The Cytoplasmic Inclusions of a Mammalian Sympathetic Neurone: A Histochemical Study

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SUMMARY

1. The neurones studied were those of the anterior mesenteric and coeliac ganglia of immature rabbits.

2. Ectoplasm and endoplasm can be distinguished in these cells.

3. Two kinds of cytoplasmic inclusions occur. These are (*a*) spherical or spheroid lipid globules or lipochondria, which are confined to the endoplasm; and (*b*) very minute threads and granules, regarded as mitochondria.

4. A wide variety of histochemical tests was used. The results indicate that the lipochondria consist of galactolipid (cerebrosid) and phospholipid, though the smallest ones may perhaps consist of galactolipid only.

5. The objects regarded as mitochondria are too small for accurate investigation by *in situ* histochemical methods, but they give positive reactions for phospholipid and protein.

OUR object has been to reveal the chemical composition of the cytoplasmic inclusions of the sympathetic nerve-cells of a mammal by the application of reliable histochemical tests. We have not undertaken any enzymological studies. The anterior mesenteric and coeliac ganglia of the rabbit were chosen, partly because they are of convenient size, partly because the former ganglion was used in the earlier studies by one of us (Baker, 1944). We have not detected any difference between the cells of the two ganglia, and the description of our results refers equally to both.

We have not studied the lipofuscin granules that sometimes occur in clumps in the nerve-cells of old animals, the whole of our work having been done on immature specimens, which have no pigment in these ganglia.

MATERIAL AND METHODS

The mean weight of the fourteen rabbits used in the investigation was 0·87 kg. (maximum 1·56, minimum 0·42 kg.). The animals were all killed by breaking the neck suddenly. The ganglia were removed as quickly as possible after death, care being taken not to damage them mechanically. They were at once placed in fixatives. Some (not all) were divided with fine scissors to allow especially rapid access of the fixatives to the nerve-cells.

Sudan black B was used for the localization of lipids. We shall use the expression 'standard Sudan black' to mean that fixation, postchroming, and colouring were done in exact accordance with the instructions given previously by one of us (Baker, 1949). The Nile blue test for the differentiation

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of acidic and neutral lipids (Cain, 1947*a*, 1948) was applied only after fixation in formaldehyde-calcium without postchroming. The acid haematein test for phospholipids (Baker, 1946, 1947*b*; Cain, 1947*b*; Casselman, 1952) and its pyridine extraction control were applied to tissues fixed in formaldehyde-calcium and weak Bouin's fluid respectively. The acid haematein test was also used on ganglia fixed and extracted with cold or hot acetone before being treated with formaldehyde-calcium and the other reagents. Windaus's test for cholesterol (Leulier and Revol, 1926, 1930; Lison, 1953) and Liebermann's for cholesterol and its esters (Schultz, 1924; Schultz and Löhr, 1925) were applied to gelatine sections of ganglia fixed in formaldehyde-calcium.

For the periodic acid / Schiff (PAS) test (McManus, 1946), use was made of gelatine sections that had been fixed in formaldehyde-calcium, Da Fano's, or Regaud's fluid, saturated aqueous mercuric chloride, or Heidenhain's mercuric chloride / saline, or fixed in weak Bouin's fluid and extracted with pyridine (Baker, 1946), or simultaneously fixed and extracted with cold or hot acetone (the treatment with hot acetone being for 18–20 hours in a semi-micro Soxhlet extractor). The PAS test was also done on paraffin sections of ganglia fixed in Carnoy's or Zenker's fluid. Control sections were first acetylated with acetic anhydride in glacial acetic acid (Casselman, Macrae, Simmons, 1954). The performic acid / Schiff (PFAS) test (Lillie, 1952) was applied to gelatine sections of ganglia fixed in formaldehyde-calcium, or treated with hot or cold acetone, or extracted with pyridine after weak Bouin fixation. Control sections were immersed in saturated aqueous bromine for 3 hours. With both tests, any interference from formaldehyde was prevented by first treating the sections for at least 3 hours with a saturated solution of dimedone in 1 N acetic acid.

Proteins were studied by the Sakaguchi (Baker, 1947*a*) and coupled tetrazonium (Pearse, 1953) tests, which were carried out on gelatine sections of ganglia fixed in Heidenhain's mercuric chloride / formalin or mercuric chloride / saline, in saturated aqueous mercuric chloride, or in Da Fano's or Regaud's fluid, and on paraffin sections of a ganglion fixed in 80 per cent. ethanol.

Observations

The ground cytoplasm

The whole of the ground cytoplasm of the cell is thrown by some of the fixatives into the form of a very fine reticulum, the threads of which are near the limit of microscopical resolution. This appearance is particularly clearly given by fixation in 80 per cent. ethanol and colouring by the coupled tetrazonium test.

The cytoplasm is sometimes seen to contain oval, spindle-shaped, or crescentic spaces, commonly about $2\frac{1}{2}\mu$ long and $\frac{3}{4}\mu$ wide at the widest point. They appear to be empty, for none of our tests has ever given a positive reaction with them. They are most often seen in preparations that have been fixed with solutions containing strong protein precipitants (Carnoy's fluid,

saturated aqueous mercuric chloride, and the mercuric chloride / saline or mercuric chloride / formaldehyde fluids of Heidenhain). They are rendered particularly evident if the cytoplasm is strongly coloured by the coupled

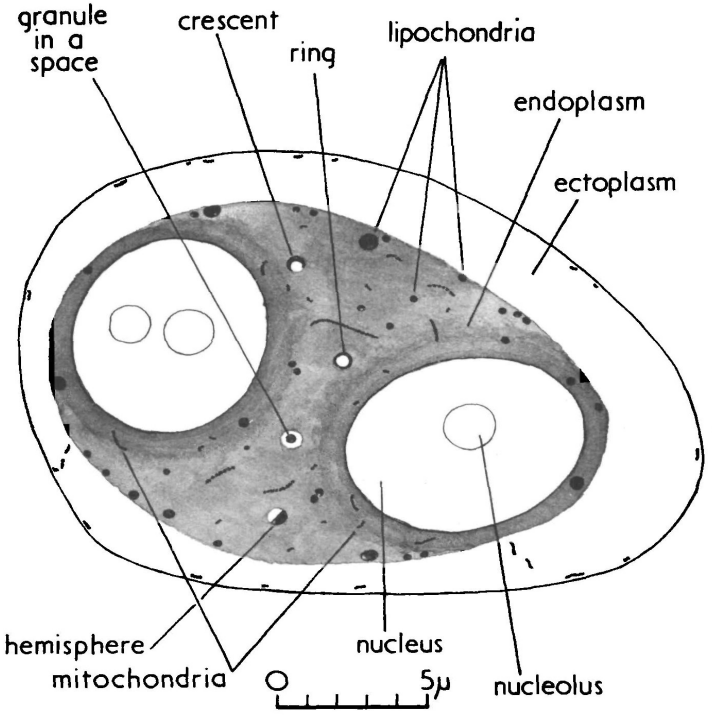

FIG. 1. Diagram of a section through a sympathetic cell (coenocyte) of the immature rabbit. The diagram is based upon what can be seen in a standard Sudan black preparation, but the mitochondria are added from what can be seen in an Altmann-Metzner preparation. The following are regarded as artificially modified lipochondria: granule in a space, crescent, ring, hemisphere.

tetrazonium test. We have not seen them in preparations fixed in formaldehyde-saline, formaldehyde-calcium, or Champy's fluid. It seems probable that they are fixation-artifacts of an unusual kind.

An outer and an inner region can be distinguished in the cytoplasm (fig. 1). This was pointed out in the earlier paper by one of us (Baker, 1944), in which

the inner region was described as the sudanophil or lipoid-containing part. The distinction between the outer and inner parts is particularly easily seen in gelatine sections. We shall call the outer region the ectoplasm and the inner the endoplasm. The width of the ectoplasm appears to vary a good deal in different parts of the same cell.

In our present studies we have found that the ectoplasm generally appears very pale pink in Sudan black / carmalum preparations. It is usually relatively homogeneous in fixed microscopical preparations, and contains few inclusions. It must be mentioned, however, that the ectoplasm tends to be somewhat obscured in many preparations by the lipid globules present in the adjacent capsular or satellite cells. It is sometimes difficult to be sure whether a particle is in the ectoplasm of a neurone or in a capsular cell. This must be borne in mind in reading what follows.

The ectoplasm is negative to the PAS test. The external part of the cytoplasm often reacts somewhat more strongly to the coupled tetrazonium and PFAS tests than the internal part. The two zones are not differentiated by the Sakaguchi reaction.

The endoplasm appears pale blue or blueish grey in Sudan black / carmalum preparations, and is seen to contain many inclusions.

In unstained sections examined by positive phase-contrast microscopy, the cytoplasm appears darker in the immediate vicinity of each of the two nuclei usually contained in the cell (or coenocyte) than elsewhere. These parts of the cytoplasm also tend to react slightly more strongly than the general endoplasm to the coupled tetrazonium test. The modified cytoplasm in the vicinity of the nuclei shades off gradually into the rest.

The cytoplasmic inclusions

Lipochondria. The endoplasm contains colourless lipid globules or lipochondria. They are distributed nearly at random in it, but are more abundant near its outer border than elsewhere. There is no special accumulation of them in the axon hillock. These bodies, most of which are spherical or subspherical, vary considerably in size. Few (if any) of them exceed 1μ in diameter in the immature rabbits we have used. The smallest are below the limit at which reliable measurements can be made; these appear to be $0{\cdot}2\mu$ or less in diameter. In fixed preparations that have not been treated with any colorant, the lipochondria cannot be distinctly seen by phase-contrast microscopy; they must have nearly the same refractive index as the cytoplasm, after fixation of the cell. They are most easily exhibited by the standard Sudan black technique, which colours them blue-black. Each blue-black globule is sometimes surrounded by a clear space, as though the method of preparation had caused the lipid globule and the surrounding cytoplasm to pull slightly apart from one another. In other cases the lipid seems to have retracted to the edge of a spherical or spheroidal space which presumably represents the original form of the lipochondrion. The coloured material then appears as a hemi-

sphere, crescent, or ring in optical section. The larger lipochondria tend particularly to assume these forms. (See fig. 1.)

After fixation in formaldehyde-calcium or Champy, or fixation in formaldehyde-saline or formaldehyde-calcium with subsequent postchroming, the lipochondria are readily coloured by Sudan black B in gelatine sections. The colorant can be entirely extracted by 70 per cent. ethanol, there being no stable sudanophilia. With Nile blue, the lipochondria cannot be differentiated from the surrounding basiphil cytoplasm. The larger lipochondria give rather a weak, truly positive reaction (AH+, PE—) with the acid haematein test, the reacting material often presenting the forms mentioned in the preceding paragraph. The smaller ones are not stained by acid haematein. When the PAS test is applied to the cell, positively reacting granules are seen which resemble the lipochondria in distribution, number, size, and shape. There seems to be no reason to doubt that they are the same objects as are coloured by Sudan black. The evidence from solubilities, given below, supports this view, and they will be called lipochondria in what follows. The lipochondria cannot be differentiated by the PFAS test nor by the coupled tetrazonium or Sakaguchi tests. They are poorly preserved, if at all, by saturated aqueous mercuric chloride or mercuric chloride / saline, and do not resist paraffin embedding after fixation in Carnoy's or Zenker's fluid or in 80 per cent. ethanol.

The following experiment was done to make certain that none of the lipochondria seen with Sudan black B was merely due to precipitation of the reagent. A standard Sudan black preparation was mounted in glycerine. One particular cell was selected and the distribution of the twenty lipochondria seen in one plane of focus was recorded in a careful drawing. The lipid colorant was then removed with 70 per cent. ethanol. The section was again mounted in glycerine and the complete removal of the Sudan black from the cell confirmed. It was again coloured with Sudan black B as before. Re-examination of the same cell in the same plane of focus showed the same twenty lipochondria that had been recorded in the drawing prepared earlier, each characterized by its position and size, and in some cases by its shape.

The lipochondria respond negatively to the tests for cholesterol and for its esters.

When a freshly removed ganglion is placed in acetone at room temperature, left in this overnight, and then subjected to the acid haematein test, the positively reacting material appears in a diffuse form, not as separate lipochondria and mitochondria. The phospholipid content of the cell appears to be pushed inwards by the invading acetone, but it cannot pass through the cell-membranes and therefore tends to accumulate on the side of each cell nearest the centre of the piece of tissue. There is a gradient in the intensity of the blue-black reaction, which falls off to nothing on the side of each cell that is nearest to the outside of the piece of tissue. We have made no attempt at quantitative studies, but it appears that more phospholipid is revealed after the previous action of acetone than is seen in all the lipochondria and mitochondria after the application of the ordinary acid haematein test without previous treatment

with acetone. This suggests that there is a considerable amount of histochemically unreactive phospholipid in the cell, and that acetone sets it free.

When a ganglion that has been fixed as above in cold acetone is subsequently fixed and coloured according to the standard Sudan black technique, the diffuse lipid is coloured pale grey. The lipochondria are coloured by the Sudan black in some of the cells, but they often appear as crescents, rings, or very small dots, as though part of their material had been extracted. They give a positive PAS reaction, scarcely diminished in intensity from that seen in sections of ganglia not treated with acetone.

The lipochondria are not demonstrable by any method in sections of ganglia treated overnight with hot acetone in a Soxhlet extractor. The displaced phospholipid resists such treatment and retains its histochemical properties. Neither lipochondria nor displaced lipids are present after pyridine extraction.

The lipochondria are not coloured by Metzner's method after Altmann fixation.

Mitochondria. It is usually difficult to make clear preparations of the mitochondria of the nerve-cells of mammals, and the cells that are the subject of the present paper constitute no exception in this respect. If ganglia are fixed for 24 hours in Altmann's fluid, sectioned at 2 or 3μ in paraffin, and stained by Metzner's method (Metzner and Krause, 1928), with very careful differentiation, the cytoplasm is seen to be yellow, with numerous very small pink or red objects in it. These objects appear to be extremely thin threads and tiny granules, but the threads are so thin and the granules so minute that the resolution obtainable by the light-microscope does not permit any exact description of their form or width, though the threads sometimes appear to be made up of rows of granules. The longest threads are as much as 3μ in length. These bodies, which are not seen in routine preparations of any kind, are presumably mitochondria. The threads resemble the mitochondria seen by Thomas (1948) in the living neurone of the anterior mesenteric ganglion of the mouse.

It would appear that the mitochondria occur mainly in the endoplasm, but that there are also some in the ectoplasm, especially in its outermost part. Ectoplasm and endoplasm are not clearly differentiated, however, in Altmann-Metzner preparations, and the ectoplasm seems to be considerably shrunken.

Only two of the many histochemical tests that we have applied show any objects that could be regarded as mitochondria. These are the acid haematein and coupled tetrazonium techniques. In acid haematein preparations the cytoplasm appears yellowish with extremely fine blue or blue-grey markings. When these markings are examined critically with the highest useful powers of the microscope, they can just be resolved into threads and granules, which appear to have the same distribution as the objects seen in Metzner preparations. Exceedingly thin, positively reacting threads are also seen in the cytoplasm of cells that have been fixed in mercuric chloride solution, sectioned in gelatine, and subjected to the coupled tetrazonium test. The positive reactions

with acid haematein and coupled tetrazonium are consistent with the identification of these bodies as mitochondria.

Other cytoplasmic inclusions. A supposedly canalicular object has been reported by Lacy (1954*a*) in the exocrine cell of the mammalian pancreas, and it has been thought that this may represent a cytoplasmic inclusion of quite general occurrence in the cells of vertebrates. We have used Lacy's method: that is to say, we have fixed and postchromed ganglia as for the standard Sudan black method, cut very thin gelatine sections, and coloured them with Sudan black B. We have prolonged the period of colouring to 20 minutes (instead of the usual $2\frac{1}{2}$), because this is said to make it easier to see the canals in the pancreas (Lacy, 1954*b*). A careful study of these preparations has not revealed any canalicular object resembling what Lacy reports in the pancreatic cell.

In all our histochemical preparations, very variously fixed and coloured, we have looked attentively for any cytoplasmic inclusion having the form of a network, but have not found anything of this kind.

Discussion

The lipochondria described in this paper are clearly the same as those already described by one of us in the anterior mesenteric ganglion of the rabbit (Baker, 1944, p. 30). They correspond to the 'spheroids' studied by Thomas (1948) in the same ganglion of the mouse. Perhaps they correspond also to the bodies figured by Ciaccio in spinal ganglion cells of the dog (1910, his plate XIX, figs. 28 and 29), though these might have been lipofuscin pigment.

From the histochemical observations, we conclude that the lipochondria have two main components. The more plentiful one is sudanophil and insoluble in cold acetone but soluble in hot acetone or hot pyridine, and gives a positive reaction with the PAS test which is blocked by acetylation but a negative one with the acid haematein test. On the basis of these properties, it can be classified as a galactolipid (cerebroside). The other main component, which cannot be demonstrated with certainty in the smallest lipochondria, is sudanophil, insoluble in hot or cold acetone but soluble in hot pyridine, and gives a negative reaction with the PAS test but a truly positive one with the acid haematein test. This can be classified as phospholipid. Thus the composition of at least the larger lipochondria in the rabbit sympathetic neurone studied by us is qualitatively similar to that of the lipochondria in the locust neurone (Shafiq and Casselman, 1954). Although quantitative interpretation of histochemical tests should be undertaken with great caution, it appears that in the lipochondria of the rabbit neurones, galactolipid predominates (or actually may occur alone in the smaller globules), whereas in the locust neurones the reverse is true. It is likely that the 'diffuse' glycolipid described by Dixon and Herbertson (1950*b*, 1951) as occurring in various cells of the brain of the rabbit, corresponds to the cerebroside component of the lipochondria we have studied. They themselves note (1950*b*) that it can sometimes

be recognized as occurring in the form of 'extremely fine cytoplasmic granules'.

There is no reason to believe that the lipochondria which we have studied are identical with the sudanophil and PAS-positive granules described by Dixon and Herbertson (1950, *a* and *b*) in human and rabbit neurones. As these authors point out, their granules vary in colour from pale yellow to dark brown, owing to the presence of lipofuscin pigment in them. Further, their granules are very resistant to lipid-solvents, and indeed were studied in paraffin sections, generally after simple fixation in formaldehyde-saline, without postchroming. It must be mentioned that lipofuscin granules have a strong tendency to be aggregated in a particular part of the cell, and indeed this is shown in most of the figures of Dixon and Herbertson (1950*b*), while lipochondria are distributed throughout the endoplasm. It is possible, and even probable, that lipofuscin develops in certain lipochondria as the animal ages by autoxidation. It was for this reason that we used immature rabbits exclusively.

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