The Histochemical Recognition of Lipid in the Cytoplasmic Network of Neurones of Vertebrates

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With two plates (figs. 1 and 2)

Summary

1. A method for the histochemical demonstration of the lipid of the cytoplasmic network ('Golgi apparatus', 'Nissl complex') of the neurones of vertebrates was accidentally discovered.

2. Very small pieces of tissue are fixed in Lewitsky/calcium or Flemming/calcium, postchromed, and embedded in gelatine. Frozen sections are cut at 0·5 to 5 μ, and coloured in Sudan black after bleaching.

3. The intensity of the colouring of lipid by Sudan black can be increased by centrifuging the tissue before fixation.

4. The method described in this paper demonstrates lipid in the strands and bodies of the cytoplasmic network of vertebrate neurones. It was shown, in sections subsequently decolorized and then stained in a basic dye, that the sudanophil reticular objects are the Nissl complex.

Introduction

MALHOTRA (1959, 1960) demonstrated by interference microscopy a cytoplasmic network in living vertebrate neurones, and presented evidence that this network corresponded to the Nissl complex (Nissl, 1894), the Golgi apparatus (Golgi, 1898), and the endoplasmic reticulum (Palade, 1955; Palay and Palade, 1955; Palay, 1956) of the fixed cell. Malhotra's findings have been confirmed and extended in this laboratory (David, Mallion, and Brown, 1960; David and Brown, 1961 *a*, *b*; David, Brown, and Mallion, 1961). The results of recent biochemical studies suggest that the endoplasmic reticulum of vertebrate neurones contains a complex of ribonucleic acid (RNA) conjugated with basic proteins and phospholipids (Waelsch, 1957; Clouet, 1957; Clouet and Richter, 1959). Yet proven methods for the histochemical recognition of lipid, such as colouring in Sudan black after fixation in formaldehyde/calcium, generally demonstrate only small separate globules in the cytoplasm of vertebrate neurones (Baker, 1944, 1949, 1957 *a*, *b*, 1959; Thomas, 1948; Casselman and Baker, 1955; Malhotra, 1959; David and Brown, 1961 *a*). In the course of preparing vertebrate neurones for other work (David and Brown, 1961 *a*, *b*; David, Brown, and Mallion, 1961), we found that the lipid of the cytoplasmic network could be demonstrated when the neurones were fixed in two new reagents; this lipid could be coloured in Sudan black quite strongly if 'unmasked' in a certain way.

This paper is restricted to the description of the improved methods of

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fixation and 'unmasking' which enabled us to recognize the presence of lipid in the network by the use of Sudan black: the detailed characterization of this lipid will be considered in another paper.

Materials

The neurones of the dorsal root ganglia of immature cats and rabbits were used. There is no reason to suppose that the results would have been different had other vertebrate neurones been chosen.

Methods

Fixation

The original aim of the experiments on fixation was to improve the preservation of the form of lipid globules. Baker (1944, 1958*a*) and Chou and Meek (1958) showed that the addition of calcium ions to certain fixatives rendered them less liable to extract lipid and to distort cytoplasmic inclusions containing lipid. Baker (1956) also introduced a modified Flemming solution, Lewitsky / saline or LS. This does not contain calcium ions, but is a superb cytological fixative. It occurred to us that calcium ions might be beneficial in LS as well, and made this up to contain 1% w/v anhydrous calcium chloride instead of the 0·75% sodium chloride of the original formula. We call this fluid Lewitsky / calcium or L/Ca.

L/Ca, though an excellent fixative for lipids and proteins (see fig. 1), does not fix RNA adequately. In studies of the cytoplasmic network of vertebrate neurones, in which we may wish to demonstrate the Nissl complex by the use of basic dyes in the same piece used for the demonstration of lipids, this is a serious drawback. For this reason we tried adding 1% anhydrous calcium chloride to Flemming's original fluid (Flemming, 1882), which contains 5% acetic acid and therefore can be expected to fix RNA adequately. We call this modified fluid Flemming / calcium or Fl/Ca.

Since the constituents of L/Ca and Fl/Ca penetrate tissues at different rates, it is important that the pieces to be fixed should be as small as possible. Tissues should be fixed overnight (15–18 h), and washed in running water for 5 to 6 h.

After-treatments

Postchroming should be carried out exactly as in the acid haematein technique (Baker, 1946). Lipid is sufficiently well fixed by L/Ca and Fl/Ca, with postchroming, not to be extracted by the after-treatments required to produce paraffin sections. But, since the tissue then becomes severely shrunken and distorted, embedding in gelatine is preferable. Baker's (1944) method gives excellent results.

The cytoplasm of vertebrate neurones is crowded with inclusions of various kinds. For this reason it is best to cut extremely thin frozen sections. It is not difficult to cut 3 μ frozen sections with an ordinary sledge microtome. We use a Sartorius no. 31a microtome, equipped with a knife having a cutting bevel of

35°. Thinner sections, down to 0·5 μ, are cut with a Cambridge rocker microtome in a cryostat at −20° C.

Before treating sections of tissues fixed in L/Ca or Fl/Ca with colouring agents, it is necessary to oxidize away the osmium combined with the tissue. A mixture containing 99 ml of 1% w/v aq. potassium permanganate and 1 ml of concentrated sulphuric acid bleaches sections in 30 min. The permanganate is then removed in 2% w/v aq. oxalic acid, and the sections are washed in running water for 30 min.

Colouring and dyeing methods

Lipids of all kinds are best demonstrated by Baker's standard Sudan black (Baker, 1944, 1949). We colour for 10 min at 60° C. Control sections are subsequently extracted in 90% v/v ethanol for 15 min (Baker, 1958*b*). Berg's very sensitive method of fluorochroming lipids in 3:4-benzpyrene (Berg, 1951) could not be used, since the osmium remaining in the tissue after the permanganate bleach effectively quenches the induced fluorescence. The acid haematein test for phospholipids (Baker, 1946) can be used satisfactorily (David and Brown, 1961*b*).

The Nissl complex is best demonstrated by dyeing in cresyl violet monomer (David, 1955). This method was applied directly, and to sections that had been coloured in Sudan black, with subsequent removal of the lysochrome in 90% ethanol. Other methods of dyeing the Nissl complex, such as gallocyanine / chrome alum (Einarson, 1932, 1951), gave disappointing results.

Method of unmasking lipid

A number of unfixed dorsal root ganglia were ultra-centrifuged in order to stratify the contents of the neurones (David and Brown, 1961*a*; David, Brown, and Mallion, 1961). The contents of neurones resist stratification to a surprising extent: living neurones centrifuged at 33,000 *g* for 30 min could not be distinguished from untreated neurones by interference microscopy. Some of the centrifuged neurones were subsequently fixed in L/Ca or Fl/Ca, and coloured in Sudan black. Much to our surprise, the cytoplasmic network now coloured much more vigorously than in untreated neurones. The network remains strongly sudanophil after stratification at 95,000 *g*. The following procedure is recommended for increasing the sudanophilia of the network.

Suspend small fragments of dorsal root ganglia in about 10 ml of ice-cold Baker's (1944), Ringer's, or Krebs's saline. Transfer the suspension to a chilled centrifuge-tube, containing a 1 cm layer of 30% w/v bovine plasma albumin (either crystallized or 'fraction V') dissolved in the same saline. Avoid stirring up the albumin. The tissue should settle at the interface between the two liquids, where a density-gradient gradually forms by diffusion of the albumin. The albumin prevents the crushing of the neurones against the bottom of the tube during centrifugation. Centrifuge at 33,000 *g* for 30 min at 2° C. Then fix in L/Ca or Fl/Ca.

Results

Neurones fixed in L/Ca after centrifugation are illustrated in fig. 1. The form of the neurones and the cytoplasmic inclusions are faithfully preserved. Centrifuged neurones fixed in Fl/Ca are shown in fig. 2.

A sudanophil cytoplasmic network, similar in form to that described by Malhotra (1959), can be seen in neurones fixed in either reagent (figs. 1; 2, A). It is composed of irregular bodies and thin strands. Some of the bodies are small and nearly spherical (figs. 1, A; 2, A); others are massive objects with rather indistinct edges, as much as 8 μ long and about 2 to 4 μ wide (fig. 1, B). The strands are about 0·5 μ in diameter, and are peppered with minute granules that are too small to be measured accurately. The strands are associated with the bodies in such a way that the bodies give the impression of being enlarged nodal points in a three-dimensional network. Sudanophobe elongate spaces, about 2 to 5 μ long and 1 to 3 μ wide, are sometimes seen between adjacent strands.

Both fixatives dissolve away a large proportion of the nuclear contents. The plasmosome and chromatin clumps within the nucleus are sudanophil. The general preservation of form is rather better after fixation in L/Ca than in Fl/Ca. However, the cytoplasmic network stands out more sharply in neurones fixed in Fl/Ca, and it can be stained adequately in basic dyes (fig. 2, B). The results are qualitatively the same in neurones that are fixed without having been centrifuged, but then the cytoplasmic network and other inclusions are much less sudanophil.

In view of the consistent finding by previous workers that the cytoplasmic network of vertebrate neurones gives negative results to tests for the histochemical recognition of lipid (for references see p. 391), it is necessary to provide clear evidence that Sudan black acted as a lysochrome for the lipid of the network and not as a weak basic dye. Sections coloured in Sudan black were immersed in 90% ethanol for 15 min; this solvent extracted all the colour from the cytoplasmic network. This suggests that the Sudan black must have coloured the network by lysochromy (Baker, 1958*b*). Other pieces of dorsal root ganglia were extracted in pyridine at 60° C for 24 h, after fixation in weak Bouin (Baker, 1946). In neurones from these ganglia it was not possible to colour the cytoplasmic network in Sudan black. The findings of these control-experiments leave no doubt that Sudan black coloured the lipid of the cytoplasmic network of the neurones fixed in L/Ca and Fl/Ca (see figs. 1; 2, A). These findings will be discussed in greater detail in a later publication.

In view of the immense number and variety of the cytoplasmic inclusions of the neurones of dorsal root ganglia, it is necessary to provide direct evidence that the sudanophil network is the Nissl complex, that is to say the network that stains in basic dyes. Neurones coloured in Sudan black were mounted in

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Fig. 1 (plate). Neurones of the dorsal root ganglia of the cat, fixed in L/Ca after slight centrifugation, coloured in Sudan black and photographed in monochromatic light of 600 mμ. A is a neurone in a 3-μ gelatine section, and B in a thinner section (about 1 μ).

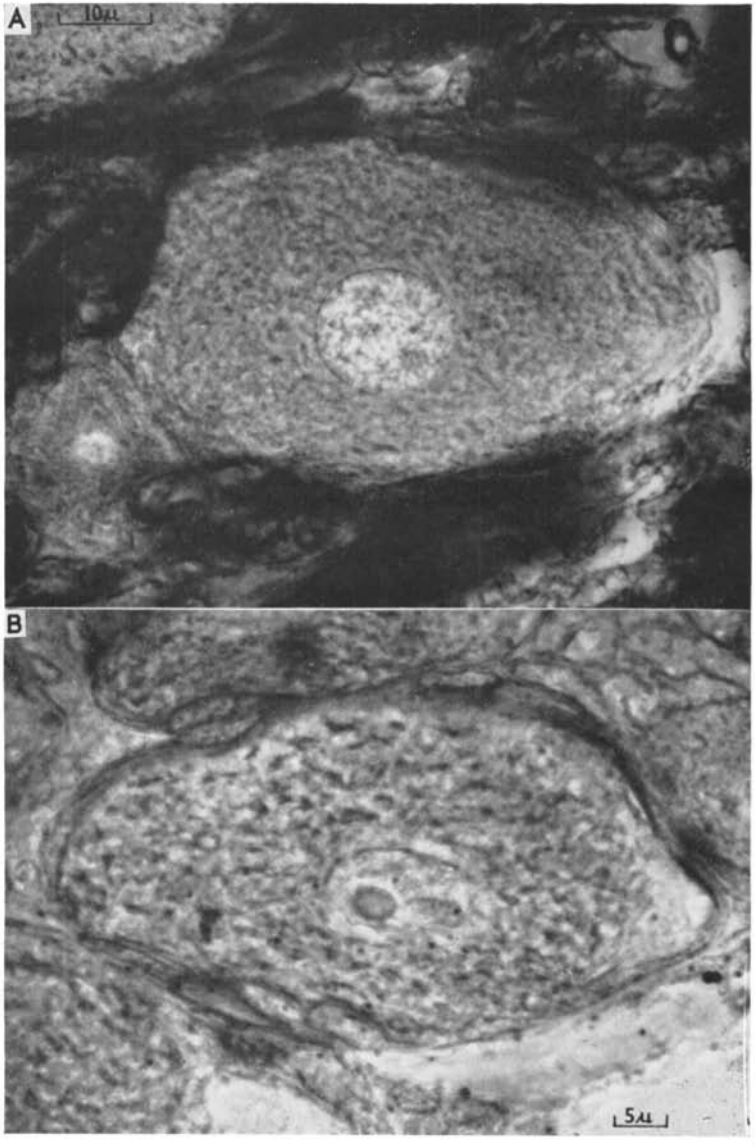

FIG. 1

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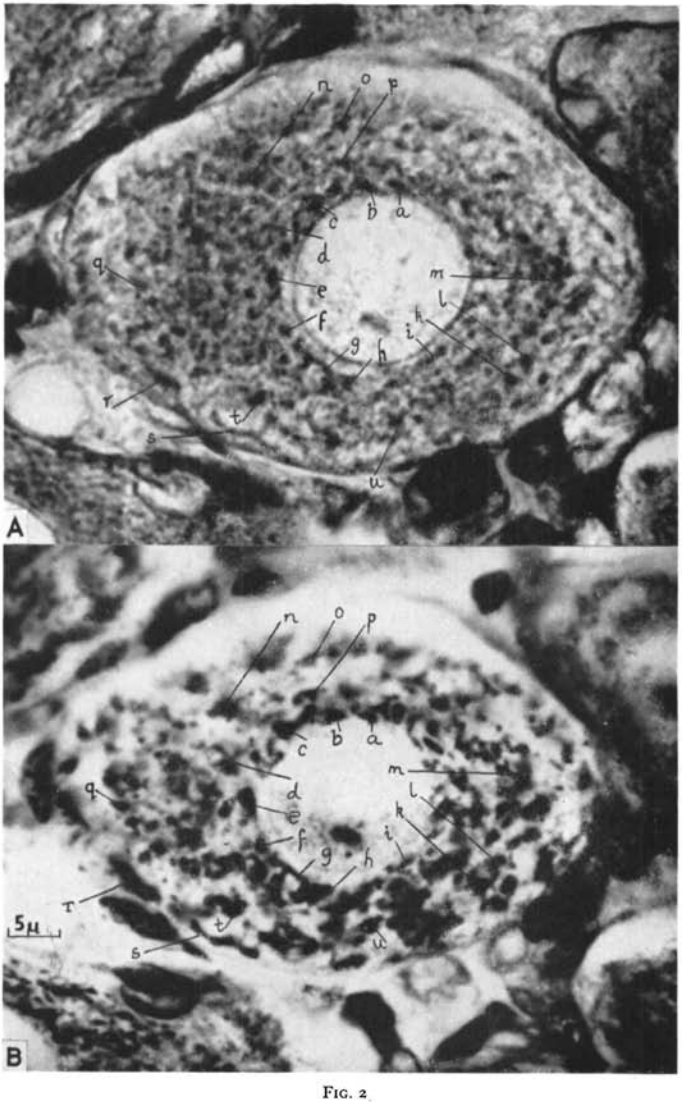

FIG. 2

anhydrous glycerol, and photographed (fig. 2, A). Then, the Sudan black was completely extracted in 90% ethanol, and the sections were dyed in cresyl violet. The same neurones were found and photographed again (fig. 2, B). The sudanophil network is also basiphil; therefore, it is the Nissl complex. This is not to say that all sudanophil objects are part of the Nissl complex: lipid globules are not, though they are strongly sudanophil.

Discussion

It is not clear why the addition of calcium ions to modified Flemming solutions should facilitate the colouring of the lipid of the cytoplasmic network. Calcium ions prevent the emulsification of lipids, but one would not have thought that the failure of previous techniques to show lipid in the network was due to lipid being removed. Formaldehyde / calcium (Baker, 1944), as used for the histochemical study of lipid, extracts some glycerophosphate (Chayen, Gahan, and LaCour, 1959); but LS does not. Yet the cytoplasmic network is sudanophobe after fixation in either formaldehyde / calcium or LS. Is there any reason to suppose that LS allows the escape of lipids that are fixed by L/Ca? The action of calcium ions in Fl/Ca is even more obscure. 'Indifferent' salts appear to have little or no effect when used in coagulating fixatives that contain much acetic acid (Baker, 1958*b*).

It is not immediately obvious why centrifugation should have any effect on the ease with which lipid is coloured by Sudan black. It is true that during centrifugation the neurones were subjected to a pressure of between 3,000 and 13,000 lb/sq. in. Is it conceivable that some of the bonds between lipids and proteins or RNA are sheared by these pressures? On the other hand, the lipid may have been masked by a small quantity of a substance of either higher or lower specific gravity than that of the cell as a whole; this substance might have been displaced by centrifugation. We have not detected the accumulation of a substance at either pole of the cell after centrifugation adequate to render the network sudanophil, but not to stratify it. But, if this substance were present in very small amounts, it might easily be overlooked. Further experiments are planned to clarify these problems.

After the work described in this paper was completed, we heard that Prof. O. L. Thomas and Dr. S. K. Malhotra had independently succeeded in demonstrating histochemically the lipid of the cytoplasmic network of vertebrate neurones by a different method.

Fig. 2 (plate). These are two photographs of the same dorsal root neurone, fixed in Fl/Ca after slight centrifugation, embedded in gelatine, and cut at 3 μ.

A, coloured in Sudan black, mounted in glycerol, and photographed at 600 mμ, to demonstrate lipid.

B, dyed in cresyl violet and photographed at 546 mμ after extraction of the lysochrome, to show that the sudanophil network is the Nissl complex.

The small letters indicate structures that are visible in both photographs. Compare *a* with *a*, *b* with *b*, and so on. Since extraction of the lysochrome and dyeing produce shrinkage and distortion, it is not possible to obtain a perfect correspondence of all parts of a cell by this method.

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APPENDIX

The formulae given below will be found convenient for the preparation of L/Ca and Fl/Ca. The stock solutions are quite stable, as is L/Ca; Fl/Ca deteriorates fairly rapidly.

Stock solutions	*Working solutions*	
	L/Ca	*Fl/Ca*
	(ml)	(ml)
Osmium tetroxide, 2% w/v aq.	2·0	2·0
Chromium trioxide, 5% w/v aq.	1·5	1·5
Acetic acid, 20% v/v aq.	—	2·5
Anhydrous calcium chloride, 10% w/v aq. . .	1·0	1·0
Distilled or deionized water	5·5	3·0
	10·0	10·0

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