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Microprobe Analysis of Element Distribution in Rabbit and Dog Erythrocytes as Examples of "High" and "Low" Potassium Cells

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MICROPROBE ANALYSIS OF ELEMENT DISTRIBUTION IN RABBIT AND DOG ERYTHROCYTES AS EXAMPLES OF "HIGH" AND "LOW" POTASSIUM CELLS

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Abstract

The concentrations of Na, Mg, P, S, Cl, K and Fe were determined by microprobe in near 100% hematocrit suspensions of rabbit and dog erythrocytes prepared by freezing and drying. These cells are representative, respectively, of "high" potassium, "low" sodium, and "high" sodium, "low" potassium cells. Water contents of the cells were the same, as were, approximately, the levels of Cl, S and Fe. Rabbit P was nearly double that of the dog. For the rabbit, the cell Na/K ratio was 0.21 and for the dog 15.4, illustrating the major diffusible electrolyte difference between these two types of cell. The rabbit erythrocytes showed an apparent negative immobile charge density of 95 meq/kg of cell water and the dog 56 meq/kg cell water, a distinct difference. Serum electrolytes in the two species are exactly comparable (Standard Tables). Ionic distribution in these cell types was treated by the Gibbs-Duhem equation representing two heterogeneous systems in thermodynamic equilibrium with the blood serum. Factors to be considered are: (1) the composition of the erythrocyte and its net immobile charge; (2) the physicochemical properties of the individual ions (charge, ionic radius, hydration energy, standard chemical potential); (3) the dielectric constant of the dispersion medium (in this case, water); and (4) the binding constants of the ions. The hypothesis of "active transport" (the sodium-potassium pump) is specifically rejected as an explanation of ionic differences.

Key Words: Dog and rabbit erythrocytes, high and low K erythrocytes, microprobe analysis of erythrocytes, Na, K, distribution.

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Introduction

The distribution of the ions Na, K, Ca, Mg and Cl in cells and tissues represents a fundamental aspect of their physiological behavior. Scanning electron microscopy (SEM) and X-ray analysis can provide conditions in which a given cell content of elements can be determined simultaneously on the same specimen. When combined with adequate methods of cryofixation, a reasonably ideal situation in which to examine such distributions is available [5].

While most nucleated cells have a higher concentration of potassium than sodium, the adult erythrocytes of some species show the reverse situation of high sodium and low potassium. Of the common species, rabbit and human erythrocytes show the high potassium and low sodium common to vertebrate cells. Erythrocytes of dog, cattle and some breeds of sheep have high sodium and low potassium. An explanation of this species differences has usually depended on some adaptation of the theories of ionic distributions in cells generally. Since the nineteen forties, the actions of ionic pumps, "powered" by adenosine triphosphate (ATP), have tended to become the dominant form of hypothesis. In most cells, it is supposed, a sodium/potassium ATPase "pump" continually consumes energy to transfer sodium from cell to blood and potassium from blood to cell, against their "concentration gradients" [9, 19]. In erythrocytes, which show high sodium levels, this pump is supposed to be inoperative, missing in part (i.e., too few pumps) or abnormally leaky. No generally accepted mechanism of action has been proposed for any ionic pump, and the status of those erythrocytes which fail to take advantage of such postulated devices remains correspondingly unsettled [23].

We have, however, rejected this popular view which links ionic distribution to metabolic pumping [14, 15, 16] and have approached the subject from the standpoint of known physicochemical properties of the cell ions and those of their surrounding fluids, together with adherence to a modern concept of the cell as a microtrabecular lattice [24] with an ordered state of the cell water.
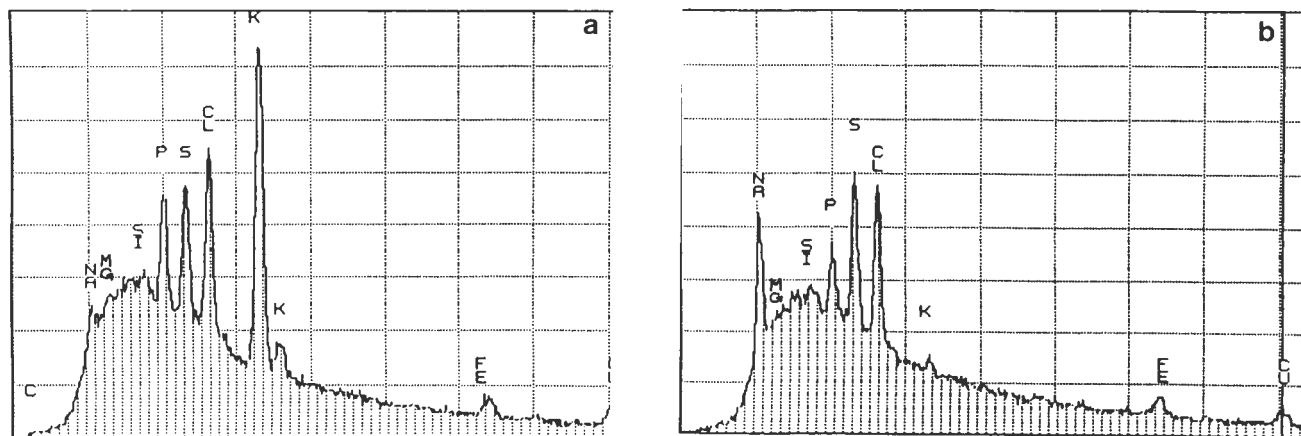
Figure 1. Microprobe spectra from (a) rabbit erythrocytes and (b) dog erythrocytes.

Biological structures, including erythrocytes, are heterogeneous systems of at least two phases, originally labeled by us as colloid-rich, water-poor, and water-rich colloid-poor, in thermodynamic equilibrium with the blood serum [6]. Such systems necessarily show deviations from the ideal solution treatments of Donnan and Nernst, on which ion pump concepts rely.

Overall factors to be considered in explaining ionic distribution in heterogeneous systems of more than one phase are: (1) the fixed negative charge, denoted x⁻, of the cell; (2) the physicochemical properties of the individual ions (Na, K, Ca, Mg, Cl), namely their charge, ionic radii, hydration energies and standard chemical potentials; (3) the dielectric constant (D) of the dispersion medium (water, in biological systems); and (4) the binding constants of the ions.

A theoretical basis for this treatment is provided by the Gibbs-Duhem equation [8, 13]. Intracellular changes of electrolyte composition occur under conditions of constant chemical potential as established by the constant ionic composition of the blood serum. In systems of more than one phase, alterations in cellular states of composition or aggregation are expressed by means of changes in standard chemical potentials and binding constants of the intracellular ions. These, and not solely ion concentrations, are the determining factors in the equilibrium of cells and the blood plasma.

Materials and Methods

Blood was collected from 4 animals of each species, by venipuncture (dog) and directly from the heart (rabbit) and immediately defibrinated by gently shaking with glass beads. The red cells were then sedimented in a refrigerated centrifuge at 1-2°C at 4000 g for 20 minutes. The cells were examined under the microscope to determine that they were intact, and a sample of the sedimented cells was transferred to a hematocrit tube to determine the volume of packed cells. This was usually 98-99%. The water content of the packed cells was determined by drying on filter paper to constant weight at a temperature of 50-55°C.

For microprobe analysis, a typical preparation was made by placing a drop of packed cells on a carbon planchet. The planchet was immediately and rapidly immersed in isopentane chilled to -150°C in liquid nitrogen. The planchet was then transferred to a vacuum chamber maintained at -32°C for 48 hours and the sublimed water vapor removed by a P_2O_5 T-trap. The dried samples were exposed to anhydrous paraformaldehyde vapor [7] in a sealed vessel at 37°C for 2 hours to stabilize the cells against the destructive effects of carbon coating and the electron beam. The specimens were then coated with a carbon film in a vacuum evaporator to facilitate conduction.

The cells were examined in a SEM equipped with a energy dispersive X-ray detector. The detector was coupled to an analyzer and computer to record the element distribution and element counts. The SEM was operated at an accelerating voltage of 15 kV and the gun current was maintained at 80-100 μA. The operating conditions were standardized using a copper grid and adjusting the condenser lens to yield 2,000 counts per 50 seconds.

The resolution laterally and in depth was calculated to be around 5 μm. The analysis was localized to a group of overlying erythrocytes at a magnification of 4000X. A limited raster of approximately 16 square micrometers was scanned for a live time of 200 seconds, and a total of 20-30 observations were recorded for erythrocytes of each species (Fig. 1). The Tracor (Noran Instruments, Middleton, WI) bulk program, SQ, subtracts the background, deconvolutes and strips the peaks, and identifies the elements. The individual elements identified were Na, Mg, P, Cl, S, K, and Fe.

Table 1. Element distribution (in Mm/kg water) in rabbit and dog erythrocytes.[*]

	Na	K	Mg	Fe	Cl	P	S
Rabbit (E & C)[†]	29 ± 2	140 ± 3	10 ± 0.5	21	74 ± 2	49 ± 1	61 ± 2
Rabbit (Bernstein)	22	142			80		
Man (Zierold)	10	100	5	21	62	24	62
Dog (E & C)	123 ± 5	8 ± 1	7.5 ± 0.5	21	75 ± 1	28 ± 1	71 ± 1
Dog (Bernstein)	135	10			87		
Mammalian Serum (Mm/l)	140	5.0	2.5		105		

[*]Also included for comparison are some results both for the same species and for man (high potassium, low sodium) and characteristic values for mammalian blood serum.

[†](E & C), present results, values are given as means and standard errors (SE) of the means; SE less than 0.5 omitted.

Counts for Ca were below the sensitivity of our equipment. The counts were converted to millimoles per kg of tissue water using the gelatin-element standards prepared according to the method of Roomans [26], together with the values obtained for the water content of the erythrocytes.

Results

Serum values of the electrolytes Na, Mg, K, and Cl are virtually the same in rabbit and dog and in the totality of mammals. However, in erythrocytes of rabbit and dog, the concentrations particularly of Na, Mg, and K are different (Fig. 1, Table 1). The results obtained by the SEM method and the present preparative technique, using essentially freezing and drying and avoiding the use of anti-clotting agents, should avoid errors implicit in methods using separated and/or washed cells, cells chemically fixed, and cells also exposed to various anti-clotting agents. Comparisons are included with the results of Bernstein [1] who used heparinized cells and flame photometry, and also with Zierold's [27] microprobe analyses of human cells fixed by several procedures including freeze-drying. Our results are in reasonable agreement with Bernstein. Both show certain unexplained differences from Zierold in the human versus rabbit comparison of two high potassium erythrocytes. Our results agree with Zierold's iron and sulfur values.

For dog erythrocytes, the Na/K ratio is 15.4; for the rabbit it is 0.21, a difference of 73-fold generally characteristic of the "low potassium" versus the "high potassium" species, which requires explanation. However, the figures show other differences as well as similarities. The chloride and iron values tend to be the same in both rabbit and dog. Magnesium tends to follow potassium and is lower in dog than in rabbit. Sulfur values are comparable but phosphorus for the rabbit is 1.8 times that of the dog.

Discussion

General features of element distribution

The water content of rabbit and dog erythrocytes is the same (65% and 64%, respectively). The elements P and S are mainly components of macromolecules. Phosphorus is present in phosphoproteins and S in the cystine and cysteine of proteins. The negatively charged macromolecules include components of the cell matrix and cell membrane and hemoglobin, and are represented by phosphate and carboxylate groups. Neutralization is effected mainly by the two major cations, Na^+ and K^+. The presence of fixed negative charge establishes the electrostatic conditions for equilibrium, requiring an excess of mobile cations over mobile anions (principally Cl^-) for electrical neutrality. Then, as a rough approximation, cell ($Na^+ + K^+ - Cl^-$) can be taken as a measure of the apparent negative colloidal charge density (x^-). For the rabbit, $x^- = 95$ meq/kg cell water and for the dog $x^- = 56$ meq/kg cell water; the significantly higher rabbit value agrees with the higher content of erythrocyte phosphorus.

Theoretical treatment of cell ion results

Standard chemical potentials and dielectric constants Treatment of ionic equilibrium in cells adheres to the notation and equations of Gibbs' heterogeneous equilibrium [8] as developed in previous publications [11, 17]. The invariant environment of erythrocytes is

Table 2. Calculated parameters for ions in rabbit and dog erythrocytes.[*]

Ion	Rabbit, $D'' = 45$			Dog, $D'' = 63$		
	$\Delta\mu_i^0$ kcal	$(\Delta\mu_i^0)'$ kcal	β	$\Delta\mu_i^0$ kcal	$(\Delta\mu_i^0)'$ kcal	β
Na	1.28	1.28	1.0	0.45	0.45	1.0
K	0.97	-1.75	0.012	0.34	0.08	.66
Mg	6.54	-0.67	10^{-5}	2.27	-0.37	10^{-2}
Cl	0.70	-0.09	0.28	0.24	-0.16	0.52

[*]See **Appendix I** for an explanation of Table 2.

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equivalent to a fixed mammalian Ringer solution of the physiological ions which defines a physiological standard state whose dielectric constant may be taken as 80, the dielectric constant of water. Cells are heterogeneous systems of at least two phases in equilibrium with the blood serum. This implies constant chemical potentials of water and electrolyte in all phases. The variable properties are the **standard** chemical potentials, μ_i^0, of the cell ions and the binding constants of the ions (considered in the following section). Taken together, standard chemical potential and binding give an **apparent standard chemical potential**, $(\mu_i^0)'$, for each ion. These are calculated from the data in Table 1 to give the values in Table 2 (see also **Appendix I**) together with an estimate of the apparent dielectric constants of rabbit and dog erythrocytes. The latter are 45 for the rabbit and 63 for the dog, agreeing with estimates made from the data of Bernstein [1] of 40 and 65 respectively. Rabbit erythrocytes tend to the lower dielectric constant values characteristic of muscle cells which can be 30 or less [13]. Dog erythrocytes tend to the higher values of 60 or more, characteristic of some connective tissues [5]. An explanation for the differing behavior of potassium and sodium in phases of low and high dielectric constant can be found in the free energy of hydration of the ions, which are for potassium 73.5 kcal, and for sodium 89.7 kcal [16]. Potassium will have a greater tendency than sodium to form unhydrated ions and to neutralize fixed charges in the low dielectric constant environment of the rabbit erythrocyte. The reverse occurs in the high dielectric constant erythrocyte of the dog, where the highly hydrated sodium ion is preferentially accepted to neutralize available fixed charge.

Ion binding in cells Besides the changes in standard chemical potentials of ions in cells, deviations from the ideal distribution are produced by chemical binding of the ions depending on their unique physicochemical properties, including their ionic radii and charge and their reactions to the cell composition (e.g., conformation and relative access to the charged sites). From the data, a binding constant (β) is calculated for K^+, Mg^{2+}, and Cl^- ions based on the theoretical formulae cited (Table 2, **Appendix I**). Intracellular Na^+ is assumed to be completely ionized [12], i.e., for sodium $\beta = 1$. The ions K^+, Mg^{2+} and Cl^- are present as dissociated and bound species, e.g., total Mg = ionic Mg^{2+} + bound Mg. The weakest binding appears to be by Cl^- and the strongest by Mg^{2+}, and in general the amount of binding is considerably higher in the rabbit erythrocyte than in the dog cell. Distribution ratios for cell/plasma sodium and potassium are respectively for the rabbit 0.21 and 28, and for the dog 0.88 and 1.6. The apparent standard free energies of the K and Na distributions ($[\Delta\mu^0_K]' - [\Delta\mu^0_{Na}]'$) are -3.03 kcal for the rabbit and -0.37 kcal for the dog, representing an 8-fold difference in driving force determining the major disparity in behavior of these two ions. Binding constants for potassium are 0.012 for the rabbit and 0.66 for the dog. Ion binding and standard chemical potential act in a complex way to produce the apparent standard chemical potential values cited in Table 2.

Membrane potential of erythrocytes The intracellular potential (membrane potential) of erythrocytes is smaller than that of many cells, lying in the range of -10 to -15 millivolts [20] and holding for both rabbit and dog red cells. Using our values for the distribution of Na, Mg, K, and Cl in cells and standard values for serum, application of the Nernst equation yields a wide range of electromotive force (EMF) values (Table 3, see also **Appendix II**). For the rabbit, these vary from -90 mV (for K) to +41 mV (for Na). For the dog, the range is from +3 mV (for Na) to -24 mV (for Mg). However, the ideal Nernst equation suffers from the same limitations as does the Donnan equilibrium in describing the behavior of ions in cells: both apply to aqueous solutions separated by semipermeable membranes in which the

Table 3. Corrected intracellular EMF and Nernst equation values in rabbit and dog erythrocytes.

	Ion	Corrected EMFs[1] E (mV)	Nernst Potentials[2] E_{ideal} (mV)
Rabbit	Na	-14	+42
	K	-14	-90
	Mg	-13	-28
	Cl	-14	-10
	Mean -14 ± 0.5*		
Dog	Na	-17	+3
	K	-16	-13
	Mg	-16	-24
	Cl	-16	-9
	Mean -16 ± 0.5*		

*Standard error of mean.

[1,2]See **Appendix II** for determination of the reversible EMF (E_{real}) and the Nernst potential.

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solutions would mix freely in the absence of the membrane. Most biological systems are heterogeneous, immiscible and non-ideal, and contain a term related to the standard chemical potential of the ions. Applying this correction, the calculated values of the EMFs (Table 3) are -14 ± 0.5 mV for the rabbit and -16 ± 0.5 mV for the dog, in adequate agreement with measured values.

Cell water Most recent treatments of cell water have abandoned the concept of cell cytoplasm as an aqueous solution, and by a variety of procedures distinguish between its properties and those of "bulk water" [3, 4, 10, 21, 22]. Unfortunately, the theoretical "aqueous solution" treatment of cellular ions has been retained in physiological and biophysical texts, together with the necessity of rationalizing ion distribution by postulating ionic pumps and active transport based on metabolism.

Opposed to such concepts is one of a distinction between water-rich and colloid-rich phases of connective tissues [6] and cells [16]. Biological structures are immiscible heterogeneous aggregates for which standard chemical potentials of ions, ion binding and apparent dielectric constants can be estimated. Cell water is present as successively more highly structured hydration layers as charged colloidal surfaces are approached [25]. The cell is in equilibrium with the blood serum as a continuous dielectric, the system being amenable to the

Gibbs-Duhem theoretical treatment without postulates of non-existent entities.

Appendix I: Explanation of Table 2

The values given in Table 2 were calculated from the results of Table 1 using the equations below [13, 17]. At equilibrium in a heterogeneous system, such as the cell, the change in chemical potential, $\Delta\mu_i$, for any ion between two phases (cell and extracellular environment) is given by:

$$\Delta\mu_{Na} = \Delta\mu_K = (1/2)\Delta\mu_{Mg} = -\Delta\mu_{Cl}, \text{ etc.,} \quad (1)$$

{$\Delta\mu_{Na}$ = 0.31 kcal for the rabbit, and 0.37 kcal for the dog, calculated from eqs. (3) and (10)}.

In an ideal system, at equilibrium, where ions are distributed between two phases and are not bound:

$$\Delta\mu_i = RT \ln(c''_i/c'_i); \quad (2)$$

but, in any non-ideal heterogeneous systems, in which electrolytes are completely ionized:

$$\Delta\mu_i = \Delta\mu_i^o + RT \ln(c''_i/c'_i) \quad (3)$$

(superscripts ' and " denote outside and inside the cell, respectively).

The **invariant** $\Delta\mu_i$ is non-specific for the ions but $\Delta\mu_i^o$ (the change in standard chemical potential) is specific for each ion. The distribution between cells and extracellular environment at equilibrium must include the binding of ions; thus, eq. (3) is modified to:

$$\Delta\mu_i = \Delta\mu_i^o + \{RT \ln(\alpha''c''_{i(total)} / \alpha'c'_{i(total)})\} \quad (4)$$

where α is the ionized fraction. Then:

$$\Delta\mu_i = \Delta\mu_i^o + RT \ln(\alpha''/\alpha') + RT \ln(c''_i/c'_i). \quad (5)$$

The ratio (α''/α') is denoted β, a binding constant, and represents {(ionized/total cell ions) / (ionized/total plasma ions)}. Then:

$$\Delta\mu_i = \Delta\mu_i^o + RT \ln(\beta) + RT \ln(c''_i/c'_i). \quad (6)$$

We define $\Delta\mu_i^0 + RT \ln(\beta)$ as the change in apparent standard chemical potential: $(\Delta\mu_i^0)'$ (7)

Generalizing:

$$\Delta\mu_i = (\Delta\mu_i^0)' + RT \ln(c''_i/c'_i). \qquad (8)$$

Since $\Delta\mu_i$ and $RT \ln(c''_i/c'_i)$ are known, $(\Delta\mu_i^0)'$ can be determined. Then, the value of β can be calculated from eq. (7):

$$\ln \beta_i = \{(\Delta\mu_i^0)' - \Delta\mu_i^0\} / RT. \qquad (9)$$

The intracellular dielectric constant D'', can be evaluated as follows:

$$\Delta\mu_{Na}^0 = RT \{\Sigma c' - (\Sigma c'')\} / c''_{Na}. \qquad (10)$$

From the Born equation, as modified by Laidler and Pegis [2, 18]:

$$D'' = \{131 / (1.64 + \Delta\mu_{Na}^0)\} \qquad (11)$$

For the other intracellular ions, i, (i.e., K, Mg and Cl):

$$\Delta\mu_i^0 = (164 Z^2 / b_i) \{(1/D'')-(1/80)\} \qquad (12)$$

where, R = thermodynamic constant; T = absolute temperature; $\Sigma c'$ = sum of serum ions (from freezing point); $(\Sigma c'')$ = sum of cell ions ($Na^+ + K^+ + Cl^-$); c''_{Na} = cell Na concentration; b_i = corrected ionic radius; and Z = ionic charge, negative for Cl.

Appendix II: Explanation of Table 3

The reversible EMF (E_{real}) in a heterogeneous system is determined as follows [17]:

$$-23060 Z_i E_{(volts)} = \Delta\mu_i \qquad (13)$$

Then:

$$E = -\Delta\mu_i / 23060 Z_i \qquad (14)$$

Substituting for $\Delta\mu_i$ from eq. (8):

$$E = \{-[(\Delta\mu_i^0)'/23060 Z_i] - [RT/23060 Z_i][\ln(c''_i/c'_i)]\} \qquad (15)$$

Where $-[(\Delta\mu_i^0)'/23060 Z_i]$ is designated E_{excess}.
The Nernst potential:

$$E_{ideal} = [-RT/23060 Z_i] [\ln(c''_i/c'_i)] \qquad (16)$$

Then:

$$E_{real} = E_{excess} + E_{ideal} \qquad (17)$$

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Discussion with Reviewers

A. Ring: The paper is provocative in not accepting, for any cell, the well established mechanism of active transport. The paper does not correctly present and discuss the vast literature which supports the hypothesis of the existence of active transport and is therefore misleading to uninitiated readers.

Authors: Our rejection of active transport early in the paper is necessary to clear the way for an alternative treatment of the results. High and low potassium cells are high on the list of anybody concerning themselves with the topic of ion distribution, and how it comes about. In this area, active transport has been the postulate of choice. But, surely we did not attempt to mislead uninitiated readers. We quote, up front, a major review [9] which mentions 245 references supporting active transport and zero opposed. We quote a recent publication [19] which mentions no alternative to metabolically fueled pumps. Our more limited task was to consider our results as a whole; to include the significance of an apparent colloidal charge of the cell, and to treat the ion distribution as a result of an equilibrium state in a heterogeneous system (a cell is not a solution), involving application of the Gibbs-Duhem equation [8, 13] and concepts of chemical potential and dielectric constant. It happens that these concepts are absent from the postulates of active transport.

A. Ring: The authors could subject their hypothesis to a minimal experimental test, for example, by predicting the equilibrium distribution of test-ions, such as alkali cations (i.e., determining the Eisenman [30] selectivity sequence). Please comment.

Authors: We are aware of ion selectivity hypotheses as advocated by Eisenman [30] and also by Ling [21, 22]. These involve postulates and experiments. The postulates, we are not prepared to make, except as a part of ion-binding as described in the text, where we calculate the degree of binding of K, Mg, and Cl.

The experiments, both by temperament and training, we are not prepared to do. Our method is based on Willard Gibbs' [8] formulation of the conditions for heterogeneous equilibrium and thus differs from all other treatments of ion distribution in cells and tissues [11, 12, 13, 14, 15, 16].

A. Ring: The authors ignore completely the vast number of experiments which have shown how the cell membrane potential relates to patch-clamp findings of ion channel activity and reversal potentials, modulation of active transport with ouabain, cotransport inhibitors, ionophores, etc. Please comment.

Authors: Channels as disclosed by voltage clamp and related methods are to be distinguished from ion transport devices, such as the Na, K-ATP-ase (sodium pump) which is considered as necessary to re-establish baseline levels of cell ions and baseline potentials (Hille [33]). We would, likewise, keep our own equilibrium treatment separate from "channel" mechanisms. However, we can comment on "modulation" of active transport using ouabain (incidentally, one of the proofs of active transport), although, we did not personally use this particular drug. We found that use of nucleophilic anions, such as dinitrophenol, iodoacetate, and cyanide, powerfully affect the titration curves of frog muscle, and therefore the ion distribution in any biological situation (Engel *et al.* [31]) and of macromolecules generally (as shown by experiments in wool and dye chemistry [28, 32, 37]). We questioned the conclusions reached for active transport based on the use of such agents, including ouabain, and continue to be suspicious of their application in any experiment involving ion movement or distribution.

G.M. Roomans: It is not clear what the experimental data, while adequate, add to our knowledge. I feel that the paper could have been written from the literature data.

Authors: While all agree that erythrocytes high and low

in potassium, and correspondingly low and high in sodium, exist, figures show considerable variability both between species and within dimorphic species, for example, Tormey's [36] "Typical Electrolyte Concentrations" in high and low potassium cells (apparently sheep erythrocytes) both agree and disagree with the literature, and with our own mean values in rabbit and dog. There is also the question of treatment of cells: We do not consider prior addition of chemicals to prevent clotting, or washing of cells with assorted solvents or embedding procedures as ideal methods of pursuing valid ion concentrations. This applies also to Kirk *et al.* [34] and many other studies. We defend our methods of freezing-drying and simultaneous analyses of several cell components in 2 species, rabbit and dog, on which our discussion is based, as unobtainable from the literature. Zierold's results (for human erythrocytes), which we quote, show reasonable agreement with the literature through methods we consider superior. But the literature is not a substitute in either case.

Editor: Please provide references to the works of Donnan and Nernst.

Authors: These are classic papers covered by refs. [29, 35].

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