

INTRACELLULAR IONIC DISTRIBUTION, CELL MEMBRANE PERMEABILITY AND MEMBRANE POTENTIAL OF THE *XENOPUS* EGG DURING FIRST CLEAVAGE

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SUMMARY

Previously we have presented evidence for a direct relationship between post-mitotic new membrane formation and changes in the electrical membrane characteristics during cytokinesis of *Xenopus* eggs [1, 2]. In the present study the phenomena underlying the hyperpolarization of the electrical membrane potential during cytokinesis were investigated. Total Na^+ and K^+ contents at the onset of the first and second cleavage were measured independently by flame spectrophotometry and by means of ion-selective electrodes. Total Cl^- content was measured by the latter method only. The water content was determined from the difference between wet weight and dry weight. $^3\text{H}_2\text{O}$ -influx experiments yielded an independent estimate of the water content ($0.737 \mu\text{l/egg}$), a rate constant for the influx of $1.412 \times 10^{-3} \text{ sec}^{-1}$ and a low water permeability of $1.87 \times 10^{-5} \text{ cm sec}^{-1}$. They furthermore revealed the absence of intracellular water compartmentation. The Na^+ , K^+ and Cl^- concentrations remained constant during first cleavage at 58.6, 87.3 and 62.6 mM/l cell water, respectively. The membrane potential (E_m), the membrane resistance (R_m), and the intracellular ion activities of Na^+ , K^+ and Cl^- (a_{Na}^i , a_{K}^i and a_{Cl}^i) were measured simultaneously and continuously during cleavage, using conventional glass microelectrodes and ion-selective microelectrodes. a_{Na}^i showed an increase from 19.4 to 22.4 mM concomitant with the hyperpolarization of E_m and the decline of R_m . a_{K}^i and a_{Cl}^i remained constant at 51.4 and 53.1 mM, respectively. From the calculated activity coefficients it was concluded that all Cl^- ions were free, whereas 30 % of the K^+ ions and 60 % of the Na^+ ions were bound. The influence of changes in the ionic composition of the medium on E_m was analysed in the uncleaved egg, in normally cleaving eggs, and in eggs cleaving outside the vitelline membrane. The latter conditions leads to exposure of the whole area of newly formed membrane to the medium. The cell membrane of the uncleaved egg exhibits no permselectivity. In normally cleaving eggs the relative permeability $P_{\text{Na}}/P_{\text{K}}$ was 0.73, while in eggs cleaving outside the vitelline membrane it was 0.19. It was concluded that the changes of E_m during cytokinesis are due to the insertion of a part of the newly formed membrane into the cell surface. The K^+ permeability of this new membrane is at least five times greater than that of the pre-existing membrane. The possible role of the hyperpolarization of E_m in the regulation of the cell cycle is briefly discussed.

In previous reports [2, 3] the characteristic changes of the electrical membrane potential (E_m) and the electrical membrane resistance (R_m) during the first cytokinetic cycle of *Xenopus laevis* eggs were described. A temporal and causal relationship was found between these changes and new membrane formation. The electrical membrane characteristics start

to change at 6–8 min from the onset of the cleavage. From that time on morphological signs of the incorporation of new membrane material in the bottom of the cleavage furrow are visible at the ultrastructural level [1]. Mainly on the basis of measurements of R_m and of the geometrical surface area of the new membrane under conditions which lead

to various extents of exposure of this new material, it was concluded that the changes in R_m during cleavage can be explained by the insertion of new membrane having a low specific resistance (1–2 kOhm cm²) as compared with the specific resistance of the pre-existing membrane (74 kOhm cm²). Concomitant with the decrease of R_m a hyperpolarization of E_m was observed. In normally cleaving eggs E_m changes from approx. –10 to –20 mV. However, in eggs cleaving outside the vitelline membrane or in the presence of cytochalasin B, in which the entire new membrane area, including its normally intercellular portion, is exposed to the medium, E_m can reach values as high as –60 mV. This indicates that it is not only the absolute ionic permeabilities that increase during cleavage. In general, E_m is a function of the relative ionic permeabilities of the cell membrane, the electrochemical gradients across the membrane of the ions determining E_m and, if present, the activity of electrogenic ionic pumps. Since E_m changes towards the equilibrium potential of K⁺ ions (approx. –115 mV) it was assumed that at least a relative increase in K⁺ permeability is involved [2].

The hyperpolarization of E_m , as observed in cleaving *Xenopus* eggs is a strikingly universal phenomenon. It has been reported for a variety of developing embryos, e.g. amphibians [2–10], echinoderms [11, 12], fucoid algae [13, 14], teleosts [15]. In most of these cases it was either suggested or various degrees of quantitative evidence were presented that the hyperpolarization involves an increase in K⁺ permeability. Probably the most extensive analyses of this hyperpolarization have been made in *Asterias* [11, 12] and *Pelvetia* [14, 16], but in these cases measurements of the intracellular activities of the ions involved are lacking so that estimates had to be used. Unfortunately

these investigations were not focused on the changes in cell membrane permeability during a single cell cycle. Whether also in these systems a relationship exists between the changes in E_m and post-mitotic new membrane formation remains to be clarified. Only in amphibians was evidence for this relationship presented, i.e. for cleaving eggs of *Rana pipiens* [7], *Triturus pyrrhogaster* [6], and *Xenopus laevis* [1–3, 10, 17]. In the latter two studies the attention was focused on changes in intra-embryonic ionic distribution, on their possible relation to blastocoel formation, and on a qualitative description of the dependence of E_m on external K⁺ concentration. Intracellular Na⁺ activities were measured using Na⁺-selective microelectrodes, but measurements of K⁺ activity necessary for a quantitative description of E_m were not given.

In the present study a detailed description is given of the intracellular distributions of Na⁺, K⁺ and Cl[–] ions, the changes in relative ionic permeabilities, and their relationship to the hyperpolarization of E_m during the first cytokinetic cycle of *Xenopus laevis* eggs. The total contents of both Na⁺ and K⁺ were determined independently by flame spectrophotometry and by means of ion-selective electrodes, while total Cl[–] content was measured by the latter method only. Water content was determined by measuring wet weight and dry weight, while the possibility of intracellular water compartmentation was studied by influx experiments using ³H₂O. Simultaneous and continuous measurements of E_m , R_m and intracellular activities of Na⁺, K⁺ and Cl[–] were made during first cleavage, using conventional electrophysiological techniques and ion-selective microelectrodes. Similar measurements in media of different ionic composition provided the data for the calculation of the relative ionic permeabilities. Eggs cleaving outside the vitelline

membrane were used to specifically study the properties of the new membrane formed during cleavage.

MATERIALS AND METHODS

Fertilized eggs of *Xenopus laevis* were obtained from hormonally stimulated couples, chemically decapsulated, and handled as described earlier [3]. Eggs were kept in Steinberg solution [18] unless otherwise indicated. For some experiments the vitelline membrane was removed with fine forceps. Experiments were carried out at room temperature (20–24°C).

Construction of Na^+ microelectrodes

The construction was based on the procedure described by Hinke [19]. Clean capillaries of Na^+ -selective NAS 11–18 glass (Corning Glass Works, Corning, N.Y.) were manually pulled in an air–butane flame into microcapillaries of tip diameter smaller than 5 μm and 5–10 mm length. Insulating micropipettes were pulled from lead glass on a horizontal microelectrode puller [20]. Appropriate tip diameters were obtained by cutting or grinding the micropipette under microscopic control.

A microcapillary was pushed down a micropipette, under the microscope, using a glass rod. It is important that the inner diameter of the tip of the micropipette approximately equals the outer diameter of the microcapillary 50 μm from its tip. The tip of the inner capillary was closed in a microforge with Pt–Rh (90:10) heating wire (de Fonbrune/Beaudouin, Paris). When carefully done, the lead glass melts and collapses around the microcapillary, forming a glass-to-glass seal and leaving the end of the microcapillary uninsulated. When the protruding end of the microcapillary was too long, first the glass-to-glass seal was made and the end of the microcapillary was then pulled to the appropriate dimensions using the microforge. In this way tip diameters smaller than 2 μm could be obtained. The sealing was checked by submerging the tip of the microelectrode in a drop of distilled water. The microelectrode was filled by boiling in methanol under low pressure. The methanol was replaced by a Tris-buffered 0.1 M NaCl solution (pH = 7.4) by placing the microelectrode, tip up, in a beaker of filling solution for 2 days. The microelectrode was mounted in a perspex holder containing a Pt–Ag–AgCl electrode and filled with the same NaCl solution. The microelectrodes were stored (tip down) in 0.01 M NaCl with only the sensitive tip submerged in the solution. They remained usable for more than a month.

Construction of K^+ and Cl^- microelectrodes

Glass capillaries of Pyrex or Duran-50 glass (outer diameter 1.5 ± 0.1 mm) were cleaned for at least 24 h in chromic acid followed by boiling 3 times in distilled water and drying at 60°C. They were pulled into micropipettes (tip diameter smaller than 1 μm) on a

horizontal microelectrode puller [20]. The tip was siliconized by dipping in a 0.5% solution of silicon oil (Dow Corning no. 1107; Dow Corning Corp. Midland, Mich.) in trichlorethylene until a column of 300–500 μm appeared in the tip. The micropipettes were then heated for 2 h at 200°C. To obtain K^+ or Cl^- microelectrodes the tips were filled with K^+ or Cl^- specific liquid ion-exchanger (Corning no. 476132 and no. 476131 respectively; Corning-EEL, Medfield, Mass.) by submerging them until a column of approx. 300 μm was present in the tip. The remainder was filled with 0.1 M KCl using a syringe. Air bubbles in the “neck” of the micropipette were sucked away via a long, thin glass capillary inserted from the back end.

The K^+ and Cl^- microelectrodes were mounted in a perspex holder containing a Pt–Ag–AgCl electrode and filled with 0.1 M KCl. With a store of siliconized micropipettes at hand, microelectrodes could be prepared within 15 min. They were ready for use after 30 min equilibration in 0.1 M KCl. Similar K^+ and Cl^- microelectrodes have been described previously [21, 22].

Calibration and properties of ion-selective microelectrodes

In general the ion-selective microelectrodes were calibrated before and after each measurement, the configuration of the electrodes being identical to that in the actual measurements. The latter is important to avoid errors due to liquid-junction potentials.

When used for the determination of ion activities in a physiological environment at an approximately neutral pH one can expect some interference of K^+ ions with the response of the Na^+ microelectrode and the converse. The behavior of such electrodes can be described by:

$$E_{\text{Na}} = E_{\text{Na}}^{\circ} + S_{\text{Na}} \log (a_{\text{Na}} + k_{\text{K/Na}} a_{\text{K}}) \quad (1)$$

$$E_{\text{K}} = E_{\text{K}}^{\circ} + S_{\text{K}} \log (a_{\text{K}} + k_{\text{Na/K}} a_{\text{Na}}) \quad (2)$$

where a_{Na} and a_{K} represent the activities of Na^+ and K^+ , respectively, E_{Na}° and E_{K}° are constants depending on the concentration of the internal solution of the microelectrodes and on liquid-junction potentials in the electrode circuit, S_{Na} and S_{K} are the changes in electrode potential for a 10-fold change of the activity of Na^+ and K^+ , respectively (theoretically 58 mV at room temperature), $k_{\text{K/Na}}$ and $k_{\text{Na/K}}$ are the respective selectivity constants, and E_{Na} and E_{K} represent the potential difference between the reference electrode and the Na^+ and K^+ microelectrode, respectively, in a solution that contains both Na^+ and K^+ ions.

For determinations of Cl^- activities with Cl^- microelectrodes under physiological conditions at approximately neutral pH no significant interference with other anions is to be expected. Thus, theoretically the electrode response will follow the Nernst equation, rewritten as follows:

$$E_{\text{Cl}} = E_{\text{Cl}}^{\circ} + S_{\text{Cl}} \log a_{\text{Cl}} \quad (3)$$

Table 1. *Solutions used for calibrating the ion-selective (micro)electrodes: concentrations and (in parentheses) activities*

		Na ⁺ electrode	K ⁺ electrode	Cl ⁻ electrode
A	I	10 (9.2) mM NaCl	10 (9.2) mM KCl	10 (9.2) mM KCl
	II	50 (41.0) mM NaCl	50 (40.5) mM KCl	50 (40.5) mM KCl
	III	100 (78.0) mM NaCl	100 (77.0) mM KCl	100 (77.0) mM KCl
	IV	50 (35.5) mM NaCl	50 (37.5) mM KCl	
		200 (142.0) mM KCl	200 (150.0) mM NaCl	
B	I	0.1 (0.10) mM NaCl	0.1 (0.10) mM KCl	
	II	0.5 (0.49) mM NaCl	0.5 (0.49) mM KCl	
	III	1.0 (0.96) mM NaCl	1.0 (0.96) mM KCl	as for K ⁺ electrode
	IV	5.0 (4.7) mM NaCl	5.0 (4.7) mM KCl	
	V	10.0 (9.2) mM NaCl	10.0 (9.2) mM KCl	

A, Solutions used when calibrating for intracellular ion activity measurement.

B, Solutions used when calibrating for total ion content measurement.

where the symbols have meanings corresponding to those above. For the three types of ion-selective microelectrodes E° and S were determined from semilogarithmic plots of E against ion activity. The plot was made from the responses of the respective microelectrodes in a series of solutions of different concentrations containing no interfering ions. The selectivity constants $k_{K/Na}$ and $k_{Na/K}$ were calculated from the electrode response in a solution that contained both NaCl and KCl according to eqs (1) and (2). Table 1 gives the concentrations and activities of the solutions used for the calibration procedure. Activity coefficients were derived from MacInnes [23].

Different series of solutions were used for the calibration for the determination of intracellular Na⁺, K⁺ and Cl⁻ activities on the one hand, and of total Na⁺, K⁺ and Cl⁻ content per egg on the other, since the range of activities to be measured differed substantially (see below).

For the determination of total Na⁺ content a commercially available Na⁺-selective combined electrode was used (Orion 96-11; Orion Research Inc. Cambridge, Mass). No correction for interference was made for the determination of total Na⁺ and K⁺ content since the Na⁺ and K⁺ activities in the solution obtained after extraction of ions from the egg appeared to be in the same order of magnitude. The selectivity constants $k_{K/Na}$ and $k_{Na/K}$ of the Na⁺ and K⁺ (micro) electrodes were ca 0.03, so that the possible error due to interference was about 3%. Ten of each type of ion-selective microelectrode were tested in solutions of 0.1–100 mM. The Na⁺ microelectrodes showed a semilogarithmic behavior in the range of 1–100 mM NaCl, S_{Na} being 58.2 ± 0.8 mV (mean \pm S.E.M.) and $k_{K/Na}$ being 0.028 ± 0.007 with a minimum of 0.001. The electrical resistance of the tip was $1.2 \times 10^{10} \pm 0.6 \times 10^{10}$ Ohm. The K⁺ microelectrodes showed a semilogarithmic response in the range of 0.1–100 mM KCl, S_K being 58.1 ± 0.4 mV and $k_{Na/K}$ being 0.027 ± 0.003 with a minimum of 0.018. Their tip resistance was $7.8 \times 10^8 \pm 0.9 \times 10^8$ Ohm. The Cl⁻ microelectrodes behaved semilogarithmically in the range of 0.5–100 mM KCl, S_{Cl} being 51.7 ± 1.3 mV. Their tip resistance was $5.6 \times 10^9 \pm 1.6 \times 10^9$ Ohm.

Dry weight and water content

Adhering water was removed from decapsulated eggs by blotting them on hardened filter paper while they were held in a small ring of silver wire. They were then individually weighed on pre-weighed vials using a Cahn model G electrobalance (Cahn Instrument Corp., Paramount, Calif.), dried at 120°C, and weighed again. The difference yielded the water content.

Determination of total Na⁺ and K⁺ content by flame spectrophotometry

Individual decapsulated eggs at the proper developmental stage were washed 3 times in 10 ml deionized distilled water (dd-water) and digested in 1 ml concentrated HNO₃ at 100°C. The digests were diluted by adding 9 ml dd-water and analysed for Na⁺ and K⁺ by means of a Unicam SP 900 flame spectrophotometer. As a routine the last wash and samples of dd-water were also analysed.

Determination of total Na⁺, K⁺, and Cl⁻ content by ion-selective (micro) electrodes

Groups of 20 decapsulated eggs at the proper developmental stage were washed 3 times in dd-water and transferred to a plastic vial in approx. 1 ml dd-water. After drying the samples at 100°C, 0.5 ml dd-water was added and the samples were soaked for 2 days. Using flame spectrophotometry it was found that this extraction method gave results identical to those of eggs digested in HNO₃. A 100 μ l sample was taken for the determination of Na⁺ with the Orion 96-11 Na⁺ electrode, while samples of 50 μ l were used for the K⁺ and Cl⁻ measurements with the liquid ion-exchange-type microelectrodes. In some samples the activity coefficient was determined. After measuring the K⁺ activity a small volume of KCl of known concentration was added to the sample and the K⁺ activity was measured again. The activity coefficient was calculated from the difference in K⁺ activity between the first and second measurement and from the

concentration that was added. In these dilute solutions it did not differ significantly from unity. The respective ion activities measured were converted into μg per egg.

Tritiated water influx

Measurement of $^3\text{H}_2\text{O}$ influx gave information on the rate constant of the water flux, the water permeability of the membrane, the possible intracellular compartmentation of water, and the total water content. To retard development slightly the experiments were carried out at 15°C . Per experiment (total of 5 experiments) 20 decapsulated eggs not older than 30 min after oviposition were transferred to a small Petri dish in 200 μl Steinberg solution. At zero time 2 ml Steinberg solution containing 60 μCi $^3\text{H}_2\text{O}$ per ml (The Radiochemical Centre, Amersham, Bucks.) was added. At certain intervals during the next 2 h two eggs in 10 μl were removed from the incubation medium and washed twice in 30 ml distilled water. Each egg was transferred with 10 μl water to a counting vial containing 500 μl Soluene-350 (Packard Instrument Comp. Inc., Downers Grove, Ill.) and digested overnight at room temperature. Radioactivity was measured as described previously [24] using a Packard Model 2450 liquid scintillation counter. Measurements of radioactivity (cpm) were converted into disintegrations per minute (dpm) using the external standard ratio method. For the transfer of eggs Eppendorf pipettes (Eppendorf, Hamburg) with modified tips were used.

To check for possible incorporation of the label into macromolecules during the 2 h incubation period, at the end of each experiment two eggs were washed as described and cold TCA-insoluble radioactivity was determined.

Measurement of membrane potential (E_m), membrane resistance (R_m), and intracellular activities of Na^+ , K^+ and Cl^- ($a_{\text{Na}^+}^i$, $a_{\text{K}^+}^i$ and $a_{\text{Cl}^-}^i$)

Fortunately eggs of *Xenopus laevis* are so large (diameter 1.3 mm) and have such excellent surface healing properties that they allow for the measurement of all five parameters simultaneously without interference with subsequent development. Thus five microelectrodes were simultaneously inserted into the egg. Separate glass microelectrodes filled with 3 M KCl (5–10 M Ω dc-resistance) connected to Pt–Ag–AgCl electrodes via Steinberg solution bridges were used to measure intracellular voltages and to pass current. The bath was grounded via a separate Pt–Ag–AgCl electrode. The membrane potential was measured with respect to an indifferent Pt–Ag–AgCl electrode by means of a differential preamplifier (Grass model P18; Grass Instruments, Quincy, Mass.) modified for unity gain. A constant-current stimulator delivered a current pulse of 1.2 sec duration and 3×10^{-8} A amplitude every 30 sec. The polarity of the pulses was alternated to prevent net current flow. The current was measured by means of a current-to-voltage converter in the ground circuit. The responses of the Na^+ , K^+ and Cl^- microelectrodes were measured via electrometers (Keithley 602 for Na^+

Table 2. Dry weight and water content per egg at the onset of first and second cleavage: mean values \pm S.E.M.

	Dry weight (μg)	Water content (μg)
First cleavage	443 ± 5 ($n=45$)	782 ± 28 ($n=43$)
Second cleavage	454 ± 5 ($n=43$)	769 ± 25 ($n=42$)

microelectrode, Keithley 600 B for K^+ and Cl^- microelectrode; Keithley Instruments, Cleveland, Ohio). The intracellular voltage as measured with respect to ground via a second Grass P18 preamplifier in single-ended configuration, was subtracted from the unity gain output of the electrometers to obtain the net voltage response due to the intracellular ion activities. These voltages as well as the membrane potential, upon which a voltage pulse proportional to the membrane resistance was superimposed every 30 sec, were recorded on pen-recorders. Every 10 sec their values were digitalized via A-D converters (Analog Devices type AD 2003; Analog Devices, Norwood, Mass.) in such a way that each third digital measurement was taken 1 sec after onset of the current pulse. The BCD-outputs of the A-D converters were recorded on punch tape (Facit 4070; Facit AB, Åtvidaberg, Sweden). The timing of this system was controlled by a Devices digitimer, type 3291A-Tu (Devices Instruments Ltd., Welwyn Garden City, UK).

Calculations of the ion activities were made off line on a Wang 600 system (Wang International Trade, Tewksbury, Mass.) using eqs 1, 2 and 3. The Nernst potentials for each ion and the membrane resistance were also calculated. The average values per minute of the Na^+ , K^+ and Cl^- activities, of their Nernst potentials, and of E_m and R_m were plotted as functions of time on the Wang 600 system. Details of the positioning of the eggs were described previously [2].

RESULTS

Dry weight and water content

Using the weighing procedure described, dry weight and water content of eggs from four different batches were determined at the onset of the first and second cleavage. The results are summarized in table 2. An analysis of variance [25] showed that neither the dry weight nor the water content changed significantly during first cleavage ($P > 0.1$ and $P > 0.5$, respectively). Neither were significant differences found between the results of individual batches ($P > 0.25$).

Table 3. Total content ($\mu\text{g}/\text{egg}$) and concentration (mM/l cell water) of Na^+ , K^+ and Cl^- per egg at the onset of the first (I) and second (II) cleavageContents are given as mean value \pm S.E.M. (number of observations in parentheses)

			Content (μg)			Concentration (mM) I + II ^b
Ion			I	II	I + II ^a	
Na^+	Ion-sel.electr.	+ VM	1.06 ± 0.04 (10)	1.12 ± 0.04 (9)	0.99 ± 0.03 (89)	58.6
		- VM	1.03 ± 0.05 (11)			
K^+	Flame spectroph.	+ VM	0.97 ± 0.07 (30)	0.94 ± 0.07 (29)	2.52 ± 0.04 (112)	87.3
		- VM				
	Ion-sel.electr.	+ VM	2.58 ± 0.06 (20)	2.49 ± 0.08 (19)		
		- VM	2.59 ± 0.10 (13)			
Cl^-	Ion-sel.electr.	+ VM	1.62 ± 0.03 (17)	1.63 ± 0.03 (14)	1.64 ± 0.02 (40)	62.6
		- VM	1.69 ± 0.03 (9)			

^a Results of different stages and methods are pooled, since no significant differences were found.^b Calculated from mean ion content and a water content of $0.737 \mu\text{l}$.

+ VM, within vitelline membrane.

- VM, vitelline membrane removed.

Total content of Na^+ , K^+ and Cl^-

Na^+ and K^+ contents were measured at the onset of the first and second cleavage both by flame spectrophotometry and with ion-selective (micro) electrodes. Cl^- content at the two stages was only measured with Cl^- microelectrodes. In a separate series the contents of all ions were determined with ion-selective (micro) electrodes at the onset of the first cleavage after removal of the vitelline membrane. This was done to check whether significant amounts of these ions are present within the vitelline space. Using Student's *t*-test [25] the results obtained for each ion species were compared by stage and by method. The mean values obtained by flame spectrophotometry and with ion-selective (micro) electrodes showed no significant differences. Neither were significant differences found when the results were compared by stage, nor when the ion contents of eggs within the vitelline membrane and of eggs devoid of the vitelline membrane were compared. However, the variances of the results obtained by flame spectrophotometry were significantly greater than those

obtained with ion-selective (micro) electrodes ($P < 0.005$), as could be concluded from the *F*-test [25]. One reason for this could be that in the latter method the ion content was measured in extracts of groups of 20 eggs, whereas extracts of individual eggs were used in the former. The ion contents ($\mu\text{g}/\text{egg}$) were converted into concentrations (mM/l cell water) using a value of $0.737 \mu\text{l}$ water per egg. This value was calculated from the influx of $^3\text{H}_2\text{O}$ (see below). Table 3 gives a summary of the results.

Tritiated water influx

Considering the uncleaved *Xenopus* egg for simplicity as a sphere with a diameter of $1.3 \times 10^{-1} \text{ cm}$ one obtains an egg volume of $1.15 \times 10^{-3} \text{ cm}^3$. Twenty eggs were incubated in a volume of 2.2 cm^3 . Thus the ratio total egg volume/volume of medium was approx. 1/100. Under these conditions the uptake of $^3\text{H}_2\text{O}$ can be treated as uptake from a constant pool. Assuming steady state conditions and uptake by a single compartment the following equations will be applicable.

$$R_i(t) = R_i(\infty)(1 - e^{-kt}) \quad (4)$$

or

$$-\ln\left(1 - \frac{R_i(t)}{R_i(\infty)}\right) = kt \quad (5)$$

where R_i is the amount of intracellular radioactivity at time t (dpm), k is the rate constant (sec^{-1}) and t is time (sec). Since we are dealing with uptake from an infinitely large constant pool $J_i = k(C_i V_i)$, where J_i is the influx ($M \times \text{sec}^{-1}$) and $C_i V_i$ is the product of intracellular concentration and cell volume, i.e. the total amount of intracellular water (M).

The total amount of exchangeable water was calculated from the amount of radioactivity at equilibrium. It appeared to be $737 \pm 31 \mu\text{g H}_2\text{O}$ per egg (mean \pm S.E.M.). This is not significantly different from the total amount of intracellular water as determined by weighing. The water space can be calculated to be approx. 70 %.

Figs 1 and 2 show the results of the influx experiments plotted according to eqs (4) and (5), respectively.

A linear regression analysis [25] was made on the points of fig. 2 and showed a perfect linear relationship between $-\ln(1 - R_i(t)/R_i(\infty))$ and t ($P < 0.001$), k being $1.412 \times 10^{-3} \text{ sec}^{-1}$. The lines drawn through the points of figs 1 and 2 are derived from this linear regression analysis.

The surface area of the egg has been measured previously [1]; it has a mean value of $8.69 \times 10^{-2} \text{ cm}^2$ (corrected for surface foldings). By multiplying the rate constant with the appropriate volume/surface ratio the water permeability was calculated to be $1.87 \times 10^{-5} \text{ cm sec}^{-1}$. The unidirectional water flux J was $5.78 \times 10^{-11} \text{ M sec}^{-1}$ or $0.66 \times 10^9 \text{ M cm}^{-2} \text{ sec}^{-1}$.

No radioactivity above background levels could be detected in the cold TCA-insoluble fraction after the 2 h incubation period.

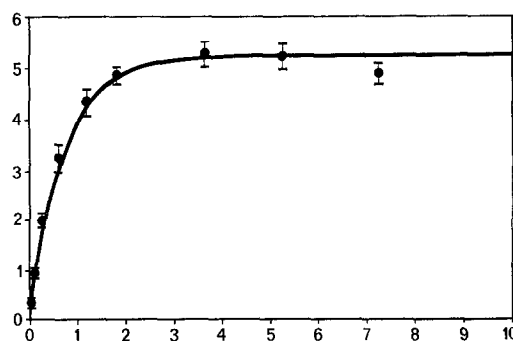


Fig. 1. Abscissa: incubation time ($\text{sec} \times 10^{-3}$); ordinate: intracellular radioactivity ($\text{dpm} \times 10^{-4}$).

$^3\text{H}_2\text{O}$ influx into uncleaved eggs of *Xenopus laevis*: mean values \pm S.E.M. The line is drawn according to eq. (4), k being $1.412 \times 10^{-3} \text{ sec}^{-1}$. Also see text.

Therefore, incorporation of tritium into macromolecules did not have to be taken into account.

E_m , R_m and intracellular ion activities during first cleavage

In a first series of experiments simultaneous measurements of E_m , R_m , and a_{Na}^i , a_{K}^i and a_{Cl}^i were made continuously during the first cleavage of eggs inside the vitelline membrane, while bathed in Steinberg solution. Fig. 3a and b show a typical example of such an experiment and table 4 summarizes the

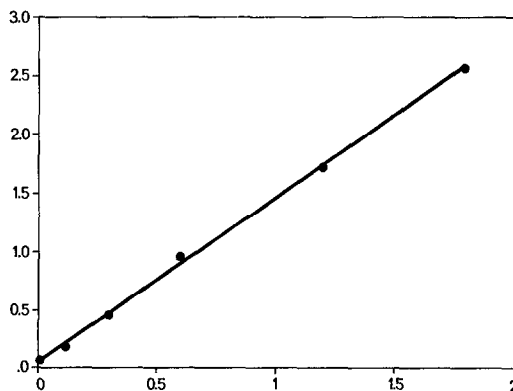


Fig. 2. Abscissa: incubation time ($\text{sec} \times 10^{-3}$); ordinate: $-\ln(1 - R_i(t)/R_i(\infty))$.

Linear regression line obtained from the data of fig. 1, showing the absence of intracellular water compartmentation. Also see text.

Table 4. Intracellular ion activities (mM/l cell water) and activity coefficients of Na^+ , K^+ and Cl^- at the onset of the first and second cleavage

Ion	Ion activity			Activity coefficient ^b		
	I	II	I + II ^a	I	II	I + II ^a
Na^+	19.4 ± 0.4 (82)	22.4 ± 1.1 (18)		0.33	0.38	
K^+	51.6 ± 0.6 (97)	50.4 ± 20 (12)	51.4 ± 0.5 (109)			0.59
Cl^-	53.5 ± 0.8 (84)	50.1 ± 1.5 (11)	53.1 ± 0.7 (95)			0.85

^a When no significant differences were found between the mean values at the onset of the first and second cleavage, the data were pooled (I + II).

^b Determined as the ratio of mean ion activity/mean ion concentration.

results. As described earlier [2, 3] E_m and R_m showed a characteristic hyperpolarization and decrease, respectively, starting 6–8 min after the onset of first cleavage. These changes were shown earlier to be due to the insertion of a small fraction of the newly formed intercellular membrane into the surface membrane of the egg. This newly formed membrane had permeability properties different from those of the pre-existing cell membrane of the uncleaved egg [2]. a_{K}^i and a_{Cl}^i remained unchanged during first cleavage, whereas a_{Na}^i showed a small but significant increase ($P < 0.005$).

The activity coefficients of the three ion species showed marked differences. Probably all Cl^- ions are present in the free state, since the activity coefficient approaches that of a 50 mM KCl solution [23]. However, ca 30 % of the K^+ ions and ca 60 % of the Na^+ ions appeared to be bound. Whether these fractions are bound to macromolecules or accumulated in cell organelles, or both, is not known at present. The Nernst potentials of Na^+ , K^+ and Cl^- were 19 mV, -116 mV and 0 mV, respectively, with a decrease of about 1.5 mV for the equilibrium potential of Na^+ during cleavage. Thus in eggs bathed in Steinberg solution Na^+ and particularly K^+ ions are far from equilibrium. The change in a_{Na}^i was too small to account for the

hyperpolarization of E_m . However, since E_m changed towards the Nernst potential of K^+ an increase in K^+ permeability could be expected during cleavage.

Ionic permeability of the cell membrane

Measurements of E_m in the uncleaved *Xenopus* egg have revealed a small potential difference between the cell interior and the external medium, the interior being negative [2, 3, 8, 10]. The specific resistance of the cell membrane of the uncleaved egg was shown to be very high (74 kOhm cm^2), indicating a very low ionic permeability [2]. To investigate the permeability to the different ion species, E_m , R_m , and a_{Na}^i , a_{K}^i and a_{Cl}^i were measured simultaneously in media of different ionic composition. R_m was measured primarily as an indicator of proper surface healing. A detailed description of the changes of R_m during cleavage has been given previously [2].

Neither equimolar replacement of Na^+ by K^+ (solutions A–F, see table 5), nor replacement of Cl^- by SO_4 (solution G), nor changes in pH ranging from 4.2 to 8.25 had any significant effect on the parameters measured. The same was true when ouabain (Calbiochem., Los Angeles, Calif.) or ethacrynic acid (Merck Sharp & Dohme, Nederland N. V., Haarlem, The Netherlands), both

Table 5. Ion composition expressed in mM/l^a of the different solutions used for studying the ionic permeability of the cell membrane

All solutions contained 0.560 g/l Tris

	NaCl	KCl	Na ₂ SO ₄	Ca(NO ₃) ₂ ·4H ₂ O	MgSO ₄ ·7H ₂ O	pH
Steinberg ^b	58.20	0.67	—	0.34	0.83	7.4
A	56.97	1.90	—	0.34	0.83	7.4
B	53.84	5.00	—	0.34	0.83	7.4
C	46.34	12.50	—	0.34	0.83	7.4
D	30.14	28.80	—	0.34	0.83	7.4
E	15.14	43.70	—	0.34	0.83	7.4
F	0.67	58.20	—	0.34	0.83	7.4
G	—	0.67	29.1	0.34	0.83	7.4

^a The concentrations given of Na⁺, K⁺ and Cl⁻ can be converted into activities by multiplying them by an activity coefficient of 0.814, as was found by measuring the actual K⁺ activity.

^b Also used were Steinberg solutions with pH = 4.2, 5.2, 6.25 and 8.25, and Steinberg solutions to which ouabain or ethacrynic acid was added in concentrations of 1×10^{-4} , 5×10^{-4} and 1×10^{-3} M.

inhibitors of active transport, were added to the Steinberg solution in concentrations up to 10^{-3} M. Similar results were reported earlier [10]. The absolute permeabilities of the uncleaved egg are apparently extremely low, while at the same time any permselectivity is absent. In fact, according to the constant field equation [26, 27], E_m across a cell membrane with a relative permeability $P_{Na}/P_K=1$ would be -9.8 mV, taking into account the intracellular activities of Na⁺ and K⁺ as measured in *Xenopus* eggs (table 4) and their activities in Steinberg solution. This is in accordance with the measured mean value of E_m of -9.7 mV [2].

In cleaving eggs the situation is rather different due to the insertion of new membrane with ionic permeability properties different from those of the pre-existing membrane.

To study these permeability properties in more detail, E_m , R_m and a_{Na}^i , a_K^i and a_{Cl}^i were measured simultaneously in eggs bathed in media of different ionic composition (table 5) during cleavage, both with and without the vitelline membrane. Removal of the vitelline membrane leads to exposure of the entire surface area of the membrane newly formed

during cleavage. It provides a way to study in detail the permeability properties of this new membrane, which normally forms the intercellular membrane. Under such conditions far more pronounced changes of E_m and R_m had previously been observed during cleavage [2]. Under both conditions E_m appeared to be dependent on the ratio of the external K⁺ and Na⁺ activities (a_K^o and a_{Na}^o , respectively). Changes in the external Cl⁻ activity or in pH or addition of 10^{-3} M ouabain or 10^{-3} M ethacrynic acid, had no effect. a_{Na}^i , a_K^i and a_{Cl}^i were not significantly altered by the changes in the composition of the medium. To determine whether E_m in the cleaved egg could be described by the constant field equation, Na⁺ and K⁺ being the permeable ions, the relationship between E_m and a under conditions of equimolar replacement of Na⁺ by K⁺ was analysed for eggs cleaving inside as well as for eggs cleaving outside the vitelline membrane. The values of E_m were taken at the time of onset of second cleavage. According to the constant field equation E_m , under the conditions mentioned, can be described by the following equation:

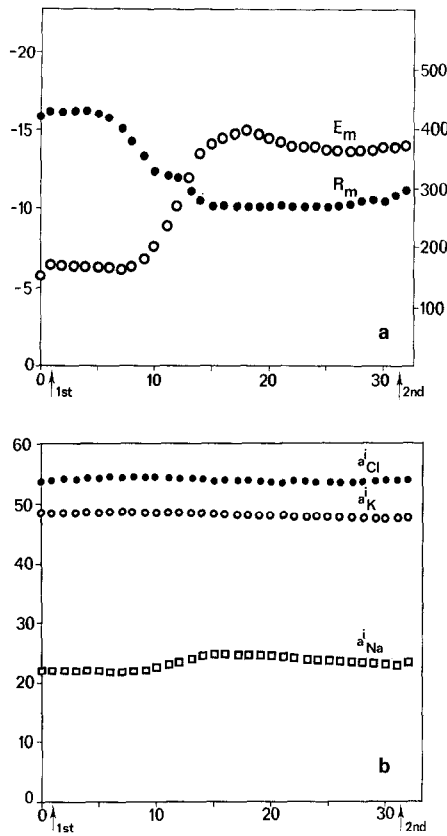


Fig. 3. Abscissa: time (min); ordinate: (a) (left) membrane potential (E_m) in mV; (right) membrane resistance (R_m) in kOhm; (b) ion activity (mM).

A typical example of a measurement of the changes of E_m , R_m , and the intracellular activities of Na^+ , K^+ and Cl^- (a_{Na}^i , a_K^i , and a_{Cl}^i) during first cleavage of a *Xenopus* egg. The arrows marked 1st and 2nd indicate the onset of the 1st and 2nd cleavage, respectively.

$$E_m = 58.1 \log \frac{p \cdot a_{Na}^o + a_K^o}{p \cdot a_{Na}^i + a_K^i} \quad (6)$$

where p represents the ratio of the permeabilities to Na^+ and K^+ ($p = P_{Na}/P_K$); a_{Na}^o and a_K^o represent the activities in the medium of Na^+ and K^+ , respectively, and a_{Na}^i and a_K^i the corresponding intracellular activities. E_m is expressed in mV.

Insertion of the measured values of a_{Na}^i and a_K^i (see table 4) and of the sum of $a_{Na}^o + a_K^o$ (47.9 mM) and subsequent rearrangement of

eq. (6) yields the following relationship between E_m and a_K^o :

$$10^{E_m/58.1} = \frac{47.9 p}{22.4 p + 52.4} + \frac{(1-p) a_K^o}{22.4 p + 52.4} \quad (7)$$

or

$$10^{E_m/58.1} = a + b a_K^o \quad (8)$$

where

$$a = \frac{47.9 p}{22.4 p + 52.4} \quad (9)$$

and

$$b = \frac{1-p}{22.4 p + 52.4} \quad (10)$$

If eq. (6) is applicable, a linear regression analysis [25] performed on the relationship between a_K^o and $10^{E_m/58.1}$ (eq. 8) should yield a statistically significant line and, if so, this will provide two coefficients a and b from which p can be calculated. For eggs cleaving outside the vitelline membrane the results are shown in figs 4 and 5. Each point represents the mean of at least three measure-

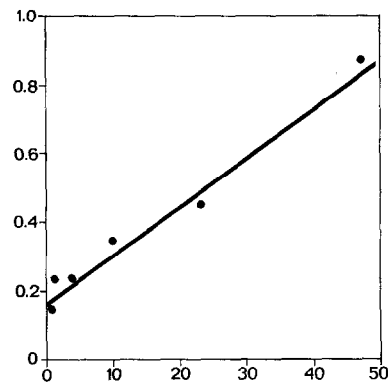


Fig. 4. Abscissa: external K^+ activity (mM); ordinate: $10^{E_m/58.1}$.

The relationship between the membrane potential (E_m) and the external K^+ activity in *Xenopus* eggs cleaving outside the vitelline membrane. Each point represents the mean of at least three measurements. The linear regression line shows that this relationship can be described by a Goldman equation (eq. 7), the relative permeability P_{Na}/P_K being 0.19. Also see text.

ments. The regression line obtained from the points of fig. 4, $10^{E_m/58.1} = 0.175 + 0.143 a_K^\circ$ is highly significant ($P < 0.001$) and is drawn in fig. 4. Calculation of p from the coefficients a and b (eqs 9, 10) gives values of 0.21 and 0.19, respectively. The latter value was considered to be the more accurate estimate since the standard error of the regression coefficient b (0.001) was relatively smaller than that of the elevation a (0.022). The line drawn in fig. 5 was calculated from eq. (6), using $p = 0.19$.

Although in eggs cleaving inside the vitelline membrane E_m was to some extent dependent on the relative activities of K^+ and Na^+ , the relationship between E_m and a_K° could not be described by the constant field equation (eq. 7). Increasing a_K° from 0.545 mM (Steinberg solution) to 4.07 mM (solution B) resulted in a decrease of E_m from -17.1 mV to -12.3 mV. At $a_K^\circ = 10.17$ mM (solution C) or greater, E_m was not significantly different from E_m measured in the uncleaved egg. In other words, no further depolarization could be observed, although this could be expected from eq. (6). Two explanations for this phenomenon can be put forward. Either we are dealing with a K^+ permeability of the new membrane which depends on a_K° , or the cleaving egg alters its morphology due to the change in ionic environment in such a way that the small fraction of new membrane normally exposed to the medium is no longer in contact with the bathing solution. We favour the second explanation, firstly because eggs cleaving outside the vitelline membrane failed to show the same phenomenon, and secondly because the blastomeres of cleaving eggs in media containing high K^+ activities did indeed tend to flatten against each other. However, knowing the dependence of E_m on the relative values of a_K° and a_{Na}° in solutions not much different from Steinberg solution, and having measured

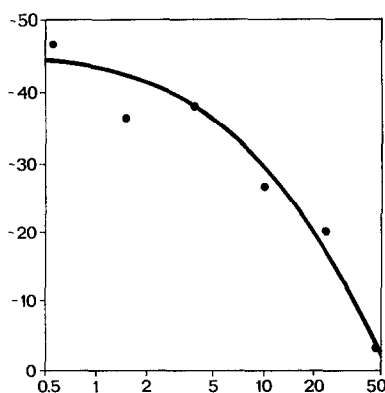


Fig. 5. Abscissa: external K^+ activity (mM); ordinate: membrane potential (mV).

Semilogarithmic plot of the membrane potential against the external K^+ activity for eggs cleaving outside the vitelline membrane. Each point represents the mean of at least three measurements. The drawn line was calculated from eq. (6) with $a_{Na}^i = 22.4$ mM, $a_K^i = 52.4$ (table 4), $p = 0.19$ (fig. 4), and $a_{Na}^\circ + a_K^\circ = 47.9$ mM. Also see text.

E_m , a_K^i and a_{Na}^i in Steinberg solution, it is possible to calculate directly from eq. (7) the relative permeability p for eggs cleaving inside the vitelline membrane in Steinberg solution. This yields a value $p = 0.73$.

DISCUSSION

To investigate the phenomena underlying the hyperpolarization of the membrane during the process of cytokinesis in *Xenopus laevis* eggs, the intracellular distribution of Na^+ , K^+ and Cl^- ions, the possible compartmentation of the intracellular H_2O content, and the relative ionic permeabilities of the cell membrane were studied during cleavage. From measurements of the total Na^+ , K^+ and Cl^- contents it was concluded that no net uptake or release of any of these ions takes place during this period. The total H_2O content also remained constant during cleavage. The influx of 3H_2O was consistent with the presence of a single intracellular water compartment.

The permeability to water was found to be very low (1.87×10^{-5} cm sec⁻¹), which is in accordance with the suggestion made by Slack et al. [17] in their hypothesis of blastocoel formation. From the values of ion content and water content the mean concentrations of Na⁺, K⁺ and Cl⁻ were calculated to be 58.6, 87.3 and 62.6 mM/l cell water, respectively. No changes could be detected during cleavage. The only comparable data are those on Na⁺ and K⁺ concentrations of Slack et al. [17] for the uncleaved egg and the 2-cell stage of *Xenopus laevis*. These authors give Na⁺ concentrations of 93.2 and 77.5 mM/l cell water and K⁺ concentrations of 88.5 and 96.2 mM/l cell water for the two stages. Particularly their Na⁺ concentrations are much higher than those found by us. Several reasons convince us that our data are more realistic. (1) Their measurements of wet and dry weight do not tally with those of egg volume; (2) in calculating the water content they assume the specific gravity of the dry material to be equal to unity; (3) they mentioned contamination problems, particularly for sodium; (4) we measured both the Na⁺ and K⁺ content and the water content by two independent methods in different eggs and obtained identical results.

E_m , R_m , and a_{Na}^i , a_K^i and a_{Cl}^i were measured simultaneously and continuously during first cleavage. This means that three ion-selective microelectrodes and two conventional 3 M KCl-filled glass microelectrodes were inserted into one cell and were left there for periods of up to 1 h without interfering with subsequent development. In this respect the amphibian egg is a rather unique cell, due to its large diameter (1.3 mm) and its excellent surface healing properties. a_K^i and a_{Cl}^i remained constant during cleavage at approx. 50 mM. a_{Na}^i was low (ca 20 mM) and showed an increase of ca 3 mM at the time E_m

hyperpolarized. All Cl⁻ ions appeared to be in the free state but 30 % of the K⁺ ions and 60 % of the Na⁺ ions were bound (see also table 4 and fig. 3a, 3b). Slack et al. [17] found somewhat smaller values for a_{Na}^i (14 mM) and measured a transient rise in a_{Na}^i during later cell cycles. They provided evidence that during later cleavages Na⁺ is released from an intracellular store to keep pace with the movement of Na⁺ towards the intercellular space. Probably this process of release starts already at first cleavage but the released fraction cannot yet be transported to the intercellular space. The activity coefficient of Na⁺ (0.33) corresponds to the value found in *Bufo bufo* oocytes by Dick & McLaughlin [28] but in the latter the total concentration of Na⁺ is lower than in *Xenopus* eggs (25.8 mM as against 58.6 mM). The same authors reported a K⁺ concentration of 113 mM for these oocytes and assumed that all K⁺ ions are in the free state. The K⁺ concentration in *Xenopus* eggs is lower (87.3 mM) while part of it seems to be bound. It would be interesting to know whether these differences are due to fertilization or to differences in intracellular ionic environment between the two species. Morrill et al. [9], using *Rana pipiens* eggs, showed a decline in the total Na⁺ and K⁺ concentrations at fertilization, which makes it difficult as yet to present a unifying picture of the ionic changes occurring at fertilization in amphibian embryos.

The cell membrane of the uncleaved *Xenopus* egg is very impermeable to ions as judged from its high specific resistance [2]. The measurements of E_m in media of different ionic composition revealed the absence of any permselectivity. In normally cleaving eggs E_m was consistent with a permeability ratio $P_{Na}/P_K = 0.73$. In eggs cleaving outside the vitelline membrane, which have a much larger area of new membrane exposed to the

medium [1], E_m behaved according to a simplified Goldman equation with a permeability ratio $P_{Na}/P_K=0.19$. These results, together with the finding that the permeability properties of the pre-existing membrane most probably remain constant during cleavage [2], make it clear that P_K of the newly formed membrane is at least five times greater than that of the cell membrane of the uncleaved egg. The absolute values of P_{Na} and P_K can only be determined by tracer-flux experiments. However, such experiments are difficult to perform under constant conditions since the duration of the cell cycle is only ca 30 min.

The only detectable difference in the intracellular ionic environment during cleavage was a small increase in a_{Na}^i , which in itself would have a negligible influence on E_m . No evidence was found for the existence of electrogenic ionic pumps. Thus we conclude, as suggested earlier [2, 7, 10], that the hyperpolarization of E_m during cleavage is due to the insertion of a small part of the newly formed membrane, which has a high K^+ permeability as compared with the pre-existing membrane.

Further investigations are necessary to see whether the hyperpolarization found in other embryonic systems (see p. 2) is also related to new membrane formation. At this time a hypothesis on the possible role of the change in E_m during the cell cycle can only be speculative. In this respect it is interesting to mention that a control of E_m over DNA synthesis has been suggested for Baby Hamster Kidney (BHK) cells [29]. In Chinese hamster V79 cells a transient rise of E_m was reported to be correlated with the onset of DNA synthesis [30]. Burger et al. [31] presented evidence that cyclic surface alterations may control DNA synthesis by influencing intracellular cAMP levels. Since in *Xenopus* eggs post-mitotic new membrane

formation leads to cyclic surface alterations [1] and cyclic changes of E_m , it seems plausible to suggest that these changes may regulate DNA synthesis. As an intermediate step E_m could alter the intracellular cAMP level by influencing the activity of membrane-bound enzymes such as adenylate-cyclase. At present we use this simple scheme as a working hypothesis for analysing the possible role of post-mitotic new membrane formation, and the hyperpolarization of E_m resulting from it, in the regulation of the cell cycle.

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