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SEROTONIN AND THE CONTROL OF SALIVATION IN THE BLOWFLY *CALLIPHORA*

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SUMMARY

The possibility that serotonin acts as a neurohormone stimulating salivation in the blowfly *Calliphora vicina* was studied by investigation of salivation induced by injections of high-potassium saline. Induced salivation is rapid and appears to be mediated by an active factor released into the haemolymph (High Potassium Salivary Gland Factor: HKSGF) since it is antagonized by cadmium (a calcium channel blocker) and by gramine (a serotonin-receptor blocker). The action of HKSGF on salivary glands *in vitro* is indistinguishable from that of serotonin: (a) it generates serotonin-like transepithelial potential changes, (b) its effect on salivation is antagonized by gramine, (c) it is as heat stable as serotonin, (d) it has the same solubility in a variety of organic solvents, (e) it is unaffected by incubation with leucine aminopeptidase or trypsin and (d) it is inactivated by rat liver monoamine oxidase type A (a serotonin deaminating enzyme).

Radioenzyme assay of haemolymph from high-potassium injected flies shows that the amount of serotonin present could account for all of the retrievable bioactivity.

Significant amounts of serotonin were found in the cerebral ganglion, the thoracic ganglion and nerves attached to the thoracic ganglion. Nerve sectioning experiments demonstrated that the abdominal nerves and the anterior nerves supplying the neck muscles are not involved in the normal salivatory response. However the cerebral-thoracic connective must be intact and it is suggested that release of serotonin is effected close to the main body of the thoracic ganglion. Some of the implications of the neurohormonal role of serotonin are discussed.

INTRODUCTION

The secretory portion of the salivary glands of *Calliphora vicina* (formerly *C. erythrocephala*) have been used extensively as a model system to study the mechanisms of cellular activation and fluid secretion (Berridge, 1972, 1981; Berridge & Fain, 1979*a*,*b*; Dalton, 1977). In these studies serotonin has been used as the primary secretagogue even though there is little evidence that this amine regulates salivation *in vivo*. It is known that a blood-borne factor stimulates the

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glands, and its release is apparently mediated by the nervous system (Hansen-Bay, 1978). It has also been shown that the brain contains a factor which stimulates secretion, is not destroyed by pronase or pepsin, is soluble in 80 % ethanol and has similar chromatographic properties to serotonin (Berridge & Patel, 1968). Furthermore, sugar-induced salivation can be blocked by injecting a known serotonin antagonist, gramine, at doses appropriate for similar serotonin stimulated rates of salivation (Hansen-Bay, 1976). This inhibition demonstrates that the physiological factor and serotonin probably share the same receptor site. If the factor is serotonin then it is likely to circulate at exceedingly low concentrations during salivation ($10\,\text{nmol}\,l^{-1}$ serotonin evokes maximal secretion rates *in vitro*: Berridge & Patel, 1968). Because only 1–2 μl of haemolymph can be obtained from a single fly, direct measurement, even using the most sensitive assay available, is not possible. The problem is compounded by the rapid inactivation of serotonin in the haemolymph (B. A. Trimmer, in preparation).

The present work investigates the possible role of serotonin in controlling salivation in the blowfly. Injections of high-potassium saline into adult *C. vicina* have been used to release large amounts of an active factor into the haemolymph. This factor stimulates isolated salivary glands in a manner indistinguishable from that of serotonin. High potassium levels have been shown to release diuretic hormone from exposed nerve endings (neurohaemal-areas) in *Rhodnius* and *Glossina* without contamination from central neurotransmitters, provided that the perineurium remains intact (Maddrell & Gee, 1974). High-potassium stimulated release is calcium dependent and is thought to release physiological secretory products rather than active prohormones (Aston & White, 1974; Aston, 1979). The factor investigated in the present studies has been named High Potassium Salivary Gland Factor (HKSGF) and is thought to be the natural hormone effecting salivation. On the basis of thin-layer chromatography, its heat stability, solubility and enzyme lability, the factor has been identiied as serotonin. A specific and sensitive radioenzyme assay procedure has confirmed that the amount of serotonin in the haemolymph of high-potassium injected flies is sufficient to account for all the bioactivity. This assay has also been used to measure serotonin in various regions of the nervous system and has been combined with microsurgical procedures in an attempt to locate release sites involved in salivation.

METHODS

Unless otherwise stated, flies were obtained from laboratory stock cultures reared on sugar, water and pig heart *ad libitum*. Both male and female flies (1–3 days old) were used. For the experiments involving sugar stimulated salivation, flies were deprived of food for 24 h prior to the experiment but were supplied with water *ad libitum*.

'In vivo' *salivation preparation*

Saliva was collected at 1-min intervals from the common collecting duct, in the manner of Hansen-Bay (1978). The diameter of the droplets was measured under silicon oil against a calibrated scale to estimate the saliva volume and the rate of

secretion. Physiological salivation could be induced by placing a crystal of sucrose on the labellum and allowing the fly to hold this on the oral lobes.

Injection and sampling procedure

C. vicina salines were routinely injected into the abdomen between the 4th and 5th abdominal tergites. Injection volumes were typically 1 or 5 μl except for the retrieval of HKSGF where 10 μl was injected. These volumes had no obviously harmful effect on the flies. Haemolymph was collected from the wing articulation. The standard saline (pH 7·3) had the following composition (in mmol l^{-1}): NaCl, 128; KCl, 10; $MgCl_2$, 2; $CaCl_2$, 2; NaH_2PO_4, 5; malic acid, 2·8; sodium glutamate, 3; Tris, 10; glucose, 10. Salines with higher potassium concentrations were made by substitution of an equivalent amount of sodium.

Microsurgery

Nerves were carefully exposed in the *in vivo* preparation by removing the ventral cuticle immediately above the thoracic ganglion. When exposing the abdominal nerves in the thorax the dissection was carried out on a cooled microscope stage (PelCool) at 2 °C. This was found to be essential to preserve the normal behavioural responses of the fly when offered sucrose. The insect was allowed to recover from the cold anaesthesia for 10 min prior to the experiment. Nerve bundles were severed rapidly and without damage to the surrounding tissue using a high frequency radiomicrocautery (Unwin, 1978). Insects treated in this fashion continued to show normal proboscis extension in response to sugar crystals.

In vitro *salivary gland bioassay*

Samples were bioassayed on the abdominal portions of salivary glands as described by Berridge & Patel (1968). These were diluted an appropriate amount to avoid stimulation of the glands by contaminating ions (Berridge, Lindley & Prince, 1975).

Measuring the transepithelial potential (TEP) of salivary glands

The transepithelial potential of salivary glands *in vitro* was measured by recording the potential difference between the secreted fluid and the salivary gland bathing medium using glass microelectrodes filled with 3 mol l^{-1} KCl and chloridized silver wire contacts. Readings made in this fashion were found to bear a close resemblance to the more precise measurements made by Berridge & Prince (1972) and Berridge (1981) using a steadily flowing external medium in a Perspex perfusion chamber. This flow cell technique could not be used here because of the small amounts of test sample available. When the bathing fluid volume was sufficiently large several salivary glands could be accommodated and the secretory rates of these glands monitored in parallel with the TEP recordings.

The radioenzyme assay for serotonin

Tissue preparation

C. vicina tissues were dissected in a chilled (2 °C) saline. The cerebral ganglion, together with the optic lobes and the nerve mass ventral to the gut, were carefully

cleaned of other tissues (gut and retina in particular) before immersion in the homogenization buffer. The thoracic ganglion was dissected with as much of the thoracic and abdominal nerve trunks as could be traced. These nerves were severed from some ganglia to estimate their contribution to the total serotonin content of the nervous system. Tissues were placed immediately into 70 μl of 0·05 mol l^{-1} sodium phosphate buffer pH 7·2 at 75 °C. This procedure was used because extraction into cold 0·01 mol l^{-1} formic acid was found to result in most of the endogenous serotonin being recovered as *N*-acetylserotonin. This is presumed to emanate from catabolism of serotonin since the recovered counts measured in the *N*-acetylserotonin blanks and the samples were inversely related depending on the extraction procedure. It was found that by heating the tissues in buffer for 10 min the *N*-acetylserotonin blanks were reduced close to the overall background of the assay (see Discussion). After heating, the tissue was homogenized with a glass rod and stored at −20 °C for no more than 14 days before the assay. To avoid delay and possible loss of serotonin the tissues were not weighed individually. Mean weights were calculated for tissues dissected in an identical manner from the same batch of insects, blotted carefully on filter paper and weighed.

Assay procedure

The protocol was the same as that of Hussain & Sole (1981) except for the following procedural differences.

The acetylation step. Twenty microlitres of the tissue extract was placed into each assay tube. After adding acetic anhydride, all the tubes (including the blanks) were transferred to a water bath at room temperature (20–24 °C) for 10 min. The water temperature was then raised to 65 °C over a 20-min period and maintained at this temperature for 10 min to evaporate the acetone, to complete the acetylation, and to decompose remaining acetic anhydride (Hammel, Naot, David & Ginsberg, 1978). The tubes were then placed in a vacuum dessicator and evacuated for 60 min at 37 °C.

The methylation step. Sixty-five microlitres of the following mixture was added to each tube at 0 °C (the quantities are sufficient for 60 assay tubes): 900 μl 0·05 mol l^{-1} sodium phosphate buffer pH 7·2, 2280 μl 0·5 mol l^{-1} sodium phosphate buffer pH 7·9, 126 μl S-[^{3}H-methyl]-enosyl-methionine (SAM) (78·5 Ci $mmol^{-1}$, $1{\cdot}638\times10^{-9}$ mol l^{-1}), 6·32 μl 0·5 mmol l^{-1} SAM in 0·05 mol l^{-1} sodium phosphate buffer, 600 μl hydroxyindole-*O*-methyl transferase (HIOMT) extracted from frozen bovine pineal glands (Pel-Freeze Biologicals Inc. Rogers, Arkansas) using the procedure of Saavadra, Brownstein & Axelrod (1973), (4 units/sample, Boireau *et al.* 1976). These were incubated and extracted in the manner described by Hussain & Sole (1981).

Solubility studies

Two types of haemolymph were extracted and treated in parallel. For each solvent to be tested 10 flies were injected with 10 μl of 120 mmol l^{-1} potassium saline (HK group) and 10 flies were injected with 10 μl of 2 mmol l^{-1} potassium saline (LK group). Twenty seconds after injection, 5 μl of haemolymph was

retrieved from each insect and ejected into an equivalent volume of the converse saline kept on ice. The haemolymph from the 10 flies in each group was pooled and dried *in vacuo*. The dried residues were vortexed for 30 s with 100 μl of the test organic solvent and then centrifuged at 1700 ***g*** at 4°C for 10 min. The supernatant from the HK group was then added to the residue of the LK group and *vice versa*, thus producing two samples with approximately equivalent ionic composition. The organic solvent was dried *in vacuo* and the residue reconstituted in 50 μl of distilled water and 75 μl of low-potassium saline. This process yielded a final potassium concentration of approximately 24 mmol l^{-1} and an overall isotonic dilution of the active factor of ×0·2. The haemolymph from the LK group was used simply to compensate for the differential solubilities of the various ions in the solvents, and contained no active factor (Trimmer, 1983). In the Results and Discussion, 'supernatant' and 'residue' refer to the respective fractions from the high-potassium injected flies. As a comparison, the solubility of serotonin in each of the solvents was tested in the same manner using serotonin dissolved initially in 24 mmol l^{-1} potassium *C. vicina* saline.

Thin layer chromatography (TLC)

HKSGF was de-lipidated by shaking the dried 80% ethanol residue with chloroform and discarding the supernatant. The residue was redissolved in 80% ethanol and spotted along the origin of a pre-run cellulose TLC plate (Polygram CEL 300, 0·1 mm layer). Separation was carried out with 70% ethanol (see Results). Plates were cut into sections and each section eluted with distilled water, filtered, freeze-dried and reconstituted in saline for bioassay. ^{3}H-serotonin (12 Ci mmol $^{-1}$, Amersham Radiochemicals Ltd) was added to HKSGF and run in parallel with the HKSGF sample under test. The distribution of serotonin in this chromatogram was determined by cutting it into sections and placing these in scintillation vials with 0·3 ml of 0·1 mol l^{-1} HCl. Scintillation fluid (Biofluor NEF 961) was added and the radioactivity counted in a Packard series 3000 scintillation counter. Authentic serotonin and its metabolites were visualized with Ehrlich's reagent (Smith, 1969).

Enzyme degradation

Fresh samples of HKSGF were divided into two. One part was incubated with the enzyme and then bioassayed, the other part served as a control and was incubated with heat-inactivated enzyme (10 min at 100°C) under the same conditions before bioassay. The conditions for each enzyme are described below. In those cases where no degradation of HKSGF was detectable the activity of the enzyme was checked with suitable substrates.

Leucine aminopeptidase (LAP) E.C. 3.4.1

The conditions used were as described by Brown (1977). LAP (78 units mg^{-1}, Sigma) was dissolved in 0·02 mol l^{-1} Tris buffer pH 8·5 to a concentration of 2 mg ml^{-1}. Two microlitres of this was incubated in a mixture containing 10 μl HKSGF (diluted 1:1 with water), 2 μl 0·5 mol l^{-1} Tris buffer pH 8·5, 0·5 μl

0·125 mol l^{-1} $MgCl_2$. The samples were incubated for 2 h at 35 °C and the reaction stopped by freezing in liquid nitrogen. Samples were bioassayed by adding a mixture formulated to produce a normal saline when added to the incubation mix. The neuropeptide proctolin (L-arginyl-L-tyrosyl-L-leucyl-L-prolyl-L-threonine: 1×10^{-8} mol l^{-1}) was inactivated by LAP under these conditions as ascertained by bioassay on the myogenic muscle bundle of the hind femora of *Schistocerca americana gregaria* as described by Evans & O'Shea (1978). The frequency of muscle contractions was used as a measure of the proctolin concentration (Piek & Mantel, 1977; May, Brown & Clements, 1979).

Trypsin E.C. 3.4.2.1.(4)

The incubation mixture consisted of 4 μl HKSGF, 10 μl 0·01 mol l^{-1} potassium phosphate buffer pH 7·4, 2 μl trypsin (0·08 mg ml^{-1}) (Aston, 1979). This was incubated for 1 h at 35 °C and the reaction stopped by the addition of 16 μl of absolute ethanol. The precipitate was removed by centrifugation and the supernatant dried *in vacuo*. The sample was reconstituted in an appropriate saline for bioassay. Under these conditions *Rhodnius* diuretic hormone, prepared by incubating the metathoracic ganglion in 60 mmol l^{-1} potassium saline, was degraded. This was determined using the rate of fluid secretion by *Rhodnius* Malpighian tubules as an assay (Maddrell, Pilcher & Gardiner, 1971). The diuretic hormone reaction was not stopped with ethanol but by freezing because the hormone is inactivated by ethanol (Aston, 1979).

Monoamine oxidase (MAO)

A crude rat liver mitochondrial fraction was prepared as described by Fowler & Tipton (1982). The MAO properties of this fraction are the same as those found in more highly purified preparations (Fowler, 1978). This preparation is known to contain both A and B type MAO and many other sorts of enzyme activity. Because of this, the broad spectrum MAO inhibitor Pargyline was used as a further control. The incubation mixture contained 10 μl HKSGF, 20 μl 0·01 mol l^{-1} potassium phosphate buffer pH 7·4, 2 μl MAO, 1 μl Pargyline-HCl (3×10^{-3} mol l^{-1}, controls only). The samples were incubated for 6 h at 35 °C. The incubation was stopped by rapid freezing in liquid nitrogen. The samples were then bioassayed on salivary glands by adding an equal volume of a complimentary saline (in mmol l^{-1}: NaCl, 247; $CaCl_2$, 4; $MgCl_2$, 4; malic acid, 5·6; sodium glutamate, 5·6; glucose, 20; and NaOH to adjust the pH to 7·9). Serotonin was incubated and assayed under the same conditions.

Selective inhibition of MAO types A and B

The conditions required for the selective inhibition of MAO types A and B were determined using ^{3}H-serotonin in high-potassium saline as substrate and the selective inhibitors Chlorgyline and Deprenil. The mitochondrial fraction was diluted with an equal volume of 0·01 mol l^{-1} potassium phosphate buffer pH 7·2 to approximately 9 mg ml^{-1} protein and this was assayed according to the procedure described by Fowler & Tipton (1982). Chlorgyline and Deprenil were preincubated

with the MAO at 37 °C for 60 min. In the control an equivalent amount of water had been added instead of inhibitor. This incubation ensures that the inhibitors form stable adducts with the MAO (Fowler & Tipton, 1982). The enzymatic breakdown of ^{3}H-serotonin (at both 1×10^{-8} mol l^{-1} and $2{\cdot}4\times10^{-4}$ mol l^{-1}) was found to be complete after an incubation time of 60 min at an enzyme dilution of 0·5.

The effect of MAO on HKSGF

HKSGF was collected in the usual manner, extracted in 80 % ethanol and dried. The residue was reconstituted in distilled water and divided into 20 μl aliquots. Diluted (×0·5) MAO preparation (5 μl) was added to the sample with 25 μl potassium phosphate buffer (0·01 mol l^{-1}, pH 7·2) and incubated for 60 min at 37 °C. The reaction was stopped by the addition of 100 μl ice cold ethanol. The MAO had been preincubated with either Deprenil (3×10^{-3} mol l^{-1}), Chlorgyline (3×10^{-3} mol l^{-1}) or water. Three samples of each treatment were prepared. Two aliquots had ethanol added to the MAO and served as blanks. The precipitate that formed after stopping the reaction was removed by centrifugation at 2000 ***g*** for 10 min. It was found that a precipitate formed when ethanol was added directly to the buffer/saline mixture in the absence of enzyme. To compensate for this loss of saline components the residue from a mixture of 20 μl high-potassium saline, 25 μl buffer, 5 μl water and 100 μl ethanol was added to each vial before drying off the ethanol and bioassaying the sample at an isotonic dilution of one-fifth.

Chemicals

S-[^{3}H-methyl]-adenosyl-methionine (^{3}H-SAM), specific activity 78·5 Ci mmol^{-1}, and scintillation fluid (Biofluor, NEF 961) were obtained from New England Nuclear. Non radioactive SAM, melatonin, *N*-acetylserotonin and serotonin (creatinine sulphate) were obtained from Sigma Chemical Company Ltd. Gramine (3-[dimethylaminomethyl]-indole) was obtained from Koch Light Laboratories Ltd. Chlorgyline and Deprenil were kind gifts from Dr B. Callingham. All other reagents were of the highest purity available commercially.

RESULTS

The release of High Potassium Salivary Gland Factor (HKSGF)

High-potassium saline injections were found to stimulate salivation in the intact fly, presumably through a depolarizing effect on neurohaemal regions, whereas low-potassium saline had no such effect (Fig. 1).

This effect of elevated potassium could be inhibited by cadmium (5 mmol l^{-1}) in the injection saline. When flies were injected with two successive doses of high potassium saline the second injection produced a response (total saliva secreted in 3 min) that was 73 ± 18 % (standard error of the mean; S.E.; $N = 6$) that of the first. If the second injection contained cadmium then the response was reduced to 21 ± 11 % ($N = 6$) that of the initial (control) injection. The secretory rate of serotonin-stimulated salivary glands *in vitro* is unaffected by cadmium at this concentration. Cadmium is a highly effective calcium channel blocker (Hagiwara &

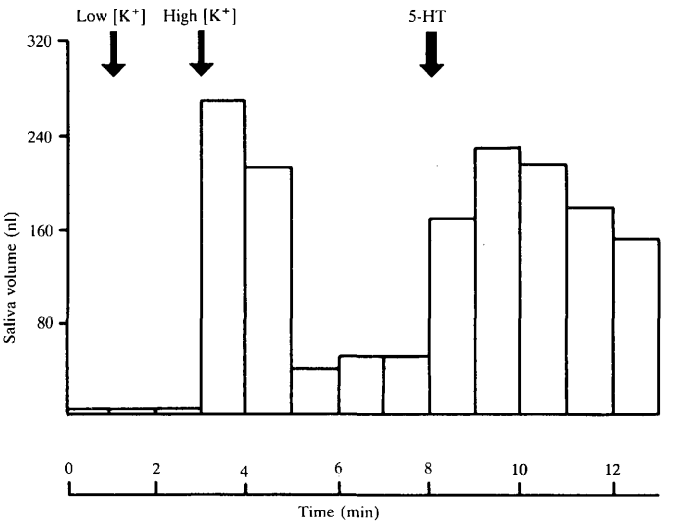

Fig. 1. The effect of injecting high- and low-potassium saline on the rate of salivation *in vivo*. The figure shows a typical response of a single fly injected with 5 μl high-potassium (60 mmol l^{-1}) saline or low-potassium (2 mmol l^{-1}) saline. For comparison 1 μl of serotonin (1×10^{-5} mol l^{-1}) was injected at the end of the response (5-HT).

Byerly, 1981; Pellmar, 1981; Ashcroft & Stanfield, 1982) and its effect on high-potassium stimulated salivation suggests that potassium's effect is calcium dependent. Potassium-evoked release of neurosecretory products is typically calcium dependent (see Discussion).

Salivation was also inhibited by gramine, which is a competitive antagonist of serotonin-stimulated fluid secretion by the salivary glands (Berridge, 1972). It is also known to block salivation *in vivo* in response to feeding (Hansen-Bay, 1976). Gramine (2×10^{-3} mol l^{-1}) inhibited salivation induced by 1-μl injections of serotonin (2×10^{-7} mol l^{-1}) (Fig. 2A), and high-potassium saline (Fig. 2C). The glands themselves were unharmed by the antagonist since injecting 1 μl of high concentration serotonin (2×10^{-3} mol l^{-1}), sufficient to override residual gramine inhibition, evoked salivation (Fig. 2A). This demonstrates that the effect of elevated potassium is mediated by serotonin-like receptors.

The collection of HKSGF and its action in vitro

After injections of high potassium saline into the fly, the haemolymph was found to contain an active factor termed High Potassium Salivary Gland Factor (HKSGF). The activity of the retrieved factor was found to be unaffected by

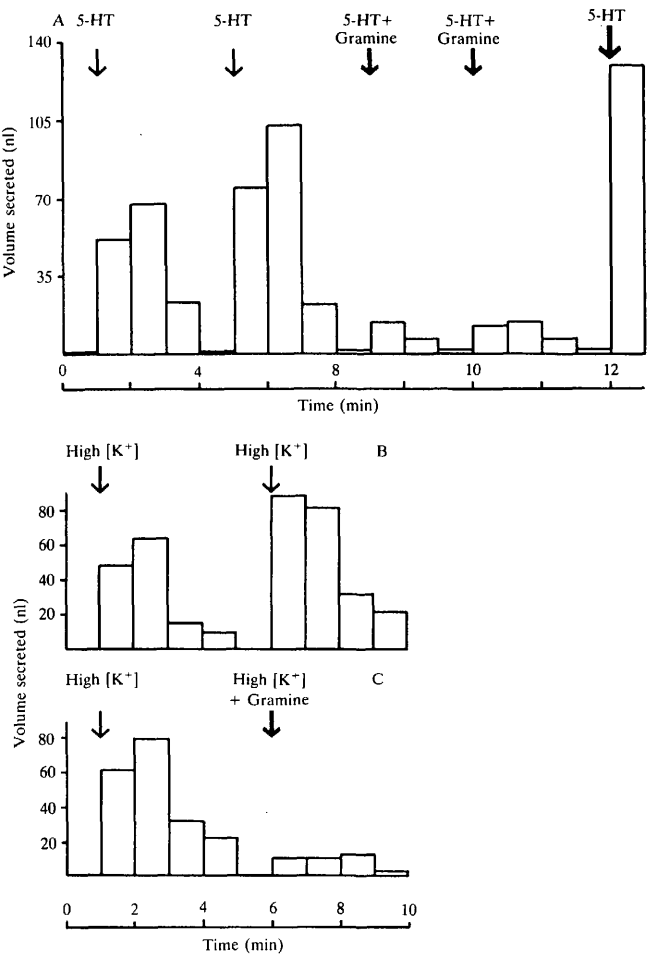

Fig. 2. The effect of gramine on serotonin and high-potassium induced salivation *in vivo*. (A) Responses of a single insect to 1-μl injections of serotonin (5-HT) (2×10^{-7} mol l^{-1}), and to the same injection containing gramine (2×10^{-3} mol l^{-1}). The final injection of serotonin contained sufficient serotonin (2×10^{-3} mol l^{-1}) to override the antagonist and demonstrates that the glands are still functional. (B) An insect injected with two successive doses of 90 mmol l^{-1} potassium saline (2 μl), salivates each time at a rate comparable to the serotonin-elicited response. (C) In another insect, inclusion of gramine in the second injection (2×10^{-3} mol l^{-1}) inhibits salivation.

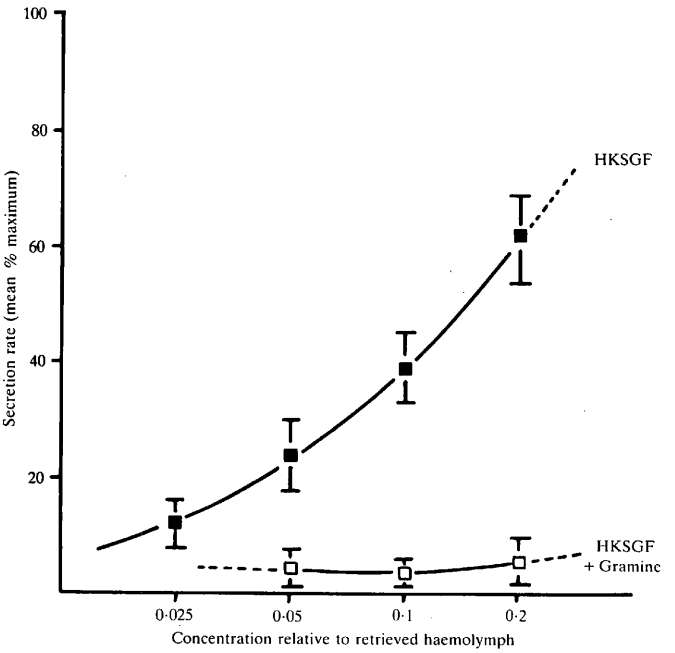

Fig. 3. The dose-response curve of HKSGF on isolated salivary glands and the effect of gramine. HKSGF was directly assayed on five salivary glands. Each point represents the mean (± standard error, S.E.) of three rate determinations for each of the glands expressed as a percentage of the maximum secretory rate in the presence of 2×10^{-8} mol l^{-1} serotonin. The gramine concentration was 1×10^{-4} mol l^{-1}.

storage on ice for a period of at least 15 min. Maximum amounts of HKSGF were obtained by taking a haemolymph sample 20 s after injecting saline. HKSGF collected in this way was bioassayed after serial dilution on salivary glands *in vitro* (Fig. 3). At the highest concentration used the maximum mean secretion rate of the glands was approximately 50–60 %. The action of the factor *in vitro* was inhibited by gramine (Fig. 3).

The action of HKSGF on the transepithelial potential (TEP) of salivary glands was compared with that of serotonin, since this is an accurate means of discriminating between certain serotonin analogues (Berridge, 1981). The resting TEP was found to be variable but on the addition of serotonin a predictable series of potential changes occurred. There were three main components to the response (Fig. 4B): (1) an initial rapid, negative potential shift, (2) a slight repolarization followed by oscillations, (3) a rapid positive-going change on washing which overshoots the

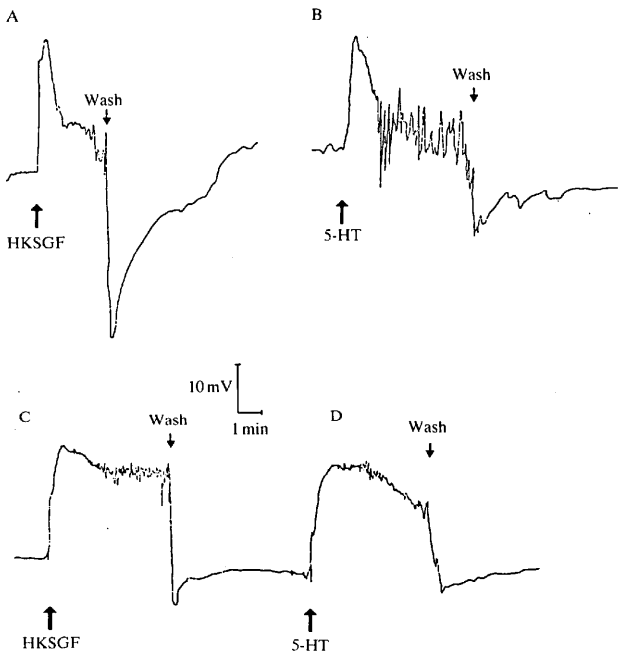

Fig. 4. The action of HKSGF and serotonin on the transepithelial potential of salivary glands *in vivo*. (A) HKSGF at a dilution of ×0·1. (B) Serotonin at a concentration of 6×10^{-9} mol l^{-1}. (C) HKSGF at a dilution of ×0·2. (D) Serotonin at a concentration of $1{\cdot}5\times10^{-8}$ mol l^{-1}. In all cases the unstimulated resting potential was 4–10 mV positive. These traces are representative of four such experiments at each concentration.

resting potential before returning to the initial potential in an asymptotic fashion.

The size of these three components varied with the concentration of serotonin applied. At low concentrations (1) disappeared and (3) was reduced. At very high serotonin concentrations the repolarization was reduced and the oscillations ceased (Fig. 4D). The response to HKSGF was indistinguishable from that to serotonin, with all three itemized characteristics at low concentrations (Fig. 4A), and a loss of the repolarization and oscillations at higher concentrations (Fig. 4C). This is highly indicative of serotonin receptor activation. Not only were the TEP responses alike but the relationship between secretion rate and electrical responses were also similar. At a dilution of HKSGF 0·1 times that of the retrieved haemolymph, as in Fig. 4A, a secretory rate of 40 % maximum is achieved (Fig. 3). The concentration of serotonin matching this TEP response (6×10^{-9} mol l^{-1}, Fig. 4B) has also been found to stimulate secretion at approximately 40 % maximum rate (see Fig. 8 and Berridge, 1981).

Biochemical properties of HKSGF

Heat stability of HKSGF

Heating to 75 °C for 10 min had no effect upon the bioactivity of HKSGF or serotonin (Table 1).

Table 1. *The effect of heating on the bioactivity of serotonin and HKSGF*

Test	Heated	Control
Serotonin	70·0 ± 4·8	63·0 ± 5·0
HKSGF	31·8 ± 4·3	25·7 ± 3·2

HKSGF was collected and diluted with an equivalent volume of low-potassium saline. The sample was divided into two. One part was heated for 10 min at 75 °C before further diluting and bioassaying. The other portion was kept on ice before diluting an identical amount with saline that had been heated for 10 min at 75 °C. A precipitate formed in both of the heated samples, only the supernatant was used in the bioassays. The data represent the mean percentage of the maximum response of four salivary glands.

The solubility of HKSGF in some organic solvents

By shaking HKSGF with various solvents, it was found that the active factor was insoluble in ethanol, acetone, ethyl acetate (Fig. 5A) and chloroform (Fig. 5C), but was soluble in methanol (Fig. 5A) and 80 % ethanol (Fig. 5D). This solubility was identical to that of serotonin under the same conditions (Fig. 5B). To reduce the decreased sensitivity of the bioassay caused by oxidation of haemolymph components (and possible long-term degradation of HKSGF) crude HKSGF was routinely extracted into ice-cold 80 % ethanol. All of the bioactivity was recovered in the supernatant (Fig. 5D).

Chromatography of HKSGF and serotonin

Thin-layer chromatography of HKSGF in 70 % ethanol revealed a single peak that corresponded with authentic serotonin run in parallel (Fig. 6). The quantity of active factor in this peak was close to that predicted by bioassay of the crude extract. No activity was retrieved from plates using haemolymph from low-potassium injected flies. Several other solvent systems were used to chromatograph HKSGF (*N*-butanol/acetic acid/water, 60/25/15; *N*-butanol/pyridine/water, 1/1/1; ethyl acetate/acetic acid, 99/1). In all of these solvents a peak corresponding to serotonin was observed with approximately the same bioactivity as that found in the crude extract. In these solvents, however, additional peaks of bioactivity appeared that had chromatographic properties different from those of serotonin. These peaks appeared to be induced in the haemolymph by the solvents since their bioactivity exceeded that measured in the original sample. These peaks (and not the 'serotonin' peak) were also generated in samples of non-bioactive haemolymph from low-potassium injected insects (see Discussion).

Enzyme degradation of HKSGF

HKSGF was not destroyed by either trypsin or leucine aminopeptidase (results not shown). This suggests that HKSGF is not a peptide that is susceptible to degradation by these particular peptidases. Incubation with a crude MAO prep-

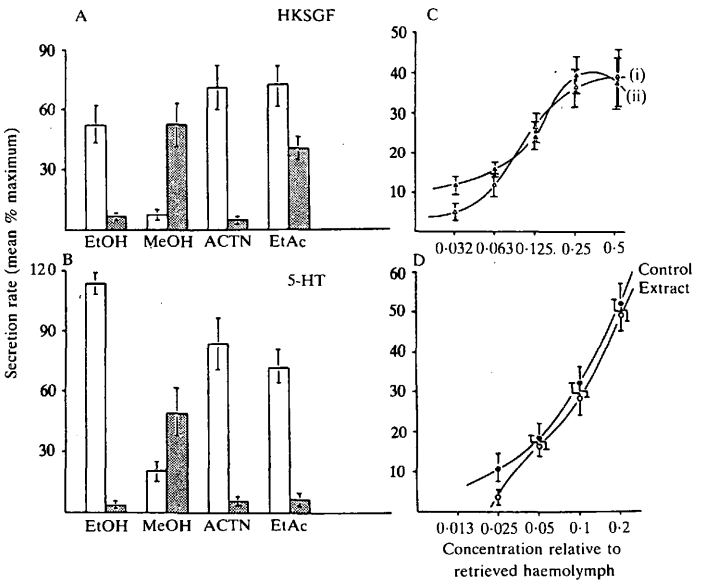

Fig. 5. The solubility of HKSGF and serotonin in several organic solvents. (A) Bioassay of the residue (open bars) and supernatant (dotted bars) fractions of HKSGF after treating with organic solvents. (B) Bioassay of the residue (open bars) and supernatant (dotted bars) fractions of serotonin treated in an identical fashion. Initial concentration of serotonin was 1×10^{-8} mol l^{-1}. EtOH, absolute ethanol; MeOH, methanol; ACTN, acetone; EtAc, ethyl acetate. (C) Comparison of the bioactivity of residue from chloroform-treated (i) and untreated (ii) HKSGF by serial dilution bioassay. This demonstrates that all of the bioactivity of HKSGF is recovered in the chloroform-insoluble residue. (D) Comparison of the bioactivity of the supernatant from 80% ethanol (Extract) and untreated (Control) HKSGF by serial dilution bioassay. All of the bioactivity in HKSGF is soluble in 80% ethanol. Each value is the mean of the secretion rate of at least four salivary glands each determined from three saliva aliquots. Results are expressed as a percentage of the maximum possible salivation rate as determined for each gland in the presence of 2×10^{-8} mol l^{-1} serotonin.

aration degraded all of the bioactivity (Table 2). This could be partially inhibited by Pargyline, suggesting that at least part of the bioactivity is attributable to monoamines.

Selective inhibition of MAO

A more precise indication of the enzyme lability of HKSGF was obtained by the selective inhibition of the two forms of MAO types A and B. These are defined by their substrate specificities and their sensitivity to the inhibitors Chlorgyline and Deprenil (Ekstedt, 1976). Serotonin is metabolized predominately by the 'A' form

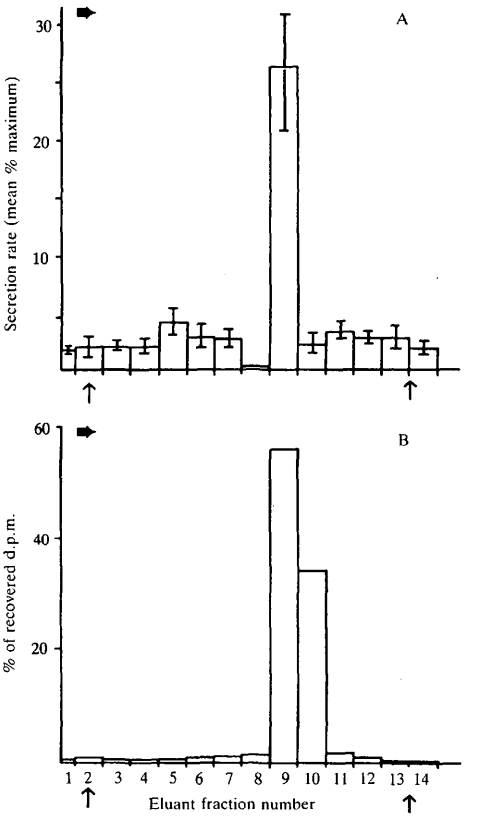

Fig. 6. Thin layer chromatography of HKSGF and serotonin in 70% ethanol. (A) Bioassay of eluants from a chromatogram of HKSGF. All eluants were five times the volume of the originally applied HKSGF. (B) Radioactivity recovered from sequential regions of a chromatogram of ^{3}H-serotonin co-chromatographed with A. The arrows above each diagram indicate the direction of solvent movement. Arrows beneath the abcissa indicate the positions of the origin and the solvent front.

(Hall, Logan & Parsons, 1969) which is particularly sensitive to Chlorgyline. The 'B' form is inhibited by Deprenil and degrades phenylethylamine. Both forms degrade tyramine. It should be remembered that neither inhibitor is entirely specific for its preferred MAO type (Johnston, 1968), and neither form of the enzyme is entirely specific for its substrate. There is, for instance, a tiny residual deamination of serotonin in Chlorgyline-inhibited MAO that is attributable to MAO type B (Fowler & Tipton, 1982). In using MAO as a probe these difficulties can be overcome by the careful selection of inhibitor concentrations and incubation

Table 2. *The effect of monoamine oxidase on the bioactivity of serotonin and HKSGF*

Test	MAO treatment		
	Active	Heated	Pargyline
Serotonin	6·3 ± 2·4	48·7 ± 9·9	16·5 ± 4·2
HKSGF	10·7 ± 3·4	82·4 ± 20·5	34·9 ± 7·4

The biological activity of serotonin and HKSGF after incubation with MAO (Active), heat inactivated MAO (Heated), or MAO in the presence of Pargyline (1×10^{-4} mol l^{-1}) (Pargyline). Bioactivity is recorded as the mean percentage of the maximum secretory rate of three or four salivary glands. All treatments were in duplicate. Incubation conditions were as described in the text.

conditions. When MAO is treated with the appropriate concentration of chlorgyline there is no measurable breakdown of authentic serotonin (Fig. 7A). The same concentration of Deprenil has no effect on the normal (control) degradation of serotonin. The ability of MAO to destroy HKSGF is inhibited by Chlorgyline but not by Deprenil (Fig. 7B).

Radioenzyme assay for serotonin

Predicted values

If the bioactivity of HKSGF is attributable to serotonin then there should be a close correlation between the amount measured by radioenzyme assay and the amount predicted from the dose-response curves of HKSGF and serotonin. Bioassay of extracted HKSGF at a dilution of ×0·2 produced an average secretion rate that was half the maximum rate *in vitro* (Fig. 3). By reference to a serotonin dose-response curve for these glands (Fig. 8) it can be seen that this rate is generated by $6{\cdot}9\times10^{-9}$ mol l^{-1} serotonin which is equivalent to 420 pg/50 μl. Four identical samples of HKSGF were prepared and assayed for serotonin. The mean serotonin content of these samples was 437 ± 50·2 pg for a 50-μl sample. Serotonin therefore accounts for all the bioactivity of HKSGF.

The localization of serotonin release

Serotonin content of nervous tissue

Serotonin was found in both the cerebral ganglion and the fused thoracic ganglion (Table 3) in roughly the same magnitude (ng serotonin/g wet weight tissue) as that found in the ganglia of most other insects (see Evans, 1980). These values are markedly higher than the levels previously measured by HPLC of pooled *Calliphora* nervous tissue (Nassel & Laxmyr, 1983). This difference is discussed later. Nerves attached to the thoracic ganglion contain substantial amounts of serotonin (Table 3), nearly half the level found in the ventral nerve system. This supports the idea that serotonin may play a peripheral role in addition to its function as a central transmitter.

Nerve sectioning

Cutting the abdominal nerves or the fine nerves supplying the neck muscles had

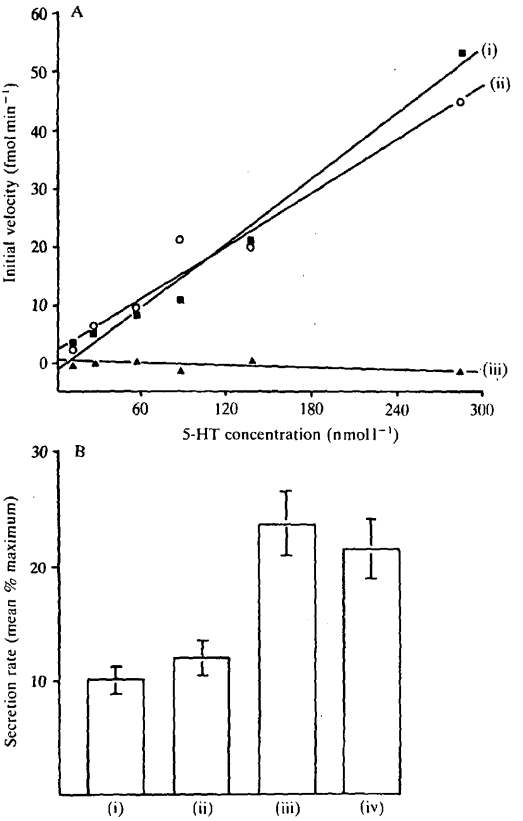

Fig. 7. The effect of Chlorgyline and Deprenil on the degradation of serotonin and HKSGF by monoamine oxidase. (A) The rate of degradation of serotonin at various initial concentrations in the presence of untreated MAO (i), Deprenil (3×10^{-7} mol l^{-1}) preincubated MAO (ii), and Chlorgyline (3×10^{-7} mol l^{-1}) preincubated MAO (iii). Units on the abcissa are femtomoles of product in the extract per minute. (B) The bioactivity of HKSGF incubated with MAO (i), Deprenil-inhibited MAO (ii), Chlorgyline-inhibited MAO (iii) or ethanol-inactivated MAO (iv). The bioactivity is expressed as the mean (± standard error, S.E.) percentage maximum secretion rate for four salivary glands *in vitro*. All experiments were carried out in triplicate.

no effect on the flies ability to salivate, but severing the cephalothoracic connective did abolish the salivatory response (Fig. 9). Attempts to expose the main leg and wing nerves disrupted normal feeding behaviour and prevented further use of this technique for studying the neurohaemal sites. These lesioning experiments suggest that the neurohormone is released close to the main body of the thoracic ganglion or possibly in the large locomotory nerves.

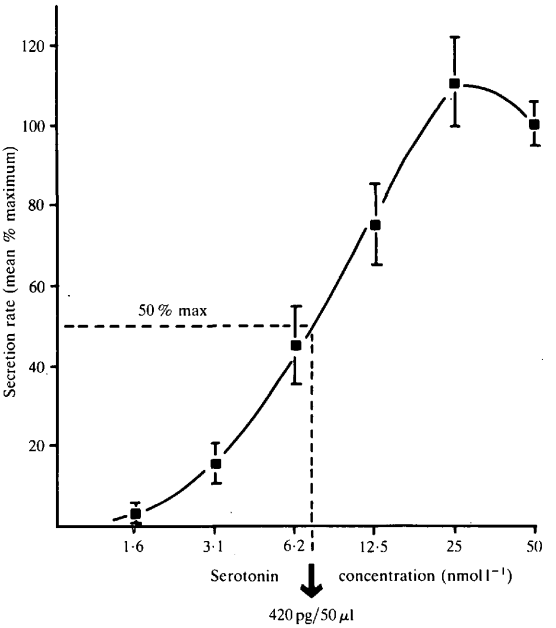

Fig. 8. Estimation of the serotonin content of HKSGF from the serotonin dose-response curve of *in vitro* salivary glands. Each point is the mean of three rate determinations on each of four salivary glands. Half-maximum secretion rate (50% max) is generated by $6{\cdot}9\times10^{-9}$ mol l^{-1} serotonin. Therefore each 50 µl of retrieved high-potassium haemolymph should contain 420 pg serotonin.

Table 3. *The serotonin content of* Calliphora *nervous tissue*

		Thoracic ganglion	
	Cerebral ganglion	With nerves	Without nerves
pg tissue^{-1}	1379 ± 130 (N = 9)	896 ± 85 (N = 12)	479 ± 76 (N = 6)
ng g^{-1} wet weight	1942	3133	ND

The serotonin content of *Calliphora vicina* nervous tissue determined by radioenzyme assay. The values show the mean (± standard error) total content per tissue and per gram wet weight of tissue. Each value was obtained from duplicate samples, one of which contained an internal standard. In all cases the *N*-acetyl-serotonin blank counts were not substantially different from the blank counts, indicating that the assay was only measuring serotonin levels. Twice background sensitivity for these assays was approximately 100 pg. ND, not determined.

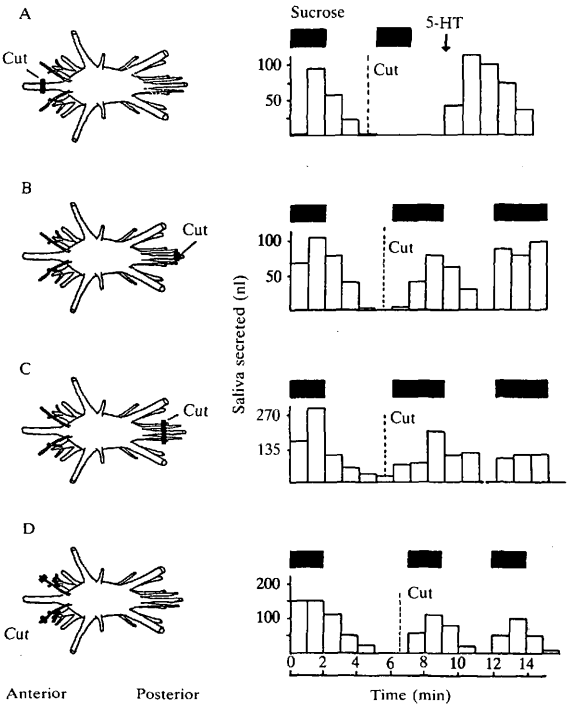

Fig. 9. The effect of severing selected nerves upon the ability of the fly to salivate in response to sucrose. Nerves were severed at the places indicated by the diagrams on the left after the response to an initial presentation of sucrose (control) had ceased. Bars above the histograms indicate the presence of sucrose on the oral lobes. In (A) serotonin (5×10^{-7} μmol) was injected at the arrow to ensure that the glands were still functional. (A) Severing the cerebrothoracic connective; (B) nervus fasiculus abdominus; (C) as (B) and including the nervus segmenti abdominus; (D) dorsal prothoracic nerve, nervus staticus, and frontal prothoracic nerve (after Vater, 1962).

DISCUSSION

Release into the haemolymph of an active factor that stimulates salivation has been achieved here by injection of high-potassium saline. Direct treatment of isolated or *in situ* tissues with elevated potassium in the manner of Maddrell & Gee (1974) or Aston (1979) was attempted but there was sufficient spontaneous release into control low-potassium saline to prevent this approach being used to localize release sites. Such release probably arose from damage to the nervous system.

The high-potassium induced salivation described here does not result from direct activation of the salivary gland itself since the injected concentration is much too low to induce such high rates of salivation. A concentration of 120–150 mmol l^{-1} potassium is required for maximal secretory rates *in vitro* (Berridge *et al.* 1975). Also the time course for induction (< 1 min) is much shorter than that expected for potassium activation (> 5 min, Berridge *et al.* 1975). The gramine inhibition of potassium-stimulated salivation demonstrates that the effect of potassium is mediated by a secretagogue acting on specific serotonin-like receptors. This factor has been presumed to emanate from neurohaemal sites, an assumption that is consistent with its calcium dependence. It is conceivable that it is released from relatively unprotected motor nerve terminals but this is considered unlikely since isolated muscle does not release HKSGF and muscle homogenates do not contain significant amounts of active factor (B. A. Trimmer, unpublished observations) or serotonin (Trimmer, 1983).

HKSGF is released and inactivated extremely quickly *in vivo* but once removed from the insect is stable enough to be studied without further purification. Its action on isolated salivary glands is indistinguishable from that of serotonin. It appears to act on gramine-sensitive serotonin receptors giving a characteristic transepithelial change. This latter observation eliminates the possibility that it is a related serotonin analogue with a substituted 5′-hydroxyl moiety, such as 5-methoxytryptamine which generates completely different TEP changes (Berridge, 1981). Certain analogues (e.g. tryptamine, 5-hydroxy-*N*-dimethyltryptamine) will activate both calcium and cAMP intracellular signals and thus generate serotonin-like TEP responses (Berridge, 1981) and it is possible that close serotonin analogues such as these may be the natural secretagogue although none is as potent as serotonin itself (Berridge, 1972, 1981). The relationship between secretory rate and TEP changes observed with HKSGF is much more like that of a serotonin response than is the relationship for any of the serotonin analogues that have been tested.

Although high-potassium injection does not release sufficient material for direct chemical analysis of the active factor, the factor was indistinguishable from serotonin in all of the physical and biochemical tests applied. The heat stability, solubility and peptidase susceptibility suggest that the active constituent is not a protein, but do not rule out the possibility that it is a small peptide (see Stone & Mordue, 1980). This possibility is extremely unlikely in view of the thin layer chromatography results and the loss of activity induced specifically by MAO type A. Thin layer chromatography in solvents other than ethanol revealed peaks of activity that had greater and lesser mobility than serotonin in the butanol based solvents, and peaks that, unlike serotonin, migrated away from the origin in acidic ethyl acetate. These peaks, although of interest in themselves, were not related to the effect of high-potassium injection since they were also generated in chromatograms of haemolymph from low-potassium injected flies. The total activity in these peaks exceeded the bioactivity of the freshly withdrawn haemolymph and they would therefore appear to be artifacts created by the chromatographic procedure. This effect may also account for the 'additional' bioactivity in the supernatant of ethylacetate treated HKSGF (see Fig. 5A). These supernumary peaks could not be attributed to salt accumulation or any similar extraneous effect on the bioassay. The nature of these substances remains to be elucidated.

The most compelling evidence for HKSGF being serotonin is the demonstration that all of the bioactivity can be accounted for by the radioenzymatically determined serotonin content. This also supports the view that serotonin is the physiological hormone since it is the only biologically active factor that can be readily released into the haemolymph.

The site of this neurohaemal release has proved difficult to identify. Serotonin has been demonstrated in all parts of the nervous system including the nerve trunks attached to the thoracic ganglion. The quantity of serotonin per unit wet weight of tissue is of the same magnitude as that reported in the ganglionic masses of other insects (see Evans, 1980; Taylor & Newburgh, 1979; Clark & Donnellan, 1982; Omar, Murdock & Hollingworth, 1982) but is significantly greater than the values reported by Nassel & Laxmyr (1983) for adult *Calliphora* ganglia. The amounts reported in the present study are considered to be accurate representations of the endogenous serotonin content for several reasons. Firstly they are derived from individual ganglia and not from pooled tissues as reported by Nassel & Laxmyr. In individual cerebral ganglia of *Peripleneta americana*, serotonin has been found, using high performance liquid chromatography, at a concentration of $1{\cdot}8\,\mu g\,g^{-1}$ (wet weight) (Omar *et al.* 1982). Secondly, it has already been pointed out that in using the present radioenzyme assay procedure, acid extraction leads to significant increases in the levels of *N*-acetylserotonin measured at the expense of serotonin. It is possible that this loss of serotonin would not have been detected in the HPLC system used by Nassel & Laxmyr. The latter authors use of an extremely powerful oxidising agent ($0{\cdot}4\,mol\,l^{-1}$ perchloric acid) may also lead to degradative loss of endogenous serotonin. These authors do not detail their characterization of the HPLC peaks which they admit can be affected by unknown compounds of the retina and lamina. The radioenzyme assay used in the present study carefully characterizes the stable reaction product (melatonin) by solvent extraction and TLC and can be used to measure *N*-acetylserotonin and serotonin in the same tissue sample. The difference between the values obtained by Nassel & Laxmyr's HPLC method and the radioenzyme assay reported here may also be due to differences in the strain of fly used or variables such as rearing conditions. This remains to be resolved.

Although serotonin is present in all parts of the nervous system it is not readily released from the intact cerebral ganglion (M. J. Berridge, personal communication) and the results of the nerve sectioning experiments suggest that the release of the hormone requires the cerebral thoracic connective to be intact. In the present study it was found that the abdominal nerves are not required for salivation to take place, a finding in contradiction to the results of Hansen-Bay (1978). This is probably due to the use of cold anaesthesia during surgery and the rapid highly-localized lesions created by radiofrequency microcautery. Release of serotonin from sites in the thorax would enable it to pass over the complete length of the secretory portion of the salivary glands and allow it to be inactivated and excreted from the abdomen (B. A. Trimmer, in preparation). It is worth mentioning that the haemolymph in the blowfly forms a thin layer of rapidly circulating fluid adhering to the organs. Hormones released into this capillary layer will be transported exceptionally quickly to the target organs. Insects with larger blood volumes are

probably unable to use neurohormones as fast effectors because mass flow in these animals is likely to be substantially slower. For the blowfly to make best use of this flow, serotonin would have to be released into the posteriorly-flowing haemolymph of the ventral canal in which the thoracic ganglion lies. Preliminary studies using a serotonin antibody have revealed a network of immunoreactive processes in the dorsal perineural layer of the thoracic ganglion and it is possible that these are the neurohaemal release sites for serotonin.

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REFERENCES

Ashcroft, F. M. & Stanfield, P. R. (1982). Calcium and potassium currents in muscle fibres of an insect (*Carausius morosus*). *J. Physiol., Lond.* **323**, 93–115.

Aston, R. J. (1979). Studies on the diuretic hormone of *Rhodnius prolixus*. Some observations on the purification and nature of the hormone and the dynamics of its release *in vitro*. *Insect Biochem.* **9**, 163–176.

Aston, R. J. & White, A. F. (1974). Isolation and purification of the diuretic hormone from *Rhodnius prolixus*. *J. Insect Physiol.* **20**, 1673–1682.

Berridge, M. J. (1972). The mode of action of 5HT. *J. exp. Biol.* **56**, 311–321.

Berridge, M. J. (1981). Electrophysiological evidence for the existence of separate receptor mechanisms mediating the action of 5-hydroxytryptamine. *Mol. cell. Endocr.* **23**, 91–104.

Berridge, M. J. & Fain, J. (1979*a*). Inhibition of phosphatidylinositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5-hydroxytryptamine. *Biochem. J.* **178**, 58–69.

Berridge, M. J. & Fain, J. (1979*b*). Phosphatidylinositol metabolism and calcium gating. *Med. Chem.* **6**, 117–125.

Berridge, M. J., Lindley, B. D. & Prince, W. T. (1975). Stimulus-secretion coupling in an insect salivary gland: cell activation by elevated potassium concentrations. *J. exp. Biol.* **62**, 629–636.

Berridge, M. J. & Patel, N. G. (1968). Insect salivary glands: stimulation of fluid secretion by 5HT and cAMP. *Science, N.Y.* **162**, 462–463.

Berridge, M. J. & Prince, W. T. (1972). Transepithelial potential changes during stimulation of isolated salivary glands with 5-hydroxytryptamine and cyclic AMP. *J. exp. Biol.* **56**, 139–153.

Boireau, A., Ternaux, J. P., Bourgoin, S., Hery, F., Glowinski, J. & Hamon, M. (1976). The determination of picogram levels of 5HT in biological fluids. *J. Neurochem.* **26**, 201–205.

Brown, B. E. (1977). Occurrence of proctolin in six orders of insects. *J. Insect Physiol.* **21**, 1879–1881.

Clarke, B. S. & Donnellan, J. F. (1982). Concentrations of some putative neurotransmitters in the CNS of quick-frozen insects. *Insect Biochem.* **12**, 623–638.

Dalton, T. (1977). Threshold and receptor reserve in the action of 5HT on the salivary gland of *Calliphora erythrocephala*. *J. Insect Physiol.* **23**, 625–631.

Ekstedt, B. (1976). Substrate specificity of the different forms of MAO in rat liver mitochondria. *Biochem. Pharmacol.* **25**, 1133–1138.

Evans, P. D. (1980). Biogenic amines in the insect nervous system. *Adv. Insect Physiol.* **15**, 317–473.

Evans, P. D. & O'Shea, M. (1978). The identification of an octopaminergic neuron and the modulation of a myogenic rhythm in the locust. *J. exp. Biol.* **73**, 235–260.

Fowler, C. J. (1978). Studies on rat liver monoamine oxidase. Ph.D. thesis, University of Cambridge.

Fowler, C. J. & Tipton, K. F. (1982). Deamination of 5-hydroxytryptamine by both forms of monoamine oxidase in brain tissue. *J. Neurochem* **38**, 733–736.

Hagiwara, S. & Byerly, L. (1981). Calcium channel. *A. Rev. Neurosci.* **4**, 69–125.

9302 (Chlorgyline). I. *Biochem. Pharmac.* **18**, 1447.

Hammel, I., Naot, Y., David, B. E. & Ginsberg, H. (1978). A simplified microassay for 5HT: modification of the enzymatic isotopic assay. *Analyt. Biochem.* **90**, 840–843.

Hansen-Bay, C. M. (1976). Secretory control mechanisms of salivary glands of adult *Calliphora*. Ph.D. thesis, University of Cambridge.

HANSEN-BAY, C. M. (1978). Control of salivation in the blowfly *Calliphora*. *J. exp. Biol.* **75**, 189–201.

HUSSAIN, M. N. & SOLE, M. J. (1981). A simple, specific radioenzymatic assay for picogram quantities of serotonin or *N*-acetylserotonin in biological fluids or tissues. *Analyt. Biochem.* **111**, 105–110.

JOHNSTON, J. P. (1968). Some observations upon a new inhibitor of MAO in brain tissue. *Biochem. Pharmac.* **17**, 1285–1287.

MADDRELL, S. H. P. & GEE, J. D. (1974). Release of the diuretic hormones of *Rhodnius prolixus* and *Glossina austeni* induced by potassium-rich solutions. Calcium dependence, time course of release and localisation of neurohaemal areas. *J. exp. Biol.* **61**, 155–171.

MADDRELL, S. H. P., PILCHER, D. E. M. & GARDINER, B. O. C. (1971). Pharmacology of Malpighian tubules of *Rhodnius* and *Carausius*. The structure-activity relationships of tryptamine analogues and the role of cyclic AMP. *J. exp. Biol.* **54**, 779–804.

MAY, T. E., BROWN, B. E. & CLEMENTS, A. N. (1979). Experimental studies upon a bundle of tonic fibres in the locust extensor tibialis muscle. *J. Insect Physiol.* **25**, 169–181.

NASSEL, D. R. & LAXMYR, L. (1983). Quantitative determination of biogenic amines and DOPA in the CNS of adult and larval blowflies, *Calliphora erythrocephala*. *Comp. Biochem. Physiol.* **75**C, 259–265.

OMAR, D., MURDOCK, L. & HOLLINGWORTH, R. M. (1982). Actions of pharmacologic agents on 5HT and dopamine in the cockroach (*Periplaneta americana*) nervous system. *Comp. Biochem. Physiol.* **73**C, 423–429.

PELLMAR, T. C. (1981). Ionic mechanisms of a voltage-dependent current elicited by cAMP. *Cell. Mol. Neurobiol.* **1**, 87–97.

PIEK, T. & MANTEL, P. (1977). Myogenic contractions in locust muscle induced by proctolin and by wasp venom. *J. Insect Physiol.* **23**, 321–325.

SAAVADRA, J. M., BROWNSTEIN, M. & AXELROD, J. (1973). A specific and sensitive enzymatic isotopic microassay for serotonin in tissues. *J. Pharmac. exp. Ther.* **186**, 508–515.

SMITH, I. (1969). *Chromatographic and Electrophoretic Techniques*. London: W. Heinemann Books Ltd.

STONE, J. V. & MORDUE, W. (1980). Isolation of insect neuropeptides. *Insect Biochem.* **10**, 229–239.

TAYLOR, D. P. & NEWBURGH, R. W. (1979). The synthesis and content of neurotransmitters and their effect on cyclic nucleotide accumulation in the CNS of *Manduca sexta*. *Insect Biochem.* **9**, 265–272.

TRIMMER, B. A. (1983). Serotonin and the control of salivation in *Calliphora*. *Ph.D thesis, University of Cambridge*.

UNWIN, D. M. (1978). A versatile high frequency radio microcautery. *Physiol. Ent.* **3**, 71.

VATER, G. (1962). Untersuchungen uber die Morphologie des Nervensystems der Dipteran. *Z. wiss. Zool.* **167**, 137–196.