STUDIES ON LIVING PROTOPLASM

I. STREAMING MOVEMENTS IN THE PROTOPLASM OF THE EGG OF SABELLARIA ALVEOLATA (L.).

By J. E. HARRIS.

(From the Laboratory of Experimental Zoology, Cambridge.)

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(With One Plate and Seven Text-figures.)

In recent years many attempts have been made to determine the absolute coefficient of viscosity of "protoplasm." The values for this constant obtained by different workers differ widely, and while it is probable that the protoplasm from different sources is never the same in its composition, and cannot be expected to be constant in its viscosity, many sources of error in such determinations have not been sufficiently appreciated. One of the most serious of these, the presence of powerful currents in the protoplasm, forms the subject of this communication.

The method employed by Heilbrunn (1926 a, b) has generally been regarded as yielding the most accurate viscosity values, though certain of his measurements have been questioned by later writers. As a general objection to his technique, it has been urged that the enormous centrifugal forces employed in such determinations are likely to affect very greatly the properties of the protoplasm. However, confirmation of his results has been provided recently by methods which do not involve any disturbance of the cells, other than that occasioned by the observational technique. In particular, Pekarek (1930, 1931) has studied the Brownian movement of natural cell inclusions, and has obtained values for both plant and animal cells which closely agree with those of Heilbronn (1914) and Heilbrunn.

In 1930 the writer, with L. W. R. Cox, was engaged in an attempt to determine the viscosity of the protoplasm of the eggs of the marine polychaete, Sabellaria alveolata (L.), by cinematographing the Brownian movement of the "granules" in the cytoplasm. As the normal egg is not sufficiently transparent for such observations, it was first centrifuged so that most of the granules were thrown to the poles of the egg, and the movements of the few granules remaining in the clear space in the centre could easily be followed. The results obtained, however, were so erratic that the work was temporarily discontinued until a visit to the laboratory at Plymouth in the summer of 1931 provided the opportunity for further investigation.

The worms were obtained fresh from their sand tubes by breaking the tubes with the fingers and carefully extracting the animals. The ripe females can readily be distinguished by their bright pink colour, which is due to the masses of eggs which are visible through the body wall. As the experiments were to be carried out on unfertilised eggs, the females were washed in two or three changes of fresh water in
order to kill any sperm adhering to the cuticle, and they were then placed separately in small finger bowls full of sea water. Accidental fertilisation of the eggs by sperm already present in the sea water was avoided by previously passing the water through a Berkfeld filter. The disturbance caused by removing the worms from their tubes was usually sufficient to induce the immediate discharge of their genital products, which were then handled by means of a glass pipette. The precautions mentioned above were, of course, unnecessary in obtaining the sperm used for control experiments.

The eggs when laid are irregular in shape, and possess a large conspicuous germinal vesicle and a distinct nucleolus. Within about 30 min. they round up, apparently swelling slightly in the process, and the germinal vesicle disappears. After a variable period of time, a wrinkled membrane¹, identical in appearance with the normal fertilisation membrane, becomes gradually elevated from the surface of the egg, which then remains in this state until cytolysis ensues, 24-48 hours afterwards.

Faure-Fremiet (1921) states that the unfertilised egg of *Sabellaria* develops with the disappearance of the germinal vesicle as far as the equatorial plate stage of the first maturation division, and stops at this point. The commencement of these maturation phenomena is postponed in hypertonic solutions, and is completely prevented on acidifying the sea water. This work is paralleled by the studies of Horstadius (1923) on the maturation of the ovum in *Pomatoceros*. The point is of considerable interest, and will be discussed later in the paper.

The mature unfertilised eggs were centrifuged in the haematocrit head of an ordinary hand centrifuge, the radius of turn being 7 cm., and the speed of rotation approximately 6000 r.p.m. It was found that under this force (about 3000 g) 30 sec. of centrifuging was usually sufficient to displace the heavy granules into one hemisphere of the egg, but as the eggs varied slightly, they all received the centrifugal treatment for 1 min. before being photographed.

If the eggs were centrifuged before disappearance of the germinal vesicle, this duration of treatment was not sufficient to move the granules to any appreciable extent, and in order to obtain a comparable displacement, centrifugal speeds in excess of 9000 r.p.m. at 10 cm. radius for 5 min. were required.

While it is not suggested that the values obtained for the viscosity will be very accurate, it is interesting to compare these figures with those of Heilbrunn (1926) for *Arbacia* protoplasm. Assuming the same values for the specific gravities of the granules and the hyaloplasm in both species of eggs, and taking into account the fact that the concentration of granules is roughly the same, the viscosity of the unfertilised egg after the breakdown of the germinal vesicle would be about 0.2 and before the disappearance of the vesicle about 10 c.c.s. units. The very high viscosity of the oocyte has been confirmed for several other marine worms (including *Nereis diversicolor*, *Perinereis cultrifera*, *Branchiomma versiculosemus* and *Arenicola marina*). Echinoderms generally seem to possess a much thinner membrane surrounding the ova, and they are usually unable to withstand centrifugal treatment of this order of magnitude.

¹ The elevation of this membrane in the healthy unfertilised egg has been questioned by Wilson (1929 and personal communication), though it was described by de Quatrefages (1847, 1848, 1850), and subsequently confirmed by Ziegler (1914) and Faure-Fremiet (1921). Wilson suggests that healthy unfertilised eggs never throw off this membrane, but the experience of the writer does not support this conclusion.
Immediately after centrifuging, the eggs were placed in a Leitz irrigating compressorium, which was screwed down until just sufficient pressure was applied to prevent them rolling away under the slow current of irrigating sea water which was employed in all these experiments. On account of the small size of the eggs, this was one of the most difficult operations to perform satisfactorily, as mechanical pressure might prove a serious complicating factor in the interpretation of the results. A suitable egg was then selected and brought into the field of the cinema camera.

The cinematographic arrangements were essentially those of the standard Leitz microcinematographic apparatus, with additional modifications necessary for the slow driving speed which was employed. Approximately six exposures per minute were made, a timing device being fitted to the camera to record on the film the exact frequency of photography. The temperature of the water circulating around the egg was indicated by a small copper-constantan thermocouple placed near the exit tube of the compressorium, and actually under the cover-slip used to compress the eggs.

The size of the granules in the cytoplasm was measured by crushing a number of eggs in sea water, and measuring the diameter of the particles with a 2 mm. immersion lens and a filar micrometer eyepiece. The process is very difficult, as the dimensions are near to the limit of microscopic visibility, and the particles are also in active Brownian movement in the sea water, but it is possible to estimate their diameter with an error of not more than 50 per cent. In actual fact, measurements of about 200 of these granules yielded a good approximation to a normal distribution curve, indicating a mean diameter of 1.77\(\mu\) with a standard deviation of 0.14\(\mu\). It is therefore probable that the granules are appreciably uniform in size.

From the negatives of the cinematograph film, enlargements were made at a known magnification, and the movements of the granules were studied on these
enlargements with reference to fixed co-ordinate axes, great care being taken to eliminate the possibility of movements of the egg as a whole.

It was immediately obvious that the movement, instead of being completely random in character, as demanded by the theory of Brownian movement, was to a large extent directed in its nature, due to streaming of the protoplasm. An attempt was therefore made to discover the magnitude and direction of this streaming motion, so that the random component could be isolated, and a value for the viscosity obtained. This was done by studying the movements of a number of granules within a comparatively small area of the cell, over which the motion might reasonably be assumed to be constant in character. The average displacement of all the granules between two successive photographs was taken to represent the directed component of their motion during this interval of time.

An example is illustrated in Text-fig. 2, which represents the average displacement, plotted in this manner, of four granules initially within about 10 µ of one another. (This figure represents their separation in a horizontal plane, but the small
Studies on Living Protoplasm

In the later stages of the graph, the directed component apparently becomes a rectilinear streaming motion of approximately constant velocity. Successive points on the graph are separated by 30 sec. and the velocity of the stream is therefore about $4\mu$ per minute. It is interesting to notice that the direction of this streaming motion is parallel to the edge of the original layer, and is in consequence not directly connected with any mass movement of the granules back to their normal distribution.

When this streaming component is eliminated from the movements of the individual granules, the resultant motion of each granule is shown in Text-fig. 3, and appears to be random in character. This is confirmed when the square of the displacements (actually the sum of the squares of the individual displacements) is plotted against the time, the points falling approximately on a straight line, according to the expression derived by Einstein (1905):

$$\Delta x^2 = \frac{RT}{3\pi N \eta} t.$$

The value of the absolute coefficient of viscosity indicated by this graph is approximately 0.2 C.G.S. units, but no very great accuracy can be claimed for a result based upon so few observations.

The streaming movement found in the egg is not generally so simple as that seen in the above results. A second case subjected to detailed graphical analysis gave the extraordinary figure shown in Text-fig. 5 for the directed component of the motion. The general trend of the movement is in this case at right angles to the granular
boundary, and is vortical rather than rectilinear in character. The first vortex is described in an anti-clockwise sense with an average velocity of 2·0 μ per minute, and is followed by a period of more or less simple rectilinear motion of velocity 0·5 μ per minute. The last portion of the path is a small vortex described in an opposite sense to that of the first, and at the slower rate of 1·0 μ per minute.

Text-fig. 4. Displacement graph for the four particles of Text-fig. 3. The ordinates of the graph are the sum of the squares of the displacements of each granule from its initial position, measured by their projections on a horizontal plane (the plane of photography), i.e. the term \( \sum (\Delta x)^2 = 8 (\Delta x)^3 \) where \( \Delta x \) represents the linear displacement of any one particle along one coordinate axis of measurement.

At first sight it seems probable that the two vortices may be only apparent ones, due to the superposition of the random Brownian movement upon the rectilinear motion present in the middle portion of the path. The small number of granules used to determine this average path might quite easily lead to the interpretation of coincidental movements of the particles as being due to definite streaming movements.
Yet when the path of each individual granule of the four is plotted out, there is found a striking similarity between the separate motions and the complicated average one just described. This is illustrated in Text-fig. 6, in which the path of each of the four particles is represented in rather greater detail. The resemblance between the four curves and the generalised one in Text-fig. 5 is a striking one.

The three separate portions of the average path are present in each, though the velocities and directions differ slightly. Even here, however, the differences are not entirely random, but follow well-defined trends. Without going into great detail, it may be pointed out that the directions and velocities of movement of the four particles at any instant change progressively as one passes across the diagram. The initial direction of motion of the particle, the length (i.e. velocity) of the rectilinear portion of the path and the position of the second vortex all show these progressive changes, and while there is not sufficient data to deduce the configuration of the hyaloplasmic streams producing these motions, this regularity provides very strong evidence for the reality of these rather fantastic curved paths.

Text-fig. 6 also illustrates another feature of this streaming motion—the extraordinarily narrow region to which a certain motion is localised. The fifth granule in the lower right-hand corner of the diagram is describing quite a different path for the first part of its cycle, the initial vortex being clockwise instead of anti-clockwise. The latter part of its path is, however, very similar to that of the other four granules, the rectilinear motion being followed by the description of a small clockwise vortex. The distance of this particle from the other four is only 15–20 μ.

As the photographs had been taken at regular intervals, it was possible to project them on an ordinary cinematograph projector, and to examine all these phenomena
visually speeded up 100 times. Seen in this way, the motion illustrated in Text-fig. 2 became immediately obvious to the eye. Particles just outside the granular boundary appeared to stream across the egg at a rapid rate. It was not found possible to follow the more intricate paths of Text-fig. 5 by this means, as the speed of projection is so rapid that even the simpler motions can only be appreciated by projecting the same portion of film over and over again, and examining a certain region of the egg with great care.

When the photographs were examined in this way, it became apparent that the *small vortices whose existence had been demonstrated by graphical methods formed a part of a much more general streaming which was taking place over the whole egg. Text-fig. 7 illustrates these movements (which may be called, for the sake of convenience “large scale movements”; Text-fig. 5 illustrating “small scale movements”) for three of the eggs photographed.*

This large-scale streaming seems to involve the slow rotation of the protoplasm of the egg within the cell membrane, the speed of rotation being less than 0.5 μ per second. In Text-fig. 7, though the movements seem to be very different in the three cases, on closer examination it will be seen that all three can be assumed to be the same motion, photographed in three different planes. The directions of viewing in Nos. 1 and 3 would be mutually perpendicular, while No. 2 would represent a view slightly inclined to the position in No. 1.
It will be seen from the diagram that the amount of movement is always greater at the end of the egg containing the lighter fatty particles, even though they are much larger in size than the other granules, and should therefore move much less rapidly by Brownian movement. The general appearance of the motion is best described by saying that the layer of fat particles at the lighter end of the egg appears to push out a blunt process along the surface of the egg, and that this is followed by an outbreak of immense streaming activity on the part of the whole protoplasm of the egg. This results in a more or less complete mixing of the separated constituents of the egg in a much shorter time than would be the case if Brownian movement were the only

Text-fig. 7. Large scale streaming movements for three of the eggs photographed. The initial appearance of the egg is represented by the full-line drawing on the left; the dotted boundaries show an intermediate, and the figures on the right a later stage of the same three eggs. Arrows indicate the directions of streaming.
factor involved in the redistribution. Actually, taking into account the hyaloplasmic viscosity and the size of the granules, complete redistribution should require about 20 hours; but recent photographs show almost complete mixing in an hour from the time of centrifuging.

DISCUSSION.

Before attempting to draw any conclusions as to the nature of these streaming movements, it is first necessary to consider how far the eggs studied can be regarded as normal, and how far abnormalities, if any, may arise during the treatment to which they are subjected. It was found quite impossible to obtain photographs of the eggs in their natural state, the high concentration of the granules in the protoplasm making it almost opaque, and although streaming movements are visible in such photographs when projected upon the screen, they are not sufficiently clearly defined to be measurable.

The centrifugal treatment and its effect on the ovum has been discussed in detail by Heilbrunn. Here it will be sufficient to say that the author made several control experiments, including the centrifuging of fertilised Sabellaria ova, and the centrifuging and subsequent fertilisation of other mature ova, and in every case there was as high a percentage of normal swimming blastulae developed from the centrifuged eggs as from their uncentrifuged controls. (In some cases the percentage developing was even higher after centrifugal treatment, but the counts were not sufficiently large to eliminate the possibility of experimental and statistical errors of a random nature.) In so far, therefore, as subsequent development could indicate, these eggs were not injured by the centrifuge. The first cleavage of the centrifuged fertilised eggs does not take place until about an hour after that of the uncentrifuged control, but this is explicable if we assume that centrifuging destroys the astral structures, which must reform before division can take place.

Such streaming movements as were observed might conceivably be due to external factors, such as change in temperature of the sea water surrounding the egg; osmotic phenomena, or purely gravitational force acting on the granules. But the thermocouple indicated that the temperature throughout the observations remained constant within 0.25°C., a difference which would be quite insufficient to produce any convection currents in the egg. Aerated sea water was constantly flowing through the apparatus, so that the osmotic properties of the environment were the same throughout the experiment, and oxygen-lack or CO₂ accumulation was unlikely. Finally, the difference in the direction of the streaming movements indicated in Text-fig. 7 shows that the orientation of the egg has no effect on the motion, which must therefore be independent of gravitational influence.

The observation of streaming movements in the eggs of animals is by no means a new one. Gardiner (1895) described them in the eggs of the turbellarian Polychoerus, and two years later von Erlanger (1897) made a detailed study of the movements in the dividing eggs of nematodes. His work has more recently been extended by Spek (1918), who developed a technique of studying the eggs at a high temperature in order to increase the rate of streaming and facilitate visual observation.
McClendon has studied the movement of the chromatophores in the echinoderm, *Arbacia*, and Conklin (1899) has worked on the mollusc, *Crepidula*.

Brownian movement, however, has been detected at a very much earlier date than any of these observations. In 1844, Grube, working on the embryology of the leech, *Cladone*, noted the movement of small granules in the developing egg, though he did not recognise the phenomenon as Brownian movement. It is interesting to see that although Grube did not understand the nature of the movements, he deduced that the protoplasm must be of a very fluid consistency, a result which was not confirmed until almost 80 years later.

The directed streaming movements observed by the numerous writers quoted above bear a close relationship to the division of the cell, since they all seem to involve a concentration of the granules of the ovum over the surface which is about to become the cleavage plane. Indeed, it has often been suggested that these streaming movements are the forces which produce cell division. Gray (1931) is of the opinion that the movements are caused by the increase in size of the semi-rigid asters, which he regards as the real sources of energy of the division. The elongation of the polar axis of the ovum, due to the pressure exerted by the expanding asters would naturally produce a flow of the peripheral cytoplasm towards the equatorial plane of the egg. Such a hypothesis would imply that the forces concerned in cell division are localised within the cell, and are not derived from alterations in the tension at the cell surface, as suggested by Robertson (1909, 1911, 1913), McClendon (1912, 1913), and Spek (1918).

Careful measurement of the photographs obtained by the author indicates that there is no appreciable change in the size or shape of the egg in the course of an experiment, and it seems unlikely that surface forces are the cause of the streaming movements observed. At least two other possibilities remain to be considered. It may be that the streaming movements associated with cell division are not produced by changes in the shape of the ovum, but are initiated even in the early stages of the formation of the asters. The formation of polar bodies of very small magnitude in comparison with the parent oocyte is not associated with the production of asters which entirely fill the oocyte, and we are forced to the conclusion that maturation division is of an entirely different nature from normal cleavage, or else that it is possible that localised streaming movements of sufficient intensity to produce cleavage can be set up even in the presence of very small amphiasters, provided the amphiaster is located near the surface of the cell.

It is known that mechanical agitation will cause the disappearance of an aster by liquefaction, and the delayed development of the fertilised ovum after centrifuging is easily explicable on these grounds. It seems reasonable to assume that the maturation amphiaster is similarly sensitive to mechanical agitation, and its reformation in the centrifuged oocyte could presumably induce streaming movements. In support of this suggestion, it may be pointed out that the outbreak of this intense streaming activity starts near the surface of the "fatty" layer of the centrifuged egg. Other experiments have shown that the nucleus is always displaced into this region during centrifugal treatment, and it seems likely that the reformation of the asters is also started at this point. The high viscosity value (0·2 c.g.s. units) obtained in
these results agree excellently with the value found by Heilbrunn (1921) during the first maturation division in *Cumingia*, and is probably associated with the production of the comparatively viscous asters.

These large-scale streaming movements have also been observed by the author in the course of some work involving the cinematography of uncentrifuged oocytes of *Marthasterias glacialis*. Measurements of the characteristics of the movement in these eggs could not be obtained, as it is impossible to separate the granular layers by centrifuging, and the evidence rests only on the observation of the films when projected. The oocytes in question were ripe for shedding, and were in process of maturation in the sea water. Certainly the maturation of the oocyte is attended by considerable streaming activity.

This large-scale streaming movement is very similar in many respects to the endoplasmic streaming shown by *Amoeba blattae* (see Schaeffer (1920)) and to the cyclosis observed in plant cells, and it is possible that it may be quite unconnected with the formation of asters within the cell. This alternative hypothesis would imply that the energy supply for these movements is localised in the endoplasm, and it is interesting to consider the possibilities of this implication. Gray (1931) has discussed in brief the differences between streaming associated with contraction of an ectoplasmic sheath, and that of a purely endoplasmic character, and the two types seem to correspond in many respects with the alternative hypotheses of streaming movement produced by expansion of gelated asters, and purely endoplasmic streaming.

It remains to consider the source of energy of such endoplasmic streaming. Here the evidence obtained by measurement of the "small-scale" movements is of considerable interest. These movements have been photographed in normal uncentrifuged echinoderm eggs by the author, though only in centrifuged oocytes of *Sabellaria* has it been found possible to obtain measurements. The movements in these echinoderm eggs occur even before the breakdown of the germinal vesicle, and in cases showing no signs of maturation or aster formation, *e.g.* in immature oocytes of *Echinus esculentus*, which will mature only inside the body of the parent, and not in sea water, as do those of *Asterias*. It seems reasonable to suppose that such movements are connected with the permanent life of the protoplasm, and not with its temporary physiological state at a particular stage of development in the ovum. The heavy granules of these eggs have been shown by Heilbrunn (1928) and many others to be histologically identical with the mitochondria of other animal cells, and these have been frequently regarded as the active elements in protoplasmic life. Let us examine this in the light of the evidence of the above experiments.

It may be assumed that movements of any particles in the protoplasm may be produced by any or all of three types of reactions:

1. Normal Brownian movement, brought about by the collision of the granules with the surrounding molecules.

This type of motion is completely random in character. It has been shown on theoretical and empirical grounds that the average displacement of such a single particle in any given time depends only on the size of the particle and on the viscosity of the surrounding fluid (provided that the temperature remains constant, and that
the granules are not appreciably "crowded"—both of which conditions are fulfilled in the experiments above).

(2) The movements may be due to reactions taking place at the surface of the granules. Such reactions may involve active groups located in the surface layer of the granule itself and/or substances adsorbed on the granule-hyaloplasm interface. Neither implies that there is a uniform radial distribution of this activity over the surface of the granule—there may be a definite polarisation of the molecules in the granule so as to produce higher intensities of activity at certain points. A uniform distribution of this intensity would presumably result in an increased random motion of the granule; a polarised distribution in a more or less directed motion.

(3) The movements may be produced by reactions taking place in the hyaloplasm, independently of the presence of the granules. Such reactions might be essentially of the same type as those suggested in (2), but would concern the colloidal and crystalloidal components of the optically structureless hyaloplasm. If these reactions were more intense in some regions than in others, streaming movements would be set up, which would carry the inactive protoplasmic inclusions along with them.

In the technique of measurement employed in this paper, the movements of the granules are resolved into directed and random components. If we are averaging a number of granules to obtain these components, reactions of type (2) will only serve to increase the value of the random component (since a directed tendency with respect to an individual granule, when averaged for a number of granules, becomes equivalent to a random motion). But the values obtained for the viscosity of the hyaloplasm, based upon the application of the Einstein and v. Smoluchowski expression, agree excellently with those obtained by the author for Sabellaria, and by Heilbrunn (1926 b) for Cumingia, using the centrifugal technique only. It therefore seems reasonable to suppose that the reactions of type (2) are negligible. On the other hand, the directed movements are an indication of reactions taking place in the hyaloplasm, and such reactions are therefore comparatively intense.

Many students of living protoplasm have suggested with Regaud (1909) that the reactions characteristic of living matter take place at the surface of the mitochondria, the hyaloplasm serving mainly to transport the participants in the reactions. The writer believes that this work would indicate that such is not the case, but that the basis of living protoplasm is to be found in the hyaloplasm rather than in such large cellular inclusions, whose function still remains unexplained.

SUMMARY.

1. An attempt has been made to determine the viscosity of the hyaloplasmic fluid in the unfertilised eggs of Sabellaria alveolata. The extent of the Brownian movement of granules in the centrifuged eggs was determined by microcinematography.

2. The random character of the Brownian movement was masked by very pronounced streaming movements.

3. After correcting for these directed movements, an approximate value of 0.2 C.G.S. units was obtained for the absolute viscosity, which agreed with the results obtained by means of the centrifuge.
EXPLANATION OF PLATE I.

Four photographs showing the progress of the streaming motion in a single egg.

(a) Approximately 8 min. after centrifuging.
(b) and (c) at intermediate stages.
(d) Approximately 18 min. after centrifuging.

Note that in (d), the cap of fat particles at the upper right-hand corner has been very considerably dispersed, while the heavier granules at the lower end are only just commencing their redistribution.

The wrinkled membrane, which is not a fertilisation membrane (these eggs are unfertilised) is very clearly shown in (d).