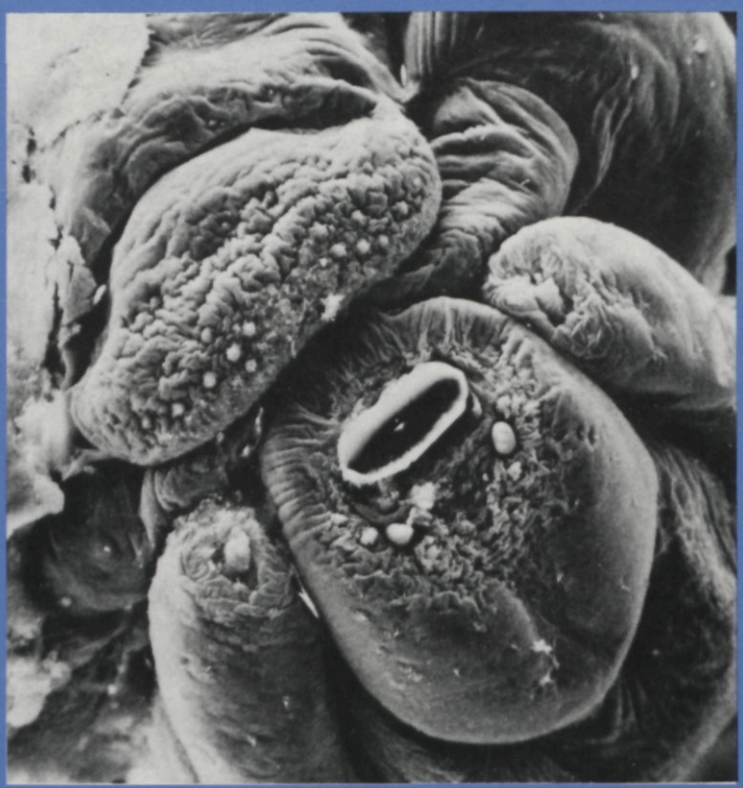
REGULATION OF CASTE DIFFERENTIATION IN THE HONEY BEE (*APIS MELLIFERA* L.)



E. A. GOEWIE

REGULATION OF CASTE DIFFERENTIATION IN THE HONEY BEE (*APIS MELLIFERA* L.)

Promotor: dr. J. de Wilde, hoogleraar in het dierkundig deel van de planteziektenkunde

595.799:591.13:591.147:591.151.3
638.12:591.151.3

E. A. GOEWIE

REGULATION OF CASTE DIFFERENTIATION IN THE HONEY BEE (*APIS MELLIFERA* L.)

(with a summary in Dutch)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. H. C. VAN DER PLAS,
HOOGLERAAR IN DE ORGANISCHE SCHEIKUNDE,
IN HET OPENBAAR TE VERDEDIGEN
OP WOENSDAG 13 SEPTEMBER 1978
DES NAMIDDAGS TE VIER UUR IN DE AULA
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN.

H. VEENMAN & ZONEN B.V.–WAGENINGEN–1978

This thesis is also published as Mededelingen Landbouwhogeschool Wageningen 78-15 (1978)
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HEAD
OF A 5TH INSTAR
QUEEN LARVA

STELLINGEN

1.

Het voedsel van honingbij larven bevat waarschijnlijk geen kaste determinerende stof.

Dit proefschrift.

2.

Ten onrechte concludeert Rembold op grond van zijn in vitro experimenten, dat het juveniel hormoon geen directe rol speelt bij de differentiatie van kasten bij de honingbij.

Rembold, H., Czoppelt, Ch. and Rao, P. J., 1974. J. Insect Physiol. *20*, 1193–1202.
Rembold, H., 1976. *In* Phase and caste determination in insects, ed. M. Lüscher, Pergamon Press, Oxford, pp. 27.

3.

Het ontstaan van supernumeraire larven als criterium voor de allatotrope activiteit van de cerebrale mediaal neurosecretoire cellen van *Galleria mellonella* L. larven is aanvechtbaar.

Granger, N. A. and Sehnal, F., 1974. Nature *251*, 415–417.
Sehnal, F. and Granger, N. A., 1975. Biol. Bull. *148*, 106–116.

4.

De gangbare opvatting dat chemoreceptoren van insecten specifieke gevoeligheden bezitten voor bepaalde verbindingen uit het voedsel, wordt door materiaal en methode bepaald.

Dethier, V. G., 1974. J. Insect Physiol. *20*, 1859–1869.

5.

Het replicatie mechanisme van planteviroïden, dient niet te worden benaderd door hybridisatie van 'verhitte' viroïden met gastheer DNA.

Hadidi, A., Jones, D. M., Gillespie, D. H., Wong-Staal, F. and Diener, T. O., 1976. Proc. Natl. Acad. Sci. USA *73*, 2453–2457.

6.

Bij de beoordeling van de toxiciteit van landbouwchemicaliën, dient de broedontwikkeling van de honingbij mede als criterium te worden gehanteerd.

Beetsma, J. and Ten Houten, A., 1975. Z. angew. Entomol. *77*, 292–300.

7.

De resultaten van kwantitatieve electronenmicroscopische onderzoekingen aan onvolledig ontwikkelde weefsels, dienen met omzichtigheid te worden geïnterpreteerd.

8.

Bij de bepaling van het bruto nationaal product verdient het aanbeveling om ook het verlies aan natuurlijke rijkdommen te calculeren.

Hueting, R., 1974. *In* Nieuwe schaarste en economische groei, Agon Elsevier, Amsterdam, Brussel, pp. 1–18.

9.

Het streven naar technisch volmaakte muziekopnamen, bewijst geen dienst aan de muzikale ontwikkeling van het publiek.

10.

Stickers voor het behoud van natuurmonumenten, geplakt op de achterruit van een auto schieten aan hun doel voorbij.

E. A. Goewie
Wageningen, 13 september 1978

1. INTRODUCTION

In the honey bee, larval nutrition during the first 72 hours determines the caste into which the female larva will develop (Perez, 1889). This has led many scientists to investigate the regulatory mechanisms of caste differentiation. For a review of literature the reader is referred to HAYDAK (1968), WIRTZ (1973), REMBOLD (1974, 1976) and DE WILDE (1976). When summarizing results from literature contradictory conceptions are found.

Some authors investigated which component in larval food is responsible for caste formation. REMBOLD (1969, 1974) reported that when royal jelly (RJ) is fractionated into its components, the dialysable fraction contains a factor which stimulates queen induction. According to the author, this substance would overcome a hormonal deficiency during the development of the honey-bee larva (REMBOLD, 1969). Rembold considered this so called 'F factor' as the caste determining principle. Dietz and HAYDAK (1970) found no evidence for the presence of such a determinator in larval food. They concluded that nurse bees may initiate caste induction by regulating the moisture content of larval food and thereby the food intake of the larva (DIETZ and HAYDAK, 1971; DIETZ, 1975). ASENCOT and LENSKY (1976) found that addition of invert-sugar to worker jelly (WJ) enhanced queen formation. LENSKY (1977) showed that addition of glucose and fructose restores the queen inducing capacity of inactive RJ. WEAVER (1962, 1974) denied that RJ contains only one factor responsible for caste differentiation. He stated that the differentiation of caste results from a complex of interacting factors. However he did not observe inhibition of queen induction after a considerable shift in the balance of nutrients or after dilution of RJ.

From the wealth of literature on the rôle of larval food on caste differentiation, it became more and more evident that honey-bee larvae can tolerate quite large distortions in the composition of their food. Therefore a direct effect of larval nutrients on the internal mechanism of caste differentiation may be doubted. DE WILDE (1966) assumed the nutritive environment of the larva to provide an external signal which affects neuroendocrine processes. The presence of substances in larval food would trigger the morphogenetic induction of castes along the neuroendocrine pathway (DE WILDE, 1976).

LUKOSCHUS (1955, 1956, 1961) found obvious differences in the volumes of Corpora Allata (CA) and Prothoracic Glands (PG) of 4th and 5th instar larvae and pupae of queens and workers. However, he did not elaborate this idea and gave no further evidence to support his opinion that hormones may be involved in caste differentiation. WIRTZ and BEETSMA (1972) and WIRTZ (1973) found caste differentiation to be a function of the larval juvenile hormone (JH) titre. Thus larval food was most likely to contain substances triggering the activity of the CA (DE WILDE, 1976).

Triggering of the CA is assumed to occur via the cerebral neurosecretory

cells (NSC). Most of the underlying evidence has been obtained by studies on adult insects, mainly with regard to the regulation of reproductive cycles, yolk deposition and adult diapause (WIGGLESWORTH, 1970; DE WILDE, 1973; GILBERT and KING, 1973; SLÁMA *et al*, 1974; MCCAFFERY and HIGHNAM, 1975 a, b; STEEL, 1975). For the wax moth larva GRANGER and SEHNAL (1974) and SEHNAL and GRANGER (1975) found evidence for a brain centered control of the CA activity.

Information from the nutritive environment of the larva is likely to be transferred to its neuroendocrine system by substances in larval food acting as environmental tokens. It has been our aim to investigate the exact nature of the external signals which larvae receive from their food. Electrophysiological methods were applied to examine the sensory effect of WJ, RJ and some important components of larval food. The effect of WJ and RJ on the activity of the cerebral NSC and the CA was studied with electron-microscopical (EM) methods. Quantitative EM studies were carried out on the NSC – CA relation in worker and queen larvae* and in worker larvae after application of JH. Finally in combination the results yield a conception of the control of the CA in relation to caste differentiation.

* Although caste characteristics are absent in larvae of queens and workers, we nevertheless will use the term queen and worker larvae. These are defined according to the type of cell in the comb in which they normally develop.

2. FOOD PERCEPTION

2.1. INTRODUCTION

The difference in growth and weight increase of queen and worker larvae would seem to indicate that food consumption of individual larvae plays an important rôle in caste differentiation (STABE, 1930; BISHOP, 1961; WANG, 1965). SMITH (1959) found that food quality is not the only factor in caste differentiation, but that the total amount of food consumed is important too. DIETZ and LAMBREMONT (1970) found that larvae of prospective queens consume 13% more food than larvae destined to become workers. Induction of intermediates or workers was triggered when larvae consumed much smaller quantities of RJ. High rates of food intake during the first 72 hours of larval development however, do not result in higher larval weights (WANG, 1965; DIETZ and LAMBREMONT, 1970). This is possibly due to higher turnover rates of the food consumed (MELAMPY and WILLIS, 1939; OSANAI and REMBOLD, 1968). Therefore we have to relate caste differentiation to the rate of food consumption and not to larval weight. The rôle of larval weight has been established only in the timing of endocrine activities in moulting (WIGGLESWORTH, 1964; NIJHOUT, 1975).

Food intake by insects is generally controlled by feeding stimulants or inhibitors (DETHIER, 1971, 1972; MA, 1972; BERNAYS and CHAPMAN, 1972; SCHOONHOVEN, 1974; MITCHEL and SCHOONHOVEN, 1974). Probably RJ contains a feeding stimulant, which controls the rate of food intake during larval development. A low rate of food intake by larvae on WJ can be explained by the absence of a feeding stimulant and/or presence of a feeding inhibitor. Electrophysiological experiments may elucidate the significance of the various food components. Evidence in favour of the existence of possible chemoreceptors on larval mouthparts is given by NELSON (1924), FYG (1966), WIRTZ (1973) and GOEWIE (1976). However detailed information about the innervation of the sensilla is lacking. We therefore first investigated the structure of the various buccal sensilla.

2.2. STRUCTURE OF THE BUCCAL SENSILLA

In this section histological data are presented on the papilla-like organs of the labrum, the Sensilla styloconica and S. trichodea on the maxillae and labium. For the purpose of comparison histological data on buccal mechanoreceptors are reported as well.

Materials and methods

Ten worker and queen larvae of the 4th and 5th instar were decapitated in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 0°C. Fixation lasted 2 hours in the same solution at 4°C. Specimen were kept overnight at 4°C in 0.1 M sodium cacodylate buffer, pH 7.2 and postfixed for 2 hours in 1% OsO_4 in 0.1 M veronal-acetate, pH 5.4 at room temperature. Dehydration took place in a graded series of ethanol dilutions. Prior to embedding the heads were impregnated with propylene oxide/Epon-Araldite mixture (LUFT, 1961). The heads were embedded in rubber boats and carefully orientated under the microscope. Ultrathin sections (400–600 Å) were cut with glass knives on a LKB UM III. Labral sensilla were cut longitudinally only, since transverse sections were difficult to obtain. Sections were contrasted with 1% uranylacetate in water during 45–60 minutes and with 1% leadcitrate during 20 minutes (modification after REYNOLDS, 1963). Tissues were examined in a Siemens Elmiskop II. Per sensillum 5 heads were sectioned and studied.

2.2.1. *Results*

Labrum

Figure 21 shows the inner structure of a labral papilla-like sensillum. These receptors are short and dôme shaped. They contain 4 dendrites: one trichogen and one tormogen cell. Probably only one pore on top of the sensillum makes contact between the larval environment and the dendrite cavity, because no other section revealed any pores through the cuticle of the sensillum.

Maxilla and labium

The internal structure of the S. styloconica on maxilla and labium is identical. Each sensillum consists of two similar parts. Each part contains one set of 5 bipolar neurons. Parts of one sensillum make contact with each other through their tormogen cells. Near the top of a sensillum a thin cuticle wall separates the two parts each ending in one papilla. Each papilla contains only one pore in its cuticle making contact between dendrites and larval environment. Figure 1 represents a topography of the inner structure of the S. styloconica on the maxillae or the labium. Figures 22 and 23 show transverse sections of these sensilla.

Mechanoreceptors

Many trichoid sense organs are found on the larval mouthparts. Of particular interest are the S. trichodea close to the chemoreceptors (Fig. 24). Histological observations from these organs revealed only one bipolar neuron (Fig. 24). The shape of the dendrite reminds of the shape of one of the dendrites in the labial and maxillar S. styloconica. Hair-like sense organs are not found on the labrum. The many rigid cuticular structures on distal and proximal parts of the labrum are not innervated.

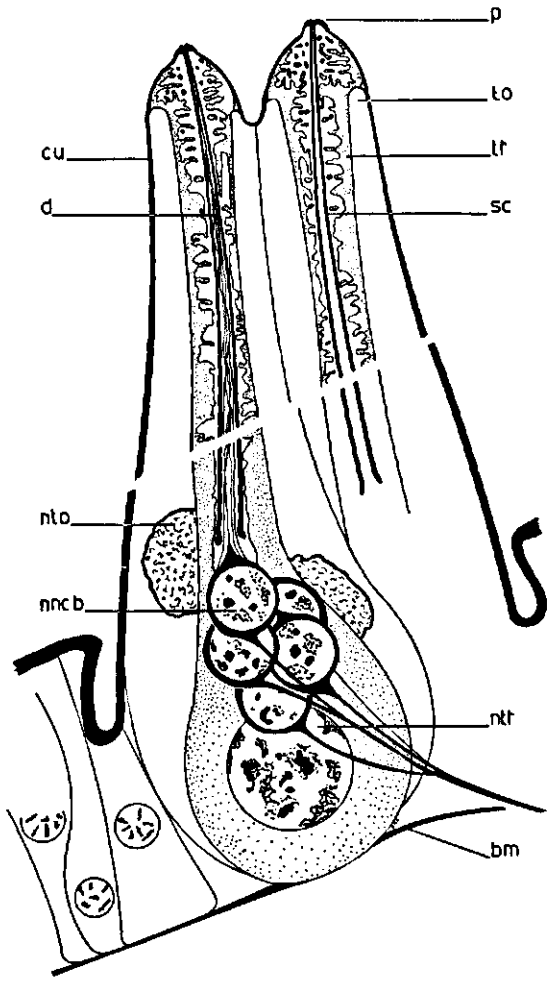


FIG. 1. Topography of the inner structure of the labial Sensillum styloconicum. A similar structure is found for the maxillar S. styloconicum. Each part of the sensillum ends in a small papilla, containing one terminal pore (p). Tormogen cells (to) surround two trichogen cells (tr) from which many microvilli penetrate an electron lucent lumen. The scolopoïd (sc) gives support to the receptor cavity, through which 5 dendrites (d) are running. Nuclei of nerve cell bodies (nncb) are seen at the level of the nucleus of the tormogen cell (nto) and the trichogen cell (ntr). A basal membrane (bm) supports the whole complex.

2.2.2. *Conclusions and discussion*

The innervation of the papilla-like sensilla and the S. styloconica on larval mouthparts, indicate a chemoreceptor function. One dendrite in the S. styloconica possibly functions as a mechanoreceptor (DETHIER, 1964; MA, 1972). The morphological difference between S. styloconica of queen and worker larvae, as described in an earlier paper (GOEWIE, 1976), was not found in all of

our later observations. However in cases where S. styloconica of the labium of queen larvae showed two papillae, a third, undeveloped scolopoïd, is sometimes found (Fig. 23). In contrast to the findings of FYG (1966) we conclude that the phenomenon may occur in queens and never in workers. It is, however, not consistent and thereby unreliable as a caste characteristic.

2.3. ELECTROPHYSIOLOGICAL STUDIES

Preliminary electrophysiological experiments on the buccal sensilla proved that action potentials are difficult to obtain when testing chemicals. Other recording techniques did not improve results either (MORITA, 1959; DEN OTTER, 1972). Moreover larvae desiccated rapidly when mounted between the electrodes. Optimal results were obtained by rinsing larval heads carefully prior to recording after the method of HODGSON and ROEDER (1956). Desiccation problems were anticipated by using mounted larval heads for short intervals of time only.

Materials and methods

Experiments were made with 4th and 5th instar worker larvae. Individuals were collected from different colonies. Frames containing dated brood were taken from the colony and placed into an incubator (32°C, RH 70–80%). Frames were thus kept up to 12 hours. At least half an hour before recording started, larvae were removed from their cells and placed in a watch glass with tap water at 32°C during 15 minutes. Then the larvae were carefully dried with tissue paper. Special attention was paid to the larval mouthparts. After drying larvae were placed in a dry Petri dish in the incubator.

Prior to recording, one head was severed in such a manner that haemolymph could not contaminate the mouthparts. Heads were mounted on a glass-pipette, filled with 0.1 M NaCl solution and connected with the indifferent electrode. A glass-capillary with tip diameter of 10 μ served as the recording electrode. An $AgNO_3$ coated silver electrode was inserted into the recording pipette and connected with a high-input impedance preamplifier (Grass P16). Action potentials were displayed on a Tektronix D11 oscilloscope and recorded with an UV recorder (SE 2006). After muscle movements in the head had ceased (within 1–5 minutes) experiments were started. Mounted heads were used for 20–30 minutes at most. Recordings from preparations used for longer periods became unreliable.

NaCl, KCl and $CaCl_2$ were used to detect possible salt receptors. The kations and anions selected for this study are present in larval food (REMBOLD, 1978). Salt receptors are generally found in insects (DETHIER, 1964) and are therefore expected to be present also in honey-bee larvae. Glucose, fructose and sucrose were used to detect sugar receptors. These sugars are found in larval food in different concentrations (LENSKY, 1977). Mixtures of glucose-fructose, glucose-sucrose and fructose-sucrose were tested to investigate a possible

synergism between the sugars. Other substances of special interest are pantothenic acid, nicotinic acid, biopterin, neopterin, γ-aminobuteric acid (GABA), L-alanin and the 'F factor'. Some of these chemicals occur in RJ only. Others are found in higher concentrations in RJ than in WJ.

Solutions were prepared in 0.1 M NaCl solutions, 1–2 days in advance and stored at 4°C. Tests were carried out by offering a series of 5 chemicals or 5 concentrations of one substance to each papilla of a sensillum. Recordings were made from at least 30 sensilla of one type. Individuals that reacted insufficiently were discarded.

Prior to testing of the various chemicals or concentrations, control tests were carried out with 0.1 M NaCl. Prior to testing the sugar mixtures, the component sugars were tested separately.

Frequency and amplitude of action potentials during the first second of each recording were measured by hand. This long period was chosen because of the low frequency with which sensilla responded. Action potentials varied considerably in amplitude. Therefore frequency distributions were made to detect the presence of specific receptors.

All chemicals were of p.a. quality. Salts and sugars were obtained from Merck, pantothenic acid, nicotinic acid and the amino acids from Sigma. Biopterin, neopterin and the 'F factor' were kindly supplied by Dr. H. Rembold (Max Planck Institute for biochemistry, Munich).

2.3.1. *Results*

Responses to salts

Chemoreceptors on all mouthparts responded to salts (Fig. 26). Firing rates were a little higher for NaCl than for KCl, but responses to NaCl were more irregular than to KCl (Fig. 2, 3, 10). Reactions to $CaCl_2$ were weak. No constant differences between the reactions of both papillae of one sensillum could be detected either. Generally frequencies and amplitudes of spikes were low. Rapid volleys of action potentials did not occur. Responses to 0.05 M concentrations occurred, but became indiscernible because of an unsatisfactory signal – noise ratio. Responses to 0.1 M salt concentrations were easily built up for long periods of time. At concentrations of 0.1 M and 0.5 M nerve cells responded with higher activities. Concentrations of 1.0 M or higher did not elicit an increased activity; responses tended to level out (Fig. 10).

Responses to sugars

Chemoreceptors on maxillae and labium responded to sugars (Fig. 4, 5, 6). There are indications that labral chemoreceptors reacted to sugars too. However responses were unsatisfactory and inconsistent. Firing rates were higher for fructose and glucose than for sucrose. Responses to 0.05 M concentrations were irregular and difficult to count. Concentrations at 0.1 M and 0.5 M evoked distinct and regular action potentials. Higher concentrations gave less satisfactory results, possibly due to rapid desiccation around the tip of the recording

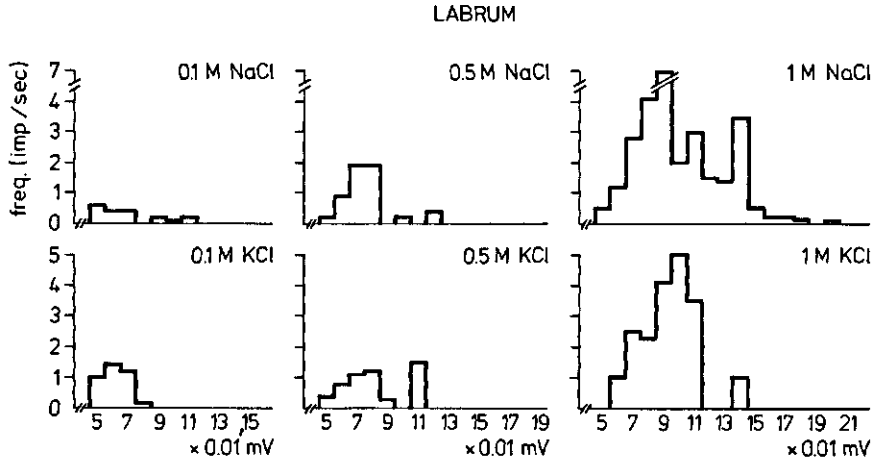


FIG. 2. Frequency distributions of spike amplitudes (falling phase) during the first second after stimulation of labral S. styloconica with NaCl or KCl. Each histogram pertains to 30 sensilla.

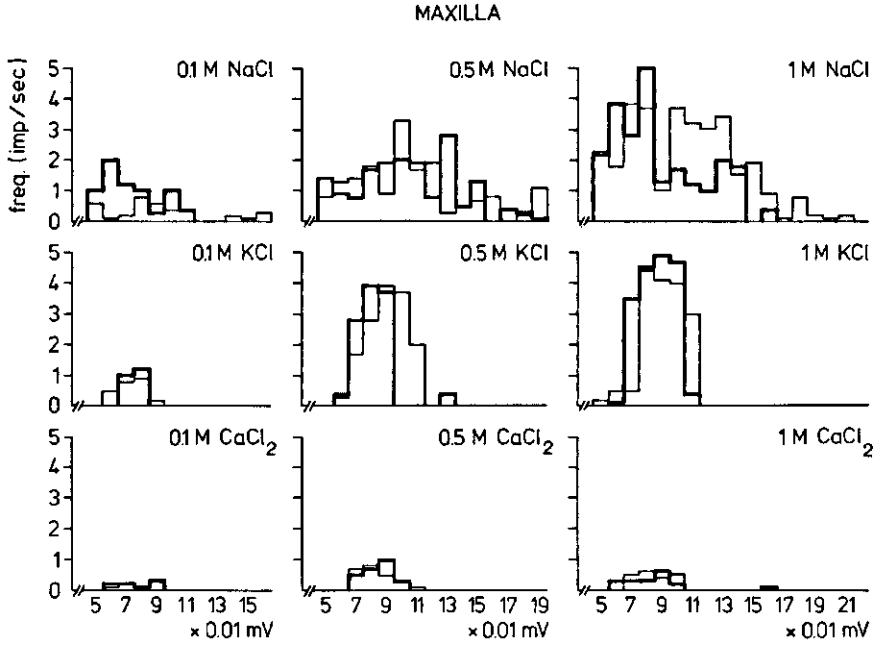


FIG. 3. Frequency distributions of spike amplitudes (falling phase) during the first second after stimulation of the maxillar S. styloconica with NaCl, KCl or $CaCl_2$. Heavy and thin lines indicate activities from, respectively, the medial and lateral part of the sensillum. Each histogram pertains to 30 sensilla. Similar results were obtained for the labial S. styloconica.

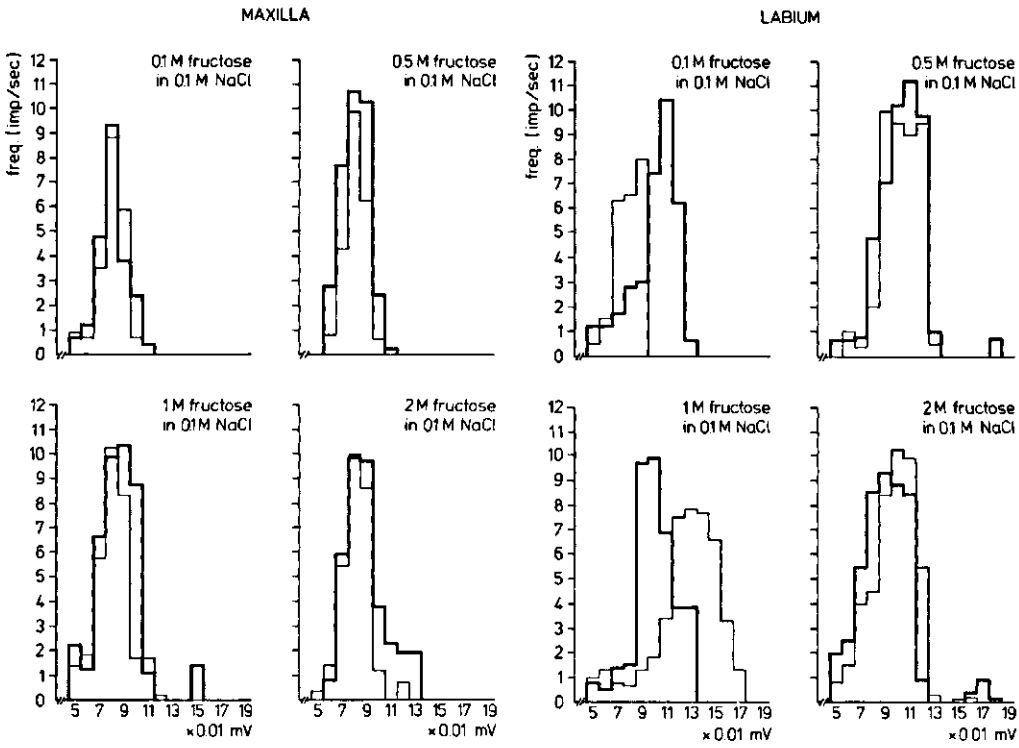


FIG. 4. Frequency distributions of spike amplitudes (falling phase) during the first second after stimulation of the maxillar and labial S. styloconica with fructose. Heavy and thin lines indicate activities from, respectively, the medial and lateral part of the sensillum. Each histogram pertains to 30 sensilla.

electrode. Under optimal conditions chemoreceptors elicited responses for considerable periods of time. No adaptation occurred, not even after stimulation with 2 M concentrations for prolonged periods. (See also Fig. 10).

Responses to mixed sugars

Impulse frequencies generated by mixtures of a 0.5 M glucose and 0.5 M fructose were similar to those evoked by the separately tested sugar concentrations (Fig. 7). However, a shift in the average spike amplitude could be observed (Fig. 8). This is also the case with the mixture of 0.5 M glucose and 0.5 M sucrose, when compared to glucose. Responses to glucose-sucrose mixtures were comparable with the added responses of the component sugars. Fructose-sucrose responses are similar to responses evoked by the fructose concentrations alone (Fig. 7).

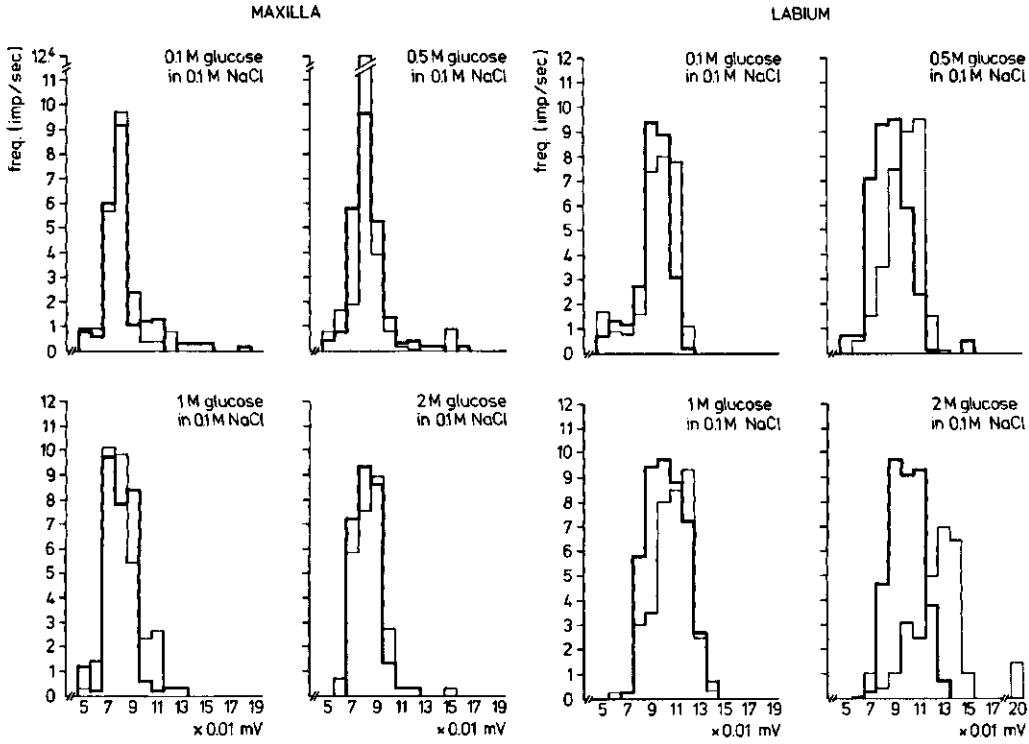


FIG. 5. Frequency distributions of spike amplitudes (falling phase) during the first second after stimulation of the maxillar and labial S. styloconica with glucose. Heavy and thin lines indicate activities from respectively, the medial and lateral part of the sensillum. Each histogram pertains to 30 sensilla.

Responses to water

No responses to water were generated in any of the buccal sensilla of the larva after stimulation with 0.05 M NaCl solutions.

Responses to other food components

Table 1 shows the various food components tested on buccal sensilla. None of the substances generated specific action potentials. Occurring spikes are due to the NaCl solution in which the various components were dissolved. Pantothenic acid elicited responses which might be aroused by Ca^{2+} ions arising from the tested Ca-pantothenate. Biopterin and neopterin were difficult to dissolve in water. The substances had to be dispersed in the conducting fluid. Thus possible responses might have been masked.

Mechanoreception

Bending of the maxillar or labial chemoreceptors results in a burst of action

TABLE 1. Chemicals tested on all types of buccal chemoreceptors of honey bee larvae. The concentrations used for the stimulation of the sensilla are indicated.

Food component	Concentration
Pantothenic acid (Ca–salt)	0.1 M in 0.05 M NaCl
Nicotinic acid	0.05 M and 0.1 M in 0.1 M NaCl
Biopterin	1 mg in 50 µl 0.1 M NaCl *
Neopterin	1 mg in 50 µl 0.1 M NaCl *
'F factor'	23 mg in 50 µl 0.1 M NaCl
GABA	0.1 M in 0.1 M NaCl
L-alanin	0.1 M in 0.1 M NaCl
L-prolin	0.1 M in 0.1 M NaCl
	* about 0.1 M concentration

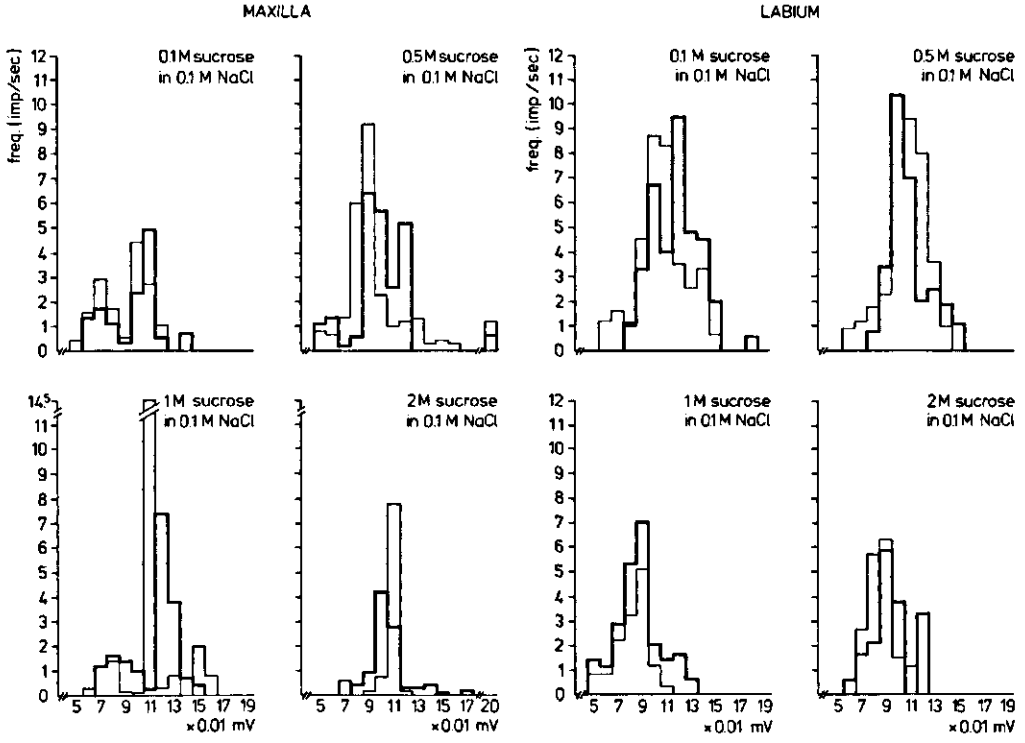


FIG. 6. Frequency distributions of spike amplitudes (falling phase) during the first second after stimulation of the maxillar and labial S. styloconica with sucrose. Heavy and thin lines indicate activities from respectively, the medial and lateral part of the sensillum. Each histogram pertains to 30 sensilla.

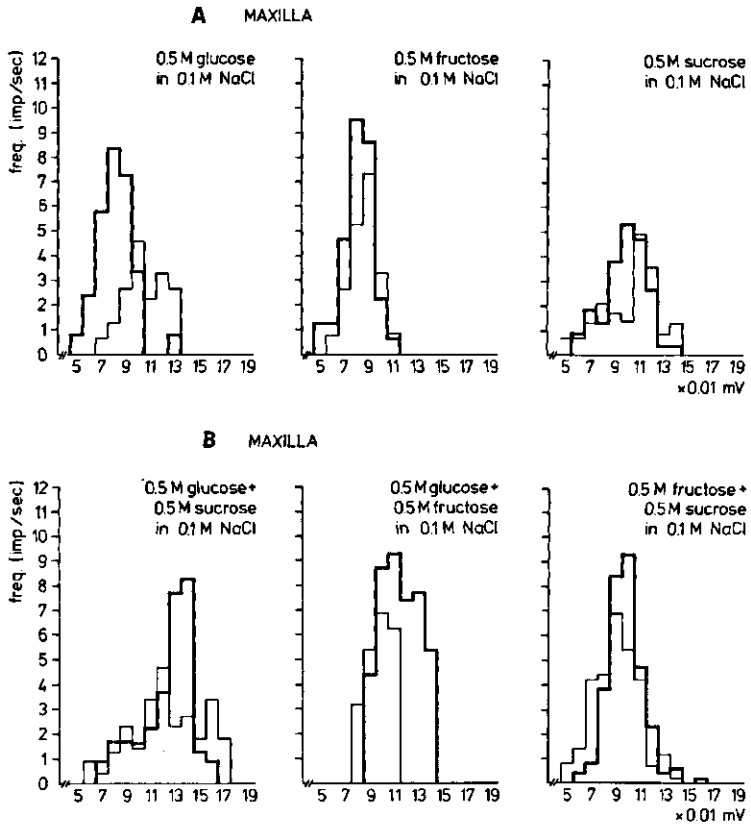


FIG. 7. Frequency distributions of spike amplitudes (falling phase) during the first second after stimulation of the maxillar S. styloconica with separate and mixed sugars. Heavy and thin lines indicate activities from respectively the medial and lateral part of the sensillum. Each histogram pertains to 20 sensilla. A. Sensillum activity on separately tested sugars, prior to testing a sugar mixture. B. Sensillum activity on different sugar mixtures.

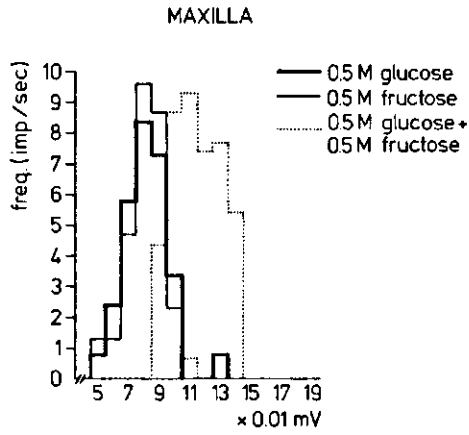


FIG. 8. Comparison of the separate responses of the maxillar S. styloconica to 0.5 M fructose, 0.5 M glucose and the equimolar mixture.

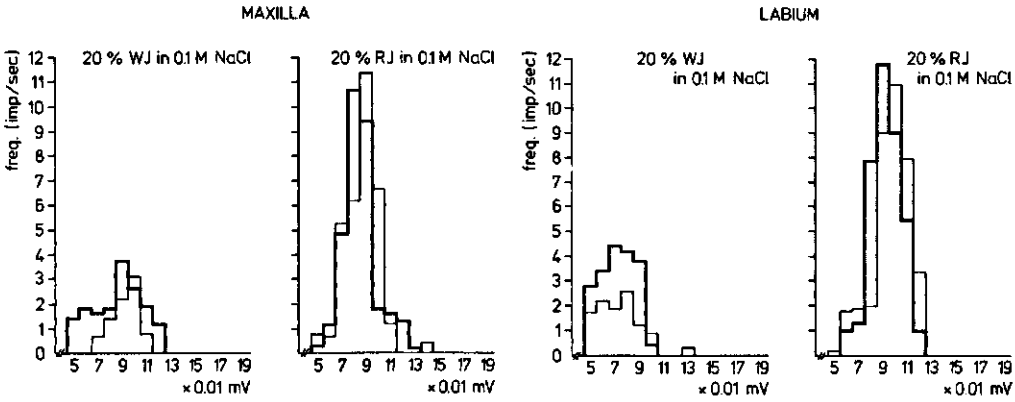


FIG. 9. Frequency distributions of spike amplitudes (falling phase) during the first second after stimulation of the maxillar and labial S. styloconica with diluted WJ or RJ. Heavy and thin lines indicate activities from, respectively, the medial and lateral part of the sensillum. Each histogram pertains to 25 sensilla. WJ and RJ were collected from cells with 2nd and 3rd instar larvae.

potentials. Repetitive activity followed, after repeatedly bending the sensillum with the recording electrode.

Responses to WJ and RJ

Responses to undiluted larval food could not be obtained, because of poor conductivity. However dilutions up to 20% gave satisfactory results. Figure 9 shows responses to 20% dilutions of WJ and RJ. Considerable differences in impuls frequency were found. Spike distribution diagrams are comparable with those obtained after stimulation with sugars alone. WJ nor RJ elicited any specific action potentials other than those induced by salts and sugars. From distribution diagrams only one type of action potential can be distinguished.

2.3.2. *Conclusions*

All buccal chemoreceptors of honey-bee larvae react to salts and sugars. In general spike amplitudes are small and frequencies are low in comparison with values found in other insects (MA, 1972; WHITEHEAD and LARSEN, 1976).

In contrast to the labral sensilla, maxillar and labial chemoreceptors have a mechanoreceptor function, in addition.

Sensitivity of labral sensilla to sugars has been indicated, however their investigation was terminated because of insufficient results. Labial chemoreceptors however gave stronger reactions to salts than chemoreceptors on other mouthparts. Reactions to monovalent ions are obvious; bivalent ions (Ca^{2+}) elicit inconspicuous action potentials. The same holds true for salt sensitivity of maxillar and labial chemoreceptors. Sensitivity is highest to fructose, less to glucose and lowest to sucrose. Probably glucose and sucrose react at the fructose receptor site of the dendrites. The respective activities disappear as

soon as the response to fructose occurs. Moreover glucose seems to amplify the respons to sucrose.

Reactions to WJ are much weaker than to RJ. Because their spike distribution diagrams are quite similar to those of fructose and of glucose-fructose mixtures we conclude that the buccal chemoreceptors reacted only to the fructose-glucose component of the larval food. Reactions to salts, present in the larval food, are masked by the reactions to sugars. Therefore reactions to RJ, showed only one type of action potential.

As far as tested no other food components generated action potentials in any of the buccal sensilla of the larva. Thus a possible relation between these substances and the initiation of caste differentiation must be rejected.

Chemoreceptor sensitivity in honey-bee larvae appears to be restricted to a few components. All sensilla show about the same properties, which is remarkable when comparing chemoreception in other insects. Honey-bee larvae depend completely on the food offered by the nursebees; they do not actively select their food. The increase of the chemoreceptive capacity might therefore be more important than the ability to react to specific components.

Spike patterns evoked by diluted RJ or WJ indicate reactions to only one, may be two, components in the food. Dilution of larval food results in a decrease of the concentration of the food components. Thus there may be concentrations that cannot be detected by electrophysiological methods.

Responses, expressed in total impulses per second, related to different concentrations of the tested substances is demonstrated by the dose-response curves (Fig. 10). Irregularities are explained by the low frequency of the action

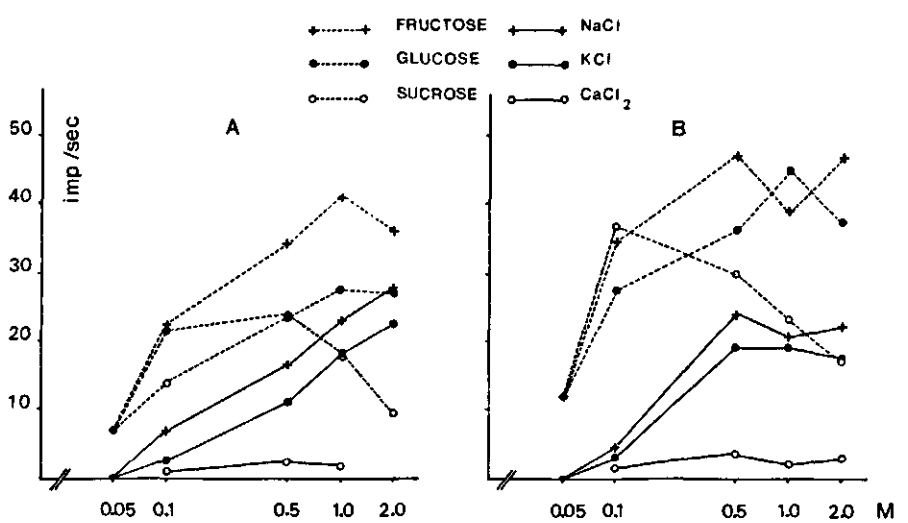


FIG. 10. Dose-response curves of sugars and salts tested on the medial part of the maxillar (A) and labial (B) S. styloconica. Points represent the total number of impulses obtained during the first second after making contact between sensillum and recording electrode.

potentials, combined with a great variation in the concerning spike amplitudes. Moreover, results are interfered with action potentials generated at the same time in the adjacent papilla by leakage from the recording electrode.

The high sensitivity to sugars of larval chemoreceptors found in these experiments, supports the findings or ASENCOT and LENSKY (1976, 1977).

Sugars may function as strong phagostimulants. However the significance of the salt sensitivity of buccal chemoreceptors is still obscure. Salts are generally considered to act as feeding inhibitors (DETHIER, 1974). Salts and sugars may therefore function in the food as phagostimulatory antagonists.

3. INHIBITION OF FOOD INTAKE AND THE EFFECT ON CASTE DIFFERENTIATION

3.1. INHIBITION BY SALTS

The quantity of food consumed within a certain time by honey-bee larvae plays an important rôle in caste formation. DIETZ and HAYDAK (1971) and ASENCOT and LENSKY (1976) proved that stimulation of larval food intake results in queen formation. On the other hand DIETZ and LAMBREMONT (1970) demonstrated that a low intake of RJ by individual larvae resulted in the development of workers. A reduced food intake might be brought about by the presence of feeding inhibitors, such as salts. WEAVER (1974) showed that the development of honey-bee queens is affected by salts. Addition of NaCl to RJ, when rearing larvae in vitro, resulted in queens with low pupal weights. Salts are considered therefore to be able to inhibit food intake.

The experiments discussed in the present chapter were aimed to investigate the effect of a reduced food intake on the differentiation of castes. Salts were used to bring about a reduction of food intake during the first 72 hours of larval development. Experiments were carried out in colonies.

Materials and methods

Experiments were carried out in 10 – framed colonies with at least 7 brood-combs. Larvae of known age were obtained after restricting the queen to a newly constructed comb for 6 hours (WIRTZ, 1973; COPIJN *et al*, in prep.). After 3 × 24 hours, eggs were controlled. Eggs not hatched within 6 hours were destroyed. Queen larvae were raised from dated brood (at the age of 12 hours) by offering strips with occupied worker cells in a hanging position in a queenless colony. Close to the larval mouthparts 3 µl of a 3% NaCl solution (diluted in distilled water, pH 3.5) was applied to the surface of the RJ. The application was repeated every 8 hours. The first treatment started with larvae at the age of 35 hours. The last treatment was given to larvae when 115 hours old. Controls received 3 µl distilled water (pH 3.5). Applications were carried out with a 50 µl Terumo syringe attached to a 1 µl Hamilton dispenser. After application frames were stored in the incubator (32°C, RH 70–80%) for 30 minutes before introducing into the colony, in order to obtain NaCl dispersion into the food and to avoid interference by the nurse bees. After all cells with treated larvae were sealed, frames were removed from the colony and placed into the incubator. One day before the expected moment of emergence, cells were opened. Pupae were weighed and their developmental state examined. Finally pupae were placed separately in Petridishes in the incubator and further development was followed. The resulting adults were immediately fixed in Carnoy 70% and dissected, to determine the rate of queenlikeness. The same experiment was carried out with 3 µl 3% KCl (pH 3.5). Both experiments were repeated 3 times.

3.1.1. *Results*

Up to an age of 90 hours larvae tolerated salt treated food. However from 90 hours on larvae started to leave the food and were found on the wall or close to the cell-opening; there was no more contact between the larva and the present food. Cells containing control larvae were sealed at the expected moment (123 hours), while cells of treated larvae were sealed between 123 and 127 hours after eclosion. Cells of treated larvae were longer (2.5–3.5 cm) than those of control larvae (2.0–2.5 cm). Table 2 shows larval and pupal survival as well as the average pupal weight gained. It is obvious that treated larvae consumed smaller quantities of food, when comparing their pupal weights with those of the controls. The percentage of intermediates, obtained after the treatment was low. Individuals with obvious worker mandibulae or hind legs were considered only. Figure 11 shows the rate of queenlikeness of all individuals with workerlike characteristics other than mandibulae or hind legs only.

Caste differentiation appeared to be affected to some extent.

3.1.2. *Conclusions and discussion*

Addition of salts to RJ appears to reduce larval food intake when compared to food intake of untreated queen larvae. Pupal weights gained on salt treated RJ, are lower than those in the control experiment, however, they are still higher than pupal weights of normal workers (WANG, 1965).

We find that addition of salts to RJ was partly effective. Larvae on salt treated food are commonly found close to the cell-opening indicating that

TABLE 2. Larval and pupal survival after application of salts to RJ in queen cells and after starvation of queen larvae during experiments in the colony. Pupal weights are determined at the same time; i.e. one day before the expected moment of emergence of the controls. Individuals with obvious worker mandibulae or hind legs are considered as to be intermediates.

Treatment	Number of treated larvae	Larval survival (%)	Pupal weight (mg)	Pupal survival (%)	Adult survival (%)	effect on queen induction	
						Intermediates (in % of the surviving adults)	Queen (in % of the surviving adults)
NaCl	63	63	201	62	43	8	92
H_2O	20	85	283	80	80	–	100
KCl	65	60	191	52	52	11	89
H_2O	18	89	249	83	83	–	100
Starvation	37	38	181	38	33	12	88
Control	10	100	286	100	100	–	100

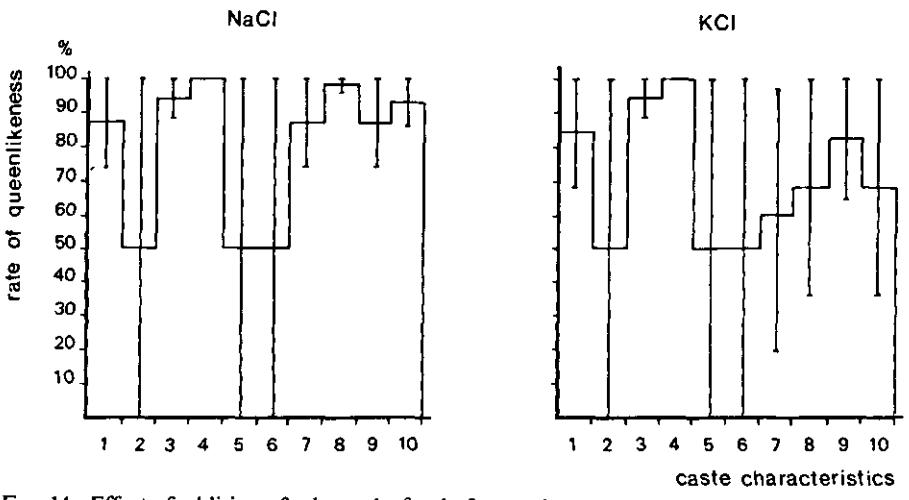


FIG. 11. Effect of addition of salts to the food of queen larvae on caste differentiation. Caste characteristics were measured from 8 and 11 workerlike individuals. The average values were expressed as percentages of the average value found in 15 newly emerged swarm queens. Explanation of figures in the abcissa: 1. length of the glossa, 2. mandibular tooth, 3. width of the head/length of the head ratio, 4. mandibular gland size, 5. chaetotaxy of the tibia, 6. basitarsal hairs (number of complete hairrows), 7. number of barbs on the sting, 8. ratio of the length of the medial and lateral venom duct.

they possibly consumed directly offered food from nurse bees. Thus a limitation of the intake of food could be more effective by preventing direct contact between nurse bees and larvae. The effect can also be reduced by the fact that nurse bees could have licked away the added amount of salts from the food. This might occur especially during the first 90 hours of larval life, because larvae do not leave the food in that period.

3.2. INHIBITION BY STARVATION

Nourishment by frequent food offerings from nurse bees was limited by placing broodcombs in the incubator for short intervals. Larvae were exposed to the colony long enough to obtain a sufficient amount of food to survive, but short enough to limit the rate of food intake.

Methods

At the age of 31 hours experimental frames were put into the incubator (32°C, RH 80%), for 12 hours. Afterwards they were introduced in the colony for 2 hours. The alternation of 12 hours in the incubator and 2 hours in the colony was repeated up to the age of 71 hours. The alternation was changed afterwards in 12 hours in the incubator and 3 hours in the colony, to improve

larval survival. Control larvae in a second frame remained in the colony during the whole experiment. After all cells were sealed, experimental frames were removed from the colony and put into the incubator continuously. Further observations were carried out as in the preceding experiment. Experiments were carried out twice.

3.2.1. *Results*

Table 2 shows larval and pupal survival as well as the average pupal weight gained during the experiment. The average pupal weight was much lower than in the control experiment and mortality was considerable.

Defining intermediates as queenlike individuals with obvious workerlike mandibulae or hind legs, we may conclude that starvation of larvae by short expositions to the colony resulted in only a small number of intermediates. Thus caste differentiation appeared to be affected partly (Fig. 12).

Cells of starved larvae are not sealed simultaneously with the control larvae. Starved queen larvae leave their food at about 120 hours and are found afterwards close to the cell-opening. Sealing occurred two to three days later than in the control experiment. Length of some cells was increased to measure up to 3.5 cm.

3.2.2. *Conclusions and discussion*

The limited number of intermediates gives some indication that starvation reduced food intake sufficiently during the first 72 hours of larval life. However, queen formation is not affected generally. Probably experimental larvae frequently received directly offered food from the nurse bees during their stay in the colony. Older instars might compensate for starvation during the prolonged larval development. Larvae extend their development up to the moment when

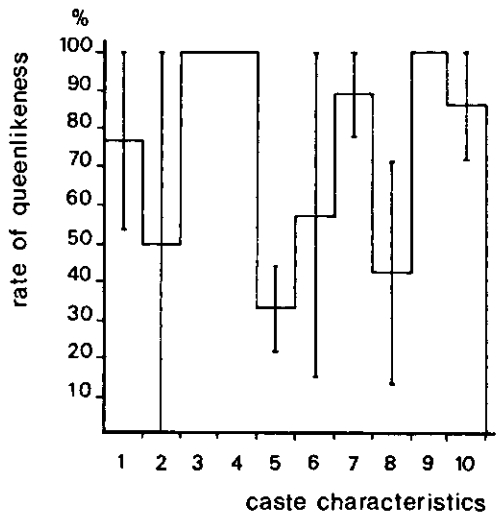


FIG. 12. Effect of starvation of queen larvae on caste differentiation. Caste characteristics were measured from 6 workerlike individuals. For further explanation, see figure 11.

physiological conditions, necessary for larval-pupal ecdysis, are fulfilled. Therefore cells seem to be sealed only when larvae are full-grown.

We have to conclude that experiments in the colony are unsuitable to test the inhibition of food consumption by salts or by starvation. Experiments in vitro do not appear to be a better alternative, since only a small number of queens are produced when rearing in vitro on *unaltered* RJ. On the other hand we might speculate that the impossibility to obtain only queens in vitro on unaltered RJ, indicate that a limitation of food occurred and thus resulted in the development of a worker. From our electrophysiological experiments it became obvious that stimulation of buccal sensilla is blocked by a thin layer of desiccated food on top of the sensilla. As soon as sensilla are washed (see chapter2), stimulation is possible again. As larval food desiccates rapidly in vitro we think that stimulation of the chemoreceptors has been cut off. Food intake will be reduced therefore, resulting in development of workers and intermediates. Queens will be induced again, as soon as chemoreception is restored. This probably occurs when RJ in vitro is diluted. From this point of view the findings of DIETZ and HAYDAK (1971) and ASENCOT and LENSKY (1977) are of great interest.

4. ANALYSIS OF THE MNSC-CA RELATION

To investigate effects on the neuro-endocrine system, detailed information on the structure of this system is required. With light microscopical (LM) and electron microscopical (EM) techniques we investigated at first the larval brain to obtain a better understanding of the NSC-CA relation.

Many investigators have shown that the activity of the medial cerebral NSC stimulates CA activity in adult insects. GRANGER and SEHNAL (1974) and GRANGER and BORG (1976) found that the activities of medial NSC (MNSC) and CA of *Galleria mellonella* larvae are related. DOGRA *et al* (1977) assumed that the MNSC control CA activity, in honey-bee larvae.

Changes in the activity of the MNSC or CA can be studied with quantitative EM techniques. Numbers of elementary granules and Golgi zones per standard area of cytoplasm are used as criteria for synthetic activity (WENDELAAR BONGA, 1971 a, b, 1972; ROUBOS, 1973, 1975).

4.1. TOPOGRAPHY OF CEREBRAL NSC

Histological studies on cerebral NSC in honey-bee larvae are mostly carried out by means of LM techniques. For further reading we refer to WIRTZ (1973).

DOGRA *et al* (1977) compared MNSC of queen and worker larvae by means of LM. The authors found that MNSC in queen larvae are activated earlier than in worker larvae. However specific staining methods in larval NSC are unreliable (DÖHMER, 1958; WIRTZ, 1973; GRANGER, pers. comm.; NAISSE, pers. comm.). EM studies might be a reliable method for the localization and recognition of NSC. This however requires initial LM studies of the larval brain. We only investigated semi-thin sections from resin embedded specimens.

Materials and methods

Dated queen and worker larvae were reared as described in section 3.1. For EM studies larval heads were prepared as described in section 2.2. Initially specimens were embedded in Epon 812 (LUFT, 1961) and later on in Epon 812 – Araldite 6005 (ANDERSON and ANDRÉ, 1968) in gelatine capsules, orientated with their frontal part downwards. Semi-thin sections (2 μ) were obtained each 10 μ from embedded specimen and attached to a clean glass slide on a drop of destilled water (GRIMLEY *et al*, 1965). After drying, sections were stained in 1% toluïdine blue in borax, pH 11.5 (SCHOONEVELD, 1974). Sections were examined in a phase-contrast Wild microscope and photographed in a Zeiss photomicroscope. After LM survey, blocks were trimmed at the appropriate area, ultra-thin sectioned and controlled in the LM as well as in the EM. Sectioning was done on a LKB UM III with a diamond knife.

4.1.1. *Results*

In LM studies, no differences are apparent between larval brains of both castes as concerns their cellular structure. Therefore results are valid for both queen and worker larvae.

Recognition of NSC in Epon embedded sections was difficult. Sections stained uniformly with toluïdine blue and differences in staining intensity could not be found. Cell shapes however were well discernible in larvae of all ages. However Epon embedded specimens appeared to be unsuitable for localization of NSC with LM during the larval stage.

When staining nervous tissue embedded in Epon – Araldite, differences in staining intensity between cells were found with the LM. Careful examination revealed two groups of purple stained cells in the pars intercerebralis of the 2nd and 3rd instar. During the last two instars these cells were not stainable any longer; they increased in size and became unrecognizable. Other regions of the brain also showed purple stained cells. These cells were also examined with EM. Figure 27 shows a part of the brain of a 4th instar larva.

Assuming that cells with at least some electron dense granules and with granule secreting Golgi zones are neurosecretory cells, EM examination revealed NSC in the pars intercerebralis only. This however does not imply that the absence of the phenomena described, denies a possible neurosecretory function of a cell. Purple stained cells in the pars intercerebralis for instance, do not show any neurosecretory activity in the EM during the 2nd and 3rd instar. They are electron dense and contain numerous free ribosomes. When these cells are activated however, cytoplasm becomes more and more electron lucent, resulting in neurosecretory phenomena.

Other purple stained cells in the brain did not show any neurosecretory activity throughout larval development.

4.1.2. *Conclusions and discussion*

NSC are found in the pars intercerebralis of the brain. No other NSC could be distinguished.

Our results do not completely agree with the findings of DOGRA *et al* (1977). Especially their description of the localization of the MNSC of 2nd instar larvae is different from our findings. Cells described by Dogra and co-workers are easily found in the LM, but do not show neurosecretory phenomena when their ultrastructure is observed in older stages. Moreover these cells are smaller than is indicated by these authors. However, differences may arise because of different experimental methods. Paraffin sectioned specimen are difficult to compare with resin embedded ones.

4.2. LIGHT MICROSCOPY OF MNSC – CA RELATION

Studies on the brain and post-cerebral complex are usually carried out with specific staining methods. The CA – MNSC relation in particular may be

revealed by cobalt ionthophoresis (PITMAN *et al*, 1973; TYRER and BELL, 1974 and RADEMAKERS, 1977). However these techniques are impracticable in honey-bee larvae, because of their small size. Vital nerve staining methods after LARIMER and ASHBY (1964), DOGRA and TANDAN (1964), MCGUIRE and OPEL (1969), STARK *et al* (1969), ITTYCHERIAH and MARKS (1971), GRANGER SEHNAL (1974) and LOUVEAUX (1975) did not give satisfactory results in honey-bee larvae. Silver staining techniques resulted in sufficient information on axonal pathways within the neuroendocrine system. The best results were obtained by a modification of the method of RIBI (1975). This method is not specific for neurosecretory axons, but is useful to stain all nerves of the neuroendocrine complex.

Materials and methods

Fifth instar larvae were collected in 0.1 M sodium cacodylate buffered glutaraldehyd-paraformaldehyd solution (KARNOVSKY, 1965) at room temperature. After fixation of the severed heads, they were rinsed in 0.1 M sodium cacodylate for one hour and fixed again in a freshly prepared Bouin solution at room temperature for 8–12 hours. Heads were rinsed in 70% ethanol, dehydrated in a series of ethanol, impregnated with methylbenzoate and methylbenzoate-celloïdine for 7 days. After embedding in paraplast, blocks were sectioned on a sledge microtome. Section thickness was 10–15 μ. After attaching sections to glass slides, they were dried for 5 days at 37°C. When deparaffinated, sections were immersed in 20% $AgNO_3$ at room temperature in darkness for 4 hours, rinsed in destilled water, immersed for 10 minutes in 2% oxalic acid at 50°C and for 10 minutes in a hydroquinone solution at 50°C (hydroquinone solution: 1 gr hydroquinone, 10 gr Na_2SO_3 in 100 ml destilled water). Slides were rinsed in destilled water for a short time. Sections were contrasted for 15 minutes in 1% AuCl in bright light, then rinsed in water again and reduced for 10 minutes in 2% oxalic acid at 40°C. When sections turned purple, the reduction was further followed by the microscope. Without rinsing slides were immersed in a 0.02% aniline blue solution for 2 minutes (GUNDERSEN and LARSEN, 1976), rinsed for a short time in destilled water and fixated for 15 minutes in 5% sodium thiosulfate. After dehydration sections were embedded in Canada balsem.

Care must be taken to avoid any contact with metal during the silver and gold impregnation and during the reduction step. A plastic or wooden pair of tweezers is required.

4.2.1. *Results*

Figure 28 shows parts of the larval brain. Numerous axons can be seen starting from different cells or groups of cells. The chiasma is formed by axons originated mainly in the MNSC area, but in other areas as well. However, no distinct courses of neurosecretory axons are detectable.

Investigation of a series of sections revealed one conspicuous axon arising from the medial part of the CA originating from the corpus cardiacum (CC).

The axon can be followed to the ventro-caudal part of the brain. From here further tracing becomes difficult.

The CA appear to be innervated also by two small axons on other sides of the CA. These axons are indicated by their cell body, close to the CA. A further tracing of their nervous contact with other organs of the head was impossible.

4.2.2. *Conclusions*

Larval CA appear to be innervated by a bundle of axons, descending from the brain, running through the CC and penetrating the CA. This bundle is supposed to be the nervus corporis allati (NCA). The CA is also innervated by two separate axons not belonging to the NCA. More axons may innervate the CA; however at the level of the LM they are invisible.

4.3. Electron microscopy on the MNSC–CA relation

To confirm the results of the preceding subsection and to obtain detailed information, EM studies were started. In this study we report results on the activity of the MNSC during larval development. MNSC activities will be expressed in indirect criteria for synthesis, cytoplasmic storage, transport and secretion. These results will be correlated with activities of the CA.

Materials and methods

Dated queen and worker larvae were obtained as described in section 3.1. The queen was allowed to lay eggs during 6 hours on a newly constructed comb. Rearing of queen larvae started at the age of 12 hours, when half of the number of cells containing worker larvae were transplanted into the queenless colony. This colony was deprived of her queen one hour before grafting. Samples were taken every 12 hours beginning at the larval age of 72 hours. From each age group and each caste 5 samples were collected in a solution of 2% glutaraldehyd in 0.1 M sodium cacodylate buffer, pH 7.2, at 0°C. After sampling larvae were immediately dissected and transferred to a fresh fixating solution. Further preparation for EM occurred as described in section 2.2. Heads were embedded in gelatine capsules in Epon – Araldite and were orientated with their dorsal part in a downward position. Experiments were carried out twice.

For quantitative EM, out of 5 collected samples, three embedded specimens were chosen at random. They were ultra-thin sectioned on a LKB UM III with glass or diamond knives. MNSC were ultra-thin sectioned at intervals of 10 μ. The next series of sections were made transversely through the middle of the CC and through the CA. Here ultra-thin sections were made at 10–20 μ intervals. Silver coloured ribbons were collected on Formvar coated grids. Finally we obtained per tissue of one animal 9 grids. Quantitative EM investigation was

done on 5 at random chosen grids. Per grid one neurosecretory cell was chosen and photographed (magnification 10.000 x).

Micrographs were made of a cytoplasmic area of 250 μ^2 (final magnification 20.000 x). From 10 random MNSC micrographs (5 cells per larva), synthesis and storage were quantified. The length of the rough endoplasmatic reticulum (RER) was calculated in μ/500 μ^2 area of cytoplasmic by means of lineal integrative analysis (LOUD *et al*, 1965), using a square grid of lines (distance between two lines: 5 mm). Counts of other organelles were expressed in numbers per 500 μ^2 area of cytoplasm (WENDELAAR BONGA, 1971); ROUBOS, 1973, 1975). Transport and release processes were described qualitatively only. Quantifications were not possible because of the phasic course of the transport of elementary granules.

CA activity was determined quantitatively from 20 random micrographs (5 cells per larva). Counts were expressed per 500 μ^2 surface area of cytoplasm. Numbers of elementary granules in the branching nerves within the CA could not be determined quantitatively, because obtaining a standard surface area of axons was impossible. Special attention was therefore given to the content of the penetrating NCA.

Diameters of elementary granules were measured at different levels of the preparation by hand, with a transparent ruler.

4.3.1. *Results*

During the experiment larvae developed synchronously. Queen and worker cells were sealed at the proper moments.

4.3.1.1. Ultrastructure of CA innervating nerves

At different moments of larval development only one CA innervating nerve contained elementary granules (Fig. 29). This axon was considered to be the NCA. It penetrates the CA and branches afterwards. However the rate of branching is limited. Fibres are frequently found only at the medial side of the CA, where the NCA enters the gland. Elsewhere in the CA, less fibres are found. Other CA innervating nerves never contained neurosecretory granules throughout the larval development. Even from EM observations these axons are small-sized; they do not penetrate the CA. They are merely attached to the surface of the gland. Figure 30 shows a non-neurosecretory axon at the ventral side of the CA.

4.3.1.2. Quantification of the MNSC activity; synthesis, transport and release

Different cytoplasmic organelles represent synthetic activity (Fig. 31). Figure 13 shows ultrastructural activity of the MNSC in queen and worker larvae. Figures are histograms in fact. However, they are represented as drawn curves facilitating comparison between results obtained in both castes.

Synthesis. The number of Golgi zones, the total length of the RER, the number of elementary granules and the number of lysosomes per cytoplasmic

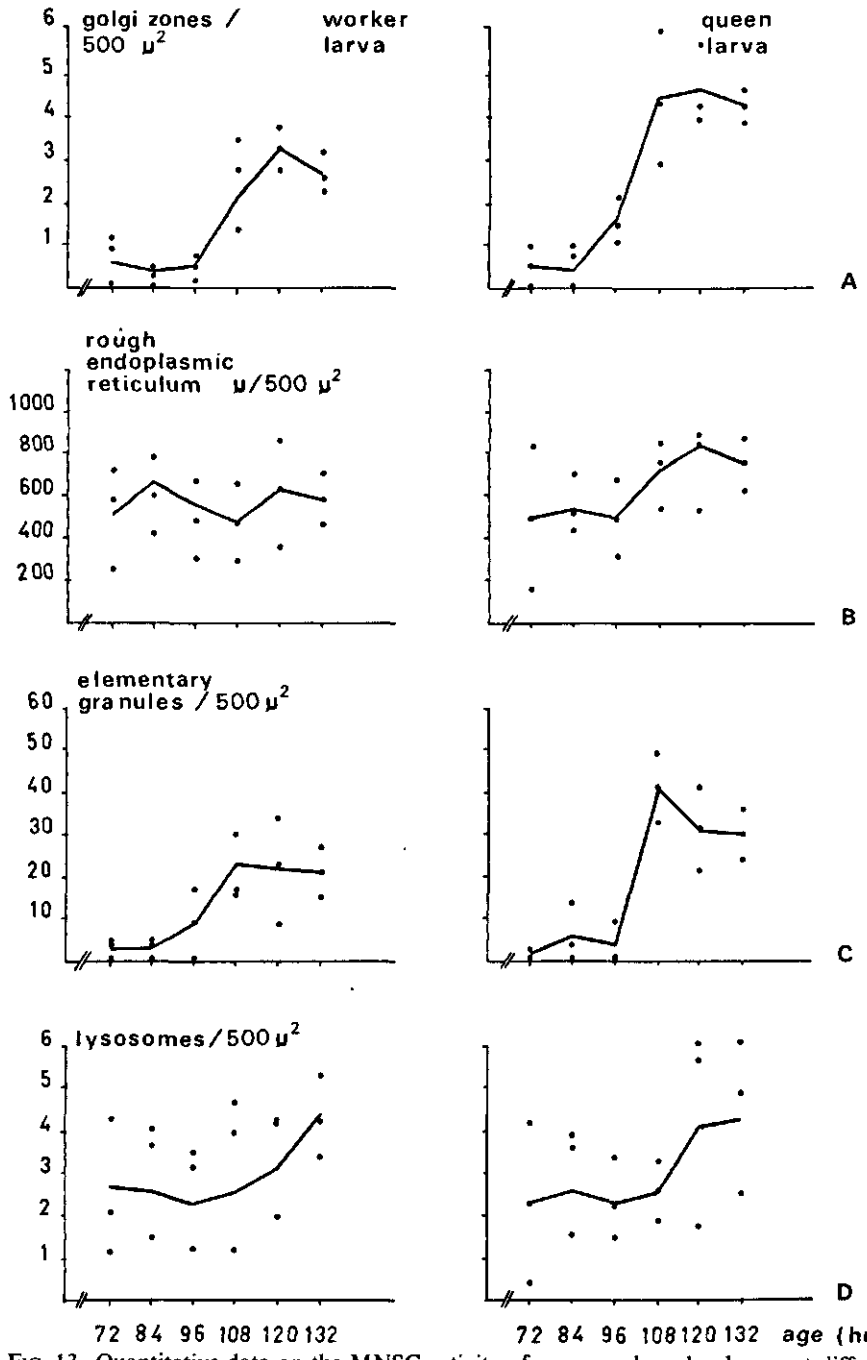


FIG. 13. Quantitative data on the MNSC activity of queen and worker larvae at different ages. Curves indicate the average values, derived from three larvae. Per larva 5 cells were measured. For further explanation, see text.

area were determined in both castes, as possible criteria for the synthetic activity of the MNSC. Up to the age of 96 hours, the number of Golgi zones in worker larvae is very low. From this moment on this number starts to increase in queen larvae. This seems to occur in worker larvae about 12 hours later.

In worker and queen larvae the number of Golgi zones increases up to the larval age of 108 hours. Afterwards no further increase is observed in queen larvae. In contrast with this, the number of Golgi zones still increases in worker larvae.

No differences are found in the total length of the RER found in the MNSC of both castes.

The accumulation of elementary granules in MNSC cytoplasm is low at the ages of 72 to 96 hours, but increases considerably afterwards. After 108 hours the rate of accumulation tends to level out.

Lysosomal frequencies do not differ in both castes. Variations are large, even at the end of the larval phase. Figure 31 shows some lysosomes in MNSC at 132 hours. In contrast to younger instars, lysosomal size is considerable.

Transport of elementary granules takes place via axons running from the MNSC to the CC. Sections through the caudo-ventral part of the brain revealed some axons with scattered elementary granules. Obviously the process is phasic. Quantification of transport phenomena is impossible, because finding of places where neurosecretory axons leave the brain is a matter of chance.

Storage and release. Half-way through the CC many neurosecretory axons were found (Fig. 32). Part of the axons run to the aorta wall against the CC. They are filled with a large amount of elementary granules.

At 72 and 96 hours of larval age peripheral axons in both castes appear to be less filled than at the other moments. Peripheral axons always show release phenomena. Different steps of exocytosis towards haemolymph can be indicated. Many microvesicles can be found against the axon membrane.

Axons surrounded by CC tissue are sometimes not completely and sometimes well filled with neurosecretory material. These axons appear to lead to the CA.

Types of neurosecretory granules. Figure 14 shows particle size of elementary granules in neurosecretory axons, cut at different levels of the preparation. No differences in types of elementary granules can be found in the brain, the CC or the NCA. Electron densities do not vary. Particle diameters are about 1500 Å at all levels of the preparation. This does not imply that biochemical properties should be identical too.

4.3.1.3. Quantification of the CA activity

CA activity will be expressed in the number of Golgi zones and in the length of the RER per 500 μ^2 cytoplasmic area. As previously stated quantification of the frequency of elementary granules in fibres after penetration of the gland is impracticable.

Synthesis. Figure 15 shows changes in the number of Golgi zones per

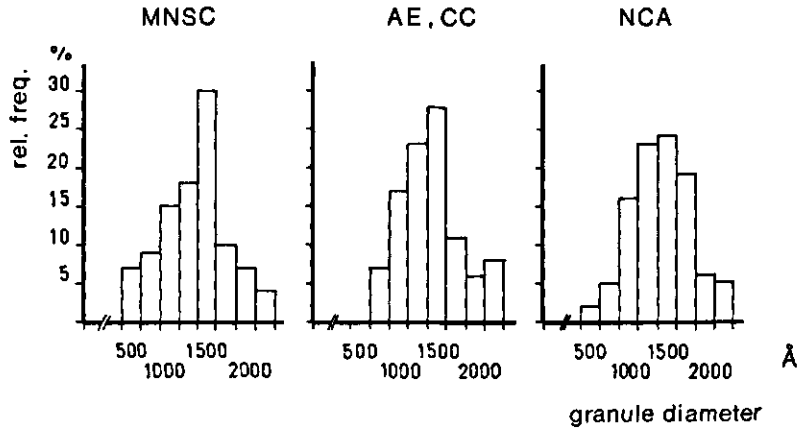


FIG. 14. Frequency distribution of granule diameters in MNSC cytoplasm, in axon endings (AE) in the CC and in the terminal part of the NCA. Per histogram 50 granules were measured from 5 larvae each.

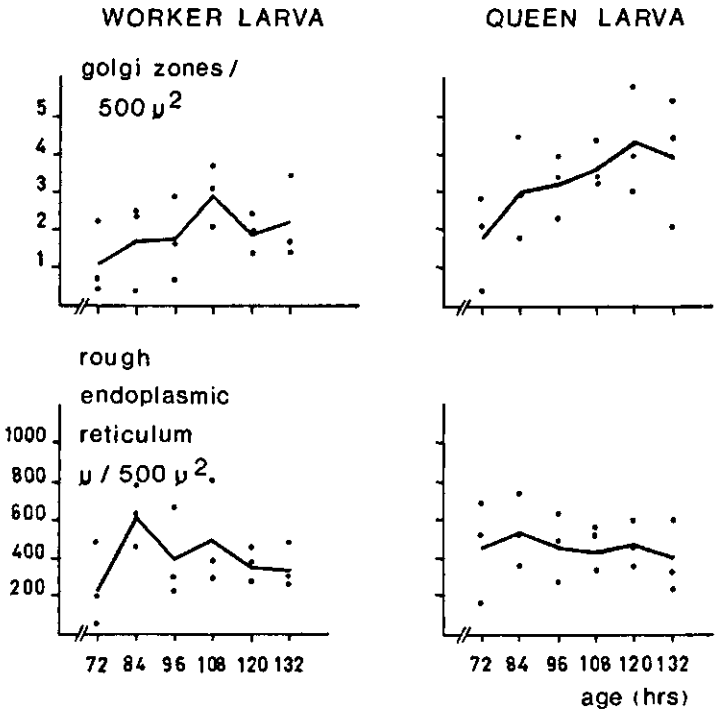
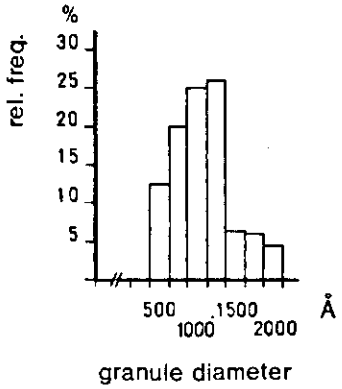


FIG. 15. Quantitative data on the CA activity of queen and worker larvae at different ages. Curves indicate the average values obtained from these larvae. Per larva 5 cells were measured. For further explanation, see text.

FIG. 16. Frequency distribution of granule diameters in the NCA, *after* penetration into the CA. Per histogram 15–25 granules were measured per larva. Total number of measured granules was 250. Compare these diameters with granule sizes in the NCA, before penetration into the CA (see figure 14).



cytoplasmic area. This number increases up to the larval age of 108 hours in worker larvae and to 120 hours in queen larvae and seems to decrease afterwards.

The length of the RER does not vary considerably between both castes. A maximal activity is manifest in 84 hours old larvae.

Elementary granules are found in the NCA, before and after penetration of the gland. Particle type and diameter do not differ from those found in the brain and in axons of the CC. However, particle size seems to decrease in the branching axons within the CA (Fig. 16). No clear release phenomena are observed in the gland. Some indication exists that granules are not released by exocytosis. Microvesicles are never found within CA fibres. Elementary granules fade away, changing their electron density. Synaptic junctions are not clearly proven either.

Granules are chiefly found in the CA at the end of a larval stage. Numbers increase considerably at the end of the 4th and during the whole 5th instar.

4.3.1.4. Correlation between the MNSC and CA activity

MNSC in queen and worker larvae show an increased acitivity along with increasing larval age. This means that neurosecretory activity chiefly functions at the end of the larval period. It is interesting to note that the CA activity decreases after an increase in the number of elementary granules in the NCA.

4.3.2. *Conclusions*

CA are innervated by neurosecretory axons originating from the MNSC in the cerebral ganglion. Some non-neurosecretory axons were found attached to the periphery of the gland. In contrast to the NCA these axons do not penetrate the CA. MNSC do not appear to trigger CA activity, but are possibly involved in the inhibition of the gland activity. However, a causal relation between the rate of neurosecretion and CA inactivation is lacking. Further evidence might be obtained from studies directly affecting MNSC or CA activities. Such effects might be brought about by changing larval nutrition or by application of juvenile hormone.

4.3.3. *Discussion*

It can be questioned in how far quantitative EM methods can reveal changes in *larval* MNSC or CA activity. Tissues in larvae are not fullgrown. Especially during younger instars large variations in activity are found, which however decrease with age. We explain this phenomenon by assuming that only some MNSC become active, while others are still inactive. Thus at 72 hours merely some cells contribute to the obtained values, while at the age of 120 hours all MNSC are involved. In this study we considered the neurosecretory complex as a whole. Each cell of this complex has a limited capacity, which is reflected by the low number of Golgi zones. When neurosecretion is further stimulated, more cells become active. Therefore variations may be understood as a measure of synchronism of the cells investigated.

Results from quantitative EM studies of undeveloped tissues may also be affected by cell activities involved in the construction and development of the cell itself. These activities have nothing to do with the physiological functioning of the cell. Probably for this reason no differences in RER length could be found.

Values obtained from quantitative EM studies on the CA are likely to be affected by changes in CA cell volume. Increasing or constant numbers of Golgi zones in figure 15 have to be interpreted therefore as an absolute increase, while decreasing numbers may merely indicate a constant number of Golgi zones.

5. MNSC AND CA ACTIVITY IN RELATION TO FOOD QUALITY

WIRTZ (1973) found an increase of the CA volume within 12–24 hours after grafting worker larvae into queen cells. He correlated this growth with an increase of the synthetic activity of the gland. When cerebral NSC are involved in this process it is likely that their activity will also change after grafting of worker larvae into queen cells.

In this study changes of MNSC and CA activities are investigated in relation to larval nourishment. Describing the very first signs of beginning gland activity we also report observations on peripheral activities in CA of worker and queen larvae.

Materials and methods

Worker cells containing dated worker larvae (see section 3.1.) were transplanted into a queenless colony at ages of 36, 48 and 72 hours (± 2 hours). After transplantation three samples were taken at 2, 4, 8 and 16 hours. Samples were collected in a 1% solution of glutaraldehyd in 0.1 M sodium cacodylate buffer, pH 7.2 at 0°C. Immediately after sampling, larvae were decapitated and further prepared for EM (see section 2.2.). Specimens were embedded in a Epon – Araldite mixture. Experiments were carried out twice.

Procedures for quantitative EM are described in section 4.3. Per tissue and per age group sampled, 6 grids were obtained and three were observed in the EM. Per grid one neurosecretory cell was chosen and observed at a magnification of 20.000x. Counts were done by direct observation and expressed per 500 μ^2.

Calculation of the elementary granule frequency in CA cells was impracticable. Therefore observations will be described only at the level of the NCA.

5.1. RESULTS

5.1.1. *Effects on MNSC*

Table 3 shows the number of elementary granules and Golgi zones per 500 μ^2 cytoplasm of MNSC at different moments after the change of food. No differences between MNSC activities in worker and queen larvae are discernible, not even at 30 hours after food change.

Larvae transplanted at 36 and 48 hours did not show obvious MNSC activities. MNSC stained deep purple in the LM at these ages and contained numerous free ribosomes in the EM. MNSC of larvae grafted at 72 hours indicate some beginning activity.

TABLE 3. Number of elementary granules (EG) and Golgi zones (GZ) per 500 μ^2 cytoplasm of MNSC at different moments after food change. Transplantations were carried out at larval ages of 36, 48 and 72 hours. Numbers are calculated from transplanted larvae (T) and from control larvae (C).

grafting age (hrs)	36				48				72			
	T		C		T		C		T		C	
samples (hrs)	EG	GZ	EG	GZ	EG	GZ	EG	GZ	EG	GZ	EG	GZ
2	0	0	0	0	0	0	0	0	9	1	3	2
4	0	0	0	0	0	0	0	0	2	0	4	1
8	0	0	0	0	4	0	1	0	2	2	7	1
16	0	0	7	1	0	1	0	0	5	2	2	1

5.1.2. *Effects on CA*

Table 4 gives comparative numbers of elementary granules in the NCA at different moments after grafting. No differences between control and treatment are found. NCA of older larvae appear to contain more neurosecretory granules than younger ones.

TABLE 4. Comparative numbers of elementary granules in the NCA at different moments after the change of food (at 36, 48 and 72 hours after eclosion). Observations were made on transplanted larvae (T) and on control larvae (C). Granulosity was scored as –, when granules were absent; as ± when granules were scarcely present and as + when nerve sections were filled with granules.

grafting age (hrs)	36		48		72	
samples (hrs)	T	C	T	C	T	C
2	–	–	–	–	+	+
4	–	–	±	±	+	+
8	–	±	+	+	±	+
16	–	–	–	±	+	+

Some remarkable phenomena were observed at the periphery of the CA. At 14 and 30 hours after the change of food, membranes started to fold and vacuoles appeared to be tied off. Figure 34 shows a tri-laminar membrane indicating that haemolymph is encapsulated by the cell membrane. Pinocytosis is found in both cases, however it occurs less frequently in worker larvae. The process seems to be responsible for the presence of the large vacuoles in the

cytoplasm. These vacuoles are transported from the border and gradually disappear in the cytoplasm of the CA of queen larvae. In worker larvae however they are maintained for a long time. They appear to increase in volume by fusion with each other. Conjugations of large vacuoles are frequently found (Fig. 35).

Processes indicating exocytosis are also found at the periphery of the CA. Here vacuoles, possibly originating from Golgi zones, are very small in comparison to the previously mentioned ones. Before exocytosis takes place vesicles are surrounded by electron dense material (Fig. 36). After exocytosis remnants are found in the cytoplasm. No relation could be established between the number of vesicles to be excreted or exocytotic activity and CA activity.

5.2. Conclusions and discussion

MNSC activity is not affected by the change of larval food. This may indicate that MNSC are not involved in triggering the CA activity.

CA activity starts previously to any obvious neurosecretory activity. First signs of glandular activity is pinocytotic activity at the periphery of the CA. Increased CA volumes might be correlated with the observed pinocytotic processes.

We do not assume that CA activity is triggered by these trophic processes. One possibility is that the CA activity is induced by bloodborne factors arising elsewhere in the body (Williams, 1975), stimulating trophic activities of the gland. Another possibility is that CA receives direct information from peripheral nerves or from the brain, before starting pinocytosis (Nijhout, 1975).

Exocytosis of vesiculae arising from active Golgi zones in CA cells, are not assumed to be indicative for JH excretion. Their presence or the exocytotic activities can not be related with other signs of activity in the CA. It is probable however, that processing techniques for EM have affected their structural appearance.

The highly convoluted plasma membrane resembles the Malpighian tubule cell in *Rhodnius prolixus* (Wigglesworth and Salpeter, 1962) and the salivary gland duct cell of *Periplaneta americana* (Kessel and Beans, 1963). In general such membranes are assumed to be correlated with active transport of water and solubles. It is of interest to note similarities between secretory cells of the CA and those mentioned above (Waku and Gilbert, 1964).

6. MNSC AND CA ACTIVITY AFTER JUVENILE HORMONE APPLICATION

Application of JH to young worker larvae induces queen development (WIRTZ and BEETSMA, 1972; WIRTZ, 1973; REMBOLD, 1974; ASENCOT and LENSKY, 1976; GOEWIE and BEETSMA, 1976).

We assume that an application of 1–2 µg of JH-I to 3¼–3½ old worker larvae increases the JH titre of larval haemolymph (COPIJN *et al*, in prep.), inducing a queenlike development of the larva. In many other cases, it has been found that JH application delays ecdysis (ABDALLAH, 1972; SLAMA *et al*, 1974). In honey bees however, the prepupal and pupal intermoult periods are shortened after JH application. Thus the pattern of neuro-endocrine activities related with the pupal moult is changed as an element of the total developmental pattern. Two pathways can be considered in this respect. JH titre might control synthetic activities of the CA directly or via the neuro-endocrine system in the larval brain.

In this chapter we report results on investigations of the MNSC and CA activity after application of JH to worker larvae. Results possibly indicate a causal relation between larval MNSC and CA.

Materials and methods

JH I (Biojine, kindly supplied by Dr. L. Lhoste, Procida SA, France) was dissolved in acetone of p.a. quality (Merck). A concentration of 1 mg JH-I per ml acetone was prepared by weighing. Solutions were kept in 10 ml injection bottles, closed with a rubber cap and sealed with an aluminium collar. Solutions were stored in deep freeze at –15°C. Control solutions (acetone only) were kept and stored in the same way.

Applications were carried out with a 50 µl Terumo syringe, attached to a 1 µl Hamilton dispenser. Before application took place, combs with dated larvae were removed from the colony. Care was taken that larvae were not touched or shaken during treatment.

Larvae were treated at the age of 78 hours (± 2 hours). Control larvae received 1 µl acetone only. For the treatments separate syringes were used. After application combs were immediately returned to the colony.

Quantitative EM studies were only carried out on JH and acetone treated larvae that were accepted completely by the colony. Therefore samples were taken 12 hours after application. Larvae were sampled during the very first hours after the 4th moult and during later moments of the 5th instar. Thus discriminating MNSC and CA activities in relation to a normal larval development and to those caused by JH applications. Three larvae aged 90, 93, 96, 102, 105 and 108 hours were collected in a 2% solution of glutaraldehyd in 0.1 M sodium cacodylate buffer, pH 7.2 at 0°C. After taking the last sample, larvae were checked every 6 hours to examine further development and to

observe the moment of cell capping. When cells of all JH treated larvae were closed, combs were removed from the colony and placed in an incubator (32°C, RH 70%). Unsealed cells of control larvae were closed artificially with a piece of wax. This was later improved by using tissue paper. Five days after placing combs in the incubator, all cells were opened and pupal development was compared.

For quantitative EM, samples were prepared, embedded and cut as described in section 4.3. For qualitative observations on neurosecretory axons in the CC, the same grids were used. Only two individuals were cut and studied in the EM.

CA surfaces were measured from larvae treated with 1 µl JH I and with 1 µl acetone at the age of 66 hours (Material was obtained from Dr. P. Wirtz). Measurements were carried out on histological preparations. With the aid of a drawing tube the largest diameter of each gland was drawn and the surface was measured with a planimeter. Each measurement was plotted against larval age.

6.1. Results

JH treated and control larvae developed simultaneously. JH treated larvae appeared to be larger in the 4th and 5th instar than untreated larvae. Cells of JH treated larvae were capped between 120 and 126 hours, whereas cells of control larvae were closed at 132 hours.

6.1.1. *MNSC activity*

Quantification of submicroscopical phenomena in MNSC in JH or acetone treated larvae is represented in figure 17. In general MNSC activities seem to increase after application of JH. In particular this is observed in larvae aged 90 to 96 hours old. In this period larvae just started their 5th instar ('pre-gorging phase'). MNSC activities appear to decrease again in larvae aged 102 to 108 hours. This period will be indicated as the 'gorging phase' (Bertholf, 1925). During the pre-gorging phase the number of elementary granules in the cytoplasm of the MNSC, increases simultaneously with the increase of the Golgi zone frequency. However, the number of elementary granules appear to decrease in JH treated larvae during the gorging phase, whereas the number of Golgi zones is still high.

Again RER lengths in acetone and JH treated larvae are not obviously different.

6.1.2. *CA activity*

Quantifications from EM studies on CA of JH and acetone treated larvae are given in figure 18. The number of Golgi zones per standard cytoplasmic area do not differ obviously in both treatments. Differences between the pre-gorging and the gorging phase are not observed either. RER length in both treatments and in both instars are very similar too. Differences are mani-

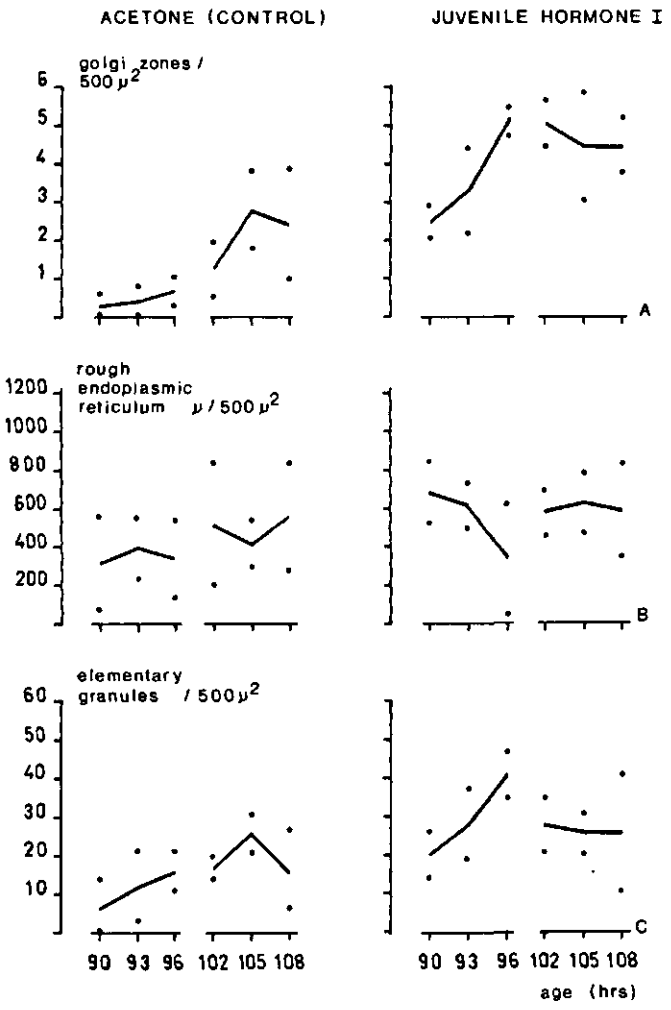


FIG. 17. Quantitative data on the MNSC activity of larvae treated with 1 μl JH-I or with 1 μl acetone (control) at the age of 78 hrs. Curves indicate the average values, derived from two larvae. Per larva 5 cells were measured. For further explanation, see text.

fest in the mitochondrium frequency per standard cytoplasmic area in CA of JH and acetone treated larvae. These frequencies varied considerably in JH treated individuals. Mitochondria in CA of JH larvae appeared to be small and were clustered in the cytoplasm. In CA of control larvae mitochondria were scattered and large.

Elementary granules are found in JH treated and control larvae. Especially nerves of the CA in JH treated larvae during the gorging phase contain more neurosecretory products than those in acetone treated ones.

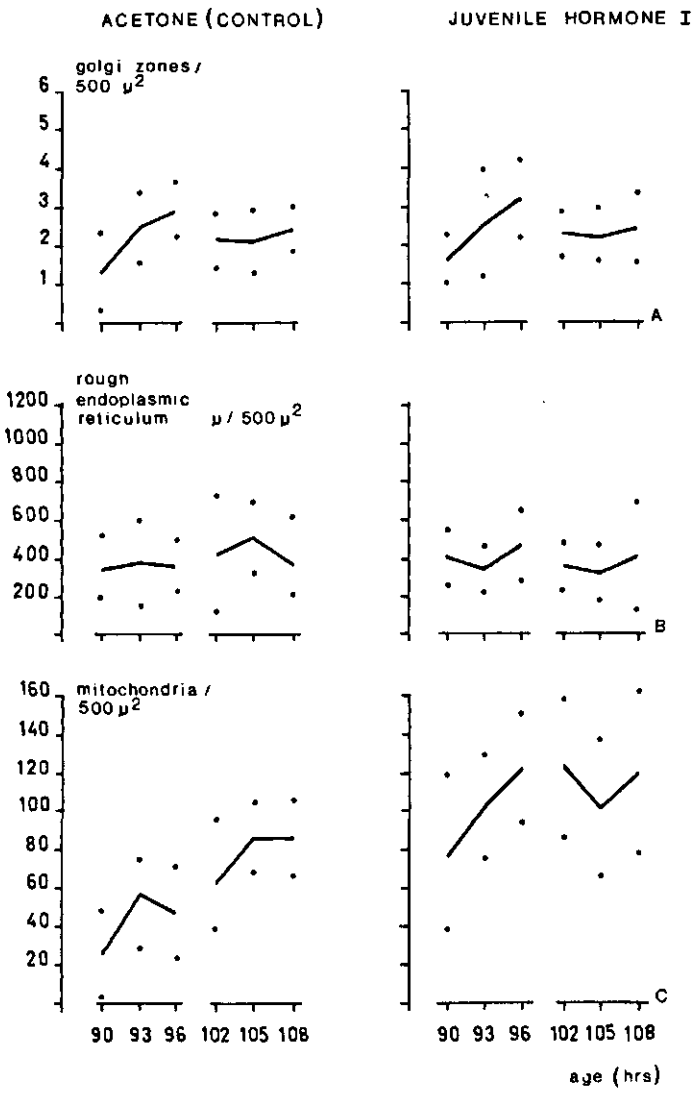


FIG. 18. Quantitative data on the CA activity of larvae treated with 1 μl JH-I or with 1 μl acetone (control) at the age of 66 hrs. Curves indicate the average values, derived from the larvae. Per larva 5 cells were measured. For further explanation, see text.

6.1.3. *CA volume*

The size of the CA in acetone and JH treated larvae, as well as the size of CA in queen larvae, is shown in figure 19. CA are small in JH treated individuals, when compared with acetone treated worker larvae or queen larvae. Irrespective of the treatment of the larvae a decrease in size of the CA follows.

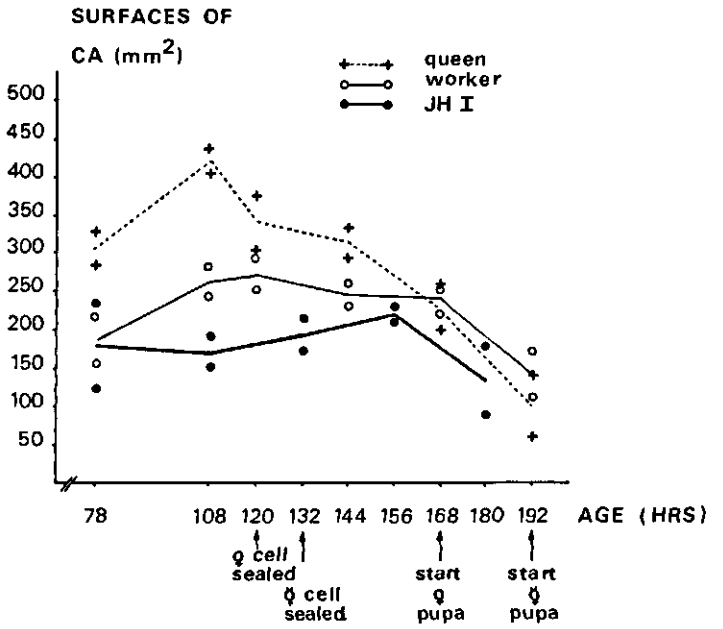


FIG. 19. Surface of the CA in queen larvae, JH-I and acetone treated larvae at different ages. Curves indicate the average values obtained from 6 CA (from 3 larvae). Variations are indicated by the extreme values.

6.2. CONCLUSIONS AND DISCUSSION

In the present study differences in the quantity of submicroscopical organelles were observed in MNSC and CA of JH and acetone treated larvae. These differences can be interpreted as follows. After JH application MNSC of worker larvae show increased numbers of actively secreting Golgi zones. Therefore an increased number of elementary granules is found in the cytoplasm of MNSC during the pre-gorging phase. This accumulation ceases after secretion of granules. Thus a decreased number of elementary granules is found during the gorging phase, whereas the number of actively secreting Golgi zones does not decrease obviously.

Results on the amount of RER are difficult to understand. It is obvious that the RER, as a criterium for the synthetic activity of the MNSC, is of no value.

Large numbers of elementary granules are found in the NCA of JH treated larvae, during the pre-gorging phase. This might indicate that MNSC transport neurosecretory granules via the NCA.

After JH application, in the CA of worker larvae no increase of the number of Golgi zones follows. Due to the decrease in CA size after JH application, a relative increase of organelle frequencies is expected to occur in the cytoplasm of CA cells. Therefore a slight increase of the frequency of the Golgi zones,

during the pre-gorging phase, merely indicates that formation of new Golgi zones did not take place. A similar explanation can be given for organelle frequencies during the gorging phase. Here constant numbers do indicate an actual decrease of the organelle frequencies. Lysosomes are found frequently during this phase.

RER length seems to be decreased in CA of JH treated larvae. As already mentioned, we do not assume that this criterium is a suitable one for expressing the activity of the CA. According to WIRTZ (1973) the amount of RER in CA cells rather indicates secreting capacity than synthetic activity.

The number of mitochondria per standard cytoplasmic area, may not be used as a criterium for CA activity either. The large number of mitochondria might indicate an increased respiratory activity of the gland. However, mitochondria in CA of JH treated larvae were small and probably not active (distinct cisternae). Moreover cluster formation of mitochondria and decreased CA volumes affect the results too. Large variations may be an indication for cluster formation of the mitochondria. Thus obvious differences in mitochondrial frequencies are not assumed to be of any significance.

Our results indicate that JH application increases the MNSC activity, inhibiting the CA activity. As large numbers of neurosecretory granules are found in the CA during the inactivation period of the CA, we may assume that MNSC do inhibit the activity of the gland.

7. THE MECHANISM OF CASTE DIFFERENTIATION

In this chapter results will be interpreted in terms of a possible mechanism of the effect of feeding on caste differentiation. This interpretation will be evaluated afterwards.

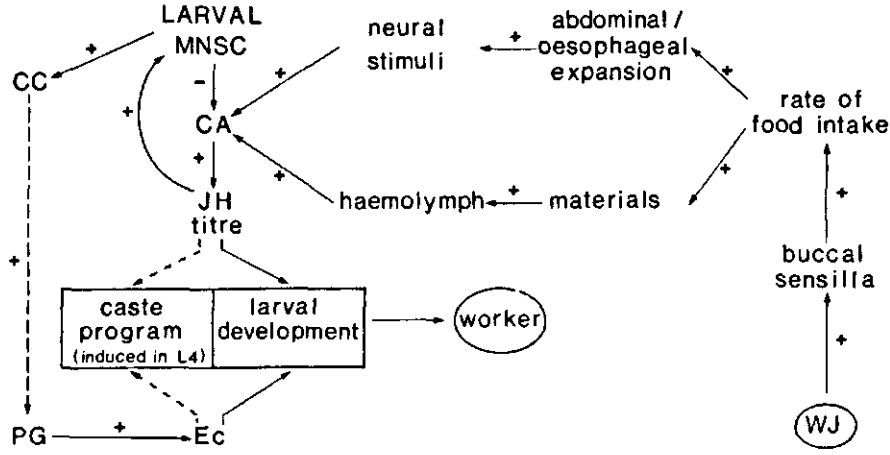
7.1. A POSSIBLE EXPLANATION FOR THE MECHANISM OF CASTE DIFFERENTIATION

Food intake is stimulated by the amount of sugars present in larval food. As WJ contains only small amounts, food intake is not stimulated strongly. The quantity of WJ consumed is sufficient to complete the whole larval development. Thus worker larvae are not short of food (BOUNHIOL, 1938).

Young larvae receiving RJ, are activated to consume large amounts of food. The high sugar concentration in RJ functions as a phagostimulant. High rates of food intake probably stimulate the CA activity. The first sign of gland activity is indicated by pinocytotic processes at the periphery of the CA. It is not likely that this activity is related with an increase of materials in larval haemolymph, caused by the intake of RJ. We merely think that pinocytosis is a consequence of some other processes, which can not be demonstrated with the EM. We think that nerves seen at the periphery of the CA receive action potentials from stretch receptors on the oesophageal or abdominal wall, responding to the rate of food intake (WIGGLESWORTH, 1934). These action potentials or those from the brain may change membrane potentials, facilitating pinocytosis in the gland.

Neural stimuli from the brain, controlling CA activity have also been proved by JOHANSSON (1958 a, b) in *Oncopeltus fasciatus*. Starvation of female adults resulted in CA inhibition, but MNSC activities did not appear to be affected at the same time. Denervation of the CA of starved individuals activated gland activity. Finally the rôle of bloodborne neurosecretory material, not visible in the EM either, must be considered as well. Membrane receptors may perceive the presence of such hormones, resulting in an increased pinocytosis. However the presence of active neurosecretory cells is rather doubtful in younger larvae. Neurosecretory cells in larval brain are undifferentiated at the time CA are fully active. We therefore prefer the first two assumptions.

In the honey bee larva JH has a dual function. On the one hand JH regulates the developmental stage after each moult and on the other it determines which 'developmental program' will be followed. When JH production is low the 'program for the development of a worker' is followed. When JH production is high, the 'program for the development of a queen' is followed. This program seems to be determined by the rate of the CA activity during the 3rd larval instar. For larvae to reach the pupal stage, JH production has to decrease quick-



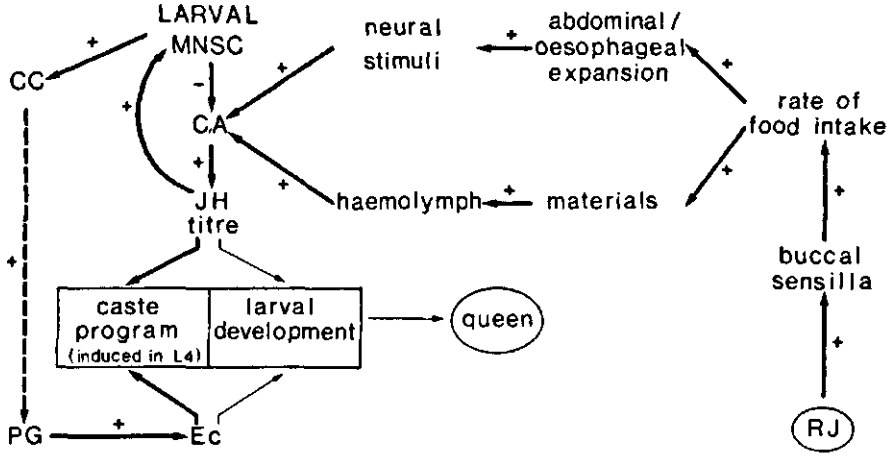


FIG. 20. Representation of a possible mechanism of the regulation of caste differentiation in the honey bee. Schemes do not pretend any completeness, but are ment to serve as an illustration of the text. Thin and heavy lines indicate respectively, weak and strong effects. For further explanation, see text.

ly. This may be achieved by the activity of the MNSC, producing allato-inhibine. At the same time, MNSC secrete neurosecretory material into the haemolymph, via the CC.

Activation of the PG takes place when axon endings in the CC are actively secreting neurosecretory material to the haemolymph. It is attractive to think that these processes are related to each other. Figure 20 gives an illustration of our interpretation of the results, trying to explain the mechanism of caste induction.

7.2. Discussion

In the preceding section, the rate of larval nutrition is assumed to affect CA activity. On the other hand it could be suggested that CA activity might also affect the rate of food intake by larvae. When larvae are induced to become a queen after activation of their CA, an increased metabolic rate will result. A rising expenditure of energy might cause an increased rate of food intake (Strangeways-Dixon, 1961). However from recent findings, it was obvious that inhibition of CA activity in queen larvae by means of Precocene II, resulted in the formation of intermediates, with workerlike characteristics but queenlike weights. This shows that the rate of RJ consumption is independent from gland activity. Caste induction is regulated obviously by the activity of the CA. The rate of food consumption by larvae is determined only by the stimulative capacity of the larval food (Goewie *et al.* in press).

Another matter of discussion is the regulation of the level of the JH titre in larval haemolymph. According to many authors this regulation is determined by both synthesis and breakdown of JH (Kramer and De Kort, 1976). For the honey-bee larva differences in the level of the JH titre in the haemolymph of queen and worker larvae are assumed to be caused mainly by differences in the rate of synthetic activity of the CA. Indeed De Kort *et al* (1977) proved that JH degradation by specific JH esterases in haemolymph and tissue of queen and worker larvae are of minor importance. In contrast with this are the findings of Mane and Rembold (1977). They found JH degrading activities in larval homogenates of queen and worker larvae of the honey bee. They concluded therefore that the level of the JH titre in larval haemolymph is regulated by breakdown of JH. However, they did not prove conclusively that specific JH esterases were involved. JH degradation might have also occured by unspecific tissue esterases, released after homogenizing larval tissue.

The rôle of the PG in caste development is not known. Observations of Lukoschus (1956) indicate a possible rôle of this gland during the late larval development of a queen. Indeed recently Hagenguth and Rembold (1978) proved that differences in ecdysone titres of queen and worker larvae of the 2nd to the 4th instar are not observable. Differences are manifest only from the 5th stage on.

Of particular interest is the rôle of the MNSC in honey-bee larvae. Their function as CA inhibiting centres appears to be in contrast with the MNSC functions in other insects. It is however, also possible that in honey-bee larvae, the MNSC are exclusively associated with the activation of the PG, and that this gland is activated when the CA cease to function. This is not astonishing, as honey-bee larvae grow up under constant micro climatical conditions. From this point of view, they can be compared for instance with parasitic insects.

SUMMARY

The nutritional environment of honey-bee larvae affects the juvenile hormone (JH) titre of larval haemolymph and tissues. In this investigation the mechanism for the regulation of caste differentiation has been studied.

Chemo- and mechanoreceptors are found on larval mouthparts. Chemoreceptors on maxillae and labium are innervated by 5 bipolar neurons, making contact with the larval environment through one pore. Labral chemoreceptors are innervated by 4 bipolar neurons, probably also making contact with the environment through one pore.

All chemoreceptors studied are sensitive to sugars and salts. No sensitivity to other separately tested food components is found. This result is confirmed when considering receptor responses generated by diluted worker jelly (WJ) and royal jelly (RJ). Thus larval food components other than sugars and salts may not affect directly caste differentiation. Sugars possibly function as phagostimulants, regulating the amount of food consumed. The rate of food intake is considered to be the releasing factor for corpus allatum (CA) activity. Possibly neural stimuli arising from stretch receptors on the oesophageal or abdominal wall or indirectly from the brain may be involved.

Salts possibly function as inhibitors of the food intake. However, when adding salts to the food of queen larvae in the colony, the larvae leave the food deposited on the bottom of the cell. Food consumption is not restricted to the present food, but larvae receive also directly offered food from nurse bees. Therefore inhibition of food consumption by addition of salt in colony experiments resulted in a small number of intermediates only. Inhibition of food intake by starvation did not improve results to a large extent. A high rate of mortality and a small number of intermediates were obtained. Though not conclusive, some indication exists that limitation of food intake during the first three days of larval life, inhibits queen induction.

The neuroendocrine system of the larva corresponds in its main features with the classical pattern. In the pars intercerebralis 12 MNSC on both parts of the brain are found. Their axons innervate for one part the corpora cardiaca (CC) and for the other the CA, transporting neurosecretory granules, synthesized in the MNSC. Neurosecretory granules are accumulated in axon endings in the CC and in the neurosecretory fibres within the CA. Release phenomena are observed in the axon endings in the CC. CA are also innervated by two other axons. These axons never contained neurosecretory material throughout larval development.

Ultrastructural studies of CA in younger larvae reveal cycles of synthetic activities. MNSC however show little differentiation at that time. MNSC become active in larvae from about 60 hours of age. At the end of larval development CA innervating axons from the MNSC (nervus corporis allati or NCA) contain considerable amounts of neurosecretory material. Therefore

MNSC can not be considered to trigger CA activity. Studies of synthesis, transport and secretion of elementary granules correlated with synthetic activities of the CA rather suggest that the neurosecretory substance inhibits CA activity.

These results are investigated further in detailed studies on MNSC and CA after changing the food of worker larvae into RJ and after application of JH to worker larvae. The activities of MNSC and CA are studied with quantitative electron-microscopical techniques. The effect of the change of food on the activity of MNSC and CA was determined after different periods. The change of larval nutrition appears not to affect the activity of the MNSC, but to stimulate CA activity. Prior to the increase of the CA activity pinocytosis at the periphery of the CA is observed. This process seems to be responsible for the increase in volume of the CA after intake of RJ by the larva. However pinocytosis is not considered as the trigger for the CA activity. Other processes not observed with the electron microscope might precede this phenomenon.

In JH treated worker larvae MNSC become activated at first. Increased amounts of neurosecretory material can be observed in the NCA, which coïncides with the inhibition of CA activity. The decrease in CA activity after JH application can be explained by a feed back mechanism via the MNSC.

Results and conclusions are interpreted and discussed, resulting in a possible scheme of the mechanism of the caste differentiation in the honey bee.

SAMENVATTING

Het voedselregime van de larve van de honingbij beïnvloedt het niveau van juveniel hormoon (JH) titer in de hemolymfe en de weefsels en als gevolg daarvan de kastendifferentiatie. Dit onderzoek heeft tot doel het mechanisme van deze beïnvloeding op te helderen.

Larvale monddelen dragen chemo- en mechanoreceptoren. De Sensilla styloconica op de maxillen en het labium worden door 5 bipolaire zenuwcellen geïnnerveerd, die tezamen via één pore met de buitenwereld in verbinding staan. Chemoreceptoren op het labium worden door 4 bipolaire zenuwcellen geïnnerveerd. Mogelijk staan ook deze neuronen tezamen via één pore met de buitenwereld in verbinding. Alle onderzochte chemoreceptoren zijn gevoelig voor suikers en zouten. Zij blijken ongevoelig te zijn voor een aantal andere apart getoetste componenten uit het larvale voedsel. Dit resultaat wordt bevestigd door de activiteiten die het voedsel van koninginne- en werksterlarven in de chemoreceptoren oproepen. Dit betekent dat voedselcomponenten, anders dan suikers en zouten, de kastendifferentiatie niet direkt beïnvloeden. Vermoedelijk fungeren suikers als vraatstimulatoren. Zij bepalen de vraatactiviteit van de larve. De snelheid waarmee het voedsel wordt opgenomen wordt beschouwd als de stimulus voor de toename van de corpus allatum (CA) activiteit. Bij dit proces zijn mogelijkerwijs actiepotentialen betrokken die rechtstreeks in strekreceptoren op de oesofagus of in de wand van het abdomen worden opgewekt, dan wel indirect in het cerebrale ganglion.

Zouten hebben mogelijk een remmend effect op de voedselopname. Echter wanneer bij experimenten in het bijenvolk zouten aan het voedsel van koninginnelarve worden toegevoegd, verlaten zij het aanwezige voedsel. Mogelijkerwijs ontvangen de larven dan hoofdzakelijk voedsel dat direct door de voedsterbijen wordt aangeboden. Vraatremming door toediening van zouten leidde derhalve slechts tot het ontstaan van een klein aantal intermediairen. Remming van de voedselopname activiteit door voedselonthouding, leidde niet tot een verbetering van het resultaat. Experimenten resulteerden in een toename van de mortaliteit en in de vorming van een klein aantal intermediairen. Ofschoon geen overtuigend bewijs kon worden geleverd, blijkt dat een beperking van de vraatactiviteit gedurende de eerste drie dagen van de larvale ontwikkeling, in een aantal gevallen werkbij-kenmerken induceerde.

Het neuroendocriene systeem van de larve beantwoordt in hoofdzaak aan het klassieke schema. In de pars intercerebralis worden mediaal neurosecretoire cellen (MNSC) aangetroffen. Hun uitlopers innerveren enerzijds de corpora cardiaca (CC) en anderzijds de CA. Via deze axonen worden neurosecretorische substanties, gevormd in de MNSC, getransporteerd. Neurosecretorisch materiaal wordt in axon uiteinden in de CC en de CA opgeslagen. In het CC worden afgifte verschijnselen waargenomen. Behalve door de MNSC axonen, wordt het CA geïnnerveerd door 2 kleine zenuwuitlopers. Tijdens de gehele larvale

ontwikkeling werd in deze axonen geen neurosecreet aangetoond.

Onderzoek van de ultrastructuur van de CA van jonge larven suggereert een cyclische activiteit. De MNSC blijken dan nog niet te zijn ontwikkeld. Pas in 60 uur oude larven worden deze cellen functioneel. Aan het einde van de larvale ontwikkeling bevatten de MNSC axonen (nervus corporis allati: NCA) grote hoeveelheden neurosecreet. Derhalve kunnen de MNSC niet worden beschouwd als allatotrope centra. Onderzoek naar de synthese, het transport en de afgifte van elementaire granula, gecorreleerd met de synthese activiteit van het CA, tonen aan dat het neurosecreet de CA activiteit afremt.

Bovengenoemde resultaten werden in detail onderzocht aan de MNSC en CA na vervanging van het voedsel van werksterlarven door koninginnegelei en na toediening van JH aan werksterlarven. De activiteit van de MNSC en CA werden met behulp van kwantitatieve EM methoden onderzocht. Het effect van de verandering van voedsel op de activiteit van de MNSC en CA werd na verschillende perioden onderzocht. Uit dit onderzoek bleek dat een verandering van het voedsel geen invloed had op de MNSC activiteit, maar wel op die van de CA. Een toename van de CA activiteit bleek allereerst uit een toenemend aantal pinocytosen aan de periferie van het CA. Het CA volume nam als gevolg van deze activiteiten toe. Pinocytose wordt niet beschouwd als de stimulus die de CA activiteit opgang brengt. Aangenomen wordt dat andere processen aan dit verschijnsel voorafgaan. Deze processen zijn met behulp van electronen microscopie niet te onderzoeken.

In larven die met JH zijn behandeld blijkt de MNSC activiteit eerst toe te nemen. Grote hoeveelheden neurosecreet verschijnen in de NCA, hetgeen samenvalt met een remming van de CA activiteit. Deze remming dient daarom te worden verklaard vanuit een terugkoppeling op de MNSC.

De resultaten en conclusies worden tenslotte in een discussie geïnterpreteerd, hetgeen leidt tot een mogelijke opvatting van de regulatie van de kastendifferentiatie in de honingbij.

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LIST OF ABBREVIATIONS

bm	basal membrane
CA	corpus allatum
CC	corpus cardiacum
cu	cuticle
d	dendrite
EG	elementary granule(s)
EM	electron microscopy
g	glycocalyx
GABA	γ-amino buteric acid
go	golgi zone
JH	juvenile hormone
LM	light microscopy
lo	lobus opticus
MNSC	medial neurosecretory cells
mt	microtubules
n	nucleus
NCA	nervus corporis allatum
nncb	nucleus of nerve cell body
nr	nervus recurrens
nto	nucleus tormogen cell
ntr	nucleus trichogen cell
NSC	neurosecretory cell(s)
oe	oesophagus
p	papillum
pG	prothoracic gland
pi	pinocytosis
RER	rough endoplasmic reticulum
RJ	royal jelly
s	scolopoïd
sj	septate junction
to	tormogen cell
tr	trichogen cell
v	vesicle
WJ	worker jelly

REFERENCES

ABDALLAH, M. D. (1972) Juvenile hormone morphogenetic activity of sesquiterpenoïds and non-sesquiterpenoïds in last stage larvae of *Adoxophyes orana* (Lep.: Tortricidae). Ent. exp. appl. **15**: 411–416.

ANDERSON, W. A. and ANDRÉ, J. (1968) The extraction of some cell components with pronase and pepsin from thin sections of tissue embedded in an Epon-Araldite mixture. J. Microsc. **7**: 343–354.

ASENCOT, M. and LENSKY, Y. (1976) The effect of sugars and juvenile hormone on the differentation of the female honey-bee larvae (*Apis mellifera*) to queens. Life Sci. **18**: 693–700.

ASENCOT, M. and LENSKY, Y. (1977) The effect of sugar crystals in stored royal jelly and juvenile hormone on the differentation of female honeybee (*Apis mellifera* L.) larvae to queens. Proc. VIII Int. Congr. IUSSI, september 5–10, Wageningen, supplement.

BERNAYS, E. A. and CHAPMAN, R. F. (1972) The control of changes in peripheral sensilla associated with feeding in *Locusta migratoria* L. J. exp. Biol. **57**: 755–763.

BISHOP, G. H. (1961) Growth rates of honeybee larvae. J. exp. Zool. **146**: 11–20.

BOUNHIOL, J. J. (1938) Recherches expérimentales sur la déterminisme de la metamorphose chez les lépidoptères. Bull. biol. Fr. Belg. Suppl. **24**, 1.

CHAUVIN, R. (1968) Traité de biologie de l'Abeille. (Masson et Cie., eds.), Paris.

COPIJN, G. M., BEETSMA, J. and WIRTZ, P. (in prep.) Queen differentation and mortality after topical application of different juvenile hormone analogues on worker larvae of the honey bee.

DETHIER, V. G. (1964) In: The physiology of Insect Senses. Methuen and Co. Ltd., London.

DETHIER, V. G. (1971) A surfeit of stimuli: A paucity of receptors. Am. Scient. **59**: 706–715.

DETHIER, V. G. (1972) Electrophysiological studies of gustation in Lepidopterous larvae. II. Taste spectra in relation to food-plant discrimination. J. comp. Physiol. **82**: 103–134.

DETHIER, V. G. (1974) The specificity of the labellar chemoreceptors of the blowfly and the response to natural foods. J. insect Physiol. **20**: 1859–1869.

DIETZ, A. (1972) The nutritional basis of caste differentation in honeybees. In: Insect and mite nutrition. (Rodriguez, I.G., ed.), pp. 272–279, North-Holland, Amsterdam.

DIETZ, A. (1975) Effect of three relative humidities and homogenizing royal jelly on body weight and survival of honey bee larvae in the laboratory. J. Ga. ent. Soc. **10**: 261–264.

DIETZ, A. and HAYDAK, M. H. (1970) The effect of refrigerated royal jelly stored for several years on larval growth and development of female honey bees. J. Ga. ent. Soc. **5**: 205–206.

DIETZ, A. and HAYDAK, M. H. (1971) Caste determination in honey bees. I. The significance of moisture in larval food. J. exp. Zool. **177**: 353–358.

DIETZ, A. and LAMBREMONT, E. N. (1970) Caste determination in honey bees. II. Food consumption of individual honey bee larvae determined with ^{32}P labelled royal jelly. Ann. ent. Soc. Am. **63**: 1342–1345.

DOGRA, G. S. and TANDAN, B. K. (1964) Adaptation of certain histological techniques for in situ demonstration of the neuro-endocrine system of insects and other animals. Quart. J. micr. Sci. **105**: 455–466.

DOGRA, G. S., ULRICH, G. M. and REMBOLD, H. (1977) A comparative study of the endocrine system of the honey bee larva under normal and experimental conditions. Z. Naturforsch. **32**: 637–642.

DÖHMER, I. (1958) Das Neurosekretorische System der Honigbiene, insbesondere der Arbeiterinnen verschiedenen Alters. Diss. Univ. Berlin, pp. 10.

FYG, W. (1966) Beitrag zur Kenntnis der larvalen Hautsinnesorgane der Honigbiene (*Apis mellifera* L.). Mitt. Schweiz. ent. Gesell. **38**: 183–192.

GILBERT, L. I. and KING, D. S. (1973) In: The physiology of Insecta, 2nd. ed., Vol. I. (M. Rockstein, ed.), pp. 249, Academic Press, New York.

GOEWIE, E. A. (1976) Early queen-worker differentiation in the honeybee (*Apis mellifera* L.). Proc. Kon. Ned. Acad. Wetensch., series C, **79**: 470–472.

GOEWIE, E. A. and BEETSMA, J. (1976) Induction of caste differentiation in the honeybee (*Apis mellifera* L.) after topical application of JH-III. Proc. Kon. Ned. Acad. Wetensch., series C, **79**: 466–469.

GOEWIE, E. A., BEETSMA, J. and WILDE, J. de (in press) Effects of Precocene II on caste differentiation in the honey bee (*Apis mellifera* L.).

GOORDEN, A., GOEWIE, E. A. and BEETSMA, J. (in prep.) Effects of juvenile hormone application on development of the prothoracic gland and oenocytes of the honey bee larva (*Apis mellifera* L.).

GRANGER, N. A. and SEHNAL, F. (1974) Regulation of larval corpora allata in *Galleria mellonella*. Nature **251**: 415–417.

GRANGER, N. A. and BORG, T. K. (1976) The allatotropic activity of the larval brain of Galleria mellonella cultured in vitro. Gen. Comp. Endocrinol. **29**: 349–359.

GRIMLEY, P. M., ALBRECHT, J. M., MICHELITCH, H. J. (1965) Preparation of large epoxy sections for light microscopy as an adjunct to fine structure studies. Stain Technol. **40**: 357–366.

GUNDERSEN, R. W. and LARSEN, I. R. (1976) Aniline blue and silver nitrate for staining developing insect nerve tissue. Stain Technol. **51**: 206–208.

HAGENGUTH, H. and REMBOLD, H. (1978) Kastenspezifische Modulation des Ecdysontiters während der Entwicklung der Honigbiene. In: Kurzreferate der Entomologentagung. IUSSI Symp., Juvenilhormone und Juvenoïde, 7–10 März, Karlsruhe.

HAYDAK, M. H. (1968): In Chauvin (1968), pag. 302–333.

HODGSON, E. S. and ROEDER, K. D. (1956) Electrophysiological studies of arthropode chemoreception. J. cell comp. Physiol. **48**: 51–75.

ITTYCHERIAH, P. I. and MARKS, E. P. (1971) Performic acid resorcin fuchsin: a technique for the in situ demonstration of neurosecretory material in insects. Ann. ent. Soc. Am. **64**: 762–765.

JOHANSSON, A. S. (1958a) Relation of nutrition to endocrine-reproductive functions in the Milkweedbug *Oncopeltus fasciatus* (Dallas) (Heteroptera: Lygaeidae). Ph. thesis, Zoological Laboratory of the University, Blindern, Oslo, pp. 83–113.

JOHANSSON, A. S. (1958b) Hormonal regulation of reproduction in the milkweedbug, *Oncopeltus fasciatus* (Dallas). Nature **181**: 198–199.

KARNOVSKY, M. J. (1965) A paraformaldehyde-glutaraldehyde fixative of high osmolarity for use in EM. J. cell Biol. **27**: 137A–138A.

KESSEL, R. G. and BEAMS, H. W. (1963) See Waku, Y. and Gilbert, L. I. (1964).

KORT, C. A. D. DE, WIETEN, M., KRAMER, S. J. and GOEWIE, E. A. (1977) Juvenile hormone degradation and carrier proteins in honey bee larvae. Proc. Kon. Ned. Acad. Wetensch. series C, **80**: 297–301.

KRAMER, S. J. and KORT, C. A. D. DE (1976) Age-dependent changes in juvenile hormone esterase and general carboxyesterase activity in the hemolymph of the Colorado potato beetle, *Leptinotarsa decemlineata*. Mol. cell. Endocrinol. **4**: 43–53.

LARIMER, I. L. and ASHBY, E. A. (1964) Reduced methylene blue as a stain for Crustacean nerves. Stain Technol. **3a**: 369–372.

LOUD, A. V., BARANY, W. C. and PACK, B. A. (1965) Quantitative evaluation of cytoplasmic structures in electron micrographs. Lab. Invest. **14**: 258–996.

LOUVEAUX, A. (1975) Étude de l'innervation sensorielle de l'hypopharynx de larves de *Locusta migratorioides*. Insect. Soc. **22**: 3–12.

LUFT, J. H. (1961) Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol. **9**: 409–414.

LUKOSCHUS, F. (1955) Die Bedeutung des innersekretorischen Systems für die Ausbildung epidermaler Kastenmerkmale bei der Honigbiene (*Apis mellifera* L.). Insect. Soc. **2**: 221–236.

LUKOSCHUS, F. (1956) Untersuchungen zur entwicklung der Kastenmerkmale bei der Honigbiene (*Apis mellifera* L.). Z. Morph. Ökol. Tiere **45**: 157–197.

LUKOSCHUS, F. (1961) Das innersekretorische System und seine Bedeutung für die Entwicklung des Weiblichen Dimorphismus bei der Honigbiene. Proc. IV Int. Congr. IUSSI, 9–14 september, Pavia.
MA, W. C. (1972) Dynamics of feeding responses in *Pieris brassicae* L. as a function of chemoreceptory input: a behavioural, ultrastructural and electrophysiological study. Meded. Landbouwhogeschool, Wageningen, 72–11.
MANE, S. D. and REMBOLD, H. (1977) Developmental kinetics of juvenile hormone inactivation in queen and worker castes of the honey bee, *Apis mellifera*. Insect Biochem. **7**: 463–467.
MC. CAFFERTY, A. R. and HIGHNAM, K. C. (1975a) Effects of corpora allata on the activity of the cerebral neurosecretory system of *Locusta migratoria migratorioides* R & F. Gen. Comp. Endocrinol. **25**: 358–372.
MC. CAFFERTY, A. R. and HIGHNAM, K. C. (1975b) Effects of corpus allatum hormone and its mimics on the activity of the cerebral neurosecretory system of *Locusta migratoria migratorioides* R & F. Gen. Comp. Endocrinol. **25**: 373–386.
MCGUIRE, S. R. and OPEL, H. (1969) Resorcin-fuchsin staining of neurosecretory cells. Stain Technol. **44**: 235–237.
MELAMPY, R. M. and WILLIS, E. R. (1939) Respiratory metabolism during larval and pupal development of the female honeybee (*Apis mellifera* L.). Physiol. Zool. **12**: 302–311.
MITCHELL, B. K. and SCHOONHOVEN, L. M. (1974) Taste receptors in Coloradobeetle larvae. J. insect Physiol. **20**: 1787–1793.
MORDUE, W. (1967) The influence of feeding upon the activity of the neuroendocrine system during oocyte growth in *Tenebrio molitor*. Gen. Comp. Endocrinol. **9**: 406–415.
MORITA, H. (1959) Initiation of spike potentials in contact chemosensory hairs of insects. III. D.C. stimulation and generator potential of labellar chemoreceptors of *Calliphora*. J. cell. comp. Physiol. **54**: 189–204.
NELSON, I. A. (1924) Morphology of the honeybee larva. J. agric. Res. **28**: 1167–1213.
NIJHOUT, H. F. (1975) A threshold size for metamorphosis in the tobacco hornworm *Manduca sexta* L. Biol. Bull. **149**: 214–225.
OSONAI, M. and REMBOLD, H. (1968) Entwicklungsabhängige mitochondriale Enzymaktivitäten bei den Kasten der Honigbiene. Biochim. Biophys. Acta **162**: 22–31.
OTTER, C. J. DEN (1972) Differential sensitivity of insect chemoreceptors to alkali cations. J. insect Physiol. **18**: 109–131.
PEREZ, J. (1889) In Chauvin (1968) part 1, pag. 46–47.
PITMAN, R. M., TWEEDLE, C. D. and COHEN, M. J. (1972) Branching of central neurons: Intra cellular cobalt injection for light and electron microscopy. Science **176**: 412–414.
RADEMAKERS, L. H. P. M. (1977) Identification of a secretomotor centre in the brain of *Locusta migratoria*. In: Neuro-endocrine system and release of adipokinetic hormone from the corpora cardiaca of the migratory Locust. Ph. D. Thesis, Univ. Utrecht, pp. 63–81.
REMBOLD, H. (1969) Biochemie der Kastenentstehung bei der Honigbiene. Proc. VI Int. Congr. IUSSI, Bern, pp. 239–246.
REMBOLD, H. (1974) Die Kastenbildung bei der Honigbiene aus biochemischer Sicht. In: Sozialpolymorphismus bei Insekten. Probleme der Kastenbildung im Tierreich (G. H. Schmidt, ed.), pp. 351–404. Wissenschaftl. Verlag Gesellsch.
REMBOLD, H. (1976) The rôle of determinator in caste formation in the honeybee. In: Phase and caste determination in insects (M. Lüscher, ed.), pp. 21–34, Pergamon Press, Oxford.
REMBOLD, H. (1978) Vergleichende Analyse von Weiselfuttersäften. In: Kurzreferate der Entomologentagung. IUSSI Symp., Juvenil hormone und Juvenoïde, 7–10 März, Karlsruhe.
REYNOLDS, E. S. (1963) The use of lead citrate at high pH as an electron opaque stain in electronmicroscopy. J. cell Biol. **17**: 208–212.
RIBI, W. A. (1975) The neurons of the first optic ganglion of the bee (*Apis mellifera*). Advances in Anatomy Embryology and Cell Biology, **50**, pp. 8–9, Springer Verlag, Berlin.
ROUBOS, E. W. (1973) Regulation of neurosecretory activity in the freshwater pulmonate *Lymnaea stagnalis* L. A quantitative electronmicroscopical study. Z. Zellforsch. **146**: 177–205.

ROUBOS, E. W. (1975) Regulation of neurosecretory activity in the freshwater snail *Lymnaea stagnalis* (L.) with particular reference to the role of the eyes. Cell Tiss. Res. **160**: 291–314.
ROUSSEL, J. P. (1976) Activité comparée des hormones juvéniles en C-18 (JH-I) et en C-16 (JH-III) chez Locusta migratoria. J. insect Physiol. **22**: 83–88.
SCHOONEVELD, H. (1974) Ultrastructure of the neurosecretory system of the Colorado potato *Leptinotarsa decemlineata* Say. I. Characterization of the protocerebral neurosecretory cells. Cell Tiss. Res. **154**: 275–288.
SCHOONHOVEN, L. M. (1974) Comparative aspects of taste receptor specificity. In: Transduction mechanisms in chemoreception, (T. M. Poynder, ed.), pp. 189–201. Information Retrieval Ltd., London.
SEHNAL, F. and GRANGER, N. A. (1975) Control of corpora allata function in larvae of Galleria mellonella. Biol. Bull. **148**: 106.
SLÁMA, K., ROMAŇUK, M. and SORM, F. (1974) In: Insect hormones and bioanalogues. Springer Verlag, New York.
SMITH, M. V. (1959) Queen differentiation and biological testing of royal jelly. Memoir 356, Cornell University, Agric. Exp. Sta., New York State Coll. of Agric., Ithaca, New York.
STABE, H. A. (1930) The rate of growth of worker, drone and queen larvae of the honeybee, *Apis mellifera* L. J. econ. Ent. **23**: 447–453.
STARK, M. J., SMALLEY, K. N. and ROWE, E. C. (1969) Methylene blue staining of axons in the ventral nerve cord of Insects. Stain Technol. **44**: 97–102.
STEEL, C. G. H. (1975) A neuroendocrine feedback mechanisms in the insect moulting cycle. Nature **253**: 267–269.
STRANGEWAYS-DIXON, I. (1961) The relationship between nutrition, hormones and reproduction in the blowfly *Calliphora erythrocephala* (Meig.). J. exp. Biol. **38**: 225–235.
TYRER, N. M. and BELL, E. M. (1974) The intensification of cobalt-filled neurone profiles using a modification of Timm's sulfide-silver method. Brain Res. **73**: 151–155.
WAKU, Y. and GILBERT, L. I. (1964) The corpora allata of the silkmoth, *Hyalophora cecropia*: an ultrastructural study. J. Morph. **115**: 69–96.
WANG, D. I. (1965) Growth rates of young queen and worker honeybee larva. J. apicult. Res. **4**: 3–5.
WEAVER, N. (1962) Control of dimorphism in the female honeybee. Science, N.Y. **138**: 995.
WEAVER, N. (1974) Control of dimorphism in the female honeybee. 3. The balance of nutrients. J. apicult. Res. **13**: 93–101.
WENDELAAR BONGA, S. E. (1971a) Osmotically induced changes in the activity of neurosecretory cells located in the pleural ganglia of the freshwater snail *Lymnaea stagnalis* L. studied by quantitative electron microscopy. Neth. J. Zool. **21**: 127–158.
WENDELAAR BONGA, S. E. (1971b) Formation, storage and release of neurosecretory material studied by quantitative electron microscopy in the fresh-water snail *Lymnaea stagnalis* L. Z. Zellforsch. **113**: 490–517.
WHITEHEAD, A. T. and LARSEN, I. R. (1976) Electrophysiological responses of galeal contact chemoreceptors of *Apis mellifera* to selected sugars and electrolytes. J. insect Physiol. **22**: 1609–1616.
WIGGLESWORTH, V. B. (1934) The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and metamorphosis. Quart. J. micr. Sci. **77**: 191–222.
WIGGLESWORTH, V. B. (1964) The hormonal regulation of growth and reproduction in insects. Adv. Insect Physiol. **2**: 247–336.
WIGGLESWORTH, V. B. (1970) Insect hormones, Oliver and Boyd, Edinburgh and London.
WIGGLESWORTH, V. B. and Salpeter, M. M. (1962) Histology of the Malpighian tubules in *Rhodnius prolixus* (Hemiptera). J. insect Physiol. **8**: 299–307.
WILDE, J. DE (1966) Buitenwereld en binnenwereld van het insect. Kon. Ned. Acad. Wetensch. gew. vergad. Afd. Natuurk. **75** (5).
WILDE, J. DE (1973) In: The physiology of Insecta, 2nd ed., vol. I. (M. Rockstein, ed.), pp. 97, Academic Press, New York.
WILDE, J. DE (1976) Juvenile hormone and caste differentiation. In: Phase and caste determination in insects. (M. Lüscher, ed.), pp. 5–20, Pergamon Press, Oxford.

WILDE, J. DE and WIRTZ, P. (1974) Hormone und polymorphismus der Insekten. Fortschr. Zool. **22**: 93–98.
WILLIAMS, C. M. (1975) Juvenile hormone... in retrospect and in prospect. In: The juvenile hormones. (L. I. Gilbert, ed.), pp. 2, Plenum Press, N.Y., London.
WIRTZ, P. (1973) Differentiation in the honeybee larva. Meded. Landbouwhogeschool Wageningen, 73–5.
WIRTZ, P. and BEETSMA, J. (1972) Induction of caste differentiation in the honeybee (*Apis mellifera* L.) by juvenile hormone. Ent. exp. appl. **15**: 517–520.

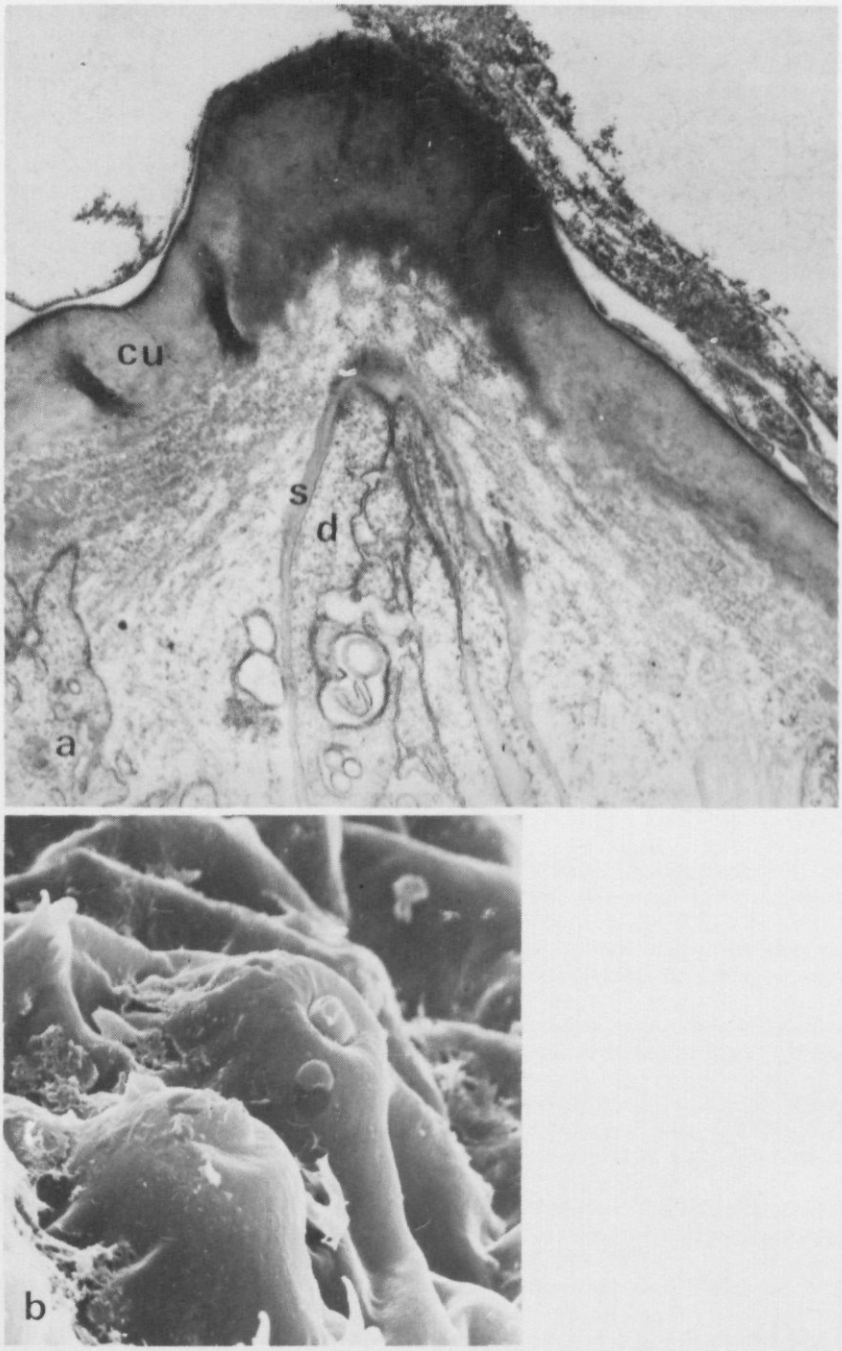

FIG. 21. Labral papilla-like sensillum of a 4th instar queen larva.
a. Longitudinal section. Note the bundle of dendrites (d) enveloped by a cuticular scolopoid (s). 20.000x. b. Scanning electron microscopical micrograph of the labral sensilla. 9000x.

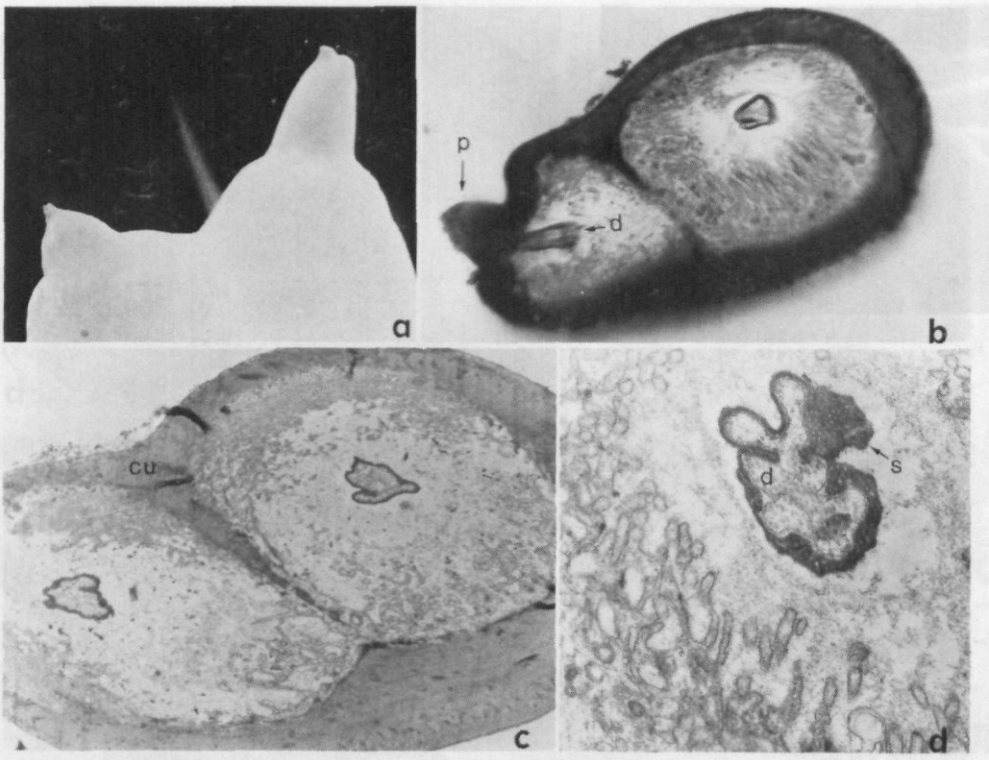

FIG. 22. Maxillar Sensillum styloconicum of a 4th instar queen larva.
a. Conical papillae on top of the sensillum. 9000x. b. Cross section through the top of the sensillum. Note a bundle of dendrites (d) leading to the medial papilla (p). 5000x. c. Cross section just below the level where papillae come together. 10.000x. d. Cross section at a lower level than before. A cuticular scolopoïd (s) envelops a bundle of dendrites (d). 20.000x.

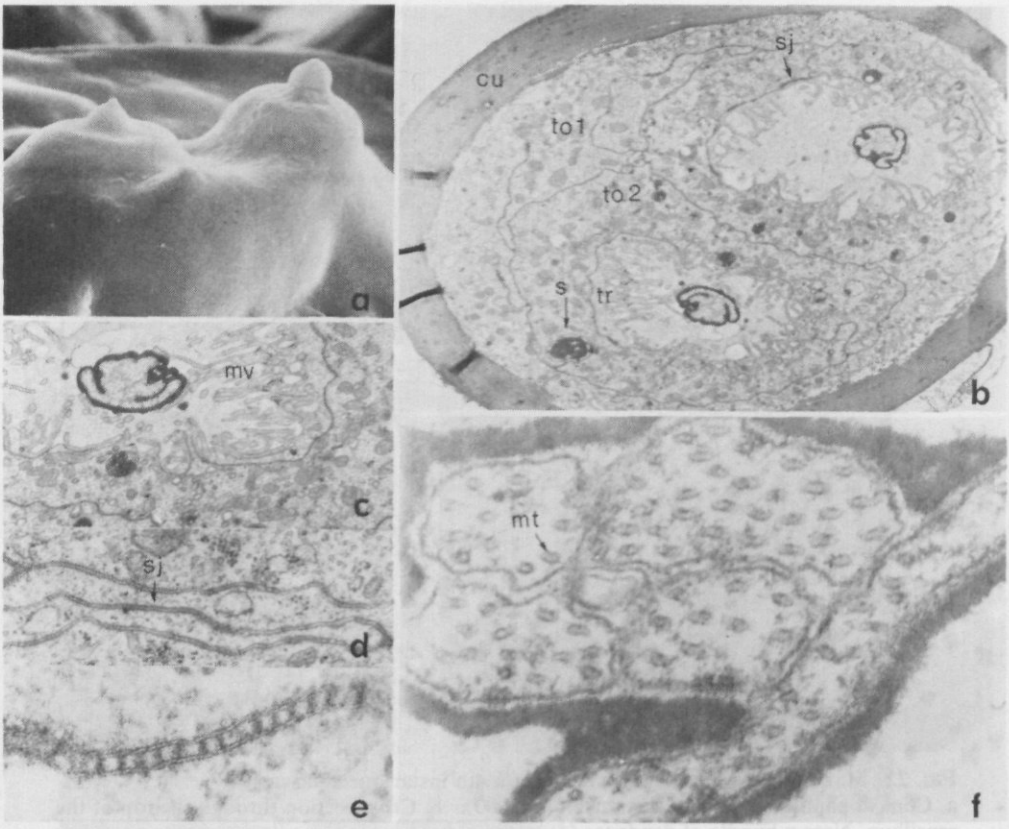

FIG. 23. Labial Sensillum styloconicum of a queen larva.
a. Scanning electron micrograph of the labial sensillum of a 3rd instar queen larva. Note the sensillum is not developed completely. A third papillum appears to be present. 3.000x. b. Cross section through the middle of a S. styloconicum of a 4th instar queen larva. Trichogen (tr) and tormogen (to 1 and to 2) cells envelop the scolopoïd. Cells make contact to each other through many septate junctions (sj). Note the presence of a third but undeveloped scolopoïd, completely with a undeveloped trichogen cell. 1.000x. c. Enlargement of a part of micrograph b. 10.000x. d. Septate junction (sj) area between enveloping cells. 30.000x. e. Enlargement of a septate junction. 120.000x. f. Scolopoïd with 5 dendrites. Note microtubules (mt) running through the dendrites. 80.000x.

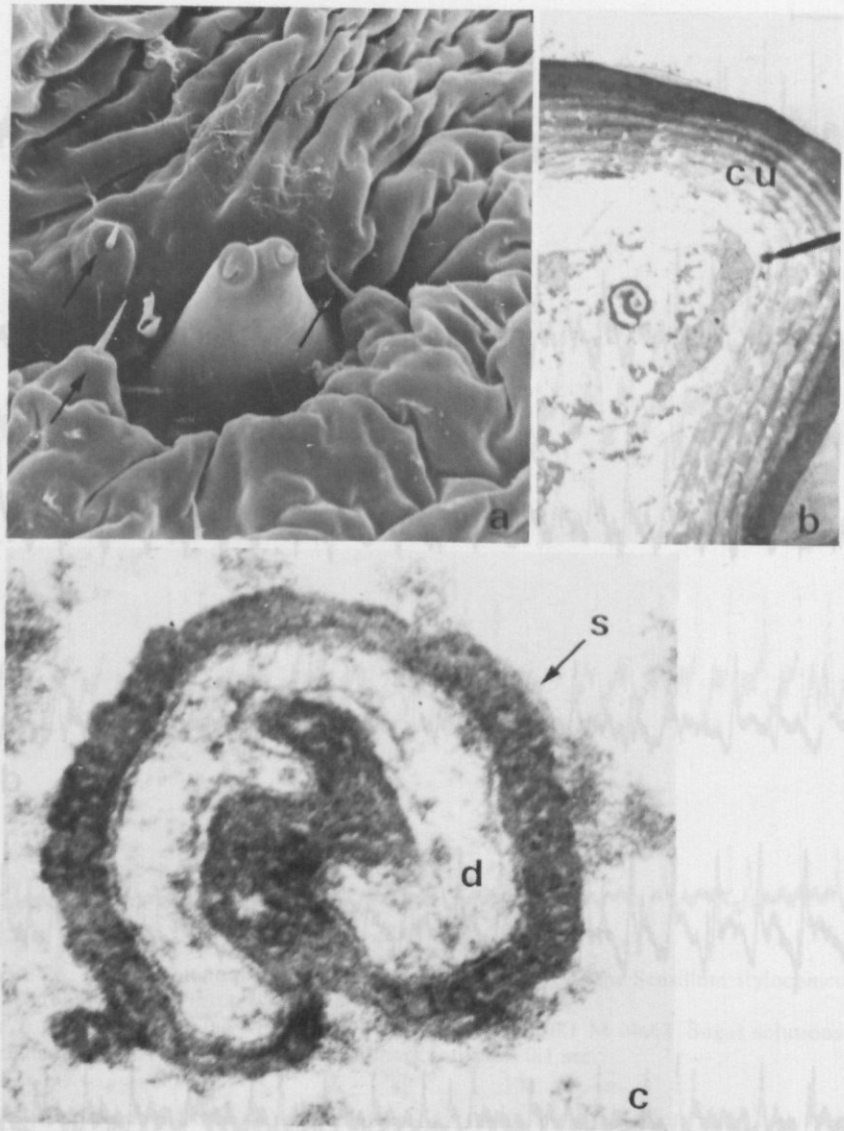

FIG. 24. Labial Sensillum trichodeum of a 5th instar queen larva.
a. Scanning micrograph of 3 S. trichodea, around the S. styloconicum of the labium. 1.000 x.
b. Cross section through the basis of the receptor. The cuticle (cu) is stratified. 2.000 x.
c. Dendrite (d) of the sensillum at high magnification surrounded by the scolopoïd (s). 40.000 x.

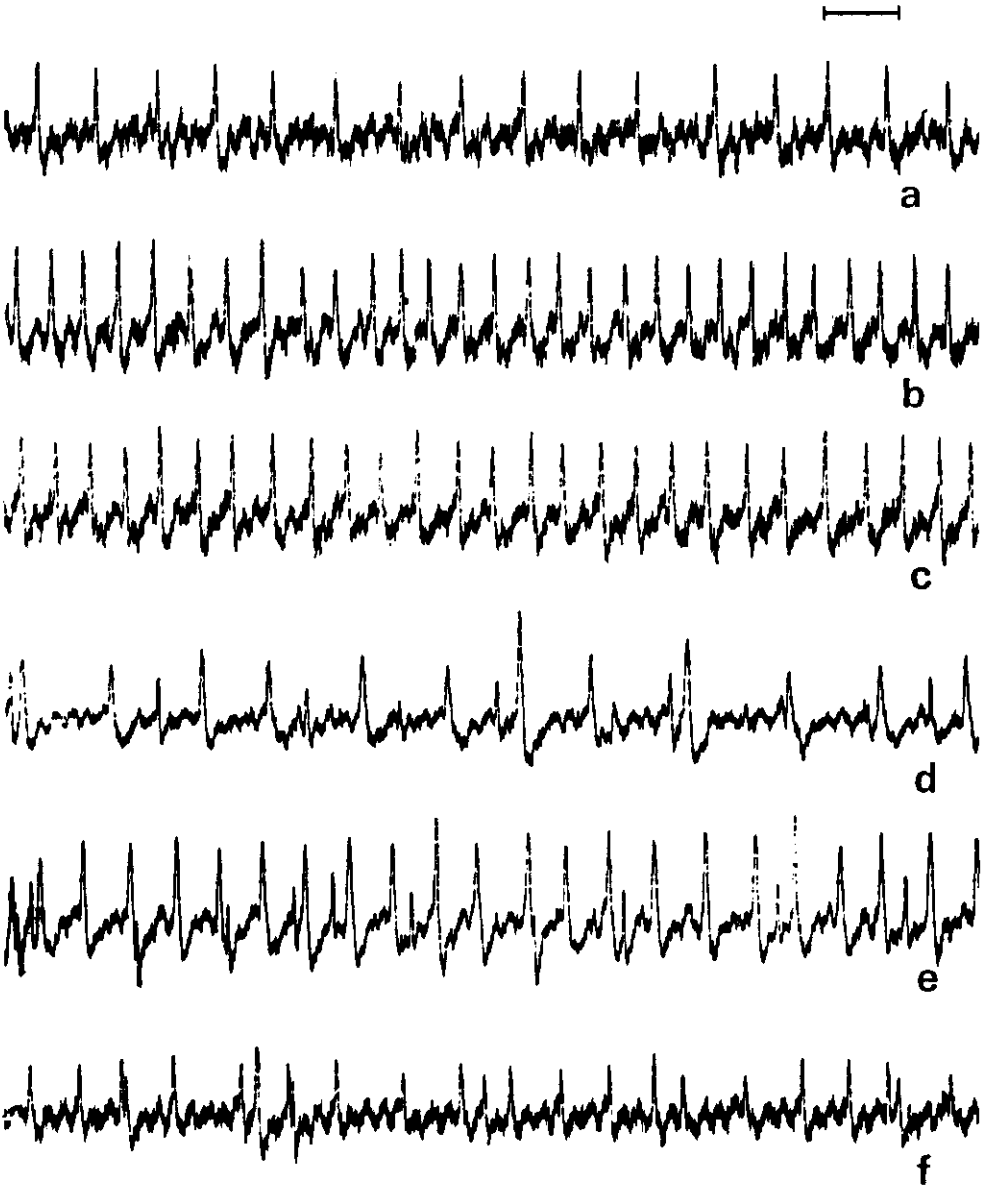

FIG. 25. Action potentials obtained from the lateral part of the Sensillum styloconicum of the labium of a 5th instar worker larvae.
a. 0.1 M fructose, b. 0.5 M fructose, c. 1 M fructose, d. 20% WJ, e. 20% RJ, f. 0.1 M NaCl. Solutions were made in 0.1 M NaCl. The calibration mark indicates 0.1 sec. WJ and RJ were obtained from cells with 2nd or 3rd instar larvae.

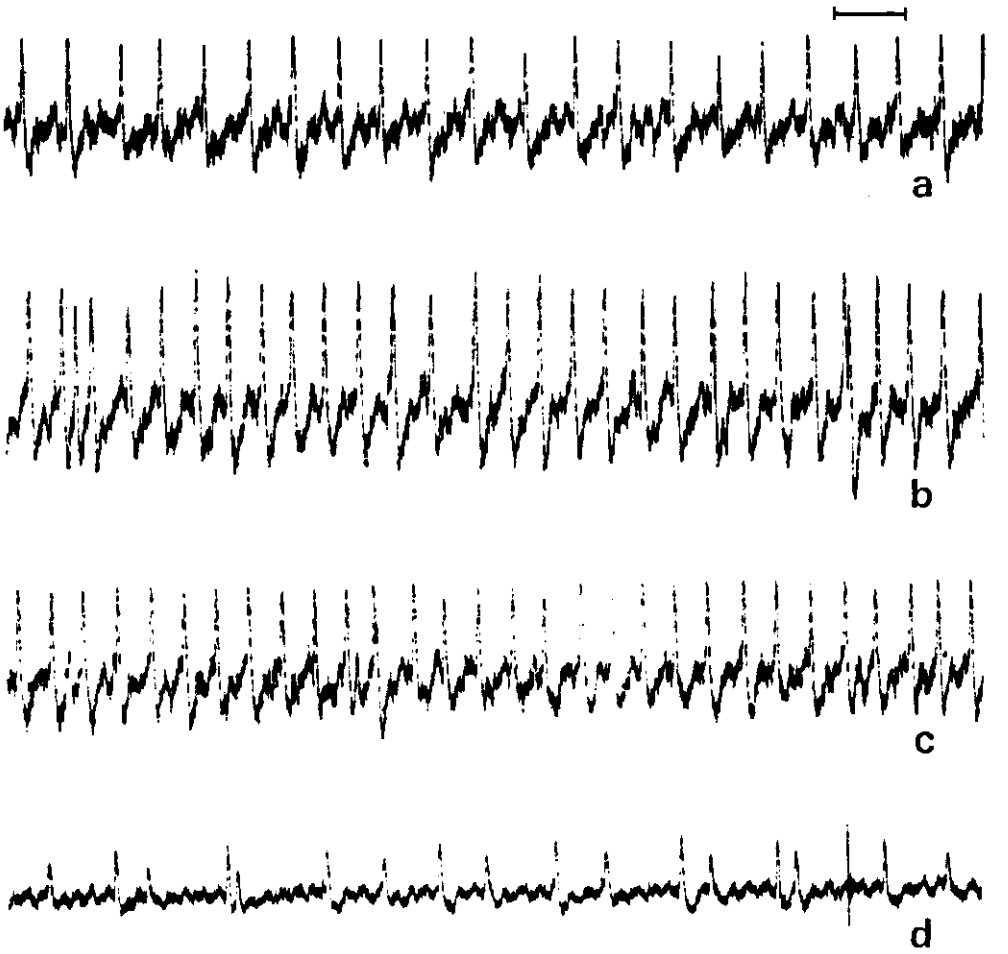

FIG. 26. Action potentials obtained from the lateral part of the Sensillum styloconicum of the labium of a 5th instar worker larvae.
a. 0.1 M sucrose, b. 0.5 M sucrose, c. 1 M sucrose, d. 0.1 M NaCl. Sugar solutions were made in 0.1 M NaCl. The calibration mark indicates 0.1 sec.

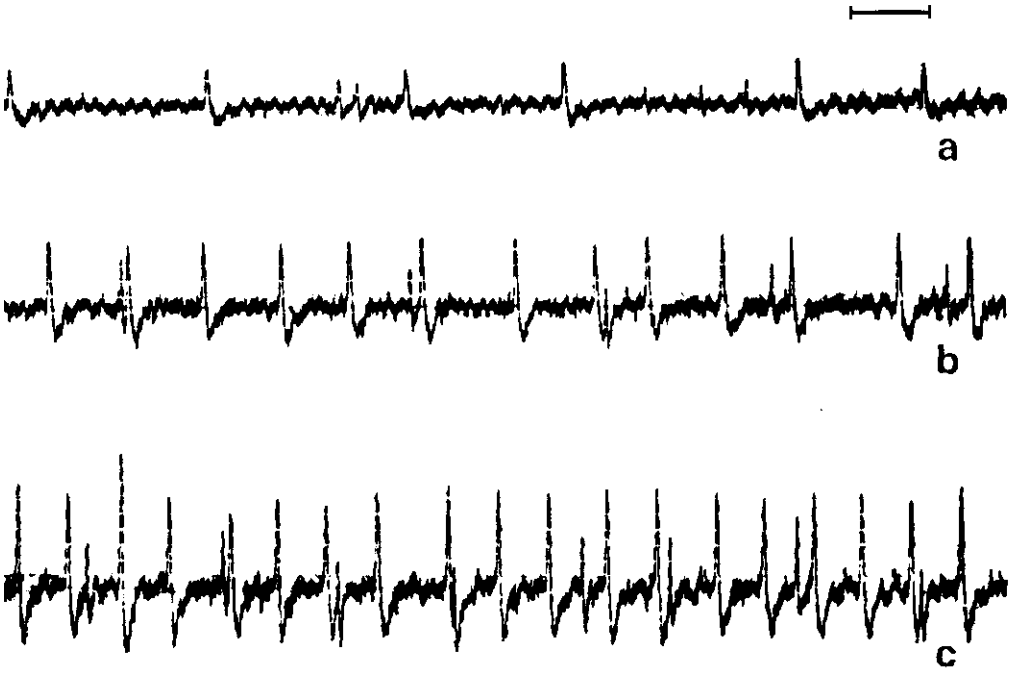

FIG. 27. Action potentials obtained from the lateral part of the Sensillum styloconicum of the labium of a 5th instar worker larvae.
a. 0.1 M KCl, b. 0.5 M KCl, c. 1 M KCl. The calibration mark indicates 0.1 sec.

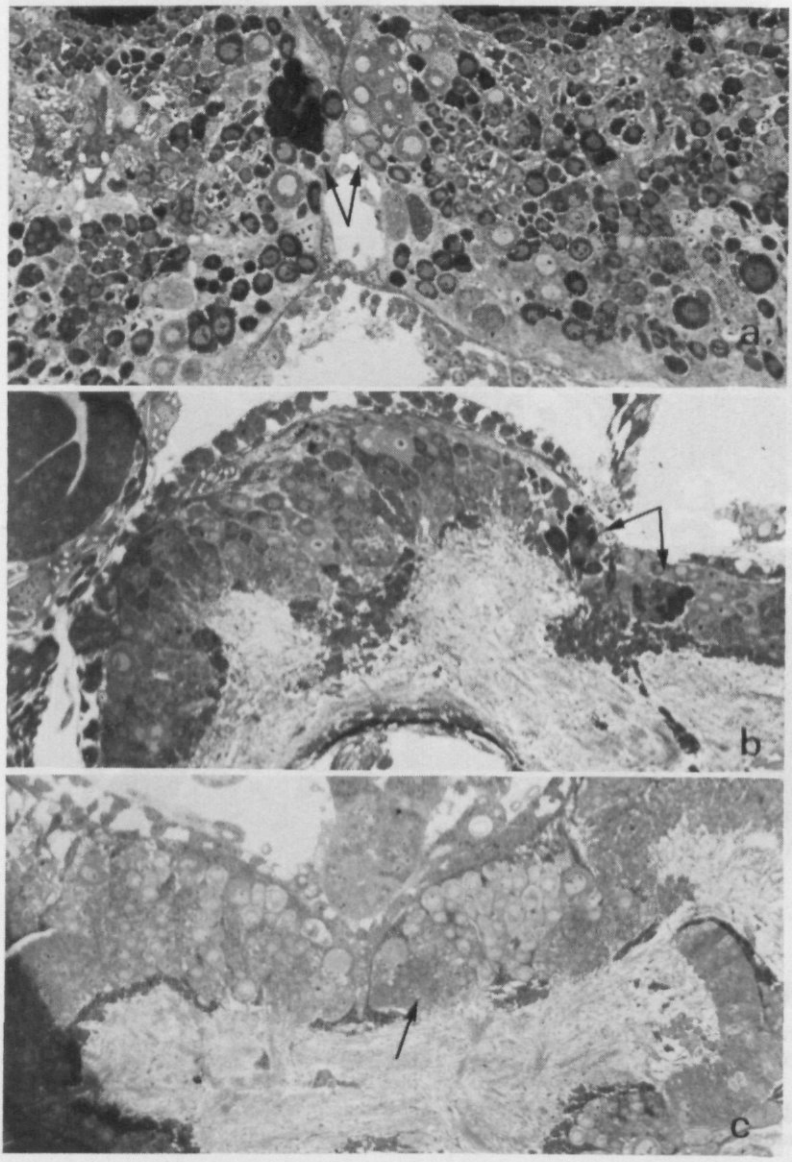

FIG. 28. Brain of queen larvae.
a. 96 hours old queen larva. Medial neurosecretory cells (see arrows). Probably the right group of cells is active. Note numerous darkly stained cell bodies in all parts of the brain. Epon-Araldite, 2 μ, 240 x. b. 48 hours old queen larva. Both groups of medial neurosecretory cells are still inactive. Epon-Araldite, 2 μ, 192 x. c. 78 hours old queen larva. Neurosecretory cells are inactive, but do not stain, probably because of the embedding medium. Epon, 2 μ, 192 x. Note axonal pathways.

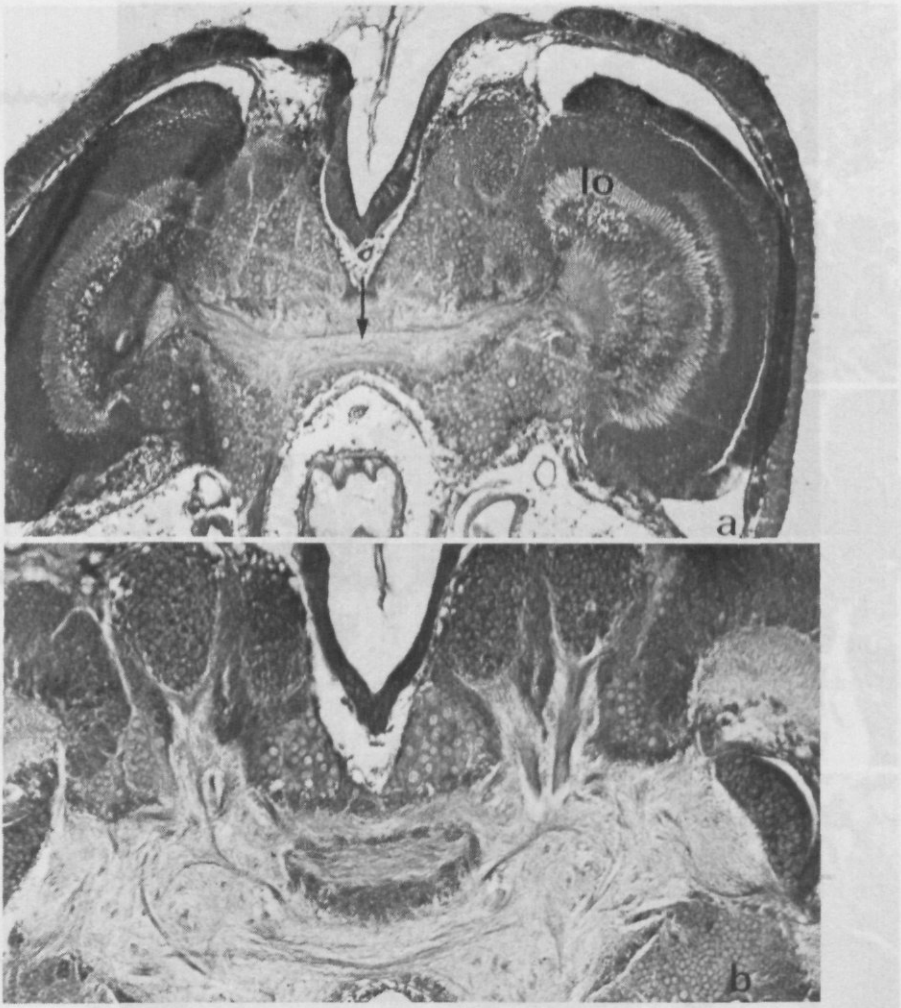

FIG. 29. Brain of queen larvae.
a. 114 hrs old queen larva. Frontal section through the brain. The lobus opticus (lo) starts to develop. Note the chiasma. Paraplast, 10 μ, 120 x. b. 82 hrs old queen larva. Frontal section through the brain. Eyes are not yet developed. Most association centres are undeveloped. Note the many axonal pathways in the neuropyle. Paraplast, 10 μ, 192 x.

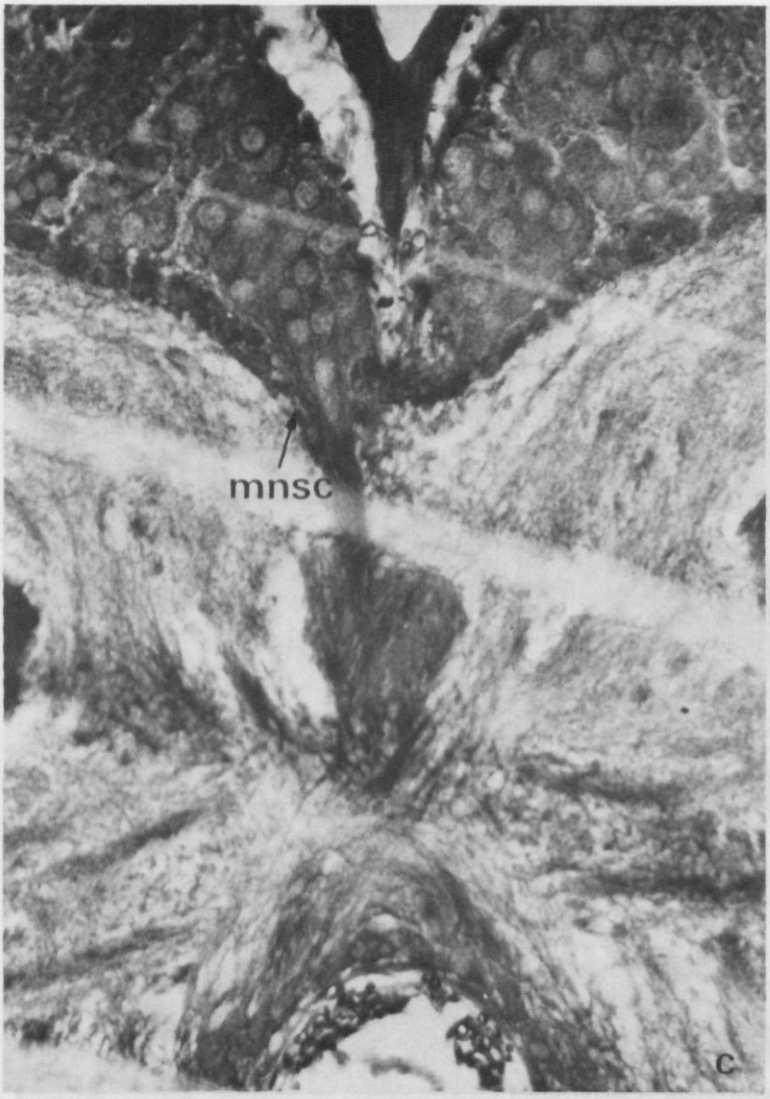

FIG. 29c. Pars intercerebralis of a 96 hours old queen larva. Medial neurosecretory cells penetrate the neuropyle, forming the chiasma. Paraplast, 10 μ, 480 x.

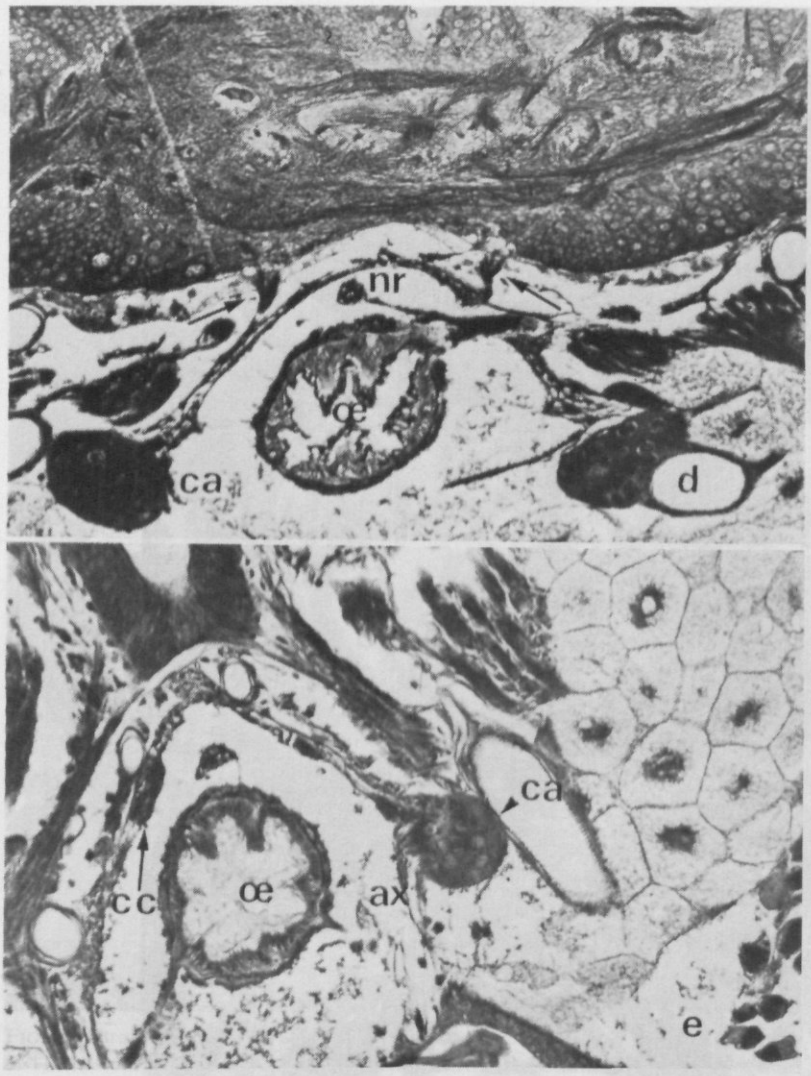

FIG. 29d, e. Innervation of larval CA.
d. 114 hours old queen larva. Ventral region of the brain, showing spots where the NCA are leaving the brain. Axons descend to the CA. Note nervus recurrens (nr) just above the oesophagus (oe). Paraplast, 15 μ, 192 x. e. 114 hours old queen larva. Oblique rostro-ventral section through the head. Nerve cell bodies ventrally of the CA possibly innervate the gland. Note remnants of the CC. Paraplast, 10 μ, 192 x.

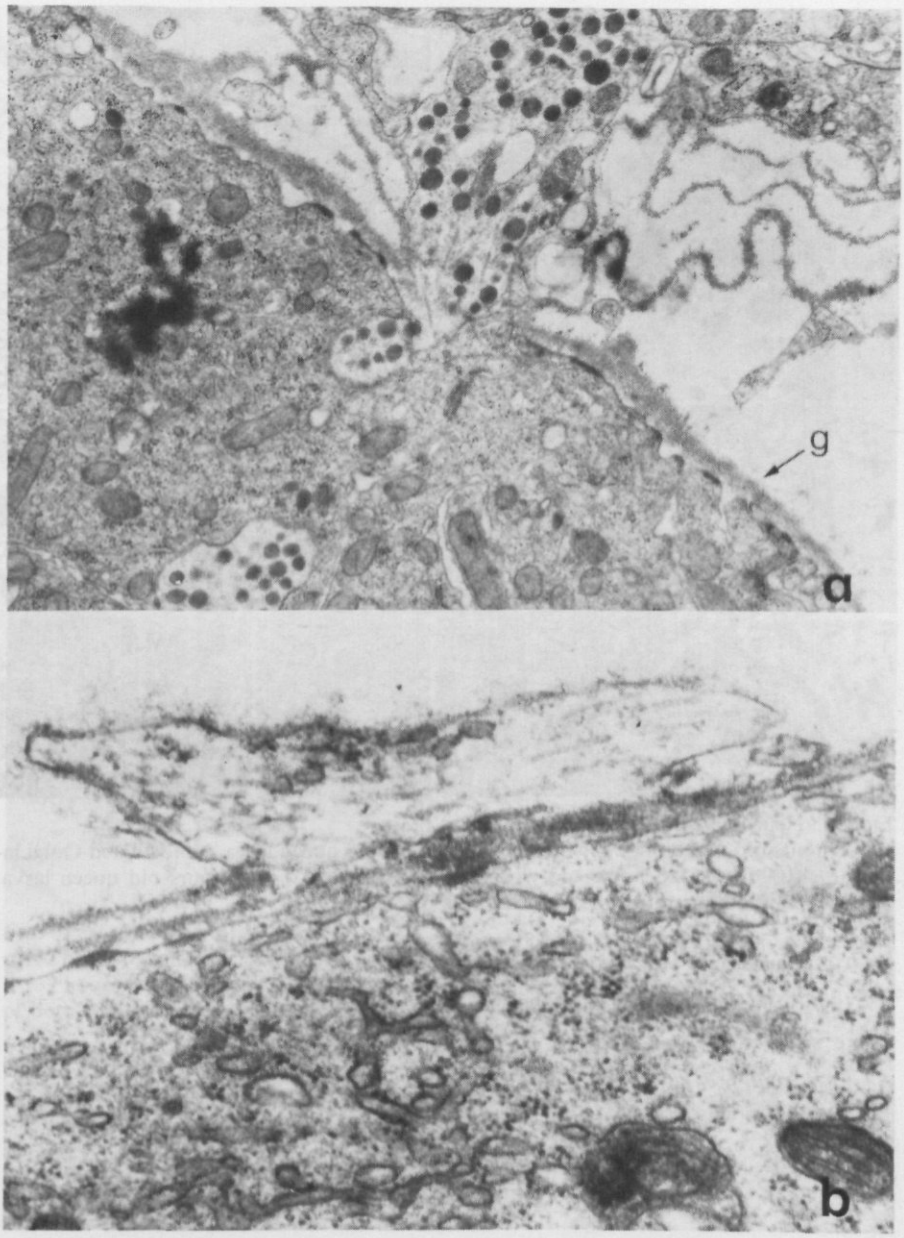

FIG. 30. Electron micrographs of the CA innervation in larvae.
a. 105 hours old queen larva. Nervus corporis allatum penetrating the CA. Many neurosecretory granules are visible. Note glycocalyx (g). 20.000 x. b. 96 hours old queen larva. Neural axon on the surface of the CA. 40.000 x.

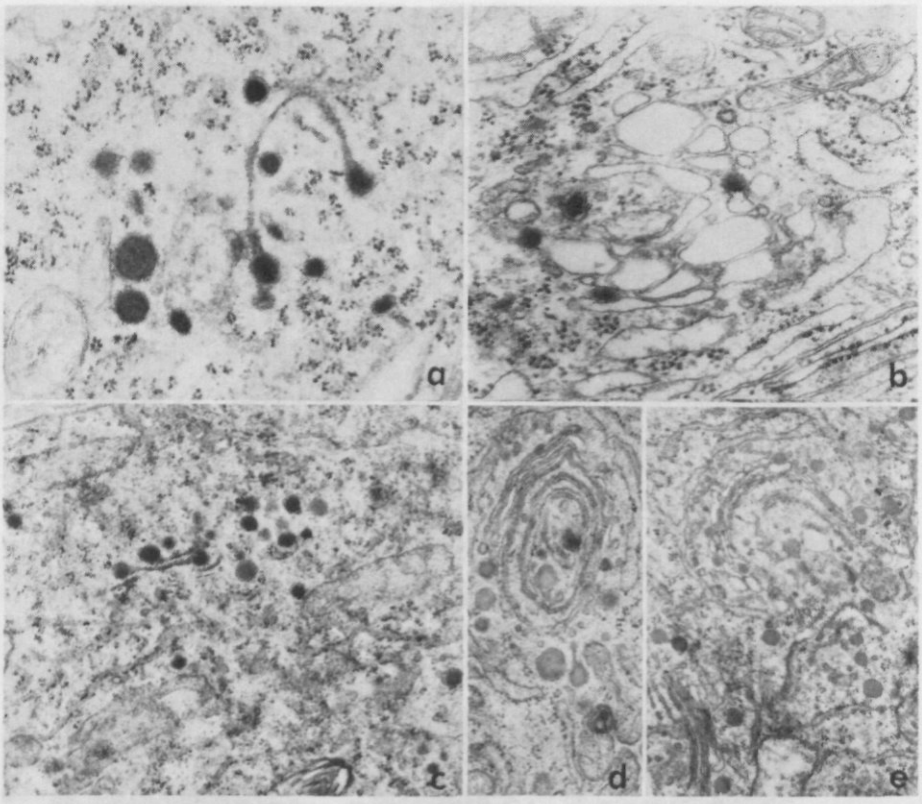

FIG. 31. Neurosecretory phenomena in larval brain.
a. 108 hours old queen larva. 35.000 x. b. 108 hours old queen larva. Note dilated Golgi lamellae. 40.000 x. c. 108 hours old worker larva. 20.000 x. d. 120 hours old queen larva. 30.000 x. e. 132 hours old worker larva. 20.000 x.

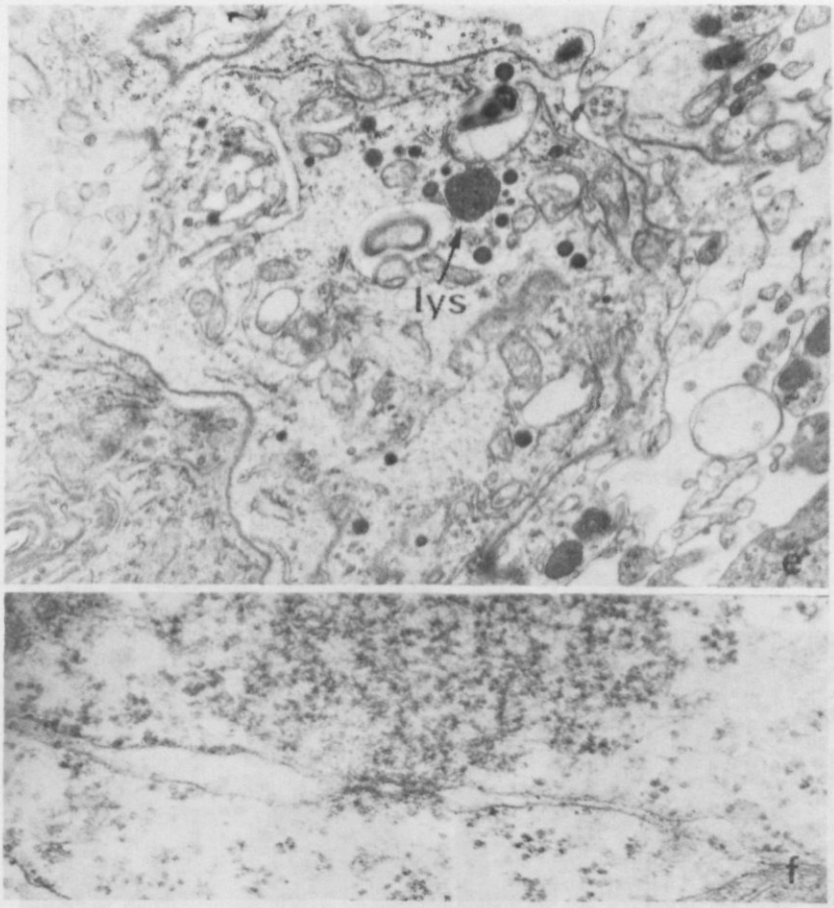

FIG. 31 e, f. Larval brain.
e. 132 hours old queen larva. Note lysosome surrounded by neurosecretory particles. 10.000 x.
f. 132 hours old queen larva. Possible synaptic activity between two cells in the brain. None of both cells showed any neurosecretory activity. 50.000 x.

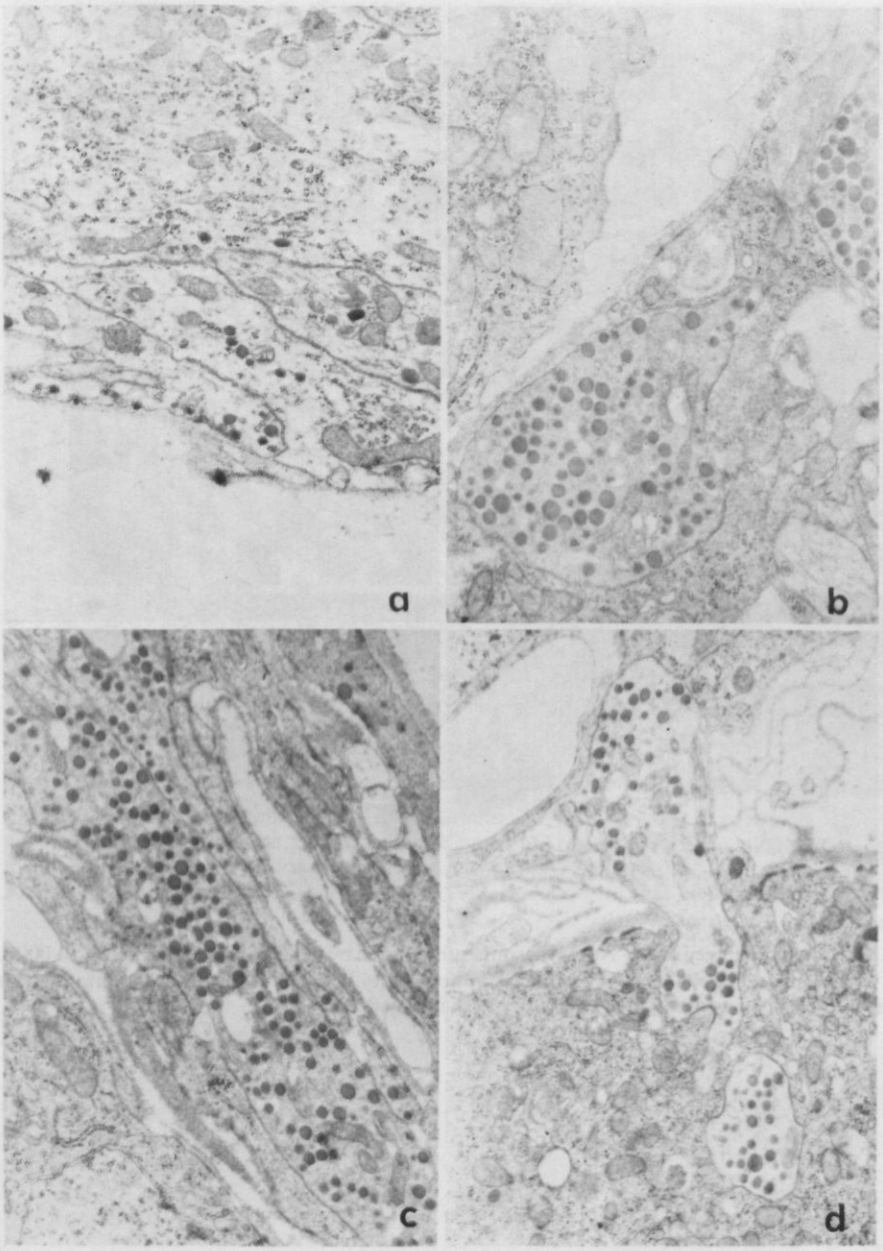

FIG. 32. Transport and release of neurosecretory granules.
a. Gland cells of CC of a 72 hours old queen larva, containing neurosecretory granules. These granules appear to be secreted to the haemolymph directly. 20.000 x. b. Neurosecretory axon endings in the CC of a 108 hours old queen larva. 20.000 x. c. Neurosecretory axons running through the lower part of the CC of a 108 hours old queen larva. 20.000 x. d. Nervus corporis allatum penetrating the CA of an 84 hours old queen larva. 20.000 x.

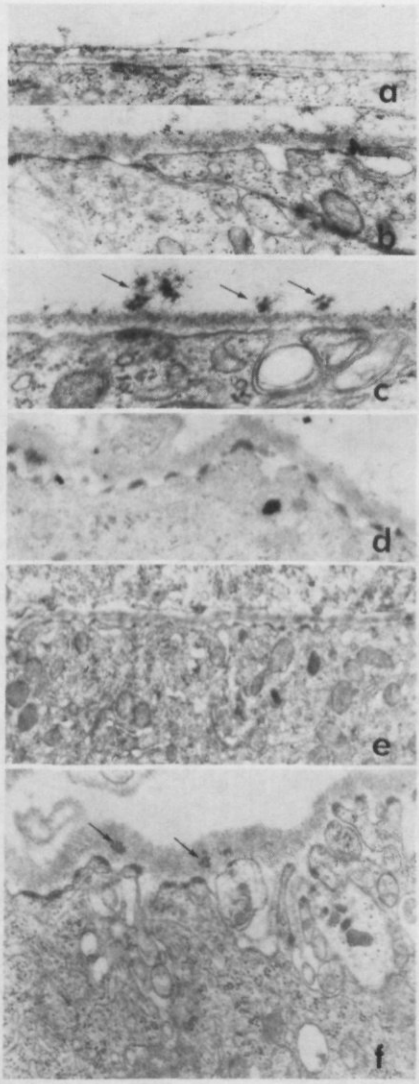

FIG. 33. Corpus allatum.
a. 60 hrs old queen larva. At the border of the CA no activity can be observed. The enveloping glycocalyx appears to be resting. 30.000x. b. 72 hrs old queen larva. More activities can be seen at the border of the gland. Formation of pinocytotic vacuoles occurs. The glycocalyx appears to be dilated. 30.000 x. c. 72 hrs old queen larva. Electron dense spots at the periphery of the gland can be seen. Electron dense matter just outside the glycocalyx occurs frequently. These phenomena seem to be related with the occurrence of pinocytosis. 30.000 x. d. 84 hrs old queen larva. High pinocytotic activity at the border of the gland. Note electron dense spots at the periphery and the dilated glycocalyx. 30.000 x. e. 84 hrs old queen larva. Formation of many vacuoles arising from the border of the CA. Vacuoles appear to migrate through the cytoplasm of the gland cells. 10.000 x. f. 108 hrs old queen larva. Highly active plasma membrane. Note electron dense spots in the dilated glycocalyx. 30.000 x.

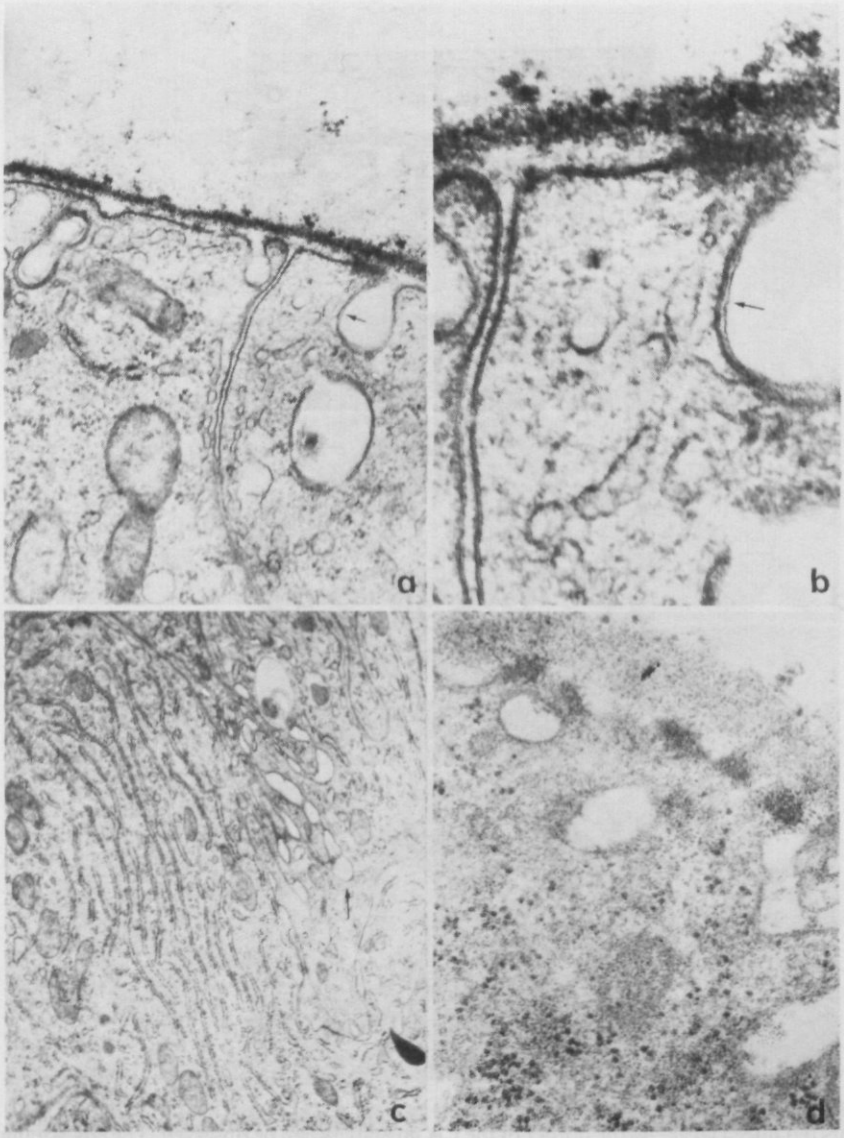

FIG. 34. Corpus allatum.
a. 78 hrs old queen larva. Formation of vacuoles at different stages. Note membrane structures. 40.000 x. b. 78 hrs old queen larva. Membrane structures before and after the formation of a vacuole at higher magnification. It is obvious that vacuoles are formed by infolding of the cell membrane. The third membrane layer in the vacuole is probably the 'border' of a drop of haemolymph. Note high electron density at the entrance of the vacuole. Membranes appear to be dilated here. 80.000 x. c. 78 hrs old queen larva. Membrane activities between two cells. 30.000 x. d. 102 hrs old queen larva. High activity at the border of the gland. Membrane structures are difficult to distinguish because of high dilatation between the infoldings. 40.000 x.

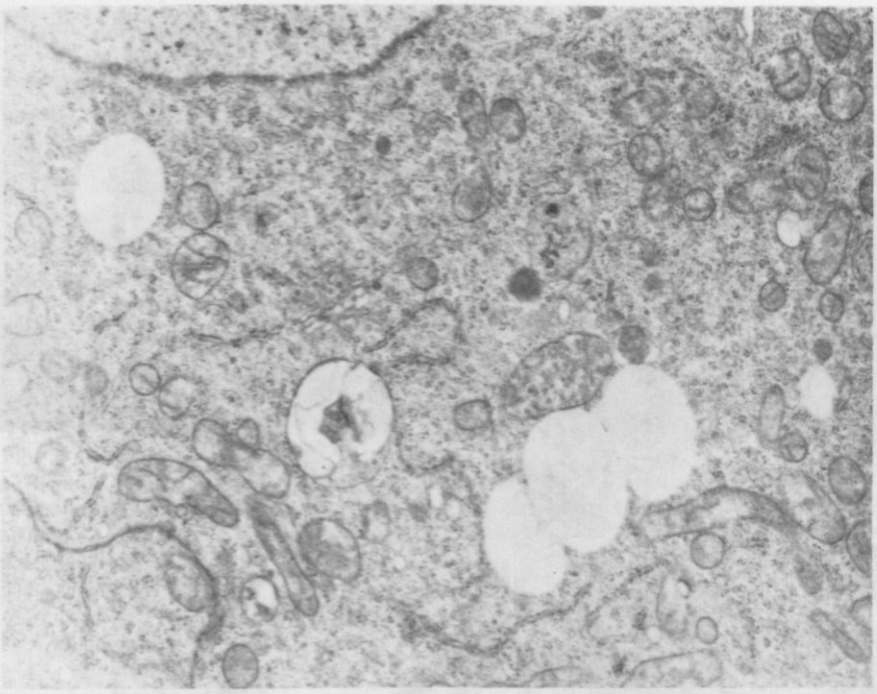

FIG. 35. Corpus allatum of a 96 hours old worker larva. Fusion of some large vacuoles. 36.000 x.

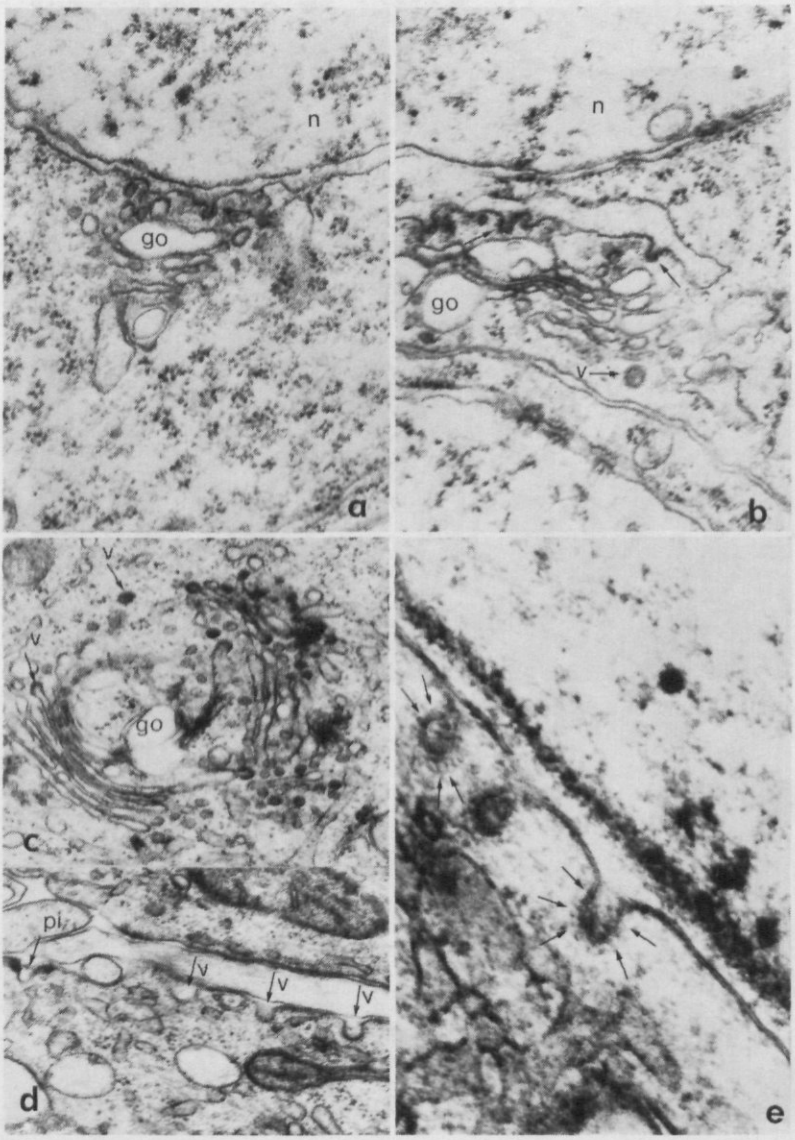

FIG. 36. Corpus allatum.
a. 78 hrs old queen larva. Nuclear (n) membrane in close relation with a Golgi zone (go). 40.000 x. b. 78 hrs old queen larva. Dilated RER in close relation with a Golgi zone. Note a small vesicle (v) with a remarkable membrane. 40.000 x. c. 78 hrs old queen larva. Small vesicles (v) are formed from highly active Golgi zones (go). Note the enveloping membranes of vesicles. 40.000 x. d. 78 hrs old queen larva. Secretion of vesicles to the intercellular space. Note differences in membrane activities at places where vesicles (v) are secreting and where pinocytosis (pi) appears to occur. 50.000 x. e. 78 hrs old queen larva. Secretion of vesicles at the border of the CA. Note differences in membrane structures. No electron dense spots at the border occur. Glycocalyx not dilated. 100.000 x.

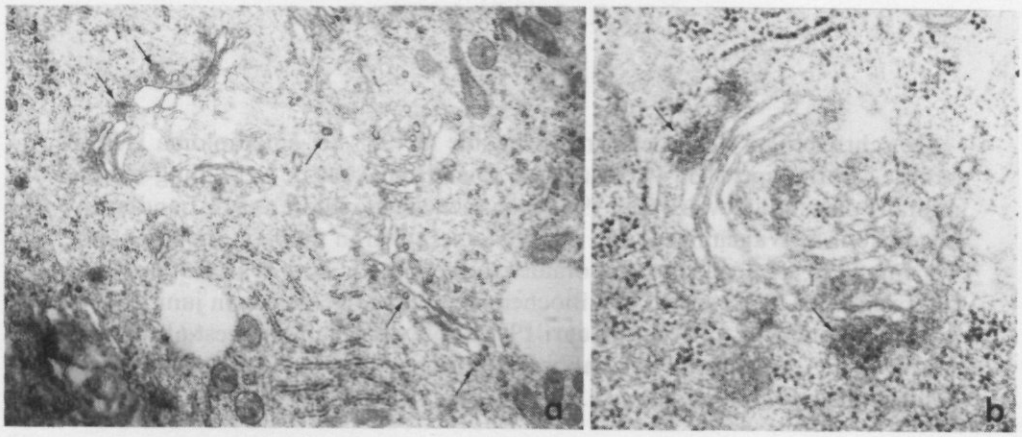

FIG. 37. Corpus allatum.
a. 78 hours old worker larva. Active Golgi zones producing small vesicles. Dense bodies close to the Golgi zones are seen frequently. 20.000 x. b. 84 hours old worker larva. Dense bodies around a Golgi zone at higher magnification. The significance of these structures is still obscure. 40.000 x.

CURRICULUM VITAE

De schrijver van dit proefschrift behaalde in 1965 het einddiploma HBS-B aan de voormalige HBS 'Beeklaan' te Den Haag. Na de vervulling van de militaire dienstplicht werd eind 1967 begonnen met de studie aan de Landbouwhogeschool te Wageningen. Het kandidaatsexamen in de planteziektenkunde werd in 1972 afgelegd; het doctoraalexamen, met als hoofdvak Entomologie (verzwaard) en als keuzevakken Biochemie en Virologie volgde in juni 1974.

Vanaf september 1974 tot januari 1978 is hij werkzaam geweest als Wetenschappelijk Assistent aan het Laboratorium voor Entomologie van de Landbouwhogeschool te Wageningen.