Cytoplasmic Inclusions of the Neurones of Gastropods

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SUMMARY

The neurones of the sub-oesophageal ganglionic mass of the Simla slug, *Anadenus altivagus*, and the Bharwain snail, *Euaustenia cassida*, have been investigated by phase-contrast microscopy of the living cells and in fixed preparations. The mitochondria are seen as granules and filaments in both living and fixed material. Alignment of granules into filamentous mitochondria has also been observed in the fixed preparations. The lipid spheroids (corresponding to the 'binary spheroids' of Thomas and the 'lipochondria' of Roque) are sudanophil, osmiophil, and argentophil. The bigger spheroids show a duplex structure, consisting of a cortical, chromophil, lipid component, which may be in the form of a complete ring (*Anadenus*) or in the form of one or two granules or a crescent (*Anadenus* and *Euaustenia*), and a chromophobe medulla (neutral red vacuome of Parat), in which lipochrome develops to form the 'mulberry spheroids' of Thomas. The small homogeneous lipid spheroids also contribute to the formation of 'mulberry spheroids'.

THE spheroid bodies (often called 'Golgi bodies') in the cytoplasm of neurones have been studied by many authors, especially in *Helix* and other gastropods. Agreement has not, however, been reached as to the structure and homologies of these bodies. It was thought desirable to investigate the cytoplasmic inclusions of the neurones of gastropods belonging to genera not previously examined in this respect.

MATERIAL AND TECHNIQUE

The slugs, *Anadenus altivagus* Theobold, were collected at Simla, a hill station in the Panjab (India), during the month of August 1955. Living neurones were studied by phase-contrast microscopy at Simla. Some material was also fixed there and brought down to Hoshiarpur, where it was sectioned and studied.

Living specimens of the snail, *Euaustenia cassida* Hutton, were collected at Bharwain, a village in the lower Shiwalik Range in Hoshiarpur District during the month of October 1955, and were brought to Hoshiarpur.

The neurones of the sub-oesophageal ganglionic mass of *Anadenus* and the pleuro-pedal ganglionic mass of *Euaustenia* were selected for the present investigations.

The living neurones of *Anadenus* were studied in saline solution (Baker, 1944) under a Zeiss phase-contrast microscope (stand 'W').

The following fixatives were employed:

Formaldehyde—osmication. Material fixed in formaldehyde saline was osmicated in 1% solution of osmium tetroxide in saline at 37° C for 24 to 48 hours. The sections were cut by Peterfi's cellodin methyl-benzoate method, recommended by Baker (1944). Some of the sections were bleached in a saturated solution of potassium persulphate before study.

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Aoyama. The material fixed in cadmium formaldehyde mixture was treated with silver nitrate for 15 to 24 hours and reduced for 4 to 6 hours. Some of the Aoyama preparations were toned in gold chloride solution.

Altmann. The sections of material fixed in Altmann were stained in iron haematoxylin for the study of mitochondria. Some of the sections were studied unstained immediately after mounting to locate the lipid contents.

Helly with postchroming. The material fixed in Helly's fixative was postchromed at 37° C in a saturated solution of potassium dichromate for 48 hours. The paraffin sections were coloured in a saturated solution of Sudan black in 70% alcohol, and mounted in Farrants's medium (Thomas, 1948). Some of the sections were also stained with Sudan IV. Some of the sections were stained in Masson's tricolour stain.

The sections of this material were used for the study of mitochondria after staining in iron haematoxylin.

Observations

The sub-oesophageal ganglionic mass of *Anadenus altivagus* and *Euaustenia cassida* is formed by the fusion of a number of ganglia, which cannot be completely separated. It is, therefore, very difficult, if not impossible, to say definitely to which particular ganglion the neurones segregated for study really belong.

Mitochondria

The mitochondria could best be studied in Helly and Altmann material, stained with 0·5% iron haematoxylin. They could also be observed in formaldehyde-osmicated slides that had been bleached and subsequently stained with iron haematoxylin (Gatenby and Moussa, 1949; Moussa, 1950), but such preparations were not found to be very satisfactory. In Sudan black preparations the mitochondria are seen as greyish bodies.

The mitochondria are in the form of granules and filaments in the neurones of both species. They are stained dark grey by haematoxylin and pink by acid fuchsin. The filamentous mitochondria are few in number and are seen as fine curled threads with a uniform contour, scattered here and there amongst the uniformly dispersed granular ones (figs. 1, A–C; 3, A). These truly filamentous mitochondria can be easily distinguished from filaments formed by the alignment of granular mitochondria in fixed preparations (figs. 1, A; 3, A, E). The latter could be compared with the 'coccoids' of *Helix* neurones (Thomas, 1947).

FIG. 1. Neurones of *Anadenus*. A, Helly / haematoxylin, showing mitochondria and small homogeneous spheroids. B, Helly / Masson, showing fuchsinophil granules forming 'mulberry spheroids'. Duplex and homogeneously stained bigger spheroids are also seen. C, Helly / haematoxylin, showing mitochondria, homogeneously stained spheroids, and 'mulberry spheroids'. D and E, formaldehyde-osmicated, showing some of the duplex spheroids with incomplete cortex having partially or completely collapsed chromophobe medulla. Various forms of spheroids, including 'mulberry spheroids', are also seen. F, Aoyama (toned), smallest observed neurone. G, Aoyama (toned), showing the formation of 'rods'. H, Aoyama, showing 'mulberry spheroids' and thick cortices of the spheroids.

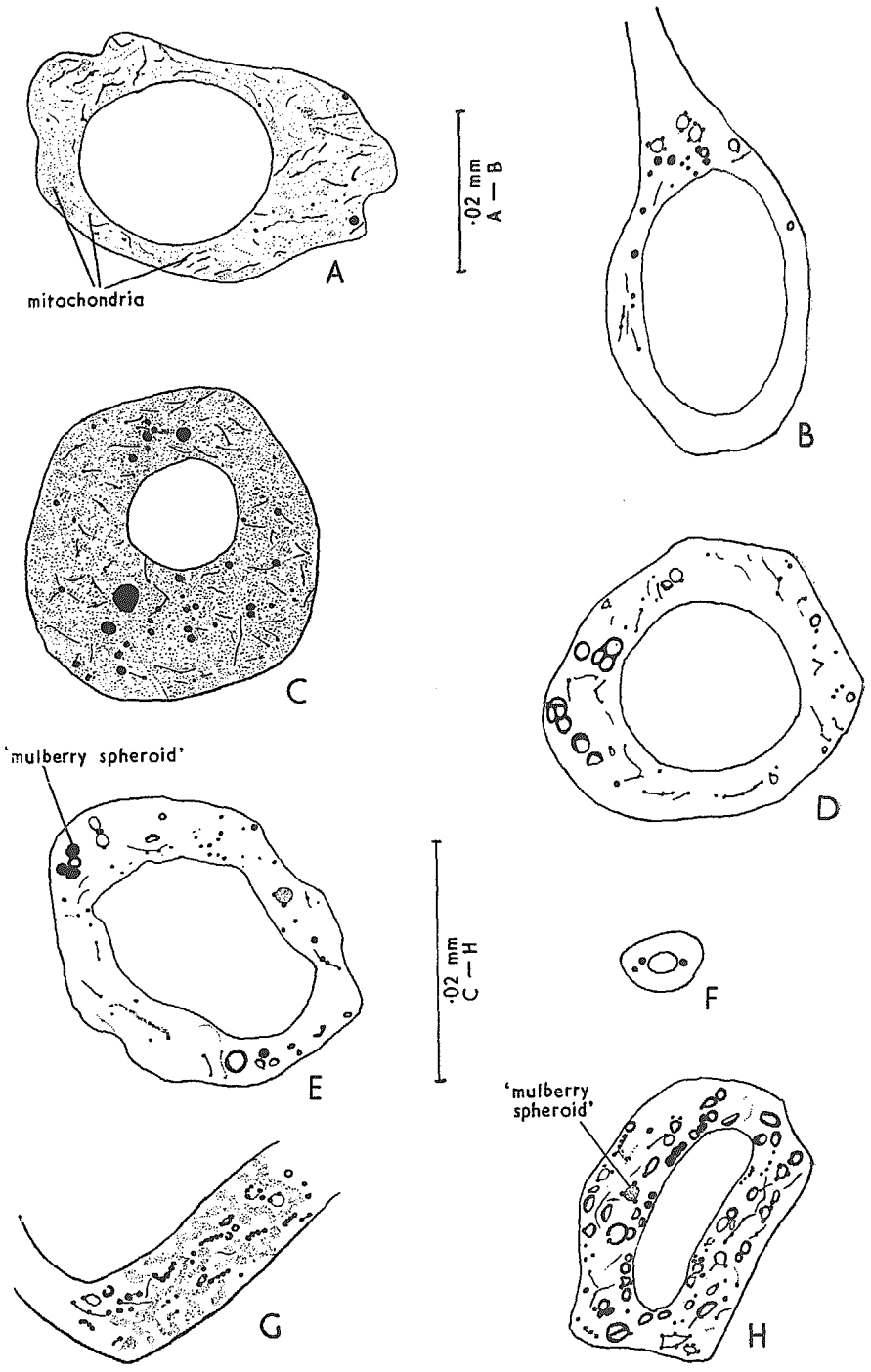
mitochondria
A
·02 mm
A — B
B
C
D
'mulberry spheroid'
E
·02 mm
C — H
F
'mulberry
spheroid'
G
H

FIG. 1

Small granules lie on the filamentous mitochondria or at their ends. These granules are bigger than the granular mitochondria and are coloured by Sudan black (fig. 2, c); it appears that they arise from the mitochondria. These lipid granules represent the intermediate granules of Shafiq (1953) and the smallest homogeneous spheroidal lipochondria of Roque (1954).

The mitochondria are seen by phase-contrast microscopy as greyish granules and filaments scattered throughout the cytoplasm in the living neurones of *Anadenus*.

Filamentous mitochondria have also been described in the neurones of *Helix* by Thomas (1947) and Roque (1954), but Boyle (1937) and Moussa (1950) described only granular mitochondria in the neurones of *Helix* and *Limnaea* respectively.

Lipid Spheroids

In view of the existing controversy regarding the nomenclature of the lipid bodies, now often described as lipochondria but originally described as 'Golgi elements' or 'Golgi apparatus' in the nerve-cells of various animals, it has been considered advisable to describe the lipid bodies of the neurones of *Anadenus* and *Euaustenia* as spheroids, in which form they exist in the neurones of these animals.

Phase-contrast study. The living neurones of *Anadenus* have been studied by phase-contrast microscopy. These neurones are rather loosely aggregated in the ganglion, and their cytoplasm does not have such a high consistency as in *Schistocerca* (Malhotra, 1955, 1956). *Anadenus* neurones, therefore, can be easily studied by simply releasing the cover-glass on a small piece of the ganglion lying in a drop of saline solution. No teasing is required; slight pressure on the coverslip flattens the nerve-cells.

Properly flattened nerve-cells show spheroids of varying sizes. There are small homogeneous ones, which give a high phase-change and are seen as dark refringent granules. These are identical with the granules that are seen lying on the mitochondria or at their ends, as described above. There are bigger spheroids, which are seen as dark homogeneous bodies. There are also spheroids of intermediate sizes. Most of these show a duplex structure by positive phase-contrast. Each has an outer dark cortex and the inner bright medulla. The medulla is generally ensheathed by the cortex, but sometimes only partially so. In the latter case the cortex appears as a crescent round the spherical medulla.

The medulla of the duplex spheroids present variable phase-changes. The medullas of a few of them present negligible phase-change, and appear almost colourless; others present various degrees of higher phase-change. The medulla of a spheroid never shows as much phase-change as the cortex. The increase in the phase-change of the medulla is due to the gradual condensation of lipochrome in it. The difference in the phase-change between the lipochrome-loaded medulla and the cortex of a duplex spheroid is generally small. Consequently such spheroids may appear as dark, homogeneous bodies.

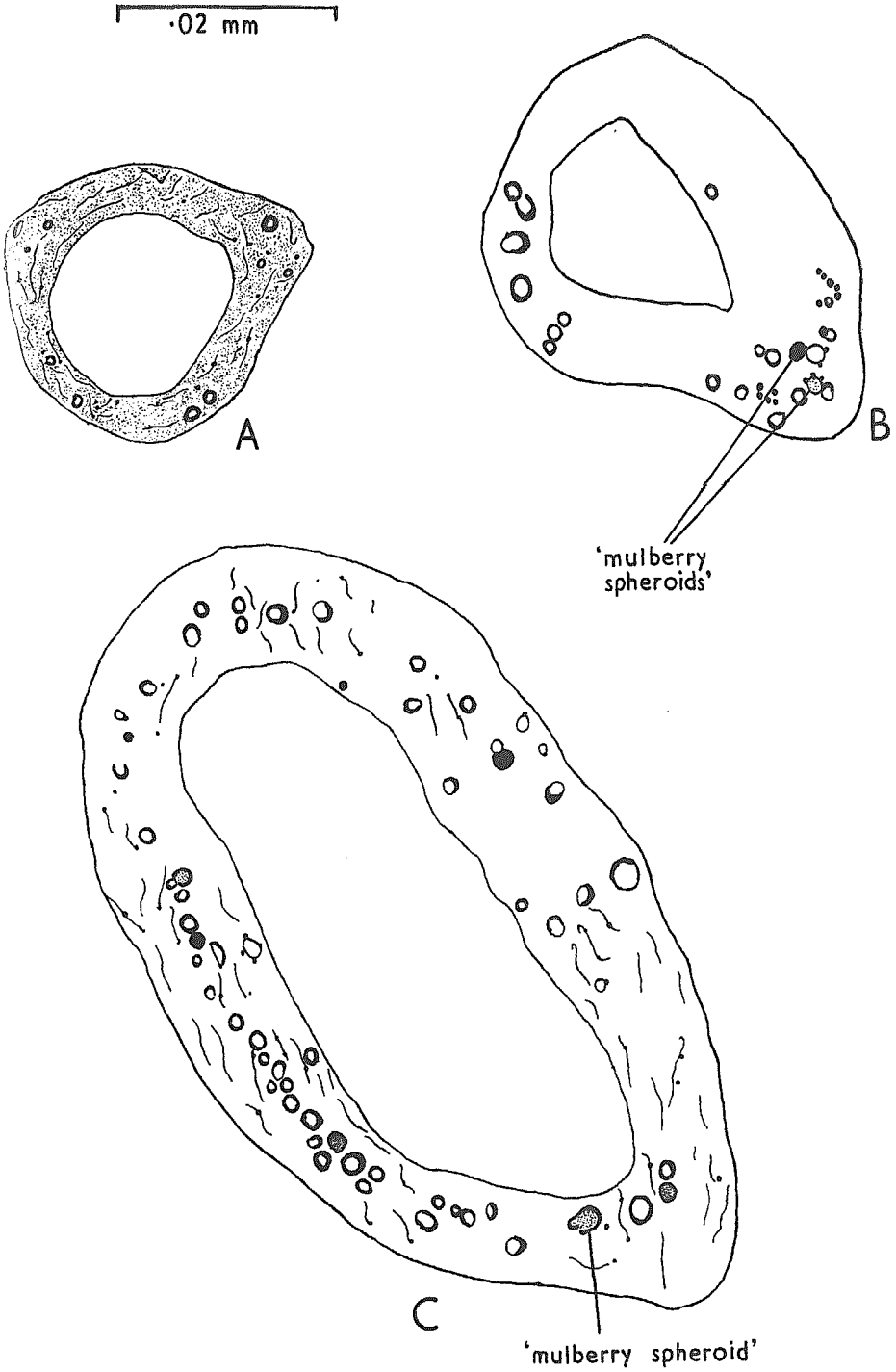

FIG. 2. Neurones of *Anadenus*. Helly / Sudan black, showing the various forms of spheroids observed, and condensation of lipochrome in figs. B and C. Mitochondria are also seen in figs. A and C.

Lipochrome-loaded spheroids are sometimes slightly distorted and resemble the 'mulberry spheroids' of Thomas (1947).

Sometimes the spheroids lie in separate groups in the living neurones, instead of being scattered at random.

Study of Fixed Cells

The observations made on the living neurones of *Anadenus* fully confirm the conclusions drawn from the study of fixed preparations. The following description applies to both species.

The spheroids are invariably present in all the nerve-cells of both the species. The size of the nerve-cells varies considerably in each of the two species. In the smallest nerve-cells observed the spheroids are few (fig. 1, F); but in the larger cells their number increases considerably, and they are seen dispersed throughout the cytoplasm. Sometimes in the largest neurones of *Anadenus* they are mostly confined to a region midway between the nuclear and cell membranes (fig. 2, C); sometimes they are confined to the two ends of the cell (fig. 2, B) or to the axon end (fig. 1, B).

The spheroids vary considerably in size even in the same cell, as has already been described in the phase-contrast study. There appears to be no relation between the size of the spheroids and the size of the cell, for the bigger spheroids are often observed in the smaller cells and conversely. Nevertheless, the number of big spheroids is smaller in the smaller cells.

The spheroids of *Anadenus* neurones are either homogeneous or duplex in structure. In the latter case the cortex may be complete or incomplete, giving the appearances of rings and crescents respectively in optical section; or the cortex may be represented by one or two granules. On the contrary, the spheroids of *Euaustenia* never show a complete cortex.

Helly and Altmann techniques. Helly preparations stained with iron haematoxylin show the spheroids as darkly stained homogeneous bodies of varying size in the neurones of *Anadenus* (fig. 1, A, C). The duplex structure of the spheroids cannot be brought out by this technique. In the neurones of *Euaustenia*, since the chromophil cortex of the spheroid is always incomplete, the medulla can be seen as a distinct, clear vacuole in such preparations (fig. 3, A).

The spheroids are stainable with acid fuchsin (fig. 1, B) when Helly preparations are stained with Masson's tricolour stain. In such preparations the spheroids may appear as homogeneous bodies, or they may show a clear differentiation between cortex and medulla. These preparations of *Anadenus* neurones also show a number of fuchsinophil granules on the surface of a large, pale granule (fig. 1, B).

The granular or crescent-shaped cortical component of the spheroids of the neurones of *Euaustenia* is blackened by Altmann fixation. When unstained Altmann preparations are studied immediately after mounting, the cortical component is seen as dark grey, and the medulla of the spheroids as a clear

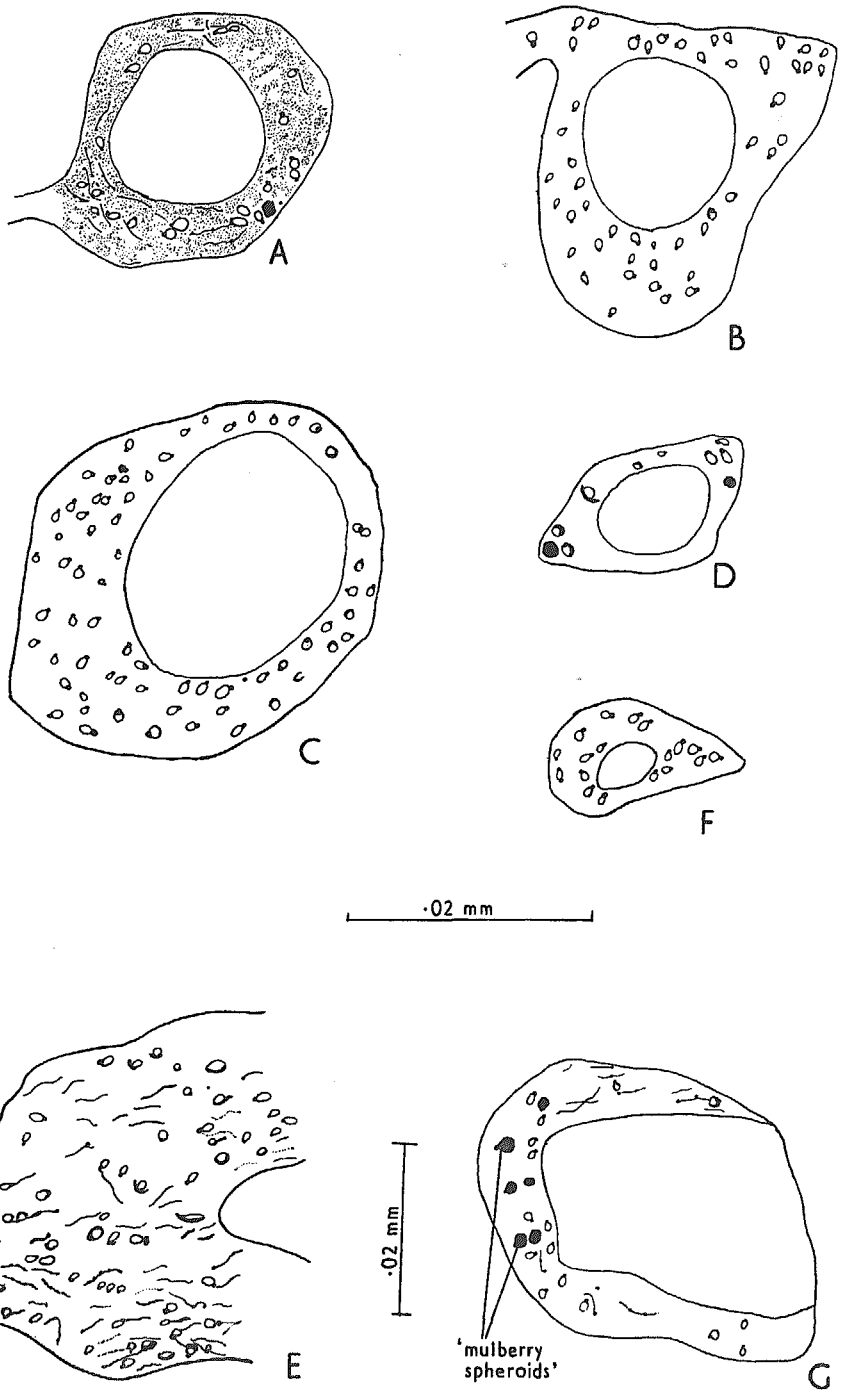

FIG. 3. Neurones of *Euaustenia*. A, Helly / haematoxylin, showing mitochondria, ordinary spheroids, and 'mulberry spheroid'. B, Altmann unstained. C, Aoyama, showing optimal impregnation. D, Aoyama, showing jet-black 'mulberry spheroids' and excessive deposition of silver. E, formaldehyde-osmicated for 48 hours, showing formation of artifacts. F and G, Helly / Sudan black.

vacuole (fig. 3, B). When stained with iron haematoxylin it is only the cortical component of the spheroid that is darkly stained.

Golgi techniques. In spite of the established fact that long osmication and treatment with silver nitrate in the Golgi techniques very often introduce serious artifacts in the cell, the picture of the spheroids obtained with these techniques in *Anadenus* and *Euaustenia* is often the same as with other methods. In formaldehyde-osmicated preparations of *Anadenus* neurones, there are seen small, dark, homogeneous spheroids (fig. 1, D, E). Besides these, there are bigger spheroids, which show a duplex structure, that is, each with an osmiophil cortex and an osmiophobe medulla (fig. 1, E). Sometimes such spheroids are seen aggregated together in small groups (figs. 1, D; 4, F, G). Some of these bigger spheroids show a condensation of a pale yellow material inside the medulla. Such spheroids appear as black, homogeneous bodies after prolonged osmication (fig. 1, E). It has also been observed that the fuchsinophil granules that surround the developing 'mulberry spheroid' are also osmiophil (fig. 1, E).

Besides the above structures, the formaldehyde-osmicated preparations of *Anadenus* also show one or two osmiophil granules or a crescent, closely associated with a rounded osmiophobe medulla (fig. 1, D, E). These forms of the spheroids are the only ones met with in *Euaustenia* (fig. 3, E). Very often the osmiophobe vacuole associated with a crescent is seen collapsed in osmicated neurones of both *Anadenus* (fig. 1, E) and *Euaustenia* (fig. 3, E). Such appearances are obviously artifacts. In extreme cases the vacuole has completely collapsed and does not show at all. It has been observed that osmium is first deposited on the cortical component of the spheroids, then round the spheroids, and, finally, on other structures unrelated to the spheroids. A spheroid round which the osmium has been partially deposited looks like a crescent with the chromophobe substance attached to it (fig. 3, E). Prolonged osmication gives rise to another artifact, namely, the formation of 'rods' without the chromophobe spheres. These rods are obviously formed by the excessive deposition of osmium on the mitochondria. The same remarks apply to silver nitrate techniques.

Aoyama preparations of the neurones of *Anadenus* (fig. 1, G, H) and *Euaustenia* (fig. 3, C, D) neurones generally show the same form of the spheroids as osmium tetroxide preparations, but the duplex spheroids of *Anadenus* neurones mostly become flattened and metallic silver deposits in the form of granules on the argentophil cortex of the spheroids. When there is heavy impregnation it gives results similar to that given by long osmication. In fig. 1, G, H, granulated rods and crescents can be seen, formed by excessive precipitation of silver. But when there is optimal impregnation of silver, the chromophobe substance of the duplex spheroids shows clearly (figs. 1, H; 3, C).

The small, homogeneous spheroids and the cortical component of the duplex spheroid (granule or crescent) appear larger in all dimensions in Golgi preparations than in Sudan black preparations. This is undoubtedly due to the excessive deposition of metallic osmium and silver on the lipid content.

Sudan black. The spheroids in the neurones of *Anadenus* and *Euaustenia* in Sudan black preparations appear essentially in the same forms as in the techniques previously described.

Paraffin sections of *Anadenus* neurones fixed in Helly and coloured with Sudan black show mostly duplex spheroids, each with a thick sudanophil cortex and a lightly staining medulla (fig. 2, A–C). In such preparations spheroids with an incomplete sudanophil cortex investing a rounded chromophobe medulla also appear (fig. 2, B, C). The sudanophil material may be even heaped up on one side of the chromophobe medulla, which shows as a thin sudanophil rim round it (figs. 2, C; 4, B), or it may be restricted to a granule closely associated with a chromophobe medulla (figs. 2, B, C; 4, H).

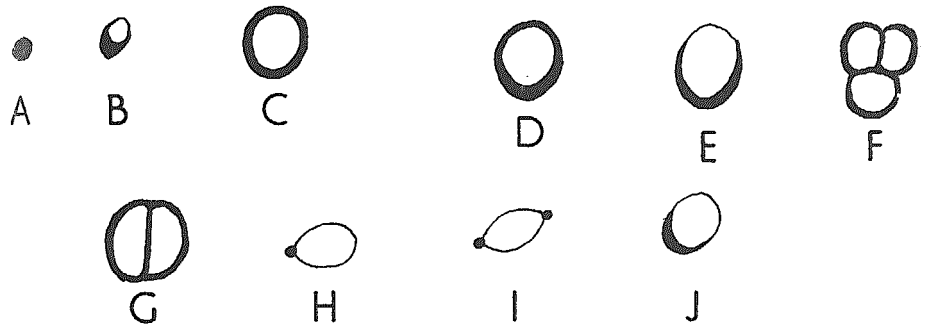

FIG. 4. Diagrammatic representation of the various forms of spheroids observed in different preparations of *Anadenus* and *Euaustenia*.

In the neurones of *Euaustenia* the cortical sudanophil component of the spheroids is mostly restricted to a single granule associated with a chromophobe medulla, but sometimes it may appear in the form of a crescent (fig. 3, F, G).

Besides the above forms of spheroids, Sudan black preparations of *Anadenus* and *Euaustenia* neurones also show small, homogeneous, sudanophil granules scattered throughout the cytoplasm and mostly lying on the mitochondrial threads (figs. 2, A, C; 3, G). Sudanophil crescents with chromophobe substance in the concavity of the crescent are also seen.

The cortical component of the duplex spheroids of the neurones of *Anadenus* and *Euaustenia* appears to be lipid, as it colours with Sudan black, reduces osmium and silver, and is darkly stained by haematoxylin after chrome-osmium and Helly fixation. It is stainable with acid fuchsin (Masson's tricolour stain).

This lipid material is variously distributed round the chromophobe substance of the spheroid (fig. 4), as shown by Baker (1949) in the neurones of the anterior mesenteric ganglion of the mouse.

It is in the chromophobe medulla of the spheroids of the neurones of *Anadenus* and *Euaustenia* that the lipochrome develops, but only few of the spheroids contain the pigment. When the lipochrome first makes its appearance, it takes up a light stain in Sudan black preparations (figs. 2, C; 3, G) in

contrast to the dark cortex. Later, when its amount increases, it colours darkly (fig. 2, C). Sometimes the lipochrome-loaded spheroids are seen as homogeneous bodies in Sudan black preparations (figs. 2, B, C; 3, G). In Golgi preparations the lipochrome-loaded spheroids are seen as black homogeneous bodies (figs. 1, E, H; 3, D), and the same is true of Helly-haemotoxylin preparations (fig. 1, C). Sometimes the spheroids which have accumulated lipochrome get so much distorted that the cortex of the spheroid may get torn on one side (fig. 2, C). The 'mulberry spheroids' of *Helix* neurones (Thomas, 1947) are formed in this way.

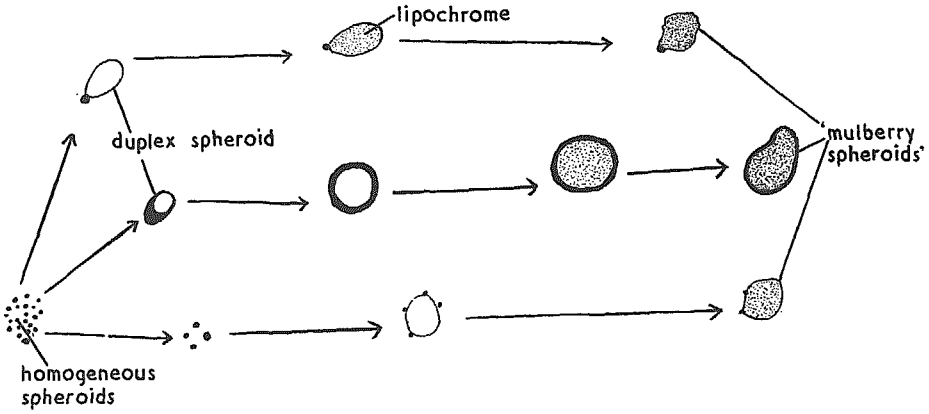

FIG. 5. Schematic illustration of the two methods of formation of 'mulberry spheroids' in *Anadenus* and *Euaustenia*.

Anadenus neurones also reveal the formation of 'mulberry spheroids' of the vertebrate type (Thomas, 1948). In this case three or four small homogeneous lipid spheroids come to lie close together and form 'mulberry spheroids' (figs. 1, B, E, H; 2, C; 5). These small homogeneous spheroids can be compared, in conformity with Thomas (1948), to the Golgi remnants of Hirsch (1939). Fig. 5 is a schematic illustration of the two methods of the formation of 'mulberry spheroids'.

Sudan IV does not colour anything in paraffin sections of neurones fixed in Helly.

DISCUSSION AND CONCLUSIONS

The spheroids of the neurones of the slug *Anadenus* and the snail *Euaustenia* exist either as small and homogeneous or bigger, duplex bodies. The former colour with Sudan black. Most of these homogeneous spheroids lie on the mitochondria. They are bigger than the granular mitochondria and seem to arise from the latter.

The duplex spheroids show an outer chromophil, lipid cortex (sudanophil, osmiophil, and argentophil) and an inner chromophobic medulla. The cortical lipid component is variously distributed round the medulla, as shown by Baker (1949) in the neurones of the anterior mesenteric ganglion of the mouse. In *Anadenus* the lipid component may be in the form of a complete or an incomplete (crescentic) sheath, or it may be restricted to one or two

granules in close association with the medulla; but in *Euaustenia* the spheroids never show a complete cortex.

The duplex spheroids, having a complete lipid sheath round the chromophobe medulla, as are seen in the neurones of *Anadenus*, are identical with the 'Golgi bodies' of Baker (1949) in the nerve-cells of the mouse, and of Malhotra (1955 and 1956) in the nerve-cells of *Schistocerca*, and with the 'lipochondria' of Shafiq (1953, 1954) in the nerve-cells of *Locusta* and Roque (1954) in the nerve-cells of *Helix*. Further, the spheroids observed in the neurones of *Euaustenia* are certainly identical with the 'binary spheroids' of Thomas (1947) in the neurones of *Helix* and the 'Golgi bodies' of the present author (1955, 1956) in the neurones of *Laccotrephes*.

Roque (1954) describes the lipochondria as homogeneous spheroids in the neurones of *Helix*. He interprets the appearance of a cortex or a crescent in the lipochondria of living neurones, as seen by phase-contrast microscopy, as optical artifacts, or as artifacts due to incomplete staining with supravital dyes. A similar view that the cortex is an optical artifact in the living neurones of *Locusta* studied with phase-contrast, and that it is an artifact due to the deposition of osmium in Golgi preparations, has been put forward by Shafiq (1953 and 1954). But the present author, supporting his phase-contrast observations by Sudan black preparations, disagrees with the interpretations of Roque and Shafiq. He described the spheroids as genuinely duplex in structure in the neurones of insects (1956). This view of the present author is supported by his studies of the neurones of *Anadenus*.

Moussa (1950) also described duplex Golgi bodies, each consisting of an osmiophil and argentophil 'dictyosome' associated with a chromophobe 'archoplasm' in the neurones of *Limnaea*. A similar form of the 'Golgi apparatus' has also been recorded by Boyle (1937) in the neurones of *Helix*; but Boyle describes the chromophobe component of the Golgi dictyosomes as the secretion product of the 'Golgi apparatus'.

Thomas (1948) described 'binary spheroids' in the sympathetic neurones of the mouse, similar to spheroids (Golgi bodies) observed in *Helix* neurones by the same author (1947). From the morphological similarity of the form of the spheroids in the neurones of the mouse and *Helix*, Thomas (1948) derived the conclusion that the spheroids of vertebrate and invertebrate nerve-cells are homologous to each other. The latter conclusion has been confirmed by Shafiq and Casselman (1954) and Casselman and Baker (1955). These authors have chemically analysed the lipochondria of the neurones of *Locusta* and the rabbit respectively, and have recorded the presence of cerebroside and phospholipid as common chemical constituents in the lipochondria of both these animals.

My observations also lead me to the conclusion that the bigger spheroids show a duplex structure in the neurones of both the gastropod species, *Anadenus* and *Euaustenia*, and that the cortical component is the true lipid (sudanophil, osmiophil, and argentophil) of the spheroids. It is this lipid material of the spheroid which represents the Golgi material.

The real form of the lipid material can be studied in Sudan black prepara-

tions, as recommended by Baker (1949). It has also been observed, in conformity with Baker (1954), that long osmium and silver techniques introduce artifacts, as the metals are precipitated on the lipid component. If there is an optimal impregnation of osmium tetroxide or silver nitrate, the picture of the lipid component seen in the finished preparation is, however, similar to that given by Sudan black. Prolonged impregnations result in the deposition of osmium or silver first on the spheroids and finally on other structures unrelated to the spheroids (Baker, 1954; Malhotra, 1956).

In silver preparations the chromophobe medulla of the spheroids becomes swollen, and most of the spheroids are seen as flattened platelets in *Anadenus* neurones. Exactly similar artifacts are produced in *Schistocerca* neurones prepared by Kolatchev's method (Malhotra, 1956).

The medulla of the duplex spheroids does not colour with Sudan black, and it is not blackened by osmium or silver. It has been established by Thomas (1947, 1948), Baker (1949), and Malhotra (1955, 1956) that the medulla of the Golgi elements is stainable in life with the basic dye, neutral red. The 'lipochondria' of Shafiq (1953, 1954) and Roque (1954) are also stainable with neutral red. It is, therefore, concluded from the above facts that the lipid material and the bodies that stain with neutral red ('vacuome' of Parat) exist closely associated with each other as duplex spheroids in the neurones of molluscs, as in the neurones of *Schistocerca* and *Laccotrephes* (Malhotra, 1955, 1956). Similarly Roque (1954) in *Helix*, Nath and Gupta (1956) in *Anadenus* and *Euaustenia*, and Nath (1956) in his comprehensive review of the cytology of spermatogenesis, have all described with phase-contrast microscopy duplex Golgi spheroids, each with a complete or incomplete cortex on the neutral red vacuole.

Young (1932) also described granular, osmiophil, and argentophil Golgi bodies and neutral red staining vacuoles as lying close to each other in the neurones of cephalopods.

It also has been observed, in conformity with the findings of Thomas (1947) in the neurones of *Helix*, and of Moussa (1950) in those of *Limnaea*, that lipochrome appears in the chromophobe medulla of the spheroids of the neurones of *Anadenus* and *Euaustenia*. Cain (1948) had demonstrated that the lipochrome contains carotenoids accumulated in the 'internum' of the spheroid of the neurones of *Limnaea*, *Planorbis*, and *Helix*.

Small homogeneous spheroids, corresponding to the Golgi remnants, also contribute to the formation of 'mulberry spheroids' by their concerted efforts in the neurones of *Anadenus*, as in the sympathetic neurones of the mouse (Thomas, 1948). Roque (1954) considered that the formation of 'mulberry spheroids' was due to pigment granules becoming attached to the lipochondria in the neurones of *Helix*.

The lipochrome may be regarded as a 'Golgi product', in accordance with Thomas (1948) and Moussa (1950).

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