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INTRACELLULAR IONIC COMPARTMENTATION, ELECTRICAL MEMBRANE PROPERTIES, AND CELL MEMBRANE PERMEABILITY BEFORE AND DURING FIRST CLEAVAGE IN THE *AMBYSTOMA* EGG

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SUMMARY

The intracellular ionic distribution in uncleaved and cleaving *Ambystoma* eggs was investigated by analysing the influx of ^3H_2O, by determining the total content of Na^+, K^+ and Cl^- in extracts of eggs at different stages by both flame spectrophotometry and ion-selective microelectrodes, and by the continuous measurement of the Na^+, K^+ and Cl^- activities (a^i_{Na}, a^i_K and a^i_{Cl}) using intracellular ion-selective microelectrodes. The electrical membrane potential (E_m) and membrane resistance (R_m) were measured continuously in uncleaved and normally cleaving eggs as well as in eggs cleaving after removal of the vitelline membrane. The latter eggs expose their newly formed cleavage membrane to the external medium. Ionic permeability of the cell membrane before and during cleavage was analysed by a statistical comparison of the experimentally determined relationship between E_m and the ionic gradients across the cell membrane with those predicted theoretically from a constant field equation in dependence on the relative permeability, through insertion of the measured intracellular ion activities.

^3H_2O influx revealed the existence of a single intracellular water compartment (3.06 μl/egg) and a low water permeability (5.35×10^{-5} cm sec^{-1}). Na^+, K^+ and Cl^- concentrations were constant at 54.1, 72.1 and 73.1 mM respectively, while a^i_{Na}, a^i_K and a^i_{Cl} were constant at 5.8, 51.8 and 59.7 mM respectively. It was concluded that all Cl^- ions are in solution, while 12.5 % of all K^+ and 86 % of all Na^+ is bound. The uncleaved egg showed a positive E_m of ca 40 mV and a specific membrane resistance of 39 kOhm cm^2. E_m could be described by a constant field equation with a permeability ratio $P_K/P_{Na} = 0.073$. Shortly after the onset of first cleavage, E_m rapidly decreased concomitant with a rise in R_m (68.5 kOhm cm^2). This was interpreted as a drop in Na^+ permeability. During the cleavage process E_m progressively hyperpolarized and R_m decreased due to the insertion of a small fraction (3.3 %) of the newly formed intercellular membrane into the cleavage furrow. This new membrane had a low specific resistance (0.69 kOhm cm^2). Both in normally cleaving eggs and in eggs cleaving in the absence of the vitelline membrane E_m behaved according to the constant field equation, P_{Na}/P_K being 0.69 and 0.39, respectively. The differences with other amphibian eggs were discussed.

In a series of previous reports [1–4] we have presented a detailed description of the changes in electrical membrane properties, cell membrane permeability, and intracellular distribution of Na^+, K^+ and Cl^- ions during the first cleavage of eggs of the amphibian *Xenopus laevis*. The uncleaved egg showed a small electrical membrane potential—E_m—(−10 mV) and a high specific membrane resistance (74 kOhm cm^2), while any permselectivity was absent. Starting at 6–8 min after the onset of first cleavage, E_m hyperpolarized from −10 to −20 mV and the electrical membrane resistance—R_m—de-

creased from 850 to 500 kOhm. The intracellular Na^+, K^+ and Cl^- concentrations 59, 87, 63 mM/l cell H_2O, respectively, and the K^+ and Cl^- activities 51 and 53 mM, respectively, remained constant during cleavage; the intracellular Na^+ activity increased from 19 to 22 mM. Consequently the activity coefficients for Na^+, K^+ and Cl^- were ca 0.3, 0.6 and 0.8 respectively. E_m of the cleaved egg could be described by a constant field equation with a permeability ratio $P_{Na}/P_K = 0.73$. Eggs devoid of the vitelline membrane expose their newly formed (normally intercellular) membrane to the medium during cleavage. The specific resistance of this new membrane was calculated to be 1–2 kOhm cm^2. In such eggs E_m hyperpolarized till about -50 mV and could be described by a constant field equation with $P_{Na}/P_K = 0.19$. It was concluded that the changes in E_m and R_m during cytokinesis are due to the insertion of part of the newly formed membrane into the cell surface. The K^+ permeability of this new membrane was at least 5 times greater than that of the pre-existing membrane.

The hyperpolarization of E_m during cleavage due to an increase in K^+ permeability seems to be a general phenomenon in developing embryos (for references see de Laat et al. [4]). Only in *Xenopus* eggs have the intracellular activities of the major ions Na^+, K^+ and Cl^- been directly measured by means of ion-selective microelectrodes [4]. This is necessary since an interpretation of E_m based on concentrations only would be misleading, due to the large differences in activity coefficients. Evidence of a relationship between the hyperpolarization of E_m and post-mitotic new membrane formation was also found in the amphibians *Rana pipiens* [5] and *Triturus pyrrhogaster* [6]. However, these studies lack data on the intracellular ionic distribution; moreover contradictory values of E_m were reported for the uncleaved *Rana* egg: -10 to -20 mV [5, 7] and 20 to 50 mV [8, 9]. This still leaves open the question whether our findings in *Xenopus* are of general validity for cleaving amphibian eggs.

In the present study we investigated the electrical membrane properties, the intracellular ionic distribution and water compartmentation, and the relative ionic permeabilities of the uncleaved and cleaving egg of *Ambystoma mexicanum*. The properties of the new membrane formed during cleavage were studied in eggs cleaving after removal of the vitelline membrane. In general, the same techniques were used as in our earlier *Xenopus* study including the simultaneous measurement of E_m, R_m and the intracellular Na^+, K^+ and Cl^- activities by glass microelectrodes and ion-selective microelectrodes. This made possible a comparison of the results obtained for both species.

MATERIALS AND METHODS

Fertilized eggs of *Ambystoma mexicanum* were obtained from temperature stimulated couples. Jelly, capsule and, for some experiments, also the vitelline membrane were removed with fine forceps. In general, eggs of 2.1 mm diameter were used. The eggs were kept in Steinberg solution [10] unless otherwise indicated. The ionic concentrations in this medium are (in mM/l H_2O): 58.2 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.83 mM $MgSO_4 \cdot 7H_2O$. The solution contains 0.560 g Tris per liter and is set to pH = 7.4 by adding HCl. The experiments were carried out at room temperature (20–24°C).

Dry weight and water content

After removal of adhering water, eggs were weighed, dried at 120°C, and weighed again. The difference yielded the water content. For details see [4].

^3H_2O influx

To obtain data on the rate constant of the water flux, the water permeability of the cell membrane, the possible intracellular compartmentation of water, and the total water content, ^3H_2O influx was measured in uncleaved *Ambystoma* eggs. Per experiment (total of 6 experiments) at zero time, 10 decapsulated eggs not older than 2 h after oviposition were transferred with 0.5 ml Steinberg solution to a small Petri dish containing 4 ml Steinberg solution including 50 μCi ^3H_2O per ml (The Radiochemical Centre, Amersham,

Buckinghamshire, UK). At certain intervals one egg in 20 μl was removed and washed twice in 60 ml distilled water. The egg was then transferred in 20 μl water to a counting vial. The eggs were digested and radioactivity was measured as previously described [4, 11]. Samples of the medium taken at zero time and after each experiment were also measured.

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

Na^+ and K^+ contents were determined by flame spectrophotometry, following the same procedure as previously [4]. Independently Na^+, K^+ and Cl^- contents were determined with ion-selective microelectrodes. Groups of 5 eggs were washed and transferred to a vial containing 1 ml deionized-distilled water. After drying the samples, 0.5 ml deionized-distilled water was added and the samples were soaked for 2 days. Samples of 100 μl were used for the determination of Na^+, K^+ and Cl^-. The details of this method were as described previously [4], except that an Na^+ glass microelectrode was used instead of a commercially available Na^+ electrode.

Measurement of membrane potential (E_m), membrane resistance (R_m), and intracellular activities of Na^+, K^+ and Cl^- ions (a^i_{Na}, a^i_K and a^i_{Cl})

Separate glass microelectrodes (5–10 MOhm d.c.-resistance) were used to measure intracellular voltages and to pass current. The bath was grounded via a Pt–Ag–AgCl electrode. E_m was measured with respect to a separate indifferent Pt–Ag–AgCl electrode by means of a differential amplifier. A single voltage-recording microelectrode was used also in cleaving eggs, since the first intercellular membrane is not yet completed at the onset of second cleavage. The blastomeres at that stage electrically still form a single compartment. Current pulses of 3.1 sec duration, 3.0×10^{-8} A in amplitude and alternating polarity were passed each 30 sec. Na^+-glass microelectrodes and K^+ and Cl^- liquid ion-exchange microelectrodes were used to measure a^i_{Na}, a^i_K and a^i_{Cl}. Their construction and properties have previously been described in detail [4]. The responses of the ion-selective microelectrodes as well as E_m, upon which a voltage pulse proportional to R_m was superimposed every 30 sec, were recorded on pen recorders. Seven seconds before and 3 sec after the onset of each current pulse their values were digitalized and recorded on punch tape. Subsequent calculations of E_m, R_m, a^i_{Na}, a^i_K and a^i_{Cl} were made off-line on a Wang 2200 B system (Wang International Trade, Tewksbury, Mass.). For further technical details see de Laat et al. [4] and fig. 1. Simultaneous and prolonged measurements with five intracellular microelectrodes appeared to be much more difficult to perform in the *Ambystoma* egg than in that of *Xenopus* [4]. In general a measurement started with the insertion of the voltage-recording microelectrode followed by the insertion of the current-passing microelectrode. After stabilization of the recorded E_m and R_m the ion-selective microelectrodes were inserted. In most cases E_m and R_m then failed to return to their initial values. Therefore, in the majority of the experiments only E_m, R_m and one or two of the intracellular ion activities were measured simultaneously, and only the data of experiments in which E_m and R_m recovered from the insertion of more microelectrodes were used. Apparently the process of surface wound healing is less efficient in the *Ambystoma* egg than in *Xenopus*. Beside this wound healing problem the relatively slow rate of development gave rise to difficulties. At room temperature the period between oviposition and the onset of first cleavage is about 6 h while the first cleavage cycle takes about 1.5 h. Reliable continuous measurements over a period of 6.5 h are almost impossible to perform because of drift in the electrode circuits. The electrode drift was checked after each measurement and a drift smaller than 1 mV over the total period of measurement was considered acceptable.

Relative ionic permeabilities

To determine the relative ionic permeabilities of the cell membrane, the dependence of E_m on the ionic gradients across the membrane was studied at the early single-cell stage and at the onset of the second cleavage. For the latter, both eggs cleaved inside the vitelline membrane and eggs cleaved after removal of the vitelline membrane were used. E_m, R_m, a^i_{Na}, a^i_K, a^i_{Cl} were measured as described. The ionic gradients were altered by equimolar replacement of Na^+ for K^+, so that the sum of the Na^+ and K^+ activities was constant in all media. At the stages used, replacement of Cl^- by $SO_4^{=}$ had no influence on E_m when the Cl^- activity was changed from 48 mM (Steinberg solution) to 10 mM. When eggs inside the vitelline membrane were studied (the normal condition), different series of eggs were used for each test solution. Eggs devoid of their vitelline membrane were difficult to handle. Therefore, in five such eggs, a different procedure was adopted. The eggs were placed on a 5% agar bottom in a small Petri dish containing about 3 ml Steinberg solution. Upon reaching the proper developmental stage 0.5 or 1.0 ml of the medium was removed and replaced by Steinberg solution containing 0.545 mM Na^+ and 47.37 mM K^+ instead of the usual activities of 47.37 mM Na^+ and 0.545 mM K^+. When E_m reached a new stable value (2–4 min) this procedure was repeated. After 3–5 such replacements a sequence of opposite replacements was made to check for reversibility, i.e. medium was replaced stepwise by normal Steinberg solution. Samples of the removed media were analysed for Na^+ and K^+ activity using ion-selective electrodes. Thus within 20–30 min the Na^+ and K^+ activities in the medium could be varied over a wide range, keeping their sum constant.

Surface area of newly formed membrane in eggs cleaving outside the vitelline membrane

Uncleaved eggs devoid of their vitelline membrane were placed on a 5% agar bottom in a small Petri

Table 1. *Dry weight and water content per egg at the onset of first (I) and second (II) cleavage (mean ± S.E.M.)*

	I	II	I+II
Dry weight (mg)	2.20±0.02 (n=29)	2.13±0.06 (n=32)	2.16±0.03 (n=61)
Water content (mg)	3.09±0.06 (n=27)	3.23±0.05 (n=24)	3.15±0.04 (n=51)

dish containing Steinberg solution. After the onset of second cleavage they were fixed in 2.5 % glutaraldehyde, 1 % acroleïne in 0.05 M cacodylate buffer (pH 7.2–7.4). The two blastomeres were gently split apart and oriented under a dissecting microscope with the newly formed, unpigmented surface in the horizontal plane. They were drawn by using a Zeiss 45° drawing attachment. The area was measured planimetrically.

RESULTS

Dry weight and water content

Eggs of three different batches were used to determine dry weight and water content by weighing. The mean diameter of the un-

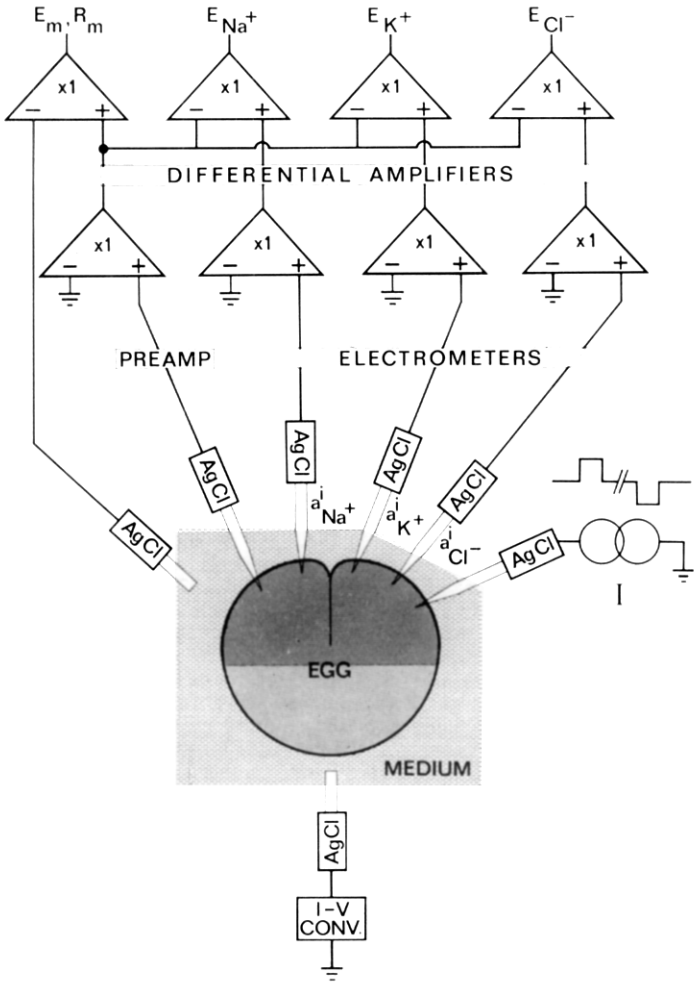

Fig. 1. Schematic diagram of the measuring configuration used for the simultaneous measurement of E_m, R_m, a^i_{Na}, a^i_K and a^i_{Cl}.

cleaved eggs in these batches was 2.1 mm. It should be noted that the egg diameter differs substantially from batch to batch (1.8–2.3 mm). The results are given in table 1. An analysis of variance [12] showed the absence of significant differences in both dry weight and water content between the onset of the first and the second cleavage ($P>0.25$ and $P=0.10$). Therefore the results of both stages have been pooled in the last column of table 1.

Tritiated water influx

Under the conditions used the uptake of tritiated water can be treated as uptake from a constant pool. Assuming steady state conditions and a single intracellular water compartment, the uptake can be described by:

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

or

$$-\ln(1-R_i(t)/R_i(\infty)) = kt \quad (2)$$

where $R_i(t)$ is the amount of intracellular radioactivity at time t (dpm), k is the rate constant (sec^{-1}), and t is time (sec) [4]. If the assumption of a single intracellular compartment is valid, a linear regression analysis performed on the results plotted according to eq. (2) should yield a significant regression line. Fig. 2 shows such a plot. The line drawn resulting from the regression analysis [12] was highly significant ($P<0.001$), k being 1.527×10^{-3} sec^{-1}. Fig. 3 gives the results plotted according to eq. (1), using the calculated k value.

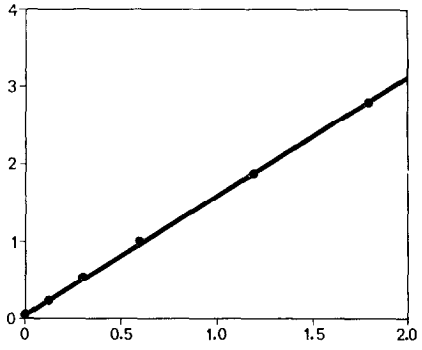

Fig. 2. Abscissa: incubation time (sec × 10^{-3}); *ordinate:* $-\ln(1-R_i(t)/R_i(\infty))$.
Linear regression line derived from the ^3H_2O influx into uncleaved *Ambystoma* eggs, showing the absence of intracellular water compartmentation.

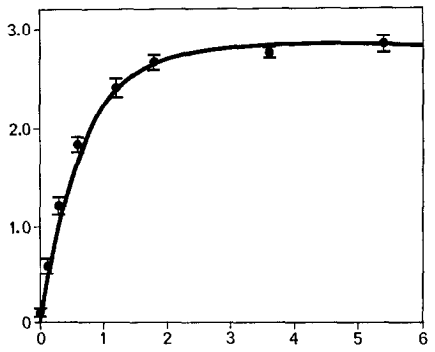

Fig. 3. Abscissa: incubation time (sec × 10^{-3}); *ordinate:* intracellular radioactivity (dpm × 10^{-5}).
^3H_2O influx into uncleaved *Ambystoma* eggs: mean values ±S.E.M. The line is derived from eq. (1), k being 1.527×10^{-3} sec^{-1}.

The amount of radioactivity at equilibrium yielded a total amount of exchangeable water of 3.06±0.036 μg H_2O per egg (mean ±S.E.M.), which was not significantly different from the total amount of intracellular water as determined by weighing ($P>0.10$). For simplicity, the uncleaved egg was considered to be a sphere (2.1 mm ∅), yielding an egg volume and surface area of 4.85×10^{-3} cm^3 and 1.38×10^{-1} cm^2, respectively. Then the water space can be calculated to be approx. 63 %. The water permeability was 5.35×10^{-5} cm sec^{-1}; the unidirectional water flux was 2.57×10^{-10} M sec^{-1} or 1.85×10^{-9} M cm^{-2} sec^{-1}. If one assumes a 70 % increase in cell surface area due to surface foldings, as was found in *Xenopus* eggs [2], the water permeability and flux would be 3.14×10^{-5} cm sec^{-1} and 1.09×10^{-9} M cm^{-2} sec^{-1}, respectively.

Table 2. *Total content (μg/egg) and concentration (mM/l cell H_2O) of Na^+, K^+ and Cl^- in the early uncleaved egg (0) and at the onset of the first (I) and the second (II) cleavage as determined with ion-selective microelectrodes (method A) and by flame spectrophotometry (method B)*

The contents are given as mean ±S.E.M., number of observations in parentheses

Ion	Method	Content (μg) 0	I	II	0+I+II[a]	Conc. (mM) 0+I+II[b]
Na^+	A	3.93±0.07 (19)	3.76±0.09 (20)	3.83±0.03 (20)	3.81±0.004 (134)	54.1
	B		3.84±0.10 (38)	3.74±0.10 (37)		
K^+	A	8.36±0.17 (10)	8.61±0.25 (10)	8.44±0.34 (10)	8.63±0.08 (103)	72.1
	B		8.71±0.12 (39)	8.68±0.12 (34)		
Cl^-	A	8.01±0.07 (10)	7.97±0.06 (10)	7.84±0.10 (10)	7.94±0.05 (30)	73.1

[a] Since no significant differences were found between the different stages or methods, the results have been pooled.
[b] Concentrations were calculated from the mean ion content and the mean water content (3.06 μl/egg).

Total contents and concentrations of Na^+, K^+ and Cl^-

Na^+, K^+ and Cl^- contents were determined with ion-selective microelectrodes in early uncleaved eggs (less than 4 h after oviposition) and at the onset of first and second cleavage. Independently, the Na^+ and K^+ contents at the latter two stages were measured by flame spectrophotometry. Table 2 summarizes the results. For each ion species the values for each stage were compared per method by an analysis of variance [12]. No significant differences were found (in all cases $P>0.25$). Therefore for each ion the results could be pooled per method after which the two methods were compared for Na^+ and K^+. Nor did this procedure yield significant differences ($P>0.25$ and $P>0.10$ for Na^+ and K^+, respectively). It was concluded that the total content of Na^+, K^+ and Cl^- per egg remains constant from the early single-cell stage till the onset of second cleavage. The ion contents were converted to concentrations (mM/l cell water), using a water content of 3.06 μl/egg as determined by the ^3H_2O influx experiments. This was permissible since no evidence of intracellular water compartmentation had been found.

Electrical membrane properties and intracellular ion activities in uncleaved and cleaving egg

In a first series of experiments, E_m, R_m, a^i_{Na}, a^i_K and a^i_{Cl} were measured continuously before and during first cleavage of eggs inside the vitelline membrane bathed in Steinberg solution. As mentioned in Materials and Methods, in the majority of the experiments, E_m, R_m and only one or two of the intracellular ion activities were measured simultaneously, because of wound healing problems. Figs 4 and 5 show a typical example of a successful experiment, and table 3 summarizes the results of all experiments. The course of E_m and R_m appeared to be very striking. The early uncleaved egg showed a high positive membrane potential of 35–45 mV which remained nearly constant till 30 min before the onset of first cleavage. A decrease of 3–4 mV preceded the onset of cleav-

age and was followed by rapid reversal of the polarity of the cell membrane starting 5–15 min later. By that time unpigmented new membrane became visible at the bottom of the cleavage furrow. At the onset of second cleavage E_m reached values of about -10 mV.

R_m remained nearly constant at ca 300 kOhm in the uncleaved egg. Just after the onset of first cleavage and concomitant with the rapid change of E_m, a transient rise of rather variable extent was observed. The maximum level of R_m during this temporary increase was 496 ± 38 kOhm ($n=9$). During further cleavage R_m decreased to ca 200 kOhm.

The intracellular ion activities were constant in the uncleaved and cleaving egg. The values obtained for a^i_{Na}, a^i_K and a^i_{Cl} in the uncleaved egg and at the onset of first and second cleavage (table 3) were compared by an analysis of variance and showed no significant differences ($P>0.25$, $P>0.25$, $P>0.10$ for a^i_{Na}, a^i_K and a^i_{Cl}, respectively). The Nernst potentials for Na^+, K^+ and Cl^- in eggs bathed in Steinberg solution were 53, -115, 5.5 mV, respectively. From the measured ion activities (table 3) and ion concentrations (table 2) the activity coefficients γ_{Na}, γ_K and γ_{Cl} were calculated to be 0.11, 0.72 and 0.82, respectively. The activity coefficient of the intracellular Cl^- ions is about equal to that of Cl^- ions in Steinberg solution (0.81); apparently all Cl^- ions were present in solution. Considering γ_{Cl} as the activity coefficient for ions in the intracellular water, we concluded that 12.5% of all K^+ ions and 86% of all Na^+ ions are not in solution and therefore must be bound to macromolecules or accumulated in cell organelles.

In a second series of experiments E_m, R_m, a^i_{Na}, a^i_K and a^i_{Cl} were measured in eggs cleaving after removal of the vitelline membrane. Due to gravity, the blastomeres fall apart during cleavage and expose their normally intercellular membrane to the surrounding

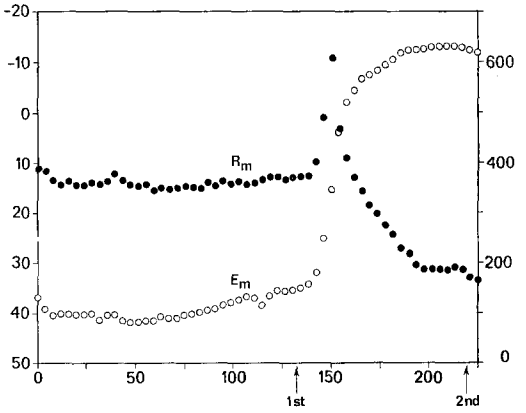

Fig. 4.

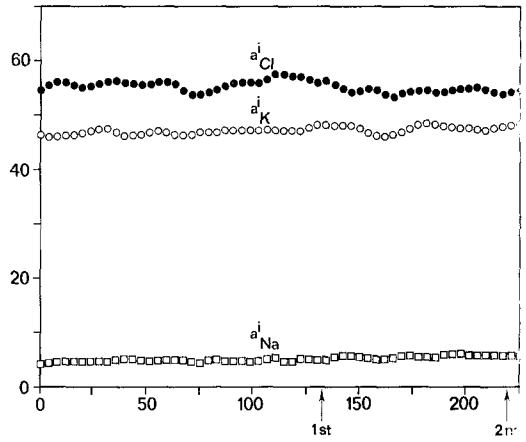

Fig. 5.

Figs 4 and 5. Abscissa: time (min); *ordinate* (*fig. 4*): (*left*) membrane potential (E_m) in mV; (*right*) membrane resistance (R_m) in kOhm; (*fig. 5*): ion activity (mM).

An example of measurements of E_m, R_m, a^i_{Na}, a^i_K and a^i_{Cl} in a normally cleaving *Ambystoma* egg. The arrows marked 1st and 2nd indicate the onset of first and second cleavage, respectively.

medium (fig. 6*b*). This provides a way of studying the properties of this newly formed membrane directly. Uncleaved and cleaving eggs devoid of the vitelline membrane showed no significant differences in intracellular ion activities as compared with eggs inside the vitelline membrane. The same was found for E_m and R_m in the uncleaved egg. However, eggs cleaving after removal of the vitelline membrane showed significant differences in

Table 3. *E_m, R_m, a^i_{Na}, a^i_K and a^i_{Cl} before and during cleavage of eggs inside the vitelline membrane. 0, I and II indicate the early uncleaved egg and the onset of the first and the second cleavage*

The results are given as mean ±S.E.M., number of observations in parentheses

	0	I	II	0+I+II[a]
E_m (mV)	37.0± 0.7 (58)	33.7± 1.5 (17)	−11.0± 1.0 (18)	0>I>II
R_m (kOhm)	277 ±14 (39)	303 ±14 (17)	187 ±17 (14)	0=I>II[b]
a^i_{Na} (mM)	6.1±0.5 (9)	5.1±0.3 (4)	5.7±0.4 (8)	5.8±0.3 (21)
a^i_K (mM)	50.8±0.9 (8)	52.0±1.1 (13)	52.3±1.0 (14)	51.8±0.6 (35)
a^i_{Cl} (mM)	54.2± 1.7 (5)	60.6± 1.6 (16)	60.6± 1.5 (17)	59.7±1.0 (38)

[a] When the analysis of variance showed no significant differences between the three stages, the results were pooled. Otherwise the significant differences (5 % level) are indicated.
[b] The pooled R_m of stage 0+I is 285±11 kOhm (n=56).

E_m and R_m as cleavage proceeded (fig. 7). Quantitatively the pattern of changes was as found in normally cleaving eggs. However, the hyperpolarization of E_m continued till −29.0±1.7 mV (n=6) at the onset of the second cleavage, while R_m concomitantly decreased to 54±5 kOhm (n=6).

A qualitative understanding of the changes in membrane properties is now possible. Based on the changes in R_m, one can distinguish three phases prior to the onset of the second cleavage. First, a period of constant R_m starting at least 1 h after oviposition and ending 5–10 min after the onset of first cleavage. During this period E_m is constant at levels close to the Nernst potential of Na^+ ions. This indicates that Na^+ permeability is probably predominant at this time. Then a short phase of 5–15 min duration follows during which R_m rapidly increases. Concomitantly E_m becomes less positive. This can be explained by an absolute and relative decrease of Na^+ permeability. E_m already starts to decrease slightly at ca 30 min before the onset of first cleavage, indicating a small decline of the relative Na^+ permeability. How-

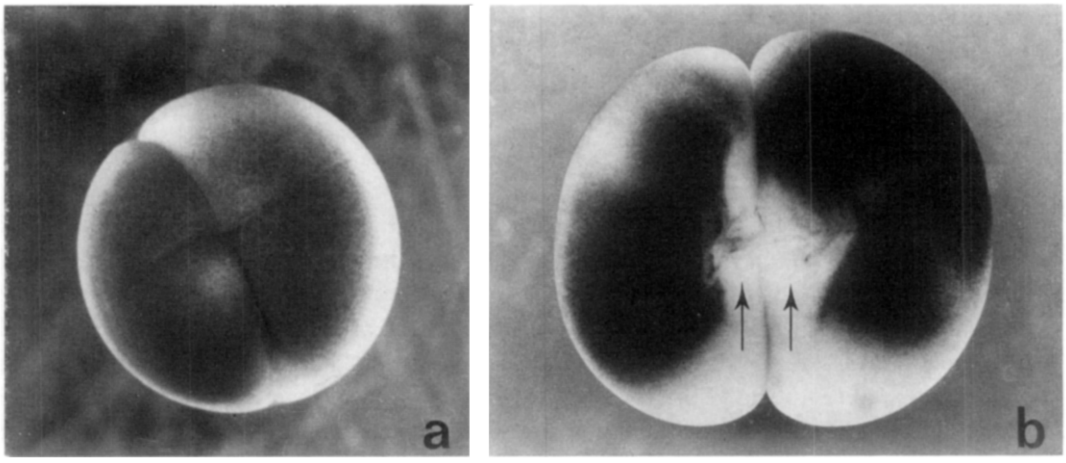

Fig. 6. Microphotographs of (*a*) a normally cleaving *Ambystoma* egg and (*b*) an *Ambystoma* egg cleaving after removal of the vitelline membrane, showing the appearance of newly formed membrane as an unpigmented area at the egg surface (*arrows*). ×20.

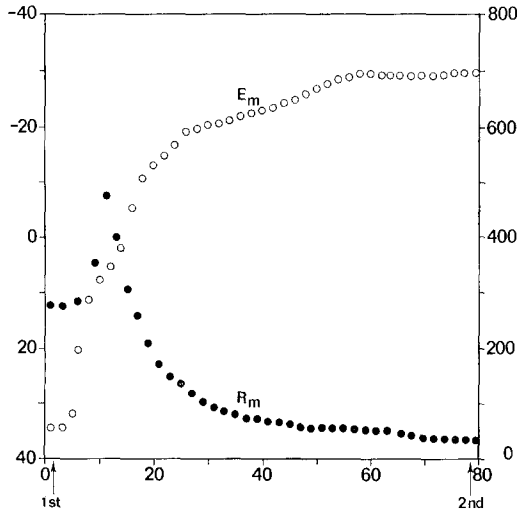

Fig. 7. Abscissa: time (min); *ordinate: (left)* E_m in mV; *(right)* R_m in kOhm.
An example of the changes in E_m and R_m in an *Ambystoma* egg cleaving after removal of the vitelline membrane. The arrows marked 1st and 2nd indicate the onset of first and second cleavage, respectively.

ever, this is not reflected in a statistically significant increase of R_m (see table 3, fig. 4). It could well be that a possible small increase of R_m is obscured by the relatively large differences in R_m among individual eggs. If this interpretation is correct, the decrease in Na^+ permeability, characteristic for the second phase would start 30 min before first cleavage and would be accelerated just after the onset of cleavage. Since removal of the vitelline membrane has no influence on the measured properties during the first two phases and since these phases cover the time before the onset of new membrane formation, we conclude that during these periods changes of the pre-existing membrane are involved. During the third phase R_m decreases while E_m hyperpolarizes. Since E_m shifts towards the Nernst potential of K^+ ions most probably an absolute and relative increase in K^+ permeability is involved.

The extent to which R_m and E_m changed is dependent on the surface area of newly formed membrane exposed to the medium (compare eggs cleaved in the presence and absence of the vitelline membrane). In analogy to our findings in *Xenopus* eggs [3] we conclude that during this period new membrane with a relatively high K^+ permeability is inserted into the bottom of the cleavage furrow.

Specific resistance of cell membrane

Considering the uncleaved egg for simplicity as a sphere of 0.21 cm diameter, the geometrical surface area is 1.38×10^{-1} cm^2. The mean value of R_m in uncleaved eggs is 285 kOhm. Thus the mean specific resistance of the cell membrane was 39 kOhm cm^2. If one assumes an increase in surface area due to surface foldings as in *Xenopus* eggs [2] the specific resistance would be 1.7×39 kOhm cm^2, i.e. 66 kOhm cm^2. Just after the onset of first cleavage R_m increases to 496 kOhm, yielding a specific resistance of the pre-existing membrane of 68.5 kOhm cm^2 or ($\times 1.7$) 116 kOhm cm^2. These latter values might well be underestimated, since the insertion of new membrane starts before R_m could reach a new constant level. The subsequent decrease of R_m is correlated to the area of new membrane exposed to the medium. This new membrane will form a low-resistance pathway parallel to the pre-existing membrane. If we put R_m of the pre-existing membrane at 496 kOhm calculation shows that the parallel resistance formed by the new membrane exposed to the medium at the onset of second cleavage has mean values of 300 and 60 kOhm for eggs cleaved in the presence and absence of the vitelline membrane, respectively. In eight eggs cleaving after removal of the vitelline membrane the geometrical surface area of new membrane exposed at the onset of the second cleavage was measured to be $1.15 \times 10^{-2} \pm 0.05 \times 10^{-2}$ cm^2 (mean $\pm$S.E.M.). Consequently the mean

specific resistance of this new membrane is 0.69 kOhm cm², which is about 100 times smaller than that of the pre-existing membrane. Dividing this specific resistance by the resistance of the new membrane exposed during normal first cleavage (300 kOhm) yields a surface area of 2.3×10^{-3} cm² for the normally exposed new membrane. If we consider the total area of intercellular surface to be a circular plane of 2.1 mm diameter, its surface area is 3.46×10^{-2} cm². In eggs cleaving inside the vitelline membrane the total new membrane area will be twice the intercellular surface (6.92×10^{-2} cm²). Thus, at the onset of second cleavage in normally cleaving eggs, 3.3 % of the newly formed membrane is in contact with the medium, which is 1.7 % of the total geometrical surface area of the uncleaved egg.

Theoretical analysis of relative ionic permeabilities of the cell membrane

The relative ionic permeabilities were studied in the early uncleaved egg and at the onset of second cleavage in normally cleaving eggs as well as in eggs cleaved after removal of the vitelline membrane. They were analysed quantitatively by investigating the dependence of E_m on the Na^+ and K^+ gradients across the cell membrane. E_m, R_m, and the intracellular ion activities were measured as before. The ionic composition of the medium was altered by equimolar replacement of Na^+ by K^+, keeping the sum of their activities equal to that of Steinberg solution (see Materials and Methods).

According to the constant field equation [13, 14] the dependence of E_m on the ionic gradients across the cell membrane can be described by the following equation:

$$E_m = \frac{RT}{F} \ln \frac{P_{Na} \cdot a^0_{Na} + P_K \cdot a^0_K}{P_{Na} \cdot a^i_{Na} + P_K \cdot a^i_K} \tag{3}$$

where P_{Na} and P_K represent the permeabilities for Na^+ and K^+, and a^0_{Na} and a^0_K the external activities of Na^+ and K^+. The other symbols have their usual meanings. The simultaneous measurement of E_m, a^i_{Na} and a^i_K leaves the relative permeability as the only unknown parameter.

Insertion of the measured mean values for a^i_{Na} and a^i_K (table 3) and of the sum of $a^0_{Na} + a^0_K$ (47.9 mM) yields the following relationships between E_m and a^0_{Na} and a^0_K, respectively (22°C; E_m expressed in mV):

$$E_m = 58.1 \log \frac{47.9\, P_K/P_{Na} + (1 - P_K/P_{Na})\, a^0_{Na}}{51.8\, P_K/P_{Na} + 5.8} \tag{4}$$

$$E_m = 58.1 \log \frac{47.9\, P_{Na}/P_K + (1 - P_{Na}/P_K)\, a^0_K}{5.8\, P_{Na}/P_K + 51.8} \tag{5}$$

Rearrangement of eqs 4 and 5 gives a linear relationship between $10^{E_m/58.1}$ and a^0_{Na} and a^0_K, respectively:

$$10^{E_m/58.1} = \frac{47.9\, P_K/P_{Na}}{51.8\, P_K/P_{Na} + 5.8} + \frac{(1 - P_K/P_{Na}) a^0_{Na}}{51.8\, P_K/P_{Na} + 5.8} \tag{6}$$

$$10^{E_m/58.1} = \frac{47.9\, P_{Na}/P_K}{5.8\, P_{Na}/P_K + 51.8} + \frac{(1 - P_{Na}/P_K)\, a^0_K}{5.8\, P_{Na}/P_K + 51.8} \tag{7}$$

Fig. 8*a–d* gives the curves calculated from eqs (4) to (7) for different relative permeabilities. An important feature of these equations is that for any value of P_K/P_{Na} (or P_{Na}/P_K) the a^0_{Na} value

$$a^0_{Na} = \frac{a^i_{Na}(a^0_{Na} + a^0_K)}{a^i_{Na} + a^i_K} = 4.8 \text{ mM} \tag{8}$$

or the corresponding a^0_K value

$$a^0_K = \frac{a^i_K(a^0_{Na} + a^0_K)}{a^i_{Na} + a^i_K} = 43.1 \text{ mM} \tag{9}$$

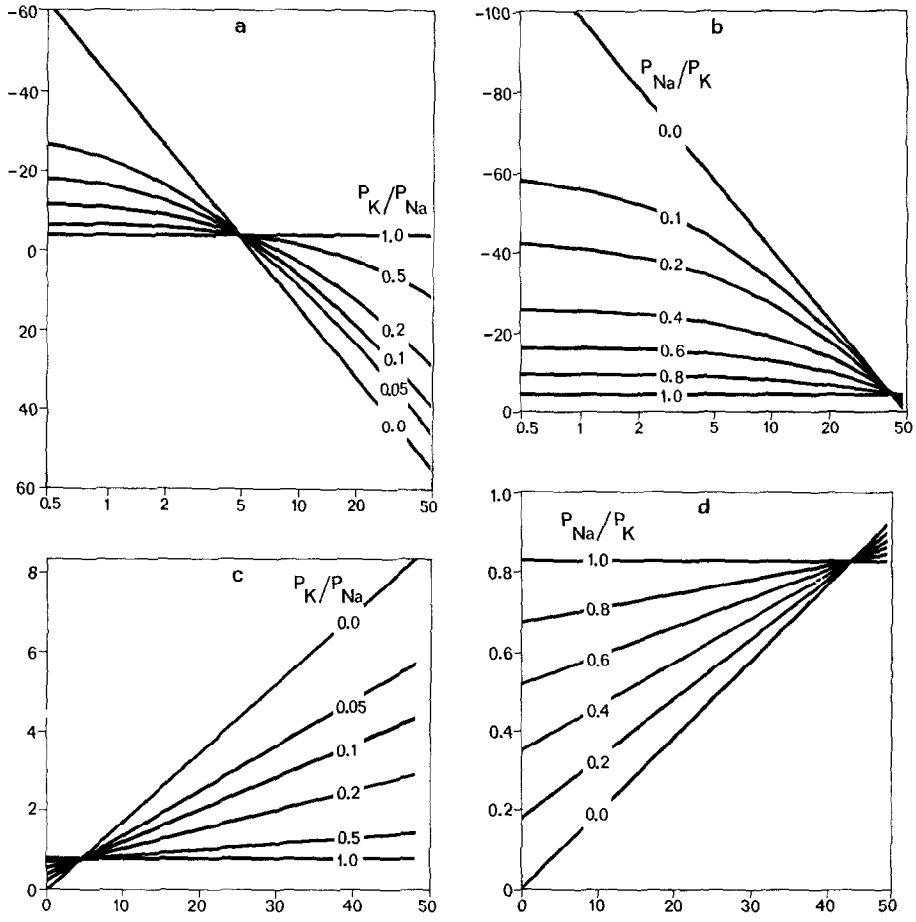

Fig. 8. (*a*, *b*) *Abscissa:* (*a*) a^0_{Na}; (*b*) a^0_K in mM on a log scale; *ordinate:* E_m in mV.

E_m as a function of (*a*) a^0_{Na}; (*b*) a^0_K in dependence of (*a*) P_K/P_{Na}; (*b*) P_{Na}/P_K. The curves were derived from the constant field equation (*a*) eq. (4); (*b*) eq. (5). $(a^0_{Na}+a^0_K)$ was constant at 47.9 mM, a^i_{Na} and a^i_K were 5.8 and 51.8 mM, as measured with ion-selective microelectrodes.

(*c*, *d*) *Abscissa:* (*a*) a^0_{Na}; (*b*) a^0_K in mM; *ordinate:* $10^{E_m/58.1}$. The linear relationship between $10^{E_m/58.1}$ and (*c*) a^0_{Na}; (*d*) a^0_K, in dependence of (*c*) P_K/P_{Na}; (*d*) P_{Na}/P_K. The lines were derived from (*c*) eq. (6); (*d*) eq. (7). $(a^0_{Na}+a^0_K)$ was constant at 47.9 mM, a^i_{Na} and a^i_K were 5.8 and 51.8 mM, as measured with ion-selective microelectrodes.

yields the same value for E_m and $10^{E_m/58.1}$, respectively:

$$E_m = 58.1 \log \frac{a^0_{Na}+a^0_K}{a^i_{Na}+a^i_K} = -4.6 \text{ mV} \qquad (10)$$

$$10^{E_m/58.1} = \frac{a^0_{Na}+a^0_{Na}}{a^i_K+a^i_K} = 0.83 \qquad (11)$$

If application of the constant field equation is valid, then a linear regression analysis [12] performed on the experimental data plotted according to eqs (6) or (7) (fig. 8*c*, *d*) should yield a statistically significant line. Moreover, the value of $10^{E_m/58.1}$ predicted from this regression line at $a^0_{Na}=4.8$ mM (or $a^0_K=43.1$ mM) should not differ significantly from 0.83 (eq. (11)). If both conditions are fulfilled, the relative permeability can be calculated from the regression coefficient; otherwise the hypothesis that E_m can be described

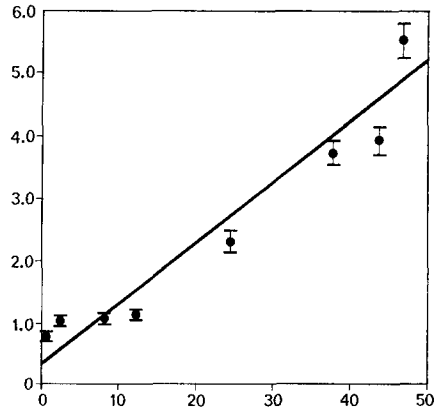

Fig. 9. Abscissa: a^0_{Na} in mM; *ordinate:* $10^{E_m/58.1}$.
Linear regression line obtained for the dependence of $10^{E_m/58.1}$ on a^0_{Na} ($a^0_{Na}+a^0_K=47.9$ mM) in uncleaved *Ambystoma* eggs. Mean ±S.E.M. are given. Also see text.

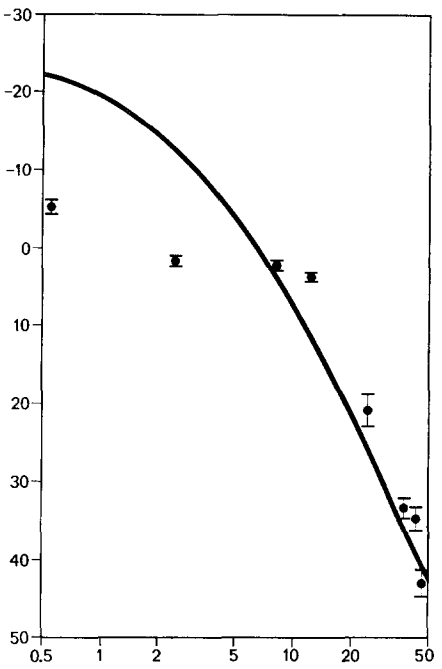

Fig. 10. Abscissa: a^0_{Na} in mM on a log scale; *ordinate:* E_m in mV.
E_m as a function of a^0_{Na} ($a^0_{Na}+a^0_K=47.9$ mM) in uncleaved *Ambystoma* eggs. Mean ±S.E.M. are given.
The curve drawn was calculated from a constant field equation (eq. (4)), P_K/P_{Na} being 0.073 as determined from the linear regression line of fig. 9. See also text.

by a simple constant field equation should be rejected. The outcome of this analysis is given below for various types of eggs. For details of the statistics see [12].

Ionic permeability of uncleaved egg

Fig. 9 shows the results of the measurements in early uncleaved eggs plotted according to eq. 6. The line drawn is the calculated regression line $10^{E_m/58.1}=0.366+9.67\times10^{-2}\,a^0_{Na}$. The sample standard deviation of the regression coefficient (s_b) was 4.9×10^{-3}. The analysis of variance (table 4) showed the regression to be significant ($P<0.005$). At $a^0_{Na}=4.8$ mM (eq. (8)) the value of $10^{E_m/58.1}$ predicted from the regression line was 0.83 with a 95% confidence interval equal to 0.57–1.09. The theoretically calculated value (eq. (11)) is exactly equal to the value predicted from the regression line. Therefore it is valid to describe E_m in the uncleaved egg by the constant field equation (eq. (4)). The relative permeability P_K/P_{Na} calculated from the regression coefficient was 0.073, thus the Na^+ permeability was about 14 times greater than the K^+ permeability. Fig. 10 shows E_m as a function of a^0_{Na}. The curve drawn was calculated from eq. (4), using $P_K/P_{Na}=0.073$. It is seen that the measured E_m deviates progressively from the curve as a^0_{Na} becomes smaller than a^i_{Na}, i.e. when eq. (4) would predict a reversal of polarity of the mem-

Table 4. *Analysis of variance for the linear regression analysis of the relationship between* $10^{E_m/58.1}$ *and* a^0_{Na} *in the early uncleaved egg*

Source of variation	Degrees of freedom	Sum of squares	Mean square
Regression	1	174.465	174.465
Deviations from regression	58	26.427	0.465
Total	59	200.892	

brane. Apparently, under these extreme conditions, the cell membrane changes its permeability properties.

Ionic permeability of normally cleaving egg

It was suggested above that in cleaving eggs the K^+ permeability becomes greater than the Na^+ permeability. We therefore analysed the relationship between E_m and a_K^0 for such eggs.

Fig. 11 gives the results of the measurements in normally cleaving eggs at the onset of second cleavage. The line drawn represents the calculated regression line $10^{E_m/58.1} = 0.596 + 5.69 \times 10^{-3}\ a_K^0$, $s_b = 8.35 \times 10^{-4}$. The analysis of variance (table 5) showed the regression to be significant ($P < 0.005$). At $a_K^0 = 43.1$ (eq. (9)) the regression line gave $10^{E_m/58.1} = 0.84$ with a 95 % confidence interval of 0.79–0.89. The theoretically calculated value (0.83) clearly falls between these limits. Thus, the constant field equation (eq. (5)) is applicable under these conditions. From the regression coefficient P_{Na}/P_K was calculated to be 0.69, so that the K^+ permeability was about 1.4 times greater than the Na^+ permeability. Fig. 12 gives E_m as a function of a_K^0. The curve was calculated from eq. (5), P_{Na}/P_K being 0.69. Clearly, this curve gives an excellent fit to the experimental data over the whole range of external ion concentrations investigated.

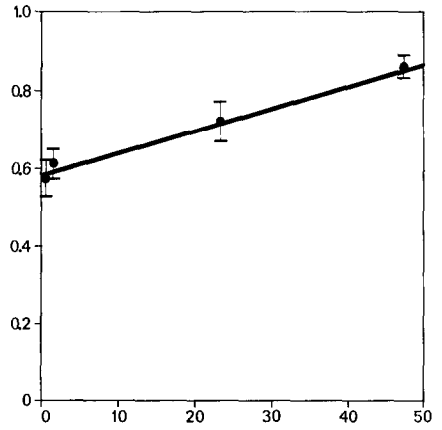

Fig. 11. Abscissa: a_K^0 in mM; *ordinate:* $10^{E_m/58.1}$.
Linear regression line obtained for the dependence of $10^{E_m/58.1}$ on a_K^0 ($a_{Na}^0 + a_K^0 = 47.9$ mM) at the onset of second cleavage in normally cleaving *Ambystoma* eggs. Mean ±S.E.M. are given. See also text.

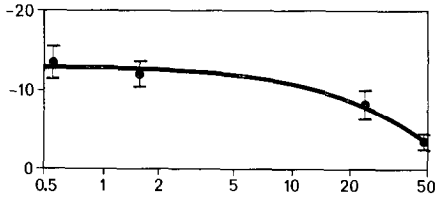

Fig. 12. Abscissa: a_K^0 in mM on a log scale; *ordinate:* E_m in mV.
E_m as a function of a_K^0 ($a_{Na}^0 + a_K^0 = 47.9$ mM) at the onset of second cleavage in normally cleaving *Ambystoma* eggs. Mean ±S.E.M. are given. The curve drawn was calculated from a constant field equation (eq. (5)), P_{Na}/P_K being 0.69 as determined from the linear regression line of fig. 11. See also text.

Ionic permeability of eggs cleaving after removal of vitelline membrane

As shown before, eggs cleaving in the absence of the vitelline membrane have electrical membrane properties which differ from normally cleaving eggs due to the exposure of the normally intercellular membrane to the medium. They provide a simple means of investigating the permeability properties of this newly formed membrane. Fig. 13 gives the dependence of $10^{E_m/58.1}$ on a_K^0 in such eggs at the onset of second cleavage. The line drawn is the resulting linear re-

Table 5. *Analysis of variance for the linear regression analysis of the relationship between* $10^{E_m/58.1}$ *and* a_K^0 *at the onset of second cleavage*

Source of variation	Degrees of freedom	Sum of squares	Mean square
Regression	1	0.242	0.242
Deviations from regression	16	8.36×10^{-2}	5.22×10^{-3}
Total	17	0.326	

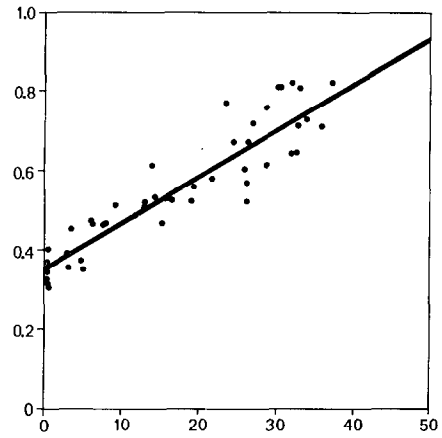

Fig. 13. Abscissa: a_K^0 in mM; *ordinate:* $10^{E_m/58.1}$.
Linear regression line obtained for the dependence of $10^{E_m/58.1}$ on a_K^0 ($a_{Na}^0 + a_K^0 = 47.9$ mM) at the onset of second cleavage in *Ambystoma* eggs devoid of the vitelline membrane. See also text.

gression line $10^{E_m/58.1} = 0.354 + 1.16 \times 10^{-2}\ a_K^0$ ($s_b = 8.91 \times 10^{-4}$). The analysis of variance given in table 6 showed the regression to be significant ($P < 0.005$). At $a_K^0 = 43.1$ (eq. (9)) the regression line gave $10^{E_m/58.1} = 0.85$, the 95% confidence interval being 0.80–0.90. This interval includes the theoretically calculated value (0.83). Therefore, also under these conditions the constant field equation (eq. (5)) is applicable. From the regression coefficient, P_{Na}/P_K was calculated to be 0.39, so that the K^+ permeability was about 2.5 times greater than the Na^+ permeability. Fig. 14 gives E_m as a function of a_K^0, the curve drawn being calculated from eq. (5) with P_{Na}/P_K equal to 0.39. As in normally cleaving eggs eq. (5) gives a good description of the relationship between E_m and the ionic gradients across the cell membrane for the whole range of external ion concentrations investigated.

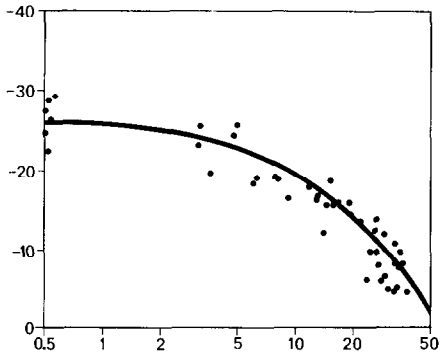

Fig. 14. Abscissa: a_K^0 in mM on a log scale; *ordinate:* E_m in mV.
E_m as a function of a_K^0 ($a_{Na}^0 + a_K^0 = 47.9$ mM) at the onset of second cleavage in *Ambystoma* eggs devoid of the vitelline membrane. The curve drawn was calculated from a constant field equation (eq. (5)), P_{Na}/P_K being 0.39 as determined from the linear regression line of fig. 13. See also text.

Table 6. *Analysis of variance for the linear regression analysis of the relationship between* $10^{E_m/58.1}$ *and* a_K^0 *at the onset of second cleavage in eggs cleaving after removal of the vitelline membrane*

Source of variation	Degrees of freedom	Sum of squares	Mean square
Regression	1	0.977	0.977
Deviations from regression	47	0.269	5.72×10^{-3}
Total	48	1.246	

DISCUSSION

From shortly after fertilization till the onset of second cleavage the intracellular ionic environment of the *Ambystoma* egg remained constant. ^3H_2O influx experiments showed the presence of a single intracellular H_2O compartment equal to the total H_2O content as determined by weighing. The water permeability is low (5.35×10^{-5} cm sec^{-1}) and in the same order of magnitude as in *Xenopus* eggs [4]. No net uptake or release of Na^+, K^+ and Cl^- can be detected; their mean concentrations are 54.1, 72.1 and 73.1 mM/l cell H_2O, respectively. For *Xenopus* eggs [4] the corresponding concentrations are 58.6, 87.3 and 62.6 mM/l cell H_2O, so that between these amphibian eggs only minor dif-

ferences exist with regard to ion concentrations.

The mean ion activities as measured by intracellular ion-selective microelectrodes are 5.8, 51.8 and 59.7 mM for Na^+, K^+ and Cl^-, respectively, they remain constant in the uncleaved and cleaving egg. The activity coefficients γ_{Na}, γ_K and γ_{Cl} are 0.11, 0.72 and 0.82, respectively. While γ_K and γ_{Cl} are about the same as those found earlier [4] in the *Xenopus* egg (0.69, 0.85, respectively), γ_{Na} is much smaller (in *Xenopus* eggs: 0.33). It is possible that the large fraction of Na^+ (86 %) which is apparently sequestered in cell organelles or bound to macromolecules provides a store of Na^+ to be released during subsequent development. According to Slack et al. [15], released Na^+ is transported to the intracellular space during the formation of the blastocoel. The very small value of γ_{Na} illustrates the necessity of determining ion activities rather than ion concentrations in studies such as the present one. The use of ion concentrations would evidently lead to erroneous conclusions.

In the *Ambystoma* egg, E_m is dependent on the external Na^+ and K^+ activities at the stages investigated. Only extreme alterations of the external activities of other ions have a significant influence on E_m. Using simultaneous measurements of E_m, R_m, a^i_{Na} and a^i_K we were in the favourable position of being able to measure directly the parameters necessary for an understanding of the ionic permeability properties of the cell membrane. This provided a way of testing statistically whether E_m behaved according to the constant field equation (eq. (3)). On the underlying assumptions of independent ion movement and a constant electric field across the cell membrane, and keeping the sum of a^0_{Na} and a^0_K constant and inserting the measured intracellular activities a^i_{Na} and a^i_K, plots were made of the theoretical dependence of E_m on a^0_{Na} and a^0_K as a function of the permeability ratio (fig. 8*a–d*).

A usually overlooked [16] but important feature of such plots is that E_m does not asymptotically approach the Nernst potential ($P_K/P_{Na}=0$ or $P_{Na}/P_K=0$) of the most permeable ion when the activity of this ion increases. On the contrary, all curves of E_m against a^0_{Na} or a^0_K derived for different permeability ratios cross at a particular external ion activity, meaning that here E_m is independent of the relative permeability (fig. 8*a–d*). Therefore it was statistically tested whether the experimentally determined relationship between E_m and a^0_{Na} or a^0_K coincides with one of the family of curves predicted from the constant field equation. Subsequently the permeability ratio could be calculated.

The uncleaved *Ambystoma* egg shows a large positive membrane potential (ca 40 mV), due to the predominance of Na^+ permeability ($P_K/P_{Na}=0.073$). Judged from the high specific membrane resistance (39 kOhm cm^2, uncorrected for surface foldings) the absolute value of the Na^+ permeability is likely to be low. The cell membrane of the uncleaved *Ambystoma* egg appears to be exceptional. Only for uncleaved *Rana* eggs were similar values of E_m reported [8, 9]. However, these results were contested by various authors [5, 7] who found E_m to range from -10 to -20 mV in these eggs. All other fertilized, uncleaved eggs investigated, including those of the amphibians *Triturus* [16, 17] and *Xenopus* [1, 3, 4, 10, 18], show a small negative E_m, probably due to a lack of permselectivity. For further references see [4].

A gradual decrease in E_m of 3 to 4 mV precedes the onset of first cleavage. Shortly hereafter, E_m rapidly reverses polarity concomitant with a sharp increase in the specific membrane resistance (68.5 kOhm cm^2, uncorrected for surface foldings) (see also figs 4, 7). We attributed these events to a

decrease in Na^+ permeability, which implies a loss of perm-selectivity of the cell membrane. During this brief period the membrane attains permeability properties similar to those observed in other fertilized amphibian eggs. At present we have no explanation for the retarded appearance in *Ambystoma* eggs of what seems to be the more common permeability properties of fertilized eggs. Differences in the fertilization mechanism may be involved. In various echinoderms [19, 20, 21] as well as in the anurans *Rana pipiens* [9] and *Bufo vulgaris* [22] a rapid transient change of E_m, the so-called 'activation potential', is observed within seconds after fertilization. In *Bufo* an increase in Cl^- permeability caused a hyperpolarization of E_m, while in the other eggs E_m changed to (more) positive values due to an increase in Na^+ permeability. All these eggs have monospermic fertilization. Urodeles such as *Ambystoma* undergo polyspermic fertilization, which does not call for rapid changes in membrane properties to prevent the penetration of more than one sperm. It is conceivable that here the changes in membrane permeability occur on a retarded time scale. It should be noted that in the urodele *Triturus pyrrhogaster*, which also has polyspermic fertilization, negative membrane potentials were reported for the uncleaved egg [6, 17]. However, these authors presented no data on E_m in early uncleaved fertilized eggs.

During the process of cleavage, E_m hyperpolarizes and R_m decreases progressively, starting with the insertion of new membrane material in the bottom of the cleavage furrow, which becomes visible as unpigmented surface. The extent of these changes is correlated with the surface area of newly formed membrane exposed to the medium. This new membrane has a low specific resistance (0.69 kOhm cm^2) and its K^+ permeability is at least 2.5 times greater than the Na^+ permeability. It was concluded that during normal cleavage, a small fraction (3.3 %) of the newly formed intercellular membrane is in contact with the medium, which by its relatively large K^+ permeability causes a hyperpolarization of E_m. An increased K^+ permeability was reported previously for a large variety of cleaving eggs (see [4]), and a correlation with new membrane formation was found or suggested for other cleaving amphibian eggs [5, 6].

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