Cytological Studies of the Neurones of *Locusta migratoria*

Part II. Cytoplasmic inclusions during the differentiation and growth of the nerve cells

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With one plate (fig. 1)

**SUMMARY**

1. The Golgi controversy as it applies to the nerve cells of *Locusta* is discussed with reference to the recent publications of Beams and others (1953) and Gatenby and others (1953). Further support has been obtained for the view that the ‘Golgi’ appearances are produced by reactions at the surface of the lipochondria.

2. Cytoplasmic inclusions during the differentiation and growth of the nerve cells are described. The early germ-band cells have two categories of inclusions—mitochondria (granular and filamentous) and lipochondria (osmiophil bodies). The neuroblasts and early ganglion-cells possess only granular mitochondria. The filamentous mitochondria and lipochondria appear again in the growing neurones, so that at the time of hatching of the embryo the nerve cells have attained the full development of their cytoplasmic inclusions. Thus, Gatenby's three-phase theory for nerve cells does not apply to *Locusta*.

3. It is concluded that the lipochondria and filamentous mitochondria are not necessarily self-reproducing bodies.

4. Masson's technique shows granules staining red with acid fuchsin in the growing neurones as well as in the different stages of the adult. These are shown to be lipochondria. No secretory cycle could be detected.

**INTRODUCTION**

THE cytoplasmic inclusions of the motor neurones of the thoracic ganglia of the adult *Locusta migratoria* were described in an earlier paper (Shafiq, 1953). Almost simultaneously Beams, Sedar, and Evans (1953) published their work on the cytology of the nerve cells of certain grasshoppers, and Gatenby, Moussa, Elbanhawy, and Gornall (1953) reviewed the Golgi controversy in nerve cells in general. The work of these authors and of Dornesco (1934) and Muliyil (1935) represents the classical views about the ‘Golgi component’ of the nerve cells of insects, and it will be desirable to give a summary of their conclusions here, so that they may be discussed later on with reference to the present work.

The opinions of Beams and his associates (1932, 1953) about the neurones of the grasshopper are that the spheroids seen after neutral red staining are new formations consisting of aggregated dye particles, and that the Golgi
element of the neurones consists of curved and circular dictyosomes only. The osmiophobe portion described earlier (Beams and King, 1932) is an optical artifact, as it is not seen by the electron microscope.

Dornesco (1934) from his studies of the neurones of dragonflies disagreed with the above authors, for he did not regard the neutral red bodies as artifacts. He considered that while these bodies existed in life, they could not be impregnated by Golgi techniques.

Muliyil (1935) repeated the work on the neurones of the Orthoptera. He studied living cells and ultracentrifuged ganglia and concluded that the neutral red bodies existed in life and could also be impregnated by Da Fano's technique; but he regarded them as different from the Golgi bodies.

Gatenby and others (1953) also believe that the Golgi apparatus (dictyosomes) of the invertebrate neurones is different from the neutral red bodies or vacuome granules, and that the latter correspond to the 'senility pigment' of vertebrate neurones.

**Material**

Cells were studied during the various embryonic stages, in the nymphal instars, and at different ages of the adult of *L. migratoria*. Specimens were kindly supplied by the Anti-locust Research Centre, London.

The following information on the embryonic stages of *Locusta* is taken from the studies of Roonwal (1936, 1937). The data on the nymphal and adult stages were given by Mr. P. Hunter Jones of the Anti-locust Research Centre, London.

The cleavage cells produced by the divisions of the zygote nucleus migrate to the periphery, especially to the posterior end of the egg, to form the germ-band at the age of about 28 hours. This becomes divided into the primary head region and the primary trunk region at the age of 42 hours. At the same time an inner layer of cells differentiates, so that the embryo becomes two-layered, having the outer ectoderm and the inner layer (mesoderm). Neuroblasts differentiate in the ectoderm of the head region at the age of 59 hours and in the trunk region at 65½ hours. They produce columns of ganglion cells by repeated unequal mitoses. The small cells budded off from the neuroblasts become concentrated into segmental ganglia at the age of 112 hours. The ganglia separate from the underlying dermogenic tissue and the cells produce nerve fibres, thus attaining their definitive form. Neuroblasts become indistinguishable on the seventh day and ganglion cells begin to grow. The embryo hatches on the thirteenth day. The stock at the Anti-locust Research Centre, however, hatches in 8–10 days at 32°C. After hatching the locusts go through five nymphal instars. If they are kept at about 28°C at night and about 35°C during the day, the total duration of the nymphal period is about 20 days, after which the adults emerge. The newly emerged adults are grey, but when they mature in about 4 to 6 weeks' time, the males become yellow and the females brown. The total adult life is about 3 months.
Neurones of Locusta migratoria. II

Methods

The methods used in this study of the cells from embryonic and nymphal stages are similar to those used in the work on adult neurones. They may be enumerated as follows:

1. Study of living cells by phase-contrast microscopy;
2. Vital staining by neutral red, Janus green, and Janus black;
3. Baker's (1944) Sudan black method for 'Golgi' component;
4. Baker's (1946) acid haematein test for phospholipines;
5. Sudan black method on paraffin sections. For this, ganglia were fixed in Helly's fluid (Thomas, 1949) and in Champy's fluid (Baker, to be published shortly);
6. Regaud's (1910) method for staining mitochondria;
7. Metzner's method for staining mitochondria (Metzner and Krause, 1928), with Altmann's and Helly's fluids as fixatives;
8. Osmium-impregnation technique of Kolatchev (1916), with Meves's and Champy's fluids as fixatives;
9. Silver-impregnation technique of Aoyama (1929);
10. Masson's tricolor staining method.

The cutting of paraffin sections was easiest when the embryos had been dissected away from the yolk. This, however, could not be done with late embryos, where the yolk is enclosed in the gut, and therefore various softening procedures were tried. Successful preparations could be made when cedarwood oil was used as the antemedium for paraffin embedding; this was followed by soaking the blocks in Baker's (1941) softening mixture. Phenol-xylene as antemedium for paraffin embedding, with soaking of the blocks in water, also gave some successful preparations.

Observations

The early cleavage cells were studied in the living state by phase-contrast, and they appeared to possess filamentous and granular mitochondria and spheroid bodies. The mitochondria could be stained by the Janus dyes and the spheroids by neutral red, but attempts to study these inclusions in fixed preparations were not successful; so these cells will not be described here.

When the cleavage cells have formed the germ-band, the cells are arranged in several layers. These cells are cuboidal in shape and are about 26μ long, their nuclei being 13μ in diameter. The germ-band was dissected out from the eggs and its cells could easily be studied in the fixed preparations as well as in the living state. They have the following inclusions in their cytoplasm.

Mitochondria

The mitochondria of these cells are of two types, granular and filamentous. The granular type are about 0.5μ in diameter and are more numerous than the...
Shafiq—Cytological Studies of the filamentous, which are thin threads about 1.2 μ long. They were seen by phase-contrast and could be stained by Janus black supra-vitally. Fixed preparations by the method of Regaud also showed them. They are evenly distributed throughout the cytoplasm.

Lipochondria (osmiophil bodies)

Dispersed among the mitochondria are seen spheroids of diameters varying from 0.7 μ to 1.2 μ (fig. 1, c). Neutral red stained these bodies when the germ-band was immersed in a 0.01 per cent. solution of the dye, dissolved in saline, for about 15 minutes. By positive phase-contrast they sometimes appear to be binary in structure, having an outer dark cortex and a lighter inner medulla (fig. 1, c); but as with the lipochondria of the nerve cells, it is not possible to assert definitely whether this is an optical illusion or not. They can be impregnated by Kolatchev's method for the Golgi apparatus (fig. 1, e). In the figure some of these spheroids are over-impregnated and appear like dense granules, while on others the osmium has been deposited on the surface only.

After this early germ-band stage, the cells greatly increase in number and the germinal layers are formed. The individual cells are now much smaller, with little cytoplasm, and are difficult material for the study of their cytoplasmic inclusions.

The embryos become very suitable for study from the stage when the neuroblasts differentiate and thereafter. They are now sufficiently big to be dissected out of the egg and manipulated in various ways. Neuroblasts of the head as well as those of the trunk region were studied; no differences could be seen between them. They are big cells measuring about 35 μ in diameter, their nuclei being 18 μ in diameter, so that there is a large amount of cytoplasm in the cells. Phase-contrast microscopy shows only one type of granule in the cytoplasm (fig. 1, d). These granules are spheroidal, measuring 0.6 μ in diameter, and are uniformly distributed throughout the cell. They appear dark

Fig. 1 (plate). All photomicrographs are at the same magnification.
A, a motor neurone from the adult locust, to show granules staining with acid fuchsin. The ganglion was fixed in Helly's fluid and 6 μ paraffin sections were stained by Masson's tricolor stain.
B, a motor neurone from the adult locust stained as A, after the ganglion had been extracted with Baker's Bouin-pyridine method.
C, cells from the early germ-band of the locust embryo as seen by phase-contrast microscopy. Spheroids and mitochondria are seen.
D, a neuroblast (in anaphase) as seen by phase-contrast. The granular mitochondria are uniformly distributed throughout the cytoplasm; lipochondria are absent.
E, cells of the early germ-band prepared by Kolatchev's method for the Golgi apparatus. The embryo was fixed in Champy's fluid and osmicated for 4 days at 37° C.; 6 μ section.
F, neuroblast and the ganglion-cells produced by the division of the neuroblast; prepared by Aoyama's method for the Golgi apparatus.
G, embryonic neurones as seen in a Sudan black preparation. The embryo was fixed in Helly's fluid and embedded in paraffin.
H, embryonic neurone of a slightly earlier stage than in G, prepared by Aoyama's method for the Golgi apparatus.
I, neurones from an embryo about to hatch. Prepared by the same method as E, except that Meves's fluid was used as fixative.
Neurones of Locusta migratoria. II

by positive phase-contrast. They stain strongly by Janus green and Janus black. Neutral red does not stain anything in the cells except in very early neuroblasts, when sometimes one or two granules were seen after staining the cells supravitally. Regaud’s method for mitochondria shows the granules clearly. Sudan black stains them in frozen gelatin sections and also in material fixed in Helly’s fluid and embedded in paraffin. They are also positive for the acid haematein test for phospholipines. The impregnation techniques of Aoyama and Kolatchev do not show any trace of ‘dictyosomes’ or ‘platelets’, but only small granules (fig. 1, F). They correspond to the granules seen in the living cell and probably represent them.

It is, therefore, concluded that there are no lipochondria in the neuroblasts and that the granules seen in the cytoplasm of the neuroblast are mitochondria. Further, the histochemically demonstrable lipide content of the cell is a constituent of the mitochondrion.

The columns of the ganglion cells produced by the division of the neuroblast were studied. Each cell is 13 μ in diameter with a nucleus 10 μ in diameter. In their cytoplasm also only one type of granules, the mitochondria, could be made out. The impregnation techniques show no ‘platelets’ or ‘dictyosomes’ (fig. 1, F); the young ganglion cells resemble the neuroblast in this respect.

When the ganglion cells have produced fibres, concentrated themselves into segmental ganglia, and begun growing, they present a different picture from the neuroblasts and the early ganglion cells. Treatment with Sudan black now reveals lipochondria of diameters up to 0.9 μ in the cells (fig. 1, G). ‘Golgi’ appearances could also be produced at this stage (fig. 1, H) by deposits on the surface of the lipochondria. These first-formed lipochondria are smaller than the lipochondria of later stages (where their diameters vary from 0.4 μ to 2.6 μ). They do not arise from any special region of the cytoplasm, but from the beginning of their appearance they are uniformly distributed throughout the cytoplasm, like the mitochondria. It is thus not possible to say whether the lipochondria arise anew in the cytoplasm or are formed by the transformation of granular mitochondria.

During the later stages of the embryo the lipochondria grow and some of them have attained almost the same size as that of the larger lipochondria in the adult neurone. These give the characteristic appearance of ‘curved and circular dictyosomes’ or ‘osmiophil platelets’, by Golgi techniques, and even in size they are comparable to the platelets of the adult neurone.

The only difference between the lipochondria of the adult and the embryonic neurones that could be found is that the lipochondria of embryonic neurones are probably more readily coloured by neutral red than those of the adult.

The mitochondria were also studied in these late embryos. In the living nerve cells the granular as well as the filamentous mitochondria are easily seen by phase-contrast; they also stain by Janus black supravitally. In the fixed preparations they were seen by Metzner’s method for mitochondria. It is thus obvious that at this stage the nerve cells in Locusta are fully differentiated
from the point of view of their cytoplasmic inclusions, their cytological picture
being essentially the same as that of the neurones of the adult.

The lipochondria were also studied in the various instars of the locust and
in immature, mature, and old locusts. For this study material was fixed in
Helly's fluid and embedded in paraffin. Sections were stained by Sudan black.
Living cells were also studied. The neurones of the thoracic ganglia were

![Diagram of neurones at various stages of development](image)

**Fig. 2.** Large neurones of locusts at various stages in development. The ganglia were fixed in
Helly's fluid and embedded in paraffin by Thomas's method; sections were coloured by Sudan
black. All the figures are camera lucida drawings at the same magnification. A, neurone from
1st instar nymph. B, from 2nd instar nymph. C, from 3rd instar nymph. D, from 4th instar
nymph. E, from 5th instar nymph. F, from an adult locust.

chosen for study. They vary greatly in size in all the different instars, as they
do in the adult. Thus it is possible to find some neurones in the ganglia of the
first instar which are bigger than the smaller neurones of the adult locust. But
taking the cell population of the ganglion as a whole, the neurones obviously
increase greatly in size. With the increase in the size of the neurones from the
first instar to the adult stage there is correspondingly a great increase in the
number of the lipochondria. The situation is depicted in the series of camera
lucida drawings of the larger neurones from the different instars of the locust
The drawings were all made from the material fixed in Helly's fluid and embedded in paraffin. A definite quantitative relationship between the number of the lipochondria and the size of the neurone could not be obtained because of the difficulty of correctly estimating the number of lipochondria of different sizes in the cells. The main conclusion derived from a general comparison of the neurones in the various instars is that during the growth of the neurone in the different nymphal instars the lipochondria increase in number, their sizes remaining more or less the same. This is clearly seen from the camera lucida drawings.

The lipochondria in immature, mature, and old locusts were also compared and no differences were found.

**Neurosecretion granules**

Wigglesworth (1950) remarked about neurosecretion, 'It is characteristic of neurosecretory cells that they contain droplets of colloid substance which stains with acid fuchsin. Cells of this type are found in the “pars intercerebralis” or medial dorsal region of the brain, in the corpus cardiaicum and in various ganglia of the nerve cord.' Thomas made a study of neurosecretion with reference to the cytology of nerve cells in molluscs and vertebrates and put forward the view (1951) that the intraneuronal granules or the neurosecretion granules are formed in the ‘spheroids’ (lipochondria).

Thoracic ganglia of immature, mature, and old locusts of both sexes and also the thoracic ganglia of the various nymphal instars were studied by Masson’s tricolor stain. Granules staining red with acid fuchsin were seen in most of the neurones of all the stages mentioned above. Their number, size, and distribution leaves no doubt that it is the lipochondria that are being stained by acid fuchsin (fig. 1, A). Lipides were extracted from some ganglia by Baker’s Bouin-pyridine method (Baker, 1946). After this treatment the characteristic lipochondria were lost, and Sudan black showed only fine granules in the section. Masson’s stain also showed only fine granules in these sections (fig. 1, B). This is thus further evidence that in Locusta the acid fuchsin in Masson’s method stains the lipochondria of various sizes.

It is important to mention, however, that though acid fuchsin stained the lipochondria more strongly in some cells than in others, a secretory cycle as described by Scharrer (1941) in the neurosecretory cells of the cockroach Leucophaea was not observed. Beams and King and Muliyil also did not find a secretory cycle of ‘Golgi bodies’ in the Orthoptera they studied.

**CONCLUSIONS**

**Lipochondria and ‘Golgi bodies’**

The classical views on the Golgi problem in insect nerve cells have been summarized in the introduction. The usual conclusion is that there are three types of inclusions in insect nerve cells—the mitochondria, the ‘neutral red’
or 'vacuome' granules, and the Golgi 'dictyosomes'. However, it was clearly shown in the earlier study (Shafiq, 1953) that neutral red stained the lipochondria and that the impregnation techniques produced the Golgi appearance in or on the lipochondria. The present work provides further evidence in support of this view. Thus, when the lipochondria are present, as in the neurones of the various nymphal instars and in various stages of the adult, the Golgi appearances can be produced on them. When, in the growing embryonic neurones, the lipochondria are smaller than in later stages, the dictyosomes formed by Golgi techniques are also small. And finally, when the lipochondria are absent, that is, in the neuroblasts and early ganglion cells, dictyosomes cannot be produced by any impregnation techniques.

Some comments can be made now on the various classical works. Thus it is interesting to see that in Muliyil's experiments (1935) with the ultracentrifuge, the neutral red granules (smaller lipochondria) and 'Golgi bodies' (i.e. artifacts on the surfaces of larger lipochondria) collected at the same (centripetal) pole and remained intermingled with each other.

Gatenby and others (1953) would homologize the senility pigment granules of the neurones of vertebrates with the 'neutral red granules' of the neurones of invertebrates. Now, the neurones of old insects sometimes contain granules of yellow pigment, and this may perhaps be formed by a modification of the contents of the lipochondria ('neutral red granules'). However, the true homologue of the insect's lipochondria are the colourless lipochondria of the neurones of vertebrates (the 'spheroids' of Thomas (1951)).

Beams and others (1953) impregnated the ganglia with osmium by Kolatchev's technique and studied the deposits of osmium by the electron microscope. They say that 'what we have described here as Golgi bodies are not gross artifacts' and that 'the Golgi bodies are relatively opaque to the electrons but they do not seem to be completely homogeneous as is evidenced by the lighter appearing areas within them'. It seems doubtful whether any useful purpose is served by making an osmium deposit on the surface of a cytoplasmic inclusion and then examining the form of that deposit with the electron microscope.

It is a pity that the methods of research developed and recommended in the pioneer studies of Baker (1944, 1946) are not applied by more workers, before drawing conclusions about the 'Golgi apparatus'. When these methods are applied to the insect nerve cells it is obvious that neutral red stains the lipochondria and that impregnation techniques produce 'dictyosome' appearances on the lipochondria.

Origin of cytoplasmic inclusions

Gatenby (1919) and Hirschler (1918) studied the early development of the gastropod Limnaea by Golgi techniques. They found that in the cleaving egg the mitochondria and 'Golgi bodies' are equally divided between the daughter cells. Gatenby and Hirschler therefore conclude that the 'Golgi bodies' are
self-reproducing bodies and as Gatenby said '... are able to assimilate, grow and divide in the cytoplasm somewhat as a protist assimilates, grows and divides in its watery medium'.

This study, however, leads to a different conclusion. Osmiophil bodies are present in the earliest stages in the Locusta, as in Limnaeae, but they disappear in the neuroblasts. In living cells and also in impregnated sections, nothing is to be seen in the cytoplasm except mitochondria. Osmiophil bodies appear again in the growing neurones. And when these characteristic lipocondria have made their appearance, the 'Golgi dictyosomes' can also be produced by impregnation techniques. These appearances cannot be produced in the neuroblast or the early ganglion cells. Gatenby and Hirschler did not study the organogeny of any tissue but studied only the very early stages and adult tissues. It would appear that they were wrong in their conclusion. The osmiophil bodies are not continuously self-reproducing organelles. They can disappear and reappear. It is not possible to say whether they appear independently in the cytoplasm, or are derived by transformation of the mitochondria.

The same conclusion may possibly be applicable to the mitochondria. There are no filamentous mitochondria in the neuroblasts and early ganglion-cells, though they are present in the early germ-band cells and in the neurones of the late embryos. Bensley (1953) does not regard the mitochondria as definite organelles and has shown that the mitochondrial substance is expendable. Harvey (1946) and Gustafson (1953) hold the same view. There is no doubt in the present study that the filamentous mitochondria disappear and reappear. It is not certain whether they originate from granular mitochondria.

**Secretion and neurosecretion**

Nerve cells in the various growth stages were studied by Rau and Ludford (1925) in the chick and by Gatenby and others (1953) in Amphibia. On the basis of this work, Gatenby and others divide the life of the neurone into an early phase, a middle or secretory phase, and a regressive phase. They consider that the neurones are not fully developed cytologically in the early phase when the neurones function as a nervous unit only, that the Golgi apparatus is fully formed only in the secretory phase, and that it degenerates into vacuoles and fatty globules in the regressive phase.

In Locusta the neurones have attained full cytological development before the embryos hatch. A secretory cycle was not seen in the cells, and as regards the lipocondria no appreciable difference is noted from the embryonic stages, through the various instars, up to the old locusts. Thus this three-phase theory does not apply to Locusta.

Gatenby, as was noted above, regards secretion as a general property of nerve cells. Many other workers hold the same view. The Scharrers (1953), however, think that neurosecretion is not a general property of nerve cells and regard neurosecretory cells as a distinct cell type. Dr. K. K. Nayar (personal communication) regards the larger neurones of Locusta (diameter about 90μ)
Shafiq—Cytological Studies of the Neurones of Locusta migratoria. II

as neurosecretory. If they are neurosecretory they appear to differ from other nerve cells only in size. Neurosecretion certainly needs careful definition. B. Scharrer is going to undertake this (personal communication.)

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