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THE OSMOTIC BEHAVIOUR OF FROGS EGGS AND YOUNG TADPOLES.

by

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With 2 figures in the text.

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In 1912 BACKMANN and RUNNSTRÖM made experiments on the development of frogs eggs in balanced salt solutions from the concentrations of frogs Ringer downwards and were surprised to find that all higher concentrations down to R/5 were unfavourable and would not allow the embryos to hatch out. They concluded that the eggs must have a very low osmotic concentration and made freezing point determinations by means of the BECKMANN method on the contents of eggs at different stages. They found a freezing point depression of 0.48° corresponding to 140 mM NaCl in the oviduct eggs, but this dropped after fertilization to 13 and at the early blastopore stage even to 12 mM which is only very slightly higher than that of the fresh-water in which the eggs normally develop. A little later a sharp rise in concentration was observed and before the blastopore was closed reached 63 mM.

BACKMANN and RUNNSTRÖM gave conclusive reasons against the assumption that the salts responsible for the osmotic concentration diffused out of the eggs by finding that these, when at the lowest concentration, could develop after transfer to distilled water which was renewed several times, and they point out also that the presence of salt seems to be necessary at every stage (e. g. to prevent precipitation of globulins). They believe therefore that the salts must become provisionally adsorbed and quote figures in support of the theoretical possibility of such adsorption.

In spite of the evidently very careful work of BACKMANN and RUNNSTRÖM we have not found it possible to accept their results, both because of the difficulties inherent in the assumption that 90% of the osmotically active substances can become thus provisionally inactivated by adsorption, and because we now know that the determination of freezing point depression on egg yolk is beset with difficulties and may easily give erroneous results (Cp. E. HOWARD 1933). We have therefore carried out a number of experiments on frogs eggs. Preliminary determinations of rates and degrees of swelling were made in 1937 and include also a few experiments with heavy water for which we are indebted to Dr. USSING, and the main series were carried out during the breeding season of the frogs in the spring of this year.

Methods.

Eggs were taken out from the "uterus" of a female frog and fertilized artificially.

Osmotic concentrations were measured on drops of egg content sucked up into a small pipette and transferred to the BALDES thermopile for vapour pressure determination. In the original BALDES model which we obtained from London the air space surrounding the thermopile is rather large and it takes 15 minutes or more for the thermal steady state to be reached. We have therefore constructed a smaller model shown in fig. 1 which embodies several improvements. All determinations are made by comparison with known solutions of NaCl and expressed by the concentration which exactly balances the unknown.

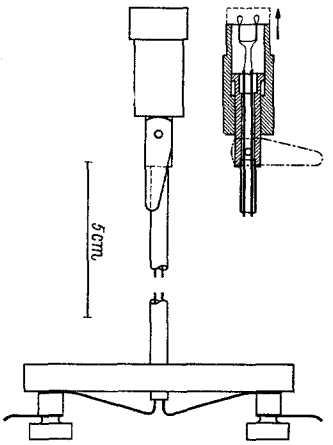

Fig. 1. A modified thermocouple for vapour pressure determinations on solutions.

Even vapour pressure determinations on yolk material are liable to grave errors which have to be guarded against. There is the possibility that autolytic processes may increase the concentration. It is improbable, however, that such processes could come to a standstill during the 10—20 minutes which must elapse before constant readings of the galvanometer are obtained, and if not they might prevent a steady state being maintained over a long period. Even constancy over a prolonged period is no proof that our determinations are free from serious systematic error. Our main argument is that we have in several cases obtained the fluid from the blastocoele or gastrocoele which is colourless, clear and practically protein free and rises by capillarity in a micropipette, and have found substantially the same values as on the yolk. The same values are also obtained when yolk material is heated to boiling temperature in a sealed capillary and centrifuged so as to give a clear liquid.

A number of Cl determinations according to the WIGGLESWORTH (1937), technique were made on the contents of a single or a few eggs which were sucked up in a micropipette, sealed and centrifuged so as to get a clear, colourless liquid.

Finally we have in some cases taken larger samples and carried out analyses of alkali and Cl [according to the methods described by KROGH (1938)].

When the eggs were to be measured they were placed on object slides provided with strips of glass 4 mm. thick on which another object slide was placed and kept in position by means of rubber bands. The

measurements were made under the microscope at a low power and care was taken to measure only such eggs as were perfectly spherical. Volumes were calculated from the measurements and averages taken for 9—12 eggs as the variations were quite small. The eggs were fertilized by an infinitesimal drop of sperma just before they were transferred with the slides to tap water or the experimental solutions. It is well known that the mucus swells greatly and as the thickness of the layer was limited to the 4 mm. between the two object slides the mucus volume could be obtained from the diameter of the circular mucus drop after lifting the slide out of the solution.

Results.

1. Osmotic concentration and Cl content of eggs.

Our determinations of total concentration on single eggs and of chloride on the mixed content of 2—3 are summarized in the curves

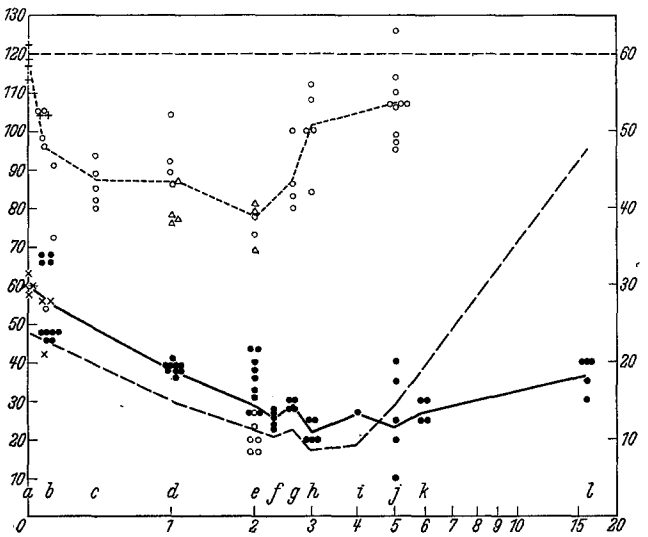

Fig. 2. Changes in total and Cl concentration in eggs of *R. temp.* during development in tap water. Abscissa — time scale, logarithmic. Fertilization and transference of eggs to tap water at 0 time. *a*—*l* Approximate developmental stages at 20—22° C. *b* First cleavage, *c* Morula, *d* Blastula, *e* Beginning of gastrulation, *f* Blastopore circular, *g* Neurula, *h* Tail budding, length 3—4 mm., *i* Gills budding, spontaneous movements, hatching, *j*—*k* External gills well developed, *l* Gills enclosed in cavity. Upper line — — — Concentration in frog. Curve - - - - - Total osmotic concentration. □ Eggs from uterus or unfertilized in tap water. Tissue fluid. ○ Fertilized eggs. Tissue fluid. △ Fertilized eggs. Blastocoele sap. Curve ——— Chloride concentration. × Unfertilized eggs from uterus or tap water. Tissue fluid. ● Fertilized eggs. Tissue fluid. ○ Fertilized eggs. Blastocoele sap. Curve — — — Total chloride per egg. Ordinate right $\frac{\mu M}{100}$ Cl content. The volume of each egg is taken as constant 4 mm.^{3} until point *i*. Increase in volume from *i* onwards according to SCHAPER's figures.

fig. 2. These show rather large individual variations (including errors). There is, however, a definite initial fall in total concentration from

isotonicity with the frogs tissues (120 mM) down to about 95 mM (20%) at the approximate time of the first cleavage. This is followed by a very gradual fall lasting 2 days (until the gastrula stage and reaching a minimum of about 80 mM when the concentration begins to rise again. In the chloride curve the initial fall is not conspicuous (due no doubt to the accidental variations). The absolute fall until stage *e* seems to be about the same as in total concentration, but the value is uncertain. The increase begins later and is much less pronounced and perhaps even doubtful. All the changes are quite small compared with those observed by BACKMANN and RUNNSTRÖM and are to be explained mainly by the volume changes taking place in the eggs when they come in contact with water.

2. *Volume changes.*

BACKMANN and RUNNSTRÖM found that the fertilized eggs swell in water and show an increase in volume amounting to 12.5—21% before the first cleavage. The later swelling is much slower and they record 1.5% from the 64 cell stage to the blastula (*d*) and a similar increase to the gastrula stage (*e*). This very slow increase after the first cleavage was confirmed by BIALASZEWICZ (1912) and we find similar figures for eggs in millimolar $CaCl_2$.

On an average we can assume a 15% increase in volume during the first few hours and a further 5% until the end of the second day. In absolute measure we have found the volume of the uterus egg to be 6 mm.^{3} of which $^1/_2$ is mucus. The egg proper swells until the gastrula stage to 3.6 mm.^{3} When the gastrula is fully formed the egg is no longer spherical and volumes become difficult to measure. SCHAPER (1902) made careful determinations at somewhat later stages (at lower temperature) beginning at our stage *h* (head and budding tail visible) and his results are summarized in table 1.

Table 1.

Days after fertilization	Temperature °C	Length mm	Weight mg	Water mg	Stage
6	17	3.75	3.9	2.6	Head and budding tail
7	15	4.5	4.3	2.9	Movements begin
8	16	5.5	4.1	2.8	Emerging from mucus. First stage gills
9	14	7	5.3	3.9	
10	15	9	6.3	4.8	Tadpoles swimming. Well developed gills
11	13	11	9.3	7.9	External gills maximally developed

It follows from the above that the initial drop in total concentration is quantitatively explained by the simultaneous osmotic uptake of water. The slow decrease until stage *e may* be due to the same cause, but the observed decrease in Cl concentration makes it probable that some salt

is lost also. The later considerable increase in total osmotic concentration and the slight increase in Cl concentration which coincide with a definite increase in volume of the embryo will be discussed below.

3. Permeability changes.

When the eggs begin by taking up water at a fairly rapid rate and the inflow is greatly reduced after a few hours it is necessary to assume a considerable decrease in water permeability. On trout eggs it was found by GRAY and confirmed by KROGH and USSING that the eggs when laid are permeable to water and later become completely impermeable through the action of the water. On frogs eggs USSING showed that heavy water would penetrate into the eggs before the first cleavage and in concentrations above 10% would delay the cleavage, while 30% was highly toxic. In continuation of these experiments USSING made for us some observations on later stages. A number of eggs were fertilized and left with a minimum of water until the first cleavage (160 minutes). To a lot of 5 was than added 0.5 ml concentrated D_2O, while another lot served as control in H_2O. In the eggs in H_2O the second cleavage took place normally after 55 minutes. In D_2O it was 5 minutes delayed and was not at right angles to the first. This result demonstrates permeability to D_2O, but of a low order compared with the initial permeability. 13 hours later the eggs in D_2O were dead. 24 hours after the fertilization when the eggs in water were in the blastula stage some were transferred to concentrated D_2O in which they showed apparently normal development with no difference from the eggs in H_2O for the next 24 hours and until the neural folds began to appear when they were brought to a standstill. This is possible only when the permeability of the eggs is of a very low order, although definitely present. Experiments on eggs of *Bufo viridis* showing that these also have a very low permeability for D_2O after the first 24 hours are described in USSING's paper.

While in the trout eggs the change in permeability is brought about by the contact with water and the fertilization is without influence the mechanism in the frogs eggs is more complicated.

In distilled water fertilized eggs will swell about 23% in 4.5 hours and go on swelling until the volume is about double after 2 days when most of them will rupture. Unfertilized eggs will reach 180% in 17 hours and many of them burst even earlier. In solutions of millimolar calcium chloride or calcium acetate the initial swelling of fertilized eggs will be about the same as in tap water, but unfertilized eggs will swell at about double the rate. Later on balanced solutions seem to be necessary if the fertilized eggs shall reach the lowest level of permeability for water.

BACKMANN (1912) supported the result of his freezing point determinations (with RUNNSTRÖM) by measuring diameters of the eggs of *Bufo* and *Triton* in salt solutions and found that fertilized eggs at the morula

stage would shrink in NaCl solutions down to 20 mM, but keep unaltered in tap water. We have made similar measurements on *Rana temporaria* eggs, from which the mucus was removed to avoid the lens action, but cannot confirm his results. As was to be expected from the very low permeability for water the changes found are small only and to make sure we have measured both vertical diameters (from the animal to the vegetative pole) and horizontal. We have obtained the concentrated solution by adding glucose to tap water, but we express the concentrations as usual by the equivalent sodium chloride solution.

According to these results the solutions 38, 23 and 50 are definitely hypotonic, 132 greatly hypertonic and 70 perhaps isotonic with the eggs.

Table 2.

Concentration mM NaCl	Average initial diameter	After 12 hour	
132	82	Eggs	
	80	Collapsed	
70	76	74.5	Not quite regular
	81.5	81 5	Spherical blastula
38	79.5	80.5	Spherical blastula
	77.5	79.5	Spherical. Gastrulation
23	83	85.5	Spherical. Gastrulation
	79.5	81	Spherical blastula
50[1]	83.5	85.5	Spherical blastula

[1] Sodium chloride in distilled water.

It is of some interest to calculate approximately the absolute water permeability of an egg during the first 4 and the next 44 hours. Assuming the volumes 3.0, 3.6 and 4.0 mm.^{3} and taking the eggs as perfect spheres we have the respective surfaces 10.1, 11.7 and 12.2 mm^2. The corresponding concentrations of the egg content are 120, 95 and 80. We take the tap water to represent 7 mM and have for the first 4 hour period an uptake of 0.6 mm^3. through an average surface of 11 mm.^{2} at a pressure difference of 100 mM = 4.5 atm. and for the second 44 hour period 0.4 mm.^{3} through 12 mm.^{2} at 80 mM = 3.6 Atm. pressure difference. The time necessary for 1 cm.^{3} to pass through 1 cm.^{2} at 1 atm. pressure difference works out for the first period as 140 days and for the second as 2000 days or $5^1/_2$ years. It seems possible that there is a very slight permeability for ions in the first brief period, but very unlikely that this should persist further on when the eggs are almost watertight. Comparative determinations of Cl and Na on a large number of eggs seem to indicate a loss of salt, but are not sufficiently reliable to prove it.

4. Osmotic regulation at later stages of development.

As mentioned above and shown in fig. 2 a rise in total osmotic concentration is observed in the eggs on the third and fourth day between the gastrulation and the budding of the gills. During this period the chloride content is unaltered and the observed rise is probably due to the production of small organic molecules which cannot escape owing

to the very low permeability. LENNERSTRAND (1933) found at this stage a definite increase in the lactic acid content of frogs eggs which will account for 5—10 mM increase in osmotic concentration.

Simultaneously with the budding of the gills the pronephros ducts open into the cloaca (ECKER-GAUPP III, 231) and from this time on a very gradual rise in total concentration and in salt concentration is observed. This coincides with a fairly rapid increase in weight and volume, and since this takes place without any food being absorbed it can be due only to the osmotic inflow of water. Whether this takes place through the integument or through the gut cannot be decided from the material available, but we think an increased permeability for water in the gills to be the more probable because it is accompanied by an active uptake of Cl and Na ions. The lowest curve (- - - - -) in fig. 2 was constructed from the analytical data combined with the weight of the embryo to represent the total amount of Cl present. It is assumed in this calculation that the weight of the egg keeps unchanged at 4 mg during the first 4 days and thereafter increases in accordance with SCHAPERs figures given in table 1.

The units in the right hand ordinate are 0.01 μM. Analyses of Cl and alcali metals made by means of the methods described in an earlier paper by one of us (KROGH 1938) on lots of 30—50 eggs picked out from the mucus have given the following results calculated also in $\frac{\mu M}{100}$ per egg.

Table 3.

	Cl	Alkali
Just after fertilization	18	16
2 days. Gastrula. Blastopore closing.	13	21
3 days. Embryo stretching	23	19
4 days. Embryo moving	18	—
6 days. Free swimming larva, well developed gills	31	40

It is the more interesting that the mechanism for active absorption is definitely present in the young tadpoles becauses one of us (KROGH 1937) failed to detect it in older tadpoles and it appears again in the frog after metamorphosis.

5. Osmotic pressure in the chorionic cavity.

Between the egg and the surrounding mucus a narrow space, the chorion cavity, appears shortly after fertilization and grows later to such a size that it does not impede the growth in length or the movements of the embryo in later stages. It has been assumed (BACKMANN and RUNNSTRÖM, BIALASZEWICZ) that osmotically active substances are discharged into this cavity from the egg and cause the increase in size by attracting water. We have made vapour tension determinations on the chorionic fluid at the time when the embryos make spontaneous movements and found on three eggs respectively 13, 10 and 18 mM, while the concentration of the tap water in which the eggs were suspended was 7 mM. The Cl concentration in the chorion cavity was found to be

only 4 mM. It is possible that the slight surplus pressure, brought about probably by organic material from the embryo, may be responsible for the increase in size of the chorion cavity.

6. The behaviour of the mucus surrounding frogs eggs.

The mucus forms on the uterus egg a firm layer approximately 0.27 mm thick, and its total volume comes very close to the volume of the egg proper viz. 3 mm.^{3}.

It is well known that this mucus swells greatly in water and to some extent even in moist air. The swelling takes place rather slowly and depends largely upon the nature of the solution surrounding the egg. We reproduce the following average results on a lot of fertilized eggs at the intervals stated.

Table 4.

	Distilled water		$CaCl_2$ 0.2 mM		$CaCl_2$ 1 mM		NaCl 1 mM		Tap water	
	Diam. mm.	Vol. mm.^{3}	Diam. mm.	Vol. mm.^{3}	Diam. mm.	Vol. mm.^{3}	Diam. mm.	Vol. mm.^{3}	Diam. mm.	Vol. mm.^{3}
$4^1/_2$ hours	12	450	9	250	7.5	180	9.5	280	5	80
21 „	15	700	10	310	8.6	230	10	310	7.5	180
45 „	16	800	10	310	9	250	11	380	8	200

In distilled water the swelling reaches 270 time the original volume and would no doubt become even larger if the water (2 liter for about 50 eggs) were changed. A very low concentration of salts will check the swelling greatly, and calcium salts are more efficient in this respect than sodium salts. Even in Ringer at p_H from 7 to 5 swelling to at least 10 times the original volume will take place.

The mucus possesses a low salt concentration to start with. An average egg weighing 5.7 mg contains 4.38 γ Cl or a concentration, calculated for the egg + mucus, of 21.7 mM. After 10 hours in 2 mM $CaCl_2$ the average content of 60 eggs picked out from the swollen mucus was 3.68 γ so that the mucus could not have contained more than 0.7 γ in 3 mm.^{3} (concentration 6.5 mM) and may have contained even less if the eggs at that time have lost some chloride.

In water the mucus comes very gradually into osmotic equilibrium with the surrounding fluid. 10.4 g. mucus recovered from 20 eggs in distilled water contained 87 γ Cl or a concentration of 0.24 mM and 1 g. from 20 eggs in Ringer/10 contained 600 γ (concentration 17 mM instead of 11) a high figure which indicates that Cl enters into combination with the mucus.

The behaviour of the mucus in solutions appears to be the same whether fertilization takes place or not.

Summary.

Determinations of total osmotic concentration on eggs of *Rana temporaria* by the vapour pressure method and of chloride by the

WIGGLESWORTH ultra micro-technique show a rapid fall during the first few hours, from 120 to 95 mM total concentration and, until the blastopore closes, a further slight fall to about 80 mM. Thereafter the total concentration rises rapidly, while the chloride concentration remains unaltered or (later) rises very gradually.

The eggs swell considerably at first and then more gradually and the changes in concentration until the budding of the gills are due at least in the main to osmotic uptake of water.

The permeability, as determined both by rate of swelling and in special experiments with heavy waterbecomes greatly reduced by fertilization.

The initial permeability is calculated to correspond to a "minute number" of 140 days, while later it is reduced to about $5^1/_2$ years.

When the gills begin to develop permeability for water again increases and the weight rises by osmotic inflow of water. The kidneys become functional and an active uptake of salt (probably located in the gills) prevents a reduction of the osmotic concentration.

The fluid in the chorionic cavity is very slightly hypertonic to the surrounding water.

The degree of swelling of the egg mucus depends upon the salt concentration in the surrounding water. The swelling is enormous in distilled water and seems to be specifically inhibited by calcium.

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