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THE INNERVATION OF THE SALIVARY GLAND OF THE MOTH, *MANDUCA SEXTA*: EVIDENCE THAT DOPAMINE IS THE TRANSMITTER

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SUMMARY

1. Using the Falck-Hillarp histochemical technique for monoamines, evidence was found for the presence of a catecholamine in the salivary gland nerves of the moth, *Manduca sexta*.

2. The innervation was studied with the electron microscope. Only the fluid-secreting region of the gland is innervated and the nerve endings are characteristic of monoamine-containing terminals.

3. Using a sensitive enzymatic-isotopic assay for catecholamines, it was found that whole salivary glands contain 0·33 μg/g dopamine but no noradrenaline.

4. It seems likely that dopamine mediates fluid-secretion in the salivary gland of *Manduca* as it does in a number of other arthropods.

INTRODUCTION

Tubular insect salivary glands are interesting because their simple construction lends itself well to studies on the mechanism and control of salivary secretion (Berridge & Prince, 1972). The salivary glands of the blowfly *Calliphora* have been especially useful in this respect, providing information on both the process by which saliva is formed (Oschman & Berridge, 1970) and on the regulation of this process by Ca^{2+} and adenosine 3′,5′-monophosphate (cyclic AMP) (Prince, Berridge & Rasmussen, 1972; Prince, Rasmussen & Berridge, 1973). The salivary glands of *Calliphora* are thought to be controlled by 5-hydroxytryptamine (5-HT) which acts as a first messenger and, as a consequence of its interaction with a specific receptor on the salivary gland cells, produces changes in the intracellular concentrations of Ca^{2+} and cyclic AMP. These changes lead to secretion of saliva (Prince *et al.* 1973). However, unlike most other insect salivary glands, those of *Calliphora* are not innervated and the exact mechanism by which 5-HT is delivered to the receptors on the salivary gland cells is unknown (Oschman & Berridge, 1970).

5-HT, has until recently, been thought to be the transmitter at the salivary gland of the cockroach (Whitehead, 1971). However, the sensitive Falck-Hillarp histochemical technique for catecholamines and 5-HT has revealed that the locust (Klemm, 1972) and cockroach (Bland *et al.* 1973) salivary glands are innervated by catechol-

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amine-containing rather than 5-HT-containing nerves. Further studies have confirmed that the substance released by the cockroach salivary gland nerves behaves in a similar fashion to catecholamines (House, Ginsborg & Silinsky, 1973) and that the salivary glands contain a measurable amount of dopamine but no noradrenaline (Fry, House & Sharman, 1974).

The salivary glands of both the cockroach and the locust are, however, of the acinar-type (Bland & House, 1971; Kendall, 1969) and receive a dual innervation (Whitehead, 1971). These features cancel out many of the advantages of working with an insect preparation. The salivary glands of the moth, *Manduca sexta*, may be a suitable preparation for further studies on insect salivary glands, combining as they do many of the advantages of innervated cockroach and locust salivary glands with the advantages of a tubular salivary gland. Tubular salivary glands consist of a single epithelial layer, and contain only a single cell-type in any given region. (Oschman & Berridge, 1970; Leslie & Robertson, 1973). It is possible therefore to study the role of individual cell types (e.g. fluid-secreting) without the complications added by the presence of more than one layer of cells or more than one type of cell. The structure of this gland has already been described in detail (Leslie & Robertson, 1973) as has the innervation of the glands (Robertson, 1974*a*). Basically, the glands consist of two long convoluted tubules which are sequentially divided into discrete functional units (a distal protein-secreting region, followed by a fluid-secreting region and an ion-reabsorptive region) (Leslie & Robertson, 1973). Only the fluid-secreting region of each tubule is innervated (Robertson, 1974*a*). The purpose of this study was to determine the nature of the transmitter in the salivary gland nerves of *Manduca*.

MATERIALS AND METHODS

Animals

Moths (*Manduca sexta* Johannson) were obtained as described previously (Robertson, 1974*a*).

Fluorescence microscopy

Salivary glands were removed from 2 to 6-day-old moths and immediately frozen in liquid freon cooled to the temperature of liquid nitrogen. The glands were then quickly transferred (on a microscope slide) to the stage of a Pearse tissue freeze-dryer (Edwards High Vacuum Co.) pre-cooled to −80 °C. The tissue was then dried for 2–4 days at −40 to −65 °C at a pressure of 0·005 torr. After drying, the tissue was heated at 80 °C for 1–2 h in a sealed container with paraformaldehyde. The paraformaldehyde had previously been equilibrated at a relative humidity of 60 % for at least a week (Hamberger, Malmfors & Sachs, 1965). Controls were treated in exactly the same manner but without the paraformaldehyde. The tissue was then covered with a non-fluorescing immersion oil and fluorescence observed with a Zeiss large fluorescence microscope equipped with a mercury light-source. BG 3 and a BG 38 filters were used as excitation filters and a Wratten 47 as a barrier filter. To minimize exposure times, Kodak Tri-X film was used to record fluorescence.

Electron microscopy

For electron microscopy, salivary glands were dissected out and fixed for 90 min in ice-cold 3 % potassium permanganate in 100 mM phosphate buffer (pH 7·0). The tissue was then rinsed in 30 % ethanol and distilled water and stained *en bloc* in 1 % uranyl acetate for 15–30 min. After dehydrating and embedding in Araldite, thin sections were cut, mounted on uncoated grids and examined with a Philips EM 200 electron microscope.

To ascertain whether incubation of salivary glands in solutions containing noradrenaline or α-methyl-noradrenaline had any effect on the number or density of vesicles in nerve endings, the technique of Hökfelt (1968) was adopted. Salivary glands were dissected and incubated in a physiological solution containing 0·2 mg/ml ascorbic acid, 0·05 mg/ml EDTA (ethylene-diamine tetracetic acid) and 100 μg/ml of either D-L-noradrenaline (Sigma) or α-methyl-noradrenaline (gift of Dr L. L. Iversen). One tubule from each gland was used as experimental while the other served as a control and was incubated in the above solution minus the catecholamine. The composition of the physiological solution for *Manduca* was as follows: (in mM) KCl, 20; $MgCl_2$, 6; NaCl, 11; $CaCl_2$, 2; tri-sodium citrate, 2; glucose 60; sucrose 250; morpholinosulphonic acid (MOPS) 10; pH adjusted to 6·8 with 3 ml of 1 M-NaOH (osmolality of this solution is about 370 osmoles/l) (Robertson, 1974*b*).

The vessels containing the tissue were incubated in a shaking water bath for 30 min. at 30 °C. The incubation medium was oxygenated throughout this period.

Enzymatic-isotopic assay for catecholamines

The results obtained with fluorescence histochemistry suggest that the salivary gland nerves contain a catecholamine. To corroborate this and to ascertain which catecholamine was present, salivary glands were assayed using a sensitive radiochemical-isotopic method for dopamine and noradrenaline. Salivary glands were removed from adult moths, weighed and frozen on dry ice. The frozen tissue was then assayed for dopamine and noradrenaline using the technique of Cuello, Hiley & Iversen (1973). Catechol-*o*-methyl transferase (E.C. 2.1.1.6) was used to catalyse the transfer of [^{3}H]methyl groups from [^{3}H]methyl-*S*-adenosyl-l-methionine to dopamine or noradrenaline. The dopamine and noradrenaline derivatives thus formed (3-methoxytyramine and normetanephrine respectively) were separated from the incubation mixture by extraction into an organic solvent. 3-Methoxytyramine and normetanephrine were then separated by paper chromatography. The spots were visualized under ultraviolet, eluted and the amount of radioactivity determined by liquid scintillation counting. The amount of each catecholamine present in the original tissue sample was determined by comparison with standards carried through the entire procedure. As a further check, known amounts of catecholamines are added to tissue samples and carried through the assay procedure to ensure that tissue factors have no effect on the enzyme activity or on extraction efficiency. With this method, as little as 100 pg of either dopamine or noradrenaline can be measured simultaneously in the same tissue sample (Robertson, 1974*b*; Megaw & Robertson, 1974).

Table 1. *Dopamine content of* Manduca sexta *salivary glands*

Determination*	Dopamine (μg/g wet weight tissue)
1	0·30
2	0·68
3	0·10
4	0·26
Mean ± S.E.M.	0·33 ± 0·12

* Each determination represents the amount of dopamine present in salivary glands pooled from at least 5 moths. Dopamine content was quantitated by comparison of tissue samples with standards.

RESULTS

A. *Fluorescence microscopy*

Treatment of salivary glands and accompanying nerves resulted in a yellow-green fluorescence of the nerves not seen in nerves or glands which were not exposed to paraformaldehyde (Fig. 1). The fluorescence was stable under ultraviolet illumination suggesting that the fluorescence observed resulted from condensation of the formaldehyde with relatively high concentrations of a catecholamine rather than 5-HT whose fluorophore is u.v.-labile and fades rapidly (Corrodi & Jonsson, 1967). The fluorescence histochemistry therefore suggests that the salivary gland nerves contain a catecholamine.

B. *Electron microscopy*

As was reported earlier (Robertson, 1974*a*), nerve terminals in salivary glands fixed for electron microscopy following the method of Hökfelt (1968) with 3 % potassium permanganate, contain small vesicles 30–40 nm in diameter (Fig. 3). The presence of small granular vesicles strongly suggests that the transmitter in the nerve endings is a monoamine (Hökfelt, 1968; Bloom, 1972). This technique cannot, however, be used to differentiate between catecholaminergic and serotoninergic nerve terminals.

Incubation of salivary glands and accompanying nerves in solutions containing noradrenaline or α-methyl-noradrenaline had no effect on either the number or density of the small granular vesicles present in nerve endings.

C. *Enzymic-isotopic assay for catecholamines*

Taken together, the results of the fluorescence histochemistry and the electron microscopy suggest that the transmitter at the salivary gland in *Manduca* is a monoamine, probably a catecholamine. The enzymic-isotopic assay for catecholamines confirms that a catecholamine, dopamine, is present in the salivary glands in concentrations expected for a transmitter (Table 1). There was no evidence for the presence of noradrenaline in salivary gland extracts.

DISCUSSION

As only the fluid-secreting region of the salivary gland of *Manduca sexta* is innervated (Robertson, 1974*a*), the present results suggest that dopamine is involved in the regulation of fluid-secretion by the salivary gland. The intense yellow-green

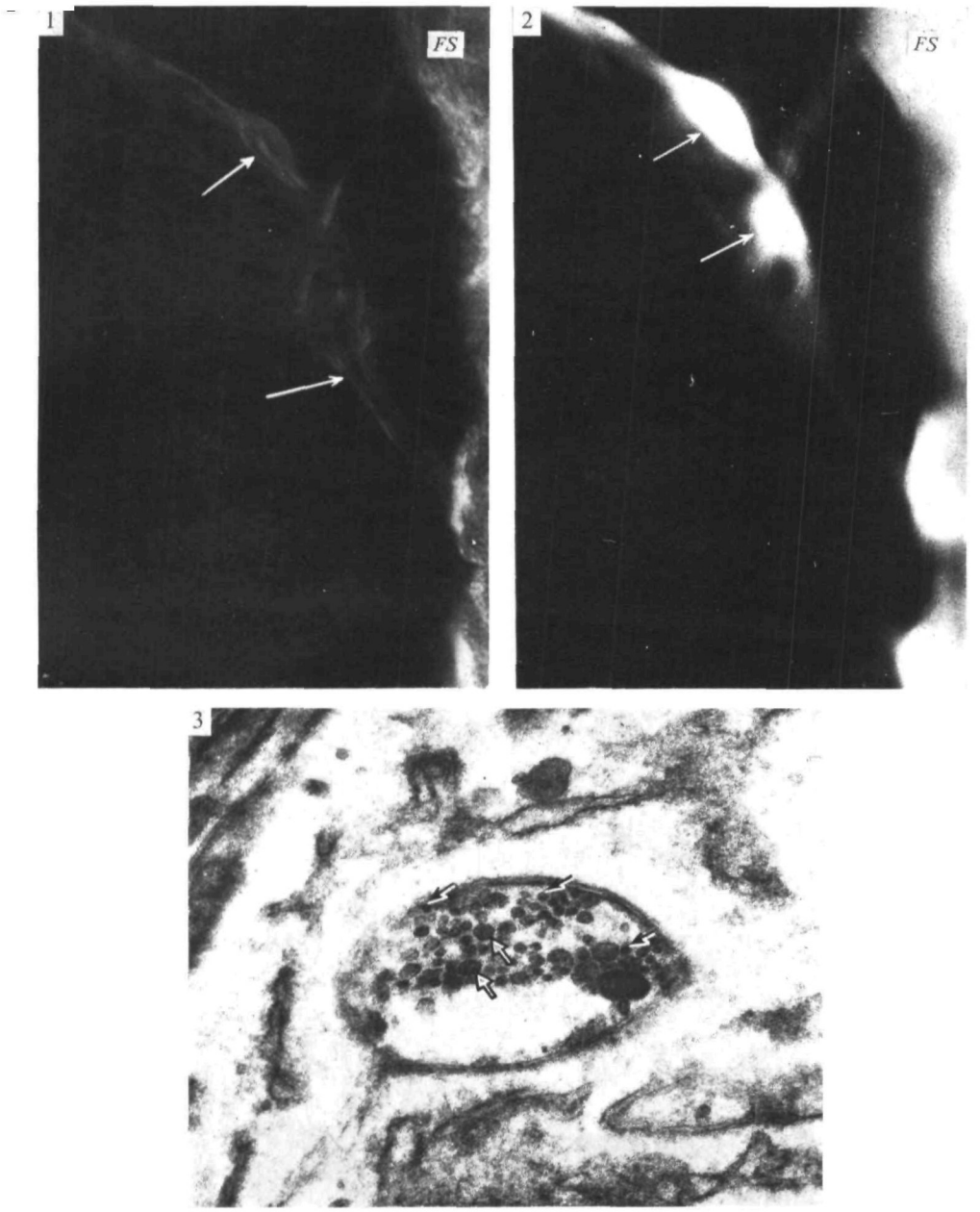

Fig. 1. Dark field micrograph of whole mount of salivary gland and accompanying nerve (arrow). This preparation has been treated with formaldehyde vapour at 80 °C (*FS*, fluid secreting region of salivary gland). ×450 approx.

Fig. 2. Same preparation as Fig. 1 but viewed under ultraviolet illumination to yield fluorescence characteristic of catecholamine-containing structures (see text for details); fluorescence is most evident in varicosities along the nerve (arrows). ×450 approx.

Fig. 3. Electron micrograph of nerve ending containing large granular vesicles (large arrows) and the small granular vesicles (small arrows) characteristic of monoamine containing nerve endings. Fixation with 3% permanganate. ×62 500.

fluorescence exhibited by the salivary gland nerve sindicate the presence of large amounts of a catecholamine and this is confirmed by the finding that the glands contain about 0·33 μg/g of dopamine. This implies that the concentration of dopamine in the nerves, which extend to only one region of the gland and are very sparse, must be quite high. The amount of dopamine in the salivary glands of *Manduca* compares favourably with the amount of noradrenaline found in mammalian salivary glands (Alm, Bloom & Carlsöö, 1973) and also with the amount present in the salivary gland of the cockroach (0·55 ng/gland) (Fry *et al.* 1974; C. R. House, personal communication). Similar amounts of dopamine are found in the salivary glands of another arthropod, the ixodid tick, *Boophilus microplus* (Megaw & Robertson, 1974).

In other studies (Robertson, 1974*b*) it has not been possible to stimulate salivary gland secretion in an *in vitro* preparation with any of a number of pharmacological agents including the catecholamines. The only means of inducing salivary secretion is by *in vivo* injection of the cholinesterase inhibitor physostigmine which acts at a more central synapse which in turn activates the dopaminergic neurons innervating the salivary gland (Robertson, 1974*a*; Megaw, 1974). The failure of exogenously applied dopamine to stimulate fluid secretion *in vitro* is thought to result from barriers which prevent catecholamines from reaching the receptor sites on the salivary gland. These receptor sites are presumably located close to the nerve endings, deep in the fluid-secreting tissue (Robertson, 1974*a*).

There are good reasons to postulate the existence of such barriers. It has long been known that a catecholamine, *N*-acetyldopamine, plays an important role in insects in the formation of a hard cuticle. Dopamine is an intermediary in the biosynthesis of *N*-acetyldopamine and is present in large amounts in insects (see review by Murdock, 1971). Barriers, either physical or chemical, may be necessary to maintain the integrity of the two roles of the catecholamines. The presence of barriers may also explain the failure to show an increase in either the density or numbers of small granular vesicles in nerve terminals from salivary glands incubated in noradrenaline or α-methyl-noradrenaline. If the noradrenaline and α-methyl-noradrenaline in the bathing medium cannot reach the re-uptake sites located on the pre-synaptic membrane, it will not be taken up into the small granular vesicles. On the other hand, it is worth pointing out that these studies are difficult to interpret. Nerve terminals in this salivary gland contain small granular vesicles even without pre-incubation in a catecholamine analogue and it may be difficult to detect any changes in granularity. Another difficulty associated with work on monoamines in insects in general is that we do not know yet how the action of monoamines on post-synaptic cells is terminated. It is tempting to assume that uptake mechanisms such as those found in vertebrates and some invertebrates (Iversen, 1974) operate in insects as well. Nevertheless, such mechanisms have not yet been demonstrated. Berridge (personal communication) has shown that the non-innervated salivary gland of *Calliphora* has an efficient 'pump' which removes 5-HT from the effector (or haemolymph) side of the gland and secretes it into the saliva. Perhaps this 'pump' is the equivalent of uptake II (non-neuronal) as described by Iversen (1974).

It now appears that dopamine is a transmitter at the salivary gland in a number of insects; cockroach (Bland *et al.* 1973); locust (Klemm, 1972) and *Manduca* (this study and Robertson, 1974*a*, *b*). There is now sufficient evidence to suggest that dopamine

is the transmitter at many of the peripheral organs in insects which are innervated by elements from the stomatogastric or visceral nervous system. Some examples are the salivary glands of various species (cited above) and the foregut of *Galleria* (Beard, 1960), *Locusta migratoria* (Freeman, 1966) and *Schistocerca gregaria* (Klemm, 1972). Another organ which may be regulated, at least in part, by the stomatogastric nervous system is the corpus cardiacum. This neurohaemal organ is innervated by dopaminergic fibres the origin of which is yet uncertain (Klemm, 1971). Recent studies have shown that dopamine may mediate the release of neurosecretory material from the corpus cardiacum, thus fulfilling another role of dopamine, that of a releasing factor (Samaranayaka, in preparation). The role of dopamine as a releasing factor has been well studied in vertebrates but has received little consideration in insects.

In many of the functions of the stomatogastric nervous system there may be a cholinergic as well as a catecholaminergic involvement. For example, salivary secretion in *Manduca* can be elicited by injections of physostigmine although acetylcholine and physostigmine have no effect of secretion by the gland *in vitro*. Similarly, in the ixodid tick *Boophilus microplus*, a number of anticholinesterases when injected cause salivary secretion (Megaw, 1974) although it seems likely that a catecholamine is the transmitter at the tick salivary gland (Megaw & Robertson, 1974).

The light organ of the firefly is another instance where physostigmine induces a response (luminescence), but transection of the light organ nerves below the ganglion interrupts this effect. In an *in vitro* preparation, only monoamines such as octopamine are effective in eliciting a light flash (Carlson, 1969). Recently, it has been demonstrated that the firefly light organ contains octopamine (Robertson, 1975).

Release of neurosecretory material from the corpus cardiacum is also produced by treatment of insects with drugs and insecticides which block cholinesterase, but this release can be prevented by pretreatment with α-adrenergic blocking drugs (M. Samaranayaka, in preparation). It is conceivable that the insect stomatogastric nervous system is composed of central cholinergic and peripheral catecholaminergic fibres, an arrangement which is similar to that found in the sympathetic division of the mammalian autonomic nervous system.

The unique structural features of the salivary glands of *Manduca* (Leslie & Robertson, 1973), together with the now well-characterized innervation of the gland, may provide a valuable model in unravelling the problems associated with monoaminergic transmission in insects.

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