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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF A PEPTIDE WHICH STIMULATES THE HINDGUT OF THE COCKROACH, *LEUCOPHAEA MADERAE* (FABR.)

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Although the corpus cardiacum of insects is known to contain a factor that stimulates the muscles of the heart and alimentary tract (Cameron, 1953), Davey (1962) was the first to describe the effect of homogenates of the corpus cardiacum on the spontaneous contractile activity of the cockroach hindgut. He found that when an isolated hindgut was exposed to 0.025 pairs of corpora cardiaca per milliliter, an increase in tonus, amplitude, frequency and coordination of contractions occurred.

Brown (1965) subsequently succeeded in separating six to nine active substances from extracts of the corpus cardiacum of *Periplaneta americana* L. by means of paper chromatography. He found that two components, Factors P_1 and P_2 were responsible for most, if not all, the effect the crude extracts had on hindgut activity. The two compounds appeared to be peptides and were heat-stable, sensitive to chymotrypsin, and dialyzable. Brown assumed that Factor P_1, by its rate of dialysis, was the larger of the two peptides and was as effective on the hindgut as on the heart; P_2 was a specific activator of the hindgut.

In an earlier paper, we described three materials present in hindgut tissue extracts of *Leucophaea maderae* (Fabr.) that stimulated the muscular activity of isolated hindgut preparations from the same cockroach (Holman and Cook, 1970). Two of the compounds were identified as L-glutamic and L-aspartic acids. An application of either of these acids to an isolated hindgut caused a single slow contraction that was indistinguishable from the response produced by electrical stimulation of the nerves innervating the hindgut. An application of the third material (not identified) resulted in a prolonged and complex stimulation of the hindgut which was similar in character to the response reported by both Davey (1962) and Brown (1965) for corpora cardiaca extracts.

We have attempted to isolate and partially characterize this active compound present in the hindgut extracts with the hope of determining its role in the control of muscular activity in this region.

Material and Methods

Extraction procedure

Extracts were made of organs and body areas from adult cockroaches, *L. maderae* and *P. americana;* from grasshoppers, *Schistocerca vaga vaga* (Scudder);

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from house flies, *Musca domestica* L.; and from fifth-instar larvae of tobacco hornworms, *Manduca sexta* (L.). All insects were reared at this laboratory under controlled conditions.

Dissected organs and body parts were homogenized in 80% ethanol (cockroaches and grasshoppers, 5/ml; fly heads, 50/ml; hornworm hindguts, 2/ml). Following centrifugation, the supernatant was evaporated to dryness with a rotary evaporator at 35° C. The residue was then partioned between equal volumes of ethyl acetate and water (25 ml of each/100 organs). The ethyl acetate was discarded and the aqueous phase lyophilized and stored in the freezer under argon.

Extracts for the centrifuge experiments were prepared by homogenizing heads and hindguts of *L. maderae* in 0.25 M sucrose (0.5 ml/organ). Following centrifugation at 1000 *g* (0° C, 15 min) to remove insoluble debris, the supernatant was re-centrifuged at 20,000 *g* (0° C, 30 min). The second supernatant was lyophilized and prepared for bioassay. The sediment was extracted twice with 5 ml of 80% ethanol which was pooled, evaporated, and prepared for bioassay.

Purification

The biologically active material in the crude extracts was purified by anion- and cation-exchange, gel filtration, and thin-layer chromatography.

Twenty grams of DEAE-Sephadex® (A-25) [mention of a proprietary product in this paper does not imply an endorsement by the U. S. Department of Agriculture.] was allowed to swell in 200 ml 50% acetic acid. The acid was decanted and replaced with fresh acid four times. The gel was then rinsed with water until the pH of the gel solution rose to 4.0, then packed with flow into a 2.5 × 45-cm column and rinsed with 1 liter of distilled water. Samples were applied to and eluted from this column with water. Four 50-ml fractions were collected, lyophilized, and stored in the freezer under argon.

Thirty grams of CM-Sephadex (C-25) was prepared and packed in the 2.5 × 45-cm column in the same fashion as the DEAE-Sephadex except that 0.5 N HCl replaced the 50% acetic acid. Samples were applied to this column in water and eluted from the column by the stepwise addition of water, 0.1 N and 1.0 N acetic acid, 0.01 N and 0.5 N HCl (300 ml each). Later, the samples were eluted with water, 0.01 N and 0.02 N HCl (300 ml each), and the 0.02 N HCl was collected as three 100-ml fractions. All fractions were lyophilized and stored in the freezer under argon.

Both G-10 and G-15 Sephadex gels were prepared in the same manner; 50 grams was stirred with 0.01 N HCl then allowed to settle for five minutes, the HCl (and fines) was decanted and replaced with fresh HCl several times. Then the gel was packed without flow into a 2.5 × 45-cm column. After packing, solvent (0.01 N HCl) was passed through the column until the gel bed stabilized. Samples were applied to the column in 0.5 ml of 0.01 N HCl containing dextran blue (to determine the V_0) and eluted with 0.01 N HCl into 4-ml fractions. The flow rate was 4 ml/cm^2/hr (about 20 ml/hr).

Thin-layer chromatography was carried out on 5 × 20-cm glass plates coated with a 250-μ layer of cellulose. A miscible solvent system (isopropanol:water: acetic acid, 25 : 10 : 1) was allowed migrate in the ascending fashion. After drying in the hood, fluorescent spots were visualized under a broad spectrum ultra-

violet lamp. Amine-containing areas were visualized with ninhydrin spray followed by heating (110° C).

Various regions of unsprayed chromatograms were scraped, and the cellulose was eluted with 0.01 N HCl. The HCl was evaporated, and the residues were dissolved in saline solution for bioassay.

Stability and sensitivity tests

Biologically active samples eluted from thin-layer plates were prepared for bioassay in screw-topped tubes and immersed in a boiling water bath for 10 minutes. After cooling, they were applied directly to the isolated hindgut preparation. Similar samples were titrated to a pH of 9.0 with 0.01 N NaOH, held for 10 minutes, titrated to pH 7.0 with 0.01 N HCl, and bioassayed.

Saline solutions of chymotrypsin and pronase (a mixture of proteolytic enzymes) were prepared at a concentration of 1 mg/ml and brought to pH 7.5 with 0.01 N NaOH. Samples of the active material in 1 ml saline solution (pH 7.5) were treated with $\frac{1}{2}$ ml of one of the enzyme solutions or with $\frac{1}{2}$ ml pH 7.5 saline solution and incubated for three hours at 37° C. After incubation, the samples were immersed in a boiling water bath for 10 minutes, cooled, and then bioassayed. Tubes containing only pronase or chymotrypsin were treated in the same manner and bioassayed.

Bioassay and various physiological preparations

Hindguts from adult male cockroaches were isolated and prepared for myographic recording as previously described (Holman and Cook, 1970). The methods of Cook, Eraker and Anderson (1969) were used for the bioassay of the foregut. On several occasions, the hindgut of fifth-instar larvae of the tobacco hornworm was isolated and suspended by threads in a chamber in a manner similar to that which was used with the cockroach. The saline solution for the tobacco hornworm consisted of 6 g sodium chloride, 0.3 g potassium chloride, 3.6 g magnesium chloride $\times$ 6 H_2O, 0.33 g calcium chloride and 54.4 g sucrose dissolved in 1 liter of water and brought to a pH of 6.5. The lyophilized residues were dissolved in 200 to 500 μl of the appropriate saline solution and adjusted to a pH of 6.5 before they were added to the aerated saline solution in the muscle chamber. Samples were left in contact with the gut from 1 to 5 minutes. The chamber was then rinsed several times and refilled with fresh saline.

Both adult *P. americana* and *L. maderae* were used for bioassays of the heart. The type B preparation described by Yeager (1939) was employed. Following several rinses with saline solution, the spontaneous contractile responses of the heart were registered by placing a glass microelectrode against the ventral lateral surface of the heart. The micropipet was used as a resistance sensor to detect the mechanical movement of the heart (Cook, Long and Owens, 1971). Samples were applied directly to the heart in saline solution (1–2 μl), and the heartbeat was monitored for 3–4 minutes to determine the effect of the applied samples.

A small suction electrode was used to record the electrical activity from the proctodaeal nerve when the terminal ganglion was exposed to the extracted peptide.

The electrode was connected to a preamplifier and an oscilloscope was used to monitor spontaneous neural activity.

Histological and cytological procedures

The *in situ* demonstration of neurosecretion in the hindgut of the cockroach was accomplished by employing the Victoria Blue method of staining described by Dogra and Tandan (1964).

Electron micrographs were made of the proctodaeal nerves obtained from adult male Madeira cockroaches that were perfused during dissection with Millonig's (1962) buffered 4% glutaraldehyde solution (pH 7.4). Fixation of the nerve segments was continued at room temperature for three hours, after which time the nerves were rinsed in buffer for one hour and placed in a 1% solution of OsO_4 for three hours. Acetone was used for dehydration, with the 70% step containing enough uranyl acetate to saturate the solution at room temperature. The nerves were left for three hours in this solution and for 20 minutes in the other dehydration steps of 50%, 95%, and twice in 100% acetone. The nerves were embedded in Araldite (Mollenhouer, 1964) and sectioned with a glass knife. Sections were stained with lead citrate (Reynolds, 1963), and micrographs were taken with an RCA EMU-3C at 50 KV.

Results

Purification

In a previous paper (Holman and Cook, 1970), we were able to demonstrate that an excitatory factor present in hindgut extracts was not retained by the DEAE-Sephadex column. Therefore, to eliminate more of the inactive residue, we increased this column to 20 g (compared with the 10-g column used previously) and collected four 50-ml water fractions. The excitatory material was present in the second of the four water fractions. Residue from this fraction was eluted through a cation exchanger (CM-Sephadex) by the stepwise addition of water, two concentrations of acetic acid, and two concentrations of HCl. Although most of the residue eluted with the early fractions, the active material appeared only in the last fraction. However, we soon discovered that 0.02 HCl removed the active material from the column and that the last 100 ml of this eluate (300 ml total) contained all the excitatory activity. Passage of the active material through a CM-Sephadex column after DEAE-Sephadex treatment did not cause loss of activity.

The excitatory compound was eluted as a single peak from G-10 and G-15 gels just after the dextran blue (V_0) and slightly ahead of reduced glutathione (molecular weight = 311). We estimated the molecular weight of the excitatory compound to be in the 400 to 600 range.

Active material from the CM-Sephadex columns was subjected to thin-layer chromatography on cellulose with the isopropanol system. Bioassay of material eluted from several areas of the plates indicated that the active material moved as a single spot and had an R_f of 0.5. When similar plates were not scraped but were sprayed with ninhydrin reagent, almost all the ninhydrin-positive material was found at a lower R_f. The active area was only weakly positive to ninhydrin,

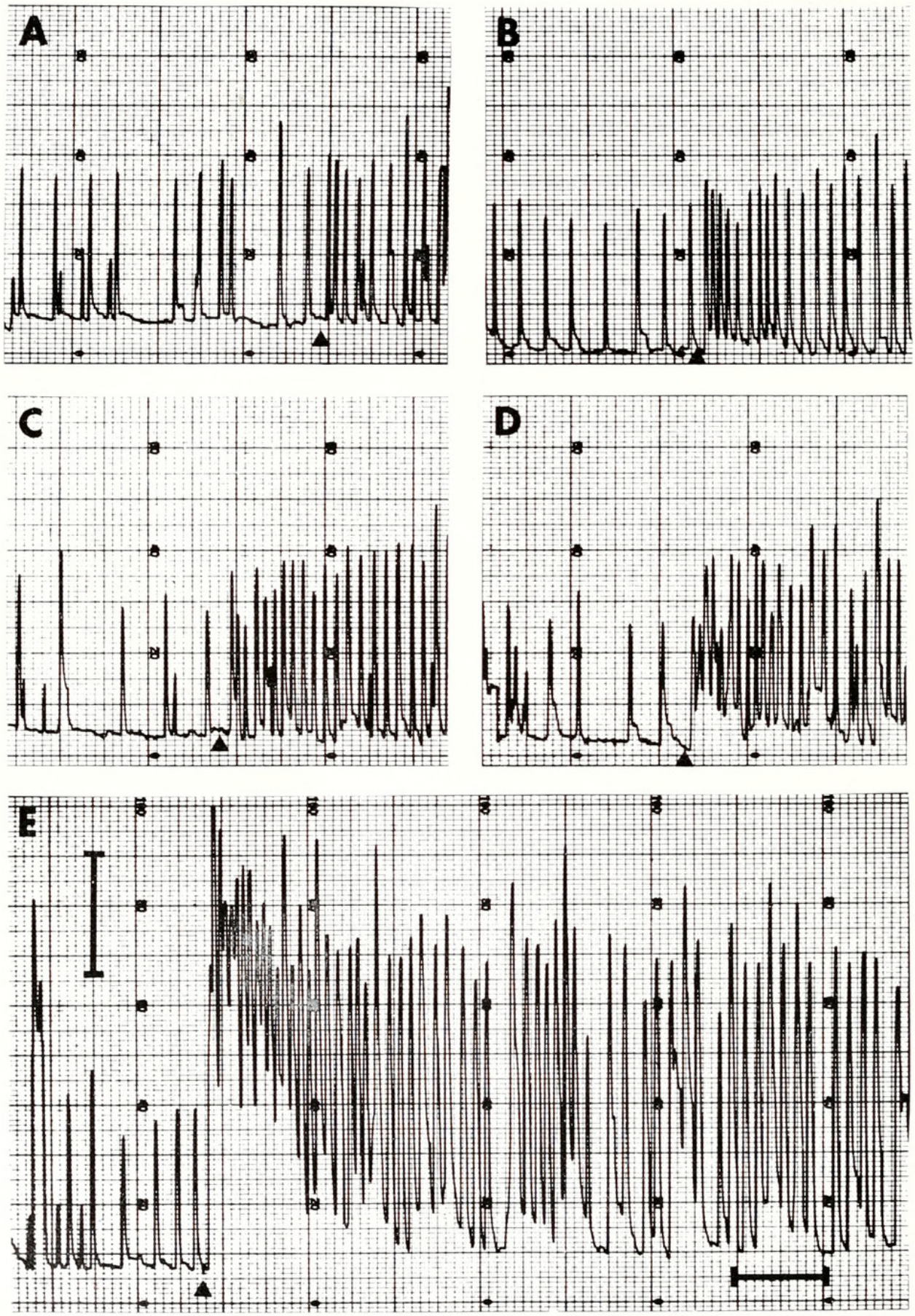

FIGURE 1. The hindgut myographic response profile to various concentrations of the extracted peptide; hindgut response to (A) 0.05 hindgut equivalents (Heq); (B) 0.12 Heq; (C) 0.25 Heq; (D) 0.50 Heq; and (E) 5 Heq. Triangle marks the addition of the peptide. Bath

and we estimated that only 10 per cent of the ninhydrin-positive material on the plate was associated with this spot.

Sensitivity to heat, pH, and proteolytic enzymes

The biologically active material was stable to heat and alkaline pH under the conditions described in the Methods.

When the active samples were incubated with chymotrypsin for three hours as described, no loss in activity was observed. The solutions incubated with pronase under the same conditions or for 30 minutes lost all biological activity. The enzymatic activity of chymotrypsin and pronase in our system was verified by the digestion of a standard albumin solution.

TABLE I

Distribution and abundance of the stimulatory peptide in the Madeira cockroach

Source of extract	Threshold conc. organ equiv./ml	μg residue/organ after purification	μg residue necessary for threshold response
Head	0.04	0.28	0.011
Foregut	No activity present		
Hindgut	0.004	3.2	0.013
Terminal ganglion	0.25	Not determined	Not determined
Cercal-proctodaeal nerve	0.25	Not determined	Not determined

Species and organ distribution

The stimulatory substance was present in head and hindgut extracts of *L. maderae, P. americana,* and *S. vaga vaga.* It was not present in heads of *M. domestica* nor in the heads and hindguts of fifth-instar larvae of *M. sexta.*

Extracts of foreguts, terminal ganglia, and cercal-proctodaeal nerves of *L. maderae* were processed through the ion exchange columns and bioassayed. Active fractions were obtained from extracts of terminal ganglia and cercal-proctodaeal nerves, but no excitatory activity was present in extracts of the foregut.

Threshold concentrations in terms of the minimum organ equivalents/ml producing the excitatory response are tabulated in Table I. A threshold response is shown in Figure 1a. Residues purified by ion exchange from 100 heads and 100 hindguts were used to determine the values in column 3 of the table. Values shown in column 4 were obtained by multiplying column 2 by column 3. Such minute amounts of residue were present after the purification of ganglia and nerve extracts that accurate weights could not be obtained. Therefore, no values were determined.

Virtually all the biological activity remained in the supernatant when sucrose extracts of heads and hindguts were centrifuged at 1000 *g* for 10 minutes. Recentrifugation of the 1000 *g* supernatant at 20,000 *g* for 30 minutes followed by semi-quantative bioassay of supernatant and sediment (extracted with 80%

volume was 12 ml, horizontal time mark, 2 min. Vertical calibration refers to 2 mm of actual movement of the hindgut.

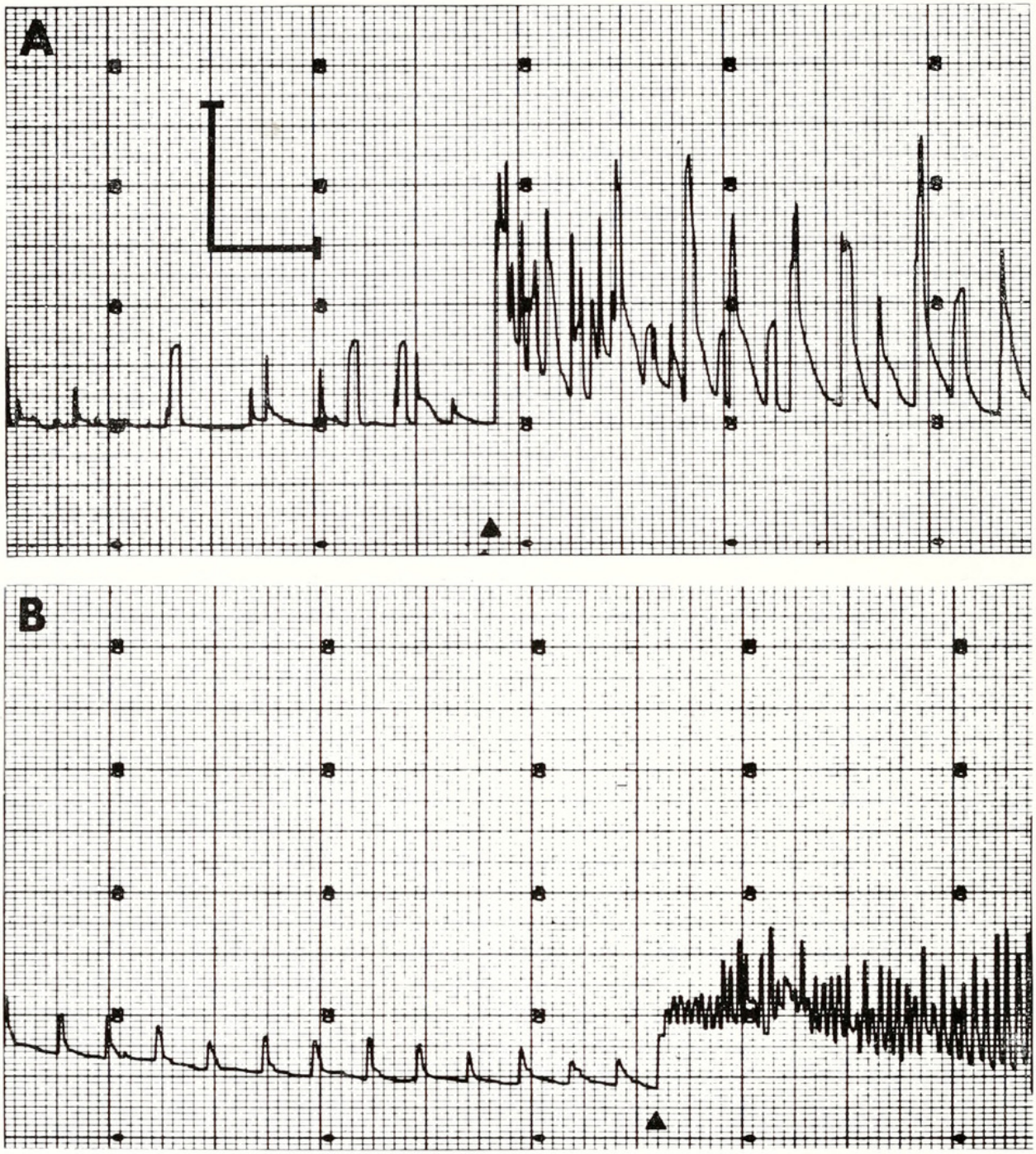

FIGURE 2. Response of the isolated rectum (A) and colon (B) to the excitatory peptide. Triangle marks the addition of 6 head equivalents to muscle chamber; horizontal time mark, 2 min; vertical calibration, 2 mm.

ethanol) showed that 97 per cent of the activity was associated with the sediment, an indication that the stimulatory substance was in the particulate fraction.

Biological properties

The extracted peptide evoked a complex response from the visceral muscles of the hindgut of the Madeira cockroach. Four distinct features were evident on the myographs: (a) an increase in tonus; this was indicated by a rise in the baseline (Fig. 1e); (b) an increase in the frequency of contractions; this was the most evident response obtained from the peptide, even at threshold concen-

trations (Fig. 1a); (c) an increase in the amplitude of contractions; and (d) an increase in the coordination of contractions; *i.e.*, a greater uniformity in the amplitude, frequency, and contour of contractile events. Only at 0.5 hindgut equivalents or above were all four features consistently present. At tissue concentrations of less than this amount, amplitude and frequency were the only prominent features.

A threshold response was obtained with only 0.004 hindgut equivalents per milliliter or 13 ng of dry residue per milliliter (Fig. 1a). A maximum response was reached with 0.4 hindgut equivalents or 1.3 μg of residue per milliliter (Fig. 1e). The excitation of the hindgut was remarkably persistent. Although not shown in Figure 1e, the response continued for 1 hour and 15 minutes. Even at a concentration of tissue residue of 31 ng/ml, the response persisted for more than six minutes. The presence or absence of the terminal ganglion with its associated motor neurons to the hindgut seemed to have no bearing on the response of the hindgut to the extracted peptide. Indeed, application of the peptide (several hindgut equivalents) to the terminal ganglion produced no change in either the frequency or the amplitude of efferent impulses in the proctodaeal nerve.

Davey (1962) showed that although the addition of homogenates of the corpora cardiaca to the isolated hindgut of the American cockroach produced an increase in tonus, amplitude, frequency, and coordination of contraction, these homogenates would not stimulate the rectum alone. He theorized that the factor in the corpora cardiaca acted by stimulating argentaffin-positive cells in the upper colon to release an indolalkylamine. In turn, this amine was proposed to induce the visceral muscles of the rectum to contract by way of a peripheral nervous system which could function in isolation from the central nervous system.

To test this hypothesis on the Maderia cockroach, we isolated the rectum from the colon and examined the myogenic response of each portion individually. Surprisingly, the extracted peptide caused nearly identical effects on both the rectum and the colon (Fig. 2a, b). The myogenic character of the two separate regions showed all four of the distinctive features mentioned previously, in spite of their individual myogenic rhythms.

Once we suspected that the excitatory substance was a peptide, we began to look for evidence of neurosecretion in the proctodaeal nerve by using Victoria Blue stain. The secertory axons in the proctodaeal nerve were stained in such a way that observations could be made in whole mounts or suitably dissected portions of the bulk-stained preparations. Figure 3b shows a portion of the proctodaeal nerve on the surface of the rectum.

The staining did not reveal continuous pathways coursing through the nerve. Rather, the Victoria Blue-positive material appeared as discrete spots that were arranged in a beaded fashion along the proctodaeal nerve near the surface. This type of staining was evident even in branches of the nerve in the anterior colon. When we observed the nerve in cross-section under the electron microscope, the axons containing the nuerosecretory granules were found close to the surface of the nerve. The motor axons were more centrally located. Figure 3a shows a neurosecretory axon near the surface of the proctodaeal nerve. In this figure, two adjacent axons are shown, one containing the characteristic neurotubules and the other showing a large cluster of neurosecretory granules. The diameter of these granules ranged from 1300 Å to 3900 Å.

From our earlier experiments, the site of action for the excitatory peptide seemed to be the visceral muscles of the hindgut. However, there was the possibility of a localized neural network which, if sensitive to the peptide, might indirectly stimulate the muscle fibers. This possibility was eliminated by treating an innervated hindgut with tetrodotoxin (10^{-6} g/ml). Four minutes after treatment, all neural function was abolished. At this time, the excitatory peptide was added to the muscle chamber, and the hindgut responded by showing all four

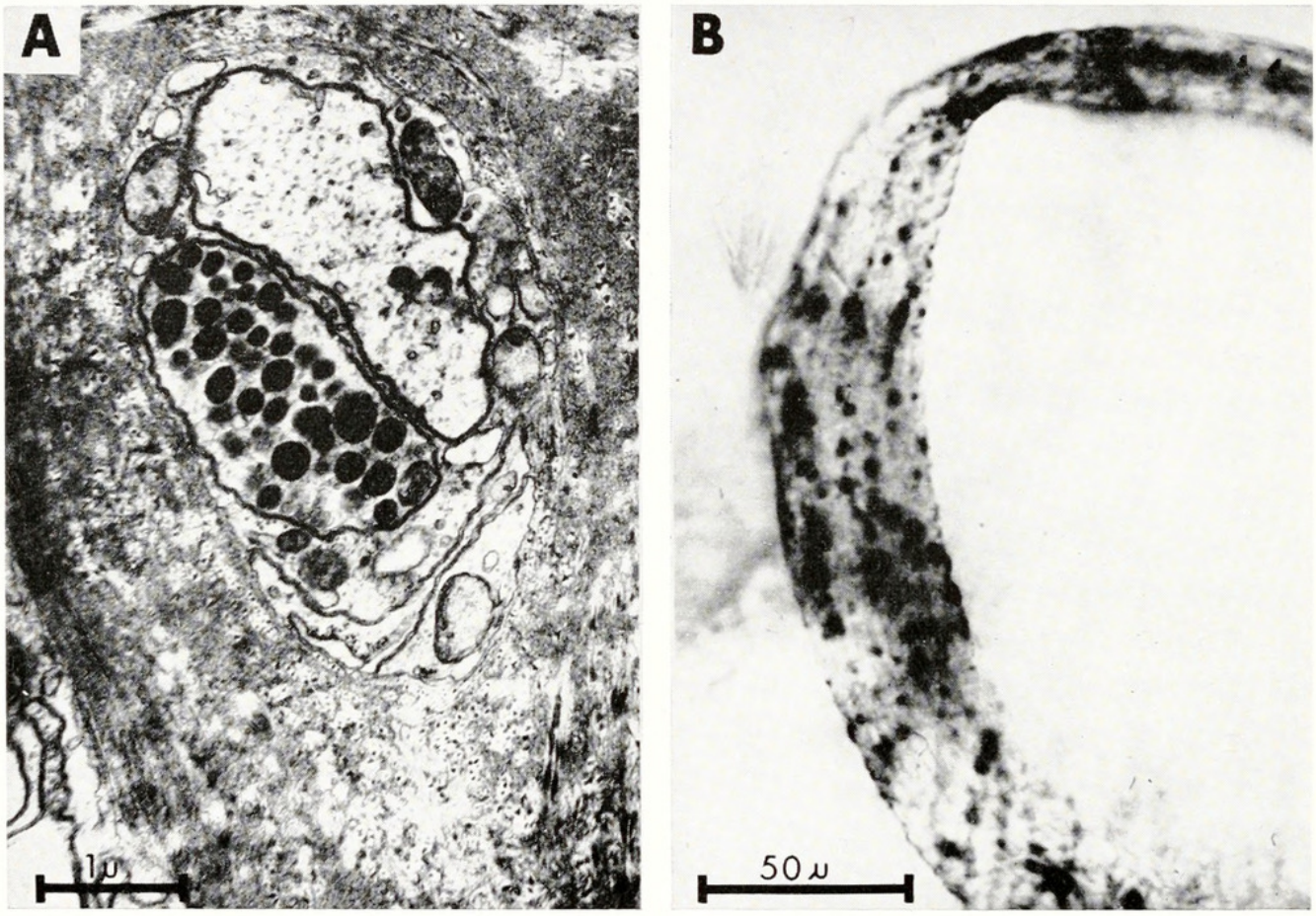

FIGURE 3. Neurosecretion in the proctodaeal nerve; (A) cross-section of 2 axons near the surface of the nerve showing neurosecretory granules in one and neurotubules in the other; (B) Victoria Blue staining of the proctodaeal nerve on the rectum.

characteristic features on the myogram (Fig. 4a). Thus, the excitatory peptide acts on the muscle fiber membrane rather than on the nerves leading to these muscles.

Since Brown (1965) found that extracts of the corpora cardiaca had an excitatory effect on the hindgut as well as the heart of the cockroach, we decided to study what effect our peptide might have on an isolated heart preparation. Figures 4b and 4c show the response of the heart to the extracted peptide. In Figure 4b, 0.3 μg of dry residue was added to the heart at the point indicated by the arrow. A noticeable increase in frequency, amplitude, and coordination was evident. In Figure 4c, 1.0 μg of dry residue was added to the heart preparation, as indicated on the chart. At this higher concentration, heart activity was markedly depressed.

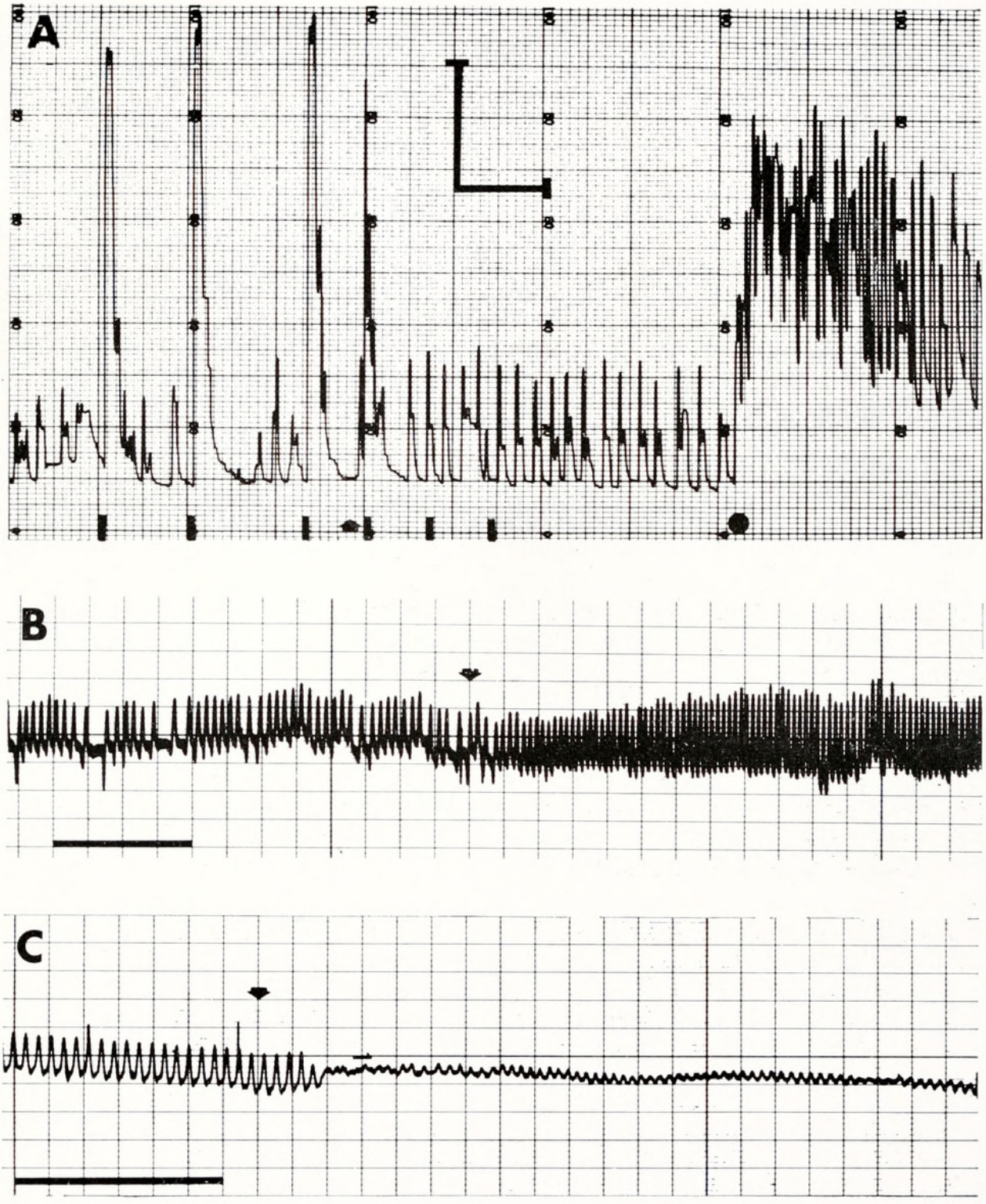

FIGURE 4. The response of tetrodotoxin treated hindgut and innervated heart to the excitatory peptide. (A) The proctodaeal nerves of the hindgut were stimulated at a pulse strength of 2 V, a duration of 1 ms, a frequency of 40 c/s, and a 2-sec train, as indicated by the vertical lines on the chart. The arrow marks the addition of tetrodotoxin (10^{-6} g/ml). The dot marks the addition of the peptide (3.2 μg of residue/ml time mark, 2 min; vertical calibration, 2 mm); (B) heart response to 0.3 μg of dry residue (1.2 μg/ml) of the extracted peptide (arrow); (C) heart response to 1.0 μg of dry residue (4 μg/ml) of the extracted peptide (arrow); time mark 40 sec. The heart was immersed in 250 μl of saline solution.

Discussion

Several investigators have extracted biologically active compounds from both the head and hindgut of the cockroach. Browne *et al.* (1961) extracted a compound(s) from *P. americana* hindgut and blood that induced contraction in the isolated rat uterus. This material was heat-labile and lost its activity when heated above 50° C for 10 min. Brown (1965), using paper chromatography, obtained two materials from extracts of the cockroach corpora cardiaca which stimulated hindgut myogenic activity. He designated these P_1 (also a stimulator of the heart) and P_2. Both materials were heat-stable and dialyzable. The dialysis rate showed P_1 to have the larger molecular weight. On the basis of biological assay, our compound is similar to P_1, as both hindgut and heart are stimulated. Factor P_1 was chymotrypsin-sensitive. Such is not the case for our substance. However, Brown (1965) described a loss of P_1 activity following incubation with heat-denatured chymotrypsin. Thus, it is difficult to assess whether the inactivation of P_1 by chymotrypsin was the result of enzymatic degradation or a non-enzymatic affect such as adsorption. Incubation of our compound with pronase completely abolished biological activity in less than 30 min, whereas incubation with heat-denatured pronase had no effect. This pronase inactivation demonstrates the amide nature of our compound.

Column chromatography provided us with a convenient method for comparing active substances extracted from different sources. Extracts of cockroach heads (including the corpora cardiaca) were found to contain an excitatory compound from hindguts. Subsequent comparison of the two active residues by thin-layer chromatography indicated that the active components had identical R_f values, whether they were chromatographed separately or as a mixture. Even with whole body extracts and a much more elaborate purification system, we have not been able to show the presence of more than one active substance that has the characteristic sustained effect on hindgut myogenic activity (Holman and Cook, unpublished data).

Natalizi, Pansa, d'Ajello, Casaglia, Bettini, and Frontali (1970) separated six biologically active materials from extracts of cockroach corpora cardiaca. Although they did not investigate the effect of these materials on the isolated hindgut, they showed four of them to be stimulators of the cockroach heart. Two of the materials also stimulated the spontaneous firing of the cockroach central nervous system (1 and 2), but the other two materials stimulated only the heart (5 and 6). Our peptide did not stimulate the spontaneous firing of the isolated cockroach nerve cord. The latter two materials (5 and 6) were retained by a carboxymethyl cation-exchange column, as was our compound. However, differences in cation-exchangers and solvents used preclude a more direct comparison between substances 5 and 6 and our peptide.

Gel filtration experiments with G-10 and G-15 Sephadex indicate a molecular weight of 400 to 600 for our compound. However, this could be misleading, as a chromatographic effect can cause certain materials to elute as lower molecular weight compounds. In fact, Natalizi *et al.* (1970) found this to be the case with all six biologically active components mentioned above. In their case, the effect was obvious, as the six materials were eluted after the salt fraction. How-

ever, their elution solvent differed from the one we used; again, direct comparison was not possible.

In summary, our evidence shows that the material we have isolated is a small, basic peptide.

Our preliminary survey of five insect species representing three orders shows that the peptide is not universally present. Thus far, it has been isolated only from representatives of the order Orthoptera. It was not present in the house fly (Diptera) or fifth-instar larvae of the tobacco hornworm (Lepidoptera).

The isolated hindgut of the American cockroach seemed to be just as responsive to the extracted peptide (regardless of the active source) as the hindgut from the Madeira cockroach. However, the isolated hindgut of the fifth-instar larvae of the tobacco hornworm showed no response to the peptide, even at several hindgut equivalents per ml.

Distribution studies of *L. maderae* showed that the hindgut contains the largest quantity of this peptide. It was also present in the head, terminal ganglion, and cercal-proctodaeal nerves. The peptide was neither present in the foregut nor did it affect the myogenic activity of that organ.

The ion-exchange chromatography of hindgut extracts resulted in a 2000-fold purification of the peptide (7.3 mg dry weight/hindgut *vs.* 3.2 μg residue/hindgut). A concentration of 0.004 hindgut equivalents/ml of this residue gave a threshold response (Fig. 1a). This corresponds to 13 ng residue/ml (Table I). The sensitivity to the pure compound is certainly much greater than this, as thin-layer chromatography of this residue showed that an estimated 90 per cent of the ninhydrin-positive materials were not associated with the R_f of the active compound.

Our extracted peptide seems to act directly on the membrane of muscle fibers. Not only was the hindgut still responsive to the peptide after treatment with tetrodotoxin, but both the isolated rectum and the colon reacted to the peptide. Thus, Davey's (1962) hypothesis of indirect stimulation of the gut through a peripheral neural network does not seem likely in the Maderia cockroach. Even in the American cockroach, a re-investigation of the neural relationships of the proctodaeal muscles (Brown and Nagai, 1969) did not reveal a peripheral neural network. However, a netlike syncytium of fibers was found which stained readily with methylene blue and had the structural characteristics of modified muscle.

Furthermore, the possible mediation of an indolalkylamine in the action of corpora cardiaca extracts on the hindgut of the cockroach as proposed by Davey seems remote. Both Colhoun (1967) and Brown quoted by Colhoun (1967) showed that bromolysergic acid diethylamine, a proven antagonist of 5-hydroxytryptamine and other indolalkylamines, failed to suppress hindgut responses to the addition of copora cardiaca extracts. Further, Davey (1962) remarked that the granules in the argentaffin-positive cells of the upper colon were positioned along the lumen side of the cell and would need some means of conduction to the muscle fibers above.

From our preliminary experiments on the innervated heart preparations, it was not possible to state whether the peptide affected the myocardium directly or indirectly via the pericardial cells, as proposed by Davey (1961). Furthermore,

the lateral cardiac nerve cord may also have been affected by the peptide. In spite of these shortcomings, both the heart and hindgut showed similar response patterns to the peptide. Heart contraction frequency and amplitude increased, and the myocardiogram showed a more coordinated pattern (Fig. 4b). Surprisingly, at concentrations of 4.0 μg dry residue/ml, the heart displayed a marked inhibition which was reversible (Fig. 4c).

The presence of neurosecretory axons in the proctodaeal nerve of the Madeira cockroach was not entirely unexpected. Numerous instances of neurosecretory axons passing into or close to a variety of internal organs in insects have been reported. In aphids, neurosecretory tracts were found running to the hindgut and various muscles (Johnson, 1963). Both the rectal papillae of the blowfly (Gupta and Berridge, 1966) and the rectal pads of the cockroach (Oschman, cited by Maddrell, 1967) receive neurosecretory axons. In a situation where a synaptic-like termination is observed in contact with the target tissue, one might assume that the products of neurosecretion have a local action. In fact, Maddrell (1967, page 108) concluded . . . "that by no means all neurosecretory axons release horomones into the general blood system. Indeed it can only be confidently stated that the hormones are in fact normally released into the general blood system in relatively few cases. Even in these cases the evidence is often indirect." In the American cockroach, there are only two hormones for which there is direct evidence of this: the diuretic hormone (Mills, 1967) and the tanning hormone bursicon (Mills, 1966).

In conclusion, there are several facts suggesting that the peptide we have isolated is a neurohormone: (1) the peptide is present in extracts of the terminal ganglion and the cercal-proctodaeal nerve complex (Table I); (2) not only did the proctodaeal nerve stain selectively with Victoria Blue, but neurosecretory granules were evident in a number of axons appearing on electron micrographs of the nerve in cross-section (Fig. 3a, b): (3) although we have no direct evidence of the presence of the peptide in neurosecretory granules, preliminary centrifugation experiments in a sucrose medium showed that 97 per cent of the peptide is present in the particulate fraction at 20,000 *g;* (4) the sustained response of the visceral muscles of the hindgut and the heart to the peptide is in striking contrast to the very short duration of the postsynaptic responses encountered with neurotransmitter (Scharrer, 1969 and Holman and Cook, 1970); and (5) neurohormones are generally peptides (*e.g.,* vasopressin and oxytocin) which often act on diverse target organs. In the present instance, the same peptide has been found in both the head and the terminal ganglion, and it has the ability to alter the contractile properties of the heart and the hindgut but not the foregut.

Finally, although the precise location of release for the peptide in the hindgut is not known, the selective staining of the proctodaeal nerve and its branches with Victoria Blue over the entire hindgut certainly suggests a localized release. If indeed this proves to be the case, the neurosecretory substance would be more like a neurotransmitter than a typical neurohormone, at least from a histological point of view. Nevertheless, the persistent physiological effects of the peptide on the hindgut, even in submicrogram amounts, seem to necessitate describing it as either a local neurohormone (Burn, 1950 and Scharrer, 1969) or neuro-

modulator (Florey, 1967) which in some manner regulates the excitability of visceral muscle.

We are indebted to Mr. John Reinecke of this laboratory for the electron microscopy of the proctodaeal nerve. We also wish to acknowledge the competent technical assistance of Mrs. Patricia Kramer.

Summary

1. Ethanol extracts of hindguts from *L. maderae* contained a material that stimulated the myogenic activity of the isolated organ. Ion-exchange chromatography of the ethanol extracts resulted in a 2000-fold purification of the active residue, and thin-layer chromatography demonstrated that the activity was associated with a single ninhydrin-positive spot.

2. The biological activity of the extracted substance was not affected by incubation for 3 hours with chymotrypsin. However, pronase completely destroyed the activity within 30 minutes, which suggests that the active material is a peptide. Gel filtration experiments indicated that the molecular weight is in the 400–600 range.

3. The peptide was isolated from extracts of terminal ganglia, proctodaeal nerves, and heads of the cockroach but not from the foregut. Interestingly, the myogenic activity of this organ was not affected by exposure to extracts from the hindgut. The peptide was found in the two cockroaches, *L. maderae* and *P. americana,* and the grasshopper, *S. vaga.* It was not found in the head of the house fly, *M. domestica* nor in fifth-instar larvae of the tobacco hornworm, *M. sexta.*

4. The characteristic response of the hindgut to this peptide was a sustained increase in the tonus, frequency, and amplitude of contractions. An excitation threshold was obtained with only 0.004 hindgut equivalents per milliliter or 13 ng of dry residue per milliliter. Both the isolated rectum and colon responded to the peptide, and although tetrodotoxin (10^{-6} g/ml) blocked neurally evoked contractions, the visceral muscle of the hindgut showed no change in sensitivity to the extracted peptide.

5. Cockroach innervated heart preparations showed a noticeable increase in frequency, amplitude, and coordination of contractile events when exposed to 0.3 μg of dry residue. If 1.0 μg or more of the dry residue was added to the heart, activity was greatly depressed.

6. Both histological and cytological evidence of neurosecretion were obtained from the proctodaeal nerve. Preliminary centrifugation experiments suggest that the extracted peptide is associated with the particulate fraction at 20,000 *g*. Finally, a number of reasons for considering our extracted peptide as a neurohormone are discussed.

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