 1971) or -8 to

−12 mV (Hempling, 1962; Aull, 1967; Smith, Mikiten & Levinson, 1972; Smith & Levinson, 1975). Determinations of the transmembrane concentrations of the presumably passively distributed Cl^- (Simonsen & Nielson, 1971; Mills & Tupper, 1975) and dibenzyldimethylammonium cation (de Cespedes & Christensen, 1974) give estimates of −20 to −30 mV. These studies, however, assume that the passively distributed ions are not compartmentalized within the cell. Finally, the fluorescent intensity of the potential sensitive dye, 3,3′-dipropylthiodicarbocyanic iodide, has been monitored in these cells (Laris, Pershadsingh & Johnstone, 1976). These studies yielded values of −18 to −42 mV, but the potentials were sensitive to changes in experimental technique.

In the present studies we report a technique for the estimation of intracellular partition of ions between the cell cytoplasm and nucleus in viable, transporting cells. We have applied the method to investigate the effects of variation of medium $[Na^+]$ and $[K^+]$ on the cytoplasmic and nuclear $[Na^+]$, $[K^+]$ and $[Cl^-]$. Concomitant measurements of the membrane potential using intracellular microelectrodes were made. Results of these studies show: (1) Na^+ is significantly more concentrated within the cell nucleus than in the cytoplasm, while K^+ and Cl^- are significantly more concentrated in the cytoplasm; (2) reduction of $[Na^+]$ in the medium by K^+ substitution results in a reduction in cytoplasmic $[Na^+]$ and an increase in cytoplasmic $[Cl^-]$; (3) the direct measurement of the membrane potential is describable by the Nernst potential of the Cl^- distribution between cytoplasm and medium; and (4) the membrane potential can be predicted by the Na^+ and K^+ distribution using measured values for cytoplasmic cation concentrations.

Materials and Methods

Experiments were performed on Ehrlich-Lettré ascites tumor cells (hyperdiploid) which were maintained in HA/ICR mice by weekly transplantation. Tumor-bearing animals with growths between eight and eleven days (average cell diameter: 12–16 μ) were used. Cells were removed from the peritoneal cavity and washed free of ascitic fluid in Na/K salt solution (154 mM NaCl, 6 mM KCl, 10 mM HEPES-NaOH buffer; pH 7.3–7.4 and 300–310 mosm) at room temperature (21–23 °C).

Solutions

Stock La^{3+} solution, 0.10 M, was prepared at least twice a week by dissolving $LaCl_3$ (K & K Laboratories) in 10 mM HEPES-NaOH (Sigma Chemical Company). The solution was clarified by titrating it with concentrated HCl; the resulting osmolarity was

305–315 mosm. Addition of 20 μl of the La^{3+} stock solution to 1.0 ml Na/K salt solution reduced the pH of the salt solution 0.1 unit. Stock salt solutions were prepared to the following compositions: Na^+-free salt solution (0 Na^+, 156 mM KCl, 10 mM HEPES-NH_4OH; pH 7.3–7.4 and 295–305 mosm); 50 mM Na^+ salt solution (50 mM NaCl, 106 mM KCl, 10 mM HEPES-NH_4OH; pH 7.3–7.4 and 300–310 mosm). ^{14}C-inulin was obtained from New England Nuclear.

Tumor Cell Electrolytes

Previous attempts to determine intracellular partition of electrolytes in Ehrlich ascites tumor cells were aimed at completely disrupting the cells and recovering intact nuclei (Pietrzyk & Heinz, 1974). These investigators were unable to accomplish disruption without damaging the nuclei. Consequently, they chose to disintegrate and fractionate the cells with nonaqueous solvents, relating ion contents of the fractions to the corresponding DNA content as a measure of ion partition.

We have adopted a different approach to study ion partition between the cell nucleus and cytoplasm. If the plasma membrane could be selectively damaged to permit loss of cytoplasmic contents without damage to the nuclear membrane, then analysis of the remaining contents should offer an estimate of nuclear electrolytes. Likewise, a comparison of total ion contents of undamaged cells to those of damaged cells will yield an estimate of cytoplasmic electrolytes. A method to achieve selective damage to the plasma membrane is suggested by investigations previously reported from our laboratories (Levinson, Mikiten & Smith, 1972; Levinson, Smith & Mikiten, 1972). In those studies we showed that exposure of cells to La^{3+} caused losses of cellular electrolytes and water. It was further shown that these losses occurred only upon mechanical resuspension of the cells during the washing procedure. We concluded (Levinson, Smith & Mikiten, 1972) that La^{3+} predisposes the cell membrane to become highly permeable when subjected to mechanical stress.

Consequently, for the present studies, we hypothesized that exposure of tumor cells to La^{3+} (2 mM) followed by mechanical stress would result in loss of cytoplasmic electrolyte content.

Experiments were performed to assess the effect of altered Na^+ and K^+ content of the incubation medium on tumor cell electrolytes. Cells, which had been washed free of ascitic fluid in Na/K salt solution, were resuspended in this same solution (25–30 ml; $1.0–1.5 \times 10^8$ cells/ml). Three 7.0 ml aliquots of the cell suspension were transferred to Ehrlenmeyer flasks containing 7.0 ml of either Na/K salt solution, 50 mM Na^+ salt solution or Na^+-free salt solution plus 50 μl of a solution containing 0.5 μmoles inulin and 0.5 μC carrier-free ^{14}C-inulin. The compositions of the resulting media, which will subsequently be referred to as Na/K HR, 100 Na HR and 75 Na HR, respectively, are given in Table 1. The flasks were placed on a gyrorotary shaker set for 48 oscillations per minute and the cell suspensions incubated under an air atmosphere at 21–23 °C for 1 hr. Preliminary experiments showed that the cells achieved steady-state electrolyte content within 45 min under these conditions.

After the incubation period, duplicate 1.0 ml aliquots of cell suspension were removed from each flask and transferred to 12 ml centrifuge tubes containing 20 μl of the appropriate incubation medium. Duplicate 1.0 ml aliquots were also transferred to 12 ml centrifuge tubes containing 20 μl 0.10 M La^{3+} stock solution (final [La^{3+}] was approximately 2 mM). The samples were mixed on a vortex mixer and allowed to stand. After 1 min 10 ml ice-cold, isosmotic choline dehydrogen citrate solution (CDHC) (30 g CDHC in 900 ml H_2O, pH adjusted to 7.3 with 58% NH_4OH and made to 1 liter) was added to each tube and the tubes centrifuged for 30 sec at $2200 \times g$ and stopped by mechanical braking.

Table 1. Composition of the incubation media

Medium	Na^+	K^+	Cl^-
		(mmoles/liter)	
Na/K HR	154.0	6.0	144.5
100 Na HR	98.9	59.7	148.6
75 Na HR	77.0	81.0	150.3

All media were buffered to pH 7.3–7.4 with 10 mM HEPES-NaOH or 10 mM HEPES-NH_4OH. The osmolarities were 295–315 mosm.

The supernatant was decanted and the packed cells rapidly washed twice with ice-cold CDHC. During the washing procedure, resuspension of the control (non-La^{3+}-treated) cells was accomplished by mixing them with wooden applicator sticks while the La^{3+}-treated cells were resuspended once by mixing on a vortex mixer and once with sticks. The entire washing procedure took 3.5–4 min. The packed, washed cell pellets were mixed with 3.5 ml of 5% (v/v) ice-cold perchloric acid and kept in an ice-bath for 1 hr. All samples were subsequently centrifuged for 5 min to remove the perchloric acid insoluble residue. Na^+ and K^+ contents of the clear perchloric acid extracts were measured by flame photometry, using $LiNO_3$ as the internal standard, while Cl^- was estimated with the Buchler-Cotlove automatic titrator.

Extracellular Fluid Volume, Wet and Dry Weights

To determine the volume of the extracellular fluid phase, four 1.0 ml aliquots of the cell suspension containing ^{14}C-inulin were transferred from each flask into preweighed plastic tubes (1.5 ml capacity; Brinkman Instrument Co.) containing 20 µl of the appropriate medium. Four 1.0 ml aliquots were also transferred from each flask into preweighed tubes containing 20 µl La^{3+} solution. The tubes were mixed using a vortex mixer and centrifuged for 4 min at $11{,}000 \times g$. The supernatants were carefully removed and saved. After weighing, half of the tubes were dried at 95–100 °C for 20 hr for dry weight determinations. The pellets in the remaining tubes, along with aliquots of the supernatants, were transferred to scintillation vials containing 10 ml scintillation fluid (20% Beckman Bio-Solv Solubilizer, 80% toluene, 7 g/liter 2,5-diphenyloxazole) and the radioactivity measured with a Beckman liquid scintillation spectrometer (Model LS-355). The volume of ^{14}C-inulin distribution (inulin space, liters/kg wet wt) was determined from

$$\text{Inulin space} = \text{CPM in Pellet}/[(\text{CPM/ml supernatant}) \times (\text{wet wt of pellet})]. \quad (1)$$

In determining inulin space it is usual to assume that cell membranes are impermeable to inulin. Thus inulin space is taken as a measure of extracellular fluid volume. In the present studies we have hypothesized that La^{3+} treatment damages the plasma membrane, rendering it permeable to inulin. Consequently, the inulin space will include any volume not bounded by intact cellular or intracellular membranes. For simplicity we will refer to the inulin space as extracellular fluid volume, recognizing that it includes cellular compartments bounded by membranes permeable to inulin.

Effect of Washing Procedure on Cell Electrolyte Contents

It is possible that control (undamaged) and La^{3+}-treated (damaged) cells exhibit differing properties of electrolyte retention during exposure to the CDHC wash procedure.

Table 2. Efflux rate coefficients (k_e) into ice-cold choline dihydrogen citrate (CDHC)

Experimental conditions	k_e ($min^{-1} \times 10^2$)		
	(Na^+)	(K^+)	(Cl^-)
Incubation in test media, washed in CDHC	2.9±0.3	2.5±0.3	4.1±0.5
Incubation in test media, exposed to La^{+3}, washed in CDHC	5.8±0.5	4.2±0.5	3.4±0.4

Errors are SEM. Values are averages for five experiments.

Consequently, in the determination of electrolyte contents of the steady-state cells and La^{3+}-treated cells, it was important to estimate nonspecific losses of electrolytes which occur during the washing procedure. Experiments were designed to measure ion losses into cold CDHC. The methods followed were the same as those described above for measurement of tumor cell electrolytes. However, instead of washing the cells as quickly as possible in ice-cold CDHC, the cells were allowed to stand for specified times, from 30 sec to 5 min after the initial centrifugation and resuspension in CDHC. The washing process was then completed, the total washing time noted and the cells analyzed for electrolyte content as before. Concomitant determinations of cell wet and dry weights were made as previously described.

Plots of the electrolyte contents of the cell were made as a function of total washing time according to the equation:

$$\ln X(t) = \ln X(0) - k_e t \tag{2}$$

where X represents the electrolyte content (mEquiv/kg dry wt) of the cells after total washing time (t). The relationships under all experimental conditions (Na^+, K^+ or Cl^-; control or La^{3+}-treated; all test media) were describable as straight lines ($r > 0.90$). Thus, the slopes of the lines (k_e) were taken as representative of the efflux rate coefficients for washout into CDHC and were used to correct all cellular electrolyte contents (mEquiv/kg dry wt) to time zero, the initiation of the washing procedure. The values are given in Table 2.

Membrane Potential

Membrane potential measurements were made using conventional Ling-Gerard glass microelectodes filled with 3 M KCl (tip diameter < 0.2 μ). The apparatus and techniques employed have been described in detail (Smith *et al.*, 1972; Smith & Levinson, 1975).

Corrections of the experimentally determined values of the membrane potential for the influence of junction potential were made by applying a modified form of the Henderson equation (Barry & Diamond, 1970). For these calculations we used the ion mobility ratios $U_{Na}/U_K/U_{Cl} = 0.682:1.0:1.038$ and activity coefficients 0.57 and 0.75 for 3 M KCl, and medium and cytoplasm concentrations, respectively (Moore, 1965). The concentrations of the ions in the incubation medium and the cell cytoplasm were those given in Tables 1 and 3C. The junction potential corrections for cells incubated in Na/K HR, 100 Na HR and 75 Na HR are −2.1 mV, −1.6 mV and −1.2 mV, respectively.

Table 3. Electrolyte and water contents of Ehrlich ascites tumor cells incubated in media of varying $[Na^+]$ and $[K^+]$

Medium	Na^+	K^+ (mEquiv/kg dry wt)	Cl^-	H_2O (liters/kg dry wt)	$[Na^+]$	$[K^+]$ (mM)	$[Cl^-]$
Section A: Control Cells							
Na/K HR	71.0±2.8	507.3±9.1	264.1±3.7	3.87±0.04	18.3±0.7	131.1±2.7	68.2±1.2
100 Na HR	53.7±3.9	514.9±5.4	310.1±6.0	4.02±0.03	13.4±1.0	128.1±1.6	77.1±1.6
75 Na HR	50.0±4.1	518.4±8.7	343.1±6.2	4.09±0.02	12.2±1.0	126.7±2.2	83.9±1.6
Section B: Cells exposed to La^{3+} for 1 min and mechanically stressed							
Na/K HR	43.5±3.6	89.4±9.1	52.0±6.5	1.44±0.02	30.2±2.5	62.1±6.4	36.1±4.5
100 Na HR	36.5±1.4	92.9±10.8	56.0±6.3	1.56±0.01	23.4±0.9	59.6±6.9	35.9±4.0
75 Na HR	39.3±2.6	103.9±12.1	60.1±8.4	1.58±0.02	24.9±1.7	65.8±7.7	38.0±5.3
Section C: Subtraction of corresponding electrolyte and water contents in Section B from those in Section A							
Na/K HR	27.5±4.6	417.9±12.9	212.1±7.5	2.43±0.04	11.3±1.9	172.0±6.0	87.3±3.4
100 Na HR	17.2±4.1	422.0±12.1	254.1±8.7	2.46±0.03	7.0±1.7	171.5±5.3	103.3±3.8
75 Na HR	10.7±4.0	414.5±14.9	283.0±10.4	2.51±0.03	4.3±2.0	165.1±6.3	112.7±4.4

Values are averages for 10 experiments, 20 observations. Errors in Sections A and B are SEM; errors in Section C are most probable errors of the calculated values.

Results

Tumor Cell Electrolyte and Water Content

The effect of incubation of tumor cells in media of varying Na^+ and K^+ concentrations on total cell ion contents is shown in columns 2–4 of Table 3A. Total cellular Na^+ decreases significantly as Na^+ is replaced by K^+ in the medium, while total Cl^- content increases significantly. Total cellular K^+ content is unaffected by variations in cationic composition of the medium at the concentrations tested. Exposure of the cells to 2 mM La^{3+} for 1 min coupled with the mechanical stresses of the washing procedure results in major changes in cellular ion contents (Table 3B, columns 2–4). For example, comparison of control cells incubated in Na/K HR (Table 3A, line 1) to cells incubated in the same medium and later exposed to La^{3+} for 1 min (Table 3B, line 1) shows that La^{3+} treatment results in losses of 39% total Na^+, 82% total K^+ and 80% total Cl^-. Similar patterns are found for the other media. Inspection of the alterations in ion contents of La^{3+}-treated cells with changes in cation concentration in the medium shows that the ion contents are less sensitive to environmental changes than are control cells.

Concomitant measurements of cell wet weight, dry weight and extracellular fluid volume were made in these cells to determine cell water content. Extracellular fluid volume for control cells in all test media was 17.6% ($n=20$) of wet weight, while in La^{3+}-treated cells in all media the extracellular fluid volume was 61.2% ($n=20$) of the wet weight. These values, along with the wet and dry weights were used to determine the ratios of cell water to dry weight, given in column 5 of Tables 3A and 3B for control and La^{3+}-treated cells, respectively. In control cells reduction of Na^+ in the medium results in small, but significant, increases in cell water; the cells were slightly swollen. Exposure of the cells to La^{3+} results in a large loss in cell H_2O. For example, cells in Na/K HR lost 63% of total cell water after 1 min exposure to La^{3+} followed by stress. As in control cells, reduction of Na^+ in the medium is accompanied by increases in cell water content.

If it is assumed that cellular electrolytes are uniformly distributed throughout the cell water, then the ion concentrations can be determined from the ion and water contents. These values are shown in the last three columns of Tables 3A and 3B. As before, reduction of Na^+ in the medium in control cells results in decreases in $[Na^+]$, no change in $[K^+]$ and increases in $[Cl^-]$. In the La^{3+}-treated cells, however, the ion concentrations are virtually insensitive to changes in the cation concentration in the medium. Moreover, comparison of control and La^{3+}-treated cells show a significantly higher $[Na^+]$ and significantly lower $[K^+]$ and $[Cl^-]$ in La^{3+}-treated cells.

These findings are not consistent with the view that La^{3+} causes disruption of some fraction of all of the cells with subsequent loss of cellular contents. If this were the case then it would be expected that the percentage losses of all electrolytes and water would be identical. However, after La^{3+} treatment there are much greater losses of K^+ and Cl^- than of Na^+. Furthermore, the electrolytes remaining after La^{3+} treatment differ from control cells both in concentration and in response to changes in cation concentration in the medium. Comparison of the residual electrolytes (Table 3B, last three columns) with their concentrations in the media (Table 1) indicates that they do not represent simple chemical equilibrium or contamination of the washed cells by extracellular medium. These findings are consistent with the hypothesis that exposure to La^{3+} and mechanical stress causes selective loss of the contents from a cellular compartment.

In the case of selective loss of contents from a cellular compartment induced by La^{3+}, then, comparison of electrolyte and water contents

of La^{3+}-treated cells to those of control cells will yield an estimate of the contents of the disrupted compartment. Table 3C shows the ion and water contents lost upon exposure to La^{3+}. The columns 2–4 give the electrolyte losses. The amount of Na^+ lost varied directly with the $[Na^+]$ in the incubation medium, while Cl^- losses were inversely related. K^+ losses were independent of the cationic composition of the medium. These losses reflect the changes in total cellular electrolytes with alterations in the incubation medium. Water loss is shown in column 5. With exposure to La^{3+} the cells lost 61–63% of total cell water. The last three columns of the Table show the ion concentrations lost assuming that the electrolytes were homogeneously distributed in a cellular compartment selectively disrupted by La^{3+} treatment. These values would then represent the ion concentrations of that compartment. $[Na^+]$ decreases with decreasing Na^+ in the medium, while $[Cl^-]$ increases. $[K^+]$ is insensitive to content of the medium. It is important to note that $[Na^+]$ lost is in all cases significantly lower than total $[Na^+]$ of control cells, while $[Cl^-]$ lost is always significantly higher than total $[Cl^-]$ in control cells. $[K^+]$ lost is also elevated in comparison to control values.

Membrane Potential

Membrane potentials of cells equilibrated in the test media were determined by microelectrode impalement. Cell suspension which had been incubated for 1 hr in the appropriate test medium was diluted 1:20 in the same medium and placed in glass petri dishes which had been rendered wettable, exposed to 20 μM La^{3+} and rinsed exhaustively to promote cell adherence (Smith & Levinson, 1975). Microelectrode impalement of the adherent cells yielded potential recordings which were stable for up to 1 min. We have previously shown that the introduction of the microelectrode into the cell does not result in a rapid discharge of the membrane potential (Smith *et al.*, 1972). The stability of the measured potential is also indicative that no significant leakage of KCl into the cells occurred.

The results of these studies are shown in Table 4. In Na/K HR the average membrane potential was -13.7 ± 0.7 mV (SEM). Reduction of $[Na^+]$ in the medium to 98.9 mM (100 Na HR) or 77.0 mM (75 Na HR) by equivalent K^+ replacement resulted in significant depolarizations of the membrane (-7.9 ± 0.8 and -6.5 ± 0.5 mV, respectively) when compared to cells in Na/K HR.

Table 4. Comparison of measured and calculated membrane potentials of Ehrlich ascites tumor cells incubated in media of varying $[Na^+]$ and $[K^+]$

Medium	Membrane potential (mV)		
	Measured[a]	Cl^- Distributions[b]	Eq. (3)[c]
Na/K HR	-13.7 ± 0.7 (50)	-12.9 ± 2.3	-13.7 ± 0.9
100 Na HR	-7.9 ± 0.8 (35)	-9.3 ± 2.2	-8.8 ± 1.8
75 Na HR	-6.5 ± 0.5 (40)	-7.4 ± 2.3	-5.9 ± 2.3

[a] Number of observations is given in parentheses. Error are SEM.
[b] Calculated from the Nernst equation for Cl^- using the values given in Table 1 and Table 3, Section C. Errors are the most probable errors.
[c] Calculated from Eq. (3) using the values for ion concentrations given in Table 1 and Table 3, Section C, and values of P_K/P_{Na} determined from the measured potential in Na/K HR (see Discussion for details). Errors are the most probable errors.

Discussion

In previous studies we have made several observations which suggest that exposure of Ehrlich ascites tumor cells to La^{3+}, followed by mechanical stress, leads to a selective disruption of the tumor cell membrane. First, La^{3+} association with the tumor cell is rapid and complete within 35 sec of the initial exposure (Levinson, Mikiten & Smith, 1972). This pattern of association is consistent with an interaction which is restricted to the cell surface. Second, determinations of the efflux rate coefficients for Na^+ and K^+ in cells exposed to La^{3+}, but not mechanically stressed, indicate that bound La^{3+} is without effect on passive K^+ movements and *inhibits* passive Na^+ efflux by 48% (Smith, 1976). These findings support our earlier observation (Levinson, Smith & Mikiten, 1972) that unwashed cells in the presence of La^{3+} undergo only minor electrolyte losses. Finally, the unwashed cells, when mechanically resuspended, lost several-fold more Na^+ than control cells similarly treated.

The present studies support the view that exposure to La^{3+} plus mechanical stress causes selective loss of cellular contents. Cells were permitted to achieve steady-state conditions in media of normal and reduced Na^+ concentrations. Control cells were then analyzed for ion and water contents, while test cells were exposed to La^{3+} (2 mM) for 1 min, separated from their environment by centrifugation and mechanically stressed by resuspension. Comparison of ion and water contents

in control and La^{3+}-treated cells equilibrated in normal Na/K HR medium (Tables 3A and 3B) shows 39% loss of Na^+, 82% loss of K^+, 80% loss of Cl^- and 63% loss of water.

These losses can be explained in terms of any of several actions of La^{3+}. First, treatment with La^{3+} could cause rupture of only a fraction of total cell population. If this were the case then we would expect the same percentage reduction in all electrolyte contents as well as equality between electrolyte concentrations in control and La^{3+}-treated cells. Neither of these expectations is observed. Second, it is possible that the tumor cell population represents a dichotomy, having vastly different cellular electrolyte contents, with La^{3+}-treatment disrupting only one of the varieties. This possibility has been suggested (Hempling, 1972) to explain two-compartment Na^+-washout kinetic in these cells. The electrolyte contents of the two varieties would be estimated by the values shown in Table 3B and C. With the vast differences in K^+ and Cl^- contents, we might also expect a dichotomy in the membrane potential. No such observation has been reported. Finally, exposure to La^{3+} and mechanical stress could cause the selective loss of a cellular compartment. For example, La^{3+} could pre-dispose the plasma membrane highly susceptible to trauma resulting in the loss of the cell cytoplasm upon stress. Since La^{3+} is restricted to the cell surface (Levinson, Mikiten & Smith, 1972), intracellular membranes (e.g., the nuclear envelope) would be spared. Thus, intracellular compartmentation would be revealed, the electrolyte and water contents lost (Table 3C) being bounded by the cell membrane and those remaining (Table 3B) being bounded by intracellular membranes. Perhaps equally as plausible is intracellular partitioning by electrolyte and water association with macromolecular contents (e.g., proteins and nucleic acids). However, estimates of nonsolvent water in cells (Pfister, 1969) give values of approximately 20% of the water content in La^{3+}-treated cells (Table 3B). Consequently, it seems unlikely that all of the residual electrolytes and water in La^{3+}-treated cells are associated with macromolecules. Furthermore, the water content of La^{3+}-treated cells represents 37–39% of total cell water, a value in reasonable agreement with approximations of the high nucleo-cytoplasmic ratio in Ehrlich ascites cells (*cf.* Pietrzyk & Heiz, 1972).

Either of these two forms of intracellular partitioning of electrolytes and water would lead to false estimates of ion electrochemical potential gradients. Comparison of control cells and La^{3+}-treated cells permits a better estimate of electrolyte concentrations within the cell cytoplasm in either form of partitioning. This view is supported by correlation

between measured membrane potential and cytoplasmic ion concentrations (*see below*).

Inspection of the data shows that the osmotic contribution of $[Na^+]$, $[K^+]$ and $[Cl^-]$ in the water lost upon La^{3+}-treatment (Table 3C) is more than twice their contribution in the water remaining (Table 3B) (ca. 270 mosM *vs.* 128 mosm). This difference may indicate that the electrolyte losses during the washing procedure are considerably larger than estimated by the rate coefficients given in Table 2. Again the agreement between membrane potential and the ion distributions would argue against this possibility (*see below*). Insufficient information is available concerning the permeability characteristics of intracellular membranes or the state of intracellular water and macromolecules in these cells to permit speculation about the basis of the apparent osmotic imbalance.

Membrane Potential of Tumor Cells

Several methods have been utilized to estimate the membrane potential of Ehrlich ascites tumor cells. Since kinetic studies of Cl^- transfer across the cell membrane indicate that this ion is passively distributed (Grobecker, Kromphardt, Mariani & Heinz, 1963; Hempling & Kromphardt, 1965; Simonsen & Nielsen, 1971), almost all estimates have directly used Cl^- distributions or have referred alternate methods to values predicted by the distribution as an index of reliability. In general, no attempt has been made to consider the possibility of intracellular compartmentation of Cl^-. Consequently, Cl^- distributions have predicted values of -20 to -30 mV (e.g., Simonson & Nielsen, 1971).

In the present investigation, the membrane potential of these cells when incubated in Na/K HR is -13.7 ± 0.7 mV (SEM) (Table 4). This agrees with the values previously reported (Aull, 1967; Smith & Levinson, 1975) for cells in similar media. Reduction of Na^+ by equivalent K^+ replacement leads to significant decreases in the measured potential (Table 4). There were concomitant changes in the ion contents lost by the cells upon La^{3+} treatment as the cation concentrations in the medium varied (Table 3C).

If Cl^- movements in these cells are not coupled to cellular metabolism or by exchange mechanisms to nonpassively distributed anions, then the Nernst potential for Cl^- should reflect the measured membrane potential. The Nernst potential can be calculated from the Cl^- concentrations in the media (Table 1) and cytoplasm (Table 3C). These values

are also shown in Table 4. There is no significant difference between corresponding Cl^- potentials and the measured values in any of the media. This agreement is consistent with: (1) passive distribution of Cl^- in these cells; and (2) cellular compartmentation of Cl^- which is separable by La^{3+} treatment. Consequently, it is necessary to consider why other investigations have shown correlation between potential estimates and Cl^- distributions without considering intracellular compartmentation.

De Cespedes and Christensen (1974) used the distribution ratio of the dibenzyldimethylammonium (DDA) cation across the cell membrane to estimate membrane potentials. They reported potential estimates slightly larger than those predicted by the Cl^- distribution. These values, of course, assume homogeneous distributions of DDA and Cl^- in cell water. However, if DDA passively distributes within the cell, then it is legitimate to consider its intracellular compartmentation. Since DDA is a cation, it would be distributed reciprocally to Cl^-. Thus, DDA would be preferentially sequestered in the compartment remaining after La^{3+} treatment (Table 3B) and deficient in the compartment lost by La^{3+} treatment (Table 3C). Taking these considerations into account, it can be shown that while the Cl^- distribution, assuming homogeneity (Table 3A), predicts a potential of -19.2 mV, the reciprocal DDA distribution predicts a slightly higher potential of -23.8 mV. Yet its cytoplasmic concentration would predict -12.9 mV, as does the Cl^- distribution (Table 4). Indeed any passively distributed cation, being preferentially sequestered in the smaller intracellular compartment, will always predict a slightly higher potential than a passive anion, if their distributions are considered homogeneous. Thus, the findings of de Cespedes and Christensen (1974) are in concert with intracellular compartmentation as indicated by our results.

Recently, Mills and Tupper (1975) have attempted to establish the ionic basis of the membrane potential of these cells. They concluded that the membrane potential could be described exclusively by the distributions and membrane permeabilities for Na^+ and K^+ according to the Goldman equation

$$V = \frac{RT}{F} \ln \frac{P_K(K)_o + P_{Na}(Na)_o}{P_K(K)_i + P_{Na}(Na)_i} \tag{3}$$

(Goldman, 1943; Hodgkin & Katz, 1949). They predicted a potential of -18 mV, compared to a Cl^- distribution potential of -21 mV. These

authors did consider the possibility of Na^+ sequestration in the nucleus, in which case their predicted potential was reduced to -14 mV. Unfortunately, intracellular compartmentation was not considered in determining cation permeabilities of the membrane.

In the present studies we have not attempted determination of the cation permeabilities. However, the applicability of the Goldman equation can be tested by using the experimental value of the membrane potential in Na/K HR to calculate the ratio P_K/P_{Na}. The calculated ratio can then be used to predict the membrane potential for both 100 Na HR and 75 Na HR solutions. This procedure yields $P_K/P_{Na}=1.56$ for Na/K HR. The predicted membrane potentials are given in Table 4 (column 4). There is no significant difference between the measured potential, the Nernst potential for Cl^- and the cation distribution potential in any of the test media. This agreement suggests that: (1) in steady-state cells, the membrane potential is primarily established by the passive movements of cations down their respective chemical potential gradients; and (2) intracellular ion compartmentation must be considered in determining energies stored in the cation gradients.

Implicit in this conclusion, of course, is that the chemical gradients must be established and maintained by active cation "pumping". However, at least in the steady state the active cation transport system cannot have significant electrogenicity. This conclusion is in agreement with the finding that ouabain is without effect on membrane potential of control tumor cells (Smith & Levinson, 1975; Laris *et al.*, 1976). It should be recognized that the present studies do not deny the possibility of contributions from an electrogenic active cation transport under non-steady state conditions. For example, Heinz, Geck and Pietrzyk (1975) have presented evidence for an electrogenic cation transport in K^+-depleted cells.

Others have reported membrane potential estimates which agreed with the Cl^- distribution potential without accounting for compartmentation. These values are more difficult to explain in terms of the present studies. Lassen *et al.* (1971), using electrophysiological techniques, found a membrane potential of -23.5 mV and concluded that it represented a Cl^- distribution potential. We have recently suggested that the lack of agreement with other electrophysiological methods (ca. -11 mV; Aull, 1967; Smith & Levinson, 1975; the present studies) may be explained by the presence or absence of Ca^{2+} in the incubation media. Ca^{2+} has been shown to hyperpolarize these cells (Smith *et al.*, 1972; Pershadsingh & Laris, 1976) and leads to an increase in K^+ permeability in

red blood cells (Romero & Whittam, 1971; Simons, 1976). Of course, if Cl^- were to remain in a passive distribution, the presence of Ca^{2+} would also require the shifting of ions between the intracellular compartments. No data are currently available to permit such a conclusion.

Potential-sensitive fluorescent dyes have also been proposed as monitors of the membrane potential in these cells (Laris *et al.*, 1976). These authors report that fluorescent intensity of the dye, 3,3′-dipropylthiodicarbocyanine iodide, was influenced by the way in which the cells were treated. However, by one treatment the estimated potential was describable by the Nernst potential for Cl^-. The fluorescent method depends upon the assumption that valinomycin increases K^+ permeability of the cell membrane without altering permeability coefficients of other ions or effecting possible electrogenic pumps. We have recently implicated a ouabain-sensitive response of the cells when exposed to valinomycin (Smith & Levinson, 1975). Valinomycin alone was without effect on the membrane potential, although K^+ permeability of the membrane increased by 30%. Addition of ouabain to the cells in the presence of valinomycin caused hyperpolarization (from -12.0 mV to -16.6 mV) of the membrane. The magnitude of this hyperpolarization is completely consistent with a 30% increase in P_K [*cf.*, Eq. (3)]. Furthermore, Laris *et al.* (1976) found a nonlinear response of fluorescence to changes in medium K^+ and suggested that the membrane potential in the presence of valinomycin is not equal to the K^+ equilibrium potential. We suggest that this is the case.

Energetic Implications of Intracellular Compartmentation

The above observations strongly support the conclusion that exposure of tumor cells to La^{3+} followed by mechanical stress can be used to estimate the extent of intracellular ion compartmentation. Comparison of control and La^{3+}-treated cells (Table 3) indicates that Na^+ is preferentially sequestered in a noncytoplasmic intracellular compartment, while Cl^- and K^+ are more highly concentrated within the cytoplasm.

For cells incubated in a physiological salt solution (Na/K HR), our results are in excellent agreement with those of Pietrzyk and Heinz (1974) with respect to cation concentrations. These investigators used ion/DNA ratios from fractionated cells as an index of nuclear sequestration. Thus, our agreement may be taken to infer that the noncytoplasmic compartment is the nucleus, as suggested by the compartment volume (*see above*).

However, our results differ from those of Pietrzyk and Heinz (1974) in two important respects. First, whereas these authors find evidence for preferential sequestration of Cl^- by the nucleus, we find a deficiency of Cl^- in the nuclear compartment. Second, our results indicate that replacement of extracellular Na^+ by K^+ leads to decreasing cytoplasmic Na^+, while Pietrzyk and Heinz (1974) report the opposite. We can offer no explanation for these differences. However, the agreement of both the Nernst potential for Cl^- and the cation basis of the membrane potential with measured membrane potentials (Table 4), favors the electrolyte patterns found in the present study.

Our results would imply that studies which require estimates of the energies available form cation gradients (e.g., tests of the "ion gradient hypothesis") have in general underestimated these energies. Although we have not reduced Na^+ in the medium to the levels necessary to achieve "reversed ion gradients", if the relatively constant levels of nuclear sequestration we have found were to be maintained, it might not be feasible to "reverse" ion gradients by replacing Na^+ with K^+ in metabolically uninhibited cells.

Taken together the results of these experiments indicate that in Ehrlich ascites tumor cells significant compartmentation of ions, specifically Na^+, K^+, and Cl^-, occurs within the cell nucleus. Na^+ is maintained in higher concentration within the nucleus than the cytoplasm, while K^+ and Cl^- are more highly concentrated within the cytoplasm. Direct measurement of the cell membrane potential supports these findings. Alterations in the cation contents in the medium show that as Na^+ is reduced cytoplasmic Na^+ also decreases, K^+ remains constant and the membrane depolarizes. This pattern of changes raises the possibility of the underestimation of cation energy gradients in "reversed ion gradient" experiments.

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