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Differences in Hydration State of Nucleus and Cytoplasm of the Amphibian Oocyte

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Abstract. Nuclear magnetic resonance (NMR) microimaging and proton relaxation times were used to monitor differences between the hydration state of the nucleus and cytoplasm in the *Rana pipiens* oocyte. Individual isolated ovarian oocytes were imaged in a drop of Ringer's solution with an in-plane resolution of 80 μm. Proton spin echo images of oocytes arrested in prophase I indicated a marked difference in contrast between nucleoplasm and cytoplasm with additional intensity gradations between the yolk platelet-rich region of the cytoplasm and regions with little yolk. Neither shortening τ_e (spin echo time) to 9 msec (from 18 msec) nor lengthening τ_r (spin recovery time) to 2 sec (from 0.5 sec) reduced the observed contrast between nucleus and cytoplasm. Water proton T_1 (spin-lattice) relaxation times of oocyte suspensions indicated three water compartments that corresponded to extracellular medium (T_1 = 3.0 sec), cytoplasm (T_1 = 0.8 sec) and nucleoplasm (T_1 = 1.6 sec). The 1.6 sec compartment disappeared at the time of nuclear breakdown. Measurements of plasma and nuclear membrane potentials with KCl-filled glass microelectrodes demonstrated that the prophase I oocyte nucleus was about 25 mV inside positive relative to the extracellular medium. A model for the prophase-arrested oocyte is proposed in which a high concentration of large impermeant ions together with small counter ions set up a Donnan-type equilibrium that results in an increased distribution of water within the nucleus in comparison with the cytosol. This study indicates: (i) a slow exchange between two or more intracellular water compartments on the NMR time-scale, (ii) an increased rotational correlation time for water molecules in both the cytoplasmic and nuclear compartments compared to bulk water, and (iii) a higher water content (per unit dry mass) of the nucleus compared to the cytoplasm, and (iv) the existence of a large (about 75 mV positive) electropotential difference between the nuclear and cytoplasmic compartments.

Key words: MRI — Nucleus — Cell water — Progesterone — Relaxation times — Membrane potential — Oocyte (*Rana pipiens*)

Introduction

The cell nucleus is generally considered to be freely permeable to water and ions as well as macromolecules such as mRNA [*reviewed in* Hinshaw, Carragher & Milligan, 1992]. However, studies on such diverse tissues as liver, kidney and ovarian oocytes have demonstrated the existence of large Na^+ and/or K^+ concentration differences between nucleus and cytoplasm [*reviewed in* (Moore & Morrill, 1976; Paine et al., 1981; Morrill & Kostellow, 1991)]. More recently, Aguayo et al. (1986) used nuclear magnetic resonance imaging on *Xenopus* oocytes, and demonstrated variations in image intensity both within the cytoplasm and between cytoplasm and nucleus. In the latter study, the water signal of the oocyte cytoplasm was distinct from the extracellular water whereas that of the nucleus was indistinguishable from that of the free water surrounding the oocyte. The differences in image contrast were proposed to result from proton density variations and/or differences in relaxation times, and implies that nucleus and cytoplasm have different hydration states. We have examined *Rana pipiens* oocytes with NMR microimaging during various stages of development and measured T_1 relaxation times for intracellular water to examine the basis of image contrast between the nucleoplasm and cytoplasm. Electropotential differences between nucleus and cytoplasm were measured as an index of charge separation (Na^+, K^+, Cl^-) between the two compartments.

Fully grown *Rana pipiens* oocytes are giant cells

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(about 1.8 mm in diameter) with a large nucleus (>0.5 mm in diameter). The ovarian oocyte is arrested in prophase I, and can be induced to resume the first meiotic division by addition of progesterone to the external medium [*reviewed in* (Morrill & Kostellow, 1986)]. Isolated amphibian ovarian oocytes can be superfused in an NMR tube and maintain normal ATP and phosphocreatine levels for several hours (Morrill et al., 1984). Following stimulation with progesterone, the oocyte resumes the first meiotic division, the nucleus rises to the surface of the animal hemisphere and the nuclear membrane fragments. In this study, nuclear-cytoplasmic hydration differences in prophase arrest were compared with oocyte hydration after the nuclear contents equilibrated with that of the cytoplasm during the first meiotic division.

Water relaxation measurements are generally complicated by the presence of varying amounts of extracellular water, which exhibits relaxation times different from those of intracellular water. We have developed a method for measuring the intracellular water content of packed amphibian oocytes using $^{35}Cl^-$ NMR as an index of extracellular volume and ^2H NMR as a measure of the total water volume so that the ratio of intra- and extracellular water volumes can be calculated (Morrill et al., 1987). Knowledge of extracellular water volume permits one to correct partially relaxed inversion-recovery FT NMR spectra of oocyte suspensions for the presence of contaminating extracellular fluid to obtain the true T_1 values for intracellular water. Because of the large size of the oocyte and slow diffusion, the exchange of intracellular water with extracellular water is slow on the NMR time scale.

KCl-filled glass microelectrodes were used to measure differences in electropotential between cytoplasmic and nuclear compartments and between these compartments and the fluid surrounding the oocyte (Ziegler & Morrill, 1977). The large nucleus was gently centrifuged for a few minutes to underlie the plasma membrane. Microelectrodes were inserted into both nucleoplasm and cytoplasm and potential differences measured.

The difference in hydration state between the oocyte nucleus and cytoplasm detected by microimaging must be the product of a Donnan type equilibrium which in turn would generate electropotential differences between nucleoplasm, cytoplasm and extracellular environment. A preliminary report has been presented (Morrill et al., 1994).

Materials and Methods

Materials

Fully grown *Rana pipiens* oocytes, arrested in first meiotic prophase, were either separated from the ovaries by fine forceps (entire follicles) or were stripped of their follicular envelopes and freed from all theca cells (denuded oocytes) by a modification (Ziegler & Morrill, 1977) of Masui's method. Progesterone (Sigma Chemical, St. Louis, MO) was the meiotic stimulus. It was dissolved in 95% ethanol; where indicated 1.0 μl was added per ml of Ringer's solution with shaking followed by a 1:10 dilution with Ringer's solution (Ziegler & Morrill, 1977). All experiments were carried out at 20–22°C.

Induction of Meiosis

Nuclear membrane breakdown was our criterion for successful induction of the meiotic divisions, determined by dissection of heat-fixed oocytes. The competence of oocytes from each female was verified by incubating samples of 20 denuded oocytes in Ringer's solution with and without 1.0 μM progesterone at 20°C for 24 hr. The oocytes used to obtain data for this report are from clutches in which 95% or more of the test oocytes displayed nuclear membrane breakdown after progesterone treatment. Spontaneous nuclear membrane breakdown was never seen in untreated oocytes. Oocyte wet weight was determined by wicking away excess fluid. Extracellular fluid has been estimated both by NMR and by [^{3}H]-labeled inulin using the oocyte wet weight corrected for adherent extracellular fluid (Morrill & Ziegler, 1980). The wet weight of individual oocytes was 2.80 ± 0.39 mg (mean ± SD for groups of 10 oocytes from each of 6 females).

Nuclear Magnetic Resonance Imaging

A denuded oocyte was placed in 50 μl of amphibian Ringer's solution in the conical tip of a 1.5 ml Eppendorf microfuge tube. The tube was centered in a 3 cm NMR tube and suspended at 9.4 Tesla in a Bruker/GE Omega-400 WB NMR Spectrometer equipped with a microimaging accessory. Oocyte images were obtained using a 1.0 mm slice thickness and with in-plane resolution of 80 μm.

Nuclear Magnetic Resonance Measurements of Intracellular and Extracellular Water

Rana pipiens follicles were preincubated in Ringer's solution containing 1% D_2O for 30 min. Approximately 50 follicles were transferred to a 5 mm NMR tube and superfused with Ringer's solution as described previously (Morrill et al., 1984). ^{35}Cl and ^2H measurements were carried out using a Varian XL-200 FT NMR instrument. The observe frequency was 19.6 MHz for the ^{35}Cl nucleus and 30.7 MHz for the ^2H nucleus. The resonance of intracellular ^{35}Cl ions in most cells is so broad as to become NMR invisible (Rayson & Gupta, 1985). Therefore, the intensity of the ^{35}Cl NMR signal of an oocyte suspension is proportional to the volume of extracellular water within the sensitive volume of the NMR receiver coil. The intensity of the ^{35}Cl NMR signal of the cell-free medium in an identical sample geometry is proportional to the total sensitive volume of the NMR coil. The ratio of the two intensities (f_{Cl}) represents the extracellular water volume as a fraction of the total sensitive volume. The ratio of the intensities (f_D) of the water ^2H-NMR signals of the cell suspension and the cell-free medium gives the oocyte water volume (extra- plus intracellular water) as a fraction of the total sensitive volume. The intracellular water content (f_W) i.e., the volume of the intracellular water as a fraction of the total cell volume, is given by the equation: $f_W = (f_D - f_{Cl})/(1 - f_{Cl})$ (Hoffman & Gupta, 1986). Measurements were carried out without changing the NMR probe configuration and they did not require perturbation of the cell system by addition of membrane-impermeable

reagents. This method is equally applicable to situations of slow and fast water exchange.

Spin-lattice Relaxation Measurements

Inversion-recovery pulse sequence was used to determine the water spin-lattice (T_1) relaxation times for groups of 50 follicles superfused with Ringer's solution at 22°C (Farrar & Becker, 1971). At least 18 data points were obtained over the range 10 msec to 10 sec and analyzed for the recovery of the magnetization via the sum of one or more exponentials. Multiexponential analysis was carried out using a computer program based on the Simplex algorithm:

$$(M_\infty - M_t) = a \exp(-\alpha t) + b \exp(-\beta t) + c \exp(-\gamma t)$$

where: a = cytoplasmic water equilibrium magnetization
α = cytoplasmic water $1/T_1$
b = nuclear water equilibrium magnitization
β = nuclear water $1/T_1$
c = extracellular water equilibrium magnetization
γ = extracellular water $1/T_1$
M_∞ = total water equilibrium magnetization
M_t = nonequilibrium magnetization at time t.

The computer program using the Simplex algorithm was tested by constructing a phantom containing three compartments using concentric NMR tubes containing 0.01, 0.1 and 1.0 mM Mn^{2+} in water with separately measured spin-lattice (T_1) relaxation times of 2.94, 0.39 and 0.056 sec, respectively. In this phantom, the relaxation times and compartment volumes could be detected with an accuracy of 10%.

Electrophysiological Measurements

Membrane potential measurements were made using a W-P Instruments M-707 microprobe system (New Haven, CT) and standard 2.5 M KCl-filled glass micropipettes. Isolated oocytes were placed with the animal hemisphere up in 2 mm depressions in a paraffin layer in the bottom of a plastic centrifuge tube containing Ringer's solution. The oocytes were centrifuged at 600 × g for 10 min. Under these conditions, the large nucleus rises to underlie the plasma membrane of the animal hemisphere and the nuclear surface and nucleoplasm can be visualized with a 30× stereomicroscope. Electrodes (0.1 μ tip) were inserted into both nucleoplasm and cytoplasm and steady-state potentials were measured for at least 60 sec. For comparison, plasma membrane potentials were measured on uncentrifuged oocytes from the same female.

Results

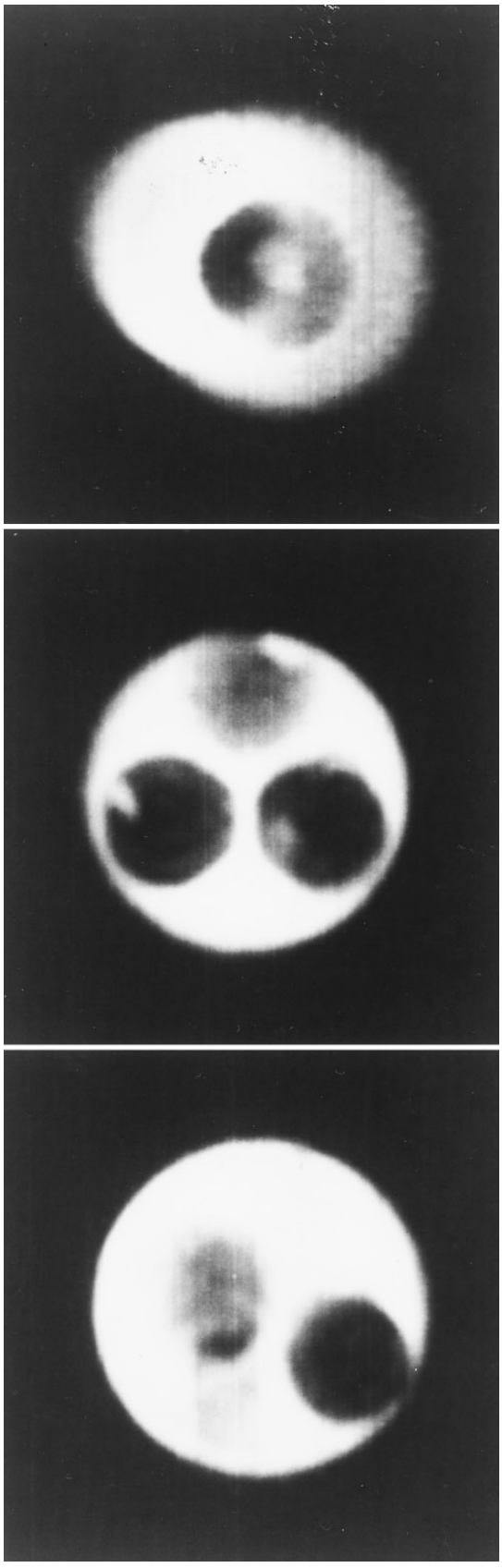
NMR Imaging

A typical proton spin echo image showing the contrast between cytoplasmic and nucleoplasmic water of a denuded amphibian oocyte in prophase I arrest is shown in the upper panel of Fig. 1. A τ_e (spin echo time) of 18 msec and τ_r of 500 msec (spin recovery time) were used to obtain this image. The bright field surrounding the oocyte represents the external drop of Ringer's solution and the intracellular bright sphere corresponds to the oocyte nucleus. A much darker region is also apparent in the yolk platelet-rich region of the cytosol (vegetal hemisphere). The mechanism underlying the observed contrast differences was investigated by varying τ_e and τ_r. We found that neither shortening τ_e to 9 msec nor lengthening τ_r to 2 sec reduced the observed contrast. The image contrast between the nucleus and the cytoplasm may therefore arise from a difference between the water proton density in the nucleus and in the cytoplasm. The oocyte can be manually enucleated by making a small slit in the animal hemisphere and pressing the sides of the oocyte with forceps. The intact nucleus is extruded from the oocyte, the plasma membrane reseals, and the resting membrane potential reappears within minutes (Ziegler & Morrill, 1977). A proton spin echo image of manually enucleated oocytes indicates a loss of the intracellular bright field.

Denuded oocytes were transferred to Ringer's solution containing 1 μM progesterone. Within 4–5 hr the nucleus begins to rise to the surface of the animal hemisphere and by 8–10 hr the nuclear membrane breaks down and the nuclear contents equilibrate with the oocyte cytoplasm. Figure 1 depicts proton spin echo images of oocytes with nuclei at the surface of the animal hemisphere (middle panel) and following nuclear breakdown (bottom panel). The middle panel illustrates the physiological state in which the nuclei have risen to underlie the cortex of the animal hemisphere. In contrast, the bottom panel shows that no bright field is present within the oocyte following nuclear breakdown. This confirms the assignment of the intracellular bright region to the nuclear compartment.

NMR Measurements of T_1

Figure 2 (lower panel) illustrates the semilog plots of inversion recovery T_1 measurement of follicular oocytes showing recovery of the magnetization, the nonlinearity indicating multiexponential relaxation. An analysis of the magnetization recovery curve indicates the presence of slow and rapid components, the slow component yielding a relaxation time (2.9 sec) close to that of the cell-free medium (3.0 sec) (Fig. 2, upper panel). After subtraction of the contribution of the extracellular medium using ^{35}Cl and ^{2}H NMR to measure extracellular volume fraction, a curve showing biexponential relaxation of the remaining magnetization was derived. The raw magnetization-recovery data before the subtraction of the extracellular compartment also showed a better fit to a sum of three exponentials (Fig. 2, lower panel) than to two exponentials, indicating distinct contributions from nuclear and cytoplasmic compartments. Table 1 presents the T_1 values for the extracellular, nuclear and cytoplasmic compartments in the prophase follicle and the relative sizes of the three compartments (coefficients of exponential terms in Eq. 1). It can be seen that the



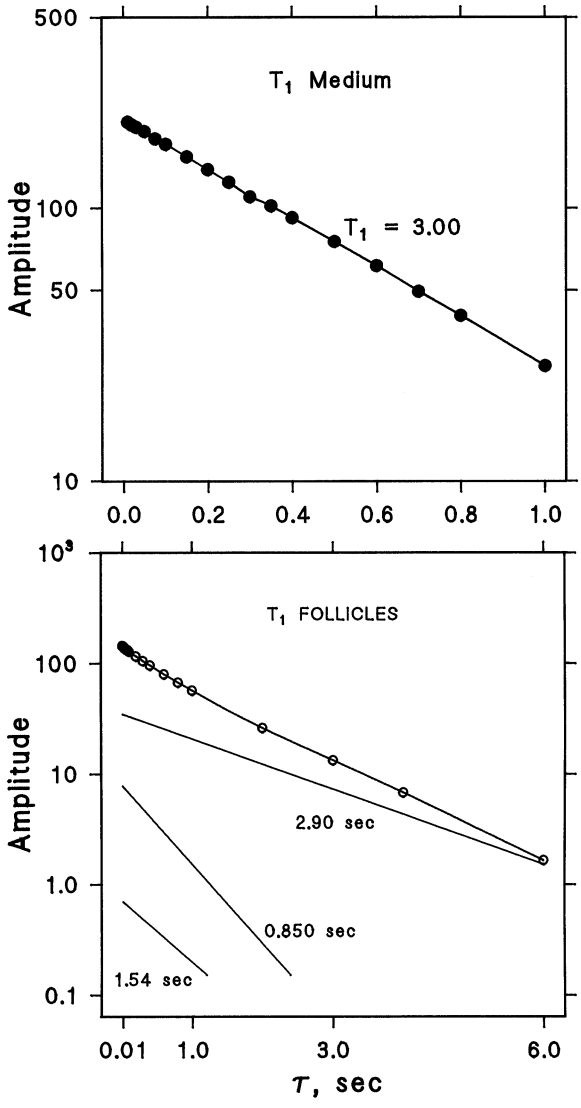


Fig. 2. Semilog plots of inversion-recovery T_1 data showing single exponential recovery of Ringer's solution (upper panel) and a multi-exponential recovery for packed *Rana* follicles (lower panel).

larger intracellular compartment has the shorter T_1 (0.85 sec) whereas the smaller intracellular compartment has a much longer relaxation time (1.54 sec). The smaller (9.4%) compartment disappeared 8–10 hr after the addition of progesterone to the medium. This corresponds to the time of nuclear breakdown (*see* Fig. 1) and indicates that this compartment is the nucleus. It is essential to obtain many data points (18 in our study) in the 10-msec to 10-sec region to detect a nuclear compart-

Fig. 1. Water proton images of *Rana pipiens* oocytes at three stagesof the first meiotic division. Upper Panel: Oocyte in prophase I arrest, Middle Panel: Oocytes following progesterone stimulation with nuclei underlying the animal cortex, and Lower Panel: oocytes following nuclear breakdown.

Table 1. T_1 and compartmentation of water in the prophase I *Rana pipiens* oocyte

Compartment	% Total H_2O Volume	T_1 (Sec)*
Cytoplasm	29.0	0.85
Nucleus	9.4	1.54
Extracellular	61.6	2.90

* Isolated ovarian follicles were superfused with Ringer's solution and the Carr-Purcell-Meiboom-Gill T_1 data were analyzed for the sum of three exponentials as described in Materials and Methods.

ment. Cameron et al. (1983) previously estimated the T_1 values of intact *Xenopus laevis* follicles in prophase arrest to be about 0.4 sec. These investigators removed most of the extracellular fluid by blotting with filter paper to eliminate the extracellular water signal but did not consider a distinct nuclear compartment.

The estimated volumes of the intracellular and extracellular water compartments measured from exponential data analysis via Eq. 1 were very similar when the T_1 was measured in follicles from different females. In three experiments the putative nuclear compartment corresponded to 9.4 ± 0.8% of the total sample water. Subtracting the contribution of the extracellular compartment, the smaller intracellular compartment corresponded to about 24% of the oocyte water. Correcting for the oocyte water content (43%), this yields a nuclear volume of about 13%, assuming nuclear contents to be 80% hydrated. This is compatible with the calculated volume occupied by the oocyte nucleus of around 10%, as measured from the ratio of relative nuclear diameter to oocyte diameter seen in Fig. 1 (upper panel).

Plasma and Nuclear Membrane Potentials

Table 2 compares the electropotential difference between the fluid surrounding the oocyte, the oocyte cytoplasm and the nucleoplasm. Nuclear potentials were measured in oocytes following centrifugation to bring the nucleus to underlie the plasma membrane. The values shown represent steady-state potentials and are means ± SD for six oocytes from the same female and are representative of data from four females.

When the tip of the recording electrode penetrated the cortex overlying the nucleus in centrifuged oocytes, there was a 40–50 mV negative deflection followed by dimpling of the nuclear membrane. Further movement of the electrode tip was followed by disappearance of the dimpling and a concomitant appearance of a steady-state potential of about 25 mV, inside positive. This potential difference was stable for 1–2 hr. A negative steady-state potential reappeared upon visual penetration of the distal side of the *in situ* nucleus. Insertion of one recording electrode into the nucleus and a second into adjacent

Table 2. Electropotential difference between the oocyte nucleoplasm, cytoplasm and extracellular medium

Compartment	E_{mV}[a]
Cytoplasm (nucleated, centrifuged)[b]	−48.7 ± 1.4
Nucleoplasm (centrifuged)	+25.7 ± 1.8
Nucleus *vs.* Cytoplasm	*ca.* + 75[c]
Cytoplasm (nucleated, not centrifuged)	−49.3 ± 1.5
Cytoplasm (Enucleated, centrifuged)	−59.5 ± 2.0

[a] Mean ± SD for 4–6 sibling oocytes. Each measurement was made on a fresh oocyte.
[b] Isolated oocytes were centrifuged as described in Materials and Methods.
[c] Estimated from the sum of the plasma membrane and nuclear membrane potentials.

cytoplasm produced an apparent 65–75 mV potential difference between nucleus and cytoplasm (nucleoplasm positive). This is consistent with the difference between separately measured electropotential differences between extracellular medium and both nucleoplasm and cytoplasm (about +75 mV). The oocyte plasma membrane depolarizes about the time of nuclear breakdown in progesterone-treated oocytes and by second metaphase arrest the oocyte cytoplasm becomes about 30 mV positive, relative to the external medium (Morrill et al., 1984).

For comparison, the cytoplasmic potential was measured both in oocytes that had not been subjected to centrifugation as well as oocytes that had been manually enucleated (Ziegler & Morrill, 1977). The oocyte cytoplasm was about 50 mV negative, relative to the extracellular fluid, with no significant change following centrifugation. As reported previously (Ziegler & Morrill, 1977), the plasma membrane quickly resealed and repolarized following enucleation. However, the enucleated oocyte plasma membrane potentials averaged 10–12 mV more negative than that of their nucleated siblings.

Discussion

Our data demonstrate that the nucleus is much more hydrated than the cytoplasm in the prophase I *Rana pipiens* oocyte. The spatial variation in cytoplasmic image intensity indicates that there are graded variations in the hydration state throughout the cytoplasmic compartment, most notably between the yolk platelet-rich region and the rest of the cytoplasm (top panel, Fig. 1). These observations are consistent with earlier MRI studies of *Xenopus* oocytes (Aguayo et al., 1986) and with reported differences in water content of intracellular organelles in *Rana* oocytes (Naora et al., 1962). The MRI image intensity gradients reported here and elsewhere (Aguayo et al., 1986) are also consistent with the low intracellular

water content reported previously (Naora et al., 1962) and the relatively anhydrous nature of the yolk platelets [*reviewed in* (Morrill & Kostellow, 1991)]. We have made measurements of oocyte water content using ^{35}Cl NMR as a measure of extracellular volume and ^{2}H NMR as a measure of the ratio of intra- and extracellular water volumes (Morrill et al., 1987). With this method, the average water content of the ovarian oocyte was only 42.6 ± 0.8% (vol/vol). For comparison, Naora et al. (1962) removed samples of cytoplasm and nucleoplasm from frozen oocytes by microdissection and reported that the water content of the nucleus was about 80% (gm H_2O per g wet wt), whereas that of the cytoplasm was only about 50%. Taken *in toto*, our results indicate that a difference in water density exists between nucleoplasm and cytoplasm in the prophase oocyte.

A number of studies indicate that, in prophase I oocytes from a wide variety of species, K^+ (but not Na^+) is much higher in the nucleus than in the cytoplasm (*reviewed in* Morrill & Kostellow, 1991). Moore and Morrill (1976) proposed a model to explain the higher K^+ ion concentrations in the oocyte nucleus compared to that in the cytoplasm. This model suggested direct channels between the nucleoplasm and the extracellular fluid, and is consistent with our finding that the nucleoplasm is essentially isopotential with respect to the extracellular fluid.

Using cryomicrodissection, Frank and Horowitz (1980) have compared the kinetics of nuclear and cytoplasmic $^{42}K^+$ exchange in *Rana pipiens* oocytes and report that whole oocyte K^+ exchange is multiphasic and reflects the presence of two intracellular K^+ fractions. Their results suggest that the nucleus behaves as an ordinary aqueous solution whose K^+ content is determined largely by the transport properties of the plasma membrane. In any case, the K^+ concentration difference between nucleus and cytoplasm does not seem to be attributable to active transport by the nuclear envelope, as this structure is thought to be too permeable to subserve such a function. For example, it has been reported that sucrose and polysaccharides as large as 5,000 Daltons move virtually unobstructed between nucleus and cytoplasm (*cf.* Paine et al., 1981). Furthermore, nuclei isolated by microdissection from oocytes in salt solutions swell irrespectively of the concentration of ions in the solution. However, when isolated in 4% bovine serum albumin solutions, swelling is negligible (Dick & Fry, 1973). These findings suggest that the prophase oocyte nucleus contains large impermeant anions and that K^+ ions are needed to neutralize the charge on these anions.

The existence of passive ionic differences between nucleus and cytoplasm is consistent with recent studies of the structure of the nuclear membrane. A supramolecular complex called the nuclear pore complex straddles the inner and outer membrane in all eukaryotes and has been isolated and characterized for amphibian oocyte nuclear membranes (*reviewed in* Hinshaw, Carragher & Milligan, 1992). The supramolecular complex is built from many distinct, interconnected subunits and the arrangement of subunits within the pore creates a large central channel, through which active nucleocytoplasmic transport of macromolecules is known to occur, and eight smaller peripheral channels that are thought to be routes for passive diffusion of ions and small molecules.

The difference in hydration state between oocyte nucleoplasm and cytoplasm might be attributed to a Donnan-type effect in which large impermeant anions and their counter ions within the nucleus produce a nearly twofold increase in nuclear water content. As shown in Fig. 1, the difference in hydration state is maintained following resumption of the first meiotic division as the nucleus swells and rises to underlie the cortex of the animal hemisphere. As the nuclear membrane breaks down, the bright spot in the image representing the nucleus disappears and is consistent with equilibration of nuclear and cytoplasmic contents late in the first meiotic division. After breakdown of the nuclear membrane, an increase in rotational correlation time for the water molecules in the nucleoplasmic environment might result in short T_2 and loss of image intensity. This change in the microenvironment of the oocyte chromatin occurs during the slow transition between the end of prophase and the lining up of the chromosomes on the spindle apparatus, and may alter both cation binding and macromolecular structure.

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