REFERENCE PHASE ANALYSIS OF FREE AND BOUND INTRACELLULAR SOLUTES

I. SODIUM AND POTASSIUM IN AMPHIBIAN OOCYTES

SAMUEL B. HOROWITZ, PHILIP L. PAINE, LOUIS TLUCZEK, AND JAMES K. REYNHOUT, *Cellular Physiology Laboratory, Department of Biology, Michigan Cancer Foundation, Detroit, Michigan 48201 U.S.A.*

ABSTRACT A method is described for the quantitative determination of free and bound solute concentrations in the cytoplasm of intact cells. The method includes (*a*) introduction of a gelatin gel reference phase (RP) into the cytoplasm; (*b*) diffusion of dissolved substances between cytoplasm and RP, (*c*) cell quenching to −196°C to prevent subsequent solute redistributions, (*d*) ultra-low temperature microdissection to isolate RP and cytoplasm samples, and (*e*) analysis of isolates for solute and water content.

In normal oocytes of the salamander, *Desmognathus ochrophaeus*, free or RP Na^+ and K^+ are 21.0 ± 1.1 and 128.8 ± 2.4 μeq/ml, respectively, and vary stoichiometrically in altered oocytes. Overall cytoplasmic concentrations are 75.2 ± 2.7 μeq Na^+/ml and 88.6 ± 1.5 μeq K^+/ml. Cytoplasmic chemical activities are 16.2 μeq Na^+/ml and 99.2 μeq K^+/ml, corresponding to activity coefficients of 0.22 and 1.12, respectively. The results demonstrate unambiguously that (*a*) oocytes actively transport Na^+ and K^+, and (*b*) cytoplasm has important binding properties which differentiate it from an ordinary aqueous solution. These cytoplasmic properties are investigated in the following paper.

INTRODUCTION

Many intracellular solutes exist in free and bound forms. This distinction is important because free solute concentrations (or the related chemical activities) determine mass-action reaction rates and equilibria in cells, whereas bound solutes serve other important functions. However, it is rarely possible to measure directly the concentrations of a solute in these two states in vivo, and in most cellular studies only total concentrations are known. A similar difficulty does not exist in cell homogenates because methods generally are available (e.g. centrifugation, dialysis, and chromatography) for separating complex mixtures of micromolecules, macromolecules, and organelles into separate phases, one or more of which are devoid of binding elements. Unfortunately, the cell pooling and disruption necessary for in vitro separation techniques introduce artifacts that compromise the relevance of the information to the living cell. Uncontrollable changes in free and bound reactant concentrations are caused by disordering and denaturation of cellular components.

A need exists for methods of distinguishing free and bound solutes within the living cell. We report here the development of such a method based on differences in the exclusion of molecular species and organelles from fibrous gels. We refer to it as the intracellular reference phase (RP) method.

In this study, we investigate sodium and potassium in cytoplasm. Although these cations,

important in many cellular processes, have been widely studied, there is no consensus on fundamental questions like whether Na^+ and K^+ have chemical activities in cytoplasm like those in ordinary aqueous solutions, and whether known Na^+ and K^+ concentration differences between intracellular regions are due to equilibrium or nonequilibrium processes (1–6). Much of this uncertainty stems from an inability to measure directly the free and bound forms of Na^+ and K^+, their isotopes, and other solutes. In this and the following paper (7) we apply the RP method to this purpose.

We will show that in oocytes free cytoplasmic cation concentrations, measured by the RP, differ from those of the cell's bathing solution. This demonstrates unambiguously that cells maintain Na^+ and K^+ activity gradients between cytoplasm and the extracellular medium, and provides proof of active cation transport, which, though widely accepted, has been questioned (1, 6).

However, comparison of RP and cytoplasm demonstrates that free and total cation concentrations in cytoplasm differ markedly for both Na^+ and K^+. In the case of Na^+, only a small proportion of the cytoplasmic cation is free, whereas for K^+ a seeming paradox exists, in that the concentration of free cation is higher than the cytoplasmic concentration of which it is putatively only a component. Thus, cytoplasm has properties that importantly distinguish it from ordinary aqueous solutions.

The physical states of cytoplasmic water and ions are the subject of a second paper (7), in which we describe the distribution of K^+, Na^+, their isotopes, and other solutes between RP and cytoplasm. Together they show that cytoplasm's water is heterogeneous; in part it acts normally, but a major fraction behaves as if it were nonsolvent. In addition, a significant portion of the cytoplasmic Na^+ and K^+ is bound. This bound cation appears to be associated with the nonsolvent water fraction and is in a mass-action equilibrium with the diffusible Na^+ and K^+. Two models of cytoplasm (compartment and sorption) are considered. In the compartment model, a nonequilibrium process, sequestration in membranous organelles, accounts for the observed water and cation heterogeneity. In the sorption model, an equilibrium model, water and cation sorption to cytoplasmic elements is posited. The data favor the latter, and a qualitative sorption model of Na^+ and K^+ is proposed. The primary adsorbing element in oocytes is identified as the yolk platelets.

RATIONALE

The RP technique involves a series of steps: A small volume of gelatin (about 2% of the cell volume) is introduced into the cell where it displaces a volume of cytoplasm. Because of its fibrous protein network, the gelatin gel excludes organelles and the structural cytoplasmic meshwork for a period of time during which the gel water comes into equilibrium with, and thus samples, the diffusible cytoplasmic solutes. The cell then is quickly frozen in liquid nitrogen to prevent subsequent artifactual solute redistribution. RP and cytoplasmic samples are isolated by ultra-low temperature microdissection (ULTM) and separately analyzed for water and solute content. The combined methods (RP-ULTM) allow the study of solutes in a biphasic quasi-equilibrium distribution: RP/cytoplasm. In addition, parallel equilibrium dialysis studies permit direct comparison of the properties of the gelatin with those of ordinary water solutions. Hence, solute behavior in cytoplasm can be referred to standard aqueous solutions.

If two phases are in diffusional equilibrium with regard to a solute, i, the electrochemical potential of the solute is the same in both phases. For an intracellular RP and cytoplasm (c), we can write:

$$RT \ln \gamma_i^{RP} C_i^{RP} + Fz_i U^{RP} = RT \ln \gamma_i^c C_i^c + Fz_i U^c \quad (1)$$

in which C is the concentration, γ is the activity coefficient, z is the net electronic charge of the solute, and U is the electrical potential of the phase. (R, T, and F have their conventional meanings.) Eq. 1 can be rearranged to give

$$\ln \frac{C_i^{RP}}{C_i^c} = \ln \frac{\gamma_i^c}{\gamma_i^{RP}} + \frac{Fz_i(U^c - U^{RP})}{RT}. \quad (2)$$

The concentrations, C_i^{RP} and C_i^c, are experimentally determined. If RP water has the properties of ordinary liquid water (i.e., $\gamma_i^{RP} \simeq \gamma_i^o$), at equilibrium the ratio of C_i^{RP}/C_i^c can be expected to reflect the interactions of solute i with cytoplasm relative to its interactions with normal water. If, furthermore, there is no electrical potential difference between cytoplasm and the intracellular RP, or the solute under investigation is neutral, the final term of Eq. 2 reduces to zero, and the ratio C_i^{RP}/C_i^c directly measures the cytoplasmic chemical activity of solute i relative to its activity in dilute aqueous solutions. (Because of cytoplasm's complexity, γ_i^c is often referred to as an apparent activity coefficient. However, for present purposes, we adhere to the formal view of this thermodynamic term which relates equilibrium concentrations in two phases without implying mechanism or phase homogeneity.)

Because 10–20% aqueous gelatin undergoes sol $\rightleftharpoons$ gel transitions in a temperature range compatible with amphibian oocyte viability, it is practicable to microinject a sol into an oocyte, cool the cell, and thereby produce an intracellular region of gelled gelatin. The solvent properties of gelatin water are similar to those of ordinary liquid water (8), and there is no electrical potential difference between the RP and cytoplasm (7). Hence, RP-ULTM allows direct determination of the chemical activities of cytoplasmic solutes.

Phase distribution interpretations are based on an analogy with the use of "ideal" and "nonideal" in thermodynamics. The RP water, which resembles the water of electrolyte solutions of physiological molality, is treated as the "ideal" phase in the belief that the "nonideal" behavior of cytoplasmic solutes will provide information on intact cytoplasm's structure and interactions. However, because of cellular complexity, the aptness of this analogy must be carefully assessed. The appearance of nonideality in solute behavior can arise as artifact in the absence of transport equilibria, and this must be a concern. For most applications of the intracellular RP technique, close attention must be given to verify two points: that diffusional equilibrium is attained between RP and cytoplasm, and that the presence of the RP does not in itself alter cytoplasm's properties.

MATERIALS AND METHODS

Solutions

The Ringer's solution used contained (mM): 104.5 NaCl, 2.5 KCl, 1.2 $MgSO_4$, 6.6 $NaHCO_3$, 2.0 NaH_2PO_4, 1.2 Na_2HPO_4, and 0.7 $CaCl_2$ (pH 7.2). Oocytes incubated in this Ringer's at 5°C remain normal, as judged by cellular K^+ and Na^+ levels, for at least 3 d.

Oocytes

Mature ovarian oocytes of the plethodontid salamander, *Desmognathus ochrophaeus* Cope, were used. These salamanders, collected from streams and seepages in the Allegheny and Monongahela National Forests (Pennsylvania and West Virginia, respectively), survive with negligible mortality in our colony. They are kept in tap water at 10°C, cleaned and fed twice weekly on 4th-instar *Chironomus thummi* larvae.

The oocytes used were 2.2–2.6 mm in diameter and weighed 8–12 mg. Descriptions can be found elsewhere (9, 10). Oocytes tolerate microinjections and in vitro incubation, and their large size facilitates manual microdissection.

In isolating oocytes, both ovaries, each containing 6–12 oocytes, are removed and placed in ice-cold Ringer's. The oocytes are carefully separated, using jeweler's forceps and iridectomy scissors, and excess ovarian tissue is trimmed with a small tab left for subsequent handling. The transparent follicular epithelium that envelops each oocyte is not removed. Cells poked or scraped during isolation and/or trimming are discarded. The remaining oocytes are then incubated 15 min and reinspected; those exhibiting abnormalities at this time also are discarded. Before injection, oocytes are kept in ice-cold Ringer's.

Reference Phase

The starting material for RP gelatin was Bacto-Gelatin (Difco Laboratories, Detroit, Mich.). A 2% (wt/vol) solution in deionized water was passed through a mixed bed, AG501-X8 ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.) at 34°C. The eluant gelatin was dialyzed extensively at 5°C against ion-free water, and then concentrated to 14% wt/wt in a rotary vacuum evaporator at 50°C. It was then filtered through 0.22 or 0.45 μm Millex filters (Millipore Corp., Beford, Mass.) at 34°C and stored in sterile polypropylene vials at 5°C until used.

Dialysis determinations of the equilibrium distributions of Na^+, K^+, and Cl^- between RP gelatin and saline were made at 5°C using viscose cellulose tubing of 3,500 mol wt cutoff. The dialysis salines contained 10 μeq/ml Na-phosphate buffer, pH 7.0, and an additional 140 μeq/ml of NaCl and KCl in varied proportions.

Just before the microinjection procedure, gelatin is warmed to 34°C, drawn into a clean syringe, and expelled through a 0.22-μm Swinnex microfilter (Millipore Corp.) into a clean glass loading tube. Loading tubes, constructed from micropipette capillary tubing, are 1 cm long with one end sealed. The exposed surface of the gelatin sol is overlayered with 20 cs silicone 200 fluid (Dow Corning Corp., Midland, Mich.) to prevent evaporative loss.

Glass micropipettes are made from acid-cleaned, precision capillary tubing, o.d. 0.85 mm, i.d. 0.55 mm (Drummond Scientific Co., Broomall, Pa.). Pipettes with long, tapered shafts are drawn on a horizontal puller, and the tips etched over hydrofluoric acid to a final diameter of 10 μm.

Micropipette loading and oocyte injection are performed on the stage of a dissecting microscope warmed to 34°C by an air curtain incubator (Sage Instruments, Div. of Orion Research Inc., Cambridge, Mass.). The injection pipette is positioned with a micromanipulator. Loading and microinjection are accomplished by hydraulic pressure from a screw-driven syringe. In loading, 50–200 nl of gelatin is drawn from the loading tube into the silicone fluid-filled micropipette, the pipette is withdrawn from the tube until its tip is in the silicone fluid overlayer, and about 1 nl of fluid is drawn up. This prevents tip drying and consequent pipette clogging. When extruded from the micropipette, the gelatin contains 0.3 μeq K^+ and 0.7 μeq Na^+/ml water.

Oocyte impalement and injection is done in about 100 μl of Ringer's on a slide that allows micropipette operation in the plane of the slide surface. Immediately after injection, oocytes are washed into a large volume of ice-cold Ringer's, where they remain 10 min to gel the injectate. Subsequent incubation is at 5°C.

It is important to limit the time that the cell is exposed to the 34°C air curtain. Exposures in excess of 90 s cause significant evaporative loss of water from the injection slide with increased harm to cells (as judged by cellular K^+ and Na^+ content). In these experiments exposure was restricted to 30 s; control experiments show this to have no detectable effect.

The choice of 5°C for incubation is dictated in part by the observation that at this temperature RP gelatin remains firm and excludes cytoplasmic elements for long times. The temperature is also close to environmental norm for *D. ochrophaeus*. Incubation is for 3 h (unless otherwise noted). This we will show is adequate for diffusional equilibrium between RP and cytoplasm to occur.

Electron micrographs (Fig. 1) show that the largest cytoplasmic elements (e.g. yolk platelets) are totally excluded from the RP. (Some interdigitization occurs at the RP periphery, but this zone is removed during microdissection.) However, the gelatin is in dispersed droplets (Fig. 1 *b*), apparently because it enters the cell through the narrow orifice of a micropipette. These droplets do not coalesce but contain in their interstitium a small amount of ground cytoplasm as well as smaller formed elements as endoplasmic reticulum, mitochondria, and ribosomes (Fig. 1 *b*, *c*). Planographic analysis shows the interstitium constitutes 13.9 ± 2.3% of the RP.

There is total exclusion of electron microscope-visible cytoplasmic elements by the gelatin itself. The exclusion persists for at least 24 h even when cytoplasm is compressed between adjacent droplets (arrow, Fig. 1 *c*). A correction for interstitial cytoplasm could be made using water and solute concentrations determined for extra-RP cytoplasm. However, we have not done so because the correction involves the doubtful assumption that interstitial cytoplasm is unmodified from whole cytoplasm. Furthermore, our calculations indicate that the correction would be small and have a negligible influence on the results.

ULTM and Analysis

The steps in ULTM are oocyte freezing to liquid nitrogen temperatures, storage, and microdissection to separate cytoplasm and RP. ULTM is described in detail by Century et al. (11) and Frank and Horowitz (12). It is reviewed briefly here, with emphasis on recent modification in the procedure.

Intracellular diffusion is terminated by placing the oocyte on a brass dissection mount, in a drop of OCT[1] to aid adherence, and rapidly freezing the assembly, first in dichlorodifluoromethane (Union Carbide Corp., New York) cooled to about −160°C in liquid nitrogen, and then directly in liquid nitrogen. The cells are stored under liquid nitrogen until microdissection.

The microdissection apparatus is similar to that described and illustrated by Frank and Horowitz (12). It is a cold stage, designed to facilitate free-hand dissection within an insulated glove box, the temperature of which is controlled by the thermostated flow of liquid nitrogen. The apparatus contains a compartment suspended below a model 4700 Electrobalance remote weighing chamber (Cahn Instruments Div., Ventron Corp., Cerritos, Calif.) in which frozen samples are weighed.

Microdissection is done at −40 to −45°C. We have shown previously using ultra-low temperature autoradiography that low temperature regimens similar to that used here prevent solute redistribution and provide spatial resolution for small solutes of 3–14 μm (13–15), fully adequate for present purposes.

Microtools are used as previously described for the nuclear isolation (12) to locate, clean, and remove the frozen RP. Similarly, chiplike samples of frozen cytoplasm are taken. (Many of the nuclei are also recovered, and will be reported on elsewhere.) The cytoplasm of *D. ochrophaeus* oocytes is yellowish and has the consistency, at this temperature, of frozen butter. The RP appears usually as a club-shaped opaque white object, whereas the nucleus is ellipsoidal and translucent. As samples are removed from the cell, they are placed in small aluminum foil (0.35 mil, Clecon Metals, Cleveland, Ohio) collecting packets, which are crimped shut to prevent water sublimation (cf. Frank and Horowitz, reference 12).

After each oocyte is dissected, the samples are transferred from collecting packets to clean, tared, foil weighing packets. During this transfer, the RP is inspected to confirm that it is free of adherent cytoplasm. The weighing packet is folded shut and transferred to the weighing chamber adjacent to

[1] OCT is a proprietary cryoembedding material (Lab-Tek Products, Div. of Miles Laboratories Inc., Naperville, Ill.) consisting of polyethylene glycol, polyvinyl alcohol, water, and Roccal. The latter is a preservative. We have found Roccal to be somewhat toxic to oocytes, and the OCT used in this laboratory is free of this substance.

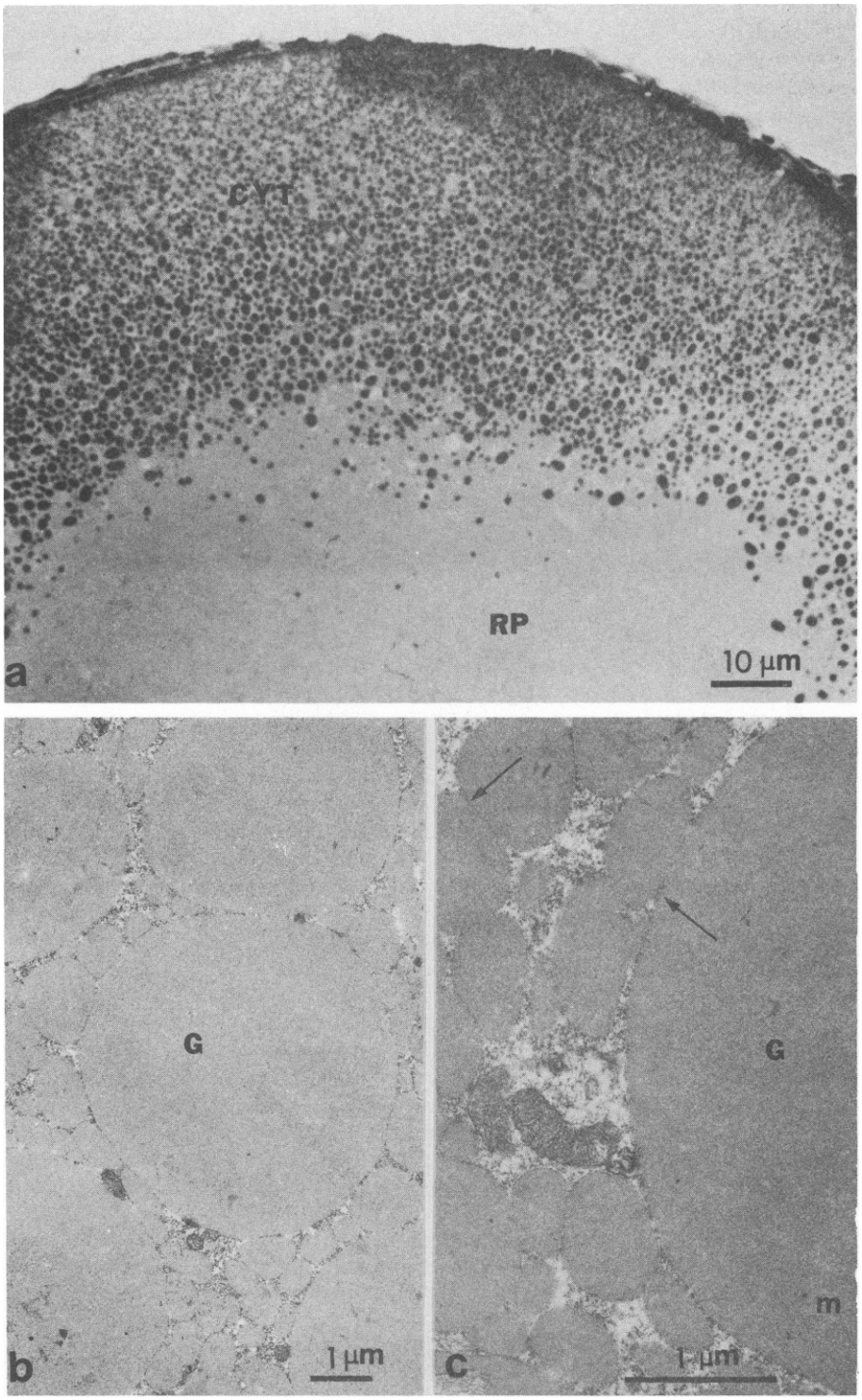

FIGURE 1 (*a*) A portion of a *D. ochrophaeus* RP oocyte maintained at 5°C for 24 h, showing the internal reference phase (RP) and adjacent cytoplasm (CYT). × 1,550. (*b*) Detail of a RP showing gelatin droplets (G) and trapped interstitial cytoplasm. × 7,000. Note that gelatin is devoid of formed cytoplasmic elements. (*c*) A RP at higher magnification. × 21,000. The arrows indicate interstitial cytoplasm compressed between abutting gelatin droplets.

the dissecting stage. Wet weights for the isolated RPs range from 50 to 200 μg; tare weights of the weighing envelopes were 400–600 μg. The isolated cytoplasmic sample is similarly transferred to a weighing packet and weighed; typical cytoplasm sample wet weights are 200–800 μg.

After wet weight determinations, samples are dried at 60°C over P_2O_5 for 16 h. Dry weights are taken, and the original water content of RP and cytoplasm calculated. The mean water content of the RP at microdissection is 82.3 ± 0.6%; that of the cytoplasm, 31.8 ± 0.3%.

The packets containing the dried samples are carefully opened under a dissecting microscope, immersed in 0.8–1.5 ml of ion-free water in polypropylene vials, and extracted over boiling water for 10 min.

Determination of the water, Na^+, and K^+ content in isolated RP and cytoplasm requires accurate wet and dry weights and minimal cation contamination. To this end, samples from each cell are bracketed and accompanied throughout the entire ULTM weighing and analysis procedure by three control foil packets. When necessary, the wet and dry weight of each sample is corrected for changes in the control packet weights, and the Na^+ and K^+ contents of the experimental samples corrected for background cation levels determined for the controls. Throughout, concentrations are expressed as microequivalents per milliliter of water in the phase (media, RP, cytoplasm, or nucleus) in question.

Everything that comes into contact with the RP, cytoplasm, or the sample collecting and weighing packets is kept scrupulously clean. This includes the microdissection tools, the foil and tools used in constructing packets, the sample and packet handling forceps, and the water and vials used for cation extraction. With these precautions, background measurements on control packet extracts are <0.0005 μeq/ml. RP samples are smaller than cytoplasmic samples, and their extracts have smaller absolute cation concentrations. Cation concentrations were measured with a model 251 atomic adsorption spectrophotometer (Instrumentation Laboratory, Inc., Lexington, Mass.). Extracts of RP samples from normal oocytes contain about 0.010 μeq/ml K^+ and 0.002 μeq/ml Na^+, corresponding to signal/noise ratios of 20/1 and 4/1 for K^+ and Na^+, respectively.

Control of Na^+ and K^+ Levels

A great advantage of the present methodology is that free (RP) and total cytoplasmic concentrations of Na^+ and K^+ are determined for each oocyte, and this provides a quantitative measure of the functional capacity of the cell to carry on active transport. Because of this, it is unnecessary to make subjective distinctions between "damaged" and "healthy" cells. Rather, every cell finds its place on a continuum that extends from those with the highest free K^+/Na^+ ratios, indicating a fully functional active Na^+-K^+ transport system, to those in which the ratio approaches that of the external medium. The behavior of cytoplasmic and nuclear Na^+ and K^+ as changes occur in the free ion continuum is extremely revealing, and those of cytoplasm are considered in detail in the following paper (7).

For convenience in describing our results, we have defined a specific range of values on the continuum as normal cells. These are those with Na^+ and K^+ unmodified from the levels prevailing before experimental manipulation. Such cells are referred to as N oocytes. (The criterion for this designation is described in Results). The proportion of N oocytes obtained in a given experiment depends on many factors, including the extent of handling, length of incubation, and incubation temperature. Under the conditions described here (incubation limited to <5 h at 5°C, and minimal handling), 82% of the RP cells are N oocytes.

RESULTS AND DISCUSSION

We found previously (8) by equilibrium dialysis that sucrose distributes uniformly between the water of an 11% gelatin gel and saline solutions (C^{gel}/C^{buffer} = 0.98), indicating that little, if any, gelatin water has abnormal solvent properties. For present purposes, ion asymmetries due to ion exchange and Donnan influences must be determined as well. Fig. 2 shows that at the ionic strength found in oocytes, these influences are negligibly small. The ratio of the cations' concentrations (determined by atomic absorption spectroscopy and ex-

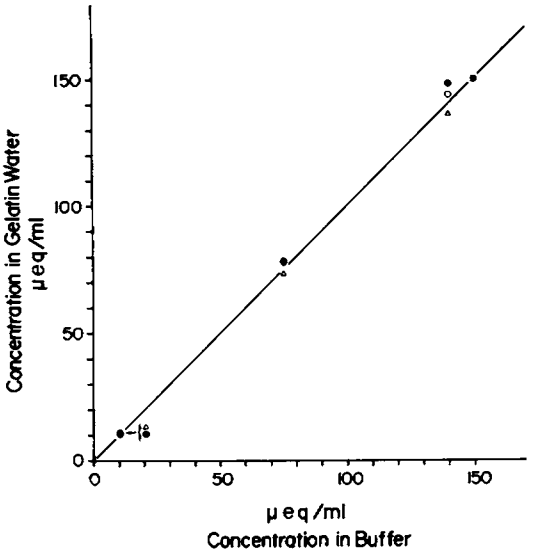

FIGURE 2 Distribution by equilibrium dialysis of Na^+ (closed circles), K^+ (triangle), $^{22}Na^+$ (open circles), and $^{36}Cl^-$ (squares) between buffer and 14% gelatin gel at 5°C. The slope of the line is unity.

pressed on a water basis) between gelatin and buffer is unity and is independent of concentration. Similarly, trace amounts of $^{22}Na^+$ and $^{36}Cl^-$ distribute uniformly between the water of gelatin and the saline solution. Hence, we conclude that at concentrations and pH appropriate to intra-oocyte studies, the concentration and activity coefficients of Na^+, K^+, and Cl^- in gelatin are similar to those of physiological saline solutions, and RP water can be assumed to resemble ordinary liquid water.

Diffusion coefficients in oocyte cytoplasm range from 0.1×10^{-5} to 2.0×10^{-5} cm^2/s for molecules <1,000 daltons (14, 16, 17) and are about one-half as large as those in 15% gelatin (18). Based on these values, 10–15 min should suffice for the diffusional equilibrium of Na^+ and K^+ between RP and cytoplasm. The mean total cation concentration in the RP 1 h after injection (the shortest period tested) is 149 ± 4 μeq/ml. This is unchanged at 3–4 h (146 ± 4 μeq/ml) and at 8–24 h (152 ± 5 μeq/ml). (When injected into the oocyte, purified gelatin contains about 1.0 μeq/ml total cation concentration ($K^+ + Na^+$), i.e. <0.01 of cytoplasmic levels.) Parallel results are obtained using isotopes (7). It should be noted, as well, that no change in the relative concentrations of RP Na^+ and K^+ between 1 and 24 h is seen. Collectively, these results eliminate diffusion or phase boundary potentials (an objection raised by Civan [19]) as contributory mechanisms in determining solute distribution at the times we have studied.

Fig. 3 shows the equilibrium RP concentrations of Na^+ (C_{Na}^{RP}) and K^+ (C_K^{RP}) of 85 cells derived from 15 experiments. The concentrations are reciprocally related, with the ratio C_K^{RP}/C_{Na}^{RP} ranging from 36 to about 0.02 (that of the extracellular Ringer's). High K^+ and low Na^+ are the norm, with RP K^+ being replaced by Na^+ in cells modified by the microinjection procedure or subsequent treatment. When the data are plotted as a frequency distribution, the modal class oocytes have $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP}) \geq 0.8$ (*inset*, Fig 3). These cells, which we refer to as normal or N oocytes, have mean values for C_K^{RP} and C_{Na}^{RP} of 129 ± 2 and 21 ± 1 μeq/ml, respectively.

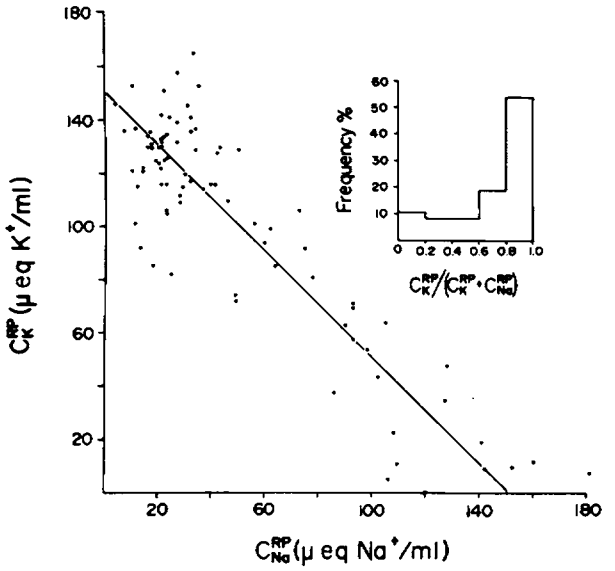

FIGURE 3 RP concentrations of sodium, C_{Na}^{RP}, and potassium, C_K^{RP}. Each point represents a single RP-containing cell. The line represents $C_K^{RP} + C_{Na}^{RP} = 150.9$ μeq/ml, the mean $C_K^{RP} + C_{Na}^{RP}$ for all cells. (*Inset*) The quantity $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP})$ as a frequency distribution.

Because the Ringer's contains 2.5 μeq/ml K^+ and 115.5 μeq/ml Na^+, N oocytes maintain approximately 50- and 5-fold concentration differences of K^+ and Na^+, respectively, between the intracellular diffusible compartment and the extracellular environment. These concentration differences are likely also to be the activity differences between the two comparments because $\gamma_i^{RP} \simeq \gamma_i^{H_2O}$. We conclude that the oocyte maintains Na^+ and K^+ activity gradients between the diffusible cytoplasmic phase and Ringer's.

Fig. 3 shows that although the free K^+ and Na^+ concentrations (C_K^{RP} and C_{Na}^{RP}) vary widely, presumably as a function of the state of the cell membrane, their sum remains constant at about 151 ± 2 μeq/ml. This is greater than the sum of the cations of the Ringer's, and we may deduce an unchanging level of intracellular Donnan anions. In this regard, it is interesting to inquire as to the identity of the RP or free cytoplasmic anions.

Studies in which RP oocytes are incubated in ^{36}Cl-Ringer's (7)[2] show that $C_{Cl}^{RP} = 43$ μeq/ml; hence chloride accounts for <30% of the necessary RP anions. As expected, membrane permselectivity (20) seems to play an important role in determining free cellular anion concentrations.

Table I shows the concentration of trichloroacetic (TCA)-soluble inorganic and total phosphate in RP to be 17.6 and 48.6 μM/ml, respectively. Without specific knowledge of the chemical activities of competing cations (e.g. H^+, Ca^{++}, Mg^{++}) and the chemical form of the 31 μM/ml of organic phosphates represented, one cannot quantitatively determine phosphate's role in providing the counteranion for RP Na^+ and K^+. Nevertheless, the magnitude of the values in Table I suggests that phosphate, in its various forms, is, in addition to Cl^-, a dominant cellular-free anion.

[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.

TABLE I

TCA-SOLUBLE INORGANIC AND TOTAL PHOSPHATE IN CYTOPLASM AND RP

	RP(C_i^{RP})	Cytoplasm (C_i^c)
	μM P/ml H_2O	
Inorganic phosphate	17.6 ± 0.8	8.0 ± 0.5
Total phosphate	48.6 ± 2.7	22.3 ± 1.4

After microdissection RP and cytoplasm from individual cells were extracted in 0.45 ml 10% TCA (wt/vol) for 36 h at 5°C. Aliquots of the TCA supernate were analyzed, with and without prior ashing (21), by the method of Chen et al. (22). Controls included gelatin and standards in TCA.

The difference between RP and cytoplasm phosphate concentrations implies that TCA-soluble phosphate is not uniformly distributed in cytoplasm; instead only part of the cytoplasmic water is accessible as solvent for the free anion. We shall show that this is true for a number of exogeneous substances (7). However, the phosphates are endogenous and therefore of special interest in that they can be assumed to be at diffusional equilibrium in the cell. Their exclusion from a significant portion of the cellular water therefore supports the conclusion, to be offered in regard to exogenous substances, that exclusion is an equilibrium or steady-state process.

Table II summarizes the Na^+ and K^+ concentrations in the cytoplasm and RP of N oocytes and in the cytoplasm of oocytes without an RP. Comparison of these two groups of cells shows that N oocytes have cytoplasmic Na^+ and K^+ concentrations indistinguishable from oocytes without an internal RP. This permits the conclusion that the RP technique, which includes gelatin microinjection, temperature transitions, and the presence of an RP, does not alter the composition of cytoplasm.

The second point evident from Table II is that Na^+ and K^+ concentrations in the RP differ markedly from those of the adjacent cytoplasm. The solvent properties of the RP are essentially those of ordinary water; hence these differences are attributable to the specific properties of cytoplasm and its inclusions. With regard to Na^+, Table II indicates that free Na^+ concentrations in cytoplasm, measured by the RP, are less than a third that of free and bound Na^+ combined.

In contrast to Na^+, the K^+ concentration of the RP is higher than in cytoplasm ($C_K^{RP}/C_K^c = 1.45$). Two explanations suggest themselves. The first is that the K^+ asymmetry reflects a Donnan distribution, implying that cytoplasm is positively charged relative to the RP. This can be ruled out, not only because of the C^{RP}/C^c asymmetry of opposite sign

TABLE II

Na^+ AND K^+ IN UNINJECTED AND RP OOCYTES

	N oocytes		Uninjected cells
	RP(C_i^{RP})	Cytoplasm (C_i^c)	Cytoplasm (C_i^c)
		μeq/ml H_2O	
K^+	128.8 ± 2.4	88.6 ± 1.5	91.6 ± 3.6
Na^+	21.0 ± 1.1	75.2 ± 2.7	69.3 ± 5.2

exhibited by Na^+ (it could be argued that specific Na^+ binding is sufficient to compensate for the nonspecific Donnan effect), but also because solute distributions closely paralleling those of K^+ are exhibited by neutral molecules and anions (7, 8) as well.

The apparent explanation for the excess RP concentration of K^+ is that this cation is excluded as a solute from a nonsolvent portion of the cytoplasmic water. In this instance, $C_K^{RP}/C_K^c > 1$ occurs because C_K^c is an average value taken over a heterogeneous phase, part of which is water-enriched relative to K^+.

The calculated quantity of the water-enriched or nonsolvent fraction depends on the assumptions made about cytoplasmic binding. If it is assumed that no K^+ is bound, the ratio $C_K^{RP}/C_K^c = 1.45$ implies that 0.31 of the cytoplasmic water is nonsolvent for K^+. Alternatively, if cytoplasmic K^+, like Na^+, is bound, then a larger fraction of cytoplasmic water must be nonsolvent. This uncertainty arises also in attempts to interpret Na^+ and K^+ activities.

Data presented in Table II permit the calculation of cytoplasmic activities and activity coefficients from the relation

$$\gamma_i^c = a_i^{RP}/C_i^c = \gamma_i^o C_i^{RP}/C_i^c. \tag{3}$$

These give $a_{Na}^{RP} = 16.2$ and $a_K^{RP} = 99.2$ μeq/ml and $\gamma_{Na}^c = 0.22$, and $\gamma_K^c = 1.12$, values lower and higher, respectively, than the activity coefficients of ordinary aqueous solutions of comparable concentrations ($\gamma_i^o \simeq 0.77$). These results agree well with the activities ($a_{Na} = 6 \pm 1, a_K = 120 \pm 3$ μeq/ml) and the activity coefficients ($\gamma_{Na} = 0.08 \pm 0.02$, $\gamma_K = 1.15 \pm 0.03$ to 1.29 ± 0.04) reported for cytoplasm by Palmer et al. (23) using ion-sensitive microelectrodes in the comparable large ovarian oocytes of *Rana pipiens*.

The chemical activities measured by microelectrodes have resulted in the widespread belief that although Na^+ is bound in cytoplasm, K^+ is not (4, 24). However, as pointed out by Lev and Armstrong (5), this interpretation is based on questionable assumptions, and the physical meaning of activity measurements is obscure without information on the solvent properties of cellular water. For this reason we defer discussion of activities to the following paper (7).

Finally, it is instructive to compare oocyte nuclear cation concentrations with those of the RP. Nucleus/cytoplasm concentrations of *D. ochrophaeus* reported by Century et al. (11) for K^+ and Na^+ were 128.9/81.1 (= 1.59) and 14.2/74.8 (= 0.19), respectively. These concentrations and ratios are similar to those for RP/cytoplasm, given in Table II. This correspondence implies that the nucleus behaves like the RP (much as a droplet of ordinary water), lacking active transport capabilities for micromolecules and in thermodynamic equilibrium with cytoplasm which has more complex cation-handling properties (8, 12, 25). It is evidence against the existence of nuclear cation transport mechanisms, but is consistent with the known dimensions of nuclear envelope pores (26). The parallel behavior of nucleus and RP is at variance with the hypotheses that attribute active micromolecular transport to the nuclear envelope (27) and those that postulate channels between the nucleus and the extracellular media (28, 29).

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