

ON THE MECHANICAL PROPERTIES OF THE VITELLINE MEMBRANE OF THE FROG EGG

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INTRODUCTION

The mechanical properties of the cortex of the echinoid egg have for a long time been the subject of investigation. In the early studies the sessile drop method was employed (Vlès, 1926; Harvey & Fankhauser, 1933) and in the interpretation of the results obtained the assumption was made that, corresponding to an interfacial tension, the membrane tension is constant and independent of the state of strain. Cole (1932) devised a method in which the egg was compressed with a known force between two parallel plates and the tension was calculated from the degree of compression. He found evidence for an elastic tension in the cortex of sea-urchin eggs. In the undeformed state the internal pressure is 40 dyn/cm². This early work has been reviewed by Harvey & Danielli (1938).

The experiments of Cole were repeated by Hiramoto (1963), who reported the prevalence of an elastic tension, and by Yoneda (1964, 1972), who came to the opposite result. Mitchison & Swann (1954*a, b*) criticized Cole's method for neglecting bending resistance. Introducing a new device, the cell elastimeter, they found that the cortex is thick enough to resist bending and that there is probably no internal pressure in the normal sea-urchin egg. Hence, for mechanical purposes the unfertilized egg can be compared to a hollow sphere filled with fluid and surrounded by a solid elastic wall (Mitchison & Swann, 1954*b*).

In the present study Cole's method has been adopted for measurements on the body-cavity egg of *Rana temporaria*. In this object the mechanical strength of the 'cortex' is primarily provided by the vitelline membrane, and it is thus the properties of the latter which have been determined. In this structure the ratio R/h between egg radius and thickness of the cortex is such that bending resistance can be neglected. The circumstance that the vitelline membrane plays an important, though not indispensable, role for the early development in many amphibian species may serve to justify the present study.

The pressure changes induced by incubation in hypotonic Ringer solutions of different concentrations have been studied as well as the effect of temperature on this parameter.

MATERIAL AND METHODS

The biological material was body-cavity eggs from *Rana temporaria*. Mature frogs were purchased from commercial dealers in Western Germany and the frogs were kept under moist conditions at 5 °C until used. The eggs were surgically removed from the body cavity after artificial ovulation induced by the method described by Rugh (1952).

According to the method of Cole (1932) the internal pressure of an egg may be estimated by compressing the egg slightly between two parallel plates with a known force F . From the contact area A between the plates and the egg the pressure p can be calculated from

$$p = F/A. \quad (1)$$

This equation is valid only if the bending of the membrane can be neglected. It is shown by Hansson Mild & Kalnins (1974) from theoretical considerations that this assumption holds for amphibian eggs.

The applied force was measured by the automatic diver balance (Bergfors, Hansson Mild & Løvtrup, 1970; Løvtrup, 1973). The basic principle of this method is as follows. A plastic diver with density less than the surrounding medium is prevented from rising to the surface by an electromagnetic force acting on a small piece of iron placed in the bottom of the diver. The current through the coil to the electromagnet is proportional to the force holding the diver in place. If an object is placed on the diver less force will be needed to hold the diver, and if the diver has been calibrated with known weights, measurements of the current through the coil will give the reduced weight (i.e. weight minus buoyancy) of the submerged object placed on the diver.

The diver employed in this study is shown in Fig. 1. The diver body is made of polypropylene, except for the top where a piece of Plexiglass is inserted to accommodate the egg. The reason for this construction is that the contact-area between the egg and the diver must be very smooth, a requirement which is satisfied by Plexiglass but not by polypropylene.

To ensure that the diver remains vertical when pressure is applied from above it is necessary that the centre of gravity is situated as far down as possible. For this reason the diver has been designed with a broad collar at the upper end enclosing an air pocket, the size of which influences the loading capacity of the diver.

At the bottom of the diver there are inserted, at equal distances from the centre of the diver, two pieces of soft iron with a length of 6–8 mm and a diameter of 0.2–0.4 mm. It is desirable to have a high length-diameter ratio of the iron pieces because of the demagnetization effect (Bozorth, 1951; Bergfors *et al.* 1970). By using two pieces of iron at equal distances the diver is prevented from rotating during the course of the experiment. To lower further the centre of gravity a piece of platinum is inserted in the middle at the lower end of the diver.

The holes, in which the iron and platinum pieces are situated, are filled with Araldite; this is done under vacuum in order to prevent air from being left around the metal pieces. The device used for this purpose is shown in Fig. 2. When the glue has hardened the lower end of the diver is polished against a blasted glass plate in order to get the surface as smooth and plane as possible.

The diver is calibrated by loading with known weights of platinum. The calibration curve depends, among other things, on the density of the liquid in the cuvette and on

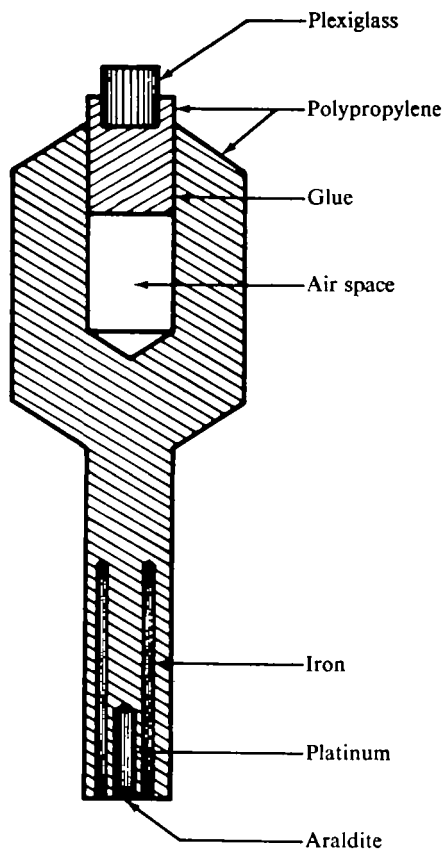


Fig. 1

Fig. 1. Section of a diver. The measurements are: length 25 mm, smallest diameter 3 mm, largest diameter 8 mm.

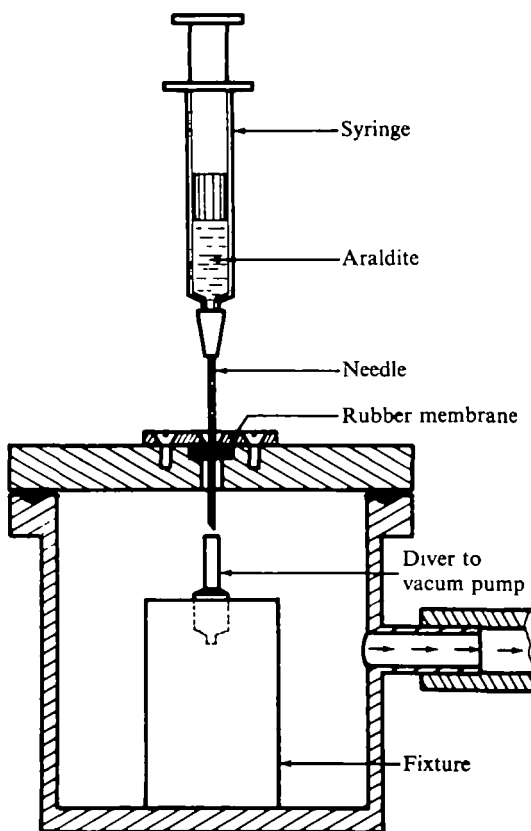


Fig. 2

Fig. 2. Apparatus employed for filling the space around the iron and platinum pieces with Araldite.

the distance between the diver and the electromagnet. It is therefore necessary to start each experimental series with a calibration of the diver. Examples of calibration curves are shown in Fig. 3 for two different divers. When the load approaches the maximum capacity of the diver, the curves deviate from a straight line for reasons outlined earlier (Bergfors *et al.* 1970).

After deposition on the diver, the egg is compressed by a Plexiglass rod which is lowered to touch the egg and which, by means of a micromanipulator, can be moved further down to get the desired degree of compression (Fig. 4). The current in the magnet coil is now a measure of the force applied by the rod.

As has been pointed out by various authors, among others Mitchison & Swann (1954*a, b*) and Yoneda (1964), the critical point in this method is to measure the contact area between the rod and the egg. Several attempts were made to overcome this difficulty. Thus, we tried to measure the diameter of the contact area from photographs taken through a microscope, but good accuracy cannot be obtained in this way because the edge of the diver is closer to the camera than is the edge of the contact zone. We also

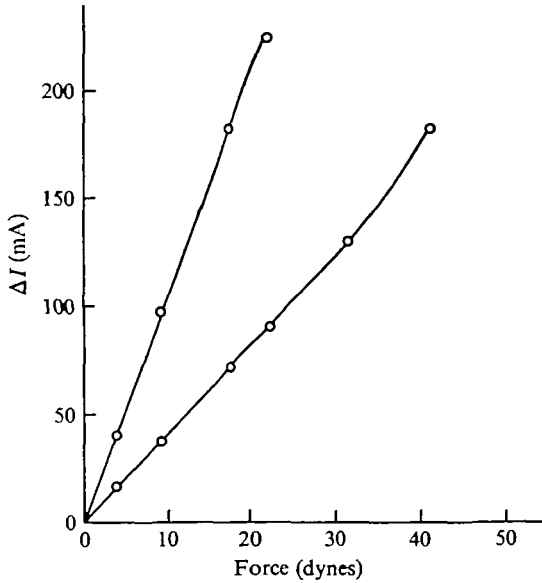


Fig. 3. Two typical calibration curves.

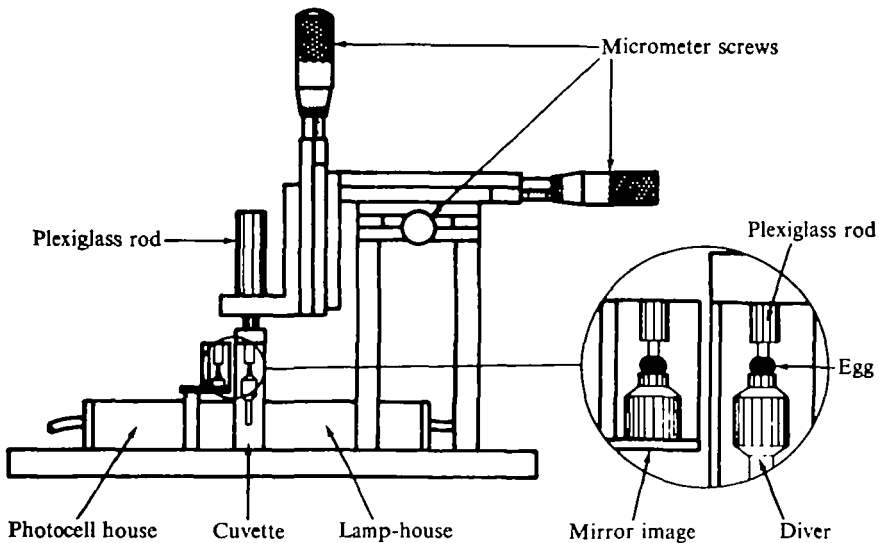


Fig. 4. Micromanipulator arrangement with mirror used for compression of the eggs. The enlarged part shows an egg compressed between rod and diver.

tried to measure the diameter through a transparent rod, but were unable to distinguish the contact zone from the rest of the egg.

We solved the problem by designing the rod, by which the egg is compressed, in a special way (Fig. 5). The part of the cylindrical rod which is in contact with the egg is carefully polished to get a smooth surface. To obtain different degrees of compression a set of rods with different diameters of the tip were employed.

The rod is placed in a three-dimensional micromanipulator so that it is possible to

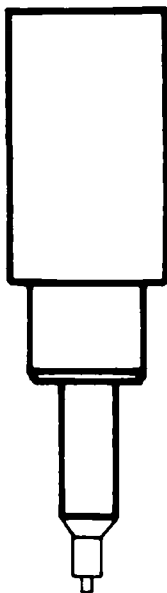


Fig. 5

Fig. 5. The design of the Plexiglass rod used for compression of the eggs. The largest diameter is 1.5 mm.

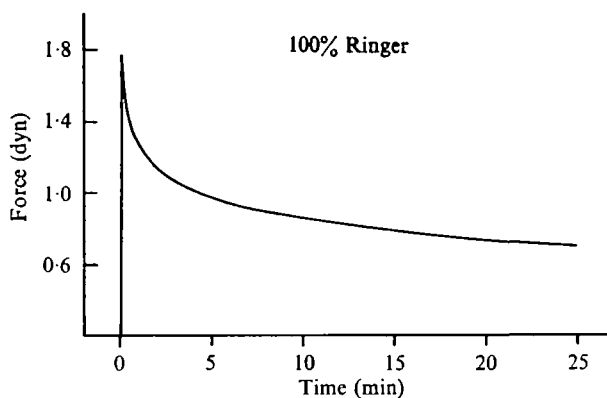


Fig. 6

Fig. 6. Changes in force recorded for an egg in isotonic solution. The initial increase in the force reflects the onset of the compression.

position the rod exactly above the centre of the egg. At the side of the cuvette a mirror is placed so that the egg can be viewed from two sides at the same time. The egg is then compressed so that most of the surface of the tip is in contact with the egg. Since the tip is not much larger than the contact zone the diameter of the latter can be measured quite accurately. The error in the diameter measurements was estimated to be $\pm 30 \mu\text{m}$. The manipulation was observed through a stereomicroscope with 25 times magnification.

The measurements on a single egg usually comprised five or six different degrees of compression. This could be achieved, with maintained accuracy in the contact area measurements, by using two or three different rods. The pressures were calculated according to equation (1) and plotted against the forces. By extrapolation the pressure in the undeformed state was obtained.

RESULTS

In each individual case the recorded force was dependent on the amount of compression, on the rate of the compression and on the incubation medium used. Examples of force recordings are shown in Figs. 6 and 7. Immediately after the rod was in position the force reached a maximum value and subsequently there was a rapid fall lasting for about 20 sec. After this stage, two things would happen, depending on the incubation medium. If isotonic solution (100% Ringer) was used, the force continued to decrease, but at a slower rate, for more than 1 h (Fig. 6). In hypotonic solutions the force began

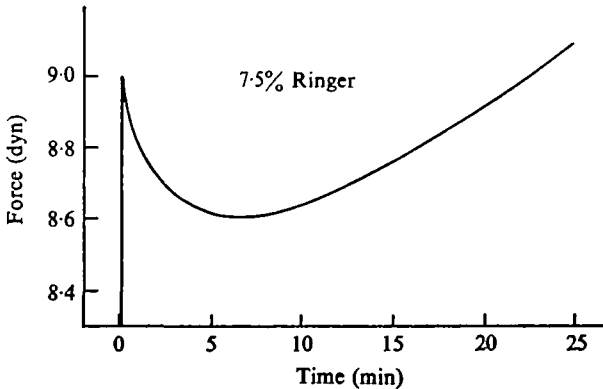


Fig. 7. Changes in force recorded for an egg in hypotonic solution.

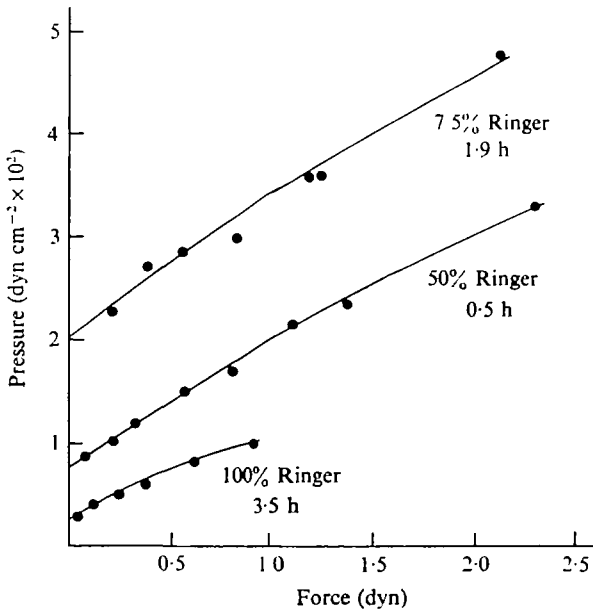


Fig. 8. Illustration of the extrapolation procedure used to estimate the pressure in the undeformed egg. The media employed and the duration of the incubation are indicated for each of the curves.

to increase at a constant rate with no tendency to diminish even after a couple of hours (Fig. 7).

In no case could any change be observed in the contact area with time. This means that the changes in pressure are directly proportional to the changes in the forces. The value for the force employed was the one recorded about 20 sec after the change of the degree of compression. This expedient implies a minimum influence on the result for the extrapolated pressure of the undeformed egg (Fig. 8). We have made no attempts to find a theoretical equation for these curves. Four different concentrations of Ringer solution were employed, namely 100% (isotonic), 50%, 25% and 7.5%. The lowest concentration corresponds approximately to fresh water.

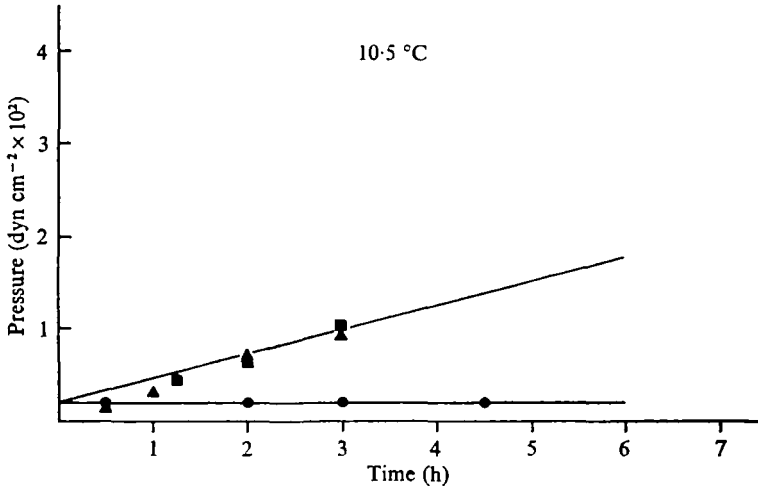


Fig. 9. Pressure versus time of incubation for different Ringer solutions at 10.5 °C. ●, 100 % Ringer; ■, 50 % Ringer; ▲, 25 % Ringer.

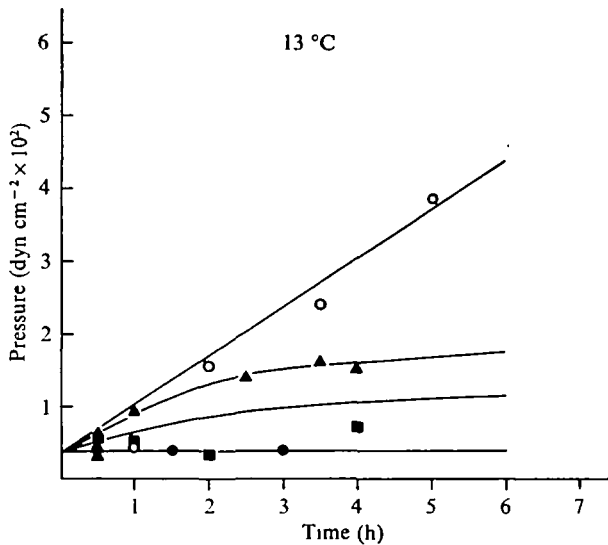


Fig. 10. Pressure versus time of incubation for different Ringer solutions at 13 °C. ●, 100 % Ringer. ■, 50 % Ringer; ▲, 25 % Ringer; ○, 7.5 % Ringer.

Figs. 9–13 show the extrapolated pressure corresponding to the undeformed spherical egg as a function of the time of incubation in the specific media and at different temperatures.

At time $t = 0$, i.e. when the eggs are removed from the frog and placed in the specific medium, the extrapolated pressure is found to be about 30 dyn/cm². After 3 h in 7.5 % Ringer at 25 °C the pressure has risen to 500 dyn/cm².

In Figs. 14–16 the pressure is plotted as a function of the temperature with time as a parameter. The curves show an anomalous behaviour at 16 °C. The pressure at 16 °C is higher than at 19 °C and this difference increases with time. This phenomena is most pronounced in 7.5 % Ringer solution.

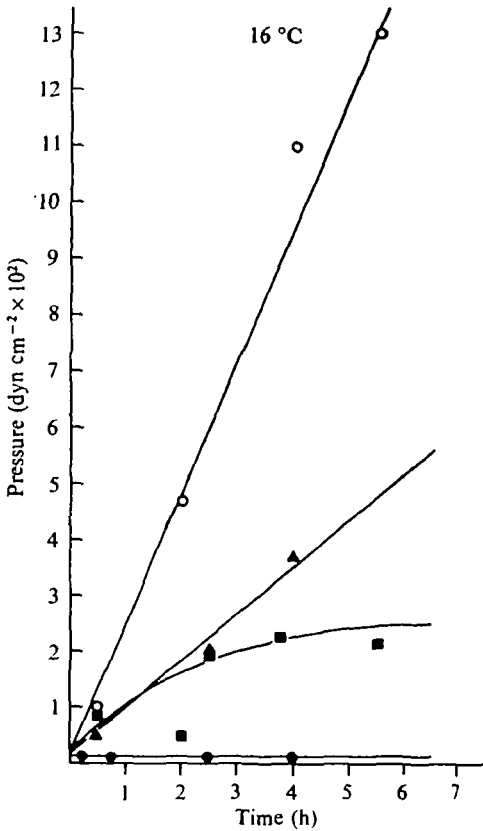


Fig. 11

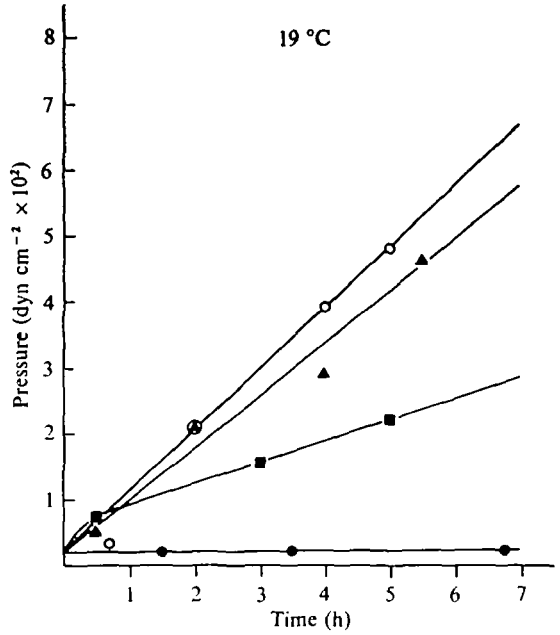


Fig. 12

Fig. 11. Pressure versus time of incubation for different Ringer solutions at 16 °C. ●, 100% Ringer; ■, 50% Ringer; ▲, 25% Ringer; ○, 7.5% Ringer.

Fig. 12. Pressure versus time of incubation for different Ringer solutions at 19 °C. ●, 100% Ringer; ■, 50% Ringer; ▲, 25% Ringer; ○, 7.5% Ringer.

Combining the relations for pressure versus time from Figs. 9–13 with separate swelling experiments in hypotonic Ringer solution where the radius was measured as a function of time, Young's modulus, E , for the egg membrane has been calculated according to the formula derived by Hansson Mild & Kalnins (1974),

$$E = \frac{1-\nu}{2h} \frac{R_0}{\Delta R} (p_0 \Delta R + R_0 \Delta p + \Delta p \Delta R), \quad (2)$$

where ν is the Poisson's ratio (assumed to be 0.5), R_0 is the radius at $t = 0$, p_0 the corresponding internal pressure, ΔR and Δp the changes in these parameters and h the thickness of the membrane. The modulus was found to be $(3.7 \pm 1.1) \times 10^5$ dyn/cm² at 23 °C (31 experiments). No significant difference could be noticed for the various experimental conditions employed.

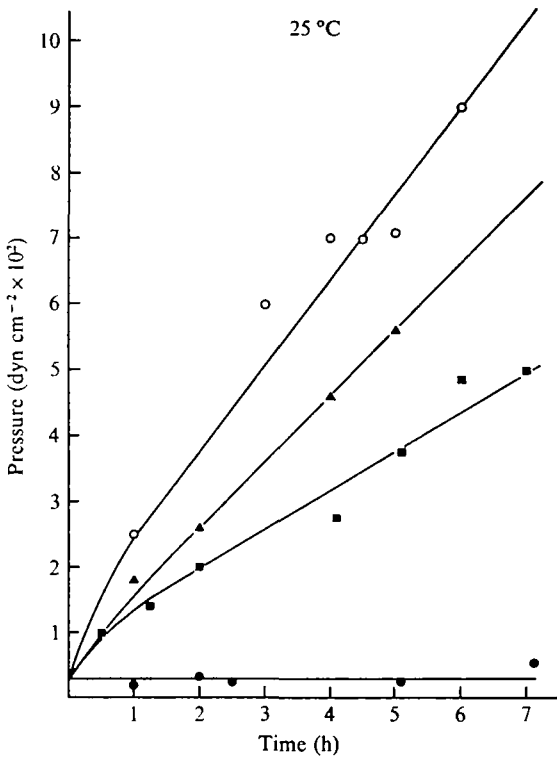


Fig. 13

Fig. 13. Pressure versus time of incubation for different Ringer solutions at 25 °C. ●, 100 % Ringer; ■, 50 % Ringer; ▲, 25 % Ringer; ○, 7.5 % Ringer.

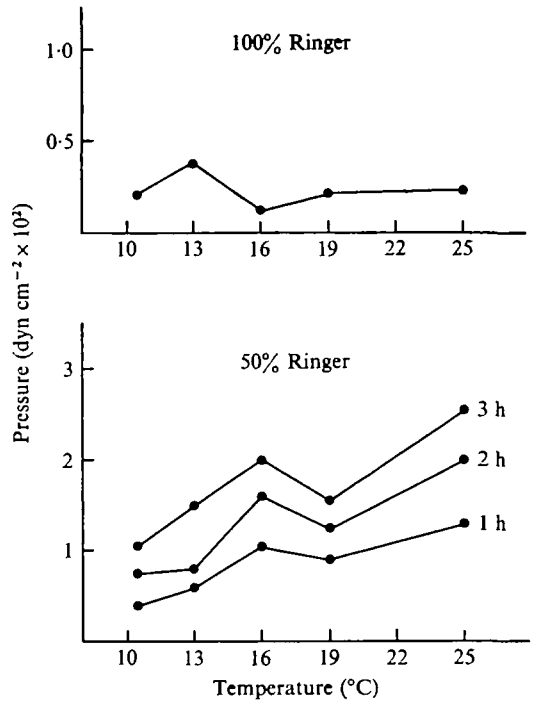


Fig. 14

Fig. 14. Pressure versus temperature for 100 % and 50 % Ringer solutions at different times of incubation. In the case of 100 % Ringer no change with time is observed.

DISCUSSION

From Fig. 6 it is seen that in isotonic solution there is a very rapid initial drop in force, followed by a much slower gradual decrease. In hypotonic solution the initial reaction, for which we can offer no explanation, is also seen, but here an increase in tension is observed to begin after about 5 min. This change may presumably be referred to the swelling of the egg which occurs under our experimental conditions.

A decrease of pressure in isotonic solution has also been observed by Yoneda (1964) and by Hiramoto (1963), both of whom employed Cole's method on sea-urchin eggs. They used a constant force for the compression and observed a decrease in the distance z , and thus an increase in the contact area. The observations were made at 5 sec, 30 sec and 5 min. From the values reported it appears that the change is most rapid at the beginning of the experiment, similar to that shown in Fig. 6. This initial decrease in force may be referred either to the viscosity of the protoplasm or to the rheological properties of the egg membrane. Hiramoto (1963), on basis of experiments with eggs in different stages of development, claims that the latter alternative is most likely. No such experiments have yet been carried out with frog's eggs.

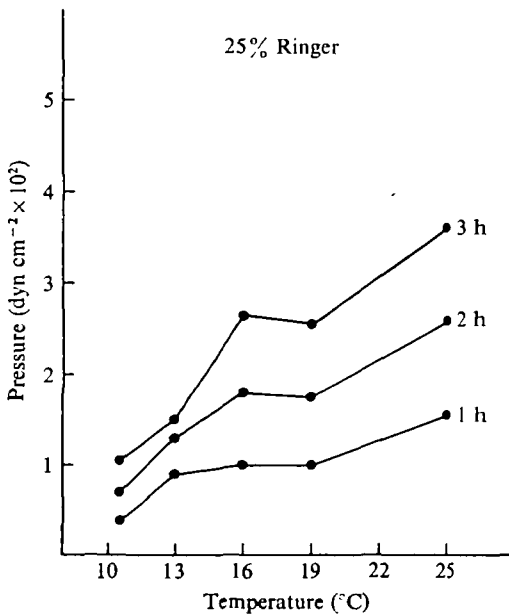


Fig. 15

Fig. 15. Pressure versus temperature for 25% Ringer at different times of incubation.

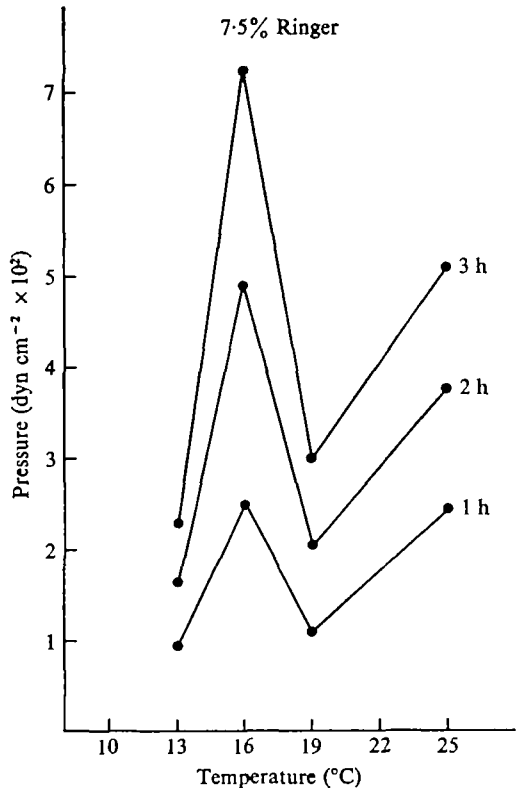


Fig. 16

Fig. 16. Pressure versus temperature for 7.5% Ringer at different times of incubation.

From the measured pressure the stress resultant (i.e. the tension) in the membrane can be calculated. For a spherical shell the tensions in the two main directions are equal and given by

$$N_1 = N_2 = \frac{pR}{2} \quad (3)$$

(Flügge, 1967). The mean radius of the egg used in this study was about 0.09 cm. This leads, at 25 °C, to a tension of 1.5 dyn/cm in 100% Ringer and 4.5 dyn/cm after 7 h in 7.5% Ringer.

Yoneda (1964, 1972), in his studies of the cortical tension in sea-urchin eggs, made the assumption that the tension is constant and independent of the deformation of and the direction on the shell. When his theory is applied to our experiments, the theoretically calculated form of the compressed egg clearly deviates from the real form. The calculated contact radius was in all cases examined considerably lower than the measured radius. Evidently, this approach must lead to a large overestimation of the internal pressure.

To obtain a satisfactory theoretical evaluation of the contact radius from the form of a compressed egg, the theory of thin elastic shells must be employed. As a first approximation the results presented by Reissner (1949) and by Updike & Kalnins (1970, 1972)

may be applied. In the latter paper the membrane tensions in the two main directions are calculated for a situation similar to the experimental arrangement presented here, and it is seen that the tensions in the two directions are equal only at the centre of the contact area. In this study no attempts have been made to calculate the contact radius; the special design of the Plexiglass rod permits an estimation of this parameter with an accuracy sufficient for our purposes.

When osmotic water permeability is studied the internal pressure p is usually neglected, because it is assumed to be very small compared to the osmotic pressure difference, $\Delta\Pi$, across the membrane. A commonly used experimental technique is to place the egg in a hypotonic solution and follow the swelling as a function of time. The equation describing the volume flow is given by Katchalsky & Curran (1965) as

$$\mathcal{J}_v = L_p (\Delta p - \sigma \Delta \Pi), \quad (4)$$

where \mathcal{J}_v is the volume flow, L_p the phenomenological coefficient for mechanical filtration and σ is the reflexion coefficient.

In order to employ this equation to evaluate the permeability coefficient from experimental data, the term Δp is neglected compared to $\Delta\Pi$. If the egg is placed in 7.5% Ringer solution, $\Delta\Pi$ is 0.205 osm at the beginning of the experiment, corresponding to an osmotic pressure difference of 4.6×10^8 dyn/cm². Hence, even with pressure differences as high as 2000 dyn/cm² (cf. Fig. 13) Δp is here negligible compared with $\Delta\Pi$. This also seems to be valid for sea-urchin eggs. Rieser (1950) found that the maximum pressure these eggs can withstand without rupture is of the order 0.01 atm (10⁴ dyn/cm²) and since this is a maximum value the pressure being built up during the swelling process can be only a fraction of this; hence, Δp can be neglected in comparison with $\Delta\Pi$ for this type of egg also. It should be noticed that this conclusion is restricted to the initial phase of the swelling and implicitly the assumption is made of a semipermeable membrane. Sigler & Janáček (1969, 1971) have recently studied the ion content of frog ovarian eggs after incubation in both hypo- and hypertonic media. They found that, due to the efflux of K⁺ and Cl⁻ and a small influx of Na⁺, the difference between the intracellular and the external osmolarity was almost zero after 3 h in hypotonic solution. The volume changes are of the order 15–20%. At this stage of the swelling process we thus have almost isosmolarity, and under these circumstances the mechanical properties of the membrane may very well play a role in the regulation of the volume since Δp in equation (4) cannot be neglected under these conditions. It has been proposed (Berntsson, Haglund & Løvtrup, 1965) that the elastical properties of the vitelline membrane play a significant role in the osmoregulation process. In view of the present findings and some preliminary experiments on ovarian eggs, it appears that the return to the original volume observed by the mentioned authors may be explained in this way, provided that the initial difference in osmotic pressure is abolished through the loss of ions from the egg.

At this stage we can offer no explanation of the anomalous temperature-dependence of the pressure, but merely discuss alternative possibilities. Theoretically it is to be expected that the curves in Figs. 14–16 should be slightly convex towards the temperature axis. This follows from the following reasoning.

In an elastic spherical shell with zero pressure initially a change in the volume causes a pressure change which according to equation (2) is proportional to ΔR . As a first

approximation ΔR may be obtained from equation (4), neglecting Δp and assuming that the reflexion coefficient σ is equal to unity,

$$\mathcal{J}_v = dR/dt = -L_p \Delta \Pi. \quad (5)$$

From equations (2) and (5) it is seen that the temperature-dependence of the pressure should be approximately the same as that of the water permeability. If the latter is presumed to follow an Arrhenius equation, the behaviour at 16 °C in Figs. 14–16 is anomalous. This, however, is not an unusual situation in biological systems. Drost-Hansen (1971, 1973) has reviewed different phenomena in which thermal anomalies occur. No exhaustive explanation at molecular level has been given so far, but some interesting attempts have been published by Drost-Hansen (1971, 1973) and Forslund (1971).

However, macroscopically there are at least three different parameters that could explain the anomaly in the pressure, namely (a) Young's modulus, (b) the water permeability and (c) the concentration difference across the membrane.

(a) If the elastic modulus of the egg membrane has a local maximum at 16 °C this would cause the pressure to rise at that temperature. Anomalous behaviour of this parameter has been observed in sea-urchin eggs (Mela, 1968), but here a minimum was found at 16 °C. The interpretation of these experiments is questionable however – a point which will be discussed later.

(b) If the water permeability is abnormally high at 16 °C proportionally more water will move across the membrane per unit time than at adjacent temperatures, thereby increasing R and hence also p . Investigations of the water permeability by means of isotopic exchange at different temperatures (Hansson Mild & Løvtrup, 1974, to be published) have revealed no anomalies in the permeability. We have reason to believe that this is true also for the osmotically measured permeability, and therefore this alternative (b) seems to be ruled out.

(c) By using a reflexion coefficient $\sigma = 1.0$ it is implicitly assumed that the membrane of the frog's egg is impermeable to the ions of the Ringer solution and that only water moves across the membrane. This may hold for the initial stage of the experiment, since the permeabilities to Na^+ and K^+ are some orders of magnitude smaller than that to water, but, as discussed above, when the swelling goes on for several hours this assumption is no longer valid (Sigler & Janáček, 1971).

The fluxes of Na^+ and K^+ in red blood cells have a paradoxical temperature-dependence (Wieth, 1970, 1971). The passive fluxes of Na^+ can be described as having two different components, one having a Q_{10} of 0.3 for the temperature range 0–18 °C and the other having a Q_{10} of 3.7 for 18–38 °C. Thus, the curve of flux versus temperature has a minimum around 18 °C. A similar effect is seen for the K^+ flux. If the ion fluxes through the frog egg membrane have this kind of temperature-dependence this could explain the peaks in the pressure seen in Figs. 14–16, since the effective concentration gradient over the membrane would have a local maximum at the particular temperature, hence causing a proportionally larger flow of water across the membrane than at slightly lower or higher temperatures.

This phenomenon could also explain the anomalous behaviour of Young's modulus found for sea-urchin eggs and oyster eggs (Mela, 1968). This author presumed that the

pressure difference is given by the osmotic pressure difference across the membrane at equilibrium in hypotonic solution.

$$p = \frac{\phi n R T}{V - V_b} - \Pi_m, \quad (6)$$

where R is the gas constant, V the volume of the egg, V_b the osmotic inactive volume, ϕ the osmotic coefficient, n the number of moles of solutes and Π_m the osmolarity of the external medium. The assumption that the product ϕn is constant during the experiment may cause the observed minimum in the value for Young's modulus since, according to his derivation of the formula for calculating the modulus, the latter is proportional to the pressure. If the temperature-dependence of the ion fluxes is that given by Wieth (1970) the product ϕn would then have a maximum at 18 °C and hence the equilibrium volume would be large, giving a low pressure. At higher or lower temperatures the product ϕn would be smaller giving a lower value of the volume and a correspondingly higher pressure.

SUMMARY

Cole's method is employed to measure the internal pressure in body cavity eggs of *Rana temporaria*. In the interpretation of the results the effect of bending resistance is considered.

The internal pressure in the undeformed egg in isotonic solution, at 25 °C, is 30 dyn/cm², corresponding to a stress resultant, or tension, in the vitelline membrane of 1.5 dyn/cm. In hypotonic solutions substantial increases in the pressure have been recorded.

When the pressure is studied as a function of temperature an anomalous high pressure is found at 16 °C for all the different incubation media employed.

A new method is applied to determine Young's modulus of the membrane. The result obtained is 3.7×10^5 dyn/cm² at 23 °C.

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Note added in proof. Recent measurements in 75% Ringer at 10.5 °C have given the following results: 1 hour, $\rho_0 = 90 \text{ dyn/cm}^2$; 2 hours, $\rho_0 = 90 \text{ dyn/cm}^2$; 3 hours, $\rho_0 = 125 \text{ dyn/cm}^2$.