Cytological Studies of the Neurones of *Locusta migratoria*

Part III. Histochemical Investigations, with Special Reference to the Lipochondria

By SAIYID AHMAD SHAFIQ AND W. G. BRUCE CASSELMAN

(*From the Cytological Laboratory, Department of Zoology and Comparative Anatomy, Oxford*)

With one plate (fig. 1)

SUMMARY

The motor neurones of the thoracic ganglia of adult *Locusta migratoria* have been studied by *in situ* histochemical techniques. Phospholipides and unsaturated cerebrosides are concentrated in the lipochondria; acetalphosphatides, cholesterol, protein, and ribonucleic acid are diffusely present throughout the cytoplasm; little nucleic acid is demonstrable in the nuclei.

INTRODUCTION

IN earlier studies of the neurones of *Locusta migratoria* (Shafiq, 1953, 1954) it was found that these cells contain lipochondria (osmiophil bodies). It was concluded that the 'neutral red' or 'vacuome' granules are these structures stained by such dyes as neutral red or methylene blue; that the 'Golgi' pictures of 'curved and circular dictyosomes' are produced by deposits of osmium or silver on the lipochondria; and that the 'secretion' or 'intraneuronal' granules staining with acid fuchsin in paraffin sections are in fact lipochondria. The present paper deals with the histochemistry of these nerve cells with special reference to the lipochondria. Such an investigation of these cells does not appear to have been reported previously.

MATERIAL AND METHODS

The motor neurones of the thoracic ganglia of mature, adult *L. migratoria* were studied. The insects were kindly supplied by the Anti-locust Research Centre, London. The ganglia were dissected out and fixed in formaldehyde-calcium (Baker, 1944), cobalt-formaldehyde-calcium (McManus, 1946*b*), or Serra's (1946), Helly's, or Zenker's fluid. Some ganglia fixed in formaldehyde-calcium were postchromed in 5 per cent. potassium dichromate for 16–18 hours at 37° C. and others were treated overnight with 0·1 per cent. 'Aerosol OT' (di-octyl ester of sodium sulphosuccinic acid, British Drug Houses Ltd., now called 'Manoxol OT'). Frozen sections of gelatin-embedded ganglia or paraffin sections were cut as required. Some fresh, unfixed ganglia were extracted with cold or hot acetone or hot ether by Keilig's (1944) method as modified by Pearse (1953). Other ganglia were fixed in weak Bouin's fluid and extracted with pyridine (Baker, 1946).

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 315-320, Sept. 1954.]

For the detection of lipides Sudan black B was most often used as a saturated solution in either 70 per cent. ethanol (Baker, 1944) or propylene glycol (Chiffelle and Putt, 1951). The reagents were allowed to act for as long as 30 minutes at room temperature, 37° C. or 60° C. Additional studies were made with saturated solutions of acetylated Sudan black B (Lillie and Burtner, 1953; Casselman, 1954) or of Sudan IV in 70 per cent. ethanol or of oil red O in *iso*-propyl alcohol (Lillie, 1944), and with 0·02 per cent. and 1 per cent. aqueous Nile blue sulphate (Cain, 1947*a*, 1948). The acid haematein test was chosen for the detection of phospholipides and phosphatidic acids (Baker, 1946, 1947*b*; Cain, 1947*b*; Casselman, 1952). For acetal-phosphatides Hayes's (1949) and Cain's (1949) variants of the plasmal reaction, with additional tests recommended by Danielli (1953), were employed. A modified Liebermann–Burchardt reaction (Romieu, 1925) was used for detecting cholesterol and its esters.

A number of investigations were made with the periodic acid Schiff (McManus, 1946*a*) and performic acid Schiff (Lillie, 1952) tests. Control sections were either acetylated with acetic anhydride in glacial acetic acid (Casselman, Macrae, and Simmons, 1954) or treated with bromine gas or a saturated aqueous solution of bromine. The Schiff's reagent for these and other tests requiring it was prepared as described by de Tomasi (1936).

The distribution of pentosenucleic acids was studied in paraffin sections of tissues fixed in Helly's, Serra's, or Zenker's fluid by using toluidine blue or pyronin/methyl green (Brachet, 1953; Jordan and Baker, unpublished). Some sections were first treated with 1 *N*. HCl (Dempsey, Singer, and Wislocki, 1950) or 4 per cent. trichloroacetic acid (Schneider, 1945). The nucleal reaction (Feulgen and Rossenbeck, 1924) was used for the study of deoxypentosenucleic acids.

Tests for proteins included the coupled tetrazonium reaction introduced by Danielli (1947, 1949) and further developed by Pearse (1953), and Millon's (Bensley and Gersh, 1933), Sakaguchi's (Baker, 1947*a*), and Chèvremont and Frederic's (1943) tests.

Observations and Discussion

The various tests were applied first to unextracted tissues and then to extracted ones.

Unextracted neurones

In frozen sections of ganglia fixed in formaldehyde-calcium or cobalt-formaldehyde-calcium the lipochondria of the neurones are coloured intensely by Sudan black B, while the cytoplasm is only weakly Sudanophil. Strongest coloration is obtained when this reagent in propylene glycol is used for 30 minutes at 60° C. Unlike those in other species (Baker, 1949) these lipochondria can be coloured readily without postchroming the tissue, although this does improve the results.

After 'Aerosol' treatment the lipochondria are no longer discernible but the cytoplasm of the motor neurones is diffusely and quite strongly Sudanophil.

At least some of the lipochondria resist paraffin embedding, regardless of the fixative used. They are best preserved when fixed in Helly's or Champy's fluid or when postchromed after formaldehyde fixation. When fixed in Bouin's fluid they are much reduced in size.

The lipochondria are coloured as intensely by acetylated Sudan black B as by the unacetylated, but whereas the acetylated derivative is readily extracted by 70 per cent. ethanol, the Sudan black B is not extracted even after 20 minutes. In this case, the 'stable Sudanophilia' is due to lipide material, because it is not demonstrable in ganglia which have been extracted with pyridine. Similarly, Sudan black B was not appreciably extracted after 35 minutes' treatment with 70 per cent. ethanol from highly purified lecithins tested on cigarette paper (Baker, 1946) or by Coujard's (1943) technique. The phenomenon of 'stable Sudanophilia' was first described by Lillie and Burtner (1953) in their studies of human neutrophil leucocytes. They attribute it to a chemical reaction between Sudan black B and some tissue-constituent rather than to the ordinary solution of the colorant in lipide.

When frozen sections are treated with oil red O or Sudan IV for 5 or 30 minutes at room temperature or at 60° C., nothing within the motor neurones is coloured, but the fat-body surrounding the ganglia gives an intensely positive reaction.

Diffuse blue staining throughout the cells results when the Nile blue test is applied to frozen sections of formaldehyde-calcium-fixed tissues, whether postchromed or not. The lipochondria cannot be distinguished.

Some tests were carried out on pairs of frozen sections. One section remained in distilled water and served as a control, while the other was extracted with cold acetone for 24 hours. After this, they were simultaneously treated with Sudan black B. There are no differences between the coloration of the lipochondria or cytoplasm in the two sections, even though the reaction of the surrounding adipose tissue is considerably reduced by the acetone extraction.

Thus, there appears to be little or no triglyceride present in the lipochondria or the cytoplasm of the motor neurones. Acetone extraction does not decrease their coloration by Sudan black B. They do not give positive reactions with oil red O, Sudan IV, or the oxazone of Nile blue. On the other hand, the presence of compound lipides is suggested by the resistance of the lipochondria to paraffin embedding and their behaviour with Sudan black B. This was verified by further studies.

The acid haematein test gives a strong positive reaction with the lipochondria and a weak diffuse reaction in the cytoplasm (fig. 1, A). This is absent from the pyridine-extracted controls (fig. 1, C) and is not obtained if the postchroming is omitted. The reaction is, therefore, a true positive one, specific for phospholipides and phosphatidic acids, and is not due to other organic substances which yield false positive reactions or to inorganic constituents acting as mordants for the haematein.

With the Liebermann–Burchardt reaction, a diffuse, weakly positive reaction is obtained which does not differentiate between the cytoplasm and the lipochondria.

The cytoplasm of the neurones also gives a diffuse weak reaction with Schiff's reagent after treatment with mercuric chloride (Cain, 1949; Hayes, 1949) or 0·1 *N.* HCl (Danielli, 1953). Neither unfixed nor formaldehyde-calcium-fixed ganglia react directly with Schiff's reagent. Therefore, neither free aldehydes nor any carbonyl compounds resulting from atmospheric oxidation or the action of formaldehyde on unsaturated lipides (Wolman and Greco, 1952) are present. The positive plasmal reaction may thus be attributed to acetalphosphatides and is not a pseudo-plasmal reaction.

In paraffin sections of Helly- or Zenker-fixed ganglia and in frozen sections of formaldehyde-calcium-fixed ones the lipochondria give positive reactions and the cytoplasm weak diffuse ones with the PAS (fig. 1, D) and PFAS tests. The PAS reactions are blocked by acetylation and the PFAS ones by bromination. Both remain positive when applied to tissues in which aldehydes produced by the plasmal reaction are first blocked by sodium bisulphite.

With toluidine blue or the pyronin/methyl green reagent the cytoplasm of the neurones is strongly basiphil. The reactions are greatly reduced by first treating with HCl or trichloracetic acid, suggesting that the basiphilia is due to pentosenucleic acids. In the motor neurones the nucleal reaction is negative, although it is positive in the nuclei of the smaller cells of the ganglia. Neither of the nucleic acids could be localized in the lipochondria.

The various protein tests give diffuse positive reactions throughout the cytoplasm, which is most intensely coloured by the tetrazonium reaction. The lipochondria could not be differentiated from the cytoplasm in any of these tests.

Extracted neurones

Differential extraction methods were used to identify further the substances in the lipochondria responsible for the reactions described above. Although the solubilities of lipides in mixtures may be appreciably different from those

---

FIG. 1 (plate). All photomicrographs are at the same magnification.

A, a motor neurone from the adult locust, showing the positive acid haematein reaction given by the lipochondria.

B, acid haematein reaction on a section of ganglion extracted with hot acetone. All the phospholipides have been displaced from the lipochondria and cytoplasm and have come to lie in one region of the cell. A similar appearance is obtained after extraction with cold acetone.

C, acid haematein reaction on ganglia fixed in weak Bouin's fluid and extracted with pyridine. All phospholipides have been extracted.

D, a neurone showing a positive PAS-reaction in the lipochondria. A similar result is obtained after the PFAS test.

E, PAS reaction on ganglion extracted with cold acetone. Some PAS-positive lipide remains in the lipochondria. Some is displaced.

F, PAS reaction on ganglion extracted with hot acetone. All the cerebroside has been removed.

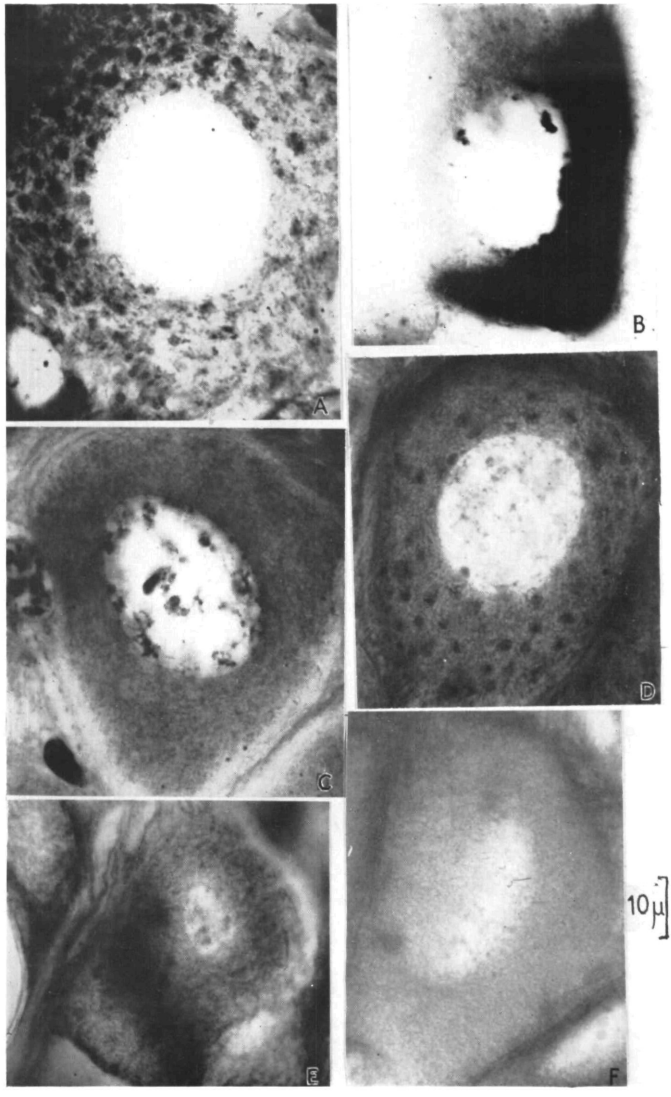

FIG. 1

S. A. SHAFIQ *and* W. G. B. CASSELMAN

of the components when in the pure state, extraction methods proved helpful in this case. They were applied to unfixed tissues because even fixation alters the extractability of the lipides. For example, when ganglia are fixed in formaldehyde-calcium and then extracted with cold acetone, no lipide displacement such as described below occurs.

After cold acetone extraction, Sudan black B shows that while some lipide remains in some of the lipochondria, a great amount has been displaced within the cells by the inward diffusion of the solvent. The lipide remaining in the lipochondria is PAS- (fig. 1, E) and PFAS-positive and acid haematein negative. The displaced lipide is strongly acid haematein positive and also weakly PAS- (fig. 1, E) and PFAS-positive. The latter reactions are appropriately blocked by acetylation and bromination respectively.

After hot acetone extraction no lipide is demonstrable in the lipochondria by Sudan black B but the displaced lipide is still present. The acid haematein test also shows only the displaced lipide (fig. 1, B). The PAS (fig. 1, F) and PFAS tests show nothing in the cells.

The ganglia were so distorted after extraction with hot ether that morphological details could not be distinguished.

In ganglia fixed in weak Bouin's fluid and extracted with pyridine, the lipochondria are not demonstrable by any of the above techniques. The cytoplasm is negative to the Sudan black B, PAS, and PFAS tests, but with the acid haematein test it is coloured diffusely dark brown and there are patches of similar colour in the nuclei (fig. 1, C), probably attributable to nucleic acids.

Only diffuse positive protein reactions are obtained in ganglia extracted with pyridine. After cold and hot acetone extraction, however, the coupled tetrazonium reaction gives a stronger colour at the site of the displaced lipides than in the rest of the cell. It may be that some of the phospholipides are associated with protein. There is not a demonstrable concentration of the latter in the lipochondria, which thus differ from the 'Golgi bodies' in the oocytes of the water-spider investigated by Krishna (1953).

From these observations it is concluded that the lipochondria contain two principal lipide components. The first is Sudanophil, acid haematein positive, PAS- and PFAS-negative, and insoluble in cold or hot acetone, but soluble in pyridine. This is identified as phospholipide. The second component is Sudanophil, acid haematein negative, PAS- and PFAS-positive, insoluble in cold acetone but soluble in hot acetone and in pyridine. This is identified as unsaturated cerebroside. The faint PAS- and PFAS-reactions noted for the phospholipides displaced by cold acetone are attributed to traces of this cerebroside being dissolved in them.

Gersh (1948) and Arzac and Flores (1952) have considered that the positive PAS-reaction given by the 'Golgi apparatus' in certain other cells is due to a carbohydrate moiety unassociated with lipides because it resists extraction with fat solvents. Such a carbohydrate is not present in the lipochondria of the locust motor neurones, where this reaction is entirely attributable to unsaturated cerebrosides. Lipide-pigments may also be PAS- and PFAS-positive

during their formation but they appear only in older locusts than were studied here, and they differ from the lipochondria in other histochemical properties.

The authors are grateful to Dr. J. R. Baker for suggesting this problem and for much helpful advice and discussion during the course of the work. It is also a pleasure to thank Professor A. C. Hardy for accommodating us in the Department of Zoology and Comparative Anatomy at Oxford. This investigation was carried out while one of us (S. A. S.) was on a Rhodes Scholarship and study leave from Dacca University, Pakistan, and the other (W. G. B. C.) was a Merck Postdoctoral Research Fellow in the Natural Sciences of the National Research Council of Canada, on leave from the University of Toronto, Ontario, Canada.

REFERENCES

ARZAC, J. P., and FLORES, L. G., 1952. Stain Tech., **27**, 9.
BAKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947*a*. Ibid., **88**, 115.
—— 1947*b*. Ibid., 463.
—— 1949. Ibid., **90**, 293.
BENSLEY, R. R., and GERSH, I., 1933. Anat. Rec., **57**, 217.
BRACHET, J., 1953. Quart. J. micr. Sci., **94**, 1.
CAIN, A. J., 1947*a*. Ibid., **88**, 383.
—— 1947*b*. Ibid., 467.
—— 1948. Ibid., **89**, 429.
—— 1949. Ibid., **90**, 411.
CASSELMAN, W. G. B., 1952. Ibid., **93**, 381.
—— 1954. Ibid., **95**, 321.
CASSELMAN, W. G. B., MACRAE, A. I., and SIMMONS, E. S., 1954. J. Path. and Bact. (in the press).
CHÈVREMONT, M., and FREDERIC, J., 1943. Arch. Biol., **54**, 589.
CHIFFELLE, T. L., and PUTT, F. A., 1951. Stain Tech., **26**, 51.
COUJARD, R., 1943. Bull. Hist. Appl., **20**, 161.
DANIELLI, J. F., 1947. Symp. Soc. exp. Biol., **1**, 101.
—— 1949. Cold Spr. Harb. Symp. quant. Biol., **14**, 32.
—— 1953. *Cytochemistry: a critical approach.* New York (Wiley).
DEMPSEY, E. W., SINGER, M., and WISLOCKI, G. B., 1950. Stain Tech., **25**, 73.
FEULGEN, R., and ROSSENBECK, H., 1924. Zeit. Physiol. Chem., **135**, 203.
GERSH, I., 1948. Arch. Path., **47**, 99.
HAYES, E. R., 1949. Stain Tech., **24**, 19.
JORDAN, B. M., and BAKER, J. R., unpublished.
KEILIG, I., 1944. Virchows Arch. Path. Anat., **312**, 405.
KRISHNA, D., 1953. Quart. J. micr. Sci., **94**, 315.
LILLIE, R. D., 1944. Stain Tech., **19**, 55.
—— 1952. Ibid., **27**, 37.
LILLIE, R. D., and BURTNER, H. J., 1953. J. Histochem. and Cytochem., **1**, 8.
MCMANUS, J. F. A., 1946*a*. Nature Lond., **158**, 202.
—— 1946*b*. J. path. Bact., **58**, 93.
PEARSE, A. G. E., 1953. *Histochemistry: theoretical and applied.* London (J. A. Churchill).
ROMIEU, M., 1925. C.R. Soc. Biol., **92**, 787.
SERRA, J. A., 1946. Stain Tech., **21**, 5.
SHAFIQ, S. A., 1953. Quart. J. micr. Sci., **94**, 319.
—— 1954. Ibid., **95**, 305.
SCHNEIDER, W. C., 1945. J. Biol. Chem., **161**, 293.
TOMASI, J. A. de, 1936. Stain Tech., **11**, 137.
WOLMAN, M., and GRECO, J., 1952. Ibid., **27**, 317.