REFERENCE PHASE ANALYSIS OF FREE AND BOUND INTRACELLULAR SOLUTES

II. Isothermal and Isotopic Studies of Cytoplasmic Sodium, Potassium, and Water

Samuel B. Horowitz and Philip L. Paine, *Cellular Physiology Laboratory, Department of Biology, Michigan Cancer Foundation, Detroit, Michigan 48201 U.S.A.*

ABSTRACT The intracellular reference phase (RP) method and ultra-low temperature microdissection were used for isothermal and isotopic phase distribution studies of Na^+, K^+, and water in amphibian oocyte cytoplasm. One-third of the cytoplasmic water is available as solvent for [^{3}H]sucrose. This fraction, designated $c1$, quantitatively coincides with the water volume in which Na^+ and K^+ are freely diffusible. Two-thirds of the cytoplasmic water is inaccessible to sucrose and is designated $c2$. The Na^+ and K^+ associated with $c2$ are extremely slowly exchanging (bound) and at different concentrations than in $c1$. The cations in $c1$ are in mass-action equilibria with those in $c2$, each described by an equation of the form

$$C_i^c = C_i^{c1} + C_i^{c2} = q_i \cdot C_i^{RP} + {}^{max}C_i^{c2} \cdot f(C_i^{RP}),$$

in which C_i^c is the cytoplasmic Na^+ or K^+ concentration, C_i^{c1} is the free, and C_i^{c2} the bound cation concentration averaged over the cytoplasmic water. q_i is the fractional free solute space, C_i^{RP} the RP concentration, ${}^{max}C_i^{c2}$ the concentration of binding sites, and the function f is satisfied by the Langmuir isotherm. Numerical values for the variables of the isotherm are determined. Activity coefficients are calculated from RP data and provide a basis for generalizing the oocyte results to other cells. The conclusion is drawn that both $c1$ and $c2$ are widely distributed in cells, and that cellular ionic activities involve two distinct systems: the cell-membrane system and an adsorbed water ion-exchange-like buffering system. Alternative explanations for the two-component cytoplasm are considered. A model is proposed in which $c1$ is a normal intracellular aqueous phase controlled by the plasma membrane, whereas $c2$ consists of water and ions adsorbed in hydrate crystalline structures. In oocytes these structures are identified with yolk platelets.

INTRODUCTION

The preceding report, which describes the intracellular reference phase (RP) method and its use in phase distribution studies of Na^+ and K^+ in amphibian oocyte cytoplasm (1), shows that the diffusible cytoplasmic concentrations of these cations differ from overall cytoplasmic

concentrations. We interpret these results to mean that cytoplasm is heterogeneous, containing both free and bound[1] ions and water.

In this paper we experimentally define the solvent and solute states. We show that sucrose, often used to measure extracellular volume, also can be used to measure an intracellular space. This space includes only one-third of the cytoplasmic water and quantitatively coincides with the volume in which Na^+ and K^+ diffuse freely. Two-thirds of the cytoplasmic water is inaccessible to sucrose. The Na^+ and K^+ associated with this water differ in concentration and isotope exchangeability from their free counterparts, with which they are, nevertheless, in equilibrium.

MATERIALS AND METHODS

Except as indicated, the materials and methods are those already described (1). These include the oocytes (of the salamander, *Desmognathus ochrophaeus*), solutions, the methods of RP introduction, oocyte incubation, ultra-low temperature microdissection (ULTM) and analysis.

Solutions

$^{22}Na^+$, $^{42}K^+$, and [^{3}H]sucrose were obtained from New England Nuclear Corp. (Boston, Mass); the cations as carrier-free chlorides. $^{36}Cl^-$ was obtained from Amersham Corp. (Arlington Heights, Ill.) as NaCl. In incubation experiments, the commercial radioisotope solutions were dried and reconstituted to provide Ringer's solution of the proper final composition (1). Final concentrations were monitored by atomic absorption spectroscopy.

Microinjection of $^{42}K^+$ and [^{3}H]sucrose was made in a medium consisting of 150 mM KCl, 25 mM NaCl, 2 mM $NaHCO_3$, and 1 mM KH_2PO_4 at pH 7.4.

Microinjection

Microinjection of gelatin to form the RP is described in the preceding paper (1). Unless otherwise noted, tracer injections were made 2–4 h after gelatin injection in 30–50 nl of injection media at room temperature.

Analysis

Radioactive tracers were measured in a volume of the boiled extraction fluids described in the preceding paper (1). $^{22}Na^+$ and $^{42}K^+$ were counted in an Intertechnique CG30 or a Searle model 1195 automatic gamma spectrometer (Searle Diagnostics Inc., Des Plaines, Ill.). [^{3}H]Sucrose and $^{36}Cl^-$ solutions were dissolved in cocktail D and counted in a Packard model 3380 liquid scintillation spectrometer with external standardization (Packard Instrument Co. Inc., Downers Grove, Ill.).

RESULTS

Intracellular Sucrose Space

When injected into the oocyte, [^{3}H]sucrose diffuses rapidly ($D \cong 2 \times 10^{-6}$ cm^2/s) from the site of injection to occupy (within the resolution of autoradiography) the entire intracellular volume (3). However, when diffusion is complete, the sucrose is not distributed

[1] Here, and throughout, we employ "bound" or "binding" when we wish to denote the range of cellular solute phenomena that shares the property of spatial localization or restricted movement. We do not wish to connote the specific mechanism responsible for the restriction. For a discussion of the uses of the terms "bound" and "binding" in cellular transport studies, see Fenichel and Horowitz (2).

TABLE I

SUCROSE SPACE IN N-OOCYTES* OF *D. OCHROPHAEUS*

	Mode of sucrose introduction	$C^{RP}_{sucrose}$	Incubation time	q_s ($C^{c}_{sucrose}/C^{RP}_{sucrose}$)
			h	
A	Gelatin	1.4 μM	4	0.33 ± 0.01
B	Separate	1.4 μM	20	0.30 ± 0.01
C	Separate	1.3 mM	20	0.32 ± 0.01

*Defined as having relative free K^+ concentration ratios, $C^{RP}_K/C^{RP}_K + C^{RP}_{Na}$), ≥ 0.8 (1).

uniformly in the cellular water. At apparent equilibrium, concentrations in nucleus and RP are higher than in cytoplasm, demonstrating that a fraction of the cytoplasmic water is inaccessible as sucrose solvent (3, 4). Moreover, because gelatin water acts as a normal solvent for sucrose (4), a RP can be used to directly measure the sucrose accessible and inaccessible spaces.

Table I presents results of experiments in which (A) [^{3}H]sucrose (55 nM) was injected, dissolved in the gelatin, and the oocyte incubated 4 h, (B) [^{3}H]sucrose (32 nM) was injected after the RP and the oocyte incubated 20 h, and (C) [^{3}H]sucrose (32 μM) was injected after the RP and the oocyte incubated 20 h. The cytoplasmic sucrose space ($C^{c}_{sucrose}/C^{RP}_{sucrose}$) is denoted by q_s. We deduce from Table I that q_s is independent of the mode of sucrose injection because the same value is achieved whether sucrose is introduced in gelatin

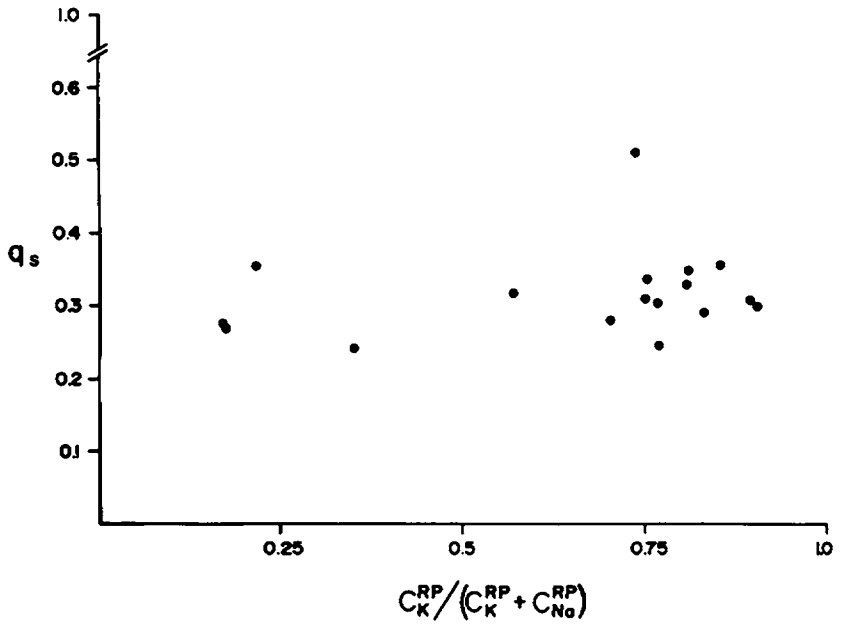

FIGURE 1 Cytoplasm/RP distribution of sucrose, q_s, as a function of $C^{RP}_K/(C^{RP}_K + C^{RP}_{Na})$. [^{3}H] Sucrose solutions were microinjected into oocytes because sucrose permeates the cell too slowly for incubation loading (3).

and followed by diffusion into cytoplasm, or introduced into cytoplasm and followed by diffusion into the RP. The value of q_s is also invariant for 4–24 h after injection. These two observations confirm the earlier conclusion, based on autoradiography, that after injection, sucrose rapidly diffuses to an intracellular equilibrium distribution. Furthermore, Table I shows that, over a 10^3-fold concentration range, q_s is constant, demonstrating that the equilibrium between RP and cytoplasm obeys Henry's law.

The average q_s for all RP oocytes is 0.32 ± 0.01. Its complement, 0.68, is the fraction of cytoplasmic water that excludes sucrose. Two mechanisms have been evoked to explain exclusion: Sucrose is less soluble in cytoplasmic water than in normal aqueous solution; and a fraction of the cytoplasmic water is sequestered in organelles into which sucrose cannot enter. We shall return to this question. For the present, it suffices to consider q_s and, for the i^{th} solute, q_i, as phenomenological coefficients which provide quantitative measures of solute exclusion from cytoplasmic water.

Fig. 1 shows that q_s is independent of $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP})$, which, as a measure of free cation ratios, indexes the oocyte's active transport functions (1). Results in ouabain-treated cells[2] extend this observation to $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP}) < 0.1$. The significance of this will become clear when the isothermal and isotopic data are discussed.

Potassium and Sodium Isotherms

Normal RP cells (N oocytes) maintain 50- and 5-fold gradients of free K^+ and free Na^+, respectively, between their cytoplasm and the outside, whereas other oocytes exhibit smaller gradients (1). Thus RP-containing cells present a spectrum of free cation contents. The isothermal relationships between the total cytoplasmic K^+ or Na^+ concentrations (C_i^c) and the free concentrations measured by the RP (C_i^{RP}) reveal the differences between cytoplasm's interactions with cations and those of an ordinary aqueous solution.

Figs. 2 and 3 show the K^+ and Na^+ concentrations in cytoplasm, C_i^c, plotted as functions of RP concentrations, C_i^{RP}. The dashed line (Fig. 2) is the isomolar line which would describe the data if cytoplasmic water had the same solvent properties as RP water ($\gamma_i^{RP} = \gamma_i^c$), and if no other cytoplasmic interaction were important; that is, if cations distributed uniformly on a water basis. Clearly this is not the case. Two distinct segments characterize the isotherms. At cytoplasmic concentrations <20 μeq/ml, the slopes are greater than unity. Above 20 μeq/ml, the slopes decrease and asymptote to values less than unity. Thus, for each cation, cytoplasm exhibits both an unsaturable fraction linearly related to the RP concentration, but with slope less than expected on a water basis, and a high affinity, saturable fraction with initial slope greater than unity.

The unsaturable cation fractions obey Henry's law for distribution between phases, resembling the behavior of sucrose. Furthermore, the least-square estimates of the limiting slopes in Figs. 2 and 3 are statistically indistinguishable from the observed q_s of 0.32. Isotopic studies described below indicate that these fractions exhibit rapid isotope exchange and quantitatively coincide with the freely diffusible Na^+ and K^+ of the cell. We postulate that the unsaturable cation fractions are dissolved in the water of the cytoplasmic sucrose space and designate this fraction $c1$.

[2]Pearson, T. W., and S. B. Horowitz. Reference phase analysis of free and bound intracellular solutes. III. The effect of ouabain. Manuscript in preparation.

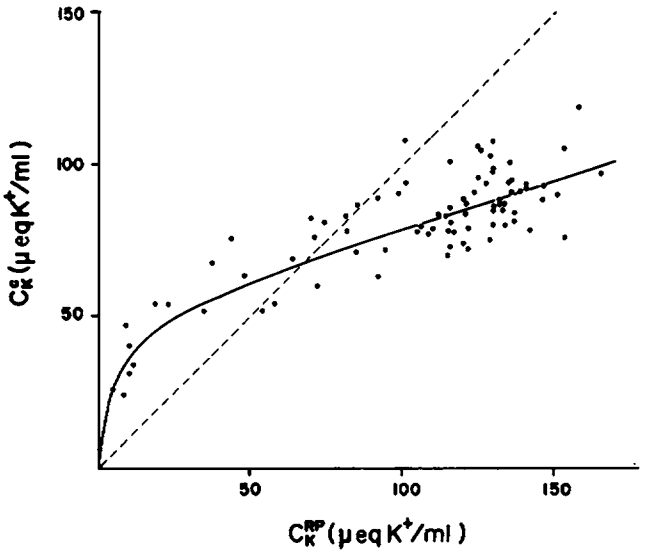

FIGURE 2 Isothermal relations for potassium between cytoplasmic concentrations, C_K^c, and RP concentrations, C_K^{RP}. Solid line is theoretical (Eq. 4) with values for A_K and ${}^{max}C_K^{c2}$ that optimize the fit; see text. Data of 85 oocytes from 15 salamanders are represented. Dashed line is a hypothetical isomolar line, $C_K^c = C_K^{RP}$.

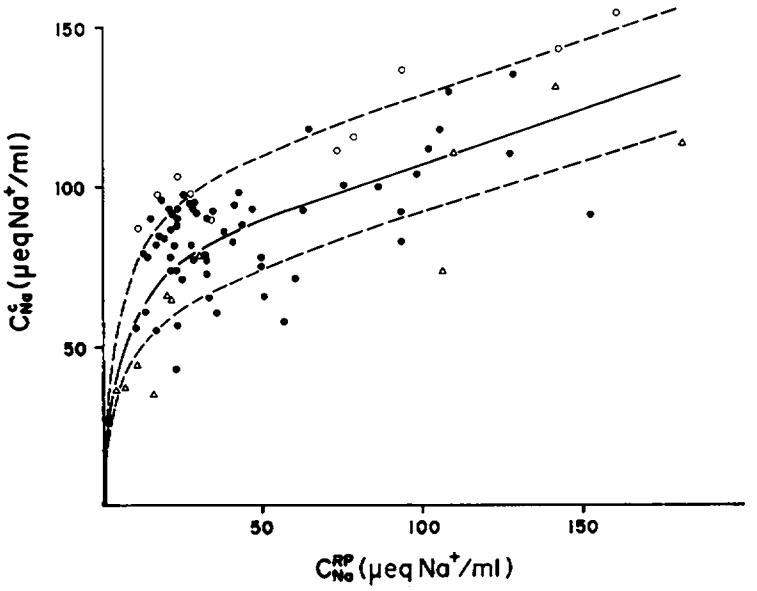

FIGURE 3 Isothermal relations for sodium between cytoplasmic concentrations, C_{Na}^c, and RP concentrations, C_{Na}^{RP}. Solid line is theoretical (Eq. 4) with values for A_{Na} and ${}^{max}C_{Na}^{c2}$ that optimize the fit; see text. ${}^{max}C_{Na}^{c2}$ varies considerably more from one salamander to another than does ${}^{max}C_K^{c2}$ (cf. Fig. 2); this is illustrated by the individual isotherms (dashed lines) for the two salamanders (Δ and 0) contributing 10 or more points. Much of this variation appears to be related to oocyte size.

The remaining cytoplasmic water is assumed (unless otherwise specified) to exclude sucrose completely and is designated as $c2$. The components of $c2$, when considered phenomenologically, are referred to as bound.

The data of Figs. 2 and 3 are fitted by theoretical isotherms derived as follows. It is assumed that the total cytoplasmic i^{th} cation concentration, C_i^c, can be expressed as the sum of two concentrations, C_i^{c1}, the free (diffusive) cation concentration, and C_i^{c2}, the bound cation concentration. Both C_i^{c1} and C_i^{c2} are average concentrations, computed as the amount of free and bound cations, respectively, divided by the total water content of the cytoplasm. When cytoplasm is in diffusional equilibrium with the RP,

$$C_i^{c1} = q_s \cdot C_i^{RP} \tag{1}$$

and

$$C_i^{c2} = {}^{max}C_i^{c2} \cdot f(C_i^{RP}). \tag{2}$$

${}^{max}C_i^{c2}$ is the maximum possible concentration of bound i^{th} cation averaged over the entire cytoplasm, and $f(C_i^{RP})$ is the fraction of ${}^{max}C_i^{c2}$ occupied.

A simple function for $f(C_i^{RP})$ which fits our data is the Langmuir isotherm (5–7)

$$f(C_i^{RP}) = \frac{k_i \cdot C_i^{RP}}{1 + k_i \cdot C_i^{RP}} \tag{3}$$

in which k_i is a binding constant for the i^{th} cation. Using this term, the total cytoplasmic concentration for each cation can be explicitly expressed as

$$C_i^c = C_i^{c1} + C_i^{c2} = q_s \cdot C_i^{RP}\left(1 + \frac{{}^{max}C_i^{c2}}{q_s C_i^{RP} + A_i}\right), \tag{4}$$

where $A_i = q_s/k_i$ and $q_s = 0.32$. In Fig. 2, the solid line represents this equation with ${}^{max}C_K^{c2} = 50\ \mu$eq/ml, $A_K = 1.64$. In Fig. 3, the solid line represents Eq. 4 with $A_{Na} = 1.28$ and a ${}^{max}C_{Na}^{c2}$ value of 80 μeq/ml.

Thus, the data are fitted by a model of cytoplasm in which about one-third of the total water is normal solvent, the remaining water is nonsolvent (or otherwise inaccessible), and the concentration of nondiffusible cytoplasmic K^+ and Na^+ can be related to the RP concentrations by simple sorption isotherms.

Isotopic Analysis

The preceding section fits a two-compartment (free and bound) model to the isotherms that relate total cation concentrations to free cation (RP-determined) concentrations in cytoplasm. Isotope exchange studies with oocytes regularly (8–12)[3] exhibit rapidly and slowly exchanging intracellular Na^+ and K^+ fractions.[4] We may expect these kinetic fractions to

[3] Frank, M., and S. B. Horowitz. Potassium exchange in the amphibian oocyte: whole, cell, cytoplasm, and nucleus. Manuscript in preparation.

[4] Morrill et al. (13) contend that the fast Na^+ and K^+ fractions observed in oocytes are extracellular in origin. This conclusion is based on the observation that if the follicle layer of cells is removed from oocytes, the kinetics of uptake are no longer biphasic. A flaw exists in their argument. Follicle removal was by the method of Masui (14), which involves extended exposure of oocytes to Ca-Mg-free Ringer's containing 1 mM EDTA and subsequent physical removal of follicle cells with forceps. No controls were provided for the Ca-free treatment, though evidence exists that such treatment itself profoundly affects cation exchange (11). We have found that manual follicle removal without media manipulation has no effect on ${}^{42}K^+$ uptake in *Rana pipiens* oocytes (see footnote 3).

TABLE II

$^{22}Na^+$ EXCHANGE AMONG RINGER'S, CYTOPLASM, AND RP

	Total Na^+ concentration	$^{22}Na^+$ concentration	$^{22}Na^+$ specific activity	Compartment specific activity / Ringer's specific activity
	(μeq/ml H_2O)	*(cpm/ml H_2O) $\times 10^{-2}$*	*(cpm/μeq Na^+) $\times 10^{-4}$*	
Ringer's	115.5	61.6	53.3	1.00
RP	23.7 ± 2.2	12.3 ± 2.6	51.8 ± 9.4	0.97
Cytoplasm	84.3 ± 5.4	5.3 ± 0.6	6.3 ± 0.4	0.12

After incubation for 4 h at 5°C in ^{22}Na-Ringer's, RP-containing oocytes were frozen, and the RP and cytoplasm isolated by ULTM. Data are for N oocytes.

arise from $c1$ and $c2$, respectively. Furthermore, the observed rates of the slow fractions are on the order of 1.0% h^{-1} or less—much slower than fast fraction exchange, which implies that $c1$ Na^+ or K^+ fully exchanges before appreciable exchange of $c2$ occurs, and consequently that isotopes may be used to directly measure C_i^{c1} and C_i^{c2}. We have found this to be true.

Table II contains data from a ^{22}Na-Ringer's incubation experiment. It shows that isotopic exchange between the RP and Ringer's is complete by 4 h, but only 0.12 of cytoplasmic Na^+ has exchanged. Because some limited exchange of $c2$ must have occurred, C_{Na}^{c1} is $\leqq 10.1$ μeq/ml (0.12 × 84.3 μeq/ml). This corresponds to a lower limit of 74.2 μeq/ml for C_{Na}^{c2}. The ratio of ^{22}Na concentrations in cytoplasm and RP, $q_{Na^*} = C_{Na^*}^{c}/C_{Na^*}^{RP}$, is 0.43. The difference between this value and q_s (0.32) may be attributed to exchange with the Na^+ of $c2$, though differences in q_s and q_{Na} cannot be excluded.

Similar experiments to determine C_K^{c1} and C_K^{c2} are complicated by the low permeability of the cell membrane to K^+. Table IIIA shows that 7.9 h incubation is insufficient for the RP to reach isotopic equilibrium with ^{42}K-Ringer's. Thus, C_K^{c1} cannot be estimated directly from the cytoplasmic isotope concentration, as was possible for Na^+. However, q_{K^*}, the ratio of the ^{42}K concentration in the cytoplasm, $C_{K^*}^{c}$, to that in the RP, $C_{K^*}^{RP}$, is constant with time. This demonstrates that intracellular diffusional equilibrium has been reached. Consequently, an upper limit for C_K^{c1} of 45.7 μeq/ml, and a lower limit of C_K^{c2} of

TABLE III

DISTRIBUTION OF $^{42}K^+$ AMONG RINGER'S, CYTOPLASM, AND RP

Mode of tracer introduction	Incubation time	$\frac{(\text{Specific activity})^{RP}}{(\text{Specific activity})^{Ringer's}}$	$C_K^{c*}/C_{K^*}^{RP}$
	h		
A. Incubation	1.9	0.12 ± 0.01	0.33 ± 0.01
	3.5	0.14 ± 0.04	0.35 ± 0.01
	7.9	0.33 ± 0.08	0.33 ± 0.01
B. Injection	1.0	—	0.35 ± 0.05
	2.0	—	0.36 ± 0.02

RP-containing oocytes were incubated continuously in ^{42}K-Ringer's (A) or microinjected (B) with ^{42}K in injection medium. After incubation at 5°C, the cells were frozen, and the RP and cytoplasm isolated by ULTM. Data are for N oocytes.

TABLE IV

EXPERIMENTALLY DETERMINED CYTOPLASMIC VARIABLES FOR N-OOCYTES

	Potassium				Sodium*			
	C_K^{c1}	C_K^{c2}	$C_K^{c1'}$	$C_K^{c2'}$	C_{Na}^{c1}	C_{Na}^{c2}	$C_{Na}^{c1'}$	$C_{Na}^{c2'}$
				(μeq/ml)				
Isothermal	40.5	48.1	126.6	70.7	8.0	67.2	25.0	98.8
Isotopic‡ (incubation)	45.7	45.3	142.8	66.6	10.1	74.2	31.6	109.1
Isotopic‡ (microinjection)	43.1	47.4	134.7	69.7	—	—	—	—

Summary of the isothermally and isotopically derived values for cytoplasmic variables C_i^{c1} and C_i^{c2} in N oocytes. In addition to these quantities (defined in the text), calculated values are shown for $C_i^{c1'}$ ($= C_i^{c1}/q_s$) and $C_i^{c2'}$ ($= C_i^{c2}/1 - q_s$), which are the concentrations in compartments $c1$ and $c2$, respectively, calculated on the basis of the water in each compartment.

*The variability of $c2$ Na^+ is discussed in the legend to Fig. 3.

‡The assumption is made that no isotopic exchange with $c2$ has occurred, that is, that $C_{i^*}^c/C_{i^*}^{RP} \equiv q_{i^*} = q_i$, consequently, values for C_i^{c1} are upper limits, and for C_i^{c2}, lower limits.

45.3 μeq/ml can be estimated using Eq. 1 if we assume no exchange with $c2$ has occurred, and substitute q_{K^*} (= 0.33) for q_s. The complementary lower limit for C_K^{c2} is 45.3 μeq/ml.

We also have microinjected ^{42}K into RP-containing oocytes to bypass the rate-limiting cell membrane. These experiments are summarized in Table IIIB, and the calculated variables are $q_{K^*} = 0.35$ and $C_K^{c1} \leq 43.1$ μeq/ml, $C_K^{c2} \geq 47.4$ μeq/ml.

Table IV summarizes the determination of C_i^{c1} and C_i^{c2} in N oocytes by three methods. The agreement among values determined by isothermal and isotopic methods is good, and would improve if a correction of a few percent were introduced to compensate for the small systematic overestimate of C_i^{c1} that results from isotopic exchange with $c2$.

The data on intracellular isotope distribution in Tables II and III are restricted to N oo-

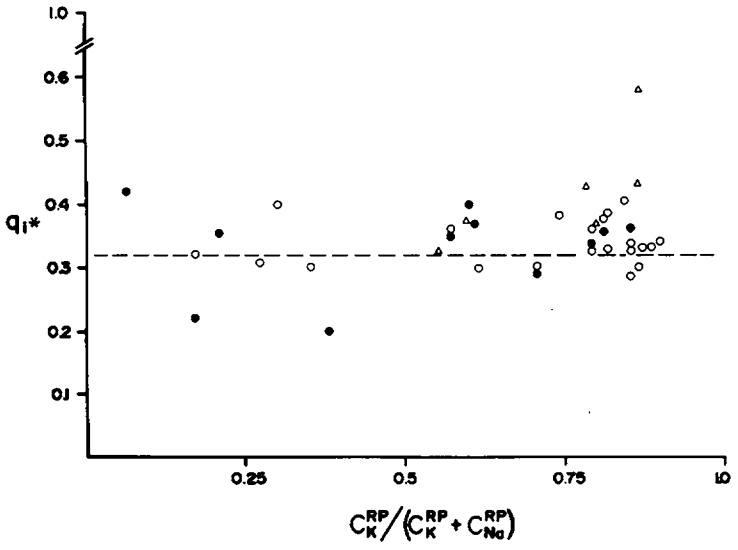

FIGURE 4 Cytoplasm/RP distribution, q_{i^*}, as a function of $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP})$ for tracer solutes: $^{42}K^+$ (o), $^{22}Na^+$ (Δ), and $^{36}Cl^-$ (•). Isotopes were introduced by incubating RP-containing oocytes in Ringer's containing tracer at 5°C, except when microinjected in some $^{42}K^+$ experiments (Table IIIB).

cytes. They indicate that freely diffusible K^+ and Na^+ are, like sucrose, excluded from about two-thirds of the cytoplasmic water. In Fig. 4, $q_{i^*}(C_{i^*}^c/C_{i^*}^{RP})$ for $^{42}K^+$, $^{22}Na^+$, and $^{36}Cl^-$ demonstrates that this exclusion is not restricted to N oocytes, and is independent of $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP})$ over a wide range of values.

The dotted line in Fig. 4 indicates 0.32, the value of q_s. Values of $C_{i^*}^c/C_{i^*}^{RP}$ for the three ions, q_{i^*}, generally fall above this line. This difference may be attributable to exchange with the bound ions in $c2$. If this is the case, then q_i, the "free" solute spaces of these ions, are equal to each other and to q_s. On the other hand, q_i may show differences between solutes. Carefully performed kinetic experiments might distinguish these possibilities.

Finally, it should be noted that C_i^f/C_i^{RP} does not depend on solute charge. Dialysis experiments described in the preceding paper (1) show that gelatin behaves macroscopically as if it were electrically neutral. Fig. 4 shows this also is true for cytoplasm.

ACTIVITY COEFFICIENTS

RP Na^+ and K^+ activities equal those of cytoplasm ($a_i^{RP} = a_i^f$), and RP activity coefficients equal those of ordinary aqueous solution ($\gamma_i^{RP} = \gamma_i^o$). Hence cytoplasmic activities can be determined from $a_i^f = C_i^{RP}\gamma_i^o$ and "apparent" activity coefficients from $\gamma_i^f = a_i^f/C_i^f$. The $\gamma_i^f - C_i^f$ relations for Na^+ and K^+ are presented in Fig. 5. At low C_i^f activity coefficients γ_i^f

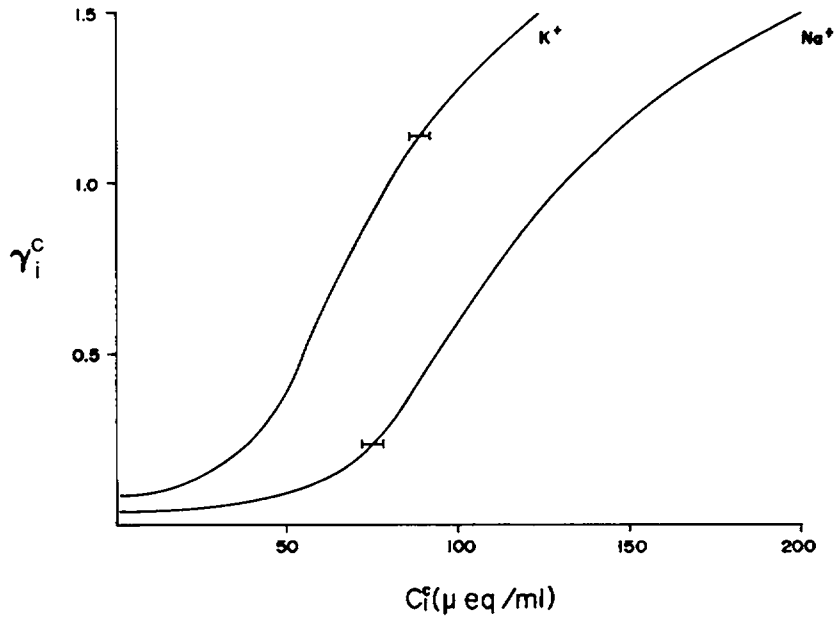

FIGURE 5 Relationship between the cytoplasmic activity coefficient, γ_i^f, and the cytoplasmic concentration, C_i^f, for K^+ and Na^+. Derived from the equation

$$C_i^f = \gamma_i^{RP}\left(\frac{^{max}C_i^{c2}}{\gamma_i^{RP} - q_s\gamma_i^c} - \frac{A_i}{q_s\gamma_i^c}\right)$$

which combines Eq. 4 and the relationship $\gamma_i^{RP}C_i^{RP} = \gamma_i^f C_i^f$ (1). Values for q_s, A_i, and $^{max}C_i^{c2}$ are given in the text. γ_i^{RP} taken as 0.77 (28). Ranges indicated on curves correspond to the mean values (±1 SEM) for C_K^c and C_{Na}^c in N-oocytes of *D. ochrophaeus*.

are low; at high C_i^f they are high and may exceed unity. The sigmoid curves of Fig. 5 reflect the changing contributions of solute exclusion and solute binding to γ_i^f as the free cation concentration varies.

This exercise is of limited importance in understanding *Desmognathus* oocytes, because γ_i^f is a derivative term which provides no information not disclosed by the isotherm and the isotopic exchange data. However, from another point of view, these activity coefficients are important in that they are comparable to determinations of γ_i^f, available for a variety of cells, based on ion-sensitive microelectrode studies. Consequently, the $\gamma_i^f - C_i^f$ relations in Fig. 5 provide a basis for extending the conclusions of RP analysis to cells in which RP analysis has not been performed.

Ion-sensitive microelectrodes have been used to measure cytoplasmic ionic activities, a_i^f, and activity coefficients, γ_i^f, in oocytes of *Rana pipiens* (15), which resemble those of *Desmognathus* (16). Palmer et al. (15) found that in *R. pipiens*, in which C_{Na}^f = 73–77 μeq/ml and C_K^f = 93–104 μeq/ml, the respective activity coefficients were γ_{Na}^f = 0.08 ± 0.02 and γ_K^f = 1.29 ± 0.04 to 1.15 ± 0.04. The values of C_{Na}^f and C_K^f from Fig. 5 predict corresponding activity coefficients of γ_{Na}^f = 0.21–0.26, and γ_K^f = 1.20–1.33. Considering that the comparisons are between oocytes of different species, these similarities are remarkable, and constitute a mutual validation of RP and ion-sensitive microelectrode methods. The former measures the ion concentration, and the latter the activity, in the freely diffusible compartment, $c1$.

The RP and microelectrode-determined values of γ_{Na}^f in amphibian oocytes resemble those in other cells. Examples are 0.36 in immature *Bufo* oocytes, 0.46 in *Planorbis* neuron, 0.17 in *Balanus* muscle, 0.48 in *Rana* epithelium, 0.19 in *Rana* muscle (17), and 0.48 in immature *Rana* oocytes (15). Combining the data of Kroeger et al. (18) and Palmer and Civan (19), we have calculated γ_{Na}^f = 0.26 in *Chironomus* salivary gland cells. Na^+ activity coefficients measured in cells are regularly less than in normal solutions of comparable concentration. Consequently, Na^+ binding in cytoplasm has been widely recognized, a conclusion fully consistent with the present results from RP analysis.

Unlike Na^+, the measured cytoplasmic activity coefficients of K^+ have been about equal to, or greater than, those of aqueous solutions ($\gamma^o \simeq 0.65$–0.85). This is true for the *Rana* oocytes and other cells. Examples are: 0.56 in *Loligo* axon, 0.71–0.79 in *Rana* muscle, 1.0 in *Rana* epithelium, 1.15 in *Balanus* muscle (17), and 1.0 in *Chironomus* salivary gland (18, 19). These values have been interpreted to mean that the state of K^+ in cells is similar to normal aqueous solution (see references 17 and 20 for notable exceptions). The RP results and Fig. 5 demonstrate that this interpretation is incorrect for the oocyte. They show that $\gamma_i^f = \gamma_i^o$ does not signify the absence of binding when solute exclusion also occurs. Direct evidence of exclusion in cells other than oocytes is provided by the values of $\gamma_i^f > \gamma_i^o$ seen in *Rana* epithelium, *Balanus* muscle, and *Chironomus* salivary gland. Collectively, the data on K^+ suggest that $c2$ is a common property of cells though differences in q_i and ${}^{max}C_i^{c2}$ may influence its contribution to C_i^f.

DISCUSSION

Our results demonstrate that, in oocytes, water and ions constitute not a single kinetic phase but (at least) two dissimilar phases. Injected sucrose rapidly distributed in the equivalent of one-third of the cytoplasmic water, whereas the remaining water is unoccupied even after

20 h. Similarly, an equal volume of cytoplasmic water is accessible to diffusible Na^+ and K^+, while the remainder is not. Isothermal and isotopic analysis shows the accessible water to contain 10% of the Na^+ and 50% of the K^+ of N oocyte cytoplasm.

The inaccessible water and ions can be treated formally as a second phase, distinct from the accessible phase, $c1$. The inaccessible phase, $c2$, contains the slowly exchanging cytoplasmic cations, about 90% of the Na^+ and 50% of the K^+ in N oocytes. $c2$ largely excludes diffusible cations. However, its "bound" or slowly exchangeable cations are in a mass-action equilibrium with the diffusible Na^+ and K^+, and this equilibrium is described by a Langmuir isotherm.

A two-phase cytoplasm, distinguished by fast and slow exchange, is not unexpected; evidence of its presence is a consistent feature of studies of K^+ and Na^+ in oocytes (8–12 and footnote 3). The RP findings appear to eliminate any uncertainty that may have existed (13). The fast exchanging fraction, $c1$, whose concentrations are measured by the RP, apparently is similar to a normal aqueous solution with its contents determined and regulated by the transport properties of the cell membrane. The question arises: what is the physical nature of $c2$, the bound fraction?

Compartment or Sorption Model

Dichotomous kinetic behavior by cellular solutes is usually explained within one of two paradigms—compartment or sorption (in Kleinzeller's nomenclature [21], structural and chemical compartmentalization, respectively).

In compartment models, cytoplasmic water and cations exist in organelles as ordinary dilute aqueous solutions. Nonideal behavior and exclusion are accounted for by intracellular membranes. The observed q_i in oocytes and the rapidly and slowly exchanging Na^+ and K^+ signify in these "vesicle" models that about two-thirds of the cytoplasmic water and a considerable fraction of the cations are sequestered in virtually impermeable compartments ($c2$) surrounded by a continuous aqueous phase ($c1$).

In sorption models, the same results imply that a substantial fraction of cytoplasmic water and cations is adsorbed to macromolecules. Cation adsorption explains the nonlinear portion of the RP cytoplasm isotherms and slow isotopic exchange. Cytoplasmic water is viewed as differing from ordinary dilute aqueous solution in exhibiting greater intermolecular association and, as a consequence, there is an enhanced expression of water's inherent nonideal properties. The observed values of q_i imply that the average solvent capacity of cytoplasmic water is only 32% that of ordinary water.

Though sorption and compartment paridigms differ in the predictions they can make about cytoplasm, a number of models are admissible within each, and it is usually difficult to make a clear distinction in practice. This is especially true because compartmentalization and sorption are not intrinsically exclusive, and cytoplasm could include both a significant fraction of sorbed water and cations, and organelles capable of sequestering water, Na^+, and K^+. Nevertheless, it would be difficult to overestimate the importance of distinguishing between these two mechanisms as the explanation of any specific cellular process.

We enumerate below observations that seem relevant to the question of compartment or sorption model. Although none is conclusive, collectively they lead us to favor the hypothesis that $c2$ consists of water and cations adsorbed to macromolecules.

(*a*) $c2$ contains about 70% of the oocyte's K^+ and Na^+ (Table IV). To maintain electroneutrality, $c2$ must contain an equal fraction of the oocyte's anions. In a sorption model these are the anionic groups of fixed macromolecules. In a compartment model they presumably are smaller anions subject to vesicle membrane transport. However, we have found that the most plausible candidate anions, the diffusible inorganic and organic phosphates, are strongly excluded from $c2$ relative to $c1$. (Table 1, reference 1). Similarly, as shown by $q_{Cl^-} \simeq 0.32$ (Fig. 4), free Cl^- is also excluded. The compartment model appears not to answer the question of which anions are neutralizing $c2$ cations.

(*b*) Isotherm data for K^+ and Na^+ show that these cations vary independently of each other in $c2$. This is comprehensible if $c2$ contains adsorbing sites of low cross-affinity. However, the observation is difficult to reconcile with a compartment model; it seems necessary to postulate a variety of organelles (compartments), each of which accumulates Na^+ and K^+ differently. The membranes of these vesicles would necessarily be functionally different from the plasma membrane.

(*c*) Isotherm data show $c2$ to have a maximum achievable K^+ and Na^+ concentration, ${}^{max}C_i^{c2}$, which means that $c2$ is saturable. In sorption models, this implies a finite number of macromolecular adsorption sites, a reasonable expectation. The implication for a compartment model seems to be a complex membrane phenomenon in which permeability is an inverse function of C_i^{c1}.

(*d*) Our data fit a known sorption model. Eq. 4 incorporates the Langmuir adsorption isotherm to describe a phase containing both adsorbing and free solute components. (Expressions similar to Eq. 4 have previously been used to describe sorption models of solute distribution between cytoplasm and extracellular medium [7, 22]). Our attempts to find a similarly uncomplicated expression with explicit meanings within the compartment paradigm have regularly failed.

These observations imply a sorption model of $c2$, if for no other reason than the difficulties they present for a compartment model. However, there are problems with acceptance of a sorption model. Most important is that no macroscopic physical system has been identified that quantitatively combines the characteristics of marked Na^+ and K^+ selectivity, exclusion of small polar solutes, and high water content of $c2$. We can measure differences in the properties of cytoplasmic water, but lack relevant, defined reference systems for their interpretation. (Recent attempts to understand the microscopic environment of cellular monovalent sorption sites, Eisenman and Krasne [23], have suggested models which at the macroscopic level would have $c2$-like properties.)

Absence of convincing macroscopic physical analogues can be taken either as evidence that sorption models are implausible or as confirmation of cytoplasm's special properties (not the least of which may be the instability of its tertiary structure). The latter view requires extrapolation from the few understood properties of water, proteins, and electrolyte solutions to the observed properties of living cells—a shaky venture because of the complexity of these systems. Nevertheless, our results indicate the importance of attempting to understand $c2$ in the context of adsorbed water and cations. There follows a qualitative description of a model which emphasizes certain striking or paradoxical aspects of the observed solute behavior, suggesting how, if exaggerated by structuring through interaction with macromolecules, water's properties may account for these.

c2 Adsorption

Water loses molecular freedom through interaction with macromolecules and becomes more crystalline (24, 25). It can be shown from the known cooperativity of H-bonding in water that this structuring is propagated outward from macromolecular surfaces, being greatest close to the surface, and decreasing with distance (26). The solvent ability of water is a function of water mobility and decreases with increasing structure. The explicit functions relating solvent ability and structure are unknown. However, it is likely that the solubility of free polar solutes, measured by q_i, will be lowest close to macromolecular surfaces and increase sharply (possibly to unity with respect to bulk water) with increasing distance from these surfaces.[5]

Low values of q_i imply low free energies of solution in $c2$ water relative to ordinary water. The molecular forces that determine these free energies are those that determine ion-pair formation. Sorption of Na^+ and K^+ can be viewed as ion-pair formation between a macromolecular fixed anion and the cation derived from solution. Thus, physical grounds exist for an explicit understanding of the relationship of q_i to both the high degree of cation sorption in cytoplasm and the kinetics of exchange of adsorbed cations.

Isotopic exchange data show that Na^+ and K^+ in $c1$ exchange very slowly with that in $c2$. Estimated rates from other amphibian oocytes are about 1.0% h^{-1} for Na^+ (9) and less for K^+ exchange (see footnote 3). This might lead one to expect that the mass-action response of $c2$ to changes in free cation concentration, C_i^{c1}, also would be slow. Is this the case? The answer for increases in C_i^{c1} is not known, but it appears that, for K^+ at least, decreases in C_K^{c1} are followed rapidly by a decrease in C_K^{c2}. If it were otherwise, we would not be able to discern, as shown in Fig. 2, the nonlinear fraction of the isotherm. These oocytes have lost $c1$ K^+ in changing from N oocytes to oocytes in which $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP}) < 0.8$, and this has occurred in minutes. Hence, if isotopic exchange rates prevail, too short a time elapses for appreciable desorption to occur. Apparently, desorption in response to changing K^+ activity is rapid, whereas isotopic exchange is slow.

Edze and Berendsen (27) have pointed out that the slow isotopic exchange rates observed in cytoplasm cannot reasonably be attributed to enthalpically determined residence times, as this would require "incredible" binding energies. However, the hypothesized crystalline solvent property of $c2$ water for polar substances, that is, very low values of q_i close to macromolecular surfaces, suggests an essentially entropic explanation. The unit process in adsorbed cation exchange requires a number of coincident events: (*a*) the presence of a free, isotopically labeled ion pair; (*b*) the dissociation of the sorbed cation from its fixed anion; (*c*) the dissociation of the free cation from its free anion; and (*d*) an exchange of partners. The probability of each of these events occurring can be expected to be strongly reduced by water structure.

Another property of water may provide an explanation for the paradoxical observations of rapid mass-action desorption and slow isotopic exchange of $c2$. Proton transport is entirely different in water from the transport of other cations because of a proton-jump mechanism (28) that more closely resembles the rapid charge transfer of semiconductors than ion

[5]Solutes, such as urea, that influence water structure might constitute an exception to this generalization.

diffusion. This difference is enhanced in crystalline water systems (29). If H^+ were the primary competing cation for K^+ and Na^+ adsorption sites, and the compensating cation when Na^+ or K^+ desorbed in response to a concentration change in $c1$, the speed with which mass-action desorption occurs could be explained.

A role for H^+ in K^+ and Na^+ exchange helps to clarify another puzzling finding. As a sorption isotherm, Eq. 3 describes a one ligand-one site model. The observation that an isotherm of this form (Langmuir) fits our data was unexpected. RP Na^+ and K^+ vary in a compensatory manner, and we anticipated the same in $c2$. However, our attempts to fit isotherms that include competition have been unsuccessful, and separate sites are indicated for the two species. This leaves the problem of accounting for electroneutrality when Na^+ or K^+ is lost from $c2$. The problem is clarified if one assumes that H^+ is the compensating cation. One need then only postulate two classes of $c2$ ligands, one that (at the Na^+, K^+, and H^+ activities that prevail in cytoplasm) adsorbs in the order $K^+ > H^+ \gg Na^+$, and a second $Na^+ > H^+ \gg K^+$. The sorption isotherm for each of these ligands would reduce to the Langmuir in aqueous systems where H^+ is available.

The Identity of c2

The properties of $c2$ suggest a model resembling the structured portion of hydrate protein crystals. In protein crystals, water exists in bulk form, occupying the solvent channel spaces, and in structural hydration shells composed of more localized water. The former "free solvent" region is accessible to external solutes; the latter is not (30). Ordered structures with crystal-like regularities are widespread in cells (31). In electron micrographs, they can appear as latticed depositions of osmium, uranium, lead, or other heavy metals used as electron-dense stains. Occasionally, as in the myofibril, such structures are sufficiently extensive and oriented to be studied directly in vivo by X-ray diffraction (32).

Yolk platelets, which are latticed structures (33), are prominent features of oocyte cytoplasm. They are regular hydrate crystals and contain 80–90% of the protein of the grown oocyte. Platelets are rich in anionic phosphate and carboxylic residues and contain about 30% water (34, 35); thus they are the obvious candidate for the primary source of $c2$ behavior. Observations demonstrating the parallel spatial and temporal distributions of $c2$ and platelets support this view.

An important difference between $c1$ and $c2$ is their Na^+/K^+ ratios. The ratio in $c1$ ($C^{c1'}_{Na}/C^{c1'}_{K}$) is about 0.2; in $c2$ ($C^{c2'}_{Na}/C^{c1'}_{K}$), it is about 1.5 (Table IV). Hence, if yolk platelets are the origin of $c2$ behavior, the ratio C_{Na}/C_K in a given phase within a N oocyte will vary progressively from about 0.2, in the complete absence of platelets, to 1.5, in a platelet-dense phase. This expectation is confirmed in a number of circumstances.

(*a*) Platelets are shown by microscopy to be absent from the oocyte nucleus and the RP (1). The ratio C_{Na}/C_K in the nucleus of *Desmognathus* has been determined to be 0.11 (16); in the RP, this ratio is 0.16 (1). Both also appear to lack $c2$.

(*b*) Platelets are the most dense organelle of the oocyte and are readily concentrated by centrifugation. The yolk platelet fraction of *Triturus cristatus* oocytes has a ratio (C_{Na}/C_K) of 1.50 (36), identical to that expected of $c2$.

(*c*) In oocytes of the anurans, *Xenopus laevis* and *R. pipiens*, platelets are more densely compacted in the vegetal than the animal hemisphere. In *Desmognathus*, they are denser in the medullary than the cortical cytoplasm. If platelets are the origin of $c2$ behavior,

C^{ξ}_{Na}/C^{ξ}_{K} should be higher in regions of dense platelet concentrations. This has been tested using ULTM, by isolating cytoplasm from specific regions. We found the expected correlation to hold in anuran oocytes.[6] Similarly, in *Desmognathus*, the ratio, C^{ξ}_{Na}/C^{ξ}_{K}, is 1.22 ± 0.14 in the medullary and 0.86 ± 0.02 in the cortical cytoplasm.

(*d*) The density of platelets in cytoplasm increases as the oocyte grows (33), and this is associated with increasing C^{ξ}_{Na}/C^{ξ}_{K}. Palmer et al. (15) determined Na^+ and K^+ in *R. pipiens* oocytes of 200–300 μm diameter and in the full-grown yolky oocyte. The ratio C^{c}_{Na}/C^{c}_{K} in the latter is 0.79, in good agreement with 0.85 in *Desmognathus* (1); in the former, the ratio is 0.3. Similarly, the ratio reported in relatively yolk-deficient, small oocytes of *Bufo bufo* is 0.23 (37).

We conclude that, in the amphibian oocyte, *c*2 is attributable primarily to yolk platelets. In fact, though it is difficult to quantitate the point, there seems to be no other material sufficiently abundant to account for this fraction. The abundance of yolk in grown oocytes masks other sources of *c*2 behavior, if they exist. However, evidence that other sources may exist is suggested by the demonstration of Palmer et al. (15) that γ_{Na} in 200–300 μm *R. pipiens* oocytes (which are presumably yolk free [33]) is 0.48 ± 0.15, lower than that expected (≃0.77) of a completely *c*1 system.

Implications

We have argued above, based on the positive and negative deviations from normal water values of γ^{ξ}_{K} and γ^{ξ}_{Na}, respectively, that *c*2 is a common feature of cells. Other evidence exists.

Lee and Armstrong (38) reported that in *R. pipiens* sartorius muscle, incubation in K^+-free Ringer's results in the loss of 30 μeq K^+/ml muscle water in 48 h, whereas microelectrode measurements of activity account for a loss of only 13 μeq K^+/ml. These investigators concluded that muscle contains free and bound K^+ fractions, and that the unaccounted 17 μeq K^+/ml was lost from a bound fraction (*c*2 in our nomenclature). Thus, in effect, a few points of a muscle cell isotherm have been established. (They have done the same for Na^+ and for muscle modified by low calcium.)

Figs. 1 and 4 show that in the oocyte q_s and q_{i^*} are not modified by altered membrane function. If comparable exclusion behavior occurs in other cells, it might explain the observation that ouabain inhibition of membrane Na^+-K^+-ATPase activity does not raise cellular Na^+ to extracellular levels (21, 39). Similarly, the appearance of "pumping" by ouabain-poisoned cells of Li^+, choline, and Tris, may be accounted for by *c*2 exclusion; this would relieve the necessity of postulating a ouabain-insensitive cell membrane transport system (21, 39).

In addition, the existence of *c*2 can be deduced in a variety of cells by other techniques. Bound water has been identified by the exclusion of solutes and as osmotically inaccessible (21, 40), nonfreezable (41), and of reduced mobility (42–45, cf. 46); bound cations have been deduced from cellular concentrations exceeding Donnan equilibrium expectations (47, 48), from nuclear magnetic resonance evidence (49, cf. 46), and from multiexponential exchange kinetics (9–12, 50–52).

We have attributed *c*2 in the amphibian oocyte to yolk platelets. However, the protein

[6]Reynhout, J. K., and S. B. Horowitz. Manuscript in preparation.

constituents of platelets, lipovitellin and phosvitin (34, 35), are not present in muscle, for example, and cannot account for the evidence of binding in this tissue (38, 40, 43, 45, 47, 51–53) nor in most cells where *c*2-like behavior is observed. We believe that the transport and equilibrium characteristics of *c*2 arise from the ordered water of the platelets. In this regard, platelets seem to be an especially suitable structure in which to demonstrate binding, because the unit cell volume occupied by water is only 22%. This is less than in any of 226 globular protein crystals recently surveyed (30). The modal hydration range among these is 40–50%, with volumes as great as 78% reported (30). The implication is that a higher proportion of platelet water may be ordered, and that this ordering may be more complete than is typical in protein crystals, resulting in greater solute exclusion, and slower, more easily distinguishable exchange rates.

It seems possible that platelets are at one end of a continuum of cellular hydrate protein structures which exhibit solute exclusion, slow cation exchange rates, and the other "binding" properties we have attributed to *c*2. In the important case of the myofibril, the crystalline structure is highly hydrate, and the average order imposed by the protein components, one can assume, is intermediate between that of yolk platelets and bulk water. Perhaps, therefore, it is to be expected that students of muscle transport find it possible to hold widely divergent views, from the position that the cell is essentially a membrane-enclosed volume of bulk water, similar to *c*1, to one in which it is a relatively homogeneous hydrate crystal, similar to *c*2 (43, 45, 49, 51–57).

Data suggesting the presence of both free and bound cytoplasmic water and monovalent cations have been known and contested for years. Unfortunately, the complexities of the cell membrane, the uncertainty introduced by an extracellular space of unknown dimensions, and the instability of cytoplasm have conspired to introduce a pervasive ambiguity into this area of research. We believe the introduction of ULTM and the RP technique will help remove some of the causes of this ambiguity.

We thank Drs. M. R. Das, G. Eisenman, P. Furmanski, K. Magleby, J. Putney, T. W. Pearson, S. Rapaport, and J. Reynhout for helpful discussions, and Mr. L. Tluczek and Dr. J. Reynhout for assistance with experiments.

This work was supported by grants GM 19548 and CA 17456 from the National Institutes of Health and an institutional grant from the United Foundation of Greater Detroit.

Received for publication 18 May 1978 and in revised form 8 September 1978.

REFERENCES

1. HOROWITZ, S. B., P. L. PAINE, L. TLUCZEK, and J. K. REYNHOUT. 1979. Reference phase analysis of free and bound intracellular solutes. I. Sodium and potassium in amphibian oocytes. *Biophys. J.* **25**:33–44.
2. FENICHEL, I. R., and S. B. HOROWITZ. 1969. Intracellular transport. *In* Biological Membranes. R. Dowben, editor. Little, Brown & Co., Inc., Boston. 177–221.
3. HOROWITZ, S. B. 1972. The permeability of the amphibian oocyte nucleus, in situ. *J. Cell Biol.* **54**:609–625.
4. HOROWITZ, S. B., and P. L. PAINE. 1976. Cytoplasmic exclusion as a basis for asymmetric nucleocytoplasmic solute distributions. *Nature (Lond.).* **260**:151–153.
5. MOORE, W. J. 1972. Physical Chemistry. Prentice-Hall Inc., Englewood Cliffs, N.J. 4th edition. 497.
6. TROSHIN, A. S. 1961. Sorption properties of protoplasm and their role in cell permeability. *In* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. Academic Press Inc. Ltd., London. 45–53.
7. KATCHALSKY, A. 1961. Discussion. *In* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. Academic Press Inc. Ltd., London. 104–105.

8. DICK, D. A. T., and D. J. FRY. 1973. Location of inexchangeable sodium in the nucleus and cytoplasm of oocytes of *Bufo bufo* exposed to sodium-free solutions. *J. Physiol. (Lond.).* **231**:19–21.
9. HOROWITZ, S. B., and I. R. FENICHEL. 1970. Analysis of sodium transport in the amphibian oocyte by extractive and radioautographic techniques. *J. Cell Biol.* **47**:120–131.
10. CENTURY, T. J., and S. B. HOROWITZ. 1974. Sodium exchange in the cytoplasm and nucleus of amphibian oocytes. *J. Cell Sci.* **16**:465–471.
11. ECKER, R. E., and L. D. SMITH. 1971. Influence of exogenous ions on the events of maturation in *Rana pipiens* oocytes. *J. Cell. Physiol.* **77**:61–70.
12. TUPPER, J. T., and B. L. MALOFF. 1973. The ionic permeability of the amphibian oocyte in the presence or absence of external calcium. *J. Exp. Zool.* **185**:133–144.
13. MORRILL, G. A., D. ZIEGLER, and V. S. ZABRENETZKY. 1977. An analysis of transport, exchange, and binding of sodium and potassium in isolated amphibian follicles and denuded oocytes. *J. Cell Sci.* **26**:311–322.
14. MASUI, Y. 1967. Relative roles of the pituitary, follicle cells, and progesterone in the induction of oocyte maturation in *Rana pipiens*. *J. Exp. Zool.* **166**:365–376.
15. PALMER, L. G., T. J. CENTURY, and M. M. CIVAN. 1978. Activity coefficients of intracellular Na^+ and K^+ during development of frog oocytes. *J. Membr. Biol.* **40**:25–38.
16. CENTURY, T. J., I. R. FENICHEL, and S. B. HOROWITZ. 1970. The concentrations of water, sodium and potassium in the nucleus and cytoplasm of amphibian oocytes. *J. Cell Sci.* **7**:5–13.
17. LEV, A. A., and W. MCD. ARMSTRONG. 1975. Ionic activities in cells. *Curr. Top. Membranes Transp.* **6**:59–123.
18. KROEGER, W. TRÖSCH, and G. MÜLLER. 1973. Changes in nuclear electrolytes of *Chironomus thummi* salivary gland cells during development. *Exp. Cell Res.* **80**:329–339.
19. PALMER, L. G., and M. M. Civan. 1977. Distribution of Na^+, K^+ and Cl^- between nucleus and cytoplasm in *Chironomus* salivary gland cells. *J. Membr Biol.* **33**:41–61.
20. HINKE, J. A. M. 1969. Glass microelectrodes in the study of binding and compartmentalization of intracellular ions. *In* Glass Microelectrodes. M. Lavallee., O. F. Schanne., and N. C. Hebert, editors. John Wiley & Sons Inc., New York. 349–357.
21. KLEINZELLER, A. 1972. Cellular transport of water. *In* Metabolic Transport. Vol. VI. L. E. Hokin, editor. Academic Press Inc., New York. 91–131.
22. NASONOV, D. N. 1959. Local Reaction of Protoplasm and Gradual Excitation. Academy of Science, U.S.S.R. Institute of Cytology. Moscow-Leningrad. Translated by Y. S. Halpern for the National Science Foundation, Washington, D.C., 1962. 116–126.
23. EISENMAN, G., and S. J. KRASNE. 1975. The ion selectivity of carrier molecules, membranes and enzymes. *MTP Inter. Rev. Sci. Biochem. Ser.* **2**:27–59.
24. TAIT, M. J., and F. FRANKS. 1971. Water in biological systems. *Nature (Lond.).* **230**:91–94.
25. BERENDSEN, H. J. C. 1975. Specific interactions of water with biopolymers. *In* Water: A Comprehensive Treatise. Vol. V. F. Franks, editor. Plenum Publishing Corporation, New York. 293–349.
26. HAGLER, A. T., and J. MOULT. 1978. Computer simulation of the solvent structure around biological macromolecules. *Nature (Lond.).* **272**:222–225.
27. EDZES, H. T., and H. J. C. BERENDSEN. 1975. The physical state of diffusible ions in cells. *Annu. Rev. Biophys. Bioeng.* **4**:265–285.
28. ROBINSON, R. A., and R. H. STOKES. 1970. Electrolyte Solutions. Butterworth & Co. Ltd., London. 2nd revised edition.
29. EIGEN., and L. DEMAEYER. 1958. Self-dissociation and protonic charge transport in water and ice. *Proc. R. Soc. (Lond.).* A **247**:505–533.
30. MATTHEWS, B. S. 1977. X-ray structure of proteins. *In* Proteins. H. Neurath and R. L. Hills, editors. Academic Press, New York. 3rd edition. 403–590.
31. FAWCETT, D. W. 1966. The Cell. W. B. Saunders Company, Philadelphia.
32. HANSON, J. 1968. Recent x-ray diffraction of muscle. *Q. Rev. Biophys.* **1**:177–216.
33. WISCHNITZER, S. 1966. The ultrastructure of the cytoplasm of the developing amphibian egg. *Adv. Morphog.* **5**:131–179.
34. WALLACE, R. A. 1963. Studies on amphibian yolk. IV. An analysis of the main-body component of yolk platelets. *Biochim. Biophys. Acta.* **74**:505–517.
35. REDSHAW, M. R., and B. K. FOLLETT. 1971. The crystalline yolk-platelet proteins and their soluble plasma precursor in an amphibian *Xenopus laevis*. *Biochem. J.* **124**:759–766.
36. RIEMANN, W., C. MUIR, and H. C. MACGREGOR. 1969. Sodium and potassium in oocytes of *Triturus cristatus*. *J. Cell Sci.* **4**:299–304.
37. DICK, D. A. T., and S. G. A. MCLAUGHLIN. 1969. The activities and concentrations of sodium and potassium in toad oocytes. *J. Physiol. (Lond.).* **205**:61–78.

38. LEE, C. O., and W. McD. ARMSTRONG. 1974. State and distribution of potassium and sodium ions in frog skeletal muscle. *J. Membr. Biol.* **15**:331–362.
39. MACKNIGHT, A.D. C., and A. LEAF. 1977. Regulation of cellular volume. *Physiol. Rev.* **57**:510–573.
40. HINKE, J. A. M. 1970. Solvent water for electrolytes in the muscle fiber of the giant barnacle. *J. Gen. Physiol.* **56**:521–541.
41. WOOD, T. H., and A. H. ROSENBERG. 1957. Freezing in yeast cells. *Biochim. Biophys. Acta.* **25**:78–87.
42. LING, G. N., M. M. OCHSENFELD., and G. KARREMAN. 1967. Is the cell membrane a universal rate-limiting barrier to the movement of water between the living cell and its surrounding medium? *J. Gen. Physiol.* **50**: 1807–1820.
43. COPE, F. W. 1969. Nuclear magnetic resonance evidence using D_2O for structured water in muscle and brain. *Biophys. J.* **9**:303–319.
44. MILD, K. H., T. L. JAMES and K. T. GILLEN. 1972. Nuclear magnetic resonance relaxation time and self diffusion coefficient measurements of water in frog ovarian eggs (*Rana pipiens*). *J. Cell. Physiol.* **80**:155–158.
45. HAZLEWOOD, C. F., D. C. CHANG., B. L. NICHOLS., and D. F. WOESSNER. 1974. Nuclear magnetic resonance transverse relaxation times of water protons in skeletal muscle. *Biophys. J.* **14**:583–606.
46. SHPORER, M., and M. M. CIVAN. 1977. The state of water and alkali cations within the intracellular fluids: The contribution of NMR spectroscopy. *Curr. Top. Membranes Transp.* **9**:1–69.
47. SIMON, S. E., F. H. SHAW., S. BENNETT., and M. MULLER. 1957. The relationship between sodium, potassium and chloride in amphibian muscle. *J. Gen. Physiol.* **40**:753–777.
48. ROBERTSON, J. D. 1961. Studies on the chemical composition of muscle tissue. II. The abdominal flexor muscle of the lobster *Nephrops norvegicus (L). J. Exp. Biol.* **38**:707–728.
49. COPE, F. W. 1970. Spin-echo nuclear magnetic resonance evidence for complexing of sodium ions in muscle, brain and kidney. *Biophys. J.* **10**:843–858.
50. DICK, D. A. T., and E. J. A. LEA. 1967. The partition of sodium fluxes in isolated toad oocytes. *J. Physiol. (Lond.).* **191**:289–308.
51. LING, G. N., C. MILLER., and M. M. OCHSENFELD. 1973. The physical state of solutes and water in living cells according to the association-induction hypothesis. *Ann. N. Y. Acad Sci.* **204**:6–47.
52. LING, G. N., and C. L. WALTON. 1975. Simultaneous efflux of K^+ and Na^+ from frog sartorius muscle freed of extracellular fluids: Evidence for rapidly exchanging Na^+ from the cells. *Physiol. Chem. Phys.* **7**:501–515.
53. LING, G. N. 1962. A Physical Theory of the Living State: The Association-Induction Hypothesis. Blaisdell Publ. Co. Division of Random House, Inc., New York.
54. SJODIN, R. A., and L. A. BEAUGÉ. 1973. An analysis of the leakages of sodium ions into and potassium ions out of striated muscle cells. *J. Gen. Physiol.* **61**:222–250.
55. KERNAN, R. P. 1972. Active transport and ionic concentration gradients in muscle. *In* Transport and Accumulation in Biological Systems. E. J. Harris, editor. Butterworths, London. 3rd edition. 193–270.
56. PALMER, L. G., and J. GULATI. 1976. Potassium accumulation in muscle: A test of the binding hypothesis. *Science (Wash. D.C.).* **194**:521–523.
57. LING, G. N. 1977. Potassium accumulation in frog muscle: The association-induction hypothesis versus the membrane theory. *Science (Wash. D.C.).* **198**:1281–1283.