The Presence of Phosphatidylcholine in Neurons Isolated from the Lateral Vestibular Nucleus of Ox Brain

By PATRICIA V. JOHNSTON AND BETTY I. ROOTS

Department of Anatomy, University College London, Gower Street, London, W.C. 1

(Received 9 July 1965)

1. Lipid was extracted from neurons isolated from the lateral vestibular nucleus of ox brain and examined by two-dimensional thin-layer chromatography. 2. The major lipids found were phosphatidylethanolamine and its plasmalogen, phosphatidylcholine, phosphatidylserine and cholesterol.

Since phosphatidylcholine has been reported as a major constituent of lipids from various fractions and subcellular particles of brain (Davison & Graham-Wolfaard, 1964; Eichberg, Whittaker & Dawson, 1964) it was surprising that we were unable to detect any appreciable amount in the lipids of neurons isolated from the lateral vestibular nuclei of ox brains (Roots & Johnston, 1965a). Though this finding could be an indication that phosphatidylcholine is largely a component of glia and myelin it could also have been due either to a peculiarity of the particular neurons studied or to a hitherto unrecognized analytical artifact. It occurred to us that the intracellular pigment, lipofuscin (Bondareff, 1964), was concentrated in the lipid extracted from pure samples of neurons and that the high proportion of this complex may have interfered with the normal thin-layer chromatographic migration patterns of the polar lipids. Previously we have found that, because of the proportion and nature of an accompanying pigmented complex, the lipids from the chloragosomes of the earthworm (*Lumbricus terrestris* L.) fail to separate into homogeneous fractions when subjected to various chromatographic procedures (Roots & Johnston, 1965b). Recently we achieved separation of these lipids by the use of two-dimensional thin-layer chromatography. We have therefore re-examined neuronal lipids by using this technique.

MATERIALS AND METHODS

Material. Neurons were isolated from the lateral vestibular nuclei of fresh ox brains by the method described by Roots & Johnston (1964). From each batch of brains approx. 1000 cells were collected and placed in Ringer-Locke solution in glass cavity slides. The solution was withdrawn and the cells were immediately frozen and stored at -25° .

Extraction of lipid. The lipid was extracted at room tem-

perature with $3 \times 100 \mu\text{l.}$ of chloroform-methanol (2:1, v/v) per slide. Three separate samples, the combined extracts from 5380, 2125 and 3600 neurons respectively, were examined. The solvent was removed under vacuum and the dry lipid was transferred to glass tubes (5 mm. $\times$ 8 mm.) by redissolving it in approx. $200 \mu\text{l.}$ of the same solvent, which was then removed under a stream of O_2 -free N_2 . The lipid was examined immediately or stored only overnight under N_2 at -10° .

Thin-layer chromatography. Two-dimensional thin-layer chromatography was carried out on 3 in. $\times$ 3 in. plates with Silica Gel G as the adsorbent. Two or three platings were obtained from each lipid sample according to the size of the neuron collection (i.e. approx. 10–35 $\mu\text{g.}$ per plate). The plates were developed in the first direction with chloroform-methanol-water (65:25:4, by vol.) and in the second direction with butan-1-ol-acetic acid-water (6:2:2, by vol.) (Rouser, Galli, Lieber, Blank & Privett, 1964). In addition to the use of acid sprays and iodine vapour to reveal all lipids, plates were sprayed with 2,4-dinitrophenylhydrazine to reveal plasmalogens (Reitsem, 1954), ninhydrin to detect phospholipids containing free amino groups and a modified Dragendorff reagent to detect choline-containing lipids (Wagner, Hörhammer & Wolff, 1961). Several mixtures of phosphoglycerides and cholesterol were used to check the resolution and limits of detection. The lipids used were synthetic dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine (both from Sigma Chemical Co., St Louis, Mo., U.S.A.), phosphatidylserine (from ox brain; Koch-Light Laboratories Ltd., Colnbrook, Bucks.) and cholesterol (British Drug Houses Ltd., Poole, Dorset). In preference to the synthetic disaturated compounds phosphatidylethanolamine (from ox brain) and phosphatidylcholine (from egg) were used in some mixtures (Koch-Light Laboratories Ltd.). The relative proportion of the lipids was varied in each mixture and 10–40 $\mu\text{g.}$ samples were plated. The lipids were always well separated and it was possible to detect 0.5 $\mu\text{g.}$ of any lipid by using acid sprays or iodine vapour. The ninhydrin spray required 0.5–1.0 $\mu\text{g.}$ and the Dragendorff reagent at least 7.0 $\mu\text{g.}$ of the appropriate compounds.

During collection of the neurons and subsequent extraction and treatment of the lipid, Perspex covers were used to protect against airborne contamination. The solvents used were either redistilled Reagent or AnalaR grades,

except methanol, which was Spectrosol grade (Hopkin and Williams Ltd., Chadwell Heath, Essex). All manipulations of the lipid including plating for thin-layer chromatography were carried out under an atmosphere of N_2 .

RESULTS

Two-dimensional thin-layer chromatograms of the neuronal lipids showed phosphatidylcholine to be one of the major constituents. It was identifiable only by its R_F when smaller samples were used (10–25 μ g.), and when larger samples were employed (25–35 μ g.) by its positive reaction to the Dragendorff reagent. The other major lipids were phosphatidylethanolamine, phosphatidylserine and cholesterol. The first two of these were identified by their R_F values and their positive reaction to the ninhydrin reagent. The phosphatidylethanolamine spot also gave a positive reaction to 2,4-dinitrophenylhydrazine reagent, indicating that part of this lipid is present as the plasmalogen. The cholesterol spot at the second solvent front was obscured by the large amount of lipofuscin. However, on heating the plate after spraying with 50% (w/v) sulphuric acid the cholesterol was clearly visible and underwent the typical colour changes from blue to a reddish purple before charring. Two other spots could not be positively identified. These components appeared only when 25–35 μ g. samples were used. One had a similar migration pattern to that of sphingomyelin but its R_F was not identical and it did not give a positive reaction to the Dragendorff reagent. The other gave a weak positive reaction to the ninhydrin reagent. It is probable that these spots are breakdown products that appeared in spite of the use of nitrogen to protect the lipids from oxidation.

In agreement with our preliminary results we found no evidence of cerebrosides in neuron lipid.

DISCUSSION

The separation of phosphatidylserine from phosphatidylethanolamine and phosphatidylcholine was one of the earlier difficulties of thin-layer chromato-

graphy. This problem was discussed by Skipski, Peterson & Barclay (1962) and by Horrocks (1963), who described procedures for successful separation. By using standard mixtures and the lipids of whole ox brain we found that unidimensional thin-layer chromatography on 'basic' plates (Skipski *et al.* 1962) gave good separation. However, it is now evident that our use of this procedure for 10–15 μ g. of neuronal lipid did not give complete separation, the phosphatidylcholine and phosphatidylserine having migrated together. Since there was insufficient of the former constituent to react to the Dragendorff reagent but sufficient of the latter to give positive reaction to ninhydrin, the phosphatidylcholine remained undetected.

Pelick, Wilson, Miller, Angeloni & Steim (1965) have described various ways in which erroneous conclusions can be drawn from thin-layer chromatography; however, failure to separate constituents owing to a relatively high concentration of a lipid-pigment complex such as lipofuscin does not appear to have been encountered previously.

This work was supported by a grant from the Nuffield Foundation.

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