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Neuromuscular Transmitter Substance in Insect Visceral Muscle

Abstract. *Stimulation of the nerves innervating the proctodeum (hindgut) of the cockroach* Periplaneta americana *(L.) causes a slow-type, graded contraction of the longitudinal muscles. An unidentified substance, or substances, present in the foregut and hindgut, the specific activity of which is highest in the nerves innervating these organs, effects a similar contraction. This "gut-factor" is depleted from the hindgut after surgical section of the proctodeal nerves. None of Factors P_1 and P_2, 5-hydroxytryptamine, acetylcholine, adrenaline, noradrenaline, α-aminobutyric acid or glutamate duplicates the pharmacological behavior of this substance. The active factor is associated with subcellular particles that require centrifugal forces of approximately 1,000,000 g-min for sedimentation. The substance is inactivated in homogenates of gut tissue in the absence of suitable precautions. It is proposed that the "gut-factor" functions as an excitatory neuromuscular transmitter substance in insect visceral muscle.*

Until recently, the characterization of excitatory neuromuscular transmitter substances in insects has been unsuccessful. However, glutamate may function as an excitatory neuromuscular transmitter in some arthropod muscle, including the somatic muscle of the cockroach *Periplaneta americana* L. (*1*). This report deals with a potential neuromuscular transmitter in the striated, visceral muscle of *Periplaneta americana* L.

Two peptides, Factors P_1 and P_2, that have pharmacological activity on the isolated hindgut of the cockroach have been extracted from the *corpus cardiacum* gland of the roach (*2*). In a study of the distribution of these peptides in various tissues of the cockroach, a substance (or substances) was extracted from the foregut (stomodeum) and hindgut (proctodeum) which also had pharmacological activity on the hindgut. This factor differs from Factors P_1 and P_2 in its action on the hindgut and in the fact that it is not inactivated by chymotrypsin. The substance is heat stable and dialyzable. The active substance appears to function as a neuromuscular transmitter in the longitudinal muscles of the proctodeum and probably elsewhere in visceral muscle of *Periplaneta*.

The "gut-factor" was extracted from foreguts or hindguts washed free of their contents with physiological saline. The viscera were homogenized in 1*N* acetic acid, and the homogenate was dialyzed against 10 volumes of water; both procedures were performed at 0° to 1°C. The dialyzate was dried by rotary evaporation at 40°C under reduced pressure. Traces of acetic acid were removed by extraction of the dry residue with ethyl acetate. For pharmacological assay, the residue was dissolved in water or .05*M* phosphate buffer, *p*H 7. The pharmacological technique with the roach hindgut has been described previously (*2*). Quantitative results in terms of "rectum equivalents" were obtained by a comparison of the heights of contractions caused by unknowns with those caused by a standard extract of rectums. Specific activities express the content of the active substance in a tissue in terms of rectum equivalents per milligram of tissue protein. Protein was determined by the Lowry method (*3*) on duplicate aliquots of extract before dialysis.

As in many insects, the roach proctodeum is divided by a constriction into the anterior intestine and the posterior intestine, or rectum. The arrangement of the longitudinal muscles is different in the two regions, but the muscles of both are innervated by the same two nerves; these nerves arise bilaterally from the two large cercal nerves just posterior to the sixth abdominal ganglion (*4*). Stimulation of these proctodeal nerves (1 volt, 1-msec duration) at frequencies exceeding 5 to 10 impulses per second results in contraction of the longitudinal muscles in the whole hindgut (the anterior intestine and the rectum) (Fig. 1A). The response of the rectum only is shown in Fig. 1B. In both cases, the contraction is of the slow type and is graded, increas-

Table 1. Rectum equivalents, protein, and specific activities of "gut-factor" (rectum equivalents per milligram protein) in various tissues of the cockroach. Determinations were performed on tissue pooled from 20 to 40 roaches.

Tissue	Rectum equivalents per organ	Protein per organ (μg)	Specific activity
Stomodeum	1.89	1332	1.4
Stomodeal nerve	0.35	9.4	37.3
Stomodeal nerve	.28	8.6	32.6
Proctodeum	1.52	1557	1.0
Proctodeal nerve	0.032	2.0	16.0
Proctodeal nerve	.029	2.9	10.0
Nerve*	.014	57.8	0.2
Ganglia†	.16	159	1.0

* Thoracic peripheral nerve. † Pro-, meso- and metathoracic ganglia.

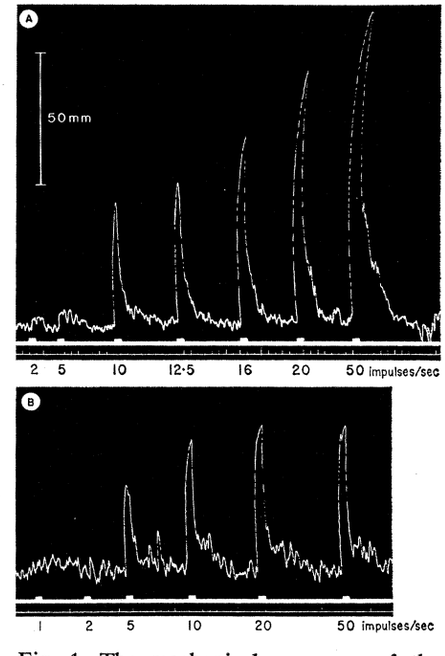

Fig. 1. The mechanical responses of the whole hindgut (A) and of the rectum only (B) to stimulation of the proctodeal nerves. Stimulation (1 volt and 1-msec duration) with platinum electrodes for 10 seconds at the frequencies indicated. Time marker is 10 seconds.

ing with the frequency of stimulation.

The active substance from the roach viscera also causes contraction of the longitudinal muscles of both the anterior intestine and the rectum. These responses are mainly tonic contractions similar to those resulting from nerve stimulation (Fig. 2, A and B). Often, however, the response to the "gut-factor" contains a pronounced rhythmic component superimposed on the tonic contraction. This complex effect resembles that caused by 5-hydroxytryptamine (5-HT) and by Factors P_1 and P_2 (*2*). However, the response to 5-HT is completely blocked by bromolysergic acid diethylamide, and Factors P_1 and P_2 are completely inactivated by incubation with chymotrypsin; neither of these conditions affects the activity of the "gut-factor." Furthermore, the pharmacological behavior of the "gut-factor" is not duplicated by any of the known or suspected neurohumors that have been tested (at concentrations up to $10^{-4}M$), including acetylcholine, adrenaline, noradrenaline, γ-aminobutyric acid, and glutamate.

After the active substance in the foregut and hindgut was found, the major nerve trunks innervating these parts of the digestive tract were excised and extracted. These nerves also yielded a substance that was dialyzable, insensitive to chymotrypsin, and active on the hindgut. The quantities and specific activities of "gut-factor" in these nerves and in some other tissues are given in Table 1. Two facts are significant. (i) The specific activities of the active substance in the proctodeal and stomodeal nerves are approximately 10 and 25 times higher than those in the viscera that they innervate; this finding indicates that the "gut-factor" is of neural origin. (ii) The concentration of "gut-factor" in the nerves innervating visceral muscle is up to 150 times greater than that in the thoracic peripheral nerves, which innervate somatic muscle. Presumably, the high specific activity in the stomodeal and proctodeal nerve tissue is a reflection of a specific function in visceral nerve to muscle relations. A substance which is similar to the "gut-factor," at least superficially, can be extracted also from the ganglia of the roach central nervous system (Table 1). However, the "gut-factor" reported in Table 1 is characterized only as being dialyzable, resistant to chymotrypsin and active on the roach hindgut; more than one substance may be involved. Therefore, it

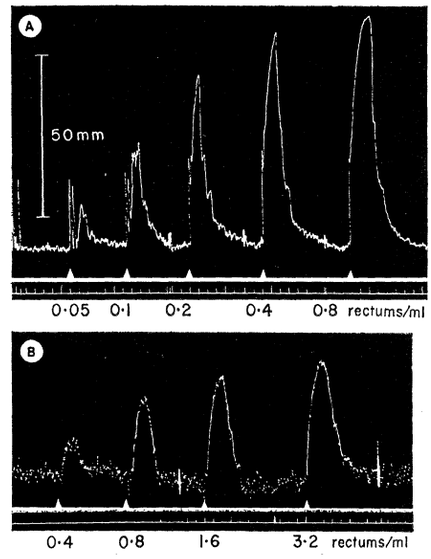

Fig. 2. The mechanical responses of the whole hindgut (A) and of the rectum only (B) to a 15-second exposure to the "gut-factor" at the concentrations indicated. Time marker is 10 seconds.

is premature to speculate on a role for the "gut-factor" within the insect central nervous system.

Surgical section of the proctodeal nerves resulted in a decreased titer of active substance in the rectum. In one experiment (Table 2) the left and right halves of the rectum were assayed at 4 and 8 days after section of the left or right cercal nerve, from which the proctodeal nerve originates. The total amount of "gut-factor" in the rectum, 8 days after unilateral nerve section, was 49 and 51 percent of the amount in the unoperated control. Of this amount, approximately 25 percent persists in the denervated side of the rectum, and 75 percent is located in the nondenervated side. These results probably reflect some degree of overlapping innervation. The same trend is shown by the results at 4 days, although depletion is incomplete at this time. The results of this experiment are similar to the loss of acetylcholine from cholinergic nerve endings following nerve section (*5*), and they strongly support the conclusion that the "gut-factor" is of neural origin.

Table 2. "Gut-factor" in the denervated and nondenervated sides of the rectum 4 and 8 days after unilateral nerve section. Each determination was performed on tissue pooled from 16 roaches.

Proctodeal nerve sectioned	Time after section (days)	Gut-factor (% control)		
		Denervated side	Nondenervated side	Total
Left	4	58	90	74
Right	4	66	95	81
Left	8	21	77	49
Right	8	26	75	51

A widely accepted theory, supported by the work of Fatt, Katz, Del Castillo, De Robertis, Bennet, and others (reviewed in reference *5*), holds that acetylcholine and other transmitters are held in presynaptic vesicles, the contents of one vesicle corresponding to a quantal unit of transmitter. A quantum of transmitter may be released spontaneously or in synchrony with others in response to the presynaptic action potential. In electron micrographs, the synaptic vesicles appear as membrane-limited sacs (about 450-Å diameter) that are usually concentrated in the presynaptic nerve ending. These submicroscopic cell particles may be preserved when the cell is disrupted in isotonic media, imparting a "particulate" nature to transmitter substances. At this laboratory, electron microscopy of the longitudinal muscles of the rectum has revealed a characteristically modified region of axon to muscle contact which almost certainly represents a neuromuscular junction (*6*). The axonal component of this junction contains synaptic vesicles similar in size and appearance to those seen at other junctions, as well as larger, dense granules of about 2000-Å diameter. Furthermore, the "gut-factor" in the rectum is associated with subcellular particles which require a centrifugal force of approximately 1,000,000 *g*-min (*7*) for complete sedimentation. Homogenization of rectums in 0.4*M* sucrose yields two cleanly separable particulate fractions. A heavy fraction can be sedimented at 10,000 *g*-min (1000*g*, 10 minutes) and may represent incompletely fragmented axons. However, a much higher centrifugal force is required to sediment the remaining particulate fraction, and it is convenient to separate the two fractions by differential centrifugation at 50,000 *g*-min (10,000*g*, 5 minutes) and 1,900,000 *g*-min (127,000*g*, 15 minutes). As expected, the recoveries of "gut-factor" in the two fractions vary with the conditions of homogenization. Homogenization in an all-glass system (*8*) for 1 to 2 minutes at 0°C has yielded an average of 63 percent (four experiments, range 56 to 71 percent) of the original "gut-factor" in the light fraction and 23 percent (two experi-

Table 3. Sedimentation of "gut-factor" from the supernatant of a homogenate centrifuged at 50,000 *g*-min. Starting material was 64 rectums. All centrifugations were for 10 minutes.

Centrifugation (No. of *g*)	Rectum equivalents in sediments	Cumulative recovery (%)
18,000	9.1	14.2
36,000	9.8	29.5
72,000	8.5	42.8
127,000	5.4	51.3
127,000	0	51.3

ments) in the heavy fraction. Differential centrifugation of the supernatant of a homogenate centrifuged at 50,000 *g*-min (Table 3) demonstrates that a centrifugal force in excess of 720,000 *g*-min is required for complete sedimentation of the light fraction. This fraction has been resolved further by means of density gradient centrifugation but has not yet been identified.

The active factor was dissociated from its particulate fraction with hypotonic solutions. Under these conditions, the "gut-factor" lost from the sedimentable fraction could not be detected in the soluble fraction and, presumably, had been inactivated. Other lines of evidence indicate that this inactivation proceeds enzymatically. Quantitative recovery of the "gut-factor" from the roach viscera was achieved only with extraction media that cause denaturation of enzymes (for example, acidic ethanol, 5 percent trichloroacetic acid, 1*N* acetic acid). Two attempts to extract foreguts with 0.1*N* acetic acid gave yields of 10 to 20 percent of the normal amount; water extractions recovered less than 10 percent of the activity. Presumably for the same reason (that is, enzymatic degradation) the yield of "gut-factor" from foreguts dissected from previously frozen roaches was only some 25 percent of normal. If such an enzyme exists and has physiological significance, then it represents a further analogy to the well-known cholinergic system.

On the basis of this preliminary evidence, it is proposed that the active factor functions as an excitatory neuromuscular transmitter substance in the longitudinal muscles of the proctodeum and probably in other visceral muscles of the cockroach.

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8. *Tenbroeck* type tissue grinder, clearance .010 to .015 cm, manufactured by Kontes Glass Company.
9. I thank J. A. Sholdice for technical assistance and Dr. W. Chefurka for criticism of the manuscript.
10. Contribution Number 328, Research Institute, Canada Agriculture, London.

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Neural Basis of the Sense of Flutter-Vibration

Abstract. *Comparison of human detection thresholds for oscillatory movement of the skin of the hand with response properties of first-order myelinated mechanoreceptive afferents from the monkey's hand, activated in an identical stimulus pattern, indicates that flutter-vibration is a dual form of mechanical sensibility, served peripherally by two different sets of fibers.*

One approach to the study of the neural mechanisms in sensation is to combine two experimental designs which developed separately and which differ in aim and scope. Electrophysiological studies provide measures of the neural encoding of sensory stimuli as trains of impulses in first-order nerve fibers, and of the successive relay and transformation of this input across subcortical sensory nuclei, and through the first stage of processing in the cerebral cortex. They have so far revealed little of those central neural mechanisms thought to lead to subjective sensory experience. Psychophysics seeks lawful relations between those experiences and certain physical aspects of the stimuli which evoke them. These laws establish: (i) the dynamic range required of the input side of the system to account for its output; (ii) the information about the stimulus which must be preserved in the initial encoding to account for that in the output; and (iii) a basis for determining which of the codes available to the pulse-operated input system may be of functional significance for the intact, behaving organism. It is thought that a continued updated comparison of findings of the two types—psychophysical and neurophysiological—will allow the design of experiments aimed at elucidating the *terra incognita* of central mechanisms.

In such a combined study we have measured the threshold for the perception of oscillatory movement of the skin of human finger pads. In monkeys we recorded the nerve impulses set up by identical stimuli in first-order myelinated mechanoreceptive afferents innervating the skin and deep tissues of the hand. The results indicate that: (i) the sense of flutter-vibration can be accounted for only on the assumption that at least two quite different sets of afferents are responsible, each for a different limb of the human frequency function; (ii) the perception of oscillatory movement requires that a periodic signal appear in the input impulse trains, with probabilities in the range 0.7 to 0.9; and (iii) the differential recognition of frequency requires a central nervous system mechanism for the measurement of the length of the period.

The mechanical stimulator is described elsewhere (*1*); the stimulus pattern for both monkey and human experiments is shown in Fig. 1. The depth of skin indentation was held constant in any given series by monitoring and adjusting the point of contact. Stimulus rates were 6 per minute in human and 12 per minute in monkey experiments. Human observers were seated at a table, right arm mounted in padding, hand impressed palm upward in plasticine. The stimulator was movable in all dimensions; it was normal to the surface of the distal pad of the forefinger or middle finger. Contact point with skin was determined microscopically (16 ×). Thresholds for the perception of oscillatory movement were determined by the method of limits; they were sharp and reproducible. A white noise masked the faint hum of the oscillating probe.

Monkeys were anesthetized with sodium pentobarbital, their body and skin