The Cytology and Histochemistry of the Neurones of *Periplaneta americana*

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With one plate (fig. 1)

Summary

The only cytoplasmic inclusions visible in the neurones by the light microscope are the lipochondria and mitochondria. It is suggested that the Golgi bodies, seen in preparations made by the Golgi techniques, are produced by the deposition of osmium or silver on the surface of the lipochondria.

The lipochondria consist of phospholipids and cerebrosides. There is also some lipid in the cytoplasm, together with carbohydrates, proteins, and RNA.

Introduction

THE neurones of insects have been the subject of innumerable cytological studies. The cell inclusions which have attracted the most attention are the Golgi bodies. In invertebrate neurones these are of different shapes—circles, crescents, and rods—which are collectively referred to as 'dictyosomes'. A controversial question arises as to whether the dictyosomes are present in the living cell or are artifacts produced by the fixation and impregnation techniques used to demonstrate them. Recent studies of insect neurones have fallen into two groups, those supporting the theory of the validity of the Golgi bodies as true cytoplasmic inclusions (Gresson, Threadgold, and Stinson, 1956) and those presenting evidence to support the theory that the dictyosomes are artifacts (Shafiq, 1953; Nath, 1957). The use of the electron microscope in the study of insect neurones has further complicated this issue, since a structure seen in electron micrographs has been identified as a Golgi body (Hess, 1958).

The cockroaches, *Periplaneta americana* and *Blatta orientalis*, have been used before for studies of insect neurones. These include the cytological work of Bialkowska and Kulikowska (1919) and Muliyil (1935) and the electron microscope studies of Hess (1958), but there appears to be no record of a histochemical study. It was, therefore, decided to undertake a histochemical study of the neurones of *P. americana* and to consider the status of the Golgi bodies in the light of the histochemical evidence.

Methods

The neurones studied here were those of the meso- and meta-thoracic ganglia. For vital staining, the ganglia were teased apart to free the neurones, which were then stained in dilute solutions of either neutral red or Janus

[Quarterly Journal of Microscopical Science, Vol. 102, part 3, pp. 399–405, 1961.]

green. Stock solutions of these dyes (0·5% aqueous) were diluted as follows: neutral red, 2 drops of stock solution to 2 ml of insect Ringer; Janus green, 1 drop of stock solution to 5 ml of insect Ringer. The neurones were stained for about 10 min and then examined.

To demonstrate the Golgi bodies, preparations were made by the standard Golgi techniques, Aoyama's silver method (Aoyama, 1929), Kolatchev's technique (Kolatchev, 1916), and the Mann–Kopsch technique (Weigl, 1910). The mitochondria were studied in preparations made by Baker's (1957*a*) 'HPO' technique. A number of preparations of the neurones were made by modifications of Bodian's protargol method (McClung Jones, 1950; Power, 1943). The histochemical tests performed on the neurones are given in the appendix.

Results

The neurones lie around the periphery of the central mass of nerve-fibres in the ganglion, forming an incomplete layer between the fibres and the connective-tissue sheath. There are two main types of neurone. Some are very large, having a diameter between 30 and 55 μ, while others are much smaller, being of diameter between 14 and 30 μ. Apart from the difference in size, the two types of neurone appear to be identical.

The most characteristic structures in invertebrate neurones are the Golgi bodies or dictyosomes (fig. 1, A). In the cockroach, these structures were seen most clearly after Kolatchev's technique, but were also visible after both the Aoyama and Mann–Kopsch methods. They appear in sections as crescent-shaped bodies, but in a few instances they form complete circles; these structures are between 1·5 and 3 μ in diameter. The interior of the dictyosome is darker than the surrounding cytoplasm. It must be mentioned that the dictyosomes are visible only after the techniques listed above and that these techniques are somewhat suspect since they are dependent upon the deposition of silver or osmium, a process which may be non-specific (Baker, 1957*b*).

The question now arises whether the crescents and rings represent structures of this shape in the cell, or are a form of artifact due to the deposition of osmium or silver on some other cell inclusion. In the neurones of some other invertebrates, e.g. *Helix aspersa* and *Locusta migratoria*, it has been shown that the Golgi bodies are formed by such deposition on the lipochondria (Chou, 1957; Shafiq, 1953). In an attempt to answer this question, the neurones were examined after vital staining, and also in preparations designed to show the presence of lipids. When the neurones are vitally stained with neutral red, the cell appears to be full of small, homogeneous, spherical bodies, varying between 0·5 and 2 μ in diameter. These correspond very closely with the distribution and size of the lipochondria seen in gelatin sections coloured with Sudan black (fig. 1, C), and also with the distribution

Fig. 1 (plate). A, section of a neurone showing the dictyosomes. Kolatchev preparation.
B, section of a neurone showing the structures coloured with Sudan black after Champy fixation.
C, section of a neurone showing the lipochondria coloured with Sudan black.

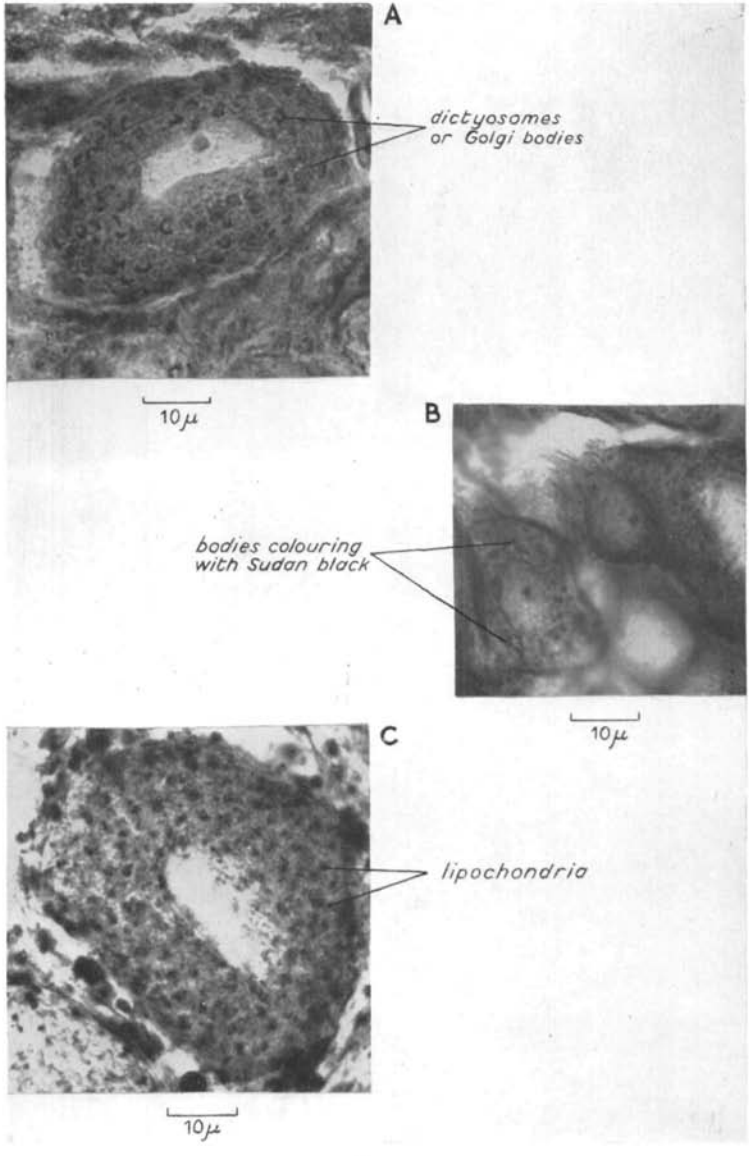

FIG. 1

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of the Golgi bodies, but the dictyosomes are sometimes 2·5 μ across, whereas the largest lipochondria are about 2 μ in diameter. Nevertheless, these results suggest that, as in *Helix* and *Locusta*, the dictyosomes may be formed by the deposition of osmium or silver on the surface of the lipochondria, the lipids inside being removed by the subsequent dehydrating and embedding procedures.

To obtain further evidence for this suggestion ganglia were fixed in Champy's fluid, as in a Kolatchev preparation, and embedded in gelatin. Sections of the ganglia were bleached in hydrogen peroxide (20 vol. diluted 1 in 4 with water) for 2 h to remove the dark coloration due to the osmium contained in the fixative, and then coloured with Sudan black; coloration at room temperature for 30 min was found to be the most satisfactory. This procedure revealed structures which are almost indistinguishable from the Golgi bodies (fig. 1, B). They have the appearance of a dark ring or crescent, with a less dense area inside. This result provides direct evidence that the osmium tetroxide used in making Golgi preparations is reduced by the lipids in the lipochondria. The crescent shapes are most probably due to an uneven deposition of osmium, and their greater diameter compared with the lipochondria may be caused by a spherical layer of osmium around a lipochondrion breaking as the tissue was dehydrated, or because the osmium is deposited on the outside of the lipochondrion, the internal diameter being that of the globule. It therefore seems reasonable to conclude that the dictyosomes are artifacts produced by the deposition of osmium or silver on the surface of the lipochondria.

The mitochondria of the neurones can be seen in cells vitally stained with Janus green and in preparations made by Baker's HPO technique. There is a large number of mitochondria scattered throughout the neurone. Some are small granules of about 0·5 μ diameter, while others are thin rods between 1·5 and 2·5 μ long. Some of the rods appear to have a granule at each end.

Neurofibrillae in the neurones of *Periplaneta* have been described by several authors (Hess, 1958; Pipa, Cook, and Richards, 1959), but they were not visible in the Bodian's protargol preparations made in this investigation. This result may be due to the origin of the protargol (G. T. Gurr), since some authorities suggest that satisfactory results are obtained only with protargol from one source (Gatenby and Beame, 1950).

The nuclei of the neurones are large, varying in diameter between 9 and 20 μ. They are roughly spherical and possess an obvious nucleolus. It will be noticed that the range in nuclear size is much smaller than the range in cell size. This results in the division of the neurones into two types, the small neurones with a relatively large nucleus and little cytoplasm, and large neurones with a nucleus of similar size, but a large amount of cytoplasm.

The cell membranes of the large neurones are indented, so that processes of the surrounding connective tissue and glial cells appear to penetrate the cytoplasm of the neurones. This phenomenon has been described by Hess (1958)

in his electron microscope study of the cockroach (*P. americana*) and by Wigglesworth (1959) in *Rhodnius prolixus*.

Histochemistry of the neurones

Carbohydrates. The neurones are only very weakly positive to the periodic acid / Schiff reaction; the individual cytoplasmic inclusions cannot be seen. The reaction was not increased when fixatives containing acridine and cetylpyridinium chloride, as recommended by Williams and Jackson (1956) for the preservation of mucopolysaccharides, were used. It is unaltered if the sections are first incubated in either diastase (0·1% aqueous) or saliva for 1¼ h at 37° C; this suggests that little if any glycogen is present. This result is further supported by the fact that Best's carmine test for glycogen is negative in the neurones.

Lipids. Histochemically, the lipochondria are the most complex constituent of the neurones. As mentioned previously, they can be coloured by Sudan black and also by Sudan IV. Some of their lipid is in the form of phospholipid since they are positive with Baker's acid haematein test.

The possibility exists that the lipochondria may also contain cerebrosides. Some ganglia were extracted in hot and others in cold acetone, and embedded in gelatin. The sections obtained were coloured with Sudan black. After hot acetone extraction no appreciable amount of lipid remains in the cell, but after cold acetone extraction the lipochondria are still visible although it is apparent that some lipid has been displaced into the cytoplasm. Phospholipids are not extracted by cold acetone, but cerebrosides are removed (Casselman and Baker, 1955). It appears, therefore, that the lipids removed by the cold acetone are cerebrosides, the phospholipids remaining in the lipochondria.

Of the fixatives suggested for 'unmasking' lipids (Bradbury and Clayton, 1958; Clayton, 1959), Flemming's fluid and mercuric chloride were used. But the subsequent colouring of the sections with Sudan black failed to reveal any 'masked' lipid in the neurones.

Proteins. The cytoplasmic inclusions could not be distinguished in tests for proteins (Barnard and Danielli's test, Sakaguchi, Hg / nitrite).

Nucleic acids. The chromatin of the nuclei gives a positive reaction with the Feulgen test. The cytoplasm is strongly basiphil; this was shown by the pyronin / methyl green test. This is probably due largely to the presence of RNA, since treatment with ribonuclease reduces the basiphilia considerably.

Phosphatases. Danielli's (1953) modification of Gomori's glycerophosphate technique for alkaline phosphatase was performed on sections of frozen-dried ganglia. No alkaline phosphatases could be detected in the neurones, even after the addition of disodium phosphate to the incubating mixture (a modification suggested for use when only minute quantities of enzyme are suspected to be present).

Discussion

The lipochondria are shown clearly after vital staining with neutral red, or after the coloration of fixed material with Sudan black. Bodies staining with

neutral red are described in the neurones of *P. americana* by Muliyil (1935), who considers that they are different from the Golgi bodies, although both go towards the centripetal pole when the cells are centrifuged. It appears probable that the neutral red bodies of Muliyil are in fact the lipochondria.

The Golgi bodies of *B. orientalis* and *P. americana* have been described previously by Bialkowska and Kulikowska (1912) and by Muliyil (1935) respectively. Their descriptions are essentially similar to the one in this paper. But they did not describe the lipochondria, and therefore the similarity in the distribution of the lipochondria and Golgi bodies within the cells was not apparent to them. This similarity of distribution suggests that the lipochondria and Golgi bodies may well be the same cell inclusions, the dictyosomes being formed as a result of the action of the Golgi techniques on the lipochondria. Further evidence for the lipid nature of the Golgi bodies is provided by their coloration with Sudan black after fixation in Champy's fluid. The main objection to this idea is that the dictyosomes are larger than the lipochondria, but this is to be expected if the osmium or silver is deposited on the outside of the lipochondrion. The crescents and rods are the result of the unevenness of the deposition of osmium and silver.

Histochemically, cockroach neurones are similar to locust neurones (Shafiq and Casselman, 1954). In both the lipochondria are all identical and may be termed 'cerephos globules' as they contain cerebroside and phospholipid (Baker, 1957*b*).

The cell inclusions seen by the use of the light microscope are also evident in electron micrographs of sections of the cockroach neurone, but in addition large numbers of small granules are found; these probably represent the basiphil material of the cytoplasm. A number of Golgi bodies are also seen in electron micrographs (Hess, 1958). These Golgi bodies are similar to those described by Gatenby, Tahmisian, Devine, and Beams (1958) in other orthopteran cells. But it has been suggested that the Golgi bodies in *Periplaneta* are artifacts produced by the deposition of osmium or silver on the lipochondria. Perhaps this can be reconciled with the electron microscope evidence; for it has been shown that the lipid globules in *H. aspersa*, which contain only phospholipid, appear in electron micrographs of material fixed in buffered osmium tetroxide as a series of curved or flat parallel lamellae with a number of scattered vesicles. If, however, calcium chloride is added to the fixative, the globules appear spherical, with concentric parallel lamellae round the periphery (Chou and Meek, 1958). It has been suggested by Schmidt (1939) that phospholipid molecules lie with their hydrophobe ends together, while the hydrophil ends associate with water. Thus in lipid globules these would form concentric layers of phospholipid and water. Baker (1958) suggests that these concentric layers remain intact during fixation if the molecular bonds are stabilized by the presence of calcium ions in the fixative, but under normal conditions of osmium fixation the layers break, with the result that a series of parallel crescent-shaped lamellae are formed. The lipochondria of *Periplaneta* contain phospholipid; so it is possible that the

typical Golgi bodies seen in the electron micrographs could be produced in this way by the action of the osmium fixative on the phospholipids in the lipochondria. If this is so, then the discrepancy between the results with the light microscope described in this paper, and those with the electron microscope, may be apparent rather than real.

I should like to thank Professor R. Dennell for his suggestions while this paper was in preparation and Mr. P. Howarth for preparing the photomicrographs. I am grateful to Professor H. Graham Cannon, F.R.S., for his encouragement and interest in this work.

REFERENCES

AOYAMA, F., 1929. Z. wiss. Mikr., **46**, 489.
BAKER, J. R., 1945. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947. Ibid., **88**, 115.
—— 1949. Ibid., **90**, 293.
—— 1956*a*. Ibid., **97**, 161.
—— 1956*b*. Ibid., **97**, 621.
—— 1957*a*. Ibid., **98**, 425.
—— 1957*b*. Symp. Soc. exp. Biol., **10**, 1.
—— 1958. J. Histochem. Cytochem., **6**, 303.
BARNARD, E. A., and DANIELLI, J. F., 1956. Nature, **178**, 1450.
BIALKOWSKA, W., and KULIKOWSKA, Z., 1912. Bull. int. Acad. Cracovie, Ser. B (no vol. number), 449.
BRADBURY, S., 1956. Quart. J. micr. Sci., **97**, 323.
—— and CLAYTON, B.-P., 1958. Nature, **181**, 1347.
CAIN, A. J., 1947. Quart. J. micr. Sci., **88**, 383.
CASSELMAN, W. G. B., 1959. *Histochemical technique.* London (Methuen).
—— and BAKER, J. R., 1955. Quart. J. micr. Sci., **96**, 49.
CHOU, J. T. Y., 1957. Ibid., **98**, 47.
—— and MEEK, G. A., 1958. Ibid., **99**, 279.
CLAYTON, B. P., 1959. Ibid., **100**, 269.
DANIELLI, J. F., 1953. *Cytochemistry. A critical approach.* New York (Wiley).
FEULGEN, R., and ROSSENBECK, H., 1924. Hoppe-Seyl. Z., **135**, 203.
GATENBY, J. B., and BEAMS, H. W., 1950. *The microtomist's vade-mecum.* London (Churchill).
—— TAHMISIAN, T. N., DEVINE, R., and BEAMS, H. W., 1958. Cellule, **59**, 25.
GRESSON, R. A. R., THREADGOLD, L. T., and STINSON, N. E., 1956. Ibid., **58**, 7.
HERXHEIMER, G., 1901. Dtsch. med. Wschr., **27**, 607.
HESS, A., 1958. J. biophys. biochem. Cytol., **4**, 731.
JORDAN, B. M., and BAKER, J. R., 1955. Quart. J. micr. Sci., **96**, 177.
KOLATCHEV, A., 1916. Arch. Russes. d'Anat. d'Hist. d'Emb., **1**, 383.
LISON, L., 1953. *Histochimie et cytochimie animales.* Paris (Gauthier-Villars).
MCCLUNG JONES, R., 1950. *McClung's handbook of microscopical technique.* London (Cassell).
MULIYIL, J. A., 1935. Z. Zellforsch., **23**, 627.
NATH, V., 1957. Nature, **180**, 967.
PEARSE, A. G. E., 1960. *Histochemistry, theoretical and applied.* London (Churchill).
PIPA, R. L., COOK, E. F., and RICHARDS, A. G., 1959. J. comp. Neurol., **113**, 401.
POWER, M. E., 1943. J. Morph., **72**, 517.
SCHMIDT, W. J., 1939. Nova Acta Leop., **7**, 1.
SHAFIQ, S. A., 1953. Quart. J. micr. Sci., **94**, 319.
—— and CASSELMAN, W. G. B., 1954. Ibid., **95**, 315.
WEIGL, R., 1910. Bull. int. Acad. Cracovie, Ser. B (no vol. number), 691.
WIGGLESWORTH, V. B., 1959. Quart. J. micr. Sci., **100**, 299.
WILLIAMS, G., and JACKSON, D. S., 1956. Stain Tech., **31**, 189.

APPENDIX

Table of methods and results

Test	*Reference*	*Neurones*	
		Cytoplasm	*Lipochondria*
Carbohydrates			
PAS	Pearse, 1960	+	not visible
PAS with no oxidation		O	,,
PAS after diastase		+	,,
PAS after saliva		+	,,
Best's carmine	Casselman, 1959	O	,,
Lipids			
Sudan IV	Herxheimer, 1901	+	++
Sudan black	Baker, 1945, 1949, 1956*b*	+	++
Sudan black after cold acetone	Casselman and Baker, 1955	+	+
Sudan black after hot acetone	Casselman and Baker, 1955	O	O
Acid haematein	Baker, 1946	O	++
Acid haematein: pyridine extraction	Baker, 1946	O	O
Nile blue	Cain, 1947	blue	blue
Liebermann	Lison, 1953	O	O
Windaus	Lison, 1953	O	O
Proteins, &c.			
Coupling reaction	Barnard and Danielli, 1956	++	not visible
Coupling reaction after benzoylation	Barnard and Danielli, 1956	O	,,
Sakaguchi	Baker, 1947	+	,,
Hg / nitrite	Baker, 1956*a*	+	,,
Nucleic acids			
Feulgen	Feulgen and Rossenbeck, 1924	chromatin+	O
Feulgen control		O	O
Pyronin / methyl green	Jordan and Baker, 1955	++	not visible
Pyronin / methyl green after RNAase	Bradbury, 1956	O	,,
Phosphatase			
Gomori's test for alkaline phosphatase	Danielli, 1953	O	,,

KEY: ++ = medium reaction; + = weak reaction; O = negative reaction.