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ION PERMEABILITY OF THE EGG OF *LIMNAEA STAGNALIS* L. ON FIXATION FOR ELECTRON MICROSCOPY

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SUMMARY

1. Changes in the permeability, especially to ions, of the cell membrane during fixation for electron microscopy are likely to have consequences for the ultrastructure of cells to be investigated.

2. Ion permeability changes of single egg cells of *Limnaea stagnalis* L. were studied by a microconductometric method.

3. To this end a conductometric cell was developed with electrode surfaces of 0.14 mm² and a cell constant of 0.05. Its volume is about 26 times that of an egg cell. Conductivity changes, depending on salt concentration of the medium, were recorded as variations of a 1000 cycles/sec current through the conductivity cell.

4. By means of electrical cytolysis of the egg cell in distilled water its salt content could be determined as to correspond to a solution of about 0.04 M NaCl.

5. On fixation with OsO₄ the cell membrane becomes completely permeable to ions in a few seconds. On fixation with glutaraldehyde, ions leave the egg cell only very slowly, as they do in distilled water.

6. On the basis of these observations the optimal ionic composition of an OsO₄ fixing solution is discussed, taking into account the establishment of a Donnan equilibrium between egg content and fixative medium.

INTRODUCTION

Light-microscopic observations *in vivo* on eggs of the sea urchin *Paracentrotus lividus* which had been treated with a solution of LiCl, led RUNNSTRÖM^{1,2} to the conclusion that the primary action of Li⁺ consists in a condensation of the cytoplasm of the egg cell caused by dehydration of certain of its colloidal components. Such a conclusion was also arrived at by DE GROOT³, RAVEN AND ROBORGH^{4,5} and RAVEN AND VAN ZEIST⁶ with regard to the effect of Li⁺ on the egg cells of *Limnaea stagnalis*, a fresh-water mollusc.

It was of course supposed that such changes in cytoplasmic structure would be more clearly demonstrated by electron microscopy. However, ELBERS⁷ could not find any evidence of the influence of Li⁺ on the cytoplasmic ultrastructure of *Limnaea* eggs at the single-cell stage, at least at subcytolytical concentrations. He tentatively concluded that Li⁺ probably did not permeate through the cell membrane at all. Ample room must be left, however, for the possibility that the method of electron-

microscopic investigation at that time was simply too crude to detect such changes in structure that would be expected from the action of ions in this experimental case.

Foreign ions such as Li^+ , if they really enter the cell, will tend to change the electrostatic interactions between positive and negative charges of macromolecules and association colloids and thus change the colloid structure of the cytoplasm. Such a role of micro-ions has recently been stressed again by BOOIJ⁸ in his model studies on the interaction of lipids and macromolecules. The changes to be expected in the cytoplasmic structure may occur almost instantaneously when the ions reach a sufficient concentration, but they may also be reversed very quickly when the concentration of the ions is lowered again. In microscopic studies on the primary action of ions under experimental conditions compatible with continued life and development of the cell, it should therefore be ascertained whether the fixation of the cytoplasmic structure has also occurred in the presence of such ions. Furthermore, it is of importance to choose a refined fixation method so as to be able to distinguish the normal structure from one that has presumably become only slightly changed by the action of the ions studied. YOUNG⁹ has already stressed the importance of the ionic composition of fixative solutions for light microscopy, while PORTER AND KALLMAN¹⁰ defined the optimum requirements for fixation in electron microscopy.

In preliminary work on the influence of Li^+ on *Limnaea* eggs⁷ a fixation method was devised which, in view of these requirements, enabled the best results to be obtained. The egg cells were fixed in a 1% solution of OsO_4 buffered at pH 8 with sodium acetate-Veronal (ref. 11), which contained, in addition, 2 mM CaCl_2 and 8.6 mM sucrose. The amount of CaCl_2 was at a physiological optimum with regard to the amount of Na^+ present in the buffer solution¹². Sucrose was added in order to raise the tonicity of the fixing solution¹³. The solution was made hypertonic to the egg cells to prevent swelling during fixation. With this fixation the cell membrane and the cytoplasmic inclusions such as mitochondria, Golgi bodies, yolk granules and fat droplets always had the same appearance. In the representation of the remaining cytoplasmic structures, however, great variations were generally encountered. The cytoplasmic matrix of the egg cell does not contain a regularly arranged endoplasmic reticulum, but only a mass of small vesicles and granules. The granules had a diameter of about 150 Å. The vesicular elements are sometimes at their outer surface in connection with the granules mentioned. It is difficult to state how far these vesicles are interconnected. With one and the same fixation method the vesicles were sometimes not seen at all and sometimes vesicles were found to form a loose network but without granules. The appearance of this undifferentiated cytoplasm was believed to be dependent on variables which were not kept under control by the fixation method mentioned. This view was supported by the fact that with KMnO_4 fixation a completely different cytoplasmic picture was found. Instead of vesicles and granules only a diffuse mass of small granules is observed, interspersed here and there by long slender membranous profiles.

The balance of forces which keep a cytoplasmic structure intact can easily be disturbed by cell fixation. This was demonstrated in the case of yeast protoplasts by ELBERS¹⁴. In this case, OsO_4 treatment leads to complete disruption of the cells owing to swelling of the cytoplasm as a result of the establishment of a Donnan equilibrium between cell content and fixation medium. This Donnan equilibrium was thought to be made possible by the fact that the cell membrane had become freely

permeable to ions and small molecules, but not to macromolecules such as the negatively charged polyelectrolytes (*e.g.* nucleic acids) with which the cells are filled. Recently Tooze¹⁵ explained with similar reasoning such a swelling effect during the fixation of amphibian erythrocytes with OsO_4 solutions. It is very likely that OsO_4 has the same effect on the ion permeability of the cell membrane of the *Limnaea* egg. An attempt was made to follow this by conductometric analysis of the solution in which the egg cell was fixed. The results obtained by this analysis could then be used in formulating a more rational fixation medium.

METHODS

Limnaea egg cells were studied at a stage between the extrusion of the second polar body and the first cleavage. Before each experiment the eggs were washed twice in distilled water to remove adhering capsule fluid. At this stage the eggs have a spherical form and a diameter of about $140\ \mu$. Their volume is $1.4 \cdot 10^6\ \mu^3$. As it is difficult to obtain many decapsulated and washed eggs of the same stage, and since a clear picture of the variability of the eggs was required, it was decided to perform the conductometric experiments with single egg cells.

To be able to follow conductivity changes with time easily, we measured the current through the conductivity cell. This was done by measuring the voltage drop across a resistor in series with the conductivity cell. For these measurements 1 kcycle per sec alternating current was available. In some instances, to induce cytolysis of the egg, a 50 cycles/sec current could be switched on.

In the design of the conductivity cell and the electrical measuring circuit many parameters had to be taken into account such as: resistivity of cell and electrode materials against OsO_4 , the voltage of the available 1 kcycle/sec source, the field strength which brings about cytolysis of the egg, the resistance of the conductivity cell with the salt content of one egg, the cell constant of the conductivity cell, the value of the measuring resistor and the minimum range of the voltmeter with regard to inductive pickup by the electrical leads. After many experiments a solution for the problems was found; the result is illustrated by Figs. 1 and 2.

The conductivity cell (Fig. 1) consists of a short length of glass capillary in which a platinum wire is inserted. This platinum wire was beforehand ground flat at its inside end. After insertion, the glass capillary was carefully melted onto the platinum wire, thus providing a watertight connection. The hole in the glass capillary then had a diameter of $420\ \mu$. Next, the glass capillary was ground down until the depth of the hole was $270\ \mu$. At the same time a flat surface was obtained, perpendicular to the axis of the capillary. This piece of glass capillary was then mounted by means of a neoprene O-ring seal in a hole milled in a plate of plexiglass. On top of the flat surface of the glass capillary, we placed a platinum diaphragm such as is used in the electron microscope. Its hole had a diameter of $300\ \mu$. This diaphragm was also ground flat. It was pressed down onto the surface of the capillary by means of the two prongs of a fork made of tantalum, which is not attacked by OsO_4 . By means of this fork, held in a sliding mechanism, the diaphragm could be centered on the hole in the glass capillary, while it was possible to look into this hole between the two prongs by means of a dissecting microscope with vertical illumination. By means of a micropipette, the hole could be filled through the diaphragm

with water and the egg placed in it. The hole was then closed by retracting the diaphragm with the tantalum fork and a conductivity microcell was thus formed. The electrical connections to the electrodes of this cell were made through the tantalum fork on the upper-side and through a spring blade on the underside of the glass capillary. From its diameter of $420\ \mu$ and its height of $270\ \mu$, it follows that the volume of the conductivity cell is $37 \cdot 10^6\ \mu^3$, that is about 26 times that of the *Limnaea* egg cell. Its cell constant is 0.05. This conductivity cell was taken up in the electrical circuit (Fig. 2).

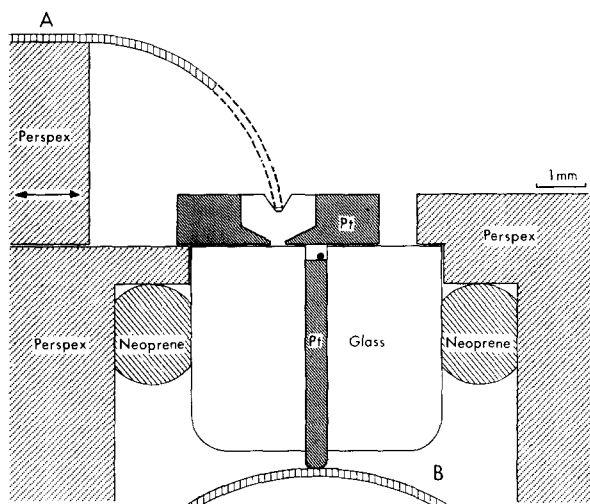


Fig. 1. Diagram of the cell used for conductivity measurements. The electrode space proper is the small rectangle in the center of the figure. The black dot represents an egg cell. Electrical connections at A and B.

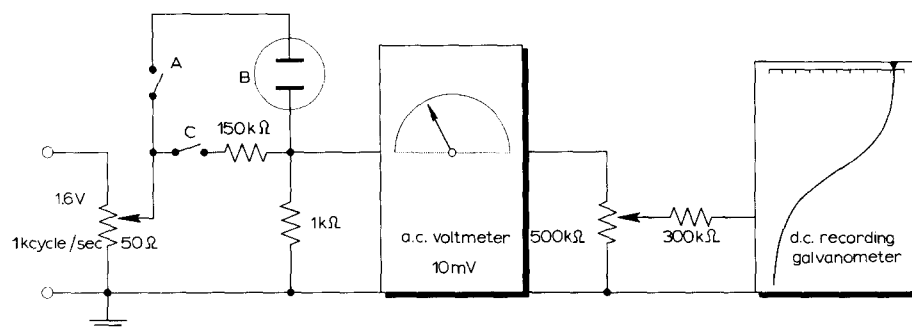


Fig. 2. Electrical circuit used in recording conductivity changes. A and C: switches for measuring and calibrating, respectively. B: conductivity cell. Further explanation in the text.

A voltage between 0 and 1.6 V at 1 kc/sec was taken from a $50\text{-}\Omega$ potentiometer and applied to the conductivity cell with a measuring resistor of $1000\ \Omega$ in series. The voltage drop across this resistor was measured by an electronic a.c. voltmeter, Philips G.M. 6012, in the 10-mV range. Part of the d.c. output of this voltmeter was fed into a 500-k Ω potentiometer which in turn was connected to a d.c. recording microvoltmeter type Kipp Micrograph BD2. The voltage across the conductivity cell

could be calibrated in the 10-mV range of the a.c. voltmeter by switching a 150-k Ω resistor in the place of this cell. For our measuring purpose the voltage drop across the measuring resistor could be neglected because this resistance is maximally 1% of that of the conductivity cell. The 500-k Ω potentiometer made it possible to use the same conductivity calibration of the recording microvoltmeter with any voltage across the conductivity cell between 0.3 and 1.6 V. The scale of the recording microvoltmeter was calibrated by filling the conductivity cell consecutively with 0.5, 1 and 2 mM solutions of NaCl (Fig. 3).

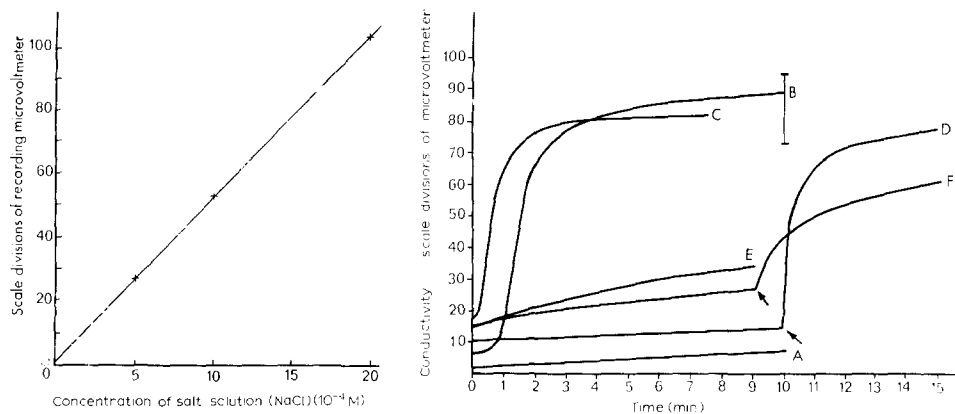


Fig. 3. Conductivity calibration curve for NaCl solution with the recording microvoltmeter.

Fig. 4. Graphical representation of the results of the experiments. Ordinate: scale divisions of recording microvoltmeter. Abscissa: time in minutes. Conductivity changes observed with one *Limnaea* egg cell in the conductometric cell. A: in distilled water; B: in distilled water and after electrical cytolysis; C: at fixation in 1% OsO₄; D: in 3% glutaraldehyde. Electrical cytolysis at arrow; E: at fixation in 1% OsO₄ after 5 min prefixation with glutaraldehyde; F: at fixation in 1% OsO₄ after 15 min prefixation with glutaraldehyde. Electrical cytolysis at arrow.

RESULTS

Cytolytic field strength

In order to avoid interference of electrical damage to the egg with the effect of OsO₄, the conductivity measurements had to be carried out at an electrical field strength much below that which would cause cytolysis. This cytolytic field strength was determined for a number of egg cells. It was found that the eggs did not cytolize within 15 min at a field strength (at 1 kcycle/sec) of 30 V/cm or lower, that is 0.8 V across the conductivity cell. At a field strength of 60 V/cm the eggs always cytolized within a few seconds. In the region between these two values variable results were obtained. At a field strength of 42 V/cm, for instance, cytolysis times of 1, 3, 4, 6, 12, 18, 24, 36 and 96 sec were noted. Microscopical inspection after cytolysis revealed that the egg cells were spread out as an irregular mass on the lower electrode of the conductivity cell.

Salt content of the egg

The cytolytic effect of the electric current provided a means of determining the salt content of the unfixed egg. To this end a *Limnaea* egg was introduced into the

cell filled with distilled water, and conductivity changes were recorded at the sub-cytolytical field strength of 24 V/cm (Fig. 4, Curve A). From this curve it is seen that, in distilled water, ions leak out of the living egg cell only very slowly. After this experiment a cytolytical field strength was used and conductivity changes were recorded again (Curve B). In this case the egg cytolized within 12 sec and ions began to diffuse out of the egg volume. The curve gives the impression that the diffusion of the ions is not hindered by the cytoplasmic mass, so that it represents the conductivity changes during homogeneous dispersion of the ions over the volume of the conductivity cell. After about 10 min the curve goes asymptotically to a maximum. This maximum varies from egg to egg, but lies within the values indicated by the vertical bar at the end of Curve B. The mean value of this maximum, from 12 measurements, is 84. This value corresponds to a salt concentration in the conductivity cell of about 1.6 mM, expressed as NaCl. This concentration is obtained by diluting the egg cell volume 26 times. Neglecting the non-solvent part of the egg, it follows that the salt concentration in the living egg cell would be about 42 mM, expressed as NaCl.

Not all of the salt in the egg will be NaCl. Unfortunately, however, the amount of different salts in the *Limnaea* egg is still unknown.

In view of further experiments a modified Earle's solution was made in which the NaHCO_3 was replaced by an equivalent amount of NaCl, and glucose was omitted. This solution contains 142 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 and 1 mM NaH_2PO_4 . A dilution series of this solution was made, and from the measurements it followed that the salt content of a *Limnaea* egg could correspond to that of a modified Earle's solution about 4 times diluted. From experiments on their osmotic properties it was shown that the eggs maintain their original volume in this solution for at least 1 h. This would imply that the *Limnaea* egg cells do not contain appreciable amounts of osmotically active solutes except salts.

Effect of fixation on salt content of the egg

What are the permeability changes manifested by egg cells when they are fixed by OsO_4 ? This question was studied by filling the conductivity cell with a 1% solution of OsO_4 made up in distilled water, introducing an egg cell into it and recording the conductivity changes (Fig. 4, Curve C). It was found that the conductivity of the medium rises very quickly in the first minute and reaches an end point after about 5 min, in the same way and at the same value as in the case of an electrically cytolized cell. With OsO_4 , however, the egg retains its spherical form. The conductivity measurements were of course done at a subcytolytical field strength (24 V/cm). Moreover, it was proved that the electric current did not interfere with the OsO_4 effect, because the same conductivity end value was also found when the current was switched on not earlier than 5 min after introducing the egg in the conductivity cell. The conclusion from this experiment is inevitably that OsO_4 renders the cell membrane of the *Limnaea* egg completely permeable to ions in a few seconds; so quickly, indeed, that fixation is not likely to have proceeded much further than the cell membrane itself.

It would be of interest to see whether this effect of fixation on permeability also occurs with other agents used in electron microscopy. Evidently, with this conductometric method, only non-ionized fixatives can be studied.

First a 3% formalin solution was tried, but this substance proved to be quickly

oxidized to formic acid by O_2 from the air in the presence of the platinum electrodes as catalyst. As a consequence, the conductivity of the solution rose in a few minutes above the salt level produced by the eggs. Fortunately glutaraldehyde, introduced by SABATINI *et al.*¹⁶ as a fixing agent, does not produce this phenomenon. A commercially available 25 % glutaraldehyde solution was freed from its acid and salt content by treatment with ion exchangers. The conductivity cell was filled with a 3 % solution made up with distilled water and an egg introduced in it. From Curve D (Fig. 4) it is seen that during 10 min of fixation, ions leave the egg cell only very slowly, virtually as they did when leaving an egg cell in distilled water.

With a few exceptions it proved to be impossible to cytolize a glutaraldehyde-fixed egg after 10 min by an electric field strength of 60 V/cm at 1 kcycle/sec. Using a shock by a field strength of 100 V/cm at 50 cycles/sec, however, these fixed cells cytolized and the ions emerged (Fig. 4, Curve D, arrow) to the same extent as the ions from eggs in distilled water. Moreover, the eggs fixed by glutaraldehyde retained their spherical form after cytolysis.

From these experiments it can be concluded that fixation by glutaraldehyde does not appreciably change the permeability to ions of the cell membrane of the *Limnaea* egg.

A post-fixation by OsO_4 of cells and tissues previously fixed with glutaraldehyde has become common practice^{16,17}. Permeability changes of the *Limnaea* egg can also be followed during this treatment. GORDON *et al.*¹⁷ indicate that, with tissue culture cells, excellent preservation of structural details is observed after 10 min fixation with glutaraldehyde. *Limnaea* eggs were therefore fixed for 5, 15 and 30 min with a 6 % glutaraldehyde solution. Afterwards they were briefly washed in distilled water and introduced in the conductivity cell which had previously been filled with a 1 % OsO_4 solution in distilled water. Curve E (Fig. 4) shows the conductivity changes after 5 min prefixation with glutaraldehyde. Ions emerge from the cell much more slowly than with direct OsO_4 fixation (Curve C), but more quickly than with direct glutaraldehyde fixation (Curve D). After 15 min prefixation with glutaraldehyde (Curve F) the permeation rate of the ions is somewhat diminished and prefixation for 30 min gave no different picture. A shock by a field strength of 100 V/cm at 50 cycles/sec applied after 9 min makes the egg cell membrane much more permeable to ions (Curve F, arrow) but they do not emerge so quickly as in previous cases of electrical cytolysis. The conductivity also reaches a lower maximum value, but naturally the eggs had already lost a certain part of their ions during the previous 15-min treatment with glutaraldehyde.

DISCUSSION

The present investigation shows that the cell membrane of the *Limnaea* egg very quickly becomes freely permeable to salts on fixation with OsO_4 . This fact has a strong bearing on the interpretation of the results of electron-microscopic structure analysis, the more so, when smaller or more labile plasma components are studied. Actually we are dealing with cytoplasmic colloid structures fixed during the absence of ions which normally have a determining influence on the spatial relations of these colloids. On the other hand, they will most probably be fixed during the

presence of quite different ions, namely those of the buffer systems which are generally incorporated in fixative formulae.

As a consequence of this enhanced permeability of the cell membrane it can safely be assumed that small molecules other than salts, for instance sucrose, are also able to penetrate from the outside medium into the cytoplasm (*cf.* ref. 14). For this reason, it is not clear what part the frequently used tonicity additives in OsO_4 fixatives could play in the fixation process of single cells.

As a point of importance in this context, preliminary mention must be made of observations on the swelling of *Limnaea* egg cells during fixation. BERENDSEN¹⁸ found a swelling of about 25 volume % when the cells are fixed in a 1 % OsO_4 solution in distilled water at pH 6.

It is quite reasonable to ascribe this also to the establishment of a Donnan equilibrium between egg and fixation medium. For the Donnan effect to occur it is necessary that the egg contains non-diffusing, macromolecular substances with a certain net charge. From the chemical viewpoint the cell content is a very complicated mixture of proteins, lipids, nucleic acids, *etc.* and it is impossible to anticipate the net charge of this complex. However, an indication of the net charge of the cell content has been obtained by cytolysis experiments in a d.c. electric field.

RAVEN¹⁹ performed such experiments with egg cells in distilled water lying on the bottom of a shallow perspex electrophoresis cell. With a d.c. field strength of 70 V/cm (current density $8 \cdot 10^{-5}$ A/cm²) these eggs generally cytolized after 5–10 min and it was observed that the outflowing cytoplasmic mass invariably moved to the anode. ELBERS AND GEILENKIRCHEN²⁰ performed electrophoresis experiments with *Limnaea* egg cells, thoroughly washed in distilled water, in which these eggs were allowed to sink freely between two electrodes 20 mm wide and 45 mm apart in a perspex electrophoresis cell filled with distilled water. The behavior of these cells during their fall was observed by means of a horizontal microscope. At the top of their falling trajectory the cells were cytolized by a 50 cycles/sec field of 25 V/cm. After cytolysis the cell content remained together as a globular mass over the whole trajectory over which its fall could be observed.

Immediately after the electrical cytolysis of the eggs, instead of the a.c. field a d.c. field was switched on, with a strength of 20 V/cm (current density $7 \cdot 10^{-5}$ A/cm²). It was then invariably observed that the whole cytoplasmic mass moved towards the anode during sinking. The whole procedure took about 20 sec. Eggs that had not previously been cytolized by an a.c. field remained intact with this low d.c. field strength and showed no visible movement in the direction of the electric field in this experimental set-up. From these experiments it may be deduced that the cytoplasmic content of the egg bears a net negative charge.

The rate of swelling of the egg cell during fixation under the conditions mentioned previously depends on the net negative charge of the cytoplasmic colloids. This Donnan swelling can be counteracted by increasing the salt concentration of the fixation medium of the eggs. For reasons set forth in the INTRODUCTION it is of prime importance to make this concentration equal to that of the egg cytoplasm itself and to do so, of course, for all ionic species occurring in the egg. Unfortunately the ionic composition of the cytoplasm is unknown, so no strictly rational experiment can be performed.

When *Limnaea* eggs are fixed in a 2 % OsO_4 solution containing the salt concen-

tration found by the conductometric experiments to be compatible with the salt concentration in the cells, a volume swelling of about 10% is still found. To explain this effect it might be possible that the net negative charge of the cytoplasmic structural system is so high that its equivalent concentration approaches that of the diffusible ions. This would mean that a much higher salt concentration in the fixation medium than in the cells themselves would be needed in order to reduce the swelling pressure to zero. At the same time this would of course lead to far from normal conditions of ionic strength under which the cytoplasmic structures are fixed.

The resulting swelling, mentioned above, could also mean that the net negative charge of the cytoplasmic colloidal structures will be significantly increased by the reaction with OsO_4 . Indications of such an effect have been reported by TOOZE¹⁵ with fixation experiments on amphibian erythrocytes. He found that the isoelectric point of new haemoglobin upon fixation is lowered from the range pH 6.9–7.2 to about pH 5.0–5.2. Such a lowering of the isoelectric point has also been observed with proteins other than haemoglobin and could very well be the case with *Limnaea* egg proteins too.

While the situation for erythrocytes is relatively simple, because their cytoplasm consists almost exclusively of haemoglobin, other cells such as the *Limnaea* egg contain many more structural elements. They often have the nature of lipids or lipid–protein complexes. There is a possibility in this case that an increase in net negative charge of the cytoplasmic structures is also due to the reaction of OsO_4 with unsaturated phospholipids. RIEMERSMA²¹ found that the second phase of this reaction consists of a partial removal of the positive charge of lecithin caused by electrostatic interaction of the positively charged part of the lipid molecule and the negatively charged colloidal osmium oxide particles formed by the reaction of OsO_4 with double bonds.

With regard to the swelling and shrinkage of cells (apart from the Donnan effect) the influence of ions on the hydration state and the electrostatic attraction of cytoplasmic colloids should be considered. The importance of this influence was stressed by FREY-WYSSLING²² in his junction theory on the cohesion of cytoplasmic proteins. He discussed at length the influence of inorganic ions on heteropolar cohesion, *i.e.* the attraction between groups of pronounced dipole character. These heteropolar cohesion bonds are very sensitive to hydration changes caused by ions. On the alkaline side of the isoelectric state of the cytoplasmic system, that is at a net negative charge, cations will usually diminish its state of hydration according to HOFMEISTER's lyotropic series. Divalent cations are in this respect effective at much lower concentration than monovalent cations. Besides the effects of single ion species, NETTER²³ stresses the importance of ion antagonism, especially of mono- and divalent cations, with regard to the state of hydration of cytoplasmic colloids.

The prevention of swelling of amphibian erythrocytes and their nuclei by the addition of Ca^{2+} to the OsO_4 fixative was indeed observed by DAVIES AND SPENCER²⁴ and TOOZE¹⁵.

In view of the influence of ion antagonism, the preservation of cytoplasmic macromolecular structures during fixation of the *Limnaea* egg cell will present great difficulties because of the complexity of the structures to be expected. Only with a knowledge of the normal ionic composition of this cell can a logical prescription for the best fixation conditions be formulated. Such an approach has already been advocated by CRAWFORD AND BARER²⁵ for phase-contrast microscopy; they discussed

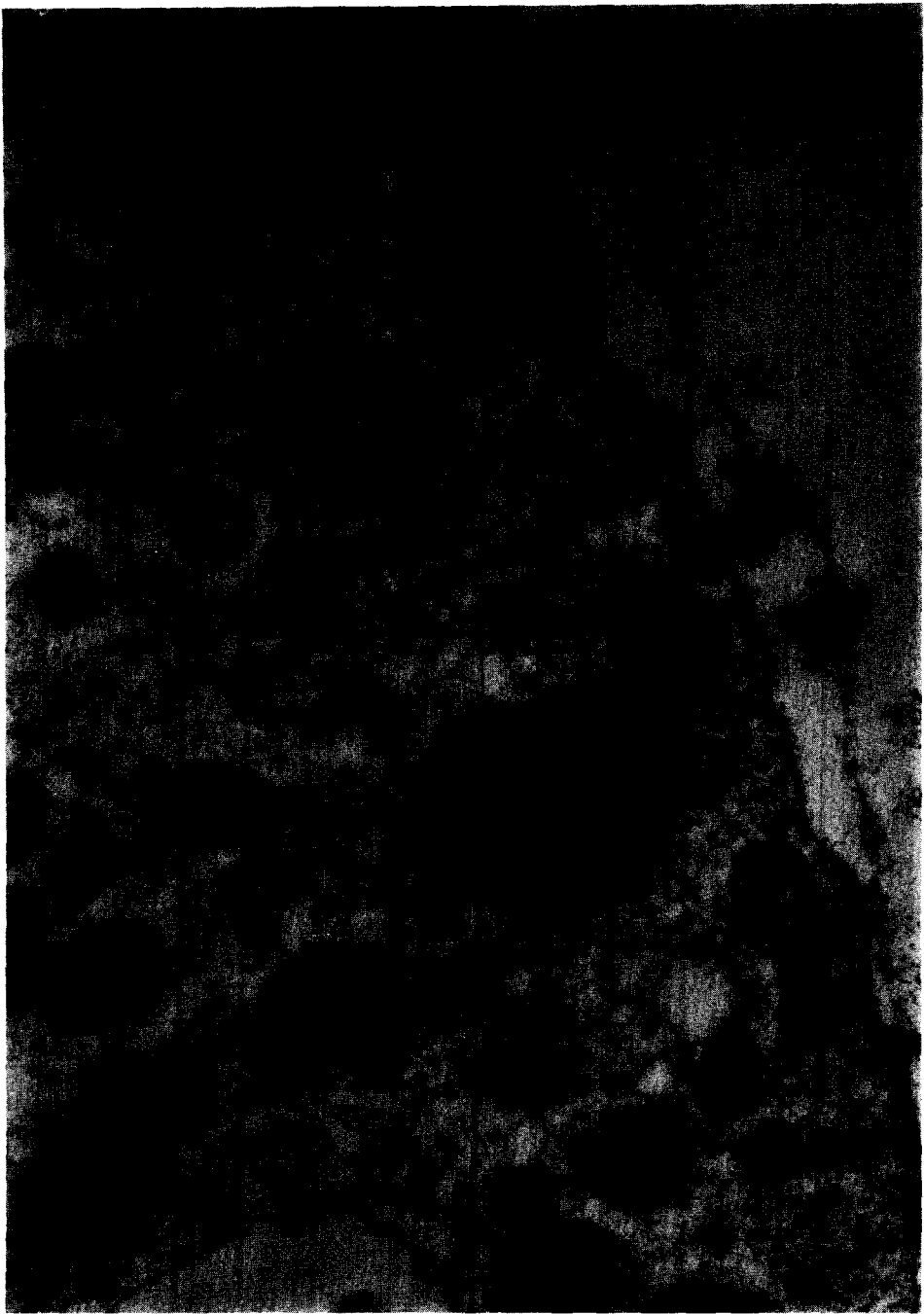


Fig. 5. Cortical part of *Limnaea* egg cell, fixed in 1% OsO_4 in a modified Earle's solution. Stained by phosphotungstic acid during dehydration. $\times 31000$.

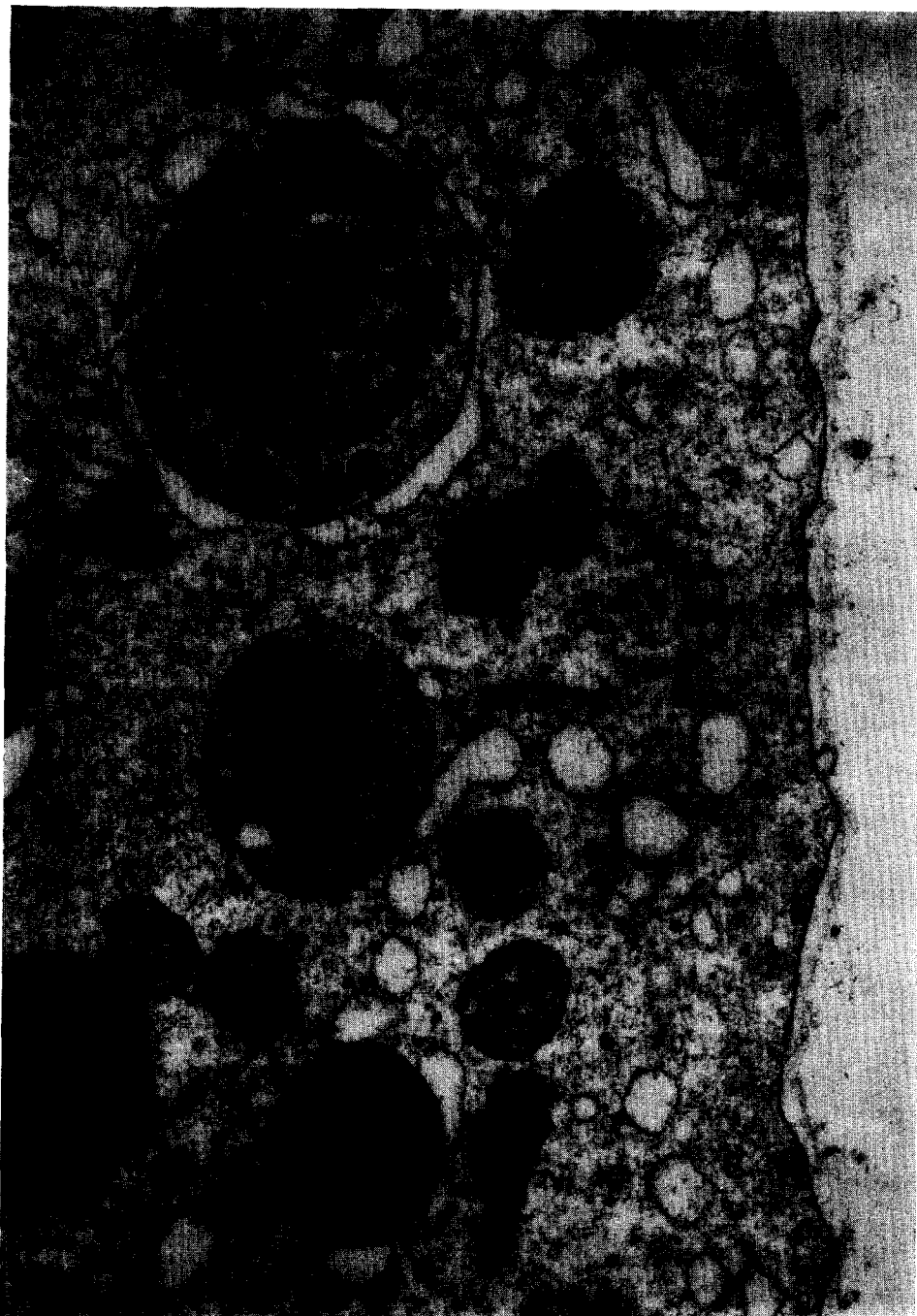


Fig. 6. Same as Fig. 5, but section-stained with lead. $\times 31000$.

the importance of many of the factors to be taken into consideration for electron microscopy.

In the present state of affairs reasonably good results with *Limnaea* eggs were obtained by using an OsO_4 fixative, the ionic composition of which corresponds to that of a four times diluted modified Earle's solution (Figs. 5 and 6).

The points developed in this DISCUSSION have stressed the difficulties of clear-cut interpretation, especially of the structures normally observed in the cytoplasmic matrix that are thought to be intimately dependent on their ionic environment. The more careful should be an approach to answering the question raised in the INTRODUCTION, namely the primary effect of ions like Li^+ on the egg cell of *Limnaea stagnalis*. It will require more data than is offered in this paper on what happens to this cell on subjecting it, in particular, to the procedures of fixation required for electron-microscopic investigation.

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