Lipids of Isolated Neurons

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1. Lipids were extracted from neurons isolated from the lateral vestibular nucleus of ox (*Bos taurus* L.) and the ganglia of *Aplysia punctata* Cuvier. 2. Thin-layer chromatography of ox-neuron lipid revealed three major fractions corresponding to neutral lipid, phosphatidylethanolamine and phosphatidylserine. Part of the phosphatidylethanolamine was present as the plasmalogen. 3. *Aplysia*-neuron lipid contained neutral lipid, phosphatidylethanolamine and phosphatidylserine. Both phospholipids appeared to be present predominantly as the plasmalogen form. 4. The fatty acids of alkali-labile lipids of ox neurons were examined by gas-liquid chromatography. The major fatty acids were oleic acid, stearic acid and palmitic acid.

Knowledge of brain lipids has been derived from studies on whole brain or gross anatomical areas of the brain. Investigations on the nervous system would be considerably facilitated if it were possible to obtain lipid analyses of particular brain-cell types. However, the sampling of neuronal or glial cells in sufficient quantity and of sufficient purity for chemical investigations presents considerable difficulties, and with the exception of the hand dissection techniques of Hyden (1959) and Lowry (1953) a procedure has been lacking. We have recently reported a method for the isolation of ox (*Bos taurus* L.) neurons in sufficient quantity for chemical analysis (Roots & Johnston, 1964), and in the present paper we report some observations on the lipids from neurons of the lateral vestibular nucleus of ox brain and from the ganglia of *Aplysia punctata* Cuvier. Because of the possible intrusion of contaminants and artifacts from various sources, the investigations of ultramicro quantities of lipids is hazardous; considerable attention, therefore, is given to precautions that must be observed in this type of study.

MATERIALS AND METHODS

Isolation of neurons. Ox brain stems were obtained immediately after slaughter and transported to the laboratory in plastic bags on ice. The lateral vestibular nuclei were excised from six brains and the neurons were collected in Ringer-Locke solution in cavity slides (Roots & Johnston, 1964). This medium contained (g./l.): NaCl, 9; KCl, 0.42; CaCl₂, 0.24; NaHCO₃, 0.2. Its pH was 7.4. About 300–500 neurons per slide were collected until a total of 2500–3500 cells was obtained. The isolation procedures were carried out at 4°. As each slide was filled, the Ringer-Locke solution

was drawn off with a fine pipette and the neurons were immediately frozen and kept at –25° until the lipid could be extracted.

The neurons of *Aplysia* were obtained by dissecting out the buccal, cerebral, pleural, pedal, parietal and visceral ganglia, cutting the sheath and ‘shelling out’ the cells into an artificial sea-water medium at 4° in a cavity slide. This medium contained (g./l.): NaCl, 24; KCl, 0.742; CaCl₂, 1.135; MgCl₂·6H₂O, 10.893; Na₂SO₄, 4.012; NaHCO₃, 0.197. Its pH was 7.2. Since there is intimate association between neurons and glia (Bullock, 1961), samples obtained in this way are not as pure as the ox-neuron collections, which are free of glial material and other extraneous matter.

Extraction of lipid. The lipid was extracted at room temperature with 3 × 150 µl. of chloroform-methanol (2:1, v/v) per slide. Extraction was facilitated by grinding the neurons with a small glass rod and the samples were checked under a microscope for complete disruption of the cells. The extracts were combined and the solvent was removed under a stream of oxygen-free nitrogen. The dry lipid was then redissolved in the same solvent and the solvent removed as before. Redissolution of the lipid was employed as a means of purification in preference to a water-washing procedure, which tends to remove some lipid material (Rouser, Kritchevsky, Heller & Lieber, 1963). Since the ox neurons were counted the amount of lipid obtained was weighed on an Oertling Q10 decimicrobalance. It was considered that the amount of lipid per cell would be more informative and accurate than any attempt to weigh the cells, which would be subject to considerable error. The lipid obtained was examined immediately if possible, but otherwise it was stored under nitrogen at –25°. All manipulations of the lipid were carried out in an atmosphere of nitrogen.

Thin-layer chromatography of lipid. Thin-layer chromatography of the lipid was carried out on microscope slides (3 in. × 1 in.), with Silica Gel G as the adsorbent. Two types of plates, ‘basic’ and ‘neutral’, were prepared as described by Skipski, Peterson & Barclay (1962). The solvent systems employed were chloroform-methanol-acetic acid-water

(50:25:8:4, by vol.) for basic plates and (65:25:8:4, by vol.) for neutral plates (Skipski *et al.* 1962). The following detection methods were employed on both types of plate:

1. All lipids were revealed by spraying with 50% (v/v) H_2SO_4 and heating over a flame, or by exposing the plates to iodine vapour in a Petri dish (Mangold & Malins, 1960).

2. An ammonium molybdate-perchloric acid spray was used as a universal reagent for the detection of phospholipids (Wagner, Hörhammer & Wolff, 1961).

3. Phospholipids with a free amino group were detected with a ninhydrin spray (Wagner *et al.* 1961).

4. A 0.5% solution of 2,4-dinitrophenylhydrazine in 2 N-HCl was used to detect plasmalogen-type lipids (Reitsem, 1954).

Standards employed were commercial preparations (L. Light and Co. Ltd.) of cholesterol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, and laboratory preparations of sphingomyelin, cerebrosides and the phosphoglycerides from ox brain. By use of standards it was determined that it was possible to detect 0.5 $\mu\text{g.}$ of any lipid with either the H_2SO_4 spray or by exposure to iodine vapour. Ninhydrin gave a weak reaction with 0.5 $\mu\text{g.}$, but the other sprays required 1–3 $\mu\text{g.}$ for positive results. Since 10–15 $\mu\text{g.}$ of sample per spot was applied the limit of detection was 3%.

Gas-liquid chromatography. The gas-liquid chromatographic analysis of the methyl esters of ester-linked fatty acids only was undertaken, since direct methanolysis in acid solution gives rise to artifacts (Johnston & Roots, 1964). In view of this source of error, methyl esters of fatty acids were obtained by transmethylation with 1–2% sodium methoxide in methanol (Riemenschneider, Herb & Nichols, 1949). Plasmalogens and amide-linked fatty acids are stable under such conditions, and therefore the methyl esters obtained must have arisen solely from phosphoglycerides or triglycerides. The methyl esters were chromatographed on a polyethylene glycol adipate column at 179° with an argon flow of 30 ml./min. Identification of peaks was made by the use of standard fatty acids and the relative composition was based on measurements of peak area.

Precautions against the intrusion of contaminants. Since microgram quantities only of lipid were examined the following precautions were carefully observed. During the collection and subsequent treatment of the neurons they were protected from air-borne contamination by the use of plastic dust covers over the working area. The investigators kept their hair covered and forceps were used for handling glassware whenever practicable. The solvents employed, AnalaR or Reagent grade, were twice redistilled before use and were subsequently checked for residue on evaporation and the presence of thin-layer-chromatographic or gas-liquid chromatographic artifacts. Immediately before use the glassware, forceps and any other tool were washed in chloroform-methanol (2:1, v/v). Filter paper, a serious source of contamination, was exhaustively extracted with the solvent employed before use. The use of rubber or plastic stoppers, cotton wool etc. was avoided. Blanks were carried through the entire experimental procedures.

RESULTS AND DISCUSSION

The cell bodies were approximately 100 μ in width and 100–200 μ long. Their dendrites extended for up to 300 μ , branching several times during this

length, and in places boutons could be seen attached to the cells. Dark areas in the cells are deposits of lipochrome pigment, which is probably an oxidized lipid-protein complex associated with aging (Chu, 1954). Electron-microscopical observations revealed that, in general, the cytoplasmic inclusions of the cells were well preserved. However, the typical electron-micrographic image of a cell surface membrane was found to be absent over the greater part of the soma and dendrites and was seen usually only where boutons remained attached. We have ascribed the loss of the plasma-membrane image to a disruption of the molecular orientation of surface molecules that may or may not be reflected in the subsequent lipid analyses. The observations on and interpretations of fine structure of the isolated cells have been reported in detail by Roots & Johnston (1964).

The weighing of the ox-neuron lipid indicated that between 7.3 and 8.6 $\mu\text{g.}$ of lipid per cell was extracted. Since no volume measurements of the neurons are available, a direct comparison with the results of others is not possible; however, these values appear compatible with the values of 4.4 $\mu\text{g.}$ per cell given by Hydén (1959) for the smaller rabbit Deiter's cells.

Thin-layer chromatography of ox-neuron lipid revealed three major spots, which were identified as neutral lipid (largely cholesterol), phosphatidylethanolamine and phosphatidylserine. Phosphatidylethanolamine appeared to be the predominant lipid since it gave the greatest positive reaction to the sulphuric acid, iodine vapour, ninhydrin and ammonium molybdate reagents. This spot also reacted positively to the 2,4-dinitrophenylhydrazine reagent, indicating that the plasmalogen form was present. The plasmalogen is known to migrate in these systems with its corresponding diacyl-phospholipid. Phosphatidylserine, though giving positive reactions to sulphuric acid, iodine, ninhydrin and ammonium molybdate, was present to a much smaller extent than the ethanolamine lipid. The serine lipid gave no reaction to 2,4-dinitrophenylhydrazine.

The yellow lipochrome pigment migrated with the neutral-lipid spot at the solvent front.

The gas-liquid-chromatographic analysis of the methyl esters of ox-neuron lipid revealed three major components, palmitic acid, stearic acid and oleic acid. Traces of longer-chain acids were observed, but were not sufficiently clear and well eluted to justify quantitation. The amounts of the major fatty acids relative to each other were calculated as: palmitic acid, 25%; stearic acid, 28.5%; oleic acid, 46.5%.

The *Aplysia*-neuron lipid was also found to contain three major fractions corresponding to neutral lipid, phosphatidylethanolamine and phosphatidyl-

serine. A smaller fraction, behaving chromatographically like an inositol phospholipid, was also noted. The serine phosphatide predominated over the ethanolamine lipid, and both the serine phosphatide and ethanolamine phosphatide spots reacted very strongly to the 2,4-dinitrophenylhydrazine reagent, indicating that these phosphoglycerides were present mainly in their plasmalogen form. This was confirmed by the fact that the *Aplysia*-neuron lipid was stable to transmethylation with sodium methoxide.

It is difficult to compare the results of the present study with previous reports on the distribution of lipids in brain tissues, since the anatomical divisions that have been employed are themselves composed of several cell types (Folch-Pi & Le Baron, 1957; McIlwain, 1963). For example, neurons often constitute only 20% of grey matter. However, it is generally agreed that cerebroside and sphingomyelin are essentially components of myelin, and hence they would not be expected to occur as major constituents of the soma and dendrites of neurons. The conclusion that sphingolipids are absent must, however, await the development of more sensitive detection reagents or a means of isolating much larger numbers of neurons. These developments are also essential to study the ganglioside content of neurons, since we have found that the present means of detecting gangliosides are too insensitive to reveal the 1-3% that may be expected. Since phosphatidylcholine has been reported as a major fraction of lipid from various anatomical areas of the brain, the fact that it was not observed in the present study is surprising. This may indicate that lecithin is largely a component of myelin and glia.

Oleic acid, stearic acid and palmitic acid have been reported as the major fatty acids of mammalian brain (Johnston & Kummerow, 1960), and the findings that they represent the major fatty acids of the alkali-labile lipids of neurons is in agreement with these observations.

There appears to be insufficient information about the lipid composition of molluscan brains to warrant any comparison with the present results. However, plasmalogen lipids have been found as major constituents of terrestrial slug phosphoglycerides (Thompson & Hanahan, 1963), and hence the finding that considerable quantities of plasmalogens exist in *Aplysia* neuron lipid is not surprising.

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REFERENCES

- Bullock, T. H. (1961). In *Nervous Inhibition*, p. 233. Ed. by Florey, E. Oxford: Pergamon Press Ltd.
- Chu, L. W. (1954). *J. comp. Neurol.* **100**, 381.
- Folch-Pi, J. & Le Baron, F. N. (1957). In *Metabolism of the Nervous System*, p. 67. Ed. by Richter, D. Oxford: Pergamon Press Ltd.
- Hydén, H. (1959). *Nature, Lond.*, **184**, 433.
- Johnston, P. V. & Kummerow, F. A. (1960) *Proc. Soc. exp. Biol., N.Y.*, **104**, 201.
- Johnston, P. V. & Roots, B. I. (1964). *J. Lipid Res.* **5**, 477.
- Lowry, O. H. (1953). *J. Histochem. Cytochem.* **1**, 420.
- McIlwain, H. (1963). *Chemical Exploration of the Brain*, p. 113. Amsterdam: Elsevier Publishing Co.
- Mangold, H. K. & Malins, D. C. (1960). *J. Amer. Oil Chem. Soc.* **37**, 383.
- Reitsem, R. H. (1954). *Analyt. Chem.* **26**, 960.
- Riemenschneider, R. W., Herb, S. F. & Nichols, P. L., jun. (1949). *J. Amer. Oil Chem. Soc.* **26**, 371.
- Roots, B. I. & Johnston, P. V. (1964). *J. Ultrastruct. Res.* **10**, 350.
- Rouser, G., Kritchevsky, G., Heller, D. & Lieber, E. (1963). *J. Amer. Oil Chem. Soc.* **40**, 425.
- Skipski, V. P., Peterson, R. F. & Barclay, M. (1962). *J. Lipid Res.* **3**, 467.
- Thompson, G. A., jun. & Hanahan, D. J. (1963). *J. biol. Chem.* **238**, 2628.
- Wagner, H., Hörhammer, L. & Wolff, P. (1961). *Biochem. Z.* **334**, 175.