The Ultra-fine Structure of Lipid Globules in the Neurones of *Helix aspersa*

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With four plates (figs. 1–4)

Summary

The three kinds of lipid globules recognizable in the living neurones of *Helix aspersa* have been examined under the electron microscope. The globules of the kind that can be stained blue with methylene blue during life are seen in electron micrographs as spheres or spheroids, with concentric lamination, after calcium-osmium fixation. After fixation with sucrose-osmium laminated crescentic bodies are seen instead; these appear to be formed by distortion of the 'blue' globules. The yellow globules contain electron-dense material, and sometimes appear reticular. It is possible that the yellow globules may originate by transformation of some of the 'blue' globules. The colourless globules generally appear as crenated objects; this appearance may be a shrinkage artifact.

Apart from the mitochondria and the three kinds of lipid globules described, no other object large enough to be identified with the light microscope has been seen in the cytoplasm.

Introduction

IT has been shown (Chou, 1957 *a*, *b*) that there are three kinds of lipid globules in the neurones of the snail, *Helix aspersa*, and that they are distinguishable from each other by the use of vital dyes and by histochemical tests. One kind, called the yellow globules, have naturally a yellow colour owing to the presence of carotenoid. Another kind, called the colourless globules have no natural colour and cannot be coloured by vital dyes. A third kind, called the 'blue' globules, have no natural colour but are readily dyed in life by methylene blue, brilliant cresyl blue, or Nile blue.

The purpose of this investigation with the electron microscope was to find evidence as to whether any of these globules, and if so which, might be concerned in the appearance called 'Golgi apparatus' (or 'dictyosomes') in Golgi preparations; or whether there might be some other cytoplasmic inclusion, not visible in life, that was responsible for appearance of the 'Golgi apparatus'.

Material and Methods

For this investigation, the cerebral ganglia of *H. aspersa* were used. After the animal had been killed by decapitation, the cerebral ganglia were removed immediately and placed in fixative. Two fixatives were used:

1. Sucrose-osmium. 1% osmium tetroxide+0·35 M sodium-veronal acetate buffer+0·22 M sucrose (Caulfield, 1957).
2. Calcium-osmium. 1% osmium+0·35 M sodium-veronal acetate buffer +1% calcium chloride.

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The ganglia consist of two groups of neurones. Aggregations of neurones were dissected out in the fixatives and left in them at 0° C for 1 h. The material was dehydrated in graded alcohols and embedded in *n*-butylmethacrylate; the latter was polymerized for 12 h at 45° C.

Thin sections were cut on a Porter–Blum microtome and mounted on carbon-coated grids. In some cases, immediately adjacent 2 μ sections were cut. These thick sections were transferred with a fine bristle brush to a clean coverslip or slide, and were then dried with warm air.

Electron micrographs of the thin sections were taken with a Siemens 'Elmiskop I' at 40 kV or 60 kV. For direct microscopy, the thick sections were placed in 70% alcohol for a few minutes and then left for 10 min in a saturated solution of Sudan black B in 70% alcohol. They were differentiated in 70% alcohol for 5 sec and in 50% alcohol for 1 min; they were washed thoroughly in distilled water and finally mounted in Farrant's medium. Two-micron sections were also mounted in unpolymerized methacrylate and examined by phase-contrast.

Results

In electron micrographs one sees mitochondria and three other kinds of cytoplasmic inclusions large enough to be studied by light microscopy. Comparative studies of adjacent thin and thick sections under electron and light microscopy were made, and the same cells were readily identified (fig. 1, A, B). It was difficult, however, to identify with any certainty the same individual lipid globules in the thick and thin sections. The three kinds of cytoplasmic inclusions can, however, be identified in electron micrographs by their positions in the cells (see fig. 1 in Chou, 1957*a*).

Colourless globules. One of the kinds of cytoplasmic inclusions is seen in electron micrographs in the axon, arranged in rows resembling beads strung together. These are obviously the colourless globules, which are known from previous studies with the light microscope to be arranged in this way. They occur also here and there in the cytoplasm of the cell.

The colourless globules are shown in fig. 2, A, B. The micrographs represent axons cut transversely (A) and longitudinally (B). The electron-dense objects shown in these micrographs are the colourless globules. They are about 1 μ in diameter. This size corresponds closely with that obtained by light microscopy. They appear in most electron micrographs as crenated objects. When calcium-osmium is used as fixative, the crenated appearance is even more pronounced (fig. 4, A). The crenation is almost certainly an artifact. The colourless globules are bounded by a simple membrane. Their

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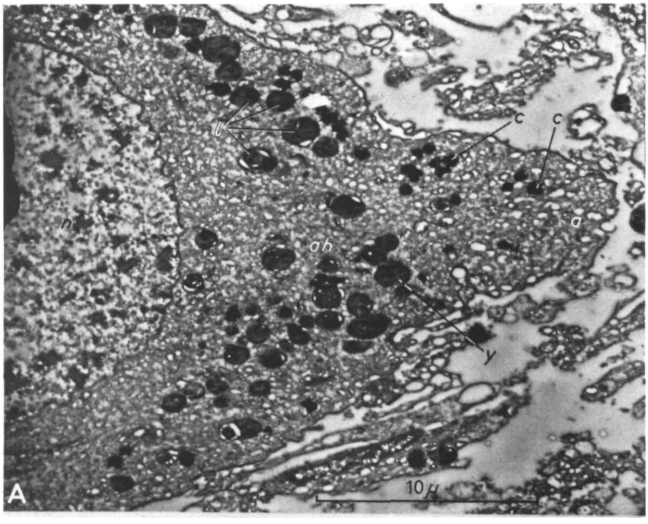
Fig. 1 (plate). A, a low-power electron micrograph of the neurone of *H. aspersa* to show that the crenated colourless globules (*c*) are distributed near the axon (*a*) and the large yellow globules (*y*) are near the axon hillock (*ah*). The nucleus (*n*) is partially shown in this micrograph.

B, a consecutive 2 μ section to show that in the same cell coloured with Sudan black and photographed by light microscopy all the lipid globules (*l*) are spherical and colour with Sudan black. Calcium-osmium fixation.



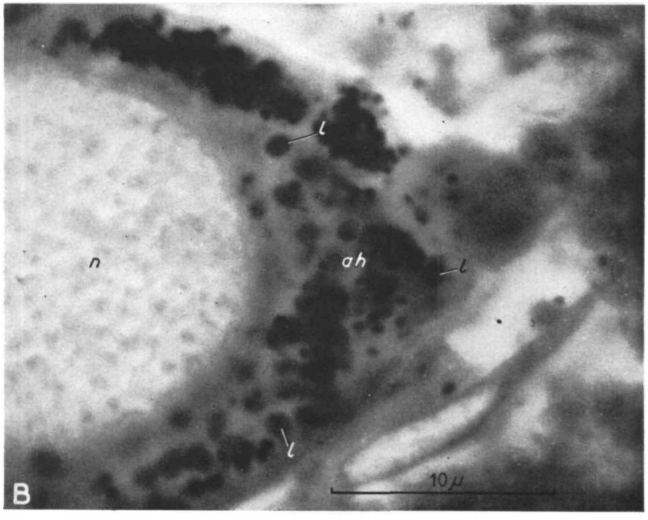


FIG. 1

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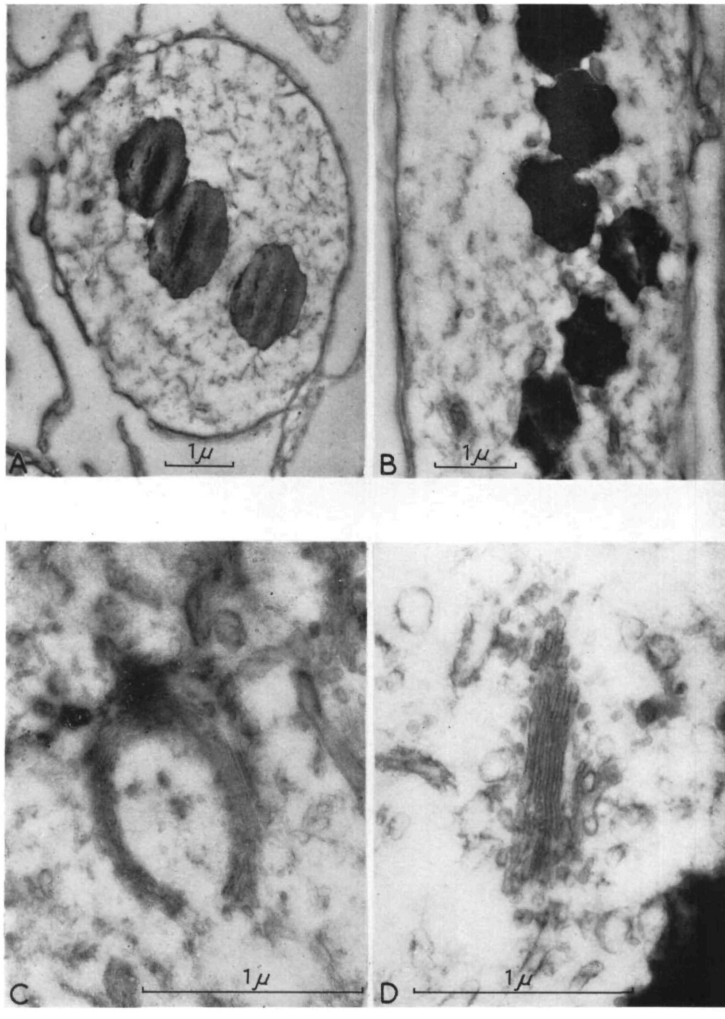

FIG. 2

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contents appear as a solid mass. The corrugated appearance generally seen in the substance of the globules is an artifact of sectioning, as the axis of corrugation is always parallel to the knife-edge.

Yellow globules. The yellow globules can be recognized in electron micrographs by three criteria already established by light microscopy. (1) They do not occur in small neurones; (2) they are particularly abundant in the axon hillock of large neurones; and (3) they are nearly always larger than the other two kinds of globules (diameter about 1·5 to 2 μ).

The yellow globules are electron-dense, but less so than the colourless globules. They appear to be bounded by lamellae. In preparations fixed with calcium-osmium these lamellae are particularly well preserved and 'stain' intensely with osmium. With this fixation lamellae are also visible in the interior of the yellow globules (fig. 3, C, D). The rest of the material of these globules appears to be structureless, or sometimes reticular.

Some of the yellow globules carry one or more 'satellites'. These are also bounded by layers of lamellae, but their internal parts are either vacuolated (fig. 3, B) or show little electron-density. These satellites resemble the 'blue' globules.

'Blue' globules. These are known from studies with the light microscope to be evenly distributed throughout the cytoplasm, but they are absent from the axon. They occur also in small neurones. These characters enable them to be distinguished in electron micrographs.

The two fixatives used in this investigation give different appearances to the 'blue' globules.

In calcium-osmium preparations the 'blue' globules appear spherical or ovoid (fig. 4, B). They consist of a laminated electron-dense external part, or cortex, and a central part or medulla that shows little electron-density. Each lamella appears to consist of two electron-dense layers with a less electron-dense layer in between, each of the three layers being about 20 Å thick. The dense layers appear to consist of irregularly arranged particles of electron-dense material. The total thickness of each lamella is about 60 Å. The distance between the lamellae varies in different globules from 100 to 200 Å. The thickness of lamellated cortex is often variable in different parts of the same globule (fig. 4, B).

When sucrose-osmium is used as fixative, the 'blue' globules do not retain their globular shape, but appear as crescentic objects (fig. 2, C) or even (in

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FIG. 2 (plate). Electron micrographs of lipid globules in neurones of *H. aspersa*. Sucrose-osmium fixation.

A, transverse section of axon showing three colourless globules.

B, longitudinal section of axon showing a row of colourless globules.

C, a 'blue' globule showing as an 'opened' ring, consisting of laminated membranes. The inner substance of the globule is not electron-dense.

D, another form of the 'blue' globules showing as a parallel double laminated membrane and associated with vacuoles. This appearance is probably due to a tangential section through the surface of the burst 'blue' globule.

section) as rods (fig. 2, D). The lamellae are similar to those seen in calcium-osmium preparations. Small vesicles are often seen in addition to the lamellae (fig. 2, D).

Thick sections, fixed in calcium-osmium, were coloured with Sudan black; all the lipid globules became homogeneously blue-black (fig. 1, B). No crescentic, curved, or cap-shaped structures appear in these preparations.

Mitochondria can be identified in the electron micrographs (fig. 3, C). They are filamentous and are found in all parts of the cytoplasm; they are better preserved in calcium-osmium preparations than in those fixed in sucrose-osmium. The limiting membrane of the mitochondrion is distinguishable, and also the matrix and cristae.

Discussion

From the present study, it appears that the crescentic laminated objects seen by electron microscopy in the nerve cells of *H. aspersa* result from an artificial modification of the 'blue' globules visible in living cells. In a previous study (Chou, 1957*b*) histochemical tests have demonstrated that these globules contain only phospholipids. From the present study, it has been shown that calcium-osmium fixation can preserve these globules as spheres comparable to those seen in living cells. However, we have seen from this present work that structures consisting of laminated membranes or lamellae tend to be formed usually at the periphery of the globules. These lamellae are probably due to the presence of phospholipids. Ross and Chou (1957) have brought forward strong evidence that the 'blue' globules contain a considerable proportion of water. This may be correlated with the fact that the central part of the 'blue' globule has a low electron-density. It cannot, however, be regarded as certain that the lamellae were situated at the periphery of the globule during life. During the processes of fixation, dehydration, and embedding for electron microscopy, water may be removed and the globules may then burst, thus forming crescentic objects. Calcium has a tendency to preserve the concentric lamellar arrangement of the phospholipids, and thus to cause the globules to retain their spherical or spheroidal shape.

Young (1932, 1953, 1956) has reported that the neutral red globules in the neurones of cephalopods can be swollen or shrunken osmotically, and he

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Fig. 3 (plate). Electron micrographs showing a series of changes of the 'blue' globules into yellow. Calcium-osmium fixation.

A, shows that certain internal substances (probably carotenoids, protein, or lipids other than phospholipids) are strongly electron-dense. The laminated membranes (*lm*) begin to become entangled in the globule with other substance. A mitochondrion (*m*) with long cristae is shown.

B, similar to A, but the electron-dense material in the globule has increased in amount. A satellite (*s*) is probably attached to this globule.

C, a fully developed yellow globule (*y*) containing very electron-dense material and also laminated structures (*lm*) (probably phospholipids). A mitochondrion is clearly shown.

D, a globule that appears to certain reticular structures and also parallel laminated membranes.

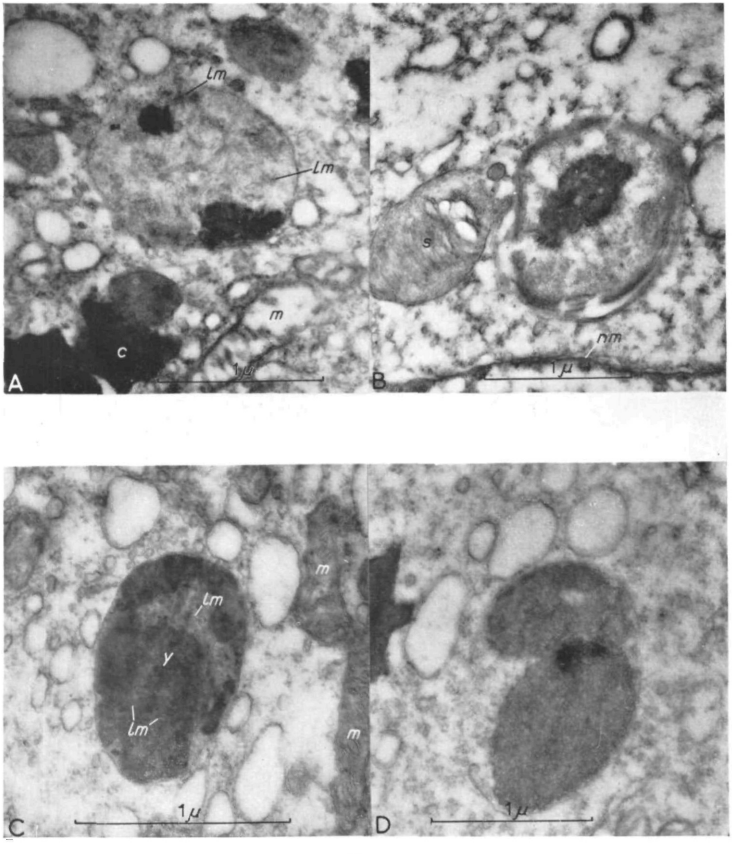

FIG. 3

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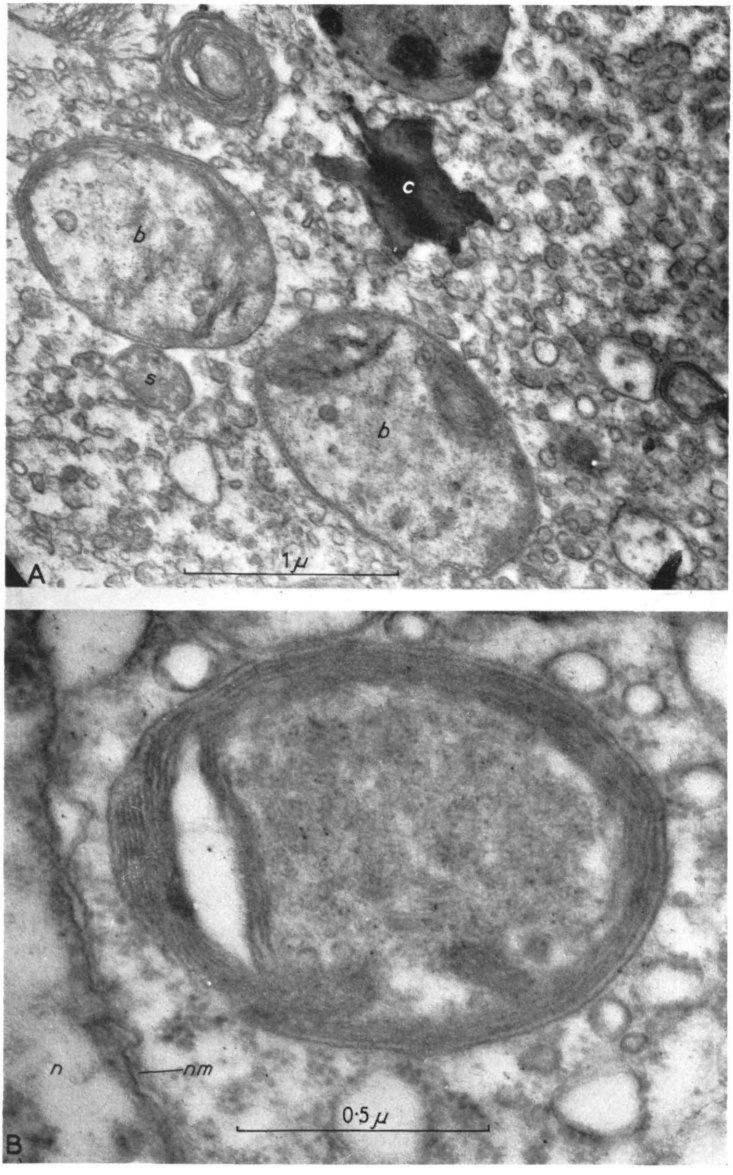

FIG. 4

concluded that a semi-permeable membrane might be present. This membrane might be formed of concentric lamellae similar to those that we have found in the neurones of *Helix*.

From the present electron microscope study, it is also interesting to note that our findings concerning the 'blue' globules agree with Schmidt's belief (1939) that, in the presence of water, certain lipids tend to arrange themselves in the form of concentric lamellae (see Baker, 1957).

The thickness of each membrane is consistent with the hypothesis that it represents a bimolecular phospholipid layer. The 'myelin' globules that exude from red blood corpuscles ingested by macrophages show a submicroscopic structure similar to that of the 'blue' globules (Policard, Bessis, and Breton-Gorius, 1957; Stoeckenius, 1957). These authors interpret the membranes of the 'myelin' globules as bimolecular layers.

Hodge (1956) and Hodge and others (1956) described similar lamellar structures in chloroplasts of *Zea mays*. These lamellae present an almost identical granular, double-membrane appearance, and are believed by them to be oriented phospholipid or lipoprotein molecules; but the whole arrangement of the lamellar membrane is not concentric like that of the 'blue' globules in *Helix*.

The structure of these three kinds of lipid globules strongly suggests that the yellow globules originate from the 'blue' globules. In calcium-osmium preparations, the 'blue' globules of the small neurones (like those of the large) appear as spherical objects consisting of strongly electron-dense, laminated membranes. These membranes enclose certain substances which, perhaps, have a high content of water and which are not 'stained' at all by osmium (fig. 4, B). During the development or growth of the cells, carbohydrate, protein, and lipids other than phospholipids, particularly cerebrosides and carotenoids, may aggregate in the 'blue' globules. These organic substances, being less saturated, may take up osmium to a greater extent. The latter fact would account for the greater electron density of the internal matrix of the globules during development from 'blue' to yellow. Fig. 3, A–C is a series of three electron micrographs showing the aggregation of electron-dense material in the body of the globules. When the 'blue' globules are completely filled with these dense substances, the laminated phospholipid membranes are no longer very prominent. It is possible that they may be dispersed or incorporated into the other substances in the globules (fig. 3, C). In many cases the layers of laminated structures enclosing a certain amount of electron-dense material may still be identified. In some globules the dense material appears to have a reticular structure (fig. 3, D).

FIG. 4 (plate). Electron micrographs of lipid globules in neurones of *H. aspersa*. All these micrographs were taken from neurones fixed in calcium-osmium preparations.

A, a group of lipid globules. The crenated colourless globule (*c*) appears to be more electron-dense than the two 'blue' globules (*b*). One of the 'blue' globules seems to bear a small satellite (*s*).

B, a 'blue' globule showing its ovoid form as seen by light microscopy. The inner substance of the globule is not electron-dense. *n*, nucleus; *nm*, nuclear membrane.

The colourless globules seem to be quite independent of the other two types of globules. They are electron-dense, possibly owing to the presence of unsaturated fatty acids, and appear to be devoid of any laminated membranes.

The duplex character of lipid globules appearing in living cell and in fixed preparations when examined by light microscopy has been summed up by Baker (1957). In our studies of these lipid globules, we have found that perfectly spherical globules can apparently be changed into crescentic forms under the influence of the particular fixative used.

In 'Golgi' preparations, the black material is presumably osmium or silver deposited in or on the lamellae of the 'blue' globules. The findings with the electron microscope confirm the conclusion reached by one of us (Chou, 1957*a*) that the 'Golgi apparatus' ('dictyosomes') of these cells is represented in life by the 'blue' lipid globules.

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