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AN INVESTIGATION OF THE PHENOMENON OF  
DIAPAUSE IN THE LARVA OF LUCILIA CAESAR L.

(DIPTERA: TACHINIDAE)

T H E S I S

for the

Degree of Doctor of Philosophy

in the

University of Glasgow

by

Alastair Fraser, B.Sc.

November, 1957

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An Investigation of the Phenomenon of Diapause in the Larva  
of Lucilia caesar L. (Diptera : Tachinidae)

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## INTRODUCTION

Recent reviews of literature on the phenomenon of diapause in insects have been produced by Andrewartha (1952), Lees (1955) and Hinton (1957). This review of current knowledge is therefore brief and confined to information directly related to this investigation.

The term "diapause" was originally used by Wheeler (1893) to define a stage in the embryogenesis of an Orthopteran Xiphidium. Henneguy (1904) is responsible for applying the term to a physiological state of rest or dormancy. Loosely, the term has been used to describe any sort of arrested development but Shelford (1929) introduced a required precision in restricting the term to cases where the arrest in development is "spontaneous" as opposed to a simple quiescent state. In the former case the diapause onset may be determined by environmental factors but the diapause itself removed in time from the receipt of the critical stimuli and may continue in conditions favourable for the rapid development of non-diapausing individuals. In the latter case the retardation of development is the direct result of adverse conditions and development proceeds as soon as the conditions are again favourable.

The termination of diapause is also governed normally by environmental factors, physical stimuli such as temperature, light/

light intensity, photoperiod being effective. For example, chilling, at temperatures lower than the minimum required for normal growth, for a duration of weeks terminates diapause in many species.

In some species diapause does not occur in every generation and may affect only a proportion of the members of a generation in which it occurs. According to the terminology of Steinberg & Kamensky (1936) such a diapause is said to be "facultative", resulting from the individual's assessment of the attributes of the environment. In other species every member of a generation will enter "obligatory" diapause, which may be a genetically fixed character of the species or maternally determined or the product of an extreme degree of sensitivity to slight variations in the physical environment.

In most species having a diapause it occurs at one stage in development only, as a halt in embryogenesis which is probably maternally determined in all cases (Hinton, 1954), or as an arrest of development in one of the larval instars or in the pupa or of the internal reproductive organs of the adult.

Larval diapause may also result from maternal effects communicated to the progeny. (Roubaud, 1928; Simmonds, 1948; Cragg & Cole, 1952.) Larval or pupal diapause can be the direct result of adverse environmental conditions providing effective diapause-provoking stimuli at the outset of the diapause/

diapause instar but, in many instances, facultative diapause in larvae or pupae is "determined" at an earlier stage in development. Several processes are implied in such cases namely, determination at stage 'a', initiation at stage 'b', maintenance of the state of diapause, termination of diapause, promotion of growth and development after diapause and there must be, as Hinton (1957) emphasizes, an accurate time-keeping mechanism between stage 'a' and stage 'b'.

The actual arrest of growth and metabolic adjustments for diapause and termination of and promotion of growth and development after diapause are governed by endocrine organs (see Lees, 1955). The humoral control of diapause is discussed in Chapter 3 of this thesis.

Diapause occurs in the genus Lucilia in the third instar after the cessation of feeding and before puparium formation. This is not, strictly speaking, a prepupal diapause since the prepupal instar is actually a fourth brief intrapuparial instar but it is usually convenient to call the stage between feeding and puparium formation the "prepupa" rather than use the longer term "mature larva". Both are used in this thesis and should be regarded as synonymous.

Raubaud (1922) first described this diapause in Lucilia sericata Mg. and outlined methods by which he thought it could be/

be terminated including chilling, heating, pricking and singeing. Cousin (1932) proved that diapause affects only the prepupa in that species and that it is facultative since it does not occur in optimal culture conditions. She could not detect a maternal influence on diapause incidence but showed that adverse environmental conditions during the feeding or the immediate post-feeding phases are important in determining diapause. Prepupae reared in optimal conditions were not sensitive to diapause inducing factors after the completion of digestion of the contents of the crop and gut. Mellanby (1938) also investigated the diapause in that species and confirmed these observations. He concluded that the real factor determining diapause is the concentration of dry matter in the prepupa, 28% being the critical limit. He also claimed to be able to end any diapause by putting the prepupa into an empty glass tube and keeping it at any temperature between 16°C and 30°C but other workers have been unable to confirm this. Indeed, the stimuli recorded as being effective in terminating diapause in L. sericata are so varied that Lees (1955) was forced to conclude that this 'instability may well be due to the fact that the endocrine centres which control pupation are less completely protected from "irrelevant" sensory stimulation than in most insects'.

A most significant observation about diapause in Lucilia was/

was produced by Cragg & Cole (1952) who, having accepted as an established fact that unfavourable environmental conditions such as cold or excessive moisture "acting on larvae of L. sericata" may produce diapause, then proved that diapause in that species may also be of maternal origin. This maternal influence is related to seasonal changes and ensures that the majority of the larvae produced late in the season enter diapause.

The experiments on diapause in Bombyx conducted by Fukuda (1951, 1952) demonstrated that a hormone produced by the sub-oesophageal ganglion of the female determines whether she produces diapause or non-diapause eggs. The egg batch is normally all of one type but "mixed" batches can be produced experimentally. It can be postulated that there is a quantitative absorption of the diapause hormone by the developing oocytes in the mother (see Hinton, 1954) resulting in the production of a mixed batch if the diapause hormone is so limited in quantity that all eggs cannot absorb sufficient to determine diapause.

A similar mechanism may operate in Lucilia. Only such a quantitative process could account for the varied maternal influence on one batch. This theory, if valid, implies a very accurate regulation of the supply of diapause hormone according to the season. It can be concluded that diapause may be induced/

induced in the larva of L. sericata by environmental factors affecting it during the feeding phase, or during the migration phase, before the digestion of food residues in crop and gut has been completed but the proportion of larvae in one egg batch which are susceptible to diapause determining factors and the level of sensitivity of susceptible larvae appears to be controlled by the mother. The diapause is undoubtedly facultative but the sensitivity to adverse factors conferred by the mother may be so high that diapause is virtually obligatory.

Assuming the validity of this hypothesis, the principal object of the investigations discussed in this thesis was a study of the endocrinology of diapause in Lucilia concomitants being studies of endocrine control of development without diapause and of the neurosecretory centres controlling the endocrine organs. Endocrine organs and their functions have been studied by experimental methods and by observing the relationship between their development and that of imaginal rudiments. Neurosecretion has been investigated by the use of selected staining procedures and histochemical tests on serial sections of the central nervous system of larvae at different stages of development. A study of the anatomy of the central nervous system and retrocerebral endocrine organs was an essential preliminary to the other work. Diapause investigations/

investigations in Lucilia have hitherto been confined to the species L. sericata but for this work Lucilia caesar L. was used since there is a higher diapause incidence in the latter species in laboratory cultures.

## Chapter 1

### Section I: Culture Methods

At the start of these investigations an attempt was made to establish a culture of Lucilia caesar from larvae bred from females trapped at Appin (Argyll). All of these larvae went into a diapause that could not be broken by any of the methods thought to be effective with L. sericata larvae. Pupation and emergence of individuals occurred at irregular intervals but a sufficient number of contemporaries for successful breeding was not obtained. The first breeding stock was obtained from Professor Cragg, Durham University, and later flies trapped in Appin, Strathblane (Dunbarton) and in the grounds of Glasgow University were added to this stock.

The flies were caged in glass tanks measuring 12" x 11" x 19" and housed in a 25°C constant temperature chamber, atmospheric humidity 80% R.H. Fluorescent tube lighting was used and, by means of a time switch, illumination was provided for 12 hour periods which alternated with 12 hour periods of darkness. Cane sugar and water were supplied ad lib. Blow-fly females require two meals of animal protein before their ovaries mature and at least one such meal before producing each egg batch after the first (Hobson, 1938). Fresh liver was, therefore, supplied daily to each new cage for the first week and/

and thereafter at 3 day intervals. The meat was supplied in a petri dish covered with moist cotton wool. Females oviposited in crevices in the liver, in the bile ducts, between the glass and meat and between the wool and meat. For routine cultures, larvae from several egg batches were allowed to hatch and feed together until they reached the second instar (about 24 hours after eclosion at 25°C) when 300 were transferred to about 100 gm. of fresh liver. The culture was contained in a breffit jar filled to a depth of 5 inches with damp peat moss, the standard damp moss being made up of 100 gm. dry granulated peat moss and 250 ml. water. The meat was laid on the moss surface and was covered with damp cotton wool. The larvae were kept at 25°C. There is a mistaken belief, implicit in many accounts of the rearing of blowflies, that the temperature of the incubator or chamber in which a larval culture is kept, is the temperature at which the larvae develop.

The larvae (and bacteria) actually raise the temperature in the meat considerably above that of the surrounding air; in one check the temperature of cultures in 25°C and 36°C incubators were found to be 32°C and 38.5°C respectively. The temperature of a culture is just one of many uncontrollable factors in supposedly controlled culture conditions.

Larvae finish feeding in about 72 hours after eclosion from the egg. At the cessation of feeding, they leave the meat/

meat and descend into the moss. At this stage the crop is distended and the gut filled with the semi-liquid food produced by the action of proteolytic enzymes, in the larval excreta, on the meat, (Hobson, 1930). During the following 24 hours, the evacuation of crop and completion of digestion of the contents of crop and gut takes place. At this cessation of feeding, meat was removed from the culture jar and the top layer of moss, saturated with excreta and products of liquefaction of the liver, was scraped off and replaced with fresh moss. If diapause did not ensue puparium formation occurred between the 4th and 7th day after departure from the meat.

Adult cultures of a number of other species of Diptera Cyclorrhapha were established from trapped flies. These were Lucilia sericata Mg., Calliphora erythrocephala Mg., Calliphora vomitoria L., Protophormia terrae-novae R-D., Musca domestica L., Muscina pabulorum Fal., Cynomyia mortuorum L. and Sarcophaga carnaria L. Using the same methods as for L. caesar all were easily bred in the laboratory except the last two. C. mortuorum was once taken successfully to the first laboratory bred adult generation which produced a few fertile eggs. The larvae from these fed well but failed to pupate and died as larvae between the 10th and 17th day. No other attempts to maintain a culture of this species was successful. The possibility that there is a larval diapause in Cynomyia remains to/

to be investigated. Captured Sarcophaga females produced larvae which fed successfully and pupated but death invariably occurred within the puparium.

L. caesar and C. vomitoria larvae were used in experimental and histological studies and the others for comparative anatomical work only.

## Section II

Larvae in diapause, larvae at the termination of diapause and non-diapause larvae at different stages of development were required for the investigations described in this work.

In a culture of L. caesar larvae some will pupate within 7 days of the departure from the meat and the rest will persist in the larval state for varying lengths of time. Of the latter some pupate after a delay of a few days but others enter a definite diapause which might last for months. The proportion of a culture, kept under standard conditions of temperature, humidity, aeration and space, which enters a diapause can vary from 10% to 90% but in the majority of cases about 50% of the larvae go into a firm diapause. Any larva un-pupated 14 days after the cessation of feeding is almost certain to be in that state. There is, therefore, no difficulty in obtaining suitable diapause material.

Some time was devoted to attempts to find sure methods of terminating diapause in order to provide the required larvae at the diapause termination stage but the results were inconclusive. Ultimately the problem solved itself for such a large number of brains of apparently diapausing larvae were sectioned that there were inevitably a few brains amongst them of early post-diapause larvae which provided the required information.

Experiments/

Experiments on diapause avoidance showed that optimal conditions at the cessation of feeding include good aeration, a high humidity to prevent desiccation and a lack of mutual disturbance. To produce non-diapause specimens large larvae, with crops still full, were selected at the cessation of feeding and transferred into 3" x 1" glass tubes which were filled with 2 inches of moist peat moss and covered with bolting silk. The moss was kept moist by the daily addition to its surface of sufficient water to restore the weight of the tube plus contents to the original weight recorded at the start of the test. Tubes were kept at 25°C. A long series of tests showed that under these conditions 92% of the larvae will pupate within 7 days and this was therefore adopted as the routine method for obtaining non-diapause larvae.

## Chapter 2

### The Anatomy of the Central Nervous System and Retrocerebral Endocrine Organs of the Larvae of Lucilia caesar L. and Certain Other Diptera - Cyclorrhapha.

The first description of the anatomy and account of the development of a Cyclorrhaphan larva, Calliphora (Musca) vomitoria L. is to be found in 'Entwicklung der Dipteren' the classical opus of Weismann (1864). Less satisfactory is the work of Lowne (1890-92) on the larva of Calliphora erythrocephala Mg. More recently Hewitt (1910) produced a comprehensive study of the house fly Musca domestica L. which includes an excellent study of the anatomy of the mature larva of that species. Others have made studies of the morphology and development of the head of these larvae without contributing to knowledge of the central nervous system but Ludwig (1949) in describing the anatomy of the anterior part of the larva of C. erythrocephala provided new information on the nerves from the cephalic and sub-oesophageal ganglia. These works formed the background of information on which this anatomical study was based.

#### (a) Techniques

Mature third instar larvae, taken at stages between the cessation of feeding and puparium formation, were used in this study/

study. Prior to dissection larvae were anaesthetised with ether until movement of the muscles of the body wall had ceased. Solid watch glasses, filled to within  $\frac{3}{16}$ " of the top with black wax, were used as dissection dishes. The dissection procedure was, first to pin the larva ventral side down to the wax with no. 20 entomological pins through the head and between the hind spiracles. The dish was then filled with a saline solution, insect Ringer's solution prepared to the formula given by Williams (1946) i.e. 7.5 gm. sodium chloride, 0.35 gm. potassium chloride, 0.21 gm. calcium chloride and 1,000 ml. distilled water. The dorsal body wall was now opened with scissor forceps, a longitudinal cut being made as far forward as the prothoracic segment and as far back as the penultimate abdominal segment. The body wall was then pinned back with two pins on each side.

For supra-vital staining of the nerves a 1:1,000 solution of methylene blue in the saline solution defined above was used following the procedure recommended by Ludwig (1949). The specimen was first rinsed with two or three changes of saline to clear blood from around the ganglion and neighbouring organs. The dish was then filled with a volume of staining solution sufficient to cover the larva and was gently agitated for five minutes. The specimen was now rinsed with one or two changes of saline to remove excess stain and, after the dish had been filled once more with saline solution, was ready for examination. The/

The nerves acquire a brilliant blue colour with this treatment and although the colour fades in time the procedure can be repeated with the same specimen. The stained nerves can be seen more easily against a white background and this was provided by slipping a small triangle of white celluloid between the ganglion and the body wall. For drawing and photographic purposes it was sometimes convenient to fix the material, after a dissection to display the point of interest, with 5% formalin solution. Another type of semi-permanent preparation for photographing was made by fixing the material in situ with aqueous Bouin and, after thorough washing, staining with Mayer's haemalum. Serial sections were prepared from material fixed in Bouin and embedded in Steedman's ester wax. Sections were cut at 6 or 8  $\mu$  and stained with a standard haemalum-eosin procedure. Tracheal systems were observed in dissections of anaesthetised larvae before and after methylene blue vital staining and in whole fresh organs mounted in glycerine.

(b) The central nervous system in the larva of *Lucilia caesar*

(The reader is referred to Figs 1, 2 and 3. The code letters suffixed to the names of certain structures in this account are used in these diagrams.) Hewitt observed that in Musca "the central nervous system of the larva has attained what would appear to be the limit of ganglionic concentration and fusion", and Ludwig found that in Calliphora "the central nervous system is a very compact mass of tissue, showing no external signs/

Fig. 1. Diagram of the dorsal aspect of the central nervous system of the larva of L. caesar showing stomato gastric nervous system, anterior nerves, etc.

BPA, bucco-pharyngeal apparatus; CH, cerebral hemisphere; PN, procurrent nerve; RN, recurrent nerve; FG, frontal ganglion; CG, crop ganglion; HG, hypocerebral ganglion; LN, labral nerve; AN, antennal nerve; MMLN, mandibular maxillary-labial nerve; APtN, accessory prothoracic nerve.

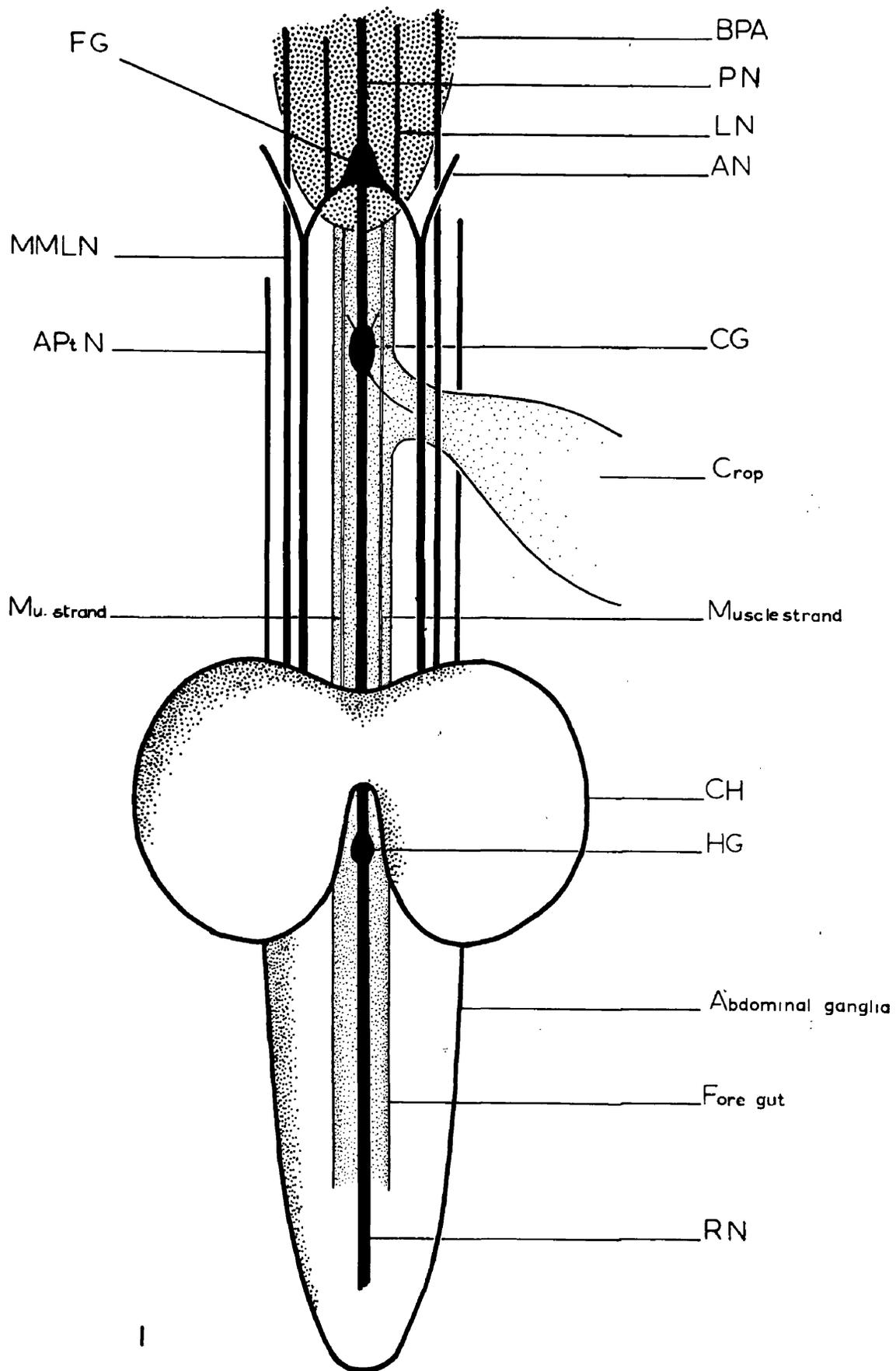


Fig. 2. Diagram of the lateral aspect of the central nervous system of the larva of L. caesar.

CH, cerebral hemisphere; A+LFN, antennal+labro-frontal nerve; MMLN, mandibular-maxillary-labial nerve; T1LD, prothoracic leg disc; T1N, prothoracic nerve; T2N, mesothoracic nerve; T2LD, mesothoracic leg disc; T3N, metathoracic nerve; A<sub>1</sub> - A<sub>8</sub>, lateral abdominal nerves; MA<sub>1</sub> - MA<sub>7</sub>, median abdominal nerves.

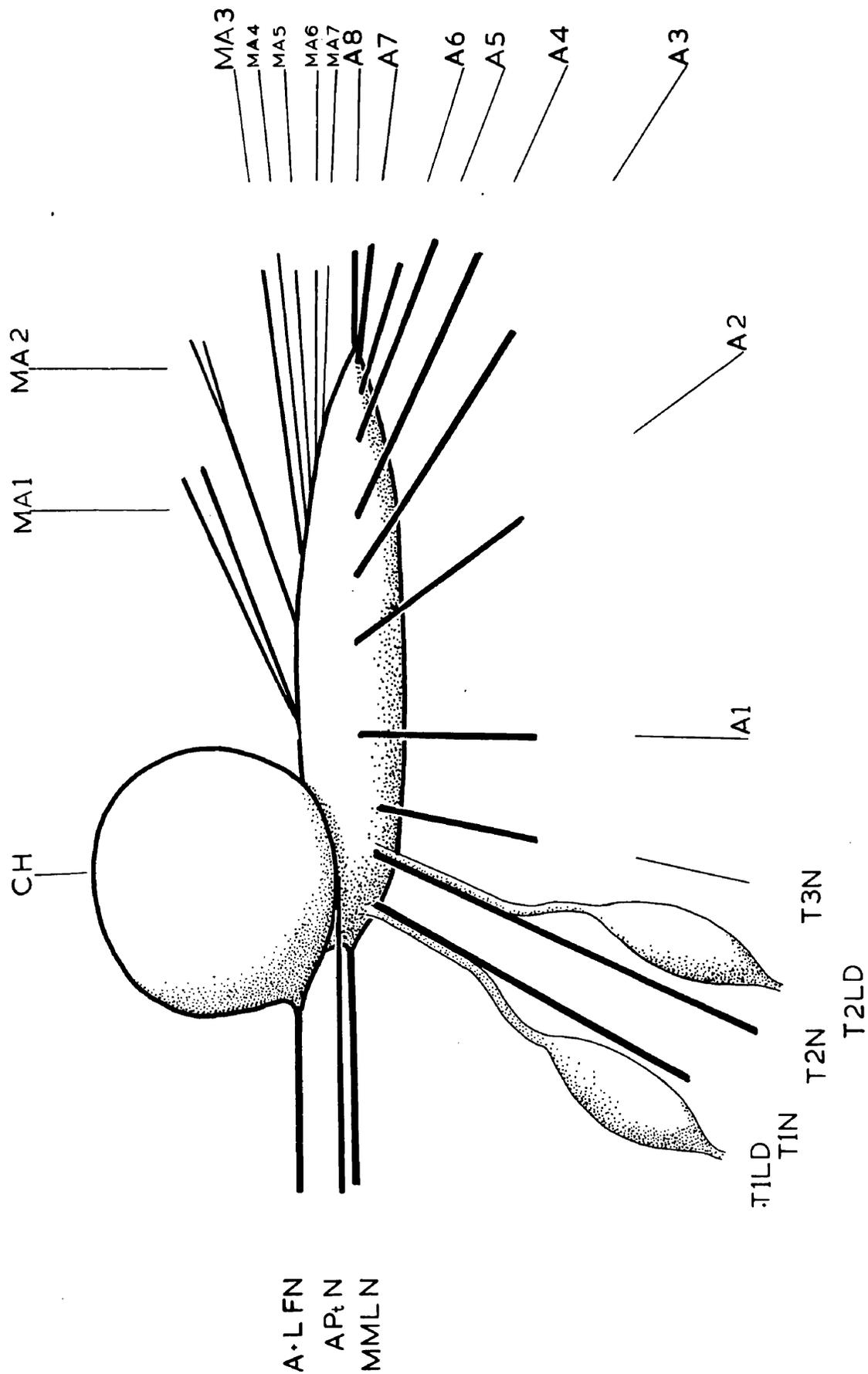


Fig. 2.

signs of segmentation. All the ganglia of the head, thorax and abdominal segments are fused into a single mass". These descriptions could apply equally to Lucilia.

The "brain" of the generalized insect is composed of three pairs of cephalic ganglia, those of the proto-, deuto- and tritocerebrum. In the larva of Lucilia caesar, as in all Cyclorrhaphan larvae anatomically investigated so far, the component ganglia of each half of the brain are fused to form the two cerebral hemispheres (CH). These are united, dorsal to the foramen traversed by the oesophagus, by the pars intercerebralis and ventrally each is fused directly to the suboesophageal ganglion which forms part of the single ventral ganglionic mass (VG). The membranous frontal sac (FS) containing the imaginal discs of the eyes (IDE) and antennae (IDA) is bifurcate posterior to the bucco-pharyngeal apparatus. Posteriorly each branch of the sac unites to the ventro-lateral surface of its corresponding hemisphere by the so-called optic stalk (OS). The eye discs fit close to the anterior surface of the hemispheres and within each branch of the sac the antennal disc lies in front of the eye disc. The dorsal aorta (DAo) passes as a closed tube over the larval brain but just in front of the pars intercerebralis it opens ventrally, the margins of this opening being fused laterally to the two branches of the frontal sac and posteriorly to the surface of the brain. In a fresh dissection of a living larva haemocytes carried forward in/

in the blood flowing along the aorta can be seen falling down from this opening between the eye discs into the body cavity. Other connections of the aorta with the brain are described later.

(c) Nerves from the brain and ventral ganglia: *L. caesar*

From the ventro-anterior surface of each hemisphere issues a thick nerve which passes forward. Its first branch (AN) innervates the larval antennal organ, its second branch (LN) innervates the labral sense organ and the nerve terminates in the frontal ganglion (FG) which lies on the surface of the pharynx concealed by the muscles of the bucco-pharyngeal apparatus (BPA). This nerve can be considered as a fusion of the antennal and labro-frontal nerves. Just below this nerve there emerges from the ventral ganglionic mass, on each side, a nerve which sends branches to the labial sense organ, to the mandibular muscles and to the maxillary palp. It can, therefore, be identified as the mandibular-maxillary-labial nerve (MMLN) from the sub-oesophageal ganglion. Just behind the root of this nerve the sac of the imaginal disc of the pro-thoracic leg (T1LD) is attached to the ventral ganglionic mass and immediately posterior to this point of attachment emerges the pro-thoracic nerve (T1N). Next to it lies the meso-thoracic nerve (T2N) and at the root of this nerve the sac of the meso-thoracic leg disc (T2LD) is attached to the ganglion. Thereafter there emerge at intervals, from the side of the ventral ganglion, the meta-thoracic nerve (T3N) and the eight abdominal/

abdominal nerves (A1 - A8). Tracheae enter the ganglia alongside each of the thoracic and abdominal nerves except those of the eighth abdominal ganglion. Another nerve emerges from the ventral ganglion from a point above and behind the root of the pro-thoracic nerve. This is found to innervate muscles of the pro-thorax and to anastomose with the pro-thoracic nerve. Ludwig's interpretation of this nerve as an accessory pro-thoracic nerve (APtN) is therefore accepted. In addition to the lateral nerves of the ventral ganglia dorsal accessory nerves have their origin in the mid-dorsal line of the ventral ganglion. According to Hewitt Musca possesses a pair and three median unpaired nerves in this position. The illustrations given by Ludwig seem to indicate three single nerves in this position in C. erythrocephala.

In Lucilia the first pair of these nerves, which can be referred to as the "median abdominal nerves", are, from their point of origin on the surface of the ganglion, separate. These nerves (MA1) serve the first abdominal segment. Behind the origin of these nerves arise in turn the other six pairs of median nerves (MA2 - MA7). The two nerves of each pair are fused for part of their length, the second abdominal pair separating soon after they leave the ganglion, the third abdominal pair separating at some distance behind the ganglion and the others at successively greater distances from their source. The median nerves of abdominal segments 4, 5, 6 and 7 are/

are much more slender than those of segments 1, 2 and 3, and it is probably because of this that they have hitherto escaped notice. It is interesting to note that there are no median nerves of the thoracic segments but such nerves are represented by three mushroom-shaped bodies attached to the dorsa of the three thoracic ganglia and lying at the loci from which median nerves should arise. The fact that they can be seen, in serial sections, to receive axons proves that these are vestiges of thoracic median nerves.

(d) The stomato-gastric nervous system: *L. caesar*

The frontal ganglion lies above the pharynx, concealed by the muscles of the bucco-pharyngeal apparatus. The single nerve running forward from the frontal ganglion and giving off rami to the cibarial muscles has been identified by Ludwig as the procurrent nerve (PN). Nerves unite the frontal ganglion to either labral nerve. A single nerve running posteriorly from the frontal ganglion, and lying above the oesophagus, terminates in the proventricular ganglion from which rami are given off to the muscles of the proventriculus. This is the recurrent nerve (RN). There are two other ganglia in the course of the recurrent nerve. The first of these is attached to the upper surface of the oesophagus near the point where the crop branches off and from this ganglion (CG) go five nerves to the muscles of the oesophagus and crop. The second is represented by a swelling in the recurrent nerve just behind the brain.

From/

Fig. 3. Lateral view of the brain and adjacent organs in the larva of L. caesar.

CT, cerebral trachea; CB, cephalopharyngeal band;  
AP, anterior prolongation; TG, thoracic gland;  
CH, cerebral hemisphere; DAo, dorsal aorta;  
HG, hypocerebral ganglion; RN, recurrent nerve;  
OS, optic stalk; IDE, eye disc; IDA, antennal disc;  
FS, frontal sac.

Fig. 4. Diagram illustrating in lateral view the location of the retrocerebral endocrine organs of the larva of L. caesar.

CT, cerebral trachea; TAT, transverse anastomosing trachea; TG, thoracic gland; CA, corpus allatum; CC, corpus cardiacum; NCC, nerve to the corpus cardiacum; MuA, anterior muscle strand; MuP, posterior muscle strands; HG, hypocerebral ganglion.

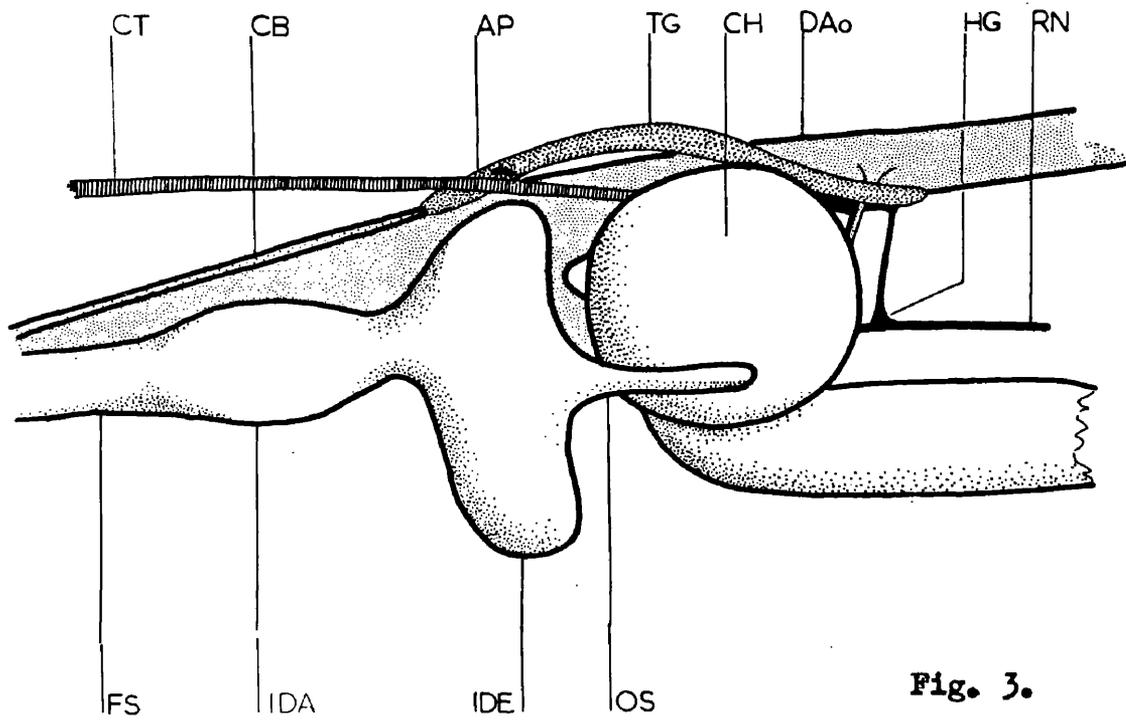


Fig. 3.

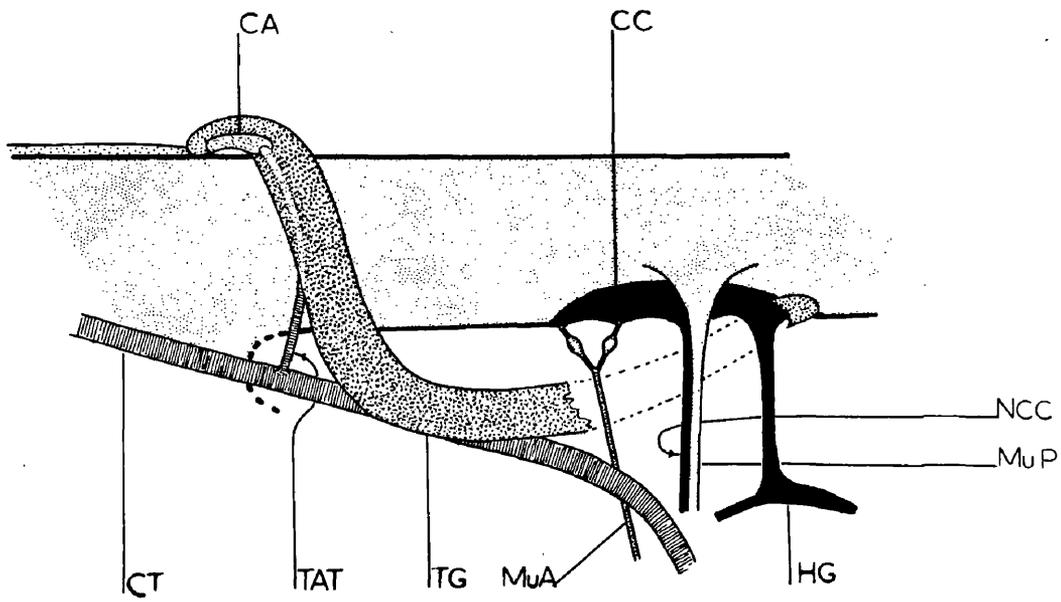


Fig. 4.

From this swelling branches the nerve to the corpus cardiacum and it is, therefore, identifiable as the hypocerebral ganglion (HG).

(e) Weismann's ring: (see Figs 3 and 4)

In his historic work, Weismann (1864) stated the following. "The dorsal vessel lies above the nerve centres and passes into the cleft between the hemispheres. Immediately in front of these there is a ring (TG) with thick cellular walls the lumen of which is sufficiently large to admit the dorsal vessel. The ring hangs freely in the body cavity and is fixed by fine tracheal vessels which pass through it. In the anterior part of the second segment a trachea springs from the main trunk and passes obliquely inwards and backwards and penetrates the hemisphere, branching in its interior (CT). In their course these tracheae are united by a transverse branch (TAT) and immediately below this they enter the ring with which their peritoneal coat is fused. The transverse branch lies in the ring itself for much of its length....." He decided that the ring was a "fixation apparatus" and did not find any connection from it to the central nervous system.

This ring usually referred to as Weismann's ring, was until recently, a problematic structure but it is now generally agreed that it formed by the fusion of three pairs of endocrine organs.

Before discussing the homologies and innervation of this structure/

structure it is appropriate to give an account of the retrocerebral endocrine organs of a generalized insect. In the generalized insect there lie behind the brain two compact spherical or ovoid bodies, the corpora allata. Nabert (1913) was probably the first to consider that these might be endocrine organs. They are ectodermal in origin. Lying between the corpora allata are the two corpora cardiaca. The latter are in intimate association with the wall of the aorta and each receives two nerves from the brain as shown first by Pflugfelder (1937) and Hanstrom (1938, 1940). A single nerve unites each corpus cardiacum to its neighbouring corpus allatum. The two inner nerves from the brain to the corpora cardiaca are the nervi corporis cardiaci I (NCCI) of Hanstrom and the two outer nerves the nervi corporis cardiaci II (NCCII). Cazal (1948) called them the internal and external nerves of the corpus cardiacum. These nerves are almost certainly formed by the axons of the neurosecretory cells in the brain of the insect. Each of the internal nerves has its origin in the opposite side of the brain to that from which it emerges and in their intracerebral course the NCCI decussate in the pars intercerebralis. The course of the NCCII is direct. Each issues from the rear of the half of the brain containing the neurosecretory cells of its origin. Various degrees of association of the corpora occur in different orders, in some the corpora allata being united, in others the corpora cardiaca fused, or the corpus allatum and corpus cardiacum of each side joined (Cazal, 1948). The role of/

of these organs in the control of growth and development of juvenile insects is discussed in Chapter 3.

The third pair of endocrine organs are the thoracic glands. Lyonet (1762) is credited, by Williams (1949), with the first discovery of these, in a Lepidopteran larva. He called them "granulated vessels". Toyama (1902) determined that they originated in the embryo of Bombyx as epithelial invaginations of the lateral parts of the labial segment. Wells (1954) confirmed that the origin is the same in Hemiptera. So far these are the only cases in which homology has been established but histologically similar organs, with the same function - and therefore almost certainly homologous - have been found in Orthoptera (Scharrer, 1948; Pflugfelder, 1949; Jones, 1953; Bodenstein, 1953), in Lepidoptera (Fukuda, 1940; Williams, 1946; Lee, 1948; Rehm, 1951), in Hemiptera (Wigglesworth, 1952; Wells, 1954 et al.). Homologies were discussed by Williams (1949) and by Pflugfelder (1949).

Although these glands originate as ingrowths of a cephalic segment they are in most orders located in the thorax and, Wigglesworth's (1952) term "thoracic glands" has been adopted in this work.

Accounts of Weismann's ring have been given by d'Herculais (1875; Volucella), Batelli (1879; Eristalis), Lowne (1890-92; C. erythrocephala),/

C. erythrocephala), Pantel (1898; Tachinidae), Hewitt (1910; M. domestica), Keilin (1917; Anthomyidae), Thompson (1921; Sarcophagidae). Since Wigglesworth's (1934) proof of humoral control of development in Rhodnius and the demonstration by Fraenkel (1935) that there is an endocrine centre, in the region of the brain of Calliphora, promoting puparium formation, attention has been centered on the ring as a possible endocrine organ in Diptera - Cyclorrhapha. Hadorn (1937) proved it by showing that ring glands from normal, mature larvae of Drosophila were competent to promote puparium formation in lethal, giant, mutant larvae. Burt (1937) arguing from the false premise established by Wigglesworth (1934), that the corpus allatum is the source of "moulting hormone", homologised the ring with two fused corpora allata. Hadorn (1937) agreed with this view but later Scharrer & Hadorn (1938) amended this interpretation considering the ring as being composed of a small cell component (the corpus cardiacum) and a large cell component (the corpus allatum), the latter forming the lateral part of the ring. Hanström (1939); Nyst (1941); Day (1943) and Poulson (1945) homologised the ring with the corpus cardiacum. Meanwhile, Vogt (1941, 1942<sub>b</sub>, 1943<sub>a</sub>) working with Drosophila and Calliphora had reached the conclusion that there were three components of the ring, a dorsal small cell component being the corpus allatum, a ventral large cell component being the corpus cardiacum and the lateral large cells of the ring which she proved/

proved to be the source of the pupation hormone. These 'Hauptzellen' can therefore be assumed to be the thoracic gland cells of Diptera - Cyclorrhapha. E. Thomsen (1942) adopted a similar interpretation and Cazal (1948) concurred. Possompes (1946, 1947, 1948 and 1953) found the homologue of these large ring cells in Chironomus, Tipula and Tabanus and he gave to this organ the name peritracheal gland which can now be considered as a synonym for thoracic gland. The descriptions of the arrangement of the retrocerebral endocrine organs in C. erythrocephala given by Possompes and by M. Thomsen (1951) confirm the three component interpretation of Vogt.

There is, however, so much general confusion between the earlier descriptions and disagreement in detail between more recent ones that a study of the structure and innervation of the ring in a number of species of Cyclorrhapha was considered justified. Since Protophormia terrae-novae R-D possesses what is apparently the most primitive arrangement of the components of the ring a description of the anatomy of this larva is given first.

(f) The retrocerebral endocrine organs of the larva of P. terrae-novae. (See Figs 5 - 9)

The single corpus cardiacum (CC) is incorporated in toto in the ventral wall of the aorta above the larval brain. It is an elongate body receiving near its posterior limit a branch from the/

the recurrent nerve (RN). M. Thomsen's (1951) identification in Calliphora of the swelling of the recurrent nerve, from which this branch arises, as the hypocerebral ganglion is confirmed by Possompes (1953). This also is the locus of the hypocerebral ganglion in Protophormia. Anterior to the junction with this nerve the corpus cardiacum receives a pair of nerves arising from the posterior inner faces of the cerebral hemispheres (CH) close to the insertions of the cerebral tracheae. At the point of emergence of each nerve there also arises a pair of muscular connective strands, the one passing forward alongside the recurrent nerve to meet the junction of the frontal sac and the functional pharynx, the other passing upward alongside the nerve to the corpus cardiacum. This latter strand is attached to the nerve for most of its length but they part before the nerve enters the corpus cardiacum and the strand continues upwards through the gap between the ring and the aorta to join the mid-lateral wall of the aorta. A second pair of connectives link the corpus cardiacum to the brain. These emerge from the hind face of the pars intercerebralis and after each has divided into two branches, join the ventral surface of the aorta at the anterior end of the corpus cardiacum. There has been some dispute about the nature of these anterior connectives, Burt (1937) stating that there were in C. vomitoria two pairs of nerves from the brain to the "ganglion on the floor of the aorta" now recognised as the corpus/

Fig. 5. Diagram illustrating the derivation of the tracheal supply to the retrocerebral endocrine organs, proventriculus etc. in the larva of P. terrae-novae.  
c.h., cerebral hemisphere; v.g., fused ventral ganglia; f.g., fore gut; prov., proventriculus; e.c., enteric caecum; m.g., mid gut; ao., aorta; con., lateral connective strands linking aorta to longitudinal tracheae; l.t., longitudinal tracheal trunk; t., single tracheal branch passing forward; r.n., recurrent nerve terminating posteriorly in the proventricular ganglion; T.G., thoracic gland; c.b., cephalo-pharyngeal band; e.d., eye disc; a.d., antennal disc; f.s., frontal sac.

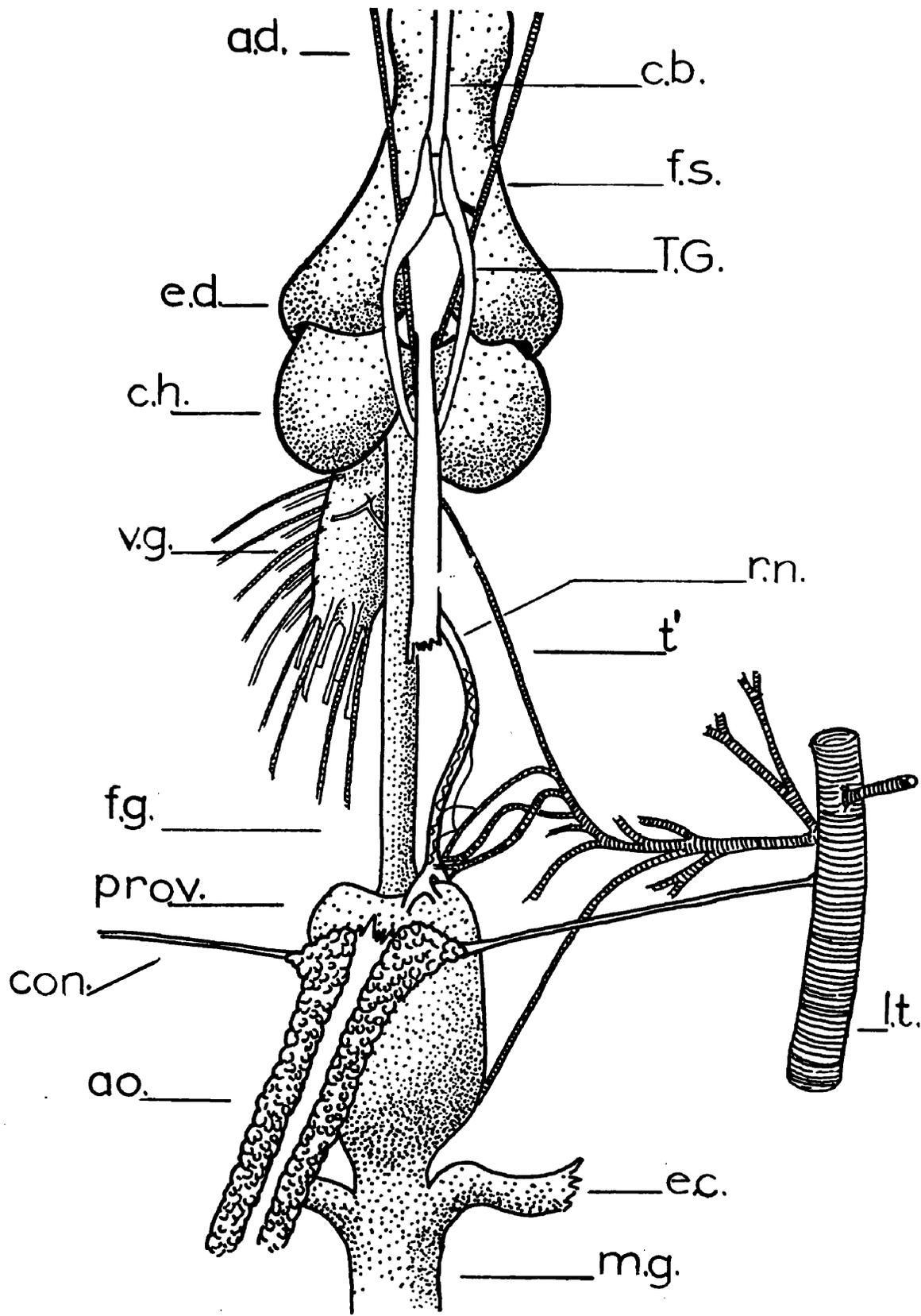


Fig. 5.

corpus cardiacum. Day (1943) stated that there was only one pair of nerves in Lucilia, which he identified as the NCC I. Thomsen (1951) having given particular attention to the matter agreed with Burt (loc cit.) that there are two pairs of NCC in C. erythrocephala. Possompes (1953) on the other hand stated that this was unacceptable saying "je n'ai jamais observe qu'une paire de liaisons de nature incontestablement nerveuse a ce niveau a vrai dire assez malaise a dechiffrer". Cazal (1948) found a single pair of NCC in Eristalis. As a result of this investigation it has now been determined that these anterior connectives are muscle strands like those parallel to the nerves. In order to demonstrate these very slender structures by vital staining or to treat the brain and neighbouring organs with fixatives before removal for sectioning stretching of the aorta and of the connectives to the brain is inevitable. As a result in sections in which muscle fibres are not specifically stained only a longitudinal fibrillation is apparent in the anterior pair, highly comparable to that seen in the posterior connectives which for most of their length are composed of an intimately associated nerve and muscle. Of the hundreds of living larvae of this species which were dissected the anterior connectives were plainly seen to be performing rhythmical contractions in one case only though such contractions were commonly seen in the two muscle strands which run forward parallel to the recurrent nerve.

The large cells, which in other *Cyclorrhapha* form a continuous ring around the aorta, form two separate thoracic glands (TG) in *P. terrae-novae*. Ventrally these are attached to each side of the corpus cardiacum above the point of entry of the branch from the recurrent nerve. Dorsally they overlie the corpus allatum (CA) and each thoracic gland forms an anterior prolongation uniting, not with the dorsal wall of the aorta, but with the cephalo-pharyngeal band (CB), a strip of cells, with large nuclei and indistinct borders, running along the dorsal wall of the aorta from the point of junction with the thoracic gland to the anterior limit of the aorta. The thoracic glands in *P. terrae-novae* are not therefore directly in contact with the aorta at any point.

The paired origin of the corpus allatum is evident. It is a V-shaped structure formed of two lobes uniting anteriorly where the corpus allatum overlies the posterior limit of the cephalo-pharyngeal band. Behind this point the separate lobes are supported by the transverse anastomosing tracheae. The position of the anterior ends of the thoracic glands relative to the corpus allatum can be seen in Figs 6 and 7.

Each cerebral hemisphere receives a trachea branching from the longitudinal trunk of its side. Just anterior to the brain there is a transverse anastomosing trachea linking these cerebral tracheae. It passes over the aorta, uniting with its dorsal/

Figs 6 - 9

- 6) Transverse section of corpus allatum, 1. P. terrae-novae.
- 7) Transverse section of corpus allatum, 2. P. terrae-novae.
- 8) Neurosecretory cells in the brain of P. terrae-novae.
- 9) Section at the junction of thoracic gland and corpus cardiacum.

CA, corpus allatum; CC corpus cardiacum; TG, thoracic gland; A, axon; AC, anterior connective to the CC; AO, aorta; CB, cephalo-pharyngeal band; ChC chromophile cell; CN, chromophobe cell nuclei; N, nucleus; NN, neurons located in the thoracic gland; S, secretory "granules"; TAT, transverse anastomosing trachea; TGN, thoracic gland cell nuclei; V, vacuoles.

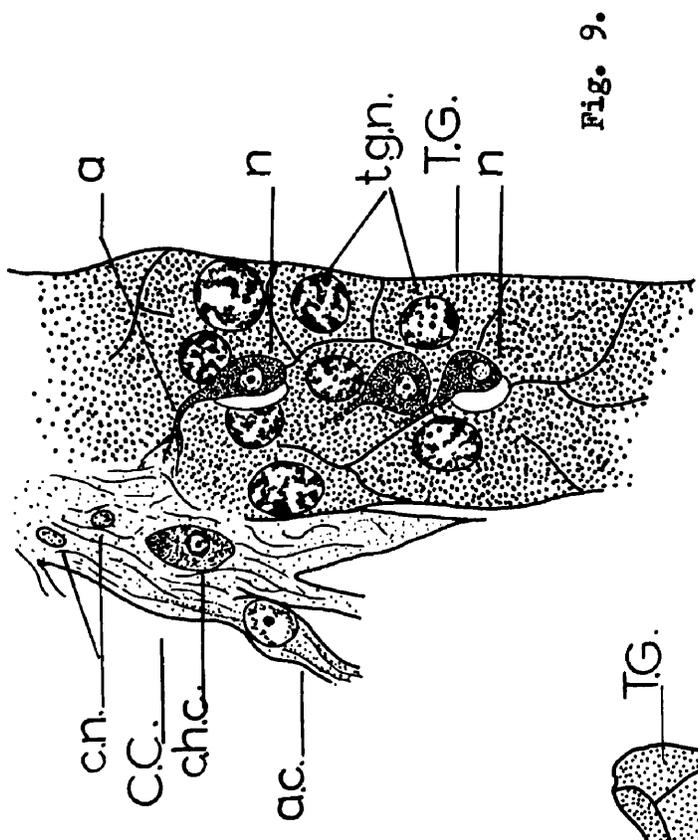


Fig. 9.

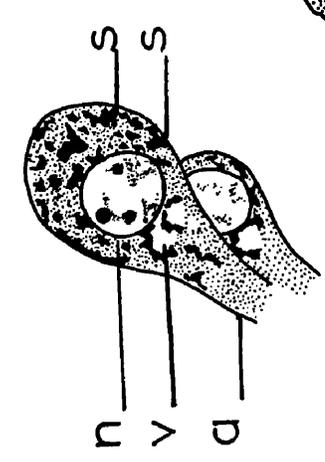


Fig. 8.

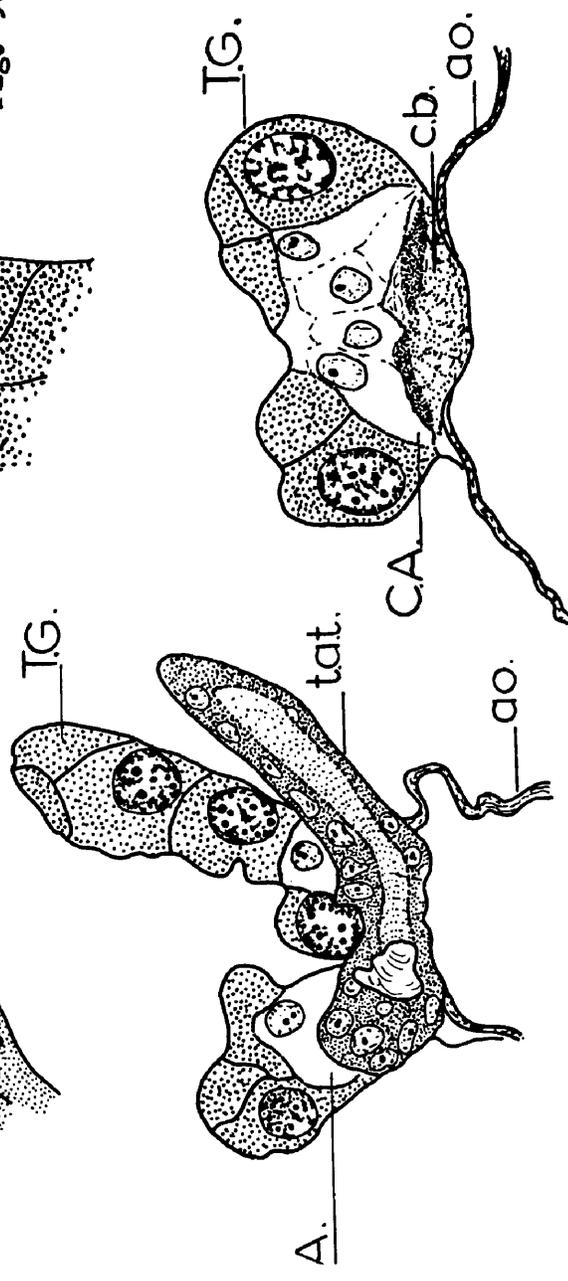


Fig. 6.

Fig. 7.

dorsal wall (Fig. 7), behind the posterior limit of the cephalopharyngeal band and supports the posterior lobes of the corpus allatum. The thoracic glands are not in contact with the cerebral tracheae in this species apart from several very fine connective strands. A trachea leaves the longitudinal tracheal trunk of each side at the level of the first abdominal segment and sends branches to the proventriculus, salivary glands, enteric caeca, fat body and Malpighian tubules. The one on the right also has branches to the proventricular ganglion and the recurrent nerve and a single branch passing forward (Fig. 5). Very rarely this group of tracheae originates from the corresponding position on the left. The single branch which passes forward divides beneath the corpus cardiacum into four trachaea, one entering each thoracic gland and one to each side of the corpus cardiacum. Minor variations occur at this level but it is usually the trachea to the right thoracic gland which sends tracheoles into the hypocerebral ganglion.

The corpus cardiacum as expected was found to contain nuclei of the two types of cells denoted by Cazal (1948) as "chromophile" and "chromophobe". The nuclei of the latter are abundant, more than a hundred being counted in the corpus cardiacum of a mature larva, and are more concentrated in the posterior part of the organ. They range in size from 4.5  $\mu$  to 16  $\mu$  in diameter with a mean size of 7.5  $\mu$ , have a single nucleolus, are relatively poor in chromatin and appear similar to/

to nuclei found in the hypocerebral ganglion. These cells appear to form a syncytium. The chromophile cells are few in number and are arranged in two lines corresponding to the two corpora cardiaca now fused. Each cell lies embedded in the syncytium and is elongated in the long axis of the aorta, a typical one at this stage measuring  $18 \mu$  by  $10 \mu$  with a round nucleus about  $7 \mu$  in diameter. As others have observed, some of the cells appear in sections to have a polygonal form but this may be a fixation artefact as vitally stained cells have a more regular ellipsoid outline. There is no evidence that these cells possess axons or dendrites. Several workers have noted cells in the thoracic glands, other than the large cells characteristic of these organs, which they consider similar to these chromophile cells. In each thoracic gland there are four cells which bear a superficial resemblance to these but which are apparently neurons with a single axon whose course can in some cases be traced as far as the corpus cardiacum (Fig. 9). These cells are pyriform and have a nucleus of average diameter  $7.5 \mu$ . Their cytoplasm has slightly different staining affinities from those of the chromophile cells. The space beside two of these cells depicted in the figure is a regular feature but is probably a fixation artefact.

The location and form of the corpus allatum has already been described. There are about twenty-eight spindle-shaped cells/

cells in this organ with subspherical nuclei 10 - 14  $\mu$  in diameter and often showing a pair of nucleoli. The existence of nerves to the corpus allatum, as described by Possompes (1953) in C. erythrocephala was not demonstrable, by the techniques used, in this or in any of the other species under investigation, though there is no reason to suppose that these nerves do not exist.

The large thoracic gland cells do not differ in detail from those described in related species. The nuclei continue to increase in size between the time of cessation of feeding and puparium formation and at the latter time may exceed 25  $\mu$  in diameter. This marked growth of thoracic gland nuclei in the mature third instar larva has been noted by Burt (1937) Vogt (1943a) and Thomsen (1951) who in discussing the state of these nuclei disagreed with the statement of Day (1943) that they are (in Lucilia) in the "polytene" condition, but admitted that the nucleolus sometimes seems to be a composite body "which may indicate that the nuclei are polyploid, perhaps due to some endomitosis".

The possession of symmetrical paired thoracic glands is a remarkably primitive feature to be found in a member of the Cyclorrhapha. It is known in some Nematocera, having been demonstrated in Chironomus plumosus by Possompes (1946) and in Ptychoptera sp. by Thomsen (1951) but not in any of the higher Diptera/

Diptera described so far. Tracheation of these organs (apart from simple superficial contact with the cerebral tracheae) is unknown in *Cyclorrhapha* but occurs in other Diptera. Possompe calling them the "peritracheal glands" in *C. plumosus*. The asymmetry of the tracheal supply to these glands in *Protophormia* is, however, unique and suggests a secondary adaptive feature. The purpose of these tracheae may be to "accelerate" the activity of the thoracic glands and of the corpus cardiacum, the duration of the phase between cessation of feeding and puparium formation being, in this species, only one third as long as that in the related genera *Lucilia* and *Calliphora* according to Cochrane (unpublished records).

The form of the corpus allatum might be likened to that of a crescent with the horns directed towards the rear. M. Thomsen (1951) described the organ as crescent-shaped in *Thereva* sp. (Diptera - Brachycera) but in that animal it is the posterior component of the retrocerebral complex and the horns of the crescent point forwards. The anterior ends of the corpus allatum in *Thereva* are connected to the thoracic glands which pass downwards and forwards to unite below the aorta. In this description of Thomsen's, the location of the corpus cardiacum is not clear but he notes a single pair of nerves from the brain to the ventro-median parts of the "ring". It is apparent that in the rearwards shift of the brain in the larvae of/

of Cyclorrhapha the relative positions of the components of the complex have been altered. The corpus allatum has become in Protophormia the anterior organ and the thoracic glands now pass downwards and backwards. While such internal anatomical details may be of doubtful taxonomic value, the divergence of the arrangement in Protophormia from that known in other members of the same sub-family suggests that an extension of this study to other Cyclorrhapha might be rewarding.

(g) The retrocerebral endocrine organs of the larva of L. caesar. (See Figs 1 - 4)

In L. caesar the corpus cardiacum (CC) is, as in Protophormia, fused with the ventral wall of the aorta (DAo). It receives at its posterior end the nerve from the hypocerebral ganglion (HG), and, just in front of this junction, the two nerves from the brain (NCC). These issue from the posterior inner faces of the cerebral hemispheres just in front of the points of entry of the cerebral tracheae. There are two muscle strands running alongside the nerves from the brain to the corpus cardiacum ( MuP ) which on diverging from these nerves pass upwards through the ring to join the lateral wall of the aorta, and two linking the anterior end of the corpus cardiacum with the cerebral commissure ( MuA ). There are again two muscle strands, arising from the same points as the two posterior muscle connectives to the aorta, which pass through/

through the cerebral foramen parallel to the recurrent nerve and terminate at the junction of the frontal sac and pharynx.

The thoracic glands (TG) form in L. caesar a complete ring around the aorta. They unite ventrally and join with the aorta just behind the posterior limit of the corpus cardiacum. Dorsally they join above the aorta and form a single anterior prolongation (AP) which is attached to the cephalopharyngeal band (CB). The corpus allatum which is fused to the underside of the prolongation is globular and not crescentic as in Protophormia. The transverse anastomosing trachea (TAT), linking the two cerebral tracheae, passes over the aorta and under the corpus allatum and appears to be fused to both these organs. There are no tracheae penetrating the thoracic glands. The lateral parts of the ring, formed by these glands, lie on top of the cerebral tracheae and are fused with them at points of contact.

Thus the ring in L. caesar is a more compact structure with a higher degree of fusion of the components than in Protophormia.

(h) Anatomy of the ring in other Cyclorrhapha

Other species examined were L. sericata, C. erythrocephala, C. vomitoria, Musca domestica, Muscina pabulorum and Cynomyia mortuorum. The structure of the ring and/

and the arrangement of nerves, connective muscle strands and tracheae in these species resemble so closely those in L. caesar that separate descriptions are not warranted. A slight variation noted in C. erythrocephala is that the corpus cardiacum is prolonged downwards towards the brain and is partly enveloped by the ventral junction of the thoracic glands. The nerves from brain to corpus cardiacum thus appear to be much shorter than in the congeneric C. vomitoria.

Histological details are similar in the seven other species to those in Protophormia. "Chromophile" cells, their cytoplasm faintly blue in the living organ and having a strong affinity for methylene blue when supra-vitally stained, form two parallel lines in the long axis of the corpus cardiacum and occur also embedded in each thoracic gland.

(i) Neurosecretory cells in the living brain

A well-known characteristic of neurosecretory cells (see E. Thomsen, 1952) is that they are the only neurons over visible in the living brain. The neurosecretory product is particulate. It has been confirmed by dark field microscopical examination of living material that the particles or granules of product, demonstrable in material which has been fixed, sectioned and stained, are not artefacts. This particulate material within the living cell causes it, when illuminated with an incident beam of light, to assume an opalescent/

opalescent bluish colour. Hence some of these cells are, when in an active state, readily located in the living brain. The loci of neurosecretory cells are dealt with in Chapter 4 and it is sufficient to state here that the cells, described later as Group 4, lying in the dorsum of each hemisphere can easily be seen in the brain of a non-diapause larva which has been dissected early in the post-feeding period. They are not evident in the diapause brain. The importance of this lies in the fact that the brain and imaginal discs of a non-diapause larva are at this stage the same size as those of a diapause larva and thus in dissections the two types of larva must be separated by the single criterion of the appearance of the Group 4 neurosecretory cells.

"Chromophile" cells in the corpus cardiacum and thoracic glands may also show a very distinct blue colour in living material. A particulate product has not been demonstrated in stained sections of fixed material but an investigation of these cells by other microscopical techniques might yield important information.

Chapter 3

Humoral Control of Metamorphosis and Diapause

Section I

(a) Humoral control of metamorphosis in insects

It was discovered by Kopec (1917, 1922) that if the pre-pupal larva of Lymantria was ligatured some time before pupation was due then only the portion anterior to the constriction would eventually pupate. If the ligature was applied nearer to the expected time for pupation both halves would pupate. Removal of the brain at the first stage also prevented pupation while removal at the second stage did not. Thus was it first demonstrated that a stimulus from the brain promotes the interconnected processes of moult and of growth and differentiation in an insect and that the stimulus is provided some time before the developmental changes become externally evident.

It was first proved by Wigglesworth (1933, 1934, 1936) by decapitation and parabiotic experiments with Rhodnius that there is a hormone which causes moulting in insects. A search then followed for the source of this hormone attention being concentrated, and thus to some extent sidetracked, on the function of the corpora allata since Wigglesworth suggested (without good evidence, as he readily admitted in 1954) that these organs/

organs were the probable source. Bounhiol (1938) showed that the corpora allata did not produce a moulting hormone in Lepidoptera since extirpation of these organs did not prevent moult. Plagge (1938) confirmed this and also showed that removal of the brain of Deilephila prepupa prevented pupation while implantation of a brain could induce it. The moulting hormone was apparently produced by the brain. Hanström (1938) demonstrated neurosecretory cells in the dorsum of Rhodnius' brain and Wigglesworth (1940) then showed that the part of Rhodnius' brain containing these cells removed from a larva after a "critical period" (i.e. after the time at which decapitation could prevent moulting) and implanted into a larva decapitated before the critical period induced moulting in the latter.

The immediate source of the hormone causing moult and pupation was shown by Fukuda (1940) to be, not the brain, but a pair of glandular structures in the larval prothorax. If a silkworm larva was ligatured behind the prothorax only the anterior half would pupate but the posterior half could be induced to pupate by implants of these glands from other pre-pupae. The function of these "thoracic glands" as the source of the moulting hormone was confirmed by, inter alia, Williams (1946) in Platysamia and by Wigglesworth (1952) in Rhodnius. Other information on these glands is given in Chapter 2, part (e).

These/

These various results were related by Williams (1948a) when he demonstrated that the thoracic glands of Platysamia only secrete their hormone if activated by a "brain hormone". His evidence indicated that the neurosecretory cells were the source of this brain secretion. The protocerebrum of Platysamia contains two median groups of eight cells and two lateral groups of three cells and experimental evidence indicated that both groups participate in the activation of the thoracic glands.

Thoracic glands degenerate in adult Pterygota (Bodenstein, 1947; Pflugfelder, 1947; Scharrer, 1948; Lhoste, 1951; Rehm, 1951; Wigglesworth, 1952, 1954). They persist in adult Apterygota, which do moult (Gabe, 1953b). Moulting hormone is probably the same substance in all insects (Wigglesworth, 1936; Becker & Plagge, 1939; Becker, 1941; Williams, 1946; Karlson & Hanser, 1952).

The corpora cardiaca play a significant part in the insect's endocrine system. Their function as neuro-haemal organs for the storage and release of the neurosecretory product is discussed in Chapter 4. It has not been established if they modify the neurosecretory product of the brain cells but their intervention is apparently essential (Possompes, 1953).

The corpora allata would appear, from the mass of published experimental evidence, to be endocrine organs of multiple/

multiple function. They act under the control of brain neurosecretory cells (see Chapter 4). Their secretion inhibits, in larvae, the differentiation of imaginal structures. Allatectomy can result in the production of premature adults or adultoids from nymphs of Exopterygota (Wigglesworth, 1934; Pflugfelder, 1937; Scharrer, 1946a) and can result in premature metamorphosis in Endopterygota (Bounhiol, 1937, 1938; Fukuda, 1944). Extra larval stages can result from the implantation of corpora allata from younger into older larvae (Wigglesworth, 1933; Bounhiol, 1938; Radtke, 1942; Fukuda, 1944; Pfeiffer, 1945b; Poisson & Sellier, 1947). As Wigglesworth (1954) stated, the corpus allatum controls metamorphosis by causing a larva or nymph to retain juvenile characters when it moults, and he proposed (1936) the term "juvenile hormone" for the secretion of this gland. In addition to this function of restraining imaginal rudiment cells the phenomena of retromorphosis in adults after implantation of young larval corpora allata (Wigglesworth, 1940) and of regression of imaginal characters in pupal integument implanted in young larvae (Piepho, 1939) suggest that corpus allatum hormone also acts as a stimulator of larval tissues. In adult females the organ produces a gonadotrophic hormone (Pfeiffer, 1936, 1939; Wigglesworth, 1936; E. Thomsen, 1940; Vogt, 1940; Joly, 1945; Scharrer, 1946b). The corpus allatum secretion may be identical in larvae and adults since adult/

adult female corpora allata implanted in juvenile insects check metamorphosis and young larval corpora allata can provoke vitellogenesis in allatectomised females (Pflugfelder, 1940; Vogt, 1943c; Pfeiffer, 1945a; Scharrer, 1946b; Wigglesworth, 1948).

From the foregoing experimental evidence, and the complementary histological evidence, reviewed in Chapter 4, the current conception of the endocrine control of growth, development and moulting in insects is formed. This can be summarised as follows. The median and lateral neurosecretory cells of the brain are activated at some point in a given instar and produce a substance which is transmitted via the axons of these cells (which form the internal and external nerves of the corpora cardiaca) to the corpora cardiaca. A hormone, probably the brain product modified by the corpora cardiaca, is released into the blood in the lumen of the aorta. When the hormone in the blood reaches a threshold titre the thoracic glands are activated and the brain is no longer required for this purpose. The time at which the brain becomes dispensable corresponds to Wigglesworth's (1940) "critical period". The thoracic gland hormone is thought actually to suppress the activity of the brain neurosecretory cells. Thoracic gland hormone itself promotes thoracic gland activity so that the two thoracic glands stimulate each other and functional/

functional unity is attained. Thoracic gland hormone promotes not only moulting but also differentiation. Progressive differentiation at moults in Exopterygota is controlled, and metamorphosis at earlier moults than the prepupal is prevented in Endopterygota, by another humoral factor, the secretion of the corpora allata. The outcome of a moult depends on the relative concentration of the thoracic gland and corpus allatum hormones at the time of the moult. There is at the end of larval life a shift in the balance between corpus allatum hormone and thoracic gland hormone in favour of the latter so that the corpus allatum hormone can no longer restrain differentiation and metamorphosis occurs. In an Exopterygote nymph this obtains at the final moult. In an Endopterygote insect the corpus allatum is relatively inactive at the penultimate and final moults. Williams (1952) stated that just prior to pupation in Platysamia there is a progressive decrease in corpus allatum activity, that it is "shut off" by some unknown influence.

Further information on this topic is contained in reviews produced by Wigglesworth (1951); Bodenstern (1951); Scharrer (1952c) and Wigglesworth (1954).

(b) Humoral control of metamorphosis in Diptera -

Cyclorrhapha

Work/

Work of the first investigators of this problem was hampered by their lack of knowledge of the homologies of components of Weismann's ring. These are discussed in Chapter 2. In the discussion which follows the results of some earlier workers are reinterpreted in the light of more recent knowledge.

Fraenkel (1935) established proof for the action of a hormone inducing puparium formation in C. erythrocephala by ligaturing experiments. He postulated that the hormone producing organ was either the "ganglion" or in its immediate neighbourhood since only that part of a ligatured larva which contained the brain would pupate. The other part remained in the larval state. This effect could only be achieved if the ligature was applied before a "critical period" of about 16 hours before puparium formation was due to occur. After this critical time both halves of a ligatured larva pupate. Hadorn (1937) by implantation of the ring from normal to "lethal giant" larvae of Drosophila, so causing the latter to pupate, established that organ as the source of the pupation hormone and Burt (1938) by extirpation or microcautery of the ring gland showed that it was the source of the hormone in C. vomitoria. Vogt (1942a, 1942b, 1943b) by implantation of Weismann's ring into the abdomen, of Drosophila larva, isolated by ligature, established that this hormone induced not only puparium formation/

formation but also metamorphosis of the gut, ovaries and fat body of the host and of eye and antennal discs of other larvae implanted with the gland. She also determined that only the large glandular cells (=thoracic gland cells) of the ring produced the active factor. Bodenstein (1943, 1947) using the adult as the culture medium showed that disc implants develop in the adult only in the presence of an active ring implant. The prepupal or mature larva of C. erythrocephala was used by Possompes (1949a, 1949b, 1950a, 1950b, 1953) to demonstrate that ablation of the ring before a critical time prevented pupation, a "permanent larva" being produced. Ablation after this time did not have this effect. Removal of the corpus allatum had no effect on pupation while removal of the thoracic gland and corpus allatum, the corpus cardiacum with its nerve connections remaining intact, did prevent pupation. He concluded that the hormone concerned was produced solely by the thoracic gland cells and proved this by inducing puparium formation in permanent larvae by the implantation of active thoracic gland fragments. He performed a masterly series of experiments involving the severing of the nerves to the corpus cardiacum, removal of all or part of the ganglionic mass and implantation from young larvae into permanent larvae of ring gland only, of brain and ring gland with nerve connections intact and of brain and ring gland with nerve connections severed. From these he concluded that/

that the brain activates the thoracic gland by means of a hormone passed to the corpus cardiacum along the nervi corporis cardiaci. He showed that the intervention of the corpus cardiacum is essential and the nerves from the brain to that organ must be intact for the brain to activate the thoracic gland. Yet several workers, notably Wigglesworth (1940) and Williams (1946) apparently found that implants of the isolated pars intercerebralis region of an active brain were alone effective. The writer now learns from Williams (personal communication) that in Rhodnius and in Platysamia new corpora cardiaca develop at the severed nerve ends of brain implants. The intervention of this organ is probably essential in all insects.

The only evidence of the inhibitory role of the corpus allatum in Dipterous larvae comes from Vogt (1943a, 1943b) who found that removal of the organ accelerates Anlagen development in *Drosophila*. Eye discs implanted in an isolated larval abdomen develop more rapidly in the presence of an implant of the thoracic gland only than in the presence of the whole ring including the corpus allatum.

Thus it has been established that the system of humoral control of pupation is the same in larvae of higher Diptera as in other insects. In conclusion it should be recalled that Wigglesworth's/

Wigglesworth's "critical period" is now known to refer to the time at which the activated thoracic gland becomes independent of the brain while Fraenkel's "critical period" refers to the time at which the titre of thoracic gland hormone in the blood is such that development and moulting can proceed without further supply of hormone by the gland.

(c) Humoral control of diapause in insects

It was first suggested by Wigglesworth (1934) that diapause in insects might be due to the temporary failure in the secretion of the growth hormone.

Our present knowledge of the humoral control of diapause stems largely from the work of Williams on pupal diapause in Platysamia (Williams, 1946, 1947, 1948a, 1948b, 1952a). This insect has an obligate diapause in the pupal instar which is terminated by exposure to a temperature of 3 - 5°C for one to two months. A chilled brain from a pupa which has received this treatment, if implanted into an unchilled diapausing pupa, terminated the diapause in the recipient. The junction of a chilled and of an unchilled pupa in parabiosis resulted in termination of diapause in the latter. Diapause is therefore apparently due, as Wigglesworth suggested, to the absence of a growth factor and not due to the presence of an inhibitor. By transection of the body Williams localised the source of a factor/

factor evoking development of tissues, the prothoracic glands, and demonstrated the action of a brain hormone which activates these glands. The effect of chilling is to activate the brain, and it does so even if all nerve connections to the brain are severed. That is to say chilling affects the brain directly and not via sense organs. It does not activate other tissues or organs. He further showed that prior conditioning of tissues by brain hormone is not required since active prothoracic gland would promote development when implanted in the isolated abdomen of a diapause pupa.

The corpus allatum is not involved in the control of diapause in Platysamia. Removal of corpora allata from larvae could result in the production of premature pupae, proving the normal inhibitory action of the corpus allatum hormone on the development of imaginal discs. This was confirmed by implanting extra corpora allata into activated pupae which resulted in the production of imperfect adultoids at the next moult.

Williams (1952b) defines two stages in the liberation of brain hormone (a) development of competence to secrete hormone and (b) release of hormone into the haemolymph. Low temperature favours the first in Platysamia and high temperature the second.

Sellier/

Sellier (1949) found that diapause in Gryllus is due to lack of a brain hormone. In Leptinotarsa active brain implants curtail diapause according to Grison (1949). Rahm (1952) has shown that the brain and thoracic glands participate in control of diapause in Sialis but found that before chilling the thoracic gland has been activated but synthesis and liberation of the hormone cannot take place until the organ had been chilled, an odd result which should be rechecked.

In most cases it seems likely that the arrest of development during diapause will prove to be the result of the failure of the brain to activate the thoracic gland. When the correct external stimulus is applied to the insect in diapause the brain is activated and it in turn activates the thoracic glands whose secretion promotes continued development and moulting. There is no need to postulate the intervention of an inhibitor substance but the possibility that an active principle, a "diapause hormone" actually arrests development can not be ignored. Hinton (1954, 1957) insists that this must be so and explains failure to demonstrate the existence of the hormone by, for example, parabiotic union of diapause and non-diapause individuals by claiming that the thoracic gland hormone "in some way blocks the action of diapause hormone". The only diapause hormone whose existence has been proved is that produced by the sub-oesophageal ganglia of adult Lepidoptera./

Lepidoptera. (See Introduction.)

In the investigations discussed in the following sections of this chapter, a dual approach is made to the problems of endocrine control of metamorphosis and diapause. In Section II the general theories outlined above are tested by observation of the size of endocrine organs and anlagen in diapause and non-diapause larvae. In Section III an experimental study of the interrelationships and functions of the brain and endocrine organs is described.

Section II: The Size of Endocrine Organs and Anlagen in  
Diapause and at Various Stages in the Development of the Larvae  
of L. caesar and C. vomitoria

(a) Introduction

Hypertrophy of the lateral parts of Weismann's ring, now recognised as the thoracic glands, was first described by Burtt (1937) in the prepupa of C. vomitoria. He and Vogt (1942a, 1942b, 1943b) related hypertrophy of the lateral ring cells to the growth of imaginal discs. Evidence produced by these workers and others, e.g. Haskins & Enzmann (1938) and Bodenstein (1943), indicates that the growth of imaginal discs in feeding Cyclorrhaphan larvae is continuous and unrelated to the humoral cycles controlling the first and second moults. Bodenstein (1950) stated that "purely larval organs grow by an increase in cell size whereas the presumptive imaginal organs grow by cell multiplication". He was referring to Drosophila but this is probably applicable to all Cyclorrhaphan larvae. In the third instar, after the cessation of feeding, the thoracic glands are activated and promote development and differentiation of anlagen and puparium formation. In the experimentally produced absence of thoracic gland hormone growth of anlagen ceases in the post-feeding third instar larva and the puparium does not form. (Burtt, 1938; Possompes, 1953.) It is apparent that the growth of anlagen is independent of humoral stimuli up to a certain stage/

stage in development but beyond this stage a humoral stimulus is required for further growth and for the differentiation of these rudiments to their adult form.

A facultative diapause occurs in third instar of L. caesar after the cessation of feeding and before puparium formation. The onset and termination of diapause are determined by factors in the external environment of the larva. Growth by cell multiplication proceeds in the imaginal discs until the onset of diapause when all mitotic activity ceases. At the termination of diapause mitotic activity is resumed. The arrest of imaginal disc growth in the diapause larva is comparable to the halt in development in the Calliphora larva deprived of thoracic gland hormone. The immediate cause of the arrested development in Lucilia diapause is, therefore, apparently due to the absence of thoracic gland hormone.

To test this hypothesis observations were made on the relationship between the development of the thoracic glands and wing buds in the non-diapause species C. vomitoria and in diapause and non-diapause larvae of L. caesar. Wing buds were chosen as representative imaginal discs because of their size, accessibility and the ease with which they can be measured. The relative sizes of corpora cardiaca and corpora allata in the Lucilia larvae were also ascertained in order to confirm the parallelism/

parallelism between the chronological evolution of neurosecretory activity and the size of the corpora innervated by neurosecretory cells which has been noted in other species by several observers. Arvy & Gabe (1952c) for example record a great increase in the volume of the corpus cardiacum at the time of the imaginal moult in Odonata. Increase in the size of the corpus cardiacum and the corpus allatum following each discharge of neurosecretory cell product is described in Bombyx mori by Bounhiol, Arvy & Gabe (1953). In Tenebrio Arvy & Gabe (1953g) observed an increase in the the size of the corpora cardiaca and allata throughout the larval life with a sudden increase during pupation at the same time as transport of neurosecretory material intensifies.

(b) A comparison of thoracic gland nuclei and wing buds of C. vomitoria at different stages in the third instar

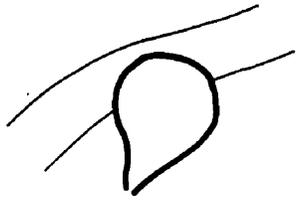
A culture of 50 C. vomitoria larvae was prepared according to the methods described in Chapter 1. Two days prior to the cessation of feeding and thereafter at 1 day intervals, until the residue had all pupated, groups of one to four larvae were removed and killed. They were dissected by the method described in Chapter 2 and the internal organs were fixed by irrigation of the dissection with Bouin's fixative. The wing buds, together with a portion of the longitudinal tracheae to which they are attached, were removed and whole mounts of them, stained/

stained with Borax carmine were prepared. The brain and the adjacent attached part of the aorta with Weismann's ring were also removed and after further fixation embedded in ester wax and sectioned. Sections were cut at  $6\ \mu$  and stained by the normal haemalum-eosin procedure.

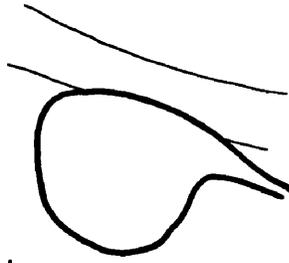
Drawings of the series of wing buds were made with the aid of a camera lucida. Comparative sizes of wing buds were ascertained by measuring the area of the outline drawings with a planimeter. The diameters of thoracic gland nuclei were measured in the serial sections by means of a callibrated graduated eyepiece. The nuclei vary in size in the different parts of the gland and it was decided to measure those in the lateral parts only. The nucleus is usually ovoid and the major axis was measured, spherical or unusually elongate types being rejected. Twenty nuclei were measured in each larva and the average diameters calculated.

Permanent larvae were obtained by severing the nerves to the corpus cardiacum in larvae taken just after the cessation of feeding, i.e. early stage 4 larvae. (See Section III of this chapter.) Wing buds of two of these were also preserved in whole mounts, drawn and measured.

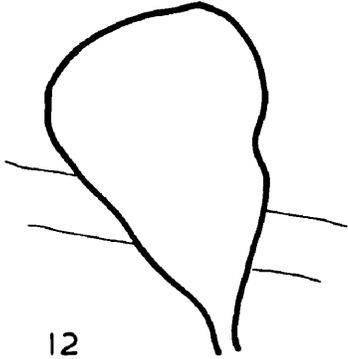
Numerical results are summarised in Tables I and II and outline drawings of wing buds representative of each stage of development/



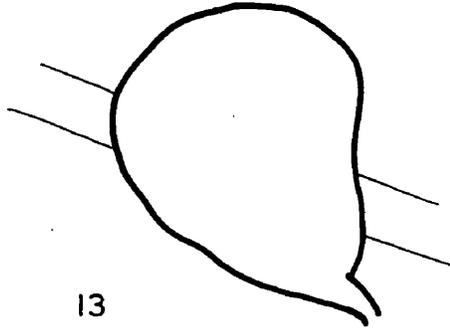
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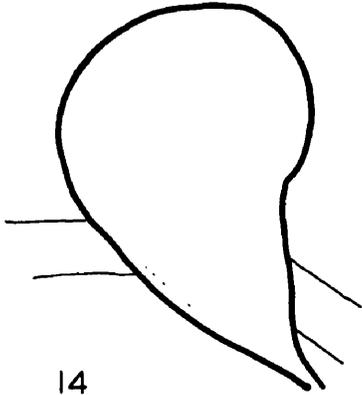
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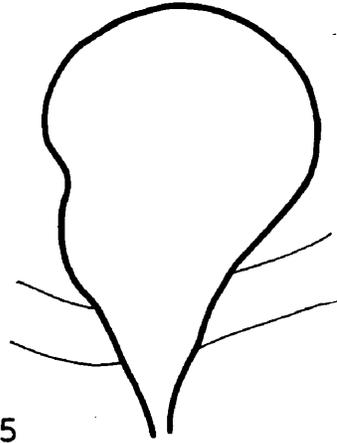
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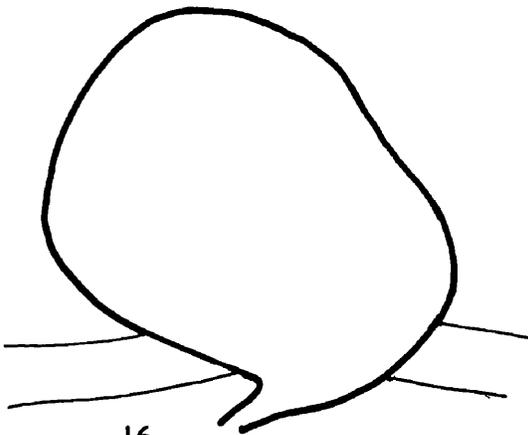
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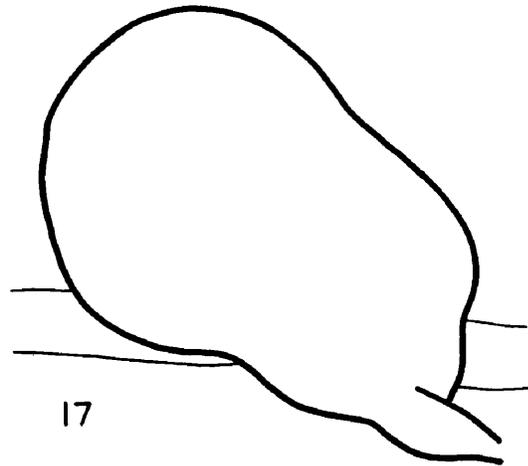
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15



16



17

TABLE I

Day dissected	Order of dissection	Wing bud size	Thoracic gland nuclei
1	1	7.3	14.4
2	2	12.4	17.0
2	3	17.0	17.5
2	4	19.0	18.8
3	5	37.0	20.8
3	6	25.0	20.5
4	7	53.0	25.3
4	8	42.0	21.8
4	9	46.0	26.0
4	10	44.0	24.5
5	11	40.0	22.8
5	12	56.0	24.3
6	13	87.5	25.0
6	14	54.0	23.5
6	15	103.0	27.3
6	16	105.0	27.5
7	17	76.5	26.0
7	18	92.5	27.0
7	19	64.0	25.8
7	20	97.0	27.3
8	Some pupae		
9	Some pupae		
10	All pupae		



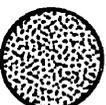
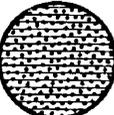
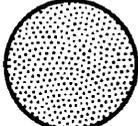
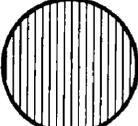
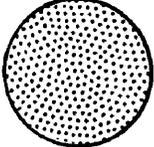
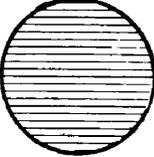
development are given in Figs 10 - 17. Table I lists the twenty larvae dissected, the day on which each was dissected, the sizes of wing buds expressed in arbitrary units and of nuclei in microns. The prepupal phase from cessation of feeding to puparium formation had a duration of 4 - 6 days at 25°C. Over the 7-day period of observation the area of the wing buds increased fourteen times.

It is clear from the list of wing bud sizes, arranged in chronological order of removal, that individuals in the same culture, in apparently optimal conditions, do not develop at the same rate. For a proper comparison the larvae must be arranged in the sequence of stages of development as indicated by wing bud sizes. This is done in Table II. They are then separated into eight groups, again according to wing bud size, each group being considered as representing a stage in development. The average sizes of wing buds and thoracic gland nuclei at each stage are calculated and the ratio of average sizes at other stages to the stage size given for wing buds and nuclei. The average sizes are illustrated in the histogram named Graph 1. In Graph 2 the average sizes of wing buds and average lengths of thoracic gland nuclei are plotted against stage in development.

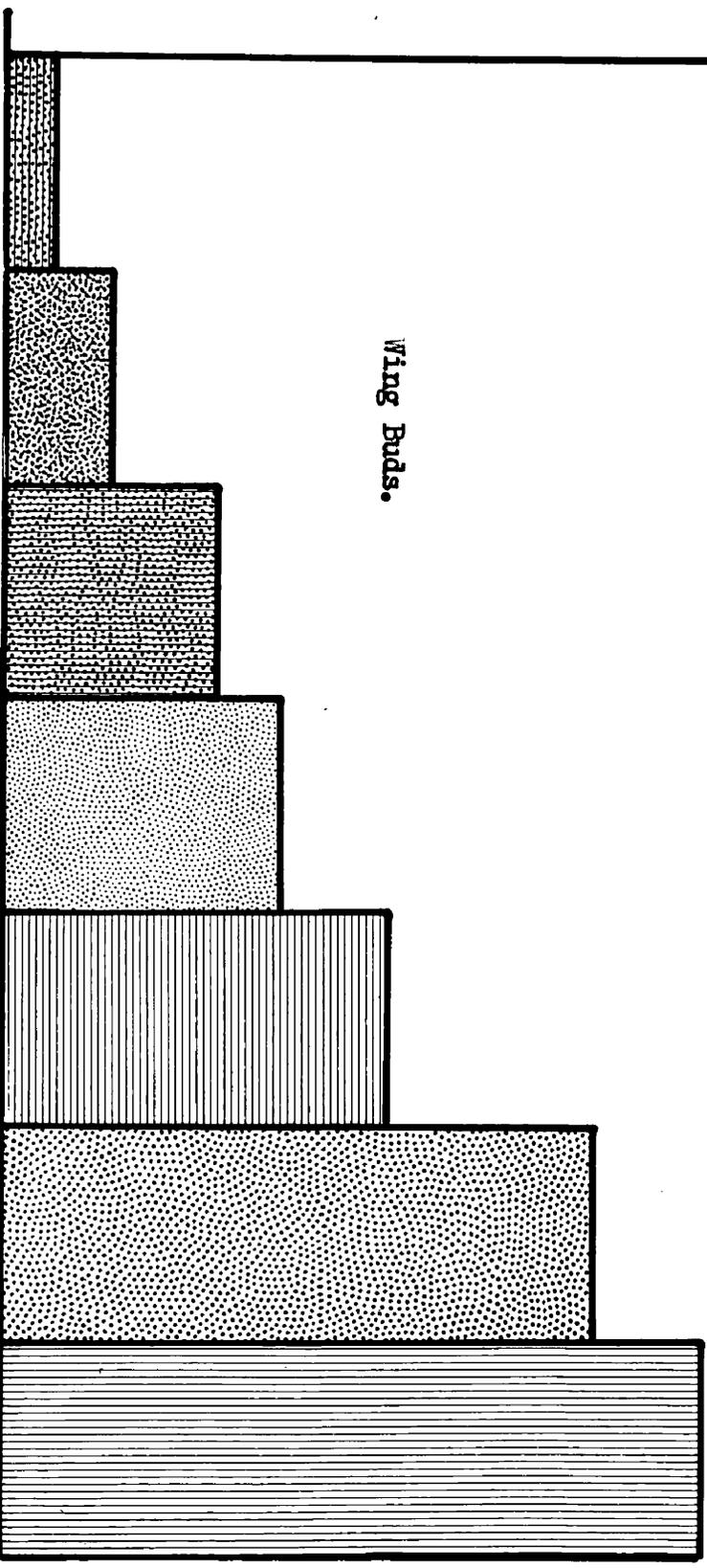
As can be seen from Graph 2 the rate of increase in size of wing buds appears to be relatively constant from the late feeding/

Thoracic Gland Nuclei.

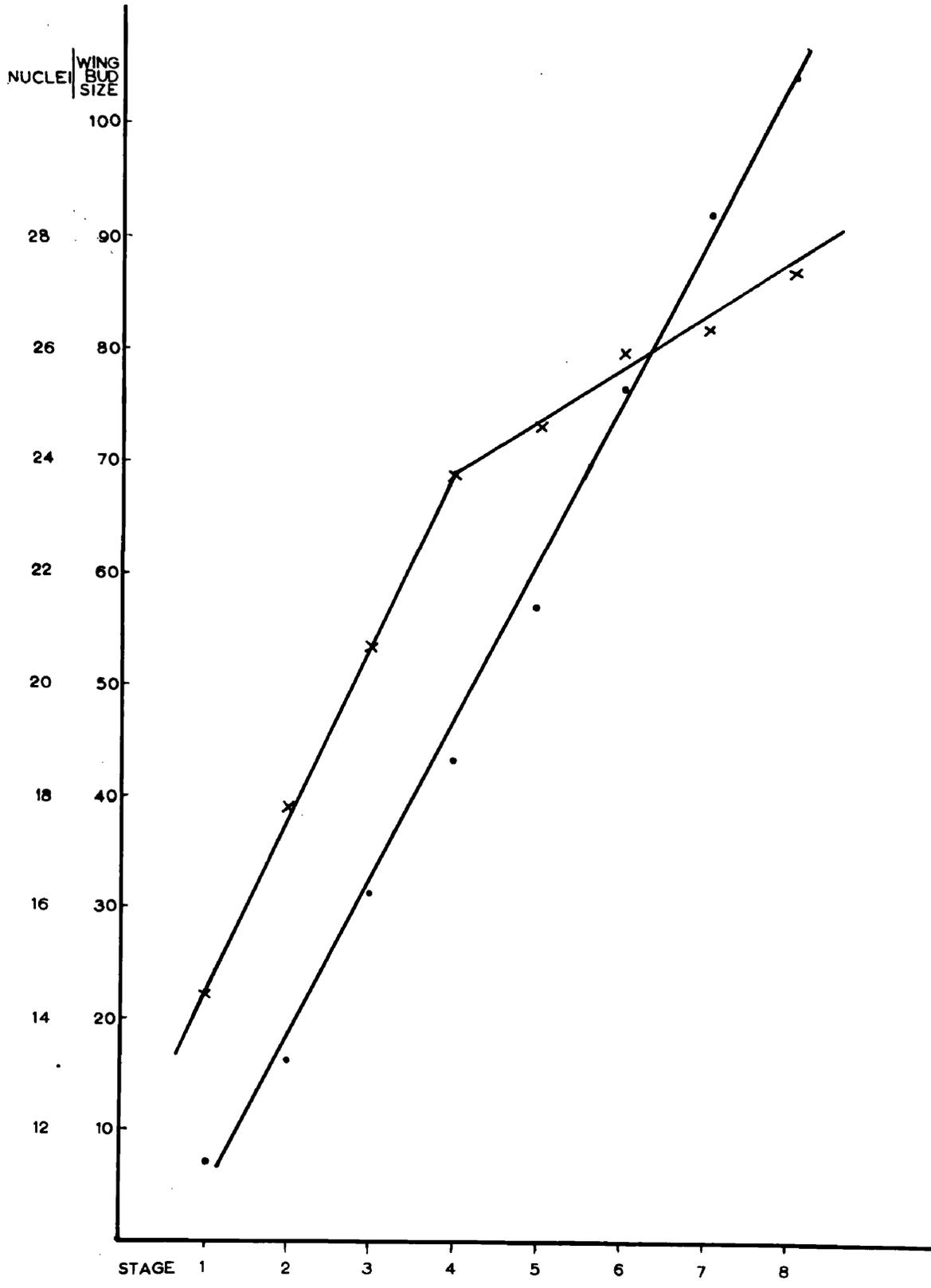
Stage

- 1 
- 2 
- 3 
- 4 
- 5 
- 6 
- 7 

Wing Buds.



Graph 2. Graph in which sizes of thoracic gland nuclei (in microns, X points) and of wing buds (• points) are plotted against stage in development, these stages representing 24-hour intervals in the life of an individual.



Graph 2.

feeding stage right through to puparium formation but it must be remembered that the method of selection and arrangement of the data would tend to mask a non-linear relationship between disc size and stage of development. In the absence of the stimulus normally provided by thoracic gland hormone (see Section III) wing bud growth halts in permanent larvae at a stage equivalent to stage 5. Thoracic gland nuclei also increase in size during the late feeding and prepupal stages. In Graph 2 it can be seen that there is a linear relationship between nuclear size and stage of development from stage 1 to stage 4 and again from stage 4 to stage 8 but there is a change in the slope of the line at stage 4 indicating a sudden change in the rate of increase of nuclei at that stage. The thoracic gland being a larval organ grows by increase in cell size, including size of nuclei (as a result of endomitoses), during larval life which can be considered as ending late in stage 4. At this point the gland is activated, and its hormone promotes the continued growth by mitoses of anlagen. There is hypertrophy of the gland cells after stage 4 but although the nuclei continue to increase in size they do so, as noted, at a slower rate than before.

Growth of anlagen, by cell multiplication, is apparently promoted by an intrinsic factor in the tissue until the end of stage 4 when intrinsic control ends and an extrinsic factor, thoracic gland hormone, comes into operation. It is remarkable that/

that the change over to extrinsic control is accomplished without interruption or alteration of the rate of growth of anlagen. That the rate of increase of the imaginal discs is the same under both types of control would indicate that the rate at which the cells can multiply is either a property of the tissue or is governed strictly by corpus allatum hormone.

(c) A comparison of sizes of thoracic gland nuclei in feeding, diapause and non-diapause larvae of *L. caesar*

For the histological investigations described in Chapter 4 serial sections were prepared of the brain and retrocerebral organs of larvae in diapause and at four stages in the third instar between late feeding and puparium formation. The stages, defined fully in Chapter 4, were as follows. Stage 1 were larvae still feeding but almost ready to leave the meat. Stage 2 had finished feeding 6 - 8 hours and their crops were almost empty but digestion of gut contents not complete. Stage 3 were mature larvae, developing without diapause, killed early in the prepupal phase. Stage 4 were similar larvae killed later in that phase.

Nuclei were measured, as described in part (b) of this section, in sections of thoracic glands of several representatives of each stage. Twenty nuclei were measured in each gland; mean sizes for each specimen and for each stage were calculated and results are listed in Table III. Calculated sizes/

TABLE III

Stage	Specimen	Thoracic gland nuclei	Mean nuclear size at each stage	Nuclear size relative to stage 1/2
1	a	12.7	12.96	1
	b	13.0		
2	a	12.9	13.97	1.08
	b	13.0		
	c	13.2		
3	a	13.4	19.7	1.52
	b	14.2		
	c	14.3		
4	a	19.5	12.9	1
	b	19.6		
	c	20.0		
Diapause	a	12.9	12.9	1
	b	13.0		
	c	12.7		
	d	13.0		
	e	12.9		

sizes for each specimen are given to the first decimal place only, since to quote a value to a smaller fraction would imply an impossible degree of accuracy of measurement but stage means are given to the second decimal place to aid comparison. The ratios of mean sizes, for other stages, to the mean size for stage 2 are also given in the table. The relative sizes of nuclei at different stages are illustrated in Fig.18.

Differences between sizes in stage 1 and stage 2 are too slight for them to be considered separately. The nuclei of glands of larvae in diapause correspond in size to those of stages 1 and 2. In the stage 3 larva, which corresponds approximately to the stage 5 larva of Calliphora, thoracic gland nuclei are only very slightly larger. A comparison of relative sizes, based on the assumption that Lucilia stage  $1/2$  is equivalent to Calliphora stage 2 shows that Lucilia thoracic gland nuclei are smaller than might be expected at stage 3 but attain the expected size at stage 4 which corresponds to Calliphora stage 7.

After activation of the thoracic gland the nuclei, as in Calliphora, continue to increase in size. In diapause larvae they remain constant in size and similar to those larvae at the cessation of feeding. It can, therefore, be deduced that the thoracic gland is not active during diapause. The thoracic gland is apparently activated in non-diapause larvae at the end of/

Fig. 18. Relative sizes of thoracic gland nuclei in L. caesar larvae at the stages of development defined in text. 1 & D, Stage 1 and diapause; 3, Stage 3; 4, Stage 4.

Figs 19 - 23.

Wing buds of L. caesar larvae at different stages in development.

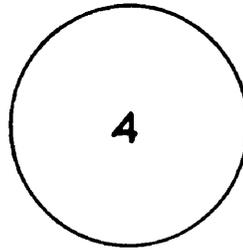
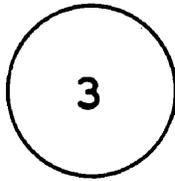
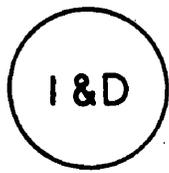
19) Stage 1.

22) Early Stage 4.

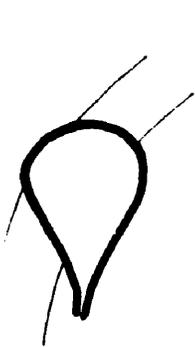
20) Stage 3.

23) Late Stage 4.

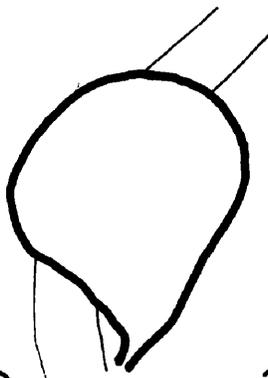
21) Diapause.



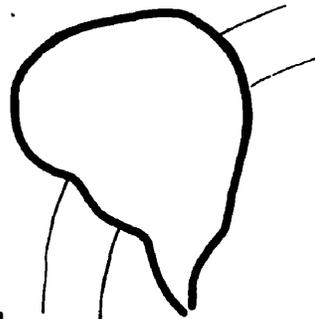
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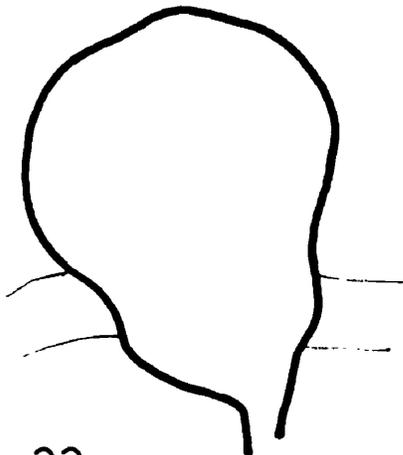
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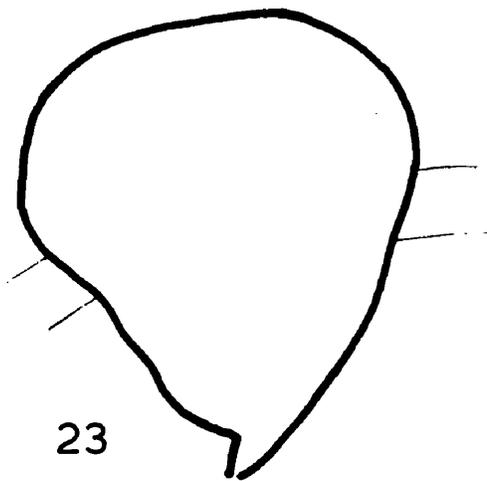
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22



23

of stage 2, at the same time as the completion of digestion of food residues in the gut.

- (d) A comparison of the sizes of thoracic glands, corpora allata, corpora cardiaca and wing buds in diapause and three stages of non-diapause larvae of *L. caesar*

It is impossible to measure the volume of such minute and enveloped organs as the corpora but a comparative method was devised using the serial sections prepared for the histological investigations. Specimens which had been cut in symmetrical frontal sections were chosen and outline drawings of the three retrocerebral organs were made with the aid of a camera lucida, care being taken to keep all drawings to the same scale. The area of each drawing was measured with a planimeter and the total area of drawings of each organ calculated. Since each organ had been sectioned in the same plane at a constant thickness of  $6 \mu$  the total area for each organ must be proportional to the volume of that organ.

The size of wing buds of larvae at each stage were compared in the manner described in part (b) of this section.

Results are given in Table IV. The sizes of organs and wing buds are given in arbitrary units of square centimetres. For confirmation of the relative accuracy of the method and as a check on constancy of size of organs at each stage, two specimens of/

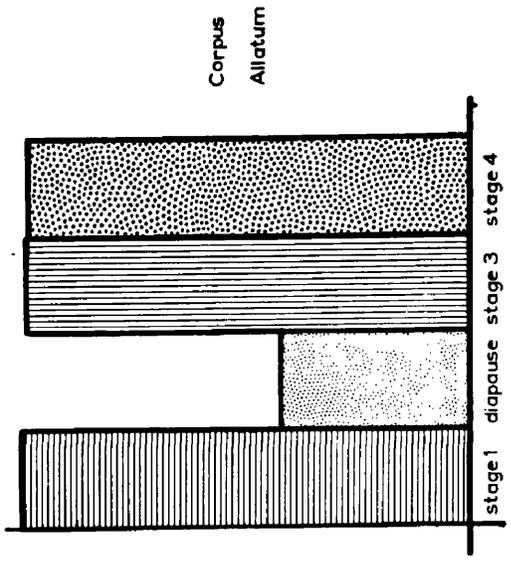
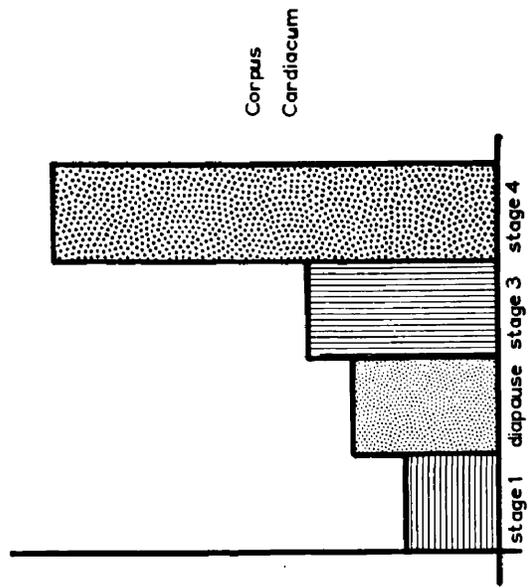
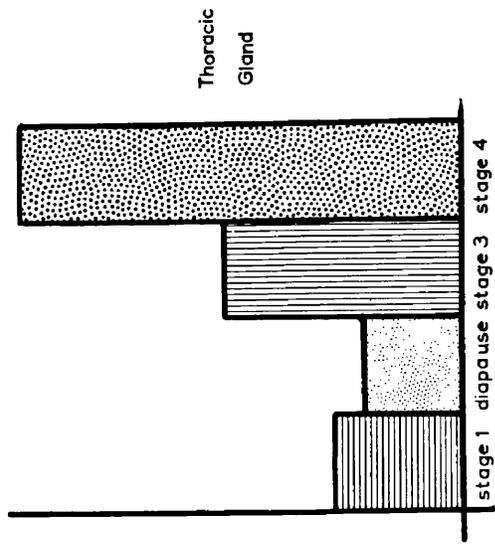
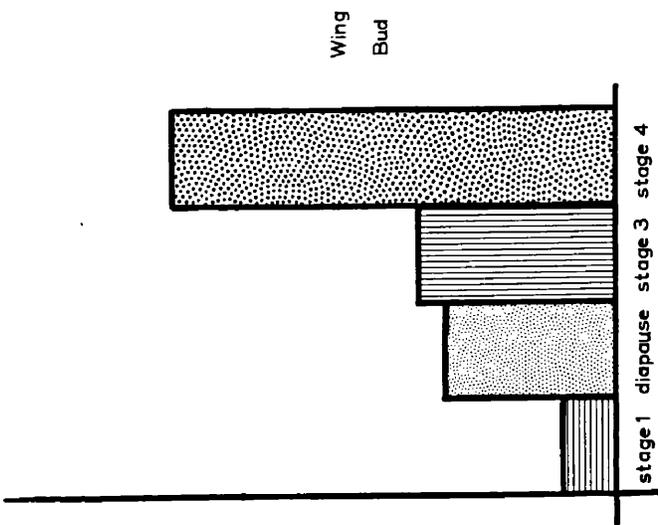


of diapause and of stage 1 larvae were measured. Wing bud sizes given for each stage are the means of sizes of buds from a number of larvae. Outline drawings of wing buds representative of each stage are given in Figs 19 - 23. In Table IV relative sizes are calculated with respect to organ sizes in the stage 4 larva and these relative sizes are illustrated in the histograms named Graphs 3 - 6. A comparison of wing bud sizes relative to stage 1 is also given and permits comparison with the Calliphora results given in part (b) of this section.

Thoracic gland; hypertrophy of this organ occurs in the non-diapause prepupal phase. The volume of the organ doubles between stages 1 and 3 and again between stages 3 and 4. If the larva goes into diapause the organ diminishes slightly in size. It is clearly not active during diapause. The contrast in size between the diapause gland and that in stage 3 indicates that it has been activated before that stage.

Corpus cardiacum; this organ is larger in diapause than in the feeding larva. This can be related to the facts that the organ is receiving neurosecretory material during diapause but not during the feeding phase of the third instar and that the chromophile cells are active during diapause. (See Chapter 4.) There is a doubling in size of the organ between stages 1 and 3 and again between stages 3 and 4, comparable to that observed in the thoracic gland. Enlargement of the corpus cardiacum/

Graphs 3 - 6. Relative sizes of wing buds, thoracic glands, corpora cardica and corpora allata in larvae of L. caesar at different stages of development. Sizes of organs have in each case been related to the size in Stage 4 larvae, and are represented as percentages of Stage 4 sizes.



cardiacum commences (Chapter 4) on receipt of neurosecretory material from the brain but hypertrophy of the organ is not simply due to the accumulation of this product in it since the chromophile cells are active in prepupae as well as in diapause larvae and the chromophobe cells may also have a function of their own, a possibility which has yet to be investigated.

Corpus allatum; there is a slight reduction in size of this organ after the cessation of feeding in the non-diapause larva but thereafter it remains constant in size in stages 3 and 4. In diapause there is a striking reduction in its size to little more than a third of its former volume. These results can be interpreted as follows. The corpus allatum is apparently active during the late feeding phase and in the non-diapause prepupa, its secretion governing the rate of development of anlagen. During diapause growth of anlagen ceases and, since corpus allatum hormone is not then required, the gland reverts to an inactive state.

Wing buds; the increase in size of wing buds between the cessation of feeding and puparium formation is relatively greater in Lucilia than in Calliphora but comparison of the ratio of sizes of stage 3 to stage 1 wing buds in Lucilia and of the ratio of sizes of stage 5 to stage 2 in Calliphora indicates that stage 3 in the former corresponds to stage 5 in the latter. Most notable is the fact that arrest of growth of wing buds in diapause/

diapause larvae of Lucilia and in the permanent larvae of Calliphora in which thoracic gland activation has been blocked occurs at the same stage in development. In Lucilia this is just before stage 3 and in Calliphora between stages 4 and 5. This evidence confirms conclusions already reached, that in the absence of diapause in Lucilia the thoracic gland is activated late in stage 2, that this gland is activated in Calliphora at the equivalent stage 4 and that the immediate cause of the arrest of development in diapause is the failure of the brain neurosecretory cells to activate the thoracic glands; intrinsic control of growth in anlagen ceases in the normal way but the extrinsic growth-promoting factor is not present to continue the process.

(e) A comparison of the size of the brain and ventral ganglia in diapause and stage 3 larvae of Lucilia

If the living larva is compressed between two pieces of glass and viewed by binocular microscope with transmitted light the ganglionic mass can be seen. It would be convenient for experimental and other purposes if the stage 3 larva, known in dissection by the appearance of Group 4 neurosecretory cells in the dorsum of the brain (see Chapters 1 and 4), could be recognised without injury to the larva by inspection of the size of the central nervous system i.e. brain and ventral ganglia. The object of this exercise was to compare the size of this organ in stage 3 and in diapause larvae.

Ten diapause larvae of a batch in that state for six months and ten stage 3 larvae, identified by their time since cessation of feeding and by appearance of Group 4 cells, were dissected and the central nervous systems removed and fixed in Bouin. These were later stained with borax carmine and mounted in cavity slides. The dimensions were measured as indicated in Fig. 24.

Results are given in Table V.

It can be seen that the dimensions are almost identical in diapause and in stage 3 specimens and that the size of this organ cannot be used to separate the two types of larvae.

Fig. 24. Dimensions used in comparing the sizes of  
central nervous systems of L. caesar larvae.

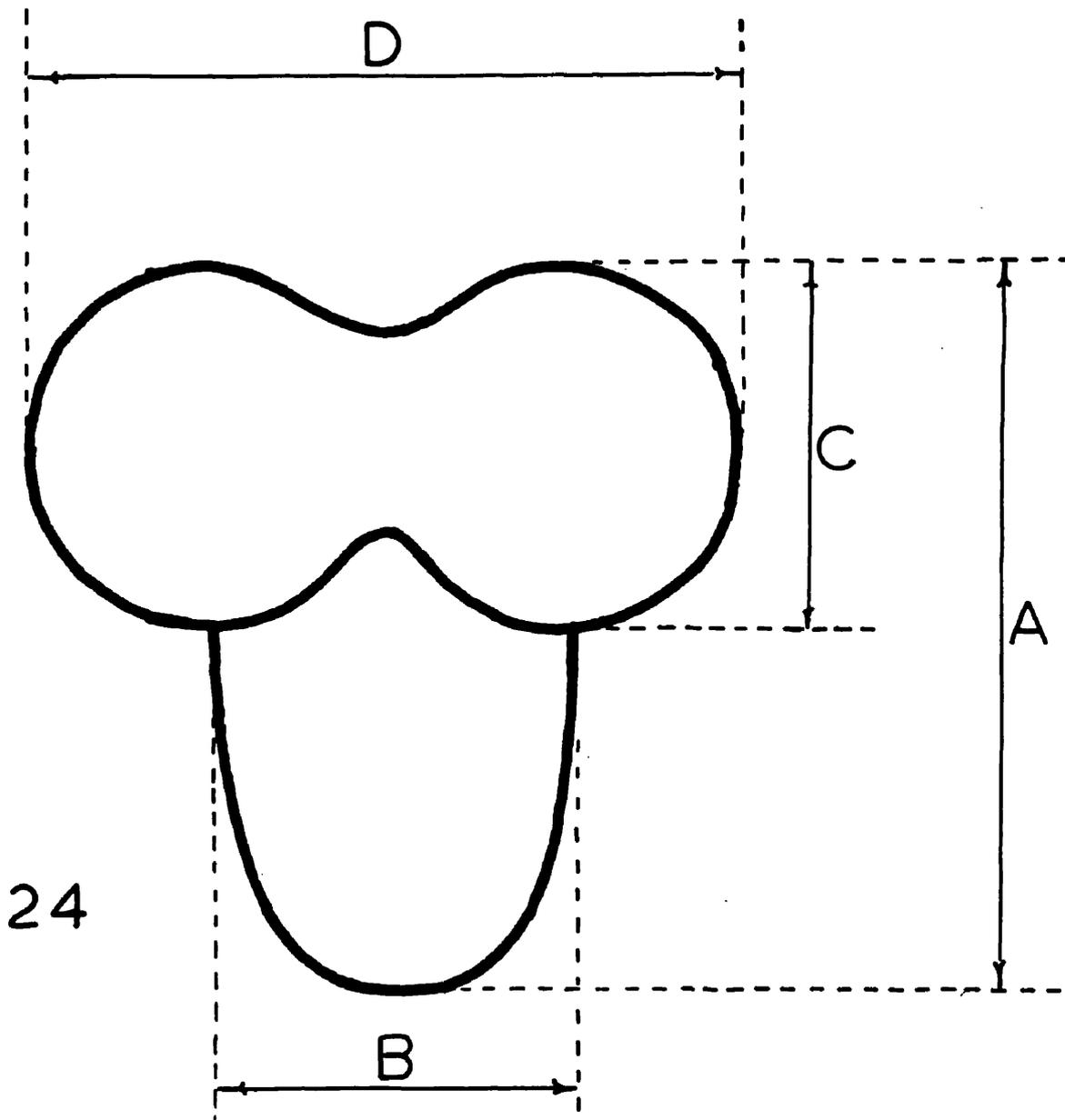


TABLE V Means of dimensions

	A	B	C	D
Diap.	39.3	17.1	15.4	33.1
Stage 3	38	17.4	15.25	32.7

Section III: An Experimental Study of Humoral Control of Metamorphosis and Diapause in Larvae of Diptera - Cyclorrhapha

The object of these investigations was to confirm certain findings of Possompes (1953) indicating that the humoral mechanism controlling pupation in Cyclorrhapha conforms to the currently accepted theory, outlined in the introduction to this chapter, and to test the theory that the arrest of development in Lucilia diapause larvae is immediately due to the absence of thoracic gland hormone.

(a) Endocrine control of pupation in Calliphora vomitoria

For extirpation a modification of the technique described in detail by Possompes was used. Larvae were anaesthetised with ether until they were completely relaxed and immobile. They were then washed in ethanol and this was followed by a rinse in water. Operations were performed in a solid watch glass, of the type used for the dissections described in Chapter 2, filled to within  $\frac{3}{16}$ " of the top with black wax. For operations a Cooke, Troughton and Sims stage binocular dissecting microscope (series M6000) was used. No micromanipulating apparatus was employed. After considerable practice and experience a degree of facility in performing the various operations was attained. The operating "table" was a rectangle of glass, measuring a  $\frac{1}{2}$ " x  $\frac{3}{16}$ " x  $\frac{1}{16}$ " thick, partly embedded in the wax. The larva to be operated on was held by "goal post"/

post" shaped wires made from no.20 entomological pins. The legs straddled the larva and were inserted into the wax. The procedure was first to clamp the larva across the table by one wire straddling the body just in front of the anterior spiracles. The wire legs were thrust down so that the larva was flattened and held against the front edge of the table by the crossbar. A second piece of glass similar in size and shape to the table was then placed on the larva exactly above the table. Pressure was applied via this glass starting at the anterior end of the larva and continuing it towards the rear so that crop, fat body and other mobile structures were expelled backwards from the compressed part of the larva. The central nervous system or ganglionic mass, with associated organs and imaginal discs, remained in place held there by the cerebral tracheae. Before pressure was relaxed a second wire was inserted close against the rear edge of the table. The pressure glass was then removed and the flattened part of the larva, stretched across the table, now contained the organs to be operated on. They are quite visible through the transparent integument. The pressure of the wire was sufficient to prevent the flow of blood, or return of organs, from anterior or posterior parts of the body into the compressed zone. The dish was now flooded with Ringer's insect saline solution and the operation proceeded.

The instruments used were scissor forceps and watchmakers' forceps/

forceps hand ground to very fine points. Instruments were boiled before use and kept in ethanol until required. They were rinsed in saline solution before actual use. Before each operation the dissecting dish, wax surface and glass table were flamed with a bunsen burner. After the operation the saline solution was poured off, wire clamps removed and a few crystals of crystalline Penicillin-G inserted in the wound to inhibit bacterial infection\*. The wound area was dusted with Penicillin powder and rapidly dried in a stream of air. When the wound was quite dry, in about 4 hours, the larva was transferred to a 3" x 1" glass tube containing moist peat moss and stoppered with a gauze cover. After the operation, larvae were kept at a constant temperature of 25°C.

Larvae were cultured according to the methods outlined in Chapter 1. Operations were performed on third instar larvae at stages between the cessation of feeding and puparium formation. To obtain even groups of larvae at similar developmental stages the larvae were not allowed to finish feeding of their own accord. Instead when judged to be ready to leave the meat the largest larvae, still with full crops, were removed and placed in peat moss in 4" x 1" glass tubes. Ten larvae were kept/

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\*Crystalline Penicillin-G (sodium salt) (1,665 units per mg.) manufactured by Distillers' Company (Biochemicals) Ltd.

kept in each tube. It is difficult to get even rates of development (see Chapter 3, Section II (b)) even in an apparently identical small group but the selection by age (in hours after removal from meat) is for such groups almost as accurate as Possompes' method of selecting by size or degree of retraction of crop. Operations were performed on groups of ten larvae at a time.

The survival rate varied in different experiments, the number surviving being given in the reports on these which follow. In most cases larvae dying within 4 days of the operation were not considered as survivors. The main causes of death were (a) excessive bleeding just after the removal of clamp wires, (b) incomplete anaesthesia resulting in early movement of larvae which caused expulsion of viscera through the incision and (c) bacterial infection via the wound. Precautions, described above, were taken to avoid the last.

### Controls

Under ideal conditions these larvae pupate within 5 to 7 days. The operated larvae lose organs and blood, the result, especially of the latter, being a reduction in size. To test that the loss of blood and injury could not alone cause failure to pupate after an operation a fragment of fat body was removed from the anterior end of control larvae. The survivors all ultimately pupated although in a longer time than unoperated controls./

controls.

Experiment I: To test the effect of removal of the entire central nervous system and Weismann's ring

Ten C. vomitoria larvae were operated on 24 hours after their removal from meat. The operation was performed by making a transverse incision in the ventral body wall, larvae being held dorsal side down. The "tail" of nerves and tracheae from the ventral ganglia was grasped with forceps and thereby the entire central nervous system was pulled out through the incision and cut off. The gut is severed in this process so the operation cannot be performed until digestion has been completed since the liberation of food material from the gut into the body cavity is invariably fatal.

Five larvae survived for 5, 6, 7, 8 and 12 days respectively without puparium formation. The removal of the central nervous system and ring does not, therefore, necessarily have an immediately fatal effect but in their absence puparium formation does not occur. This result agrees with Fraenkel's (1935) conclusion that the "pupation hormone" emanates from the "ganglion" or from an adjacent organ.

Experiment II: To test the effect of removal of the brain only, the ring and ventral ganglia remaining in the larva

Larvae 24 hours off meat were again used in this test.

The/

The object was achieved by cutting a tranverse dorsal incision above the brain. The cerebral tracheae and the muscle and nerve connections between the brain and corpus cardiacum were all severed close to the brain. The brain was held, with forceps, at the junction of the hemispheres and the connectives to the ventral ganglia cut with scissor forceps.

Only four of the ten larvae operated on survived and these remained as permanent larvae for 5, 6, 13 and 19 days respectively, before death. It can be concluded that the brain plays some part in the process of puparium formation either as the source of, or the stimulator of the source of, the pupation hormones.

Experiment III: To test the effect of removal of the entire ventral ganglionic mass, the brain and ring with their interconnections remaining in the larva

The operation, performed via a ventral incision, was similar to that in Experiment I. This time the cerebral connectives to the ventral ganglia were cut and that mass was then lifted by the "tail" and cut away. These larvae had been off meat for 36 hours.

Of the four which survived all pupated, in 4, 5, 5 and 6 days. It is apparent that the ventral ganglia do not play any part in the process of puparium formation.

Experiments IV - VIII:/.

Experiments IV - VIII: To test the effect of extirpation of Weismann's ring

A transverse dorsal incision was made in the body wall above the brain. The aorta was held by fine forceps immediately behind the ring. The cerebral tracheae, the muscle and nerve connections between the corpus cardiacum and brain and the nerve from the corpus cardiacum to hypocerebral ganglion were cut with scissor forceps. The cerebral tracheae were then cut beyond the transverse anastomosing trachea; the aorta was cut just behind the point at which it was held. The held part of the aorta complete with the ring was drawn out and cut in front of anterior prolongation of the ring and thus extirpated. The removed organ was then placed in a drop of saline solution in a cavity slide, and examined under a binocular microscope to check that the entire ring complex had been removed.

IV: Removal from larvae at the cessation of feeding

Twenty larvae were operated on in two attempts using ten larvae each time. Of the first lot only one survived, as a permanent larva, for 10 days and of the second lot three survived, as permanent larvae, for 6, 9 and 11 days.

V: Removal from larvae 24 hours off meat

The survival rate was much higher at this stage.

Thirteen/

Thirteen out of twenty larvae lived beyond the minimum test time. None of them pupated. They remained as larvae for the following times: 5 days - one; 6 days - three; 9 days - one; 10 days - three; 13 days - two; 15 days - one; 16 days - one; 20 days - one and 28 days - one.

VI: Removal from larvae 48 hours off meat

Four of ten operated larvae survived in this group, all as permanent larvae for 6, 7, 7 and 9 days.

VII: Removal from larvae 72 hours off meat

Of twenty operated on fifteen in this class survived as permanent larvae for the following times; 5 days - three; 7 days - two; 8 days - one; 9 days - one; 10 days - one; 14 days - three; 20 days - one; 22 days - one; 36 days - one; 39 days - one. This last was the longest survival of a permanent larva obtained in this series of experiments. Possompes did obtained four which survived up to 45 days.

VIII: Removal from larvae 96 hours off meat

Seven of ten larvae in this class survived the operation. Of these four pupated within 24 hours and the rest remained as permanent larvae up to 13 days.

Thus it is proved that extirpation of the ring complex can prevent puparium formation and pupation, a permanent larva resulting which can survive for at least 39 days in the larval state/

state if protected from desiccation. The operation is effective if performed at any time between the cessation of feeding and one day before puparium formation occurs. Puparium formation is not prevented if the operation is performed within about 24 hours of the time at which the change is due to occur. There is therefore a critical time before which the ring is required, and after which it is dispensable, for puparium formation to take place. This corresponds to Fraenkel's "critical period".

Experiment IX: To test the effect of removal of the corpus allatum

This organ was removed, together with part of the thoracic gland, via a transverse incision in the dorsal body wall above the larval brain. The part of the ring enveloping the corpus allatum was held with fine forceps; the thoracic glands were cut on either side of this and with them the transverse anastomosing trachea. The held organs, still attached to the aorta, were drawn upwards and the aorta cut with scissor forceps to liberate them. Twenty larvae were again used. In the first group of ten, off meat 72 hours, one pupated after 1 day, one after 2 days and two others within 4 days while three died within 24 hours and three after 5 days. Of the second ten, off meat 24 hours, two pupated after 4 days, one after 5 days and two survived as larvae for 6 and 7 days respectively, /

respectively, the other six dying soon after the operation.

The removal of the corpus allatum does not normally prevent puparium formation and indeed there is some indication of a slight acceleration of the process as a result. It cannot therefore be the source of the pupation hormone. The fact that some did not pupate but survived as larvae for several days could either be due to the removal of this organ or the result of injury to other components of the complex at the time of operation, but the fact that seven of the twelve did pupate indicates that the latter explanation is the likelier.

Experiment X: To check that severance of the cerebral tracheae does not prevent pupation

Thirteen larvae, 24 hours off meat, underwent this simple operation. Of these, five died, seven pupated within six days and one after nine days. Thus severance of the tracheae does not prevent pupation but side effects of the operation can occasionally cause a marked delay in the process.

From the foregoing experiments it can be concluded that both the brain and the ring complex play a part in promotion of puparium formation and pupation. It seems likely that the immediate source of the "pupation hormone" is the thoracic gland and this is confirmed in Experiments XV - XVII described below. The corpus allatum does not produce the pupation hormone./

hormone. The relationship between the brain and thoracic gland activity remains to be determined.

Experiments XI - XIV: To test the effects of severance of the nerves from the brain to the corpus cardiacum

This operation was performed as in the first stage of the removal of the ring. The aorta was held as described in Experiment IV and the cerebral tracheae and the muscle and nerve connections between corpus cardiacum and brain were cut with fine scissor forceps.

XI: Nerves cut at cessation of feeding

None of the survivors pupated, all remaining as permanent larvae for the following times; 7 days - three; 14 days - one; 31 days - one; 39 days - one.

XII: Nerves cut in larvae 36 hours off meat

Of thirty larvae operated on at this stage only fourteen survived longer than four days but none of these pupated. Permanent larvae lived for the following times; 5 days - two; 6 days - one; 7 days - one; 8 days - one; 9 days - two; 10 days - two; 11 days - three; 17 days - one and 20 days - one.

XIII: Nerves cut in larvae 72 hours off meat

Eight of the fifteen larvae operated on survived longer than the test time. Five of these were permanent larvae; for 6 days - one; 7 days - one; 9 days - one; 10 days - one and 15 days/

15 days - one. Two pupated, one after one day and one after two days. In a third case there was a partial hardening and darkening of the cuticle at the hind end before death on the second day.

XIV: Nerves cut in larvae 96 hours off meat

Of the seven surviving the operation one remained as a permanent larva until death after 6 days. Six pupated, four of these within 24 hours and two within 48 hours.

It can be concluded that the nerves between brain and corpus cardiacum must be intact up to a certain time before puparium formation is due, if that change is to occur. After that critical time the nerve connection is no longer required. The effect of nerve section before the critical time are similar to those of extirpation of Weismann's ring, a permanent larva being produced which survives as a mature larva for a period as long as five weeks without pupating. Some larvae do, however, pupate if the operation is performed within 48 hours of the time at which they would normally have pupated. This indicates that there is another critical time before which the nerves uniting brain and corpus cardiacum must be intact and after which this link is no longer required for pupation to follow. This critical time is at least 24 hours earlier than that discovered by extirpation of the ring and corresponds to Wigglesworth's (1940) "critical period".

It is apparent that failure to develop further is due to the fact that the thoracic gland has not been activated. Histological evidence (Chapter 4) shows that the product of brain neurosecretory cells passes via the axons of the nervi corporis cardiaci into the corpus cardiacum from which it is liberated into the blood in the lumen of the aorta. By nerve section this neurosecretory pathway has been interrupted and since nerve section blocks thoracic gland activation it can be concluded that the brain secretion contains a "thoracotrophic" hormone activating that gland.

(b) Endocrine control of diapause in *L. caesar*

Experiments XV - XVIII: To test the effect on diapause larvae of implants of thoracic glands from *C. vomitoria* prepupae

Thoracic glands were removed from donors in the following way. The normal dissection routine, described in Chapter 2, was used to expose the brain and ring. The left cerebral trachea was held gently, with fine forceps, in front of the ring and the thoracic gland cut close to its dorsal and ventral junctions with the aorta. The cerebral trachea was then severed close to the brain and, lastly, in front of the holding forceps. The left thoracic gland with its adherent fragment of trachea was then transferred to Ringer's saline solution until required. The right thoracic gland was removed in the same way.

Implantations can be performed in several ways. An injection method is preferred by some workers and indeed is the only one practicable with a small larva like that of Drosophila but for L. caesar a direct method proved to be quite satisfactory. The recipient larva was anaesthetised in the usual way, transferred to an operating dish and lightly held on the wax surface, by two goal post wires, with the left side uppermost. A transverse incision was made in the penultimate segment. The organ to be implanted was then lifted from the dish of saline with the fine forceps held in the operators right hand. The wound was distended with forceps held in the left hand and the implant thrust into the body of the larva. The process was performed "dry", that is the operating dish was not flooded with saline as in extirpation experiments. Penicillin crystals were introduced into the wound and the larva transferred to a drying tube. When the wound was 'dry and the larva recovered from anaesthesia, it was placed in damp peat moss and kept at 25°C.

Adequate controls were kept for each test. The diapause larvae were from a batch confirmed in diapause on the 27th January. Implantations were performed between the 22nd February and the 2nd March. An untreated control group was kept under observation from the 22nd February to the 8th March and in that time seven larvae out of fifty pupated. A positive result can only be claimed with certainty if pupation occurs/

occurs within 7 days of implantation. After this time other undetectable factors in the environment could be responsible for diapause termination.

XV: In this test six L. caesar larvae received the thoracic glands of six Calliphora donors 96 hours off meat. Glands were rejected if they did not have the enlarged and swollen appearance associated with the stage just prior to pupation. Five Lucilia survived and of these four pupated, two in 5 days; one in 6 days and one in 7 days. A fifth larva remained in diapause for 14 days when observations terminated. Controls were etherised and received the incision but no implants. Of seven surviving, one pupated after 6 days; one died after 7 days and the remainder were in diapause at the end of 14 days.

XVI: In the next test, each larva received the equivalent of the thoracic glands of two donors. Of six survivors five pupated within the required time and one remained in diapause. Of the etherised and wounded controls, five survivors were in diapause after 14 days.

XVII: In this test each larva again received the equivalent of two thoracic glands. The survivors numbered ten of which four pupated in 5 days; three in 6 days and one in 7 days. The other two remained in diapause. Of fourteen controls two died within 7 days, ten remained in diapause and two/

two pupated (after 10 and 11 days).

These results are summarised in the following table.

	Survi- :vors	Pupae in 7 days	Pupae in 14 days	Larvae in 14 days	Dead in 7 days
Test larvae	21	17	-	4	-
Treated controls	26	1	2	20	3

It is thus confirmed that the thoracic gland is the component of the ring which produces "pupation hormone". In addition it is demonstrated that at a certain stage in the development of the Calliphora prepupa the thoracic gland becomes capable of acting independent of the brain stimulus.

Active Calliphora thoracic glands are capable of causing Lucilia diapause larvae to pupate therefore thoracic gland hormone can itself terminate diapause and promote the continuation of metamorphosis. The postulate, that the halt in development of imaginal structures in diapause is immediately due to the absence of thoracic gland hormone, is confirmed. Dissection of recipient larvae shows that their thoracic glands are rapidly activated after the operation and that the Group 4 neurosecretory cells in their brains assume the appearance in vivo/

in vivo noted in stage 3 non-diapause larvae. It has been demonstrated by Williams (1952a) that thoracic gland hormone can activate thoracic glands. In this experiment the Lucilia thoracic gland is apparently activated by the hormone from the implant but at the same time thoracic gland hormone has activated the diapause brain. Here a diapause brain has been activated by a humoral and not by a physical stimulus. Unfortunately, no record was made of the number of recipients which ultimately emerged as adults but it can be stated that a majority did so which confirms that the hormone supplied did not merely induce puparium formation but certainly broke the diapause.

XVIII: Donors in this experiment were Calliphora larvae taken shortly after the cessation of feeding, i.e. early stage 4. Each Lucilia larva received the equivalent of two thoracic glands. Of eleven survivors one pupated after 4 days and the rest remained in diapause for the test period.

This result indicates that the thoracic gland of the stage 4 Calliphora larva has not yet been activated or is still incapable of autonomous activity in the absence of another stimulus. Histological evidence (Chapter 4) shows that the former explanation is correct.

Experiment XIX: To test the effect on diapause larvae of implants of wing buds of C. vomitoria larvae 96 hours off meat

Ten larvae were treated, receiving two fragmented wing buds each. Four of them died as larvae before the 14th day and six remained in diapause until the end of the test.

A developing imaginal organ is not capable of stimulating development in dormant tissues.

Experiment XX: To test the effect of implanting the brain and retrocerebral endocrine organs, connections intact, from *Lucilia* diapause larvae into *C. vomitoria* larvae taken shortly after the cessation of feeding

Each Calliphora received two Lucilia implants. Treated controls received Lucilia proventriculi. The operations were carried out in the same way as implants of thoracic glands from Calliphora into Lucilia. There was an untreated but etherised control group also.

Results after 7 days are summarised in the following table.

	Test larvae	Treated controls	Untreated controls
Dead	7	7	-
Pupae	9	9	20
Larvae	5	3	-
Total	21	19	20

Of/

Of the nine pupae in the test group, three pupated in 4 days, four in 5 days, one in 6 days and one in 7 days. Of the five larvae remaining in the test group after 7 days two pupated and three died within 14 days. The three treated controls remaining after 7 days pupated within 14 days.

The implantation of any organ from another larva can in some cases cause a lengthening of the prepupal period but the endocrine system of a diapause larva is not capable of preventing the pupation of the non-diapause host. One interpretation of this would be that an inhibitor substance is not produced by the diapause endocrine system but the possibility is not excluded that thoracic gland hormone is blocking a diapause hormone.

## Chapter 4

### Neurosecretion in the Larva of *Lucilia caesar* L.

#### Section I: Introduction

Weyer (1935) is credited with the first observation of neurosecretory cells in the insect brain, in *Apis mellifica*. B. Scharrer (1937) confirmed this finding in *Bombus* sp. Hanström (1938) demonstrated neurosecretory cells in the pars intercerebralis of *Rhodnius*; the physiological role of these cells in initiating the moult process in *Rhodnius* was determined by Wigglesworth (1940). Since then brain neurosecretory cells have been described in a large number of species. Investigations were in recent years facilitated by the development of two staining procedures which selectively, though not specifically, stain the product of neurosecretory cells. The chrome-haematoxylin-phloxin stain of Gomori (1941) was adapted for the study of vertebrate neurosecretory cells by Bargmann (1949) and adopted for the study of these in the insect brain by B. Scharrer (1951) and others since. The paraldehyde-fuchsin stain of Gomori (1950) was adapted by Gabe (1953a) for the same purpose and has since been widely used for the detection and study of neurosecretory cells in a diversity of invertebrate orders. A very comprehensive review of current knowledge of the phenomenon of neurosecretion in invertebrates is/

is that of Gabe (1954).

It is currently accepted (e.g. M. Thomsen, 1954; Gabe, 1954) that in the brain of a pterygote insect the neurosecretory cells lying in the protocerebrum form two median and two lateral groups. The median cells lie in the pars intercerebralis.

Hanström (1940) observed that the axons of the two median groups form two nerves which in their intracerebral course cross over each other in the pars intercerebralis and, on leaving the rear of the brain, form the nervi corporis cardiaci interni (NCCI), while the axons of the lateral cells take a direct course to the rear of the brain and externally form the nervi corporis cardiaci externi (NCCII). These observations have been confirmed by others, notably by Cazal (1948) who commented on the taxonomic significance of extracerebral fusion of the NCCI and NCCII. In larvae of Diptera - Cyclorrhapha the internal and external nerves to the corpus cardiacum are fused to form a single pair of nerves outside the brain. The axons of the neurosecretory cells of the pars intercerebralis cross over in these larvae to the opposite side of the brain from the cells of origin. M. Thomsen (1951) and Possompes (1953) described neurosecretory cells in the pars intercerebralis of third instar larvae of C. erythrocephala, cells which the former identified as belonging to the median groups, but lateral/

lateral neurosecretory cells have not been identified hitherto in *Cyclorrhaphan* larvae.

Gabe (1954), summarising the observations of many workers in this field, made the general statement that insect neurosecretory cells are pyriform, have a central nucleus and have a cytoplasm containing less RNA and shorter chondriocents than common neurones of similar size. The product of secretion is acidophil, well conserved with aqueous fixatives, eosinophil, takes acid fuchsin, azocarmin and iron haematoxylin and after permanganate oxidation has an affinity for chrome-haematoxylin and paraldehyde fuchsin. Histochemical tests show that osmiophil lipids are not detectable in the product of secretion but "le reaction Hotchkiss-McManus (i.e. PAS) permet d'y deceler un glucide different du glycogene". He claimed that the product is also PAS-positive in other invertebrates e.g. Polychaetes, Crustacea and Chilopoda. Wigglesworth (1955) on the other hand is certain that the content of neurosecretory cells is PAS-negative and quotes Rehm's (1955) conclusion that the PAS-positive reaction in neurosecretory cells in Lepidoptera is due to the presence of a bound lipid in the ground substance.

Neurosecretory cells are identified as such in this study if, at some stage in the development of the larva, they can be distinguished in vivo from common neurones by a characteristic bluish appearance and prove, in appropriately stained sections, to/

to contain a particulate product. The refractile properties of the product are responsible for the blue colour observed in vivo (E. Thomsen, 1954). In addition the product can be demonstrated in sections as passing along the axon of the neurosecretory cell and is also identifiable in the destination of that axon, the corpus cardiacum in the case of brain cells. This phenomenon of transport of neurosecretory product from the brain to the corpus cardiacum was first noted by Hanström (1940) and by B. & E. Scharrer (1944) and later by Cazal (1948) and M. Thomsen (1951) and has since been confirmed by the use of the new selective stains in a large number of species. Experimental confirmation of axonal transport of the product come from B. Scharrer's (1952a, 1952b) experiments on the interruption of the neurosecretory pathway by severing the nerve from brain to corpus cardiacum on one side, in Leucophaea maderae. The result was accumulation of the product in the part of the nerve proximal to the cut and depletion in the part distal to the cut and in the affected corpus cardiacum. E. Thomsen (1954) produced a similar result in the adult female of C. erythrocephala by ligaturing the nerve. B. Scharrer (1951) showed that this material was stored in the corpus cardiacum of Leucophaea near the lumen of the aorta and postulated that it was released from there into the blood. Stutinsky (1952) & Hanström (1953) confirmed that the neurosecretory material does not enter the corpus cardiacum cells/

cells while M. Thomsen (1954) showed that the axons of the NCC diverge in the corpus cardiacum and branch between the cells, the secretion granules being stored in the swellings of the axons. In Rhodnius Wigglesworth (1955) found the material between the cells in the medial part of the organ. The corpus cardiacum is therefore, by the definition of Carlisle & Knowles (1953), a neurohaemal organ, that is one in which nerve terminations are in contact with a blood vessel or blood sinus.

Some workers have also noted that some of the axons in the nerve to the corpus cardiacum do not terminate in that organ but pass through it and form the nerve to the corpus allatum. Transport of neurosecretory material to and accumulation of it in the corpus allatum is described by Arvy, Bounhiol & Gabe (1953), Arvy & Gabe (1953g) and E. Thomsen (1954). Each discharge of product is said to be followed by augmentation of the volume of the corpus cardiacum and corpus allatum.

The secretory cycle in these specialised neurones, in relation to post embryonic development has been studied in Ephestia by Rehm (1951) in Ephemeroptera, Odonata and Plecoptera and in Tenebrio by Arvy & Gabe (1953b, c, d, e, f and g) in Forficula by Ihoste (1953), and in Bombyx by Arvy, Bounhiol & Gabe (1953). Gabe (1954) summarises the available information as follows. 'The peak of activity of the cells of the pars intercerebralis coincides in Palaeoptera with the imaginal moult.  
In/

In Plecoptera it occurs at an earlier stage. In Forficula the peak of activity is in the imago. In Holometabola each larval stage is marked by a phase of secretion in the pars intercerebralis. That corresponding to pupation is particularly important.'

Several workers describe how the tinctorial affinities of the neurosecretory cell change in the course of its cycle of activity. Gabe (1954) describes the "product" as being at first phloxinophil (with chrome-haematoxylin-phloxin staining) and later taking the blue colour. He also insists that transport of product does not start until a certain degree of accumulation has been reached. E. Thomsen (1954) found both phloxinophil and blue coloured cells in the same section of Calliphora adult brains and decided that the secretory material could assume either a phloxinophil or a chrome-haematoxylinophil form while M. Thomsen (1954) stated that neurosecretory cells in Hymenoptera contained either dark blue granules, indistinct granules or were a uniform light red and that these appearances represented either different stages of the secretory cycle or the occurrence of two neurosecretory substances.

The objects of this study were; (a) to locate and identify the neurosecretory cells in the brain of the mature third instar larva of L. caesar with confirmatory observations in/  
in/

in the related species G. vomitoria; (b) to determine the function of the neurosecretory cell groups by relating histological evidence of activity in these at different stages in development to histological, developmental and experimental evidence of activity in endocrine organs; (c) to determine, by means of a variety of staining procedures and histochemical tests, the chemical nature of the neurosecretory product in different cell groups.

#### Techniques and staining procedures

Larvae were dissected according to the method outlined in Chapter 2. Initial fixation was effected by irrigation of the dissection with the appropriate fixative solution after which the organs required for sectioning were removed and transferred to a dish of fixative. The neurosecretory product is labile in alcoholic fixatives so these could not be employed. Several aqueous fixatives were tested and it was found that Bouin's solution gave satisfactory and consistent results. It was used for all the material sectioned except that required for Sudan black B test for which a formol-calcium fixative is recommended.

Steedman's ester wax was the embedding medium used and sections were cut at  $6 \mu$ .

The following staining procedures, of which the first two are selective stains for the neurosecretory product, were used in/

in these histological investigations.

A. The chrome-haematoxylin-phloxin stain of Gomori (1941)

Preparation of the stain

2.5 gm. haematoxylin are dissolved in 250 ml. distilled water and to this is added a solution of 7.5 gm. chrome alum in 250 ml. distilled water. To this mixture are added 10 ml. of a 5% aqueous solution of potassium dichromate and 10 ml. of N/2 sulphuric acid. The mixture is ready for use after 48 hours.

Scott's "tap water"

Since Glasgow tap water is relatively pure it is less effective as a rinse at certain stages in the procedure, for example, for blueing haematoxylin stained sections, than Scott's solution which is prepared by dissolving 3.5 gm. sodium bicarbonate and 20 gm. magnesium sulphate in 1,000 ml. distilled water.

Method

- (1) Dewax and bring sections to water.
- (2) Refix in Bouin's solution for 12 - 24 hours.
- (3) Wash in water to remove all traces of picric acid.
- (4) Oxidise sections in an aqueous solution containing 0.3% potassium permanganate and 0.3% sulphuric acid for one minute.
- (5) Decolourise by rinsing in 3% sodium bisulphite solution for five seconds.
- (6)/

- (6) Rinse in distilled water followed by Scott's tap water.
- (7) Stain sections in chrome-haematoxylin for fifteen minutes.
- (8) Differentiate in acid alcohol for twenty-five seconds.
- (9) Blue sections in Scott's tap water.
- (10) Rinse in distilled water and counterstain with 0.5% aqueous phloxin solution.
- (11) Briefly rinse in distilled water and then treat with 5% aqueous phosphotungstic acid solution for two minutes.
- (12) Rinse in distilled water and wash in Scott's tap water until the sections regain a bright red colour.
- (13) Rinse in distilled water, dehydrate rapidly in absolute alcohol, clear in xylol and mount in Canada balsam.

### Result

Granules in neurosecretory cells appear a dark blue-black colour.

B. Paraldehyde-fuchsin stain of Gomori (1950) as adapted by Gabe (1953a) with counterstaining as recommended by Clark (personal communication)

### Preparation of the stain

1 ml. concentrated hydrochloric acid and 1 ml. paraldehyde are added to 100 ml. of 0.5% aqueous solution of basic fuchsin. After 3 days storage in a stoppered bottle at room temperature a "spot test" is applied by placing a drop of solution on filter paper. At first a noticeable quantity of basic fuchsin will appear in the centre of the blot but this diminishes as the solution ages. When there is no longer a trace of basic fuchsin/

fuchsin in the blot the solution is ready for use. It normally takes about 4 days to mature. It is then filtered and the residue on the filter paper dissolved in 100 ml. of 70% alcohol. This is the stock solution. For staining it is diluted, 25 ml. of stock to 75 ml. of 70% alcohol with 1 ml. of glacial acetic acid added.

#### Preparation of Groat's haematoxylin

Mix 0.8 ml. concentrated sulphuric acid and 1 gm. iron alum in 50 ml. distilled water. Add 50 ml. 90% alcohol and 0.5 gm. haematoxylin and shake until dissolved. Filter and store in stoppered bottle.

#### Method

- (1) Dewax and bring sections to water.
- (2) Oxidise sections in an aqueous solution containing 0.3% potassium permanganate and 0.3% sulphuric acid for one minute.
- (3) Rinse in water and decolourise in a reducing rinse of 2.5% sodium bisulphite aqueous solution for forty-five seconds.
- (4) Wash in running water and stain in paraldehyde-fuchsin solution for two-and-half minutes.
- (5) Differentiate in acid alcohol for about five minutes.
- (6) Rinse in Scott's tap water.
- (7) Stain sections with Groat's haematoxylin solution for three minutes.
- (8) Wash in Scott's tap water for fifteen seconds and in distilled water for ten seconds.
- (9) Stain in a solution of 0.25 gm. indigo carmine in 100 ml. saturated/

saturated aqueous solution of picric acid for one minute.

(10) Drain sections. Blot dry. Dehydrate rapidly in absolute alcohol. Clear in xylol and mount in Canada balsam.

### Result

Chromatin, nuclear and cell membranes, general background are grey. Some nuclear material, muscle fibres are green. The granular product of neurosecretory cells, basement membranes, material in pore canals of fore-gut intima, granular material in the perineurium all stain a brilliant purple.

Gomori (1952) states that acidified potassium permanganate solution will attack glycol linkages the oxidant converting 1:2-glycol groups to dialdehydes in the first stage of oxidation. The selective colouration of neurosecretory product by chrome haematoxylin and by paraldehyde fuchsin, after acidified permanganate oxidation was considered by Gabe (1955) to be due to the affinity of these stains for aldehyde and sulphhydryl groups liberated by the oxidation. While recognising the fact that this oxidant is liable to further oxidise the resulting aldehydes it seemed a reasonable working hypothesis that these 1:2-glycol groups were associated with dehydro-glucose residues of polysaccharide molecules; that a component at least of the neurosecretory product could fit into the general classification of "carbohydrates" as used by Pearse (1953). Several "carbohydrate" staining procedures were used in an attempt to identify this component, such an attempt being justified by Gabe's/

Gabe's (1954) general statement that the product had a PAS-positive component other than glycogen.

The following procedures were used.

C. Steedman's (1950) Alcian blue

This was first developed as stain for mucin and is of use (Pearse, 1953) in differentiating acid mucopolysaccharides from other carbohydrates.

Method (a)

- (1) Dewax and hydrate sections.
- (2) Stain in a freshly filtered 1% aqueous solution of alcian blue for thirty seconds.
- (3) Rinse in distilled water.
- (4) Stain in 1% neutral red for thirty seconds.
- (5) Dehydrate in alcohol, clear in xylol and mount in Canada balsam.

Method (b)

- (1), (2) and (3) as above.
- (4) Stain in Mayer's haemalum for five minutes.
- (5) Rinse in distilled water and wash in Scott's tap water for five minutes.
- (6) Take sections up to absolute alcohol and stain in Eosin in absolute for two minutes.
- (7) Differentiate in absolute alcohol, clear in xylol and mount in Canada balsam.

Result

Positively stained material has a brilliant blue-green colour./

colour.

D. A metachromatic method for carbohydrates

The standard Toluidine blue method described by Pearse (1953) was used.

Method

- (1) Dewax and take sections to water.
- (2) Stain in 0.5% aqueous solution of Toluidine blue for six hours.
- (3) Rinse in distilled water.
- (4) Examine sections in water.

Result

Metachromatic substances are either red/pink ( $\gamma$  metachromasia) or purple ( $\beta$  metachromasia).

E. The periodic acid-Schiff test for polysaccharides

Introduced for histological work by McManus (1946) the periodic acid-Schiff reaction was adapted as a histochemical test by Hotchkiss (1948), who stated that positive results are given by substances which (1) contain 1:2-glycol groups or the equivalent amino or alkylamino derivatives or oxidation product, (2) these substances will not diffuse away during fixation nor give a diffusible oxidation product and (3) must be present in sufficient concentration to give a detectable final colour.

Exhaustive discussions on the PAS reaction are given by Gomori/

Gomori (1952) and by Pearse (1953), the latter listing polysaccharides, mucopolysaccharides, muco- and glycoproteins, glycolipids and unsaturated lipids and phospholipids as giving a positive reaction.

Briefly the periodic acid oxidant breaks C-C bonds where these are present as 1:2-glycol groups converting them to dialdehydes but does not further oxidise them so that they can be localised by combination with Schiff's reagent to give a red coloured substituted dye. The amount of colour should be proportional to the amount of reactive glycol present in the tissue. McManus (1956) cautions that a variety of substances apart from those containing 1:2-glycol linkages have been found to give a positive reaction but quotes data from organic chemistry to reinforce the belief that, provided oxidation is performed with 0.5% periodic acid solution for not longer than five minutes, positive substances are presumably carbohydrate.

Several workers have recommended a bisulphate reducing rinse immediately after periodic acid oxidation but McManus logically points out that this can only result in the reversal of the oxidation just performed.

The glycogens give a PAS-positive reaction but can be eliminated readily from sections by treating them with diastase solution or ptyalin.

Preparation of reagents/

### Preparation of reagents

Lison's version of Schiff's reagent, quoted by McManus (1948). Add 1 gm. basic fuchsin to 200 ml. boiling distilled water. Stir and cool to 50°C. Filter. Add 20 ml. N.HCl, cool to 25°C and add 1 gm. anhydrous sodium bisulphite. Store in the dark. After 1 - 2 days the solution becomes straw coloured and is now ready for use.

### Periodic acid solution

Dissolve 1.6 gm. periodic acid in 140 gm. reagent ethyl alcohol, add 20 ml. of M/5 sodium acetate and 40 ml. distilled water. Store the solution in the dark at room temperature.

### Method

- (1) Dewax and bring sections to water.
  - (2) Treat sections with diastase or ptyalin solution for twenty minutes.
  - (3) Rinse in water.
- Steps (2) and (3) may be omitted for detection of glycogen.
- (4) Rinse in 70% alcohol.
  - (5) Treat sections with periodic acid for four-and-a-half minutes.
  - (6) Wash in distilled water for ten minutes.
  - (7) Treat with Schiff's reagent for twenty minutes.
  - (8) Rinse in water.
  - (9) Treat with a 2% sodium bisulphite solution for one minute.
  - (10) Wash in running water for ten minutes.
  - (11)/

- (11) Take sections through graded series of alcohols to absolute alcohol, clear in xylol and mount in DPX or Canada balsam.

### Result

Positive material stains a rich red or purple-red.

F. Sudan black B. staining for lipids in wax sections: Pearse (1953) after McManus (1946)

The preparation of serial frozen sections from minute tissue blocks of the size of the brain of a blowfly larva is technically impossible. Lipid substances in neurosecretory cells could therefore be detected by this procedure. The tissue was fixed for three weeks in a formol-calcium solution, postchromed for 24 hours and thereafter routine embedding procedure for ester wax was used.

### Fixative solution

Dissolve 1 gm. of cobalt nitrate in 80 ml. of distilled water and add 10 ml. of 10% calcium chloride solution and 10 ml. of formalin.

A 3% potassium dichromate solution was used for post-chroming.

### Method

- (1) Dewax sections and bring down to 70% alcohol.
- (2) Stain for thirty minutes in a saturated solution of Sudan black B. in 70% alcohol.
- (3)/

- (3) Rinse quickly in 70% alcohol to remove excess dye.
- (4) Wash in running water.
- (5) Counterstain for one minute in 1% aqueous neutral red solution.
- (6) Wash in water and mount in glycerine jelly.

### Result

Lipids stain blue or blue-black.

Lillie (1950) found that the contents of cells of the mammalian adrenal medulla were phenolic and were PAS-positive. The following procedure was used to test whether certain neurosecretory cells containing PAS-positive material might contain a phenolic substance.

### G. Acid diazonium reaction for aromatic amine and phenols;

Pearse (1953)

### Method

- (1) Dewax and hydrate sections.
- (2) Immerse in 0.5M acetate buffer (pH 5.2) containing 1 mg./ml. of diazotized p-nitroaniline for forty minutes.
- (3) Wash in running water.
- (4) Counterstain with Mayer's haemalum for three minutes.
- (5) Wash in running water, thirty minutes.
- (6) Dehydrate in alcohol, clear in xylol and mount in DPX.

### Result

Positive reaction is indicated by a red or orange red colour.

Section II: Location of Neurosecretory Cells in the Brain of Larva of L. caesar

In sections the brain is seen to be invested in a sheath, formed by a layer of cells called the perineurium (B. Scharrer, 1939) which is in turn covered by a fine membrane the neural lamella. The medullary zone of the brain is composed principally of axons while in the cortical zone are found neurones, neuroglia and the anlagen cells from which structures of the adult brain develop. Certain of the neurones in the cortex can be identified as neurosecretory cells.

No fewer than six groups of neurosecretory cells can be identified in each hemisphere\* of the brain of the third instar larva of L. caesar. The groups are distinguishable by criteria of position, size, morphology and staining characteristics. The cells are all unipolar pyriform neurones located as follows. (See Figs 25, 25a and 26 - 58.)

Group 1.

This consists of two large neurones lying in an antero-dorsal position in the pars intercerebralis close to the junction of the hemispheres. These attain their maximum size of/

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\*The symmetrical arrangement of neurosecretory cells in the central nervous system has been commented on by several authors, B. Scharrer (1955) noting the fact that corresponding cells on each side are normally in phase.

Fig. 25. Diagram illustrating distribution of neurosecretory cell groups, as seen in dorsal view, in the brain of the larva of L. caesar.

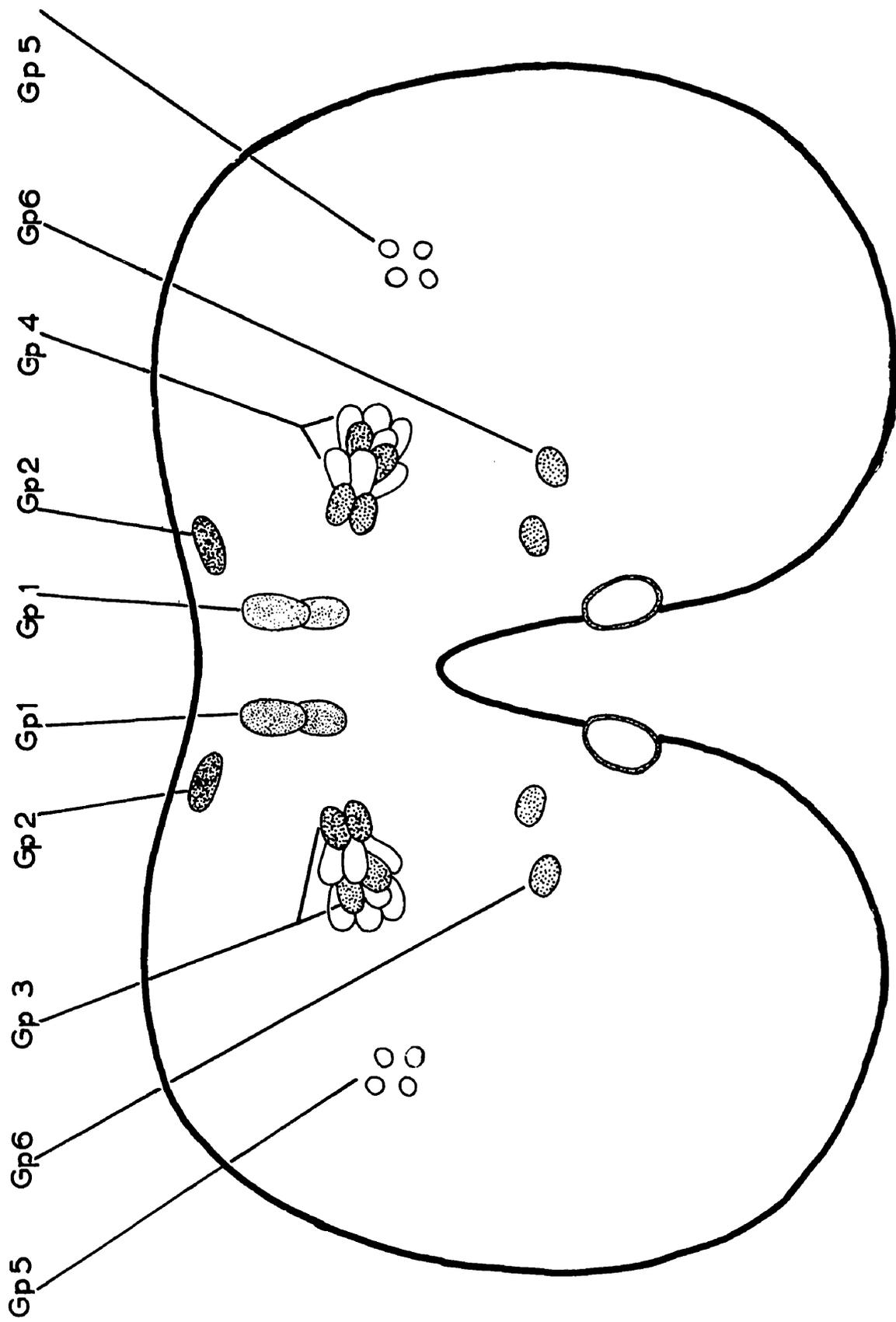


Fig. 25.

Fig. 25a. Frontal section of the brain of L. caesar larva (in diapause) showing the location of certain cell groups. (From a camera lucida drawing.)

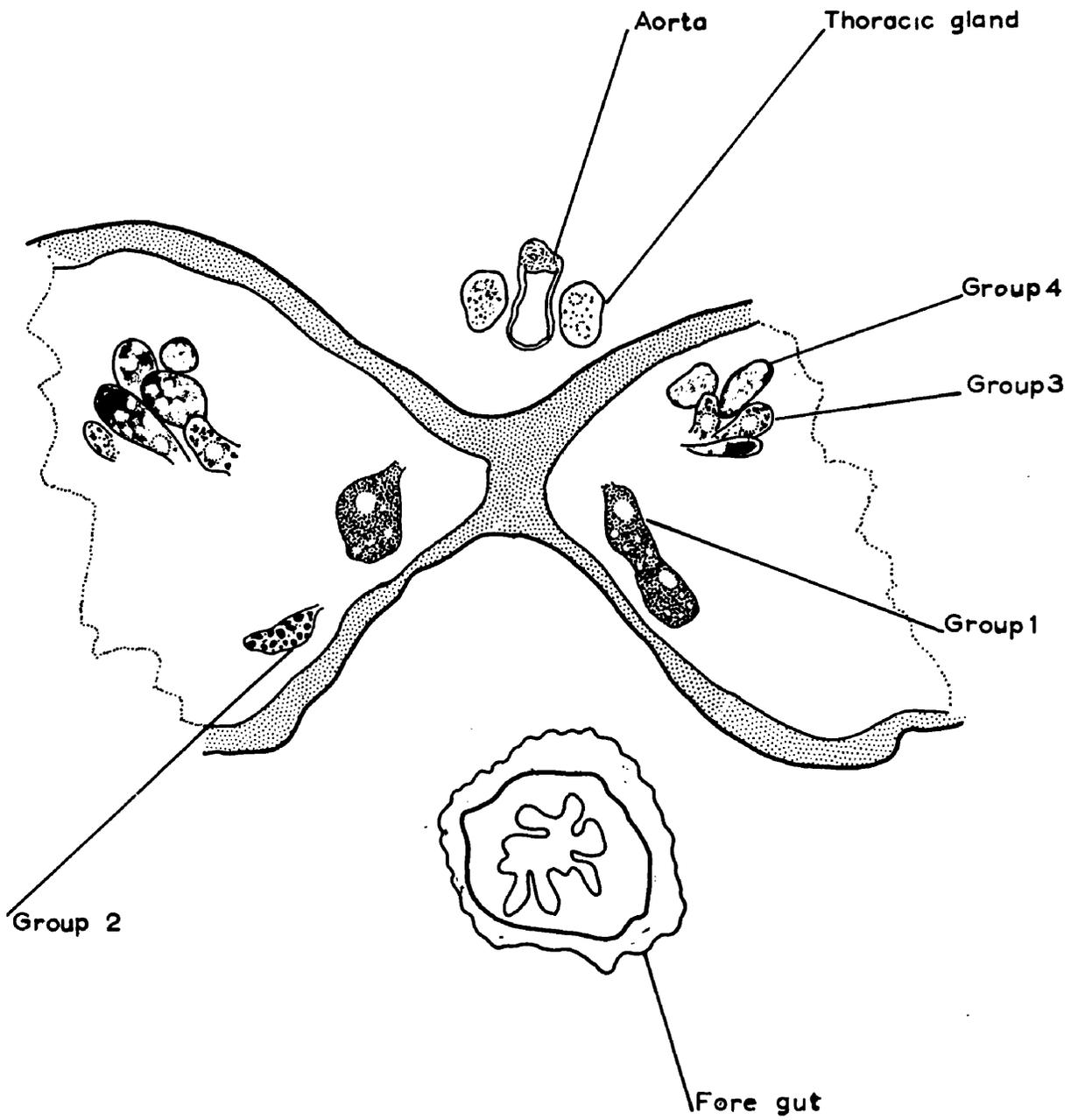


Fig. 25a.

of  $40 \mu \times 18 \mu$  in the diapause larva. They have a central subspherical or ovoid nucleus of maximum length  $10 \mu$  and contain numerous large spheroid or ovoid vacuoles ranging in size up to  $4 \mu$  in length.

#### Group 2.

This group is represented by a single cell lying anterior to Group 1 and at a lower level. It measures at its maximum, again in diapause larvae,  $20 \mu \times 15 \mu$  and has a central subspherical nucleus  $9 - 10 \mu$  in diameter. Vacuoles are not normally seen in cells of this group except in diapause brains.

#### Group 3.

Four cells can normally be counted in this group. They lie lateral to Group 1, in the dorsum of the hemisphere and measure  $20 \mu \times 15 \mu$  with a central subspherical nucleus  $8 - 9 \mu$  in diameter. There are usually two or three large vacuoles in the cytoplasm.

#### Group 4.

As indicated in Fig. 25 the cells of this group intermingle with those of Group 3, but the two groups are by other criteria quite distinct. There are usually seven Group 4 cells present, each distinctly pyriform with the nucleus,  $8 - 9 \mu$  in diameter, displaced towards the axon end of the cells. Their maximum size is approximately  $25 \mu \times 17.5 \mu$ . These are the cells which show so distinctly in the dorsum of the/

the living brain of the non-diapause larva.

Group 5.

These small, almost spherical neurones, about  $12.5 \mu$  long, lie lateral to and behind Groups 3 and 4. Several morphologically similar neurones occur in this dorso-lateral locus but the actual number of these which are differentially stained from common neurones varies from four to eight. The nucleus is of the same order of size as in other neurosecretory cells, about  $8 \mu$  in diameter, and there are one or more large vacuoles present so that there is relatively little cytoplasm in the cell.

Group 6.

This comprises two cells of irregular outline, about  $20 \mu \times 17.5 \mu$  in size with central subspherical nuclei  $9 - 10 \mu$  in diameter. These lie against the medulla at the rear of the brain close to the entry of the main cerebral trachea.

### Section III: Neurosecretory Cells in the Brain of the Diapause

#### Larva of *L. caesar*

For the purpose of identification and characterisation of the neurosecretory cell groups the diapause larva is ideal material compared with the larva of a non-diapause species since the appearance of each group is constant throughout the diapause period while in the non-diapause prepupa each group goes through a cycle of activity before pupation, the duration of this cycle and the cytological picture varying from group to group.

Brains were taken from larvae which had been in diapause for different lengths of time, the duration of the diapause being measured in days after the termination of the 14-day post-feeding test period. The main batches used had been in diapause for 24 days, 42 days, 90 days and 185 days at the time of dissection. Sections of several brains of each batch were subjected to each staining procedure.

Cells described in this section as neurosecretory do not necessarily, in the diapause state, satisfy all the criteria laid down above but as will be seen later they do satisfy all the criteria at some time in the prepupal stage.

#### A. Appearance in vivo

The cells of Groups 1 and 2 can be seen in the living diapause/

Figs 26 - 31. Neurosecretory cells in the brain of an L. caesar in diapause, after paraldehyde fuchsin staining.

26) Group 1.

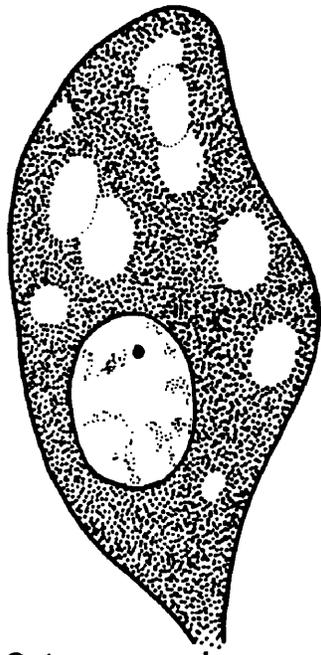
29) Group 4.

27) Group 2.

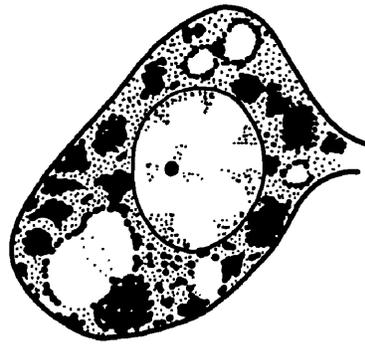
30) Group 5.

28) Group 3.

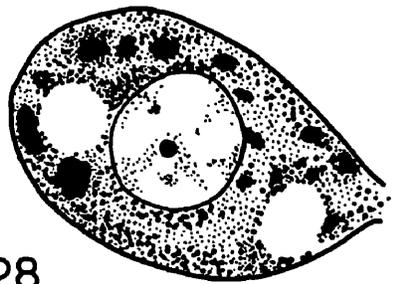
31) Group 6.



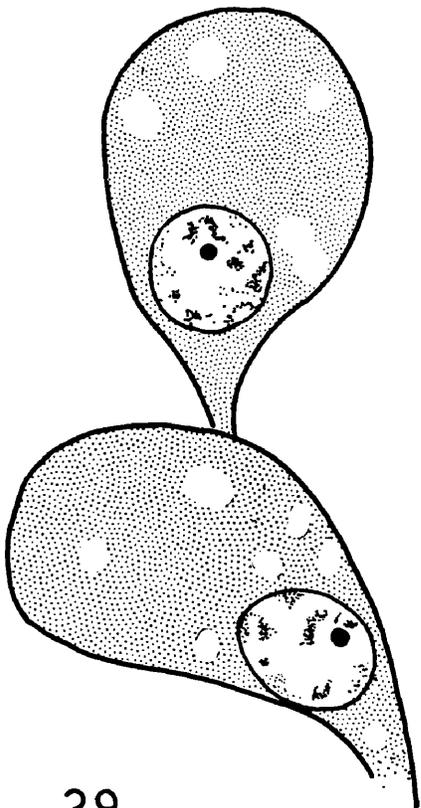
26



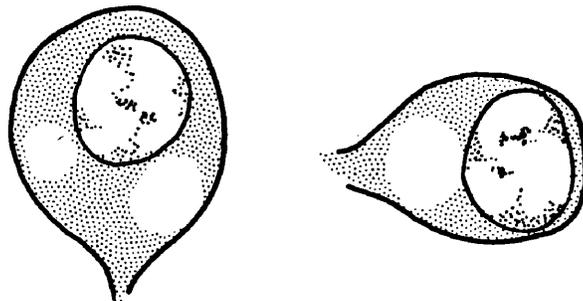
27



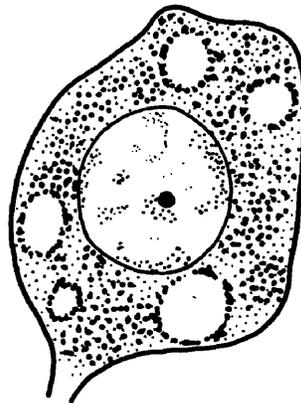
28



29



30



31

diapause brain if the aorta, ring gland etc., are dissected off. Group 3 is not distinct and other groups are not evident.

B. Appearance in stained serial sections

(a) Paraldehyde-fuchsin

A description of the results of this staining procedure is given first since all groups have some affinity for this stain.

Group 1. (Fig. 26)

At low magnifications (16 mm. achromatic objective lens) cells of this group appear to have a uniform deep purple colour broken only by the nucleus and by the several large vacuoles. At higher magnifications (3.75 mm. fluorite or 1.8 mm. achromatic objectives) this colour is resolved into minute purple particles of which the smallest are at the resolution limit of the microscope. The small particles appear to associate to form aggregates or granules of varying magnitude but none of these exceed  $1 \mu$  and most are half that size. The general impression is of a very high concentration of small, separate granules packing this distended cell.

Group 2. (Fig. 27)

The product of this group, again assuming a deep purple colour with this stain, occurs in larger granules than in Group 1. These granules associate in large, irregular masses separated/

separated by relatively clear zones of cytoplasm. In some masses the granules pack together much more closely than in others. There are several large vacuoles in the cell and granules are invariably associated with the periphery of these.

Group 3. (Fig. 28)

The product of this group is stained a less intense purple than that of Groups 1 and 2. The granules are on the average smaller than in Group 1. In some parts of the cell they remain discrete as in Group 1; in others aggregate masses of varied size are formed similar to, though smaller than, those in Group 2. In each cell there are two or three vacuoles, 3 or 4  $\mu$  in diameter; the periphery of each of these is ringed with granules.

Group 4. (Fig. 29)

The abaxonal zone of these cells stains a relatively uniform pale purple colour. No particles or granules of product can be distinguished and there are no other chondriosomes in this zone. There are several prominent vacuoles in each cell, round or oval in outline, 2 or 3  $\mu$  long.

Group 5. (Fig. 30)

No granules can be distinguished in these small cells. The same uniform pale purple colour is produced as in Group 4, a colour which seems to be property of the ground cytoplasm and not of any inclusions, though it could be produced by sub-microscopic/

sub-microscopic particles. This is not a homogeneous group. In a typical diapause brain there are in each hemisphere four cells, of a matching uniform purple, with one or two large vacuoles (as illustrated in Fig. 30) but other two larger cells with a lighter uniform colour and several smaller vacuoles can usually be found lying lateral to the others.

#### Group 6. (Fig. 31)

In these cells the granules match in size those of Group 1 but the purple colour is less intense. Granules are also discrete as in that group, distinct aggregate masses being absent, but the amount of product in the Group 6 cell is smaller. The large vacuoles have again a peripheral ring of granules.

#### Corpus cardiacum

Secretory granules occur in this organ during diapause. These could originate in any of the groups having a positive granular content at this stage. Granules occur also in the NCC. In transit in the axon the particles are minute but large granules or irregular aggregates reform in the corpus cardiacum. The granules are found in the rami of the axons between the chromophobe cells. The chromophile cells are not markedly reduced in size during diapause. They measure 25 - 30  $\mu$  by 15 - 20  $\mu$  with a central ovoid nucleus 8 - 9  $\mu$  long. They have in vivo a bluish appearance suggestive of a particulate/

particulate product and the cytoplasm has a strong affinity for methylene blue.

(b) Chrome-haematoxylin-phloxin

Group 1.

The cells of this group are clearly differentiated by this staining procedure. The granules are stained a grey-blue colour, not the deep blue-black colour accepted as positive for this stain. No part of the cytoplasm is coloured by the phloxin even in over-counterstained sections.

Group 2.

This cell is at first difficult to locate because there is no clear differentiation from common neurones with this stain. The aggregates of granules disclosed by paraldehyde-fuchsin are not in evidence at all. The cytoplasm is moderately phloxinophil and contains abundant grey chondriosomes and matches very closely that of a neighbouring giant neurone.

Group 3.

These cells are differentiated by chrome-haematoxylin. They are found to contain blue-black coloured granules associated in the manner described above. This is the only group of brain neurosecretory cells giving a characteristic positive result with this stain during diapause. The cytoplasm lacks a marked affinity for phloxin.

Group 4./

Group 4.

The contrast between this and the preceeding group is quite dramatic since Group 4 cells are strongly phloxinophil. This appears to be a property of the ground cytoplasm of the abaxonal zone. No chondriosomes or other inclusions, apart from the vacuoles already noted, can be detected in this part of the cell.

Group 5.

Again these cells resemble Group 4 in staining characteristics the cytoplasm being strongly phloxinophil with no detectable inclusions. The colour in the two types of Group 5 cell is in this case the same.

Group 6.

These cells are not differentiated from common neurones by this stain.

Corpus cardiacum. Secretory granules are rather difficult to detect in this organ in a diapause larva. The chromophile cell cytoplasm is phloxinophil and no inclusions are evident in it.

(c) Alcian blue-neutral red

The only cells with an affinity for alcian blue belong to Group 1. With this stain the granules assume a very pronounced blue-green colour. The cytoplasm is quite uncoloured by neutral red, while in Groups 4 and 5 it is evenly stained with this/

this dye. Groups 2, 5 and 6 are not in any way distinctive.

(d) Alcian blue-haemalum-eosin

Group 1 is again clearly differentiated although the colour of the granules is modified to a grey-blue which cannot be interpreted as a positive result. As expected from the notable affinity for phloxin the cytoplasm of Group 4 and 5 is strongly eosinophil. Groups 2 and 3 can be located but are not specifically stained. The Group 6 cells are not differentiated from neighbouring neurones.

(e) Sudan black B-neutral red

Group 6 was not located in these sections. The neurones of the other groups all contain some sudanophil material. In Group 2 the pattern of distribution of sudanophil granules corresponds quite well to the pattern of paraldehyde-fuchsin positive granules. Group 5 is, however, most notable for the intense uniform blackening of the cytoplasm which can certainly be interpreted as a positive result.

(f) Toluidin blue

Certain of the groups give striking metachromatic effects. Group 1 is most clearly differentiated by the deep purple colour which develops; it is definable as a strong beta metachromasia. The aggregate masses in Group 2 show a similar but slightly less intense beta metachromasia. The cytoplasm of Groups 4 and 5 also colours metachromatically.  
The/

The colour is less easy to define but after consultation with colleagues the writer decided to call it "lilac" which can be interpreted as a weak beta metachromasia. Group 6 was not detected. Group 3 does not exhibit metachromasia, the cell colour being the orthochromatic blue.

When the sections were treated with alcohol there was in all groups an instantaneous loss of metachromasia.

(g) Periodic acid-Schiff

Counter to expectation, arising from the statement of Gabe (1954) summarised in the introduction to this chapter, the granular product of these neurosecretory cells is not PAS-positive. On the other hand, the cytoplasm of Groups 4 and 5 does give a PAS-positive reaction in all the variations of the technique tested. This PAS-positive material is diastase and ptyalin fast and is not therefore glycogen. In Group 4 the positive reaction is weak but the cells are easily detected if a green light filter is used. The Group 5 cells give a strong uniform PAS-positive reaction.

(h) The acid-diazonium test

This test was applied in case, as Brunet (1952) cautions, a phenolic substance might be responsible for the PAS-positive reaction. The test was negative for all cell groups.

### Section III

#### Conclusions

Importance can be attached to the different degrees of association of the granules of the product demonstrated in sections stained with paraldehyde-fuchsin. In Group 1 the size of granules is limited and they remain discrete. In Group 2 the size is limited but larger and large aggregates of granules form. In Group 3 the granules are small and the distribution, discrete granules and aggregates, is intermediate between that seen in Groups 1 and 2. Little is yet known about the chemical nature of these granules though a similarity is indicated by their common affinity for this stain but the physical differences, shown in the varying types of association of granules, indicate a degree of chemical dissimilarity. This is clearly confirmed by the other staining procedures and histochemical tests applied.

There are clear cytochemical differences between and cytochemical identity within the six groups first located and separated on evidence provided by paraldehyde-fuchsin staining. These tests were selected with the object of identifying the carbohydrate component of the neurosecretory product recorded by Gabe (1954) and can be summarised as follows: conclusions being based on the discussions of histochemical tests for carbohydrates and lipids in Gomori (1952) and Pearse (1953).

#### Group 1./

Group 1.

The product has an affinity for alcian blue but is PAS-negative. It might therefore contain an acid mucopolysaccharide but that would give gamma metachromasia while this group gives a deep purple beta metachromasia. A lipid component of the product is not demonstrable.

Group 2.

The product is neither alcian blue nor PAS-positive. A strong metachromatic violet colour is produced with toluidin blue. There may be a lipid component but no definite conclusion can be made about the nature of the product.

Group 3.

Again the product is not selectively stained by alcian blue. If the granules have an affinity for alcian blue it is masked by the cytoplasm accepting other dyes. It is not PAS-positive and colour with toluidin blue is orthochromatic. No conclusion can be made.

Group 4.

No product is demonstrable. The cytoplasm is selectively stained with neutral red, is eosinophil and phloxinophil and is weakly PAS-positive. The PAS-positive substance in the cytoplasm is not glycogen. With toluidin blue the cytoplasm stains a weak metachromatic lilac and it is not sudanophil. These/

These results are consistent with the presence in the cytoplasm of a neutral mucopolysaccharide (or mucoprotein) not found in common neurones.

#### Group 5.

The staining of this group matches so clearly, in other respects, that of Group 4, that the PAS and Sudan black B. tests were repeated several times to make sure that the much stronger PAS-positive and the strong, and markedly uniform sudanophil reactions could be obtained consistently. They were. This result might be due to the presence of a glyco- or phospholipid, or lipoprotein not present in common neurones or in the other neurosecretory cells but such a conclusion is inconsistent with the metachromasia shown with toluidin blue.

Thus, attempts to locate a carbohydrate component other than glycogen gave inconclusive results. The product of Groups 1, 2 and 3 is not PAS-positive and cannot, therefore have a glucide component. Differences between the groups are, however, confirmed and emphasised.

Wigglesworth (1934) first suggested that diapause may be due to the temporary failure in the secretion of the growth hormone. In extensive studies of the physiology of diapause in Platysamia Williams (1946) concluded from parabiosis experiments that diapause is immediately due to the absence of growth factors and not due to the presence of an inhibitor substance./

substance. The diapause could be terminated by implanting the brain of a chilled pupa into an unchilled pupa. The relationship between brain and thoracic glands and the function of the latter organs are discussed in Chapter 3. It is sufficient to state here that the brain by means of a diffusible hormone liberated into the blood, activates the thoracic glands whose secretion promotes pupal development. Thus the unchilled diapause brain in Platysamia can be regarded as "inactive" and the chilled brain as "active". Results from experiments with Lucilia (Chapter 3) indicate that the diapause brain in that larva similarly fails to activate the thoracic glands and is in that sense "inactive".

It might therefore be expected that the neurosecretory cells in the brain of the L. caesar larva in diapause would appear to be totally inactive, devoid of a stainable product as in the feeding larva (see Chapter 4, Section IV). On the contrary, abundant granules are demonstrable in Groups 1, 2, 3 and 6, in the axons of these cells and in the corpus cardiacum. Thus there is not a suspension of neurosecretory activity during diapause. The extremely consistent unchanging appearance of the brain neurosecretory cells throughout diapause must be emphasised. The brains sectioned were from larvae which had been maintained in the diapause state at a constant temperature of 25°C for four periods of time, namely 24 days, six weeks, three months and six months. The tinctorial/

tinctorial affinities did not change during this time. It has been suggested (C.M. Williams, personal communication) that the product accumulates in certain of the cell groups at the outset of diapause but is not discharged until the state terminates. As will be seen in Section IV the enlargement of Group 1 cells as product accumulates occurs in non-diapause larvae and takes place quite rapidly. Enlargement of these cells in diapause larvae is not due to a slow accumulation without discharge. The proof that product is being discharged during diapause lies in the fact that it can be found in the nerves to the corpus cardiacum and in that organ. Since the appearance of the cells does not alter while diapause lasts, and the amount of product in the corpus cardiacum is low relative to that in a non-diapause prepupa, it must be assumed that a low rate of production is balanced by a low rate of discharge. These cells instead of going through a normal rapid cycle of accumulation and discharge lasting 3 or 4 days (see Section IV) reach a certain level of activity which is maintained for as many months as the diapause lasts. Neurosecretory cells capable of similar monotonous activity are also to be found in the ventral ganglia (see Section V). Since granules of product are present in cells of Groups 1, 2, 3 and 6 it would seem inevitable that some material must be carried out in that constant flow of axoplasm from the pericaryon first confirmed in insects by E. Thomsen (1954).

Production/

Production in the cells could be confined to compensation for this casual leakage of product. However, there is in Group 3 at least a clear "discharge" picture demonstrable with chrome-haematoxylin, the cells containing the abundant blue-black granules associated by most workers with the climax of a secretory cycle since this is the type of granule demonstrable in the corpus cardiacum with that stain (Scharrer, 1951; et al.) Moreover, after paraldehyde-fuchsin staining the Group 3 cell in the diapause larva closely resemble a Group 4 cell at its peak of activity in the non-diapause prepupa. Group 3 is, therefore, probably the major source of the neurosecretory material in the corpus cardiacum and may be the only group with a physiological function during diapause.

The absence of a stainable neurosecretory product from cells of Groups 4 and 5, in contrast to the abundance of such a product in these in a stage 3 non-diapause larva (Section IV), indicates that these are inactive during diapause.

#### Section IV: Brain Neurosecretory Cells in Non-diapause Larvae

Diapause was avoided by providing optimal conditions for larvae at all stages. When fully fed, larvae, taken with full crops, were placed in groups of five in 4" x 1¼" glass tubes containing two inches of damp peat moss. The larvae were kept at a constant temperature of 25°C. Under these conditions 92% continue to develop without diapause.

Serial sections were prepared of the brains of third instar larvae taken at the following stages in development.

Stage 1. Larvae still feeding but almost fully fed.

Stage 2. Larvae taken 6 - 8 hours after removal from food.

Stage 3. Mature larvae developing without diapause, taken at the stage when development of imaginal discs is negligible but Group 4 cells show clearly in the dorsum of each hemisphere.

Stage 4. Mature larvae developing without diapause at a stage when wing bud development, and that of other anlagen, is very noticeable. Group 4 cells no longer show clearly.

Stage 5. This is the "white pupa" stage at which the cuticle of the larva has contracted to form the barrel-shaped puparium but has not yet darkened and hardened. This is, of course, not a pupa. After puparium formation there is a third intrapuparial moult, the fourth larval instar being pharate within the puparium. This is followed within 18 hours by the fourth moult, again intrapuparial, giving rise to the pupa which is twice pharate, within the puparium and within the fourth larval integument./

integument.

The development of the retrocerebral organs and imaginal discs at these different stages is discussed in Chapter 3. Mitoses can be seen in the brain and anlagen in sections of larvae of all five stages. If the larva enters diapause mitotic activity ceases and diapause brains are characterised by its absence.

In this investigation over sixty brains were again sectioned. There are obvious limitations to the number of staining procedures and histochemical tests which can be applied to specimens of each stage, and so Sudan black B., toluidin blue and the acid diazonium tests were omitted as being unlikely to give useful information about the cycle of secretion. PAS and alcian blue were retained because it was known that in some neurosecretory cells in these larvae, (in the abdominal ganglia; see Section VI), a carbohydrate component of the product was present and it seemed possible that it might appear at a stage other than diapause in brain cells. Paraldehyde-fuchsin staining was used on sections of several brains of each stage and chrome-haematoxylin at stages 3 and 4.

The division of larvae into five stages is convenient but in fact no two brains in one stage are at the same point of development and a much more complete picture of the cycle in each cell has been obtained than might be thought possible.

A./

A. Accumulation and discharge of product in the brain  
neurosecretory cells

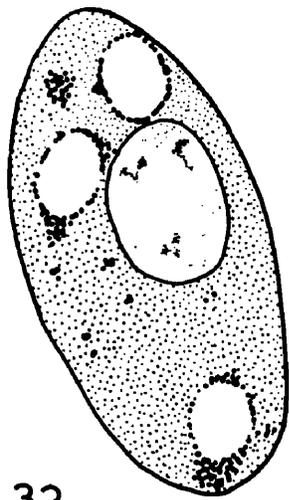
(a) A description of the cycle in each cell group as shown  
by paraldehyde fuchsin staining

Group 1.

Cells of this group have, in stage 1, a grey cytoplasm like that of common neurones. Then the first purple particles appear, just detectable around the periphery of vacuoles. At the second stage (Fig. 32) the amount of product has increased and the deep purple coloured granules around vacuole are larger. Although a few granules not associated with vacuoles are scattered in the cytoplasm they are densest in the vicinity of them. During stage 3 the granules remain limited in size but increase in abundance (Figs 33 and 34). The association of granules with vacuoles remains and these appear to be centres of production from which the material spreads out into the surrounding cytoplasm. Late in this stage, particles of product can be traced into and along the axons of several representatives of the group. Meanwhile, the cytoplasm has become clear. Presumably chondriosomes are absent and there has been a diminution in the proportion of non-labile material, precipitated normally in a finely granular state, in the ground plasm. During stage 4 there is a notable increase in the size of the cell and a slight increase in the size of the nucleus/

Figs 32 - 37. Brain neurosecretory cells of Group 1, from brains of larvae of L. caesar at different stages of development; after paraldehyde fuchsin staining.

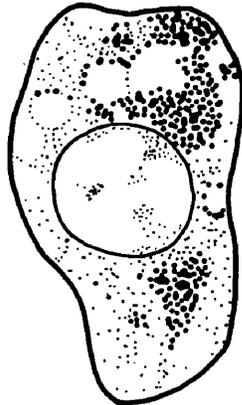
- |                    |                    |
|--------------------|--------------------|
| 32) Stage 2.       | 35) Early Stage 4. |
| 33) Early Stage 3. | 36) Late Stage 4.  |
| 34) Late Stage 3.  | 37) Stage 5.       |



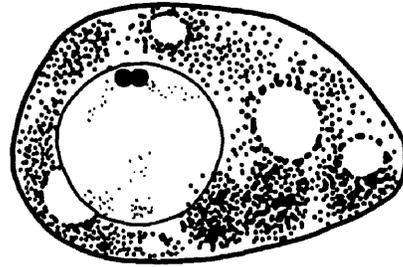
32



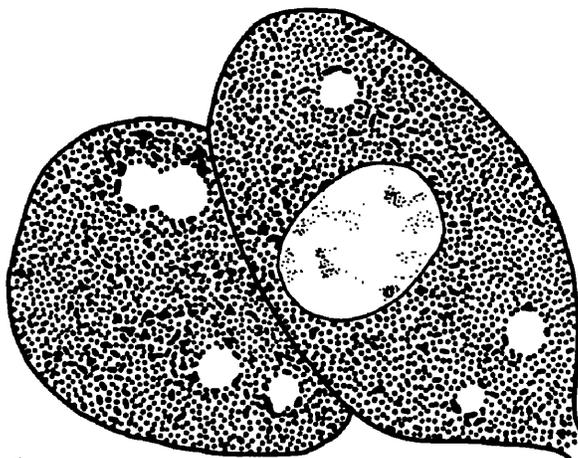
33



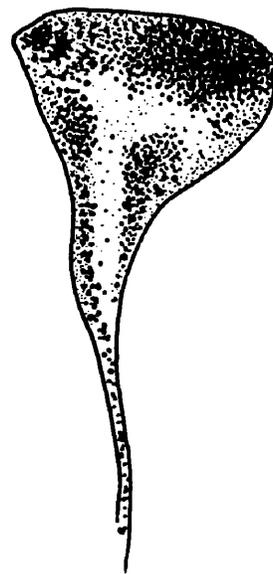
34



35



36



37

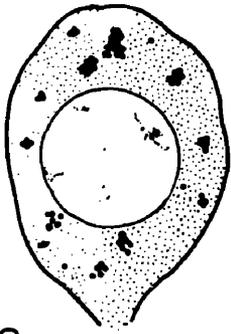
nucleus in which double nucleoli provide evidence of endomitosis. Early in stage 4 the product, which always remains discrete and never forms large aggregates (which it does in Group 2), is densest in spherical zones around vacuoles (Fig. 35). Later the entire cell, by now distended to a size of  $40 \mu \times 20 \mu$  is, except for vacuoles and nucleus, entirely filled with these discrete granules (Fig. 36). There is a pronounced similarity between cells of this group in diapause and in late stage 4 brains. It must be emphasised that granules can be seen in transit in the axons from stage 3 on and, therefore, this dramatic accumulation can be interpreted as indicating that the rate of production exceeds the rate of secretion. In the axons the product occurs in much smaller particles than in the cell. Apparently there is a physical change in the substance secreted and it is transported in this finely divided state. In the final stage 5 the cell collapses. The nucleus is pycnotic and the cell outline becomes irregular, evidence of senescence (Fig. 37). The secretion is now "pouring" along the axon in much greater amounts than at previous stages.

#### Group 2.

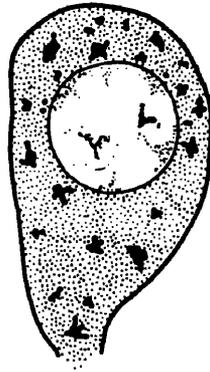
In the younger feeding larvae there is no positive material in the cells of this group. They can readily be located by position and morphology but the stain does not differentiate them from common neurones. Later a few granules, /

Figs 38 - 46. Brain neurosecretory cells of Group 2, from brains of larvae of L. caesar at different stages of development; after paraldehyde fuchsin staining.

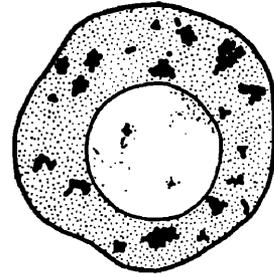
- |                    |                    |
|--------------------|--------------------|
| 38) Stage 1.       | 43) Early Stage 4. |
| 39) Early Stage 2. | 44) Mid Stage 4.   |
| 40) Late Stage 2.  | 45) Late Stage 4.  |
| 41) Early Stage 3. | 46) Stage 5.       |
| 42) Late Stage 3.  |                    |



38



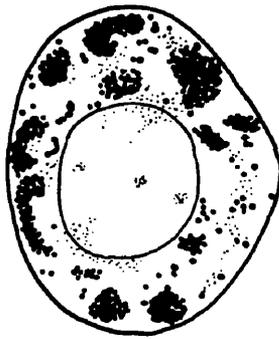
39



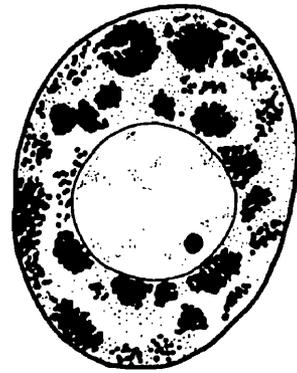
40



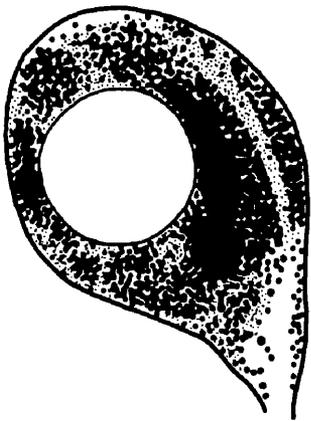
41



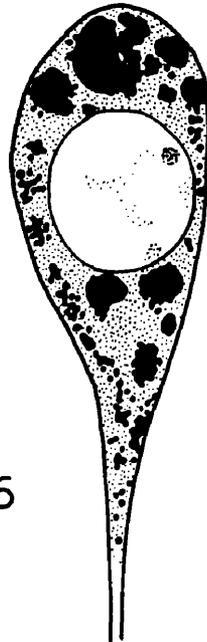
42



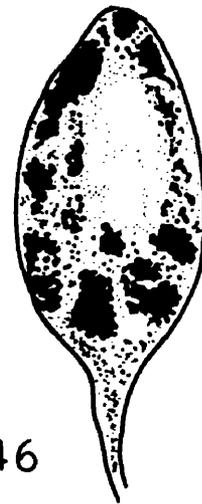
43



44



45



46

granules, of the same deep purple colour as in Group 1 cells, and small aggregates of these are seen scattered in the cytoplasm as shown in Fig. 38. This is the first group in which secretory material is formed after the cessation of feeding. At stage 2 the amount of product has increased. The granules are larger, and greater aggregates are formed. There is no marked association of granules with vacuoles (Figs 39 and 40). During stage 3 the aggregates continue to increase in size and it is notable that some are formed of tightly "clumped" granules while others are looser associations (Fig. 41). There are also numerous unassociated granules scattered in the cytoplasm; these are larger than those of Group 1. Particles of product occur in the axons at this stage but the rate of discharge appears to be low. The cytoplasm has now become clear, in contrast to the grey granular appearance of earlier stages and of common neurones. There is also a marked increase in the size of the cell and of its nucleus (Fig. 42). In some cells double nucleoli indicate amitotic division. In stage 4 accumulation of the product continues (Fig. 43), aggregates being larger and more numerous and these appear to coalesce until almost the entire cell is filled with the product (Fig. 44). At this time the small granules of the transportation state fill the axon (Fig. 45). The cell and nucleus do not increase in size after stage 3. In the last stage examined, stage 5, the cells are in senescent state.

The/

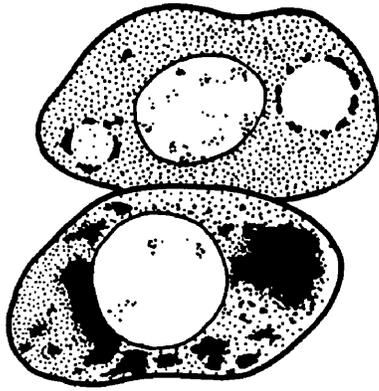
The nucleus becomes pycnotic and the cell shrinks in size. There is a diminution in the amount of product in the cell and the axon is filled with minute granules (Fig. 46). The early stage 4 cells resemble most closely the cells of this group in diapause brains.

### Group 3.

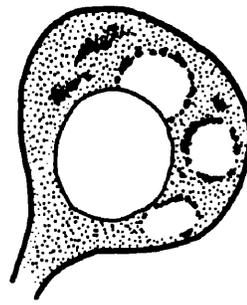
Located by position and morphology in brains of stages 1 and 2, these cells do not contain any granules until stage 3. Hitherto the cytoplasm with its two or three large vacuoles resembles that of common neurones but in stage 3 granules appear. These are at first minute, associated with the periphery of vacuoles as in early Group 1 cells, but later they enlarge and spread into the surrounding cytoplasm (Figs 47, 48 and 53). The mode of accumulation resembles that in Group 1 but the granules are on the average smaller in size and stain a much less intense purple. By stage 4 the material becomes more abundant though no higher in concentration than in an early stage 3 Group 1 cell (Fig. 49). The cytoplasm becomes clear at this stage. In the last stage the product reaches a concentration as high as that in an early stage 4 Group 1 cell. The granules remain smaller and discrete with no aggregates forming and although the colour is now deeper it never attains the dark purple of Groups 1 and 2 nor of Group 3 cells in diapause brains. At this time granules are abundant in transit in the axons (Fig. 51). The start of production and attainment/

Figs 47 - 51. Brain neurosecretory cells of Group 3, from brains of larvae of L. caesar at different stages of development; after paraldehyde fuchsin staining.

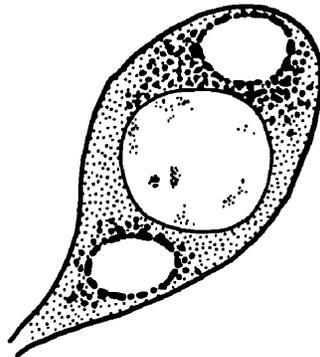
- 47) Stage 3, the Group 3 cell lying above a Group 4 cell.
- 48) Stage 3.
- 49) Stage 4.
- 50) Stage 5.
- 51) Stage 5, the Group 3 cell lying above a Group 4 cell.



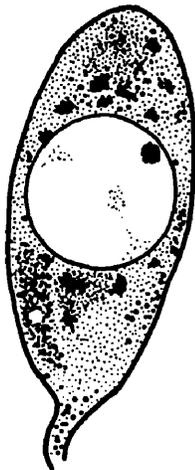
47



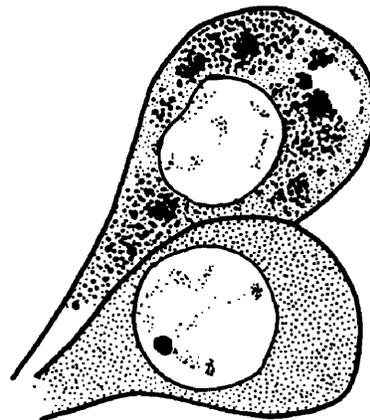
48



49



50



51

attainment of maximum concentration are later in this group than in the others. Up to the time of the intrapuparial moult they have not achieved the state noted in diapause brains. Whether they attain this later or whether the diapause condition of the cells is not repeated (as it is in Groups 1 and 2) at any stage in development, is not known but pycnotic changes observed in stage 5 nuclei indicate that the second alternative is the likier. It seems probable that they are functioning in a different way during diapause.

#### Group 4.

In the feeding third instar larva these cells can be identified by position and form but they contain no purple stained material. A few hours after the cessation of feeding, in stage 2, the first signs of neurosecretory activity appear (Fig. 52). Faint purple patches, usually of regular outline, form in the abaxonal zone of the cell. Discrete component particles cannot be distinguished but the colour is not thought to be, as in the case of the diapause cell, a property of the ground cytoplasm nor is it uniform over the whole of the zone but is restricted to these "blobs"\*. The sequence of accumulation/

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\*Shorter Oxford English Dictionary, 1933. Blob; a small, rounded mass of colour.

Fig. 53. Media neurosecretory cells in a frontal section of the right cerebral hemisphere of a larva of L. caesar, after paraldehyde fuchsin staining.

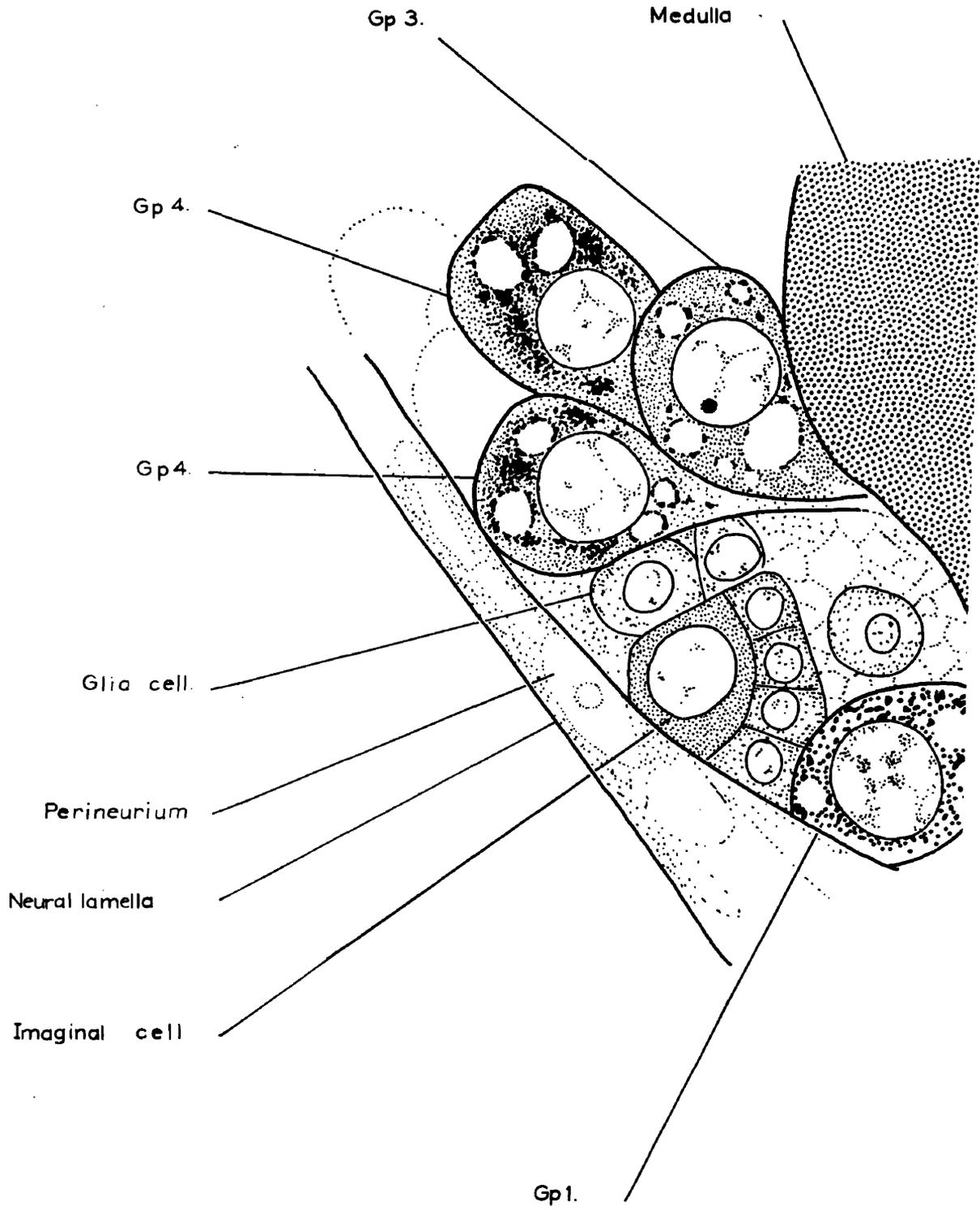


Fig. 53.

Figs 52, 54. Brain neurosecretory cells of Group 4, from brains of larvae of L. caesar at different stages of development.

52) Stage 2.

54) Stage 4.

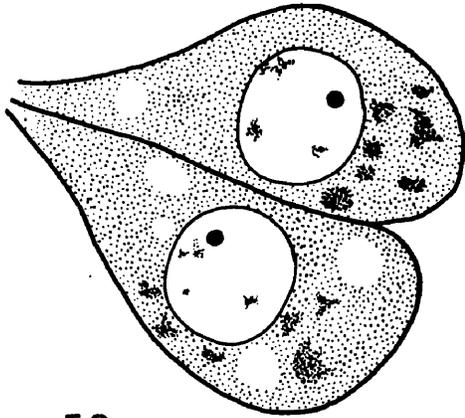
Figs 55 - 58. Cells of Group 5.

55) Stage 2.

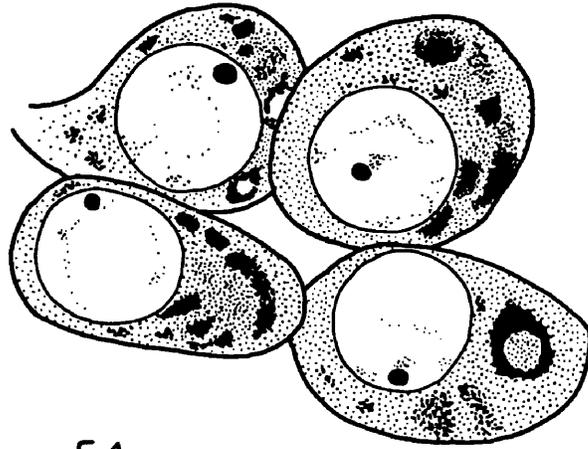
57) Late Stage 3.

56) Early Stage 3.

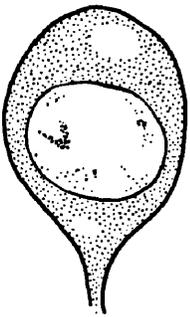
58) Stage 4.



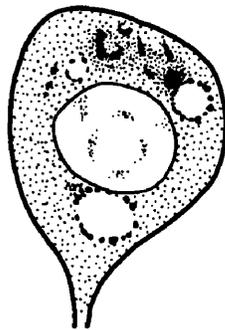
52



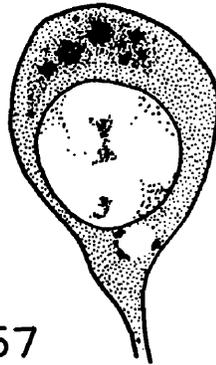
54



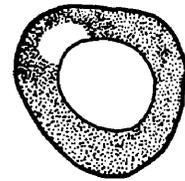
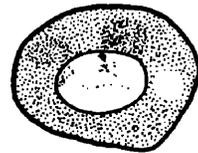
55



56



57



58

accumulation in Group 4 is simply one of increase in size and number of these blobs which by late stage 3 prove to be formed of minute purple granules of a lighter colour than those in Group 1 (Figs 47 and 53). In stage 3 the blobs tend to be associated, though not invariably, with vacuoles and in some cells the vacuoles may be entirely surrounded by a wide zone of secretory material. In others the vacuoles are ringed by distinct, rather larger, granules. Meanwhile, the material is also being discharged along the axons. The highest degree of accumulation is attained, and an almost total evacuation of this substance takes place, within stage 4 (Fig. 54) and later in this stage a few granules scattered in the cytoplasm or around vacuoles are all that remain. In stage 5 this evacuation is completed and the cell now assumes a faint uniform grey-purple colour similar to, but less distinct than, that found in cells of this group in diapause brains. Pycnotic changes in the nucleus and other signs of senescence have not been observed up to this stage in development and it is possible that this group goes through another cycle of secretion later in the pupal instar. The vacuoles which are such a prominent feature of these and other neurosecretory cells appear to remain constant in size and distribution throughout the entire cycle.

#### Group 5.

The cycle in this group is similar to that in Group 4.

No/

No colour can be observed until stage 3 (Fig. 55) when there appear round patches, of a lighter purple colour than, but similar to, the blobs of Group 4. This is succeeded by the appearance of fine granules in the cytoplasm and larger granules associated with vacuoles (Figs 56 and 57) but in stage 4 the granules disappear and the cells revert to a uniform purple colour (Fig. 58). They are no longer differentiated in stage 5. Thus the cycle is started later and is completed earlier than in Group 4.

#### Group 6.

These cells, so clearly differentiated in diapause brains, have been found to contain secretory granules in stage 3 prepupae only. The appearance is then similar to that in the diapause brain. One specimen is sectioned at such an angle that the axon can be followed for a distance equal to twice the length of the cell (c.  $40\mu$ ) and it is clearly travelling directly to the nerve to the corpus cardiacum and not via the pars intercerebralis. The cells of Group 6 are, therefore, identifiable as "lateral neurosecretory cells". The fact that they can only be found in stage 3 brains indicated a very rapid completion of the cycle of activity.

#### (b) The demonstration of prepupal neurosecretory cells by the chrome-haematoxylin-phloxin staining procedure

In this investigation brains of larvae of stages 3 and 4 were/

were examined and thus the peak of activity of each cell group was covered. Five cell groups are clearly differentiated but Group 6 is not. Dark blue secretory granules were observed in Groups 1 to 5. Group 1 cells resemble those seen in diapause brain sections treated with this stain. Group 2, unlike its diapause condition, does contain blue secretory granules though the pattern of distribution is not nearly so clear as in paraldehyde-fuchsin stained sections. Group 3 is, on the other hand, much more clearly demonstrated by this stain than by paraldehyde fuchsin and in stage 4 granules are much more abundant than the latter stain indicates. In Group 4 the blue granules are sparser and even at the peak of activity, although the cytoplasm has numerous blue-black granules in it, it retains its phloxinophil character. The phloxinophil material is not uniformly distributed but occurs in patches; it increases in quantity as the activity of the cell wanes. The faint uniform purple of the cytoplasm of these cells in diapause and stage 4 prepupal brain sections stained with paraldehyde-fuchsin therefore corresponds to the phloxinophil or acidophil condition shown by this staining procedure. This is also confirmed in Group 5. Phloxinophil inclusions do not occur in cells of the other groups.

(c) The application of histochemical tests to neurosecretory cells

I. Alcian blue; as in diapause brains the granules of neurosecretory/

neurosecretory product in Group 1 cells are, at stage 3 stained with alcian blue. This affinity is clear in sections counter-stained with neutral red but is masked to some extent by eosin. Groups 4 and 5 have eosinophil material in the cytoplasm.

II. Periodic acid-Schiff; the secretory granules of the cells in prepupal brains are not PAS-positive and therefore the material does not contain a polysaccharide component. In the cytoplasm of Group 5 cells at stage 3, patches of a positive colour occur which correspond in size, and relationship to vacuoles, to the blobs noted in paraldehyde-fuchsin sections. The material of these blobs is strongly PAS-positive but the colour is uniform and granules cannot be detected. In stage 4 brains, the cytoplasm of cells of this group is uniformly, but less strongly, PAS-positive. In Group 4 cells a weak PAS-positive colour is produced in the abaxonal zone of the cytoplasm, in stage 3 brain sections only.

Section V: Observations on the Corpora in Non-diapause LarvaeCorpus cardiacum

Secretory granules appear first in the corpus cardiacum of the post-feeding larva during stage 2 soon after the inception of activity of Groups 1 and 2. The concentration of this material in the organ increases in the successive prepupal stages. The product enters the organ in a finely granular state transported in the axoplasm of the axons of the NCC. These axons penetrate between the cells of the corpus and ovoid bodies form, swellings of nerve fibres caused by local accumulations of neurosecretory material (E. Thomsen, 1954). These are small at first but increase later in size. They contain aggregates of larger granules than those seen in transit so, presumably, the reverse of the process inferred in the description of Group 1 cells takes place. The material is abundant near the lumen of the aorta and in several specimens it can be seen quite clearly to pass through the wall of the vessel into the blood. This distribution of granules was confirmed in paraldehyde-fuchsin and in chrome-haematoxylin stained sections. It cannot be determined by histological methods whether the corpus cardiacum is modifying the product before releasing it into the blood. Indeed it is not known if the stainable material is the active principle or only a carrier substance.

Gabe (1954), summarising his investigations in a number of/

of insect orders, describes a secretion of the large cells of the corpus cardiacum which is reddish with paraldehyde-fuchsin stain or phloxinophil but the fact that chromophile cells are coloured in this way is no proof of secretory activity. One cannot decide by these techniques whether the corpus cardiacum is simply a neurohaemal organ or whether it also performs an endocrine function. Evidence in favour of a dual role was offered by Wigglesworth (1954b) and by Herlant-Meewis (1956).

### Corpus allatum

There was no evidence of the receipt of neurosecretory product by the corpus allatum in brains of any of the stages examined. Caution must be exercised in interpreting results of paraldehyde-fuchsin staining of moulting insects since the chitin of tracheae which has been attacked by moulting fluid and the chitin precursor in the tracks of new tracheae are strongly coloured by the purple stain, since the same reactive groups are presumably present in each case. In the stage 5 corpus allatum purple granules can be seen which at a cursory glance might be thought to be neurosecretory in origin but which prove in fact to be products of the breakdown of the intima of the transverse anastomosing trachea. It is not intended that this statement should be interpreted as casting doubt on observations of the receipt of material by the corpus allatum in other orders made by other workers. It is merely meant to emphasise that such receipt is not observable in this species/

species during these stages; to this can be related the fact that an augmentation of the volume of the organ in the prepupa is not observed.

Section VI: A Note on Neurosecretory Cells in Brains of Larvae at the Termination of Diapause

Mitoses were observed in paraldehyde-fuchsin stained sections of two brains of larvae which had been in diapause for six weeks. In these, it can be assumed, diapause had just terminated. The only groups differing from their diapause appearance were Groups 4 and 5. The colour of the abaxonal zone of Group 4 cells is no longer uniform. There are large, deep purple blobs which appear to coalesce and granules are also evident. In Group 5 there is a high density of fine granules similar to the late stage 3 appearance in a non-diapause larva. There is a marked increase in the amount of neurosecretory product in the corpus cardiacum but not in the size of that organ. It can be concluded that at the termination of diapause cells of Groups 4 and 5 are activated.

Conclusions

The classification of the brain neurosecretory cells of L. caesar larvae into six groups as proposed in Section III is justified by these observations. New criteria, by which the groups can be identified, are established from the information on the various ways in which product accumulated in the different groups, the differences in the timing of activity of the groups and the added information on staining characteristics.

Groups 1, 2, 3 and 4 certainly belong to the category of "medial/

171.

"medial neurosecretory cells" since their axons traverse the pars intercerebralis into the opposite hemisphere. The course of the axons of Group 5 cells could not be followed but Group 6, whose axons pass directly to the nervus corporis cardiaci of the same hemisphere as the cells of origin, can now be identified as "lateral neurosecretory cells". Since Group 5 lies near Group 4 and is separated by the entire medulla from Group 6, it seems likely that 5 belongs to the first category.

The cycle of secretion in these cells starts with the appearance of a few granules scattered in the cytoplasm (Group 2) or associated with the prominent vacuoles found in neurosecretory cells (Groups 1, 3 and 6) or developing from uniform "blobs" (Groups 4 and 5). The subsequent increase in product concentration follows a pattern in each group characteristic of that group. Accumulation and discharge are for a time synchronous processes. Shortly after the first granules are detectable in a cell they appear in the axon and in the corpus cardiacum. Product occurs in that organ in stage 2 larvae just after the start of accumulation in the two earliest Groups, 1 and 2. The possibility that a cell group is exerting its effect as soon as that group starts to produce its secretion cannot be dismissed.

A physical change in the product, or in the carrier substance of the biologically effective product, takes place when this/

this stainable material passes into the axon, the granules in transit being much smaller than those in a cell, but reformation of larger granules takes place again in the corpus cardiacum. That organ is responsible for storage and release of neurosecretory material. In the first phase of a cell's activity the concentration of product in the cell increases. In this phase the rate of accumulation must exceed the rate of discharge. In the second phase the concentration of product decreases, the rate of discharge increases and production ceases. At the end of the prepupal cycle the cell may revert to its original state (Groups 4, 5 and possibly 6) or it may become senescent with characteristic irregularity of outline, shrinkage in size and pycnosis of the nucleus (Groups 1 and 2).

At the termination of diapause there is a marked change in the appearance of Groups 4 and 5 only. They assume an appearance similar to that found in stage 3 non-diapause larvae from which it is concluded that the termination of diapause and the activation of Groups 4 and 5 are related phenomena.

M. Thomsen (1954) discussing neurosecretory material, in Hymenoptera, stained with chrome-haematoxylin-phloxin, advanced the theory that in certain cells the granules of product become subdivided the product thereby assuming colloidal properties and thus may be connected with the change in staining properties from blue to red' but in Groups 4 and 5, at the cessation of diapause, the reverse is apparently true, the particles of the/

the "colloid" appearing to coalesce to form granules. The timing and duration of activity of each group in non-diapause larvae is illustrated diagrammatically in Fig 58a. The point of origin of the line representing each group indicates the time at which product first appears in cells of the group. The ascending line, apex and descending line illustrate the build-up, climax and decline in activity of a cell. The possible function of certain groups is discussed in the final part of this thesis.

The results of the histochemical tests again controvert Gabe's (1954) general statement. There is a transient appearance of PAS-positive material in Groups 4 and 5 but in none of the cells can it be asserted that the product has a "glucide" component.

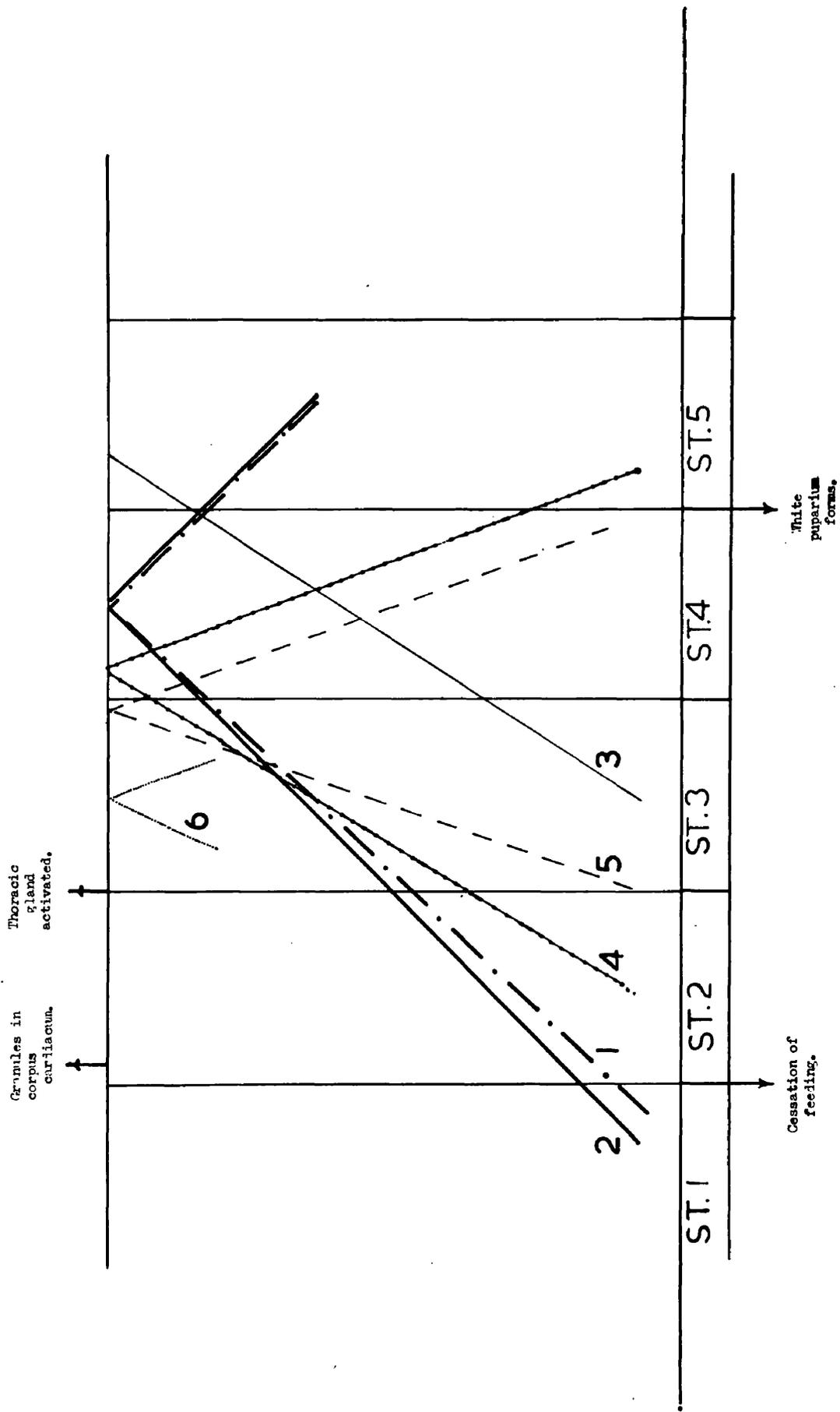


Fig. 58a.

Section VII: Neurosecretory Cells in the Abdominal Ganglia of  
Lucilia caesar

Accounts of neurosecretory cells in the ventral ganglia of insects are exceedingly rare compared with the numerous descriptions which exist of such cells in insect brains.

First noted in the ventral ganglia of Lepidoptera by Day (1940) and described in cockroaches by B. Scharrer (1941), their occurrence has since been observed in the sub-oesophageal ganglia of Ephemeroptera (Arvy & Gabe, 1952a, b), of Odonata (Arvy & Gabe, 1952c), of Tenebrio (Arvy & Gabe, 1953g), of Bombyx mori (Bounhiol, Arvy & Gabe, 1953) and of Iphita (Hemiptera) by Nayar (1955). Nayar (1953) mentions their occurrence in the thoracic ganglion of Iphita. Lhoste (1953) indicated that the ganglia of the ventral nervous system of Forficula contained neurosecretory cells but did not describe them. Wigglesworth (1955), discussing humoral stimuli concerned in the breakdown of the thoracic gland of Rhodnius suggested that 'perhaps the neurosecretory cells which are so conspicuous in the thoracic and abdominal ganglia of Rhodnius and other insects may be concerned'.

Neurones are identified, in this study, as neurosecretory cells by their morphological similarity to certain brain cells of this kind, by their staining characteristics and by the evidence in some of axonal transport of secretory granules.

Material and methods/

## Materials and methods

Since the abdominal, thoracic and sub-oesophageal ganglia are fused into a single ganglionic mass which is united with the cerebral hemispheres it is simple when preparing brain sections to produce sections of these ganglia at the same time. Sections of the ganglia of diapause larvae were stained with chrome-haematoxylin-phloxin and paraldehyde-fuchsin. Histochemical tests employed were alcian blue with neutral red or haemalum-eosin counterstaining, periodic acid-Schiff with and without pretreatment of sections with diastase, McManus' Sudan black and toluidin blue for metachromasia. Ganglia of non-diapause prepupae, of the five stages described in Section IV of this chapter, were stained with paraldehyde-fuchsin and the PAS test was applied to these.

## Location of cells in ventral ganglia

Neurosecretory cells could not be identified in the sub-oesophageal or thoracic ganglia of Lucilia larvae. A few phloxinophil cells were seen in the sub-oesophageal ganglia of diapause larvae. These might have been, like brain Group 4 cells, in a resting state but no evidence of a secretory function was produced from sections of prepupal ganglia. Neurosecretory cells occur in abdominal ganglia 1 to 5. These are pyriform unipolar neurones with the axon continuing from the conical end of the cell. They measure 20 to 25  $\mu$  long by 10 to 14  $\mu$  wide and have a central ovoid nucleus 8 or 9  $\mu$  long. They/

Fig. 59. Transverse section of the first abdominal ganglion of a larva of L. caesar showing location of neurosecretory cells. (From a camera lucida drawing.)

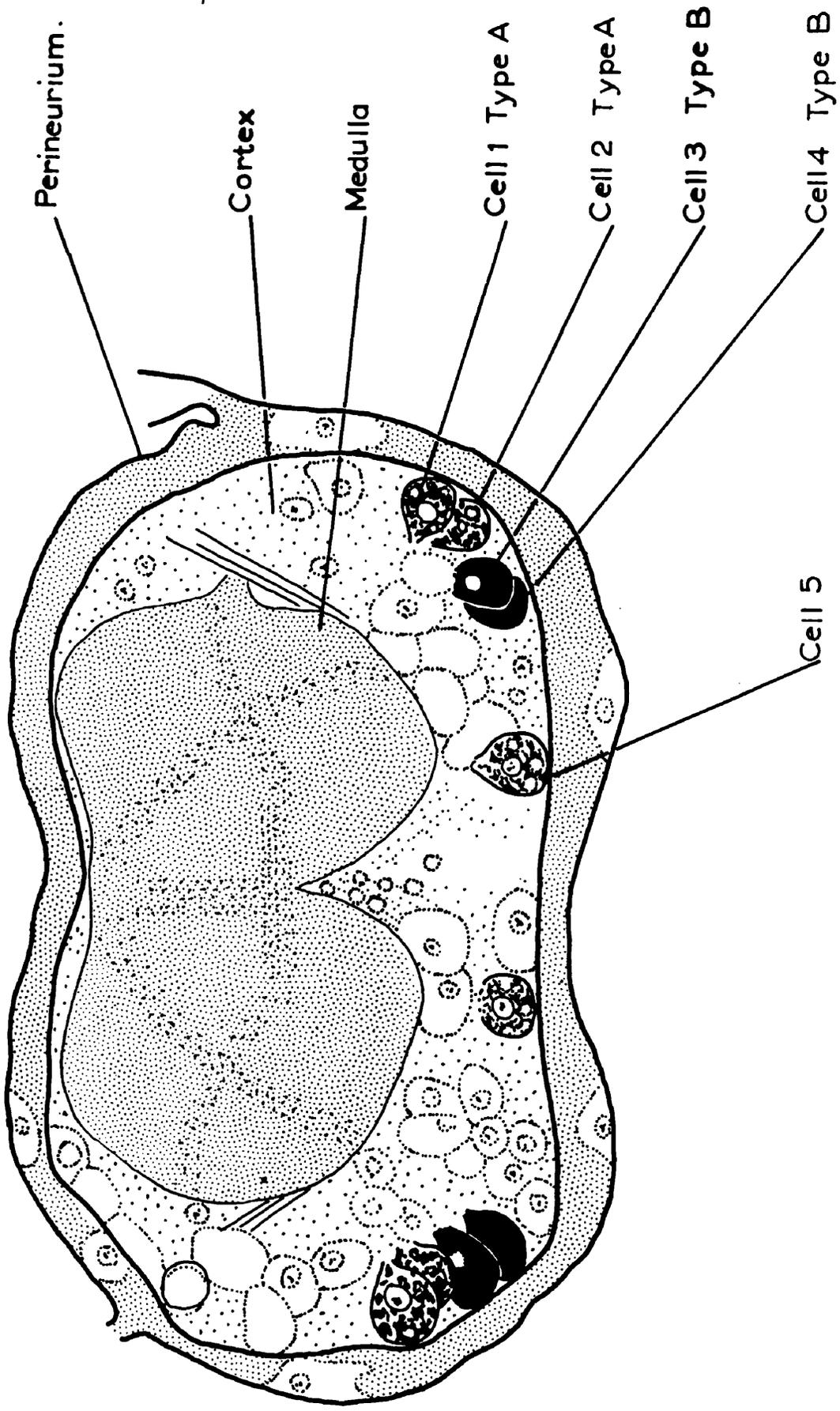


Fig. 59.

They are symmetrically arranged in each ganglion (Fig. 59). Four lie on each side at the ventro-lateral angle of the ganglion, and one on each side in a ventral position (Cell 5). The four lateral cells can be separated into an upper posterior and a lower anterior pair. Activity of the corresponding cells on each side is normally, though not invariably, in phase. Of the lateral four, two can be described from their staining characteristics as Type A. Granules of product are demonstrable in them. The other two can be considered as Type B and do not have a granular product. The Type A cells are usually the upper posterior pair (Cells 1 and 2) and the Type B cells the lower anterior pair (Cells 3 and 4) but any pair may be of the first type and, rarely, three of the four cells will be of one type (see Table XIX).

#### Neurosecretory cells in abdominal ganglia of diapause larvae

##### (a) Cells stained with paraldehyde-fuchsin

Granules of a product staining the characteristic purple colour are found in each Type A cell and in the ventral cell 5. In cell 5 the granules are relatively small (up to  $0.5\mu$ ) discrete and distinct. In general facies it resembles a brain cell belonging to Group 6. In some of the Type A cells there is a high concentration of minute granules (about  $0.25\mu$ ) while in the others the granules are large (up to  $0.75\mu$ ) and very distinct, but the total quantity of product is probably less in the/

the latter. In some cells of the latter kind these granules are discrete and dispersed while in other irregular aggregate masses as large as  $4 \mu$  by  $2 \mu$  may be found along with discrete granules. The association of granules with vacuoles, recorded in most groups of brain neurosecretory cells, is noticeable in the Type A cells. The other two cells are Type B, usually cells 3 and 4, and contain no purple granules. The cytoplasm assumes the normal grey colour of common neurones and there are several large vacuoles in each cell. Rarely these cells have a faint purple tinge like that of Group 4 brain cells of diapause or stage 5 larvae.

(b) Cells stained with chrome-haematoxylin-phloxin

With this procedure the cells described as Type B are strongly phloxinophil. The colour is remarkably uniform over the whole cell, except for the nucleus and vacuoles. They closely resemble brain Group 4 cells in the diapause state.

The Type A cells may contain very distinct blue-black granules with the rest of the cell, apart from the nucleus, completely unstained. Alternatively, the granules may be minute and less easily discernable against the grey purple colour of the ground cytoplasm. The cells with distinct large granules detected by both staining procedures undoubtedly correspond. As noted above the A cells are not always cells 1 and 2. Aggregates of granules as seen in paraldehyde-fuchsin stained/

stained cells are not seen in these sections. Abundance and size of granules may vary but they always appear to be separate. The ventral cell can be located but it is not identifiable as a neurosecretory cell by this staining method; this is also true of Group 6 brain cells.

(c) Histochemical tests

Results with alcian blue were inconclusive. The secretory granules in Type A cells do not have a selective affinity for this dye. Material is found in the lateral cells which is PAS-Positive. Pre-treatment with diastase proves that carbohydrate component is other than glycogen. The reaction is obtained in some cells from uniform patches which are weakly positive. In others there are minute granules which are distinctly positive and in a third category of cells there are large strongly positive granules and aggregates of granules. PAS-positive granules occur around vacuoles and dispersed in the cytoplasm. PAS-positive material is normally found in two of the lateral cells but not in the other two lateral nor in the ventral cell. The two cells containing the positive substance may match or may have the material in two different states. There can be no doubt that this reaction is given by the granular neurosecretory product demonstrated by the selective stains. The product of these cells has, therefore, a 'carbohydrate' component other than glycogen.

With Sudan black B staining two of the lateral neurones are/

are found to contain numerous sudanophil granules. The other two cells have a relatively uniform grey ground cytoplasm containing few scattered sudanophil droplets. Sudanophil granules occur in the large ventral neurones but the form, size, concentration and distribution of these are quite different from these features in the first two lateral cells. The ventral cell 5 is not differentiated. The product of the Type A cells is thus thought to have a lipid component.

With toluidin blue the Type A cells stain orthochromatically, containing abundant blue granules. The Type B lateral cells stain metachromatically assuming a uniform violet colour (beta metachromasia). The cytoplasm of the B cells contains an appreciable concentration of a substance not found in any other neurones in the ganglia. This substance cannot be a mucoprotein or mucopolysaccharide as it is not PAS-positive.

Combining the information on Type A neurones it can be said that the product contains a diastase fast PAS-positive substance. It also contains material which is sudanophil and which does not exhibit metachromasia with toluidin blue. These results are consistent with the presence of a glyco- or phospho-lipid or a lipoprotein.

#### Cells in the abdominal ganglia of non-diapause larvae

These were studied in sections stained with paraldehyde-fuchsin. In early stage 1 larvae no neurosecretory activity is/

is evident. In ganglia of late stage 1 only two or four Type A cells can be identified. A few scattered granules or small aggregates can be seen in each cell. The background cytoplasm has the grey colour of that in common neurones. Type B cells can be identified by position and morphology but are not selectively coloured. In stage 2 the Type A cells are more numerous and show various stages in accumulation of the product. In the third stage Type A cells, usually cells 1 and 2, contain abundant secretory granules. In some cells these form aggregates of varied size; in others the total amount of material appears to be less and the granules are separate. Cells in the ventral position are rarely seen to contain distinct positive granules though the cytoplasm in some has a faint purple colour. In one or two series of sections of ganglia at this stage the full complement of 20 Type A and 20 Type B cells can be counted, distributed eight in each abdominal ganglion from the first to the fifth. Only three or four cells with positive granules can be seen in the ventral position. The maximum number of Type A cells is seen in stages 3 and 4. In the latter these cells are mostly of the kind containing large discrete granules, 0.5 to 0.75  $\mu$  in size, which do not aggregate. Type A cells have been observed in non-diapause ganglia in positions 1 and 2, 1 and 3, and 3 and 4, the other two laterals being in each case Type B cells. In the final stage large distinct purple granules are still abundant in some cells but the product is rather sparse in most./

most. The number of Type A cells diminishes in this stage; in one count only 16 were noted, two ganglia having only a single one on each side.

A confirmatory check was made on sections of larvae of stages 3 and 4 which had been stained with chrome-haematoxylin-phloxin. As expected the granules of product in Type A cell are coloured blue-black and the cytoplasm of Type B cells is phloxinophil.

Sections of ganglia of larvae at different stages were stained with alcian blue-neutral red, alcian blue-haemalum-eosin and by the periodic acid-Schiff procedure. Only the last gave conclusive results. The distinct granules of the Type A cells are, as in diapause larvae, strongly PAS-positive.

### Conclusions

The axons of the neurosecretory cells in the abdominal ganglia take a circuitous route when they enter the medulla and it has not been possible to trace them into the nerves of the ganglia. Granules are to be seen in axons in numerous sections but the destination of this material is unknown.

The two variants of Type A cells could be functionally different cells but they probably represent different phases of a secretory cycle in similar cells. It is not certain whether Type B cells represent (a) a third phase in the secretory cycle of the Type A cell or (b) a functionally different kind of cell which/

which is either inactive in prepupae, in diapause or developing larvae, but due to function later in the individual's life or active but producing a non-granular or colloid phloxinophil product. It seems, however, reasonably certain that the Type B cells are in a resting state. While some may lack a distinct uniform purple colour after paraldehyde fuchsin staining all are strongly phloxinophil matching in this respect Groups 4 and 5 in the diapause brain.

The Type A cells and the cells in position 5 are in a state of monotonous activity during diapause. While the latter are rarely seen in the ganglia of non-diapause prepupae the former go through a cycle of activity which appears to be synchronous with the pre-pupation phase of activity in brain neurosecretory cells but whether these cells in the abdominal ganglia play any part in the promotion of metamorphosis remains to be determined.

Section VIII: The Neurosecretory Cells in the Brain of the Larva of Calliphora vomitoria

Materials and methods

Serial sections were prepared of the brains of C. vomitoria larvae killed at different stages in development in the third instar from the cessation of feeding to puparium formation. These were stained by (a) a routine haemalum-eosin procedure, (b) paraldehyde-fuchsin after permanganate oxidation.

Location and identification of groups

Group 6, observed in Lucilia was not detected in Calliphora. In the former species it is thought to have a brief phase of activity in the prepupa and it is possible that this was missed in the latter. The location of the other groups was the same as in Lucilia.

The following descriptions refer to cells of one hemisphere only.

Group 1.

This consists of two large cells lying dorsally close to the anterior limit of the junction of the hemispheres. In a larva at the stage after the cessation of feeding when crop evacuation has just been completed the cell measures about 30 μ long by 18 μ wide with a spherical or ovoid nucleus of a maximum length of 12 μ. At this time the neurosecretory product, which stains a deep purple with paraldehyde-fuchsin, is/

is already abundant as shown in Fig. 60. The particles of the product form granules of limited size. There are a number of large vacuoles in the cell and the product appears to be most highly concentrated around these. As this material increases in amount large quantities accumulate around the vacuoles, as shown in Figs 60 and 61, but the droplets or granules remain limited in size and do not fuse to form larger masses. As in the non-diapause Lucilia larva accumulation of the product, commencing at the cessation of feeding, continues right through the pre-pupation phase and is accompanied for a part of this time by an increase in cell size. The time at which discharge of product from these cells commences is not certain but fine granules of product are detectable in transit in the nerves to the corpus cardiacum and in that organ soon after the completion of crop evacuation. At this time the only possible sources of this material are cells of Groups 1 and 2 (see below) and it seems likely that product is being passed out of a cell almost from the moment at which production starts. The increase in cell size during the prepupal phase indicates that the rate of production is for a time more rapid than that of discharge.

#### Group 2.

This is represented by a single cell in each hemisphere lying anterior to and lower than Group 1, near the lower limit of the pars intercerebralis. Early in the post-feeding stage such/

such a cell measures 25  $\mu$  long by 18  $\mu$  wide with a nucleus similar in size to that of a Group 1 cell. The cell does not increase markedly in volume with accumulation of the product. This material does not originate in association with vacuoles. At first a few granules are seen scattered in the cytoplasm, larger than the individual granules in Group 1. (Fig. 63). Soon these granules come together to form larger masses (Figs 63 and 64) until the whole cell is filled with large aggregates of the product separated by clear areas of cytoplasm, though the material entering the axon and in transit reverts to a finely divided state. It seems likely that for a part of its phase of activity, which lasts until puparium formation, the processes of production and discharge of the secretion are going on at the same time.

#### Groups 3 and 4.

The cells composing these two groups intermingle. They lie in a postero-lateral position relative to Group 1. In a dissection of a living specimen of a pre-pupation larva these form an easily detected bluish patch lying in the dorsum of each hemisphere. The two groups are not so easy to distinguish from each other as in Lucilia in which larva there is greater contrast in morphology and in phases of activity. In Calliphora accumulation commences in these cells slightly later than in Groups 1 and 2 and appears to be more rapid in Group 3 than in Group 4 (Fig. 65). In Lucilia in contrast activity in Group 3/

Figs 60 - 67. Neurosecretory cells in brains of larvae of C. vomitoria at different stages of development; after paraldehyde fuchsin staining.

60) Group 1.

61) Group 1.

62) Group 2.

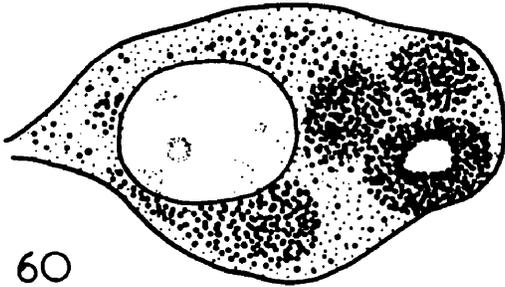
63) Group 2.

64) Group 2.

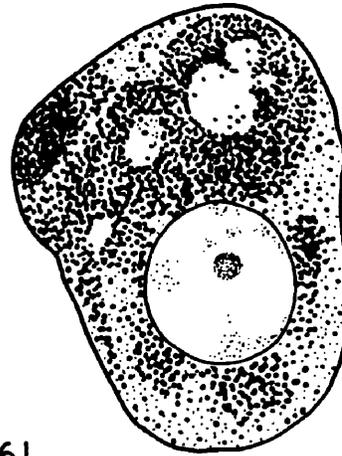
65) Group 3 (two upper cells) and Group 4  
(two lower cells)

66) Group 4.

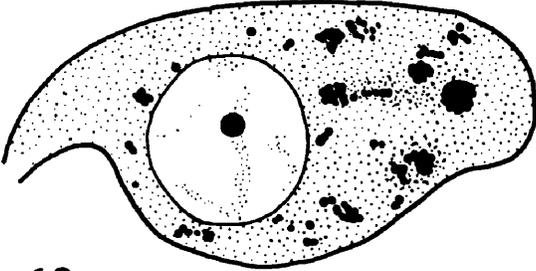
67) Group 5.



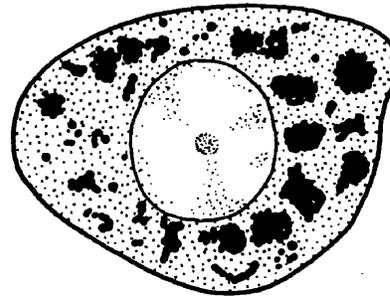
60



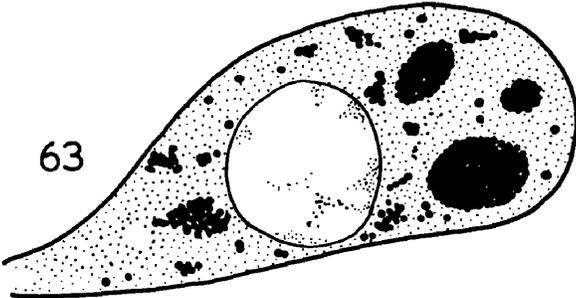
61



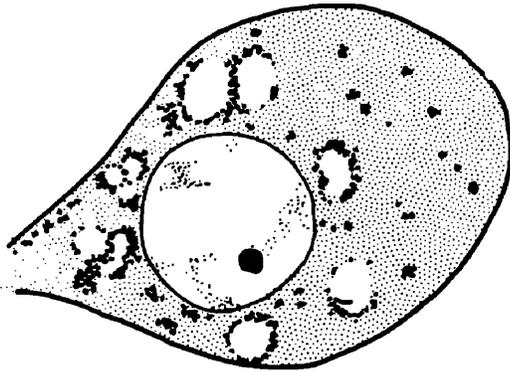
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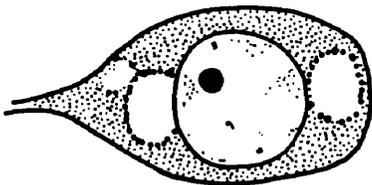
64



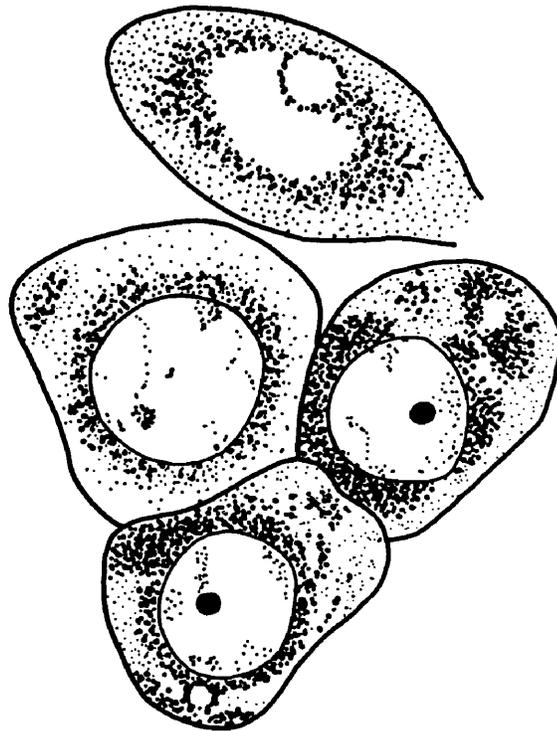
63



66



67



65

Group 3 lags behind that in Group 4 in non-diapause larvae. The Group 4 cell shown in Fig. 66 measured 35  $\mu$  long by 23  $\mu$  wide with a spherical nucleus 11.5  $\mu$  in diameter. The association of the product with vacuoles is evident.

#### Group 5.

This represented by a few small cells, of average size 18  $\mu$  long by 12  $\mu$  wide with a nucleus 8.5  $\mu$  in diameter, lying lateral to and behind Groups 3 and 4. These cells do not present such a clear picture of accumulation and discharge of product as do their homologues in Lucilia nor was the product abundant in any of the brains sectioned. In the cell illustrated in Fig. 67 the association of product and vacuoles is again evident.

#### Conclusion

While the existence of Group 6 in the brain of the Calliphora larva must remain in doubt for the moment the presence of the other five groups in the same loci as in Lucilia has been confirmed. It has also been confirmed that the mode of accumulation of the product in each of these groups is similar to that in Lucilia though there may be a difference in the sequence of activity of Groups 3 and 4.

## Discussion

During the feeding phase of the third instar of the blowfly Calliphora vomitoria and until the end of the first post-feeding phase, in which digestion of crop and gut contents is completed, growth by cell multiplication in the anlagen is independent of a humoral stimulus but in the resting mature larva this growth is promoted by a humoral factor. In the absence of this factor development of imaginal discs halts just after the cessation of feeding. The large lateral cells of Weismann's ring are the source of this hormone and constitute a gland which, on the basis of histological and functional identity, can be homologised with the "thoracic glands" of other insects. This endocrine secretion also promoted puparium formation and the two intrapuparial moults which produce first the evanescent fourth larval instar and then the true exarate pupa. Cyclorrhaphan larvae appear to be unique among insects in that a single cycle of thoracic gland activity promotes two successive moults. For these changes to take place the concentration of hormone in the larval blood is sufficient, about twenty-four hours before without further supply from the gland, puparium formation occurs.

The brain has a role in the process of pupation as the stimulator of thoracic gland activity. Since both extirpation of the ring and section of the nerves from the brain to the corpus cardiacum (NCC) halt anlagen development and prevent pupation/

pupation it is evident that the brain must stimulate thoracic gland activity, the stimulus being transmitted via the NCC. Histological study of these organs at the time of thoracic gland activation reveals that neurosecretory product originating in the brain neurosecretory cells is being passed along the axons of the cells, the axons which form the NCC. The destination of this product is the corpus cardiacum from which it is liberated into the lumen of the aorta. There is no evidence of product passing directly, via these nerves, to the thoracic glands. The brain stimulus is humoral and is liberated into the blood from an intermediary **neurohaemal** organ. The nerve connection between the brain and the corpus cardiacum must be intact up to forty-eight hours before pupation is due to occur but after that time nerve section does not block pupation since the thoracic glands have become capable of independent activity. Before this time, as observations of anlagen development indicate, thoracic gland hormone is being produced. Thus there is a first dependent but active phase and a second independent active phase in the activity of that gland. Results of experiments involving implantation of C. vomitoria thoracic glands into Lucilia caesar larvae prove that ultimate gland autonomy is not simply due to the concentration of thoracotropic hormone having reached a critical concentration in the insect's blood. The gland becomes capable of continued action in the absence of any stimulus.

It has now been established, for the first time in a Dipteran/

Dipteran insect, that the thoracic gland is inactive during the prepupal diapause in L. caesar. There are several items of evidence for this statement. Firstly, the growth of thoracic gland nuclei halts and these remain constant in size throughout diapause in contrast to their continued growth in non-diapause prepupa. Secondly, the gland cells actually shrink in size at the onset of diapause in contrast to their characteristic hypertrophy in the non-diapause prepupa. Thirdly, the arrest of development of anlagen at the onset of diapause is comparable to that in C. vomitoria larvae in which nerve section, just after the cessation of feeding, has blocked thoracic gland activation the arrest in each case being attributable to the fact that intrinsic control of anlagen development has ceased but the extrinsic growth promoting factor is not present to continue the process. The gland implantation experiments confirm these conclusions and it is notable that the hormone from gland implants not only promotes the immediate resumption of anlagen development in the recipient but also activates its thoracic glands and those brain neurosecretory cells (see below) which have been in a dormant state. The diapause brain can thus be activated by a humoral stimulus.

There is no experimental evidence for the existence of a diapause hormone with the positive role of inhibiting development. Hinton's (1954) postulate that such a hormone exists in insect larvae but that it is not demonstrable since it is blocked/

blocked by thoracic gland hormone can, in obedience to the principle of economy of hypotheses, be rejected on present evidence.

Brain neurosecretory cells have now been described in a large number of pterygote insect species. Most authors are agreed that there are two groups of these identifiable in each hemisphere of the brain, a median group lying close to the pars intercerebralis and a lateral group. In larvae of Diptera - Cyclorrhapha descriptions exist of the former only (see M. Thomsen, 1951).

Observations have now been made of the neurosecretory cells of the brain of the larva of L. caesar. Serial sections of brains of larvae in diapause and at five stages in development without diapause, from feeding third instar to cryptocephalic pupa, have been stained with chrome-haematoxylin (Gomori, 1941) and paraldehyde-fuchsin (Gomori, 1950), procedures known to colour selectively the stainable product of neurosecretory cells. Carbohydrate and fat staining techniques were also used in the hope of identifying components (particularly polysaccharide) of the product of the cells. As a result it has now been demonstrated that in the brain of the L. caesar larva there are no fewer than six groups of neurosecretory cells distinguishable by criteria of position, size, morphology and staining characteristics. The cytochemical techniques, while emphasising/

emphasising the chemical differences between groups and the identity within groups, show that the product does not contain a polysaccharide component demonstrable by these procedures. This observation is in agreement with that of Wigglesworth (1955) and controverts Gabe (1954) who claimed that there is detectable in the product a "glucide" other than glycogen.

In all the neurosecretory cell groups the granules of product in the cells are larger than those in transit in the axons. There is an apparent physical change, reflecting a presumed change at the chemical level, in the product during transit. This process is reversed in the corpus cardiacum where larger granules reform. The chemical differences between cell groups are also reflected in the physical differences in size and mode of accumulation and aggregation of the particles of product in the different groups. Since all the cell groups go through a cycle of activity in the prepupa it is certain that they are all concerned in the control of physiological processes associated with moulting and metamorphosis. Accumulation starts in the cells of Groups 1 and 2 at the cessation of feeding and continues in the non-diapause larva, with consequent distention of the cells, almost to the time of puparium formation when there is a rapid discharge of product accompanied by signs of cell death. In diapause larvae cells of Groups 1 and 2 are packed full of granules of product and closely resemble their state just prior to the total discharge in/  
in/

in non-diapause prepupae. A few small granules are found in the axons of these cells during diapause and during the accumulation phase in non-diapause brains. It is impossible to determine whether this product is passing out in sufficient quantity to be physiologically effective in diapause larvae or in the first non-diapause prepupal stages. The material in the axons at these times may represent a casual leakage, in ineffective amounts, of small particles carried from the cells in the axoplasm stream. Since thoracic gland activation occurs before any group has reached a climax of activity it must be promoted initially by relatively small amounts of the secretory substances (collectively to be considered as "thoracotrophic hormone") of the particular cell groups involved although independent activity of the gland is probably not established until the responsible cells have reached such a climax. Product may, therefore, be effective in small amounts.

Group 3 cells appear to be in a state of monotonous activity during diapause, most of the secretory material found in the corpus cardiacum during the dormancy phase apparently originating in these cells. In non-diapause larvae activity does not commence in this group until some time after the thoracic gland has been activated, and reached a peak after the formation of the puparium. This group, therefore, can be eliminated as the possible source of thoracotrophic hormone but it certainly has a definite function during diapause. If, as Hinton/

Hinton suggests, a "diapause hormone" does exist then this group is the likeliest source.

There is a marked resemblance in certain staining characteristics between Groups 4 and 5 but apart from the great morphological dissimilarity there are chemical differences, for example in lipid content during diapause and while both groups are patently inactive during diapause in the non-diapause larva Group 5 is activated after Group 4 and reaches its peak of activity and wanes before Group 4. It seems significant that Group 5 does not become active until after the first signs of thoracic gland activity and it can, therefore, be eliminated as the source of the initial thoracotrophic hormone. Group 4 is inactive during diapause when thoracic glands are inactive but becomes active at the cessation of diapause and in the non-diapause larva before thoracic gland activation. Group 4 is thus the most probable source of the humoral stimulant of that gland.

Group 6 appears to be, like Group 3, in a state of constant activity during diapause. In the developing prepupa it has a brief cycle of activity which is only evident after thoracic gland activation but since Williams (1948a) has proved that both median and lateral cells participate in that process and since Group 6 cells are the only ones identifiable as lateral neurosecretory cells in these larvae the possibility that their secretion/

secretion plays a part in the stimulation of that gland cannot be ignored.

There is as yet insufficient evidence on which to base conclusions about which group or groups activate the thoracic glands but it does seem probable that several participate in this with one, Group 4, initiating activity and others, Groups 5 and 6 at least, acting in the establishment of thoracic gland autonomy. The participation of Groups 1 and 2 seems less likely in view of their state during diapause and of the fact that they do not reach their peak of activity until after the establishment of thoracic gland autonomy. It can, however, be stated that the diapause state in the larva of L. caesar is the direct result of the failure of certain brain neurosecretory cells to activate the thoracic glands whose secretion promoted the completion of development of anlagen, puparium formation and pupation.

With the development of a microcautery technique applied to a more suitable experimental animal it should soon be possible to elucidate the physiological role of certain of the neurosecretory cell groups now that it has been established that median neurosecretory cells are not a single homogeneous group. There is no doubt that progress has been hampered by the failure of the many investigators in this field to appreciate this fact. Any descriptions of the secretory cycle which have previously been/

been produced are unsatisfactory since the varied aspects of different groups have been equated with successive stages of the secretory cycle of a homogeneous group and the assumption has been made that the varied properties of different groups are common to all neurosecretory cells.

A histological study of the brain of the larva of C. vomitoria confirms the existence of the five groups of median neurosecretory cells found in L. caesar. The lateral cells were not distinguishable from common neurones probably because their rapid secretory cycle was missed in the series of brains sectioned.

In diapause and non-diapause larvae of L. caesar there is no evidence of receipt of neurosecretory material by the corpus allatum. The reduction in size of the organ during diapause may be interpreted as indicating that the organ has lapsed into inactivity while in the late feeding larvae and in non-diapause prepupae the constant size may indicate a steady rate of activity, the corpus allatum hormone governing the rate of development of anlagen. Anlagen growth having halted in anlagen during diapause the corpus allatum is not required and the activity of the organ is suppressed by some mechanism which is probably humoral.

No evidence of multiple function of the corpus cardiacum has been produced by these investigations, its only certain function/

function being as a neurohaemal organ. It is smaller in the larva at the cessation of feeding than in the diapause larva, a fact related to the absence in the former and the presence in the latter of neurosecretory product but the enlargement of the organ is not solely due to the accumulation of product, the cells of the organ, chromophile and chromophobe, themselves increasing in size. In the non-diapause prepupa there is a great increase in the volume of the organ related to the increase in neurosecretory activity.

At the cessation of feeding whether the larva of L. caesar develops directly or enters diapause the neurosecretory system is alerted and the cells which have only been distinguishable from common neurones by position and form during the feeding phase assume distinct staining characteristics. This is equally true for those groups which in either type of post-feeding larva have a granular product and for Groups 4 and 5 which in diapause assume the appearance interpreted as the resting state. The completion of feeding and the changes in neurosecretory cells are related processes. A relationship between feeding or nutrition and the hormone cycle has long been known in insects (Wigglesworth, 1933 et seq.). It could be postulated that in a blowfly larva which stops feeding voluntarily the same mechanism which terminates feeding activity also alerts the brain neurosecretory system but if the larva of a non-diapause species such as C. vomitoria is removed from the food prematurely/

prematurely the duration of the post-feeding pre-pupation period after this non-voluntary cessation of feeding is the same as that after voluntary cessation. Therefore, the mechanisms which normally cause the larva to stop feeding voluntarily and those which activate the brain secretory cells must be separate.

At the cessation of feeding crop contraction, as its contents are evacuated without replenishment, commences. After the commencement of crop contraction come the first signs of neurosecretory cell activity. It can be concluded that the mechanism activating the neurosecretory system is directly related to the facts that feeding has ceased and the crop is contracting. Lees (1955) favoured the theory that secretion of brain hormone is induced by nervous stimuli but Munro (1956) has recently produced good evidence that in the larva of a Noctuid, Phalaenoides, the stimulus is humoral. Either is possible. It does seem significant that the stomatogastric nervous system is connected by nerves from the hypocerebral ganglion to the corpus cardiacum which in turn has nerve connections to the brain. The fact that feeding has ceased may thus be recorded by the stomatogastric nervous system, perhaps as a result of release of tension in the crop, this system then providing either a humoral stimulus liberated via the neurohaemal organ or a nervous stimulus transmitted directly to the brain. The stimulus, humoral or nervous, activates the neurosecretory/

neurosecretory system.

If environmental conditions are favourable during the time when crop evacuation is being completed and the neurosecretory system is being activated, in the L. caesar larva, the neurosecretory cycle is completed directly in all cell groups but if conditions are unfavourable then activity almost ceases when accumulation of product is complete in Groups 1 and 2, activity halts in Groups 4 and 5 before the formation of a particulate product while Groups 3 and, perhaps, 6 settle down to continuous production. It appears that another mechanism has come into operation blocking the cycle in certain groups while permitting continuous activity in others, the result being that the thoracic glands remain inactive during diapause. There is distinct evidence (Cragg & Cole, 1952) that the sensitivity of this mechanism is maternally determined.

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