The Effects of Ultra-centrifuging the Oocytes of Lumbricus terrestris.

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With Plate 17.

INTRODUCTION.

During the last fifty years a large amount of work has been carried out on the cytoplasmic inclusions of the germ-cells of invertebrates. Many of the conclusions expressed in this work were contradictory, and until the advent of the ultra-centrifuge it seemed as if some of the conflicting points would never be cleared up.

Previous Work with the Centrifuge.

Conklin in a paper in 1910 described the effects of centrifuging molluscan eggs at a speed calculated to give a force of, approximately, 600 times gravity. He found that with this force, the cytoplasm of the eggs became arranged into three layers. The light layer was composed of a grey, coarsely granular substance, the middle layer was clear, and the lower layer was formed of heavy, yellow yolk-spherules. This substance formed one-half the volume of the egg. Conklin found that the grey substance could be centrifuged out of the egg without interfering with its normal development. He suggested that both the yellow and the grey substances were not essential to development.

Gatenby (1919), working on Limnea stagnalis, found that the centrifuged egg showed three layers similar to Conklin's. He described the grey layer as being composed of yolk-spheres, and very little cytoplasm. The middle layer was clear cytoplasm. He did not agree with Conklin's statement that the yellow layer was formed of yolk, but suggested that it was formed of yellow mitochondria among which a few Golgi bodies...
were apparent, suspended in the cytoplasm. His description of the composition of the grey zone would explain why Conklin found that it was not essential for development.

Brambell (1934), working on vitellogenesis in mollusca, centrifuged the gonads of *Helix aspersa*, and *Patella vulgata*, at a force of 2,000 times gravity. He confirmed the statements of previous workers, that the oocytes were separated into three layers, which may be called light, middle, and heavy. He found that the middle layer contained the nucleus, and that the heavy layer was composed almost entirely of mitochondria. The Golgi bodies were not segregated but were found scattered throughout the cytoplasm. They consisted of rod-shaped granules which, in places, formed clouds in the cytoplasm. Brambell found, in *Helix* oocytes, that the spheres at the light pole of the cell went black or dark grey in Champy's fluid, appeared as vacuoles in Da Fano preparations, and did not show up at all in Mann-Kopsch preparations. In the centrifuged oocytes of *Patella* he found that the spheres of the light layer nearly always blackened in Mann-Kopsch, but only appeared as vacuoles in Flemming-without-acetic and Da Fano preparations. The granular mitochondria formed the heavy layer, and the Golgi bodies were again found to be distributed throughout the cytoplasm. As in *Helix* they were rod-like in form, and were found as figured by Ludford, plastered on to deutoplasmic spheres.

Experiments with the centrifuge were carried out by Nath (1930) when he was studying the Golgi apparatus in the fresh eggs of *Pheretima posthuma*. He used an ordinary hand centrifuge fitted with a motor, and centrifuged his material for 35 minutes at a speed of 1,000 revolutions a minute. The centrifuged material was then fixed in osmic acid for 2 hours. He, unlike Gatenby and Brambell, found that the Golgi bodies collected in the light layer. In agreement with these authors, he found that the mitochondria were heavy and went to the centrifugal

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1 The refringent globules in the ovarian cells of *Lumbricus*, seen by Gatenby and Nath, and believed by them to be Golgi bodies, are certainly fat. The real Golgi bodies were correctly described from Da Fano and Cajal preparations in their paper, but are not easy to see in fresh preparations.
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pole. Considering these results Nath came to the conclusion that his centrifuge experiments had proved the absence of yolk droplets in the cell, and stated that they clearly revealed the existence of only two types of inclusions, the Golgi elements and the mitochondria.

Previous Work carried out with the Ultra-centrifuge.

In 1934 Beams, King and Risley ultra-centrifuged the unfertilized eggs of Rana aurora for 2 minutes at a pressure of 40 lb. per square inch. They found that the contents of the egg became arranged into three layers: a yellow or white, fatty layer; a clear protoplasmic layer, and a centrifugal layer of heavy yolk containing the pigment granules. If an increase in pressure was applied, the eggs became split into a light fatty fragment and a heavy yolk and protoplasmic fragment. If the pressure was great enough, these fragments were flung out of the jelly envelope and burst.

The same authors working on the uterine gland-cells of the guinea-pig, in 1934, observed that the Golgi apparatus in the normal cell was situated between the nucleus and the lumen of the gland. When these cells were centrifuged, it was discovered that, if the force was acting towards the free end of the cell, the Golgi material streamed from its normal position, passed the nucleus, and collected in spherical masses at the basal or centripetal end of the cell. If the centrifugal force was acting in the opposite direction, i.e. towards the basal end of the cell, the Golgi material collected at the free end near the cell membrane, and sometimes was forced through it. If this was the case it was found in the lumen in the form of spherical masses.

Beams, Muliyil, and Gatenby in 1934 showed the effect of ultra-centrifuging on the spermatocytes of Helix. They observed that the Golgi bodies and mitochondria, which were both visible in the living cell, moved to opposite poles. The Golgi apparatus was collected at the light end, while the mitochondria were collected in a mass at the centrifugal pole.

Beams and King, again in 1934, while working on the liver of the rat, found that the mitochondria of the centrifugal hepatic cells collected at the centrifugal pole. In the normal cell the mitochondria were distributed through the cytoplasm. They
also noticed that, although the mitochondria might be packed in a dense mass against the cell membrane, they did not lose their usual form.

Continuing their experiments with the centrifuge, Beams and King in 1935 observed that the nucleus of the spinal ganglion cells of the rat became stratified into three layers; one, at the centrifugal pole, containing the nucleolus, the second, lighter than this, consisting of a chromatic layer, and a centripetal layer composed of nuclear sap.

Later in 1935, these workers investigated the effects of ultracentrifuging the root tips of the Bean. Their results showed that the nucleolus was displaced to the centrifugal pole, where it rested in contact with the nuclear membrane. The lightest substance in the cell, the lipoid material, was found collected just under the cell membrane at the centripetal pole with the vacuome immediately below. A layer of osmiophilic platelets was arranged below this, and then a clear area of cytoplasm containing the nucleus. The pseudo-chondriome was situated below this clear region. If there was no starch in the cell, the plastidome was found at the centrifugal pole. If starch was present, it was found to be the heaviest inclusion in the cell.

Later in 1935, Muliyil, centrifuging the thoracic and cerebral ganglia of Periplaneta, Blatta, and Gryllus at 300,000 times gravity, found that the Golgi bodies were always displaced to the centripetal pole. These bodies showed distinct chromophilic and chromophobic areas. He also found a large number of small dark granules, scattered between this light layer and the collection of mitochondria at the centrifugal pole. These granules readily stained in neutral red, and he considered that they were pre-existing bodies which showed a topographical relationship with the Golgi bodies in some cells.

The nucleus became elongated and moved towards the centrifugal pole, when subjected to high pressures. The nuclear substance separated out into three layers, the light nuclear sap, the chromatin, and the heavy nucleolus.

Following up the work carried out by Beams and King, and Muliyil on nerve-cells, Brown¹ made a study of the effects of

¹ This Journal, Vol. 79, Part 1.
ultra-centrifuging in vertebrate neurones. In agreement with
the former workers he found that the Golgi apparatus was light,
and that the mitochondria, granular, and rod-shaped bodies
were heavy. The neutral-red bodies were blackened by osmic,
and although light, with a similar specific gravity to the Golgi
bodies, they were distinct and separate from them.

MATERIAL AND METHODS.

The material used in these studies consisted of the ovaries of
Lumbricus terrestris. The ultra-centrifuge used was one
similar to that developed by Beams, Weed, and Pickles, and
the apparatus, used to compress the air necessary to drive the
rotor, was constructed by Mr. R. H. J. Brown, M.Sc., of this
Department.

The pH of the coelomic fluid of the earthworm was taken,
and a Hédon Fleig solution having the same pH made up.
In this solution NaHCO₃ was omitted and an equivalent amount
of NaCl substituted. This solution proved very satisfactory,
but it was found to be useless for work with neutral red, which
was precipitated in a short time when made up in such a solu-
tion. As an isotonic salt solution did not precipitate neutral
red it was just as satisfactory, and was much simpler to make
up in large quantities. This was used throughout instead of the
modified Hédon Fleig solution.

A worm was lightly anaesthetized with chloroform and dis-
sected in isotonic salt solution. The ovaries were removed, one
being placed in salt solution in the rotor of the centrifuge, and
the other left in salt solution, as a control. Several worms were
used in each experiment. The ovaries in the rotor were centri-
fuged for 4 minutes at a pressure of 10 lb. per square inch,
calculated to give a centrifugal force of about 40,000 times
gravity.

After centrifuging the ovaries were removed from the rotor
by a fine needle, and transferred immediately to a fixative.
The interval between centrifuging and fixation was made as
short as possible, so as to prevent the redistribution of the cell
inclusions. All controls were placed in the same capsule as the
centrifuged material, and received the same treatment throughout each experiment.

The methods of fixation used for the study of the Golgi apparatus were 2 per cent. osmic acid, Kolatchev's osmic method as modified by Nassanov, and Da Fano's formalin-silver technique. It was found necessary in the Kolatchev and osmic methods to incubate in osmic acid at 35° C. for 6 days. After washing, at 35° C. for 24 hours, the material was up-graded in alcohol and imbedded by the usual method. Sections were cut at 2.5 μ. In the case of the Da Fano technique, the procedure was as follows: 1-1½ hours fixation, 24 hours in 1.5 per cent. silver nitrate. The silver nitrate solution was always freshly prepared, as its action was found to be uncertain if it was more than a few days old. The material was then left for 12 hours in the reducing fluid, up-graded in alcohol, imbedded, and sectioned at 2.5 μ.

At first the ovaries were examined whole, but this method was abandoned, as it was not always possible to make an accurate study of detail. Sections toned with gold chloride were found to show up the Golgi apparatus more clearly than those left untoned.

For the study of mitochondria, Champy's fluid was used as a fixative. Sections were cut at 2.5 μ, and subsequently stained in iron-alum haematoxylin.

For vital studies the National Aniline and Chemical Company's neutral red was used. Solutions of strengths varying from 1 in 30,000 to 1 in 5,000 were made up. Of these it was found that a solution of 1 in 5,000 was the most efficacious for supra-vital staining. The weaker solutions had not the power to immobilize the coelomic epithelium cells covering the ovary, and so the dye was prevented from approaching the ova. The ovaries were stained supra-vitally for 3 hours, and centrifuged for 4 minutes at a pressure of 10 lb. per square inch.

For the identification of fats, osmic acid, Nile Blue sulphate (Grücker), Sudan IV (BDH), were used.

The method for staining in Sudan IV was that described by Ray and Whitehead (1935). It is as follows: ovaries fixed in formol made up in isotonic saline were placed in 50 per cent. alcohol for five minutes. They were then transferred to the
staining solution of Sudan IV (which had previously been prepared) for 30 minutes at 37°C. The ovaries in this solution were turned over after 15 minutes so as to ensure even staining. The ovaries were then changed to 50 per cent alcohol for a few seconds, and into distilled water for a few minutes. They were next stained in filtered haemalum, blued in alkaline tap-water for a few minutes, and mounted in glycerine jelly. The coverslip was pressed gently, the oocytes squeezed out, and examined. The globules were stained a bright red.

The Schultz cholesterol reaction, as described by Whitehead (1934), and which gives a blue-green colour when cholesterol is present, was applied in order to investigate the composition of the fat globules. Ovaries fixed in 10 per cent formol-saline for 2 days were placed in a 2.5 aqueous solution of violet iron alum and incubated at 37°C for 3 days. The ovaries were then washed in distilled water and transferred to a slide. They were blotted dry, and a few drops of a mixture, made up of equal parts of glacial acetic and concentrated sulphuric acid, were added. A cover glass was then put on and the ovaries gently squeezed. After a few minutes the fat globules showed a blue-green colour, indicating the presence of cholesterol.

The methods used for testing for glycogen were Best’s carmine, and gum iodine.

Observations.

The Uncentrifuged Cell.

The Golgi bodies in the uncentrifuged cell are distributed through the cytoplasm, but are usually most numerous in the region of the nucleus. They are most easily studied in the ripe oocytes found near the apex of the ovary. In agreement with Harvey I find the form of the Golgi bodies to be fairly stout, crescent-shaped rodlets which are visible in the living, unstained, normal egg. Their shape is shown very clearly in osmic preparations (fig. 1, Pl. 17) where the ovaries have been incubated in 2 per cent osmic acid at 35°C for 6 days. Treatment with osmic acid for any shorter period of time did not succeed in blackening the Golgi bodies.

Fat globules, as described by Harvey, 1931, are found in most
of the young oocytes, and in all the older eggs. These, in the normal egg, are large and are distributed through the cytoplasm. They are easily dissolved out of the cell by xylol. In every experiment, regardless of the fixative, the control material was found to have undergone much more severe shrinkage than the centrifuged material. This was due to the xylol dissolving out the fat globules which were distributed through the cell. In the centrifuged material, as the fat was all collected to one end, shrinkage took place only in that region.

In the normal egg the mitochondria in the living state show as a mass of very small granules, which exhibit Brownian movement. They are so numerous that they give a cloudy appearance to the cell.

In the normal living cell no arrangement of mitochondria into chains could be seen.

Effects of the Ultra-centrifuge.

In previous experiments with the ultra-centrifuge the nucleolus has always been found to go to the centrifugal pole of the nucleus.

Thus the orientation of a newly centrifuged cell is decided at first by observing the position of the nucleolus. The living, unstained, centrifuged egg is shown in fig. 2, Pl. 17, stratified into four layers. Globules can be seen collected in a mass at the centripetal or light pole. Just below them lies the nucleus with the nucleolus at its centrifugal side. Immediately below the nucleus can be seen a dense cloud of mitochondria, and heaviest of all is the collection of a non-granular substance.

As has been shown by the tests mentioned in the previous section, the substance which collects in globules at the centripetal pole is a weak sudanophil fat containing cholesterol.

When treated with osmic acid, it was found that unless the globules were very heavily blackened they were readily dissolved out by the xylol in the balsam. This suggests that the fat is very weak. Treatment of the ovaries with Nile Blue sulphate stained the globules so weakly that the test was not considered to be significant.

The Golgi bodies, which in the centrifuged cell come to lie
beside and beneath the nucleus, are shown in both osmic and silver preparations (figs. 1, 3, 4, Pl. 17) to be long crescent-shaped bodies, thicker in the centre, and tapering towards each end.

The granular mitochondria, which are heavier than the Golgi substance, lie in a dense mass below it. They are so numerous that when centrifuged they constitute approximately one-third the volume of the cell. In this study the mitochondria have always been found to be granular. In some fixed material they are arranged in lines, giving superficially the appearance of filaments.

The heaviest substance in the egg of Lumbricus is demonstrated by the ultra-centrifuge. It shows as a collection of highly refringent, globular masses, when treated with osmic acid (figs. 1, 3, Pl. 17).

These obviously are not of a fatty nature, since fat would move to the centripetal pole and would blacken with osmic acid. Tests for glycogen showed clearly that there was none present in this region.

When freshly dissected ovaries are placed in a 1 in 5,000 solution of neutral red the coelomic epithelium first takes up the stain. After about 1½ hours’ time the oocytes show a diffuse pink colour. The neutral-red bodies first appear after 2½ hours’ staining. They are distributed throughout the cell, singly or in small groups of three or four, and are bright cherry red in colour. When the ovaries have been centrifuged, these neutral-red bodies come to lie beside and beyond the nucleus (fig. 5, Pl. 17). From this observation it would seem that their specific gravity is very nearly the same as that of the Golgi apparatus. As redistribution of the cytoplasmic inclusions after centrifuging is slow, an experiment was carried out, centrifuging the ovaries first, and then staining with neutral red. After 3 hours the neutral-red bodies appeared distributed through the whole oocyte, and not as might have been expected, in a layer close to the nucleus. These results suggest that the neutral-red bodies, in this case, are not preformed, but are simply the results of segregation of the dye by the living cell.

It was found that in staining the oocytes, neutral red had the
effect of making the granular mitochondria run into chains, which tended to form a network when fixed.

**DISCUSSION.**

The observations made during this investigation prove that the egg of *Lumbricus* has a much more complicated structure than former workers have described.

The statement made by Gatenby and Nath (1926) and repeated by Nath in 1930, that the egg of *Lumbricus* contained no elements other than the mitochondria and Golgi apparatus, was contradicted by Harvey, who stated that there were three constituents, the mitochondria, the Golgi bodies, and fat. I find that Harvey's statement was correct for observations made on uncentrifuged eggs, but from the result of ultra-centrifuging I believe that there is a fourth element present in the cytoplasm. This substance is only apparent in the strongly centrifuged eggs, where it is found to be the heaviest inclusion in the cell. In living, unstained eggs, and in those stained with neutral red, it showed as a clear non-granular substance. Its reactions are negative to tests for protein, glycogen, and fats. In cells fixed by the osmic or Da Fano methods it appeared as if composed of globular masses. It seems to me that as this substance is not visible in the control oocytes, it must be a fluid present in the form of minute droplets. When centrifuged, these minute droplets collect and, being heavier than the cytoplasm, are flung towards the centrifugal pole.

Weiner in 1930 and Harvey in 1931 both mention the presence of weak fat in the oocytes. Nath himself said, in 1930, that he had discovered a point of considerable importance, namely that the spherules in *Pheretima* contained fat, but in spite of this evidence continued to deny the presence of this substance as a distinct inclusion in the cell.

Foot and Strobell describe two types of inclusion observed in their work on *Allolobophora*, the 'osmiophile' granules and the 'archoplasmic' granules.

Harvey noticed that the contents of these granules in his osmic preparations were dissolved out by xylol. In my preparations by osmic and Mann-Kopsch methods the contents of the
spherules were also dissolved out by xylol. If the blackening was very deep this process took 2 or 3 days.

Speaking of the appearance of the Golgi bodies after light impregnation with 'Kolatchev', Nath says, 'the rim of the vesicle has completely missed impregnation and the blackened fatty contents have been decolourized by xylol. Consequently the vesicle appears as a colourless or a slightly greyish body, reminding one of the so-called "yolk-droplets" of Harvey'. Nath repeatedly states that there is no yolk in the earthworm egg, either fatty or albuminous, yet except for treatment with osmic acid he does not seem to have considered making any other tests for the presence of fats.

Harvey found that the spherules became pale yellow with Sudan III and a deep scarlet after Scharlach R. He found that they were also soluble in 90 per cent. alcohol. He concluded that they were of a weak fatty nature, thus confirming Weiner's observation (1980). Staining with Sudan IV which, according to Whitehead, is the most trustworthy fat stain, I got a definitely positive reaction for fat, which on subsequent tests was found to contain cholesterol. There seems little doubt that these globules are droplets of fat, and are not connected with the Golgi bodies. Inspection of any centrifuged preparation makes this apparent. It seems unfortunate that Nath's insistence on the vesicular nature of the Golgi bodies prevented him from making full use of the discovery of what he calls his 'point of considerable importance'.

In Patella, Ludford (1921) found that the Golgi apparatus was composed of short rods surrounding the archoplasm. The Golgi elements finally became scattered through the cell. This statement was confirmed by Brambell (1924) working with the oocytes of Helix and Patella. Later, Harvey (1931) described and photographed the Golgi bodies showing that they were curved rodlets. With both osmic and silver techniques my preparations show the Golgi bodies to be similar curved rodlets. In the uncentrifuged cell they are scattered through the cytoplasm, but are most numerous around the nucleus. In the centrifuged cell they collect centrifugally to the nucleus. In his experiments with the ordinary centrifuge Brambell did not
succeed in displacing the Golgi bodies. I found that to collect these bodies a greater pressure was needed than that required to separate out the other inclusions. This seems to show that the densities of the Golgi bodies and the cytoplasm are very nearly the same.

Nath mentions that in Da Fano preparations the Golgi bodies appear invariably as solid, blackened granules. This may be due to the great amount of shrinkage which took place in these preparations, for I found that this method showed very clearly the slightly crescent-shaped form of the Golgi bodies. One of the reasons Harvey gives for his former misconception of the appearance of the Golgi elements is that they were based on Da Fano preparations which, he found later, were definitely distorted. The evidence submitted by former workers suggests that the form of the Golgi bodies in these oocytes is definitely rod-shaped. This suggestion has been made a certainty by the evidence supplied by the ultracentrifuge.

From observations made of the nucleus it seems evident that it contains three distinct substances, the nuclear sap, which is the lightest, chromatin, and the nucleolus. Every worker with the ultra-centrifuge has found that the nucleolus is very heavy. Its position in the nucleus can then be taken as a fairly safe guide to the orientation of the newly centrifuged cell.

Concerning the neutral-red bodies two conclusions can be drawn from the work reviewed. The first, illustrated in Muliyyil's material, shows that there are definite preformed bodies in the ganglia of orthopteran insects which take up neutral red. On the other hand, there is considerable evidence (Beams and Goldsmith, 1929; Gatenby, 1932; Douglas, Duthie, and Gatenby, 1933) that the neutral-red bodies are artifacts due to the segregation of the dye by the cell. Muliyyil also found this type of neutral red body in his ganglion cells when the neutral red used was of a strength of 1 in 5,000. The ultra-centrifuge has proved a very satisfactory means of giving decisive information about the identity of the neutral-red bodies. If cells, previously centrifuged, are stained with neutral red, and the red granules are seen collected into a layer, then the granules are obviously pre-
formed. In the centrifuged cell, containing no preformed granules, treatment with neutral red would show the red granules scattered throughout the cell. Since the latter type of granules are found in Lumbricus, it is evident that here we are not dealing with preformed bodies.

In 1895 the yolk-nucleus was described by Calkins as being composed of a mass of granules. Foot and Strobell (1901) called these bodies ‘archoplastic’ granules. Subsequent workers seem generally agreed that these inclusions were mitochondria. Harvey in 1925 described the mitochondria of Lumbricus as being thread-like in form. In 1931 he revised his former work, and came to the conclusion that they were filamentous and granular. Nath contradicted Harvey in 1930, and stated that there was only one type of mitochondria present in Lumbricus, and that was granular. He suggested that the granules might become arranged into linear series, and give the appearance of filaments. King observed this phenomenon when working on the oogenesis of Lithobius (1924). I have been unable to see any filamentous mitochondria in the living, unstained egg of Lumbricus. In material stained in neutral red, and fixed in osmic acid, the mitochondria show as chains of granules forming a network. This appearance is abnormal.

My thanks are due to Professor Gatenby for suggesting this problem, and for his kind help and criticism during its investigation.

**Summary.**

1. The ultra-centrifuged cell becomes stratified into four layers: fat, cytoplasm containing the nucleus and Golgi bodies, mitochondria, and a clear substance.

2. The fairly large spherical, greyish bodies seen in the living oocytes of Lumbricus are fat, not Golgi bodies. This fat contains cholesterol.

3. The Golgi bodies are slightly curved, or straight rods, found, in the ultra-centrifuged cell, in a layer placed centrifugally to the nucleus.

4. The neutral-red granules are artifacts. If centrifuged they collect beside and beyond the nucleus.
5. The mitochondria are granular and may become aligned into chains.
6. The heaviest substance in the cell is probably a fluid.

References.

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EXPLANATION OF PLATE 17.

F., fat; G., Golgi bodies; H., heavy material, M., mitochondria, N., nucleus; NL., nucleolus; NR., neutral-red granule.

Fig. 1.—Mann-Kopsch preparation.
Fig. 2.—Living, unstained centrifuged egg.
Fig. 3.—Cell, stained in neutral red, centrifuged, and post-osmicated, showing the mitochondria arranged in chains.
Fig. 4.—Da Fano preparation showing curved rod-shaped Golgi bodies.
Fig. 5.—Living cell stained with neutral red and then centrifuged.
Fig. 6.—Mann-Kopsch preparation. Control cell.
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