The Accumulation of Carotenoids in the Golgi Apparatus of Neurones of *Helix, Planorbis,* and *Limnaea*

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INTRODUCTION

THE existence of coloured granules in neurones of pulmonate gastropods is well known through the work of Legendre (1909) and others. The coloured matter has been usually described as 'lipochrome', a term that Lison (1936) regards as bad since it covers two distinct chemical classes (the carotenoids and the chromolipoids) and has been used in different ways by different authors.

Thomas (1948) has recently produced evidence for considering these coloured granules as a Golgi product, a view not in agreement with that regarding the Golgi apparatus advanced by many workers.

This paper presents the results of a histochemical examination of the coloured substance, and a repetition of some of Thomas's work. The coloured granules contain carotenoid and appear to be formed in the interna of the Golgi bodies.

MATERIAL AND METHODS

Thomas worked with the cerebral ganglia of *Helix aspersa,* in which many of the neurones are very large. The amount of coloured matter is usually small even in the largest cells; there is very much more in the neurones of *Limnaea stagnalis* (L.) and *Planorbis corneus* (L.), both very common freshwater snails. In the former the central nervous system is distinctly coloured by the pigment. In the latter the bright red coloration is due to granules inside the cells and to a certain amount of haemoglobin in the blood as well.

The following histochemical methods were employed:

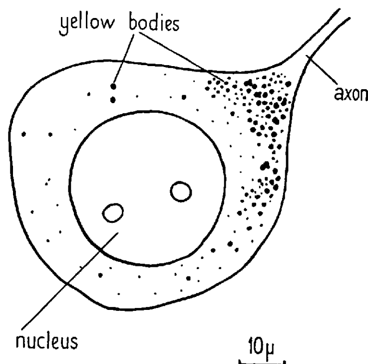
(i) For lipoids in general, material was fixed in formal-calcium, and frozen sections were cut and coloured with sudan black B (Baker, 1944). Sudan black is specific for lipoids (Lison, 1936; see also Cain, 1947*a*). It does not colour pure carotenoids (Lison, 1936, p. 245) and, of course, it does not colour *solid* lipoids. Lison includes the carotenoids under pigments. As they are soluble in lipoid-solvents, they are included here under the heading of lipoids.

(ii) Baker's acid haematein test for phospholipines (Baker, 1946, 1947; Cain, 1947*b*) was used, with pyridine extraction as control.

(iii) For detection of carotenoids the Carr–Price reaction (Carr and Price, 1926) was used. With antimony trichloride ($SbCl_3$) in chloroform carotenoids and vitamin A give a blue coloration which is not permanent.

In addition, the granules were tested with concentrated sulphuric acid, and with iodine (Lison, 1936, p. 245). With these reagents carotenoids give a deep-blue colour.

As a supplementary test, sections were exposed to light and air and the rate of fading was noted.



TEXT-FIG. 1. Diagram of the distribution of bodies visible in a neurone of *Helix*.

Living cells were observed in sodium-calcium saline (Baker, 1944), and were stained supravitally with neutral red chloride, methylene blue (BDH), nile blue, and Janus green B (Höchst).

Mann–Kopsch preparations were made of neurones from all three species, and Thomas's variant of the Mann–Kopsch technique was used with *Planorbis*.

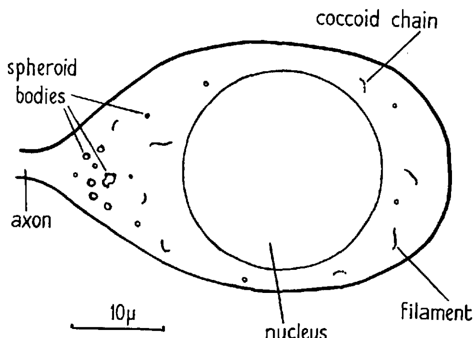
Helix neurones were fixed in Helly, postchromed, stained with Altmann's acid fuchsine, differentiated with sodium carbonate solution, and counterstained with methyl blue (Cain, 1948).

RESULTS

In living neurones of *Helix* a large number of bodies can be seen, which are either subspherical or irregular. These latter are described by Thomas as mulberry forms. The largest are often distinctly yellow in colour. These bodies are scattered throughout the cell, but more and more thickly towards the axon hillock, in which there is usually a large number (Text-fig. 1). On close examination there may be seen a cap or a granule or several granules

adhering to the rim of such bodies. In *Limnaea* and *Planorbis* these bodies are more evident, very numerous, and quite brightly coloured yellow, the largest being a faintly reddish or brownish yellow, and rather more irregular than in *Helix*. Their distribution is the same.

With methylene blue (1 in 10,000) both the bodies and their associated granules or caps are stained, the latter very deeply. A similar effect is produced, but less clearly, with neutral red chloride and with nile blue. Neutral red is the least satisfactory with *Planorbis* and *Limnaea* because in them



TEXT-FIG. 2. Diagram of the relation between the filaments and coccoid chains shown with Janus green, and the spheroid bodies.

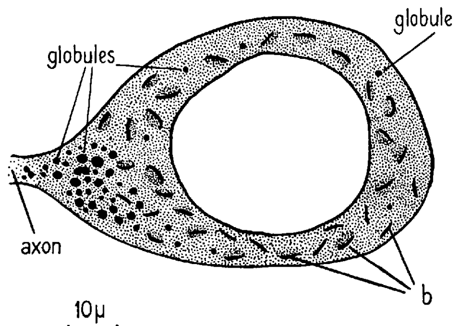
nearly all the bodies are already coloured and the contrast is not great. Methylene blue is very satisfactory.

When neurones were exposed to the vapour of osmium tetroxide, the rims of these bodies, together with their caps and granules, become blackened. Prolonged action results in the blackening of the whole body.

Minute filaments and coccoid chains were not distinctly seen in living cells. On application of Janus green B (Höchst), after many attempts, very distinct filaments were seen in living cells of *Helix* (*Planorbis* and *Limnaea* neurones were not investigated because of the multiplicity of spheroids which make for obscurity). These were very thin and short, sometimes bent or kinked. Coccoid chains were seen most distinctly in a damaged cell; it is possible that they are filaments beginning to break up. In distribution the filaments do not resemble the spheroid bodies, which are also clearly visible, and are not associated with them. They are scattered throughout the cytoplasm and show no tendency to concentration in the axon hillock (Text-fig. 2). They were seen in only a few cells, lying next to those on the outside of the teased-out cell-mass, which were dead and stained diffusely.

Mann–Kopsch preparations were made of neurones of all 3 species, 6 days being found a suitable time of osmication. In preparations from *Helix*

batonnettes as usually described were to be seen in nearly all cells, sometimes with an associated 'archoplasm' but quite often without. Sometimes the cytoplasm contained nothing else, but on occasion there were black or grey spheroidal bodies. These might occur anywhere in the cytoplasm, but where cells were cut through the nucleus and axon hillock it could sometimes be seen that there was an aggregation in the hillock (Text-fig. 3). In preparations of *Limnaea* and *Planorbis* neurones much the same pictures were obtained, but in general the batonnettes were less obvious and the globules more so.



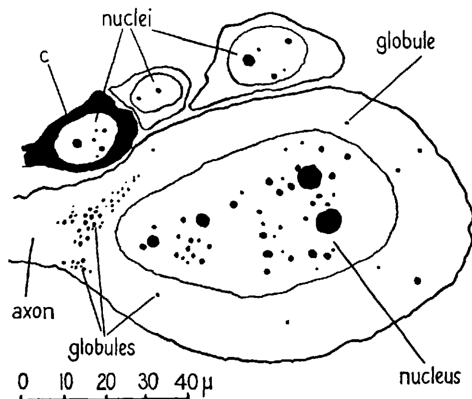
TEXT-FIG. 3. Diagram of the distribution of globules and batonnettes in a Mann–Kopsch preparation. *b*, batonnettes, mostly with archoplasm. Nucleus blank.

In distribution, the globules corresponded exactly to the coloured granules and complexes seen in the living cells. The batonnettes did not correspond to these and, as in *Helix*, were scattered throughout the cytoplasm.

Thomas describes only batonnettes in his Mann–Kopsch preparations, but was able to show globules by bleaching with Veratti's acid permanganate (followed by oxalic acid to remove the brown deposit of oxide) and then colouring with sudan black, when globules appeared in positions quite unrelated to those of the batonnettes which had been shown by osmium. When neurones of *Limnaea* and *Planorbis* were coloured with sudan black without previous bleaching, it could be seen plainly that the globules were much darker but the batonnettes were not; and when cells were bleached entirely and then coloured with sudan black, the globules reappeared in exactly their former sizes and positions but the batonnettes did not. It appears, therefore, that these globules, stained by osmium tetroxide, are the same as those produced by Thomas using sudan black after bleaching. Osmium-impregnation appears to be capricious. Either the batonnettes alone, or both batonnettes and globules may be stained. Thomas's variant (p. 455) of the Mann–Kopsch technique was tried on *Planorbis* neurones. Only globules were seen, except for a very faint indication of batonnettes in a very few cells. These globules agreed in all respects with those seen by the Mann–Kopsch technique.

With *Helix* material fixed in Helly's fluid, postchromed, stained with acid fuchsine as for mitochondria, and differentiated with sodium carbonate solution (Cain, 1948), no mitochondria were seen, but spheroid complexes with very fuchsinophil rims and colourless interna were present and agreed entirely with Thomas's description (p. 456).

With Baker's acid haematein test the picture obtained varied. The pyridine-extraction control always gave a completely negative result. The acid haematein test showed the cytoplasm either a clear yellow-brown with



TEXT-FIG. 4. Camera lucida drawing of an acid-haematein preparation of *Helix* neurones. *c*, cell with the cytoplasm staining heavily throughout.

certain blue-stained bodies or intense blue-black throughout. In the latter case the cells were often slightly shrunken, and on occasion were confined to one part of the ganglion, within which every cell was blue-black. It is considered that such cells were damaged in some way and a lipophanerosis had taken place so that phospholipines were liberated in the cytoplasm. In the cortex of rat adrenals very similar appearances have been seen in those cells of the zona reticularis lying next to the medulla; this is a region in which necrotic cells are found. In this case, the liberation of phospholipine is a sign of the death of the cell.

In neurones not blue-black throughout, coloured spheroids are occasionally visible, again tending to be concentrated in the axon hillock (Text-fig. 4). In addition there are small coloured bodies, scattered in the cytoplasm, which appear to be the caps or rims associated with the smallest spheroid complexes in the living cell, the remainder of each complex being invisible in acid-haematein preparations. Some of the larger spheroids appear to have less coloured interna, but, as Thomas remarks (p. 452), this is not obvious, and

cannot be asserted with great conviction. Clouds of phospholipine occurred in some cells.

Material fixed for 3 days in formal-calcium was cut on the freezing microtome and the sections coloured with sudan black B. This method (Baker, 1944) showed large numbers of globules in the axon hillock, and others scattered throughout the cell. In every case it was the rim or attached granule that coloured with the sudan black. The picture was almost exactly that of living cells exposed to osmium tetroxide vapour. In all 3 species the cytoplasm coloured heavily throughout, and it was necessary first to remove this general colouring which indicates the presence of lipoids throughout the cytoplasm.

The yellow or orange-yellow pigment in the cells rapidly becomes colourless under the influence of light and air; the application of concentrated sulphuric acid produces a fine blue-green colour immediately. This had been noted by Smallwood and Rogers (1908), who concluded that the pigment was 'lipochrome'. Iodine in potassium iodide solution gives a deep violet. These facts indicate that the pigment is carotenoid, and this is confirmed by the blue colour given with a solution of antimony trichloride in chloroform. This reagent gives a blue colour with both vitamin A and carotene, and in spite of statements to the contrary cannot be used to distinguish between them unless heat is used (Andersen and Levine, 1935). As the chloroform tends to dissolve out the carotenoid, and heating accelerates this, it was found impossible to distinguish any colour on heating. The white precipitate of oxychlorides formed by antimony trichloride in contact with water tends to obscure the preparation.

In cells fixed for 6 hours in formal-calcium the carotenoid-containing granules take up far less sudan black than would fat-droplets of the same size. Lison (1936) notes that pure carotenoids are negative to lipoid reagents. This suggests that the largest granules are composed of carotenoids only among the lipoids, although the presence of proteins cannot be excluded. The granules in *Planorbis* and *Limnaea* often seem much more angular than those in *Helix* and give an impression of solidity.

It appears then that the complexes contain phospholipine and perhaps other lipoids in the rims, caps, or associated granules, and carotenoid in the interna. Vitamin A being much paler in colour than carotene, and giving the same reactions, cannot be excluded by the results given above, nor is its presence established. As there is no evidence for the manufacture of carotenoid by the Golgi apparatus as against its collection from other regions, it seems best to refer to its *accumulation* therein.

Discussion

The results given above agree with those of Thomas. It seems reasonable to conclude, as he does, that the filaments stained with Janus green appear in Mann–Kopsch preparations as the batonnettes and are the mitochondria. The spheroid-complexes are the Golgi bodies with their products. There is

a coincidence of structure and distribution-patterns of the globules (spheroid-complexes) seen in life and after treatment with all the following: neutral red, nile blue, methylene blue, acid haematein, Helly and acid fuchsine, formal-calcium and sudan black, and the Mann–Kopsch technique and Thomas's variant of it. This demonstrates clearly that they are all the same bodies. From the Mann–Kopsch preparations and the living cells stained with Janus green B it is clear that they are not associated with the mitochondria. Their identification with the Golgi apparatus is discussed by Thomas (p. 456). In structure they agree very well with bodies in other cells which are undoubtedly the Golgi apparatus (Worley, 1943, 1944, and 1946; Worley and Worley, 1943; Baker, 1944; Cain, 1947*a*).

The principal arguments that might be brought against this identification are that such bodies are not shown by the standard Golgi methods, that the batonnettes with their archoplasm are the Golgi apparatus, or that the use of neutral red chloride and other supravital stains cause the production of artifacts. That in these neurones the spheroid complexes are not shown by the standard methods is a much more serious objection. The Mann–Kopsch method does show it sometimes, less readily in *Helix* than in *Limnaea* and *Planorbis*, but it is quite easy to find cells in which nothing but the batonnettes can be seen. But the standard methods are wholly empirical, and, as far as is known, no Golgi apparatus is under obligation to appear when they are used. So very little is known about conditions at the inner surfaces of the cell and the conditions under which silver and osmium precipitates form. What is known about monomolecular layers on water-surfaces indicates that their behaviour can change very greatly with small changes in pH and the concentration of ions in the water (see e.g. Langmuir, 1934) and it would not be at all surprising if structures containing layers of oriented molecules should be very similar in morphology but very diverse in their behaviour, differing, particularly, in different sorts of cell, and most divergent in the most specialized. The fact that these bodies do not usually appear with the standard Golgi methods does not rule out the possibility that they are the Golgi apparatus. The objection to the use of neutral red and similar stains can hardly be upheld in this case because it is not a question of making structures visible, but of staining structures already visible in the cell.

The view that the batonnettes are the Golgi apparatus has been very well supported. This was based on their regular appearance with the standard Golgi methods, and a supposed homology with the lepidosomes of the pulmonate primary spermatocyte. But there seems to be no special reason for carefully selecting cells which show batonnettes only and excluding one showing the spheroids as well; and the batonnettes do correspond with the mitochondria as shown by Janus green, and do not correspond with the spheroid complexes seen in the living cell and ignored by upholders of the batonnette theory. In *Limnaea* and *Planorbis* the batonnettes are less readily shown by the Mann–Kopsch method than in *Helix*, and the spheroids perhaps more readily. Perhaps if investigations on gastropod neurones had started

with *Planorbis* instead of *Helix*, as much attention might have been given to the spheroids as to the batonnettes.

Summary

Repetition of some of Thomas's (1948) work on *Helix* neurones and its extension to neurones of *Planorbis* and *Limnaea* confirms his conclusions that the batonnettes shown by standard Golgi methods are mitochondria, and the Golgi apparatus is represented by spheroid complexes, scattered throughout the cell but tending to be concentrated in the axon hillock.

The spheroid complexes appear to consist of an externum, continuous or not, which contains phospholipine and possibly other lipoids, and an internum in which carotenoids are accumulated. This accumulation is greater in *Limnaea* and *Planorbis* than in *Helix*.

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