The structure of the mitochondrial cloud
of *Xenopus laevis* oocytes

By F. S. BILLETT\(^1\) AND ELIZABETH ADAM\(^1\)

From the Department of Biology, University of Southampton

SUMMARY

The ultrastructure of the mitochondrial cloud (Balbiani body) of the pre-vitellogenic oocytes of *Xenopus laevis* has been examined using transmission and stereoscan electron microscopy. Examination of conventional thin sections confirm previous observations which suggest that the cloud consists essentially of many thousands of mitochondria and numerous small vesicles; larger clouds, in oocytes greater than 200\(\mu m\) in diameter, contain relatively more vesicles. Using a standard electron microscope at 100\(kV\) very long and coursing arrays of mitochondrial profiles can be detected. The presence of very long mitochondrial elements has been confirmed using a high voltage microscope operating at 500–1000\(kV\). Stereoscan preparations, isolated from pre-vitellogenic oocytes, lend some support to the view that the mitochondrial cloud may consist of a mass of long filamentous mitochondria and the possibility that there are large continuous regions of mitochondrial material cannot be ruled out.

INTRODUCTION

During the development of many oocytes large numbers of mitochondria are formed and frequently aggregate as well defined structures either alone or with other cytoplasmic organelles. Various names, such as ‘yolk nucleus’ and ‘Balbiani body’ have been given to these structures. However, as in many cases mitochondria are the predominant feature, the term ‘mitochondrial cloud’ is often more appropriate and has frequently been used (for references, see Raven, 1961). A good example of such a cloud may be seen in the pre-vitellogenic oocytes of *Xenopus laevis*.

Most of the descriptions of the mitochondrial aggregates seem to assume that they consist of large numbers of discrete mitochondria. However, during our study of the mitochondrial cloud of *Xenopus* we became impressed with the length, complexity, and apparently non-random arrangement of the mitochondrial profiles; the image is not quite that expected from a simple aggregate of separate mitochondria. The length of the profiles is particularly striking when 1\(\mu m\) sections are examined by transmission electron microscopy at high accelerating voltages. These observations, together with those we have made on stereoscan preparations of the isolated cloud, suggest that in several

\(^1\)Author’s address: Department of Biology, Medical and Biological Sciences, Bassett Crescent East, University of Southampton, Southampton, SO9 3TU, U.K.
regions at least the mitochondrial cloud is a continuous structure rather than a collection of discrete mitochondria. Thus the results described in this paper appear to be complementary to those reported for other cells in which large mitochondrial structures have been identified (Chlamydomonas, Schotz, Bathey, Arnold & Schimmer, 1972; Arnold, Schimmer, Schotz & Bathey, 1972; Euglena, Calvayrac, Van Lente & Butow, 1971; Osafune, 1973; yeast, Hoffman, Hoffman & Avers, 1973; Chorella, Atkinson, John & Gunning, 1974; mammalian liver, Brandt, Martin, Lucas & Vorbeck, 1971; mammalian lymphocytes, Rancourt, McKee & Pollack, 1975).

MATERIALS AND METHODS

Ovarian tissue was obtained from young female Xenopus measuring between 4 and 5 cm from the top of the head to the cloaca. The animals were starved for 2–3 days before killing them with an injection of Nembutal (6 mg in 0·1 ml). The ovaries were removed approximately 10 min after the fatal injection and placed in a culture medium (see below) before being processed for microscopic study. In some cases tissue was taken from immature ovary which had been cultured for several days at 20 °C in a culture medium consisting of 70 % Eagle’s basal medium and 10 % calf serum. The cell structure of oocytes cultured under these conditions appears to be identical with that obtained from the freshly killed animal.

For light and transmission electron microscopy small pieces of tissue, approximately 2 mm cube, and derived from the tip of an ovarian lobe, were fixed for 2 h at approximately 5 °C in 1 % osmium tetroxide contained in veronal acetate buffer at pH 7·4 (Palade, 1952). In our experience for Xenopus ovarian tissue osmium appears to be a better fixative than glutaraldehyde/formaldehyde mixtures (Karnovsky, 1965). Following normal practice the tissue was dehydrated in a graded series of alcohols and then passed successively to propylene oxide and a 50:50 mixture of propylene oxide and araldite, before finally embedding in araldite.

For light microscopy 1 μm sections were cut on a Huxley ultratome and stained with 1 % toluidine blue in 1 % Borax for 1–2 min. The sections attached to slides were then washed with distilled water to remove excess stain and dried on a hot plate.

For normal transmission electron microscopy thin sections were cut at approximately 80 nm on an LKB ultratome. These were stained with uranyl acetate followed by lead citrate (Reynolds, 1963), and examined on a Philips Em 300 at an accelerating voltage of 80 kV. For thick section electron microscopy, using high accelerating voltages, the Huxley microtome was used to cut sections varying from 0·5 to 2·0 μm. The sections were flattened by placing them on a dish of hot water (ca. 60 °C) for 30 sec before attaching them to normal electron microscope grids. Some sections were stained with hot (60 °C)
Mitochondrial cloud of *Xenopus*  

2% alcoholic phospho-tungstic acid (Locke & Krishnan, 1971), whilst others were left unstained. These sections were examined on the Philips EM 300 at an accelerating voltage of 100 kV and on the AEI high voltage machine at accelerating voltages of up to 1000 kV (Favard & Carasso, 1973; Glaubert, 1974).

Scanning electron microscope preparations were made from mitochondrial clouds removed from oocytes of known size. Fine tungsten needles, mounted in Prior micromanipulators, were used to remove the clouds from the oocytes and to manoeuvre them on to small pieces of glass prepared from coverslips. These procedures were carried out in culture medium. The isolated cloud was then fixed in 1% osmium tetroxide buffered in veronal acetate. The fixative was added dropwise in the vicinity of the preparation after the excess culture fluid had been carefully removed. After 5 min fixation the osmium was replaced by buffer and this in its turn was gradually replaced by acetone by treating the preparation with a graded series of acetone/water mixtures leading to pure acetone. The specimen was then dried by allowing the acetone to evaporate in air. The piece of glass with the specimen attached was stuck onto a stereoscan stub with double sided sellotape and the preparation was then coated with gold approximately 20 nm thick using a Polaron unit (E 5000). The specimens were examined on a Cambridge stereoscan electron microscope.

**OBSERVATIONS**

**Light microscopy**

In pre-vitellogenic oocytes the mitochondrial cloud can be readily detected in fresh tissue using normal transmitted illumination (Fig. 1). For micromanipulation however, we would recommend the use of dark field illumination such as that provided by the Wild M5 stereomicroscope; under these conditions the cloud can be detected as an opaque white patch. One micrometre sections, embedded in araldite and stained with toluidine blue (Fig. 2), clearly reveal the changes in the gross morphology of the cloud in relation to the size of the oocyte. Using approximately central sections of oocytes the toluidine blue preparations were used to relate the change in the morphology of the cloud to the size of the oocyte, the germinal vesicle and the number of nucleoli. These results are summarized in Table 1.

**Electron microscopy**

Electron micrographs of thin, approximately 80 nm sections of the cloud show that the mitochondria are the dominant feature (Fig. 3) although large numbers of small vesicles (Fig. 5), approximately 0.1–0.2 μm in diameter, are also present. Profile counts of these components indicate a relative increase in the number of vesicles, compared with mitochondria, as the cloud increases in size (Table 2). One or two electron-dense profiles, ca. 2–5 μm in diameter, can usually be detected in the cloud (Fig. 3). These are probably lipid droplets;
Table 1. *Arrangement of mitochondria in relation to oocyte size in Xenopus laevis*

<table>
<thead>
<tr>
<th>Arrangement of mitochondria</th>
<th>No. of sections measured</th>
<th>Oocyte diameter ($\mu$m) $\bar{x}$ Range</th>
<th>Germinal vesicle diameter ($\mu$m) $\bar{x}$ Range</th>
<th>No. of nucleoli in section $\bar{x}$ Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peri-nuclear</td>
<td>14</td>
<td>82 63–95</td>
<td>46 29–64</td>
<td>2 —</td>
</tr>
<tr>
<td>Cap</td>
<td>11</td>
<td>90 77–101</td>
<td>50 40–64</td>
<td>2 —</td>
</tr>
<tr>
<td>Spherical cloud</td>
<td>25</td>
<td>165 110–247</td>
<td>75 48–121</td>
<td>13 6–21</td>
</tr>
<tr>
<td>Dispersing cloud</td>
<td>19</td>
<td>238 187–321</td>
<td>109 73–143</td>
<td>24 13–49</td>
</tr>
</tbody>
</table>

Table 2. *Density of E.M. profiles in mitochondrial cloud of Xenopus laevis*

<table>
<thead>
<tr>
<th>Oocyte diameter ($\mu$m)</th>
<th>No. of sections measured</th>
<th>Profiles/100 sq. $\mu$m $\bar{x}$ ± S.E.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>60–170</td>
<td>Mitochondria</td>
<td>9</td>
<td>519 ± 80</td>
</tr>
<tr>
<td></td>
<td>Vesicles</td>
<td>9</td>
<td>140 ± 17</td>
</tr>
<tr>
<td>200–280</td>
<td>Mitochondria</td>
<td>8</td>
<td>393 ± 42</td>
</tr>
<tr>
<td></td>
<td>Vesicles</td>
<td>8</td>
<td>170 ± 19</td>
</tr>
</tbody>
</table>

they generally occur towards the edge of the cloud and may be surrounded by the vesicular component or have mitochondria closely applied to their outer surface.

Closer examination of the mitochondrial profiles reveals a fairly typical structure showing transverse cristae and mitochondrial granules. The longitudinal profiles have an average length of 2 $\mu$m but they can be as long as 5 $\mu$m. Although not frequent, branched profiles occur throughout the cloud. Counts of the number of profiles/unit area indicate a thinning out of the cloud as it increases in size (Table 2). The profiles are fairly evenly distributed but compact aggregates of profiles are dispersed throughout the cloud (Fig. 5) and groups of circular and longitudinal profiles are a regular feature (Fig. 6).

Fig. 1. Pre-vitellogenic oocyte approximately 150 $\mu$m in diameter. *mit.c.*, mitochondrial cloud *ca.* 40 $\mu$m diameter; *g.v.*, germinal vesicle. Fresh unfixed tissue. × 430.

Fig. 2. 1 $\mu$m section of lobe of immature ovary of *Xenopus* showing prominent mitochondrial clouds (*mit.c.*) in oocytes approximately 200 $\mu$m in diameter. Fixed in osmium, stained with toluidine blue. × 80.

Fig. 3. Montage of electron micrographs of thin (*ca.* 80 nm) sections covering about one-third total area of central section through a mitochondrial cloud. Oocyte diameter *ca.* 200 $\mu$m, from ovarian tissue cultured for 48 h. *L.d.*, lipid droplet. Fixed in osmium, stained with uranyl acetate and lead citrate. Accelerating voltage 80 k V. × 4000.
Mitochondrial cloud of Xenopus
Examination of thick sections, from 0·5 to 1·0 μm, with accelerating voltages ranging from 100 to 1000 kV shows that the mitochondria are much longer than might be imagined from observations on more conventional thin sections. Fig. 4 shows a montage derived from a series of low power electron micrographs of 1 μm sections (taken at 100 kV) of part of a mitochondrial cloud from an oocyte approximately 300 μm in diameter. In such sections many of the longitudinal profiles are greater than 6 μm in length and they frequently occur in groups which appear to course together over significant distances. Working at 100 kV, and at a magnification of 20000, structural detail of the mitochondria can be detected in unstained 0·5 μm sections (Fig. 7). Examination of 1·0 μm sections at 1000 kV, combined with the use of a tilt facility to obtain stereo pairs, reveals that the mitochondrial cloud contains mitochondrial structures of indefinite length (Fig. 8 A, B). The overall impression gained from such preparations is that the cloud consists of a tangled mass of very long mitochondria.

**Stereoscan preparations**

Using tungsten needles operated by micromanipulators it is a relatively easy matter to remove mitochondrial clouds from oocytes of 200 μm diameter and above. It is perhaps significant that the cloud behaves as a whole and does not fall apart during manipulation. Frequently the cloud remains attached to the germinal vesicle when the oocyte is teased apart by the micro-needles. The subsequent procedures involving the manoeuvring of the cloud onto a fragment of a glass coverslip and the dehydration process using acetone are difficult. The cloud does not stick readily to the glass surface and the rapid evaporation of the acetone solutions causes constant movement of the specimen.

Low power stereoscan pictures of the isolated cloud reveal a flattened mass which possesses filamentous structures both at the periphery and in the interior of the material (Figs. 9, 10). These filaments vary in thickness from 0·1 to 0·7 μm. Some of the larger filaments contain smaller ones (Fig. 11) which correspond in size to the mitochondrial structures seen in the transmission electron micrographs. Apart from the filaments, the isolated cloud, although generally amorphous, appears to contain strands of material whose thickness roughly corresponds to mitochondrial elements (Fig. 10).

---

Fig. 4. Montage of electron micrographs of thick (ca. 1 μm) sections of mitochondrial cloud from an oocyte ca. 300 μm in diameter. Approximately 25% of central section through cloud. Compare with Fig. 3. From tissue cultured for 8 days. Fixed in osmium and stained with phosphotungstic acid. Accelerating voltage 100 kV. Nucl., nucleolus; g.v.m., membrane of germinal vesicle. × 3200.

Figs. 5 and 6. Electron micrographs of thin sections (ca. 80 nm) of selected areas of mitochondrial cloud showing non-random arrangement of profiles. Small compact aggregates of profiles are shown in Fig. 5. Groups of circular and longitudinal profiles are illustrated in Fig. 6. Note also the presence of numerous vesicles (ves.). Fixed in osmium and stained with uranyl acetate and lead citrate. × 24000.
DISCUSSION

The electron microscope observations of Wartenburg (1962) and Balinsky & Devis (1963) clearly established that in \textit{Xenopus} pre-vitellogenic oocytes the 'yolk nucleus' or 'Balbiani body' consisted of large numbers of mitochondria associated with numerous small vesicles. Observations such as these confirmed the idea that in many cases the term yolk nucleus was ill conceived as there was little evidence that this body was actually concerned, at least directly, with the formation of yolk. Obviously, in oocytes, such as \textit{Xenopus}, where mitochondria are the dominant feature of the Balbiani body the structure must be regarded as a centre for mitochondrial replication and differentiation, and it is not surprising that in recent years much attention has been paid to the oocytes of \textit{Xenopus} by those concerned with aspects of mitochondriogenesis (Chase & Dawid, 1972; Webb, La Marca & Smith, 1975). Our own observations broadly confirm those of Wartenburg and of Balinsky & Devis, referred to above, but we feel that in some important respects they are significantly different and suggest a less conventional view of the mitochondrial cloud. Before discussing the mitochondrial cloud itself it seems worth recording some general points which arise from the present observations. These concern the choice of fixative and a note on cytoplasmic structures, other than mitochondria, in the pre-vitellogenic oocyte.

After several years experience in this laboratory working on both amphibian (Al-Mukhtar & Webb, 1971) and fish oocytes (Kanobdee, 1975) we would still recommend osmium tetroxide, buffered with veronal acetate (Palade, 1952) as the best fixative for this kind of material. The obvious alternative based on formaldehyde and glutaraldehyde mixtures (Karnovsky, 1965) although it works well on other amphibian tissues (Billett & Gould, 1971) does not, in our hands, produce satisfactory results with ovarian tissue. Other workers, however, report good results on \textit{Xenopus} oocytes using either Karnovsky's mixture (Coggins, 1973) or glutaraldehyde alone (van Gansen & Schramm, 1974).

Apart from the mitochondria the cloud contains numerous small vesicles approximately 0·1 \(\mu\)m in diameter. The profile counts indicate that they become relatively more numerous in the mitochondrial clouds of larger oocytes (Table 1). Following Balinsky & Devis these vesicles may be referred to as a form of

---

Fig. 7. An electron micrograph of a 0·5 \(\mu\)m section of part of a mitochondrial cloud. Compare with electron micrographs of thin sections shown in Figs. 5 and 6. From an unstained section examined at 100 kV. Note length of mitochondrial profiles. \(\times\) 19000.

Fig. 8A and B. A stereo pair of electron micrographs from a 1 \(\mu\)m section from the edge of a mitochondrial cloud. Note apparent continuity of mitochondrial structure. Fixed in osmium and stained with phosphotungstic acid. Accelerating voltage 1000 kV; \(\pm\) 6° tilt. \(\times\) 11300.
endoplasmic reticulum. Outside the cloud these vesicles are far less numerous. We are puzzled by the emphasis that Balinsky & Devis place on pitted membrane structures, i.e. annulate lamellae; in our material such structures are rather difficult to find in oocytes ranging in size from 50 to 300 μm diameter and we can find no evidence to suggest they are involved in the formation of vesicles of endoplasmic reticulum. It seems more likely that these vesicles are formed within the mitochondrial cloud. 'Mitochondrial cement' (André, 1962) or nuage material associated with mitochondria (Al-Mukhtar & Webb, 1971) is not a characteristic feature of mitochondrial clouds and although it may initiate mitochondrial replication (Balinsky & Devis, 1963) there is no evidence to suggest that it is necessary for the continuation of the process. As small aggregates of mitochondria associated with nuage material are found in the oogonia and primordial germ cells of Xenopus (Al-Mukhtar & Webb, 1971) it seems more reasonable to suppose that the material may be some kind of germ cell determinant (Smith, 1966; Williams & Smith, 1971; Webb, 1976). A fairly constant feature of sections of the mitochondrial cloud is the presence of one or two electron-dense profiles about 1 μm in diameter; these are probably lipid droplets.

The origin and fate of the mitochondrial cloud can be easily determined by studying sections of oocytes over the size range 50–350 μm diameter. The observations, summarized in Table 1, are broadly in agreement with Balinsky & Devis (1963). The cloud seems to arise from several small mitochondrial aggregates which are dispersed evenly around, and close to, the membrane of the germinal vesicle in small oocytes (ca. 80 μm diameter). These groups condense to form a cap-like structure on one side of the germinal vesicle. The cap grows in size and assumes the roughly spherical shape of the typical cloud. Until it begins to disperse the cloud and its precursor is closely applied to the germinal vesicle membrane. Sections indicate that the cloud is initially located in a large depression on one side of the germinal vesicle and when attempts are made to remove small clouds by micromanipulation they usually remain firmly attached to the vesicle. The cloud begins to disperse towards the periphery in oocytes of 250–300 μm diameter. This dispersal is preceded by a decrease in density of the mitochondrial packing (Table 2). Thus although the initial growth in size of the cloud is due to an increase in mitochondrial material

---

Fig. 9. A stereoscan preparation of an isolated mitochondrial cloud removed from an oocyte of approximately 200 μm in diameter. Note filamentous structures (fil.) and absence of discrete mitochondria. Acetone preparation fixed in osmium and coated with gold. × 1200.

Fig. 10. Detail from preparation illustrated in Fig. 9, showing filamentous structures (fil.) located in the interior of the cloud. × 5600.

Fig. 11. Part of a filamentous structure at the periphery of the preparation shown in Fig. 9. Note strands (str.) which correspond in thickness to the diameter of mitochondria. × 50000.
subsequent growth (oocytes greater than ca. 200 \( \mu \)m diameter) is partly due to a thinning out of the cloud before its more obvious dispersal, when it breaks up in the larger oocytes. Characteristically there is only one mitochondrial cloud in each oocyte, sometimes there are two smaller clouds but rarely more. Occasionally we have failed to detect mitochondrial clouds in oocytes of ovarian tissue from a young \textit{Xenopus}.

From electron micrographs of thin sections it is possible to make an estimate of the total number of mitochondria in the clouds of oocytes over the size range 100–300 \( \mu \)m in diameter. Assuming that the cloud is spherical and consists of randomly arranged, and evenly dispersed, mitochondria which are cylindrical in shape and of average length 2 \( \mu \)m and diameter 0.2 \( \mu \)m it can be calculated that in the small oocytes the cloud contains about 10000 mitochondria and in the larger oocytes about 120000. However, it may be more correct to refer to these figures as mitochondrial equivalents as it is not possible by examining the profiles of either thin or thick sections to come to any conclusion about the average length of the mitochondria which appear to make up the cloud. Our measurements suggest that it is not even possible to set a reasonable upper limit for the lengths of the mitochondria. The existence of very long filamentous mitochondria is revealed by stereo pairs of electron micrographs of thick sections of the cloud examined by transmission electron microscopy at 1000 kV. These observations are similar to those on mitochondria in the cells of snail mucous glands (Favard & Carasso, 1973). Taking all these observations into account and the apparent non-random arrangement of the profiles we would suggest that the best model for the cloud is a spaghetti-like mass of very long mitochondria, sometimes in parallel array, and with occasional branching regions.

The stereoscan preparations of the isolated cloud are difficult to interpret. Filamentous structures of mitochondrial size can be seen and there are larger strands which may be bundles of the smaller filaments. In general the preparations support the idea of a tangled mass of long mitochondria and the fact that during isolation and subsequent micromanipulation the cloud does not fall apart may also be significant. However, the small size of the clouds makes them very difficult to handle and we were forced to use acetone to dry the preparations rather than the preferred method of critical-point drying. We are well aware that the filamentous structures may be artifacts produced from a collapsed heap of mitochondria of a more or less conventional size and shape. Nevertheless our observations have convinced us that a three-dimensional model of the mitochondrial cloud of \textit{Xenopus} oocytes, built up from electron micrographs of serial sections, may well prove comparable to the structures which have been revealed in smaller cells.
Mitochondrial cloud of Xenopus

We gratefully acknowledge the help of Mr Woodall for instruction and help in the use of ‘Minerva’ at Imperial College. We are also indebted to Dr Audrey Glauert for advice on the techniques used in high voltage electron microscopy. The work formed part of an investigation supported by the Science Research Council.

REFERENCES


(Received 6 August 1976)