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20. The starting material for the further purification of somatomedin used in this study was an acid ethanol extract of outdated human plasma kindly supplied by Professor Bertil Åberg vice president and director of research, AB Kabi, Stockholm, Sweden. This extract was further purified by gel chromatography, cation exchange chromatography, and continuous flow liquid electrophoresis. This preparation has an activity of 187 units per milligram; 1 unit of somatomedin is defined as the amount of sulfation factor activity in 1 ml of a standard reference plasma. The somatomedin preparation contained less than 0.1 μunit of insulin per unit of somatomedin. Parathyroid hormone (180 units per milligram) was from Dr. G. D. Aurbach, and prostaglandins PGE_1 and PGE_2 from Dr. J. E. Pike.
21. Although the effects of somatomedin on adenylate cyclase activity occur in the membrane preparation of isolated chondrocytes, most of the experiments, including the data presented here, were performed with the particulate fraction of cartilage homogenates.
22. The adenylate cyclase activity of spleen lymphocyte particulate preparations, and its inhibition by somatomedin, are unaltered by the addition of 1 m*M* unlabeled cyclic AMP to the incubation medium. In the absence of an ATP-regenerating system the basal adenylate cyclase activity of these particulate preparations is decreased by about 30 percent, but the inhibition of activity by somatomedin is still clearly discernible.
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Acetylcholine: Fast Axoplasmic Transport in Insect Chemoreceptor Fibers

Abstract. *Acetylcholine is transported along insect chemoreceptor axons at a rate of 12 to 13 centimeters per day after peripheral uptake of choline. Colchicine, vinblastine sulfate, and cytochalasin B all block transport, but transport continues in axons separated surgically from the cell body. These data from an insect are in accord with many studies on vertebrates which have implicated intracellular microtubules in the transport mechanism. The peripheral uptake of choline and its acetylation and transport to nerve terminals in the brain are consistent with the suggestion that acetylcholine is an antennal sensory transmitter in insects.*

With a single exception (*1*), axoplasmic transport has not been studied in insects, although insect nerves offer a variety of unique morphological adaptations which might serve as useful experimental preparations. A case in point is the insect antennal nerve with its neural organization much reduced in size and morphological complexity as compared with most other neural systems.

The chemoreceptor fibers in the antennal nerves of insects are primary sense cells, that is, the receptor cells located in the antennal sense organs (sensilla) project from the periphery to the central nervous system without fusion and without synaptic connection through interneurons (*2*). Electron micrographs of the two main antennal nerves of the woodroach, *Leucophaea maderae*, show that more than 96 percent of the axons have a diameter of less than 0.5 μm and most lack individual glial sheaths. None of the fibers is myelinated. The small, naked fibers average 0.15 μm in diameter and are contained in fascicles of 25 to 100 axons with a common glial sheath surrounding the whole bundle. The interaxonal separation is about 100 Å, leaving little sodium space between the fibers. With glutaraldehyde-osmium fixation, only three cytoplasmic structures are consistently seen in the axons: the plasma membrane; mitochondria scattered at intervals along each axon; and 2 to 5 microtubules, typically measuring 240 Å in diameter. No neurofilaments have been observed in the small-caliber fibers of woodroach antennal nerves (*3*).

Approximately 60 percent of the antennal nerve fibers in *Leucophaea* originate from receptor cells located in thin-walled sensilla (olfactory sense organs) on the antennal surface, 30 percent from thick-walled sensilla (probable contact chemoreceptor organs or taste hairs), and the remaining 10 percent from mechanoreceptors and other receptor modalities (*3, 4*). Extensive electron microscope studies on the antennae of other insects reveal that the sensory dendrites of thin-walled sensilla are exposed to the external environment through a complex poretubule system which penetrates the cuticle of the sense organ (*5*). Dendrites of the thick-walled chemoreceptive sensilla of the cockroach antenna communicate with the external environment through an opening at the tip of the hairlike sensillum (*4, 6*). The presence of these minute openings in the cuticle of the antennal sense organs suggested that external application of isotopically labeled chemicals might result in the uptake and transport of the isotope by chemoreceptor cells.

Initial attempts to introduce an isotopic label into the cell bodies of the antennal chemoreceptors consisted of immersing the distal tip of the antenna in insect Ringer solution containing 9 μ*M* L-[^{3}H]leucine (0.5 mc/ml) or 28 μ*M* L-[^{3}H]proline (0.31 mc/ml). The average length of the antennae used was 3.5 cm ± 5 percent. Antennae were dipped for 15 minutes (proline) or 30 minutes (leucine) and thoroughly rinsed with unlabeled Ringer solution. Within 30 minutes, approximately 10 percent of the radioactivity was incorporated into a fraction of antennal homogenate which was insoluble in trichloroacetic acid (TCA), indicating that a portion of the labeled amino acids had been incorporated into protein. Tritiated leucine and [^{3}H]proline were recoverable as free amino acids after enzymatic or acid hydrolysis of the TCA-insoluble precipitate.

Attempts were made to demonstrate axoplasmic transport of the polypeptide material toward the brain by sectioning the antenna into 0.5-cm segments after a specific time interval, homogenizing the antennal segments in 0.15*M* phosphate buffer, and counting the isotopic activity in each segment with the liquid

scintillation spectrometer (*7*). It was not possible with this method to demonstrate a discrete peak or front of activity moving proximally in the antenna or the arrival of a discrete pulse of activity in the antennal brain. However, by using light microscope autoradiography (*8*) it was possible to demonstrate activity in the antennal nerves 1 to 2 cm proximal to the site of incorporation 36 hours after dipping the distal 0.5 cm of the antenna in [^{3}H]-leucine. These experiments showed that labeling of the nerves was unaccompanied by labeling of adjacent structures such as the epidermis, cuticle, blood vessel, and tracheae. It is assumed that this transport is taking place within the axons, although the autoradiographic experiments do not exclude the possibility of transport within glial cells or the nerve sheath. A tentative estimate of the rate of transport of the TCA-insoluble material based on these observations is about 1 cm/day.

Attempts to demonstrate acetylcholine transport met with considerably more success. The acetylcholine experiments were done along similar lines, but a different method of rate determination was employed. The distal 0.5 cm of the antenna was dipped for 10 minutes in insect Ringer solution containing 0.8 m*M* [methyl-^{3}H]choline chloride (0.84 mc/ml). Both right and left halves of the brain were removed from animals at ½-hour intervals after dipping and separately homogenized in 0.15*M* phosphate buffer. Each experiment utilized the pooled brain halves of four animals whose antennae were of equal length. The homogenates were air-dried on disks of glass fiber filter paper and counted by conventional liquid scintillation techniques. The criterion used for demonstration of the arrival of the labeled material was the appearance of a difference in activity between the right brain (undipped side) and the left brain (dipped side). The results of 89 experiments are shown in Fig. 1. A single, sharp peak of activity appears in the left brain 6 hours after dipping (± 15 minutes). This corresponds to a transport rate of 12 to 13 cm/day for the average length of antennae used (3.5 cm ± 5 percent).

Descending chromatography of brain homogenate (solvent system butanol : ethanol : acetic acid : water, 8 : 2 : 1 : 3; chromatographs on Whatman No. 1 strips visualized in iodine vapors) showed that 70 percent of the label present in the brain was [^{3}H]acetylcholine, and about 20 percent [^{3}H]-choline. After incubation with acetylcholine esterase, more than 90 percent of the brain radioactivity was recovered as [^{3}H]choline. Similar results were obtained with antennal homogenates, indicating that acetylcholine is the predominant molecular species transported. Further chromatographic studies of antennal homogenates have shown that the lag between the entry of the precursor choline from the outside environment and its incorporation into acetylcholine is less than 15 minutes.

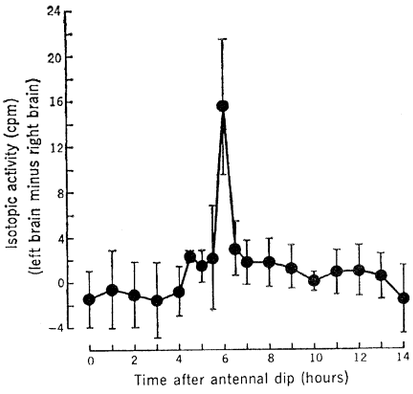

Fig. 1. Appearance of [^{3}H]acetylcholine in the left brain 6 hours after uptake by receptor cells in the distal left antenna. The averaged results of 89 experiments are shown, each experiment combining the brains of four animals. Baseline points were derived from 3 to 6 experiments each, while the peak at 6 hours is the average of 19 experiments (76 animals). The bars show the standard deviation.

The involvement of cytoplasmic microtubules in the axoplasmic transport of acetylcholine was tested by using several different agents which specifically disrupt microtubules (*9*). Colchicine (10 mg/ml) and vinblastine sulfate (10 mg/ml) resulted in the disappearance of the peak at 6 hours, but did not inhibit the incorporation of choline into acetylcholine at the periphery. The drugs were applied by immersion of the distal half of the antenna for 5 minutes before immersion in labeled choline. External application was preferred over systemic injection because of the highly debilitating effects of systemic injection of the two drugs. Surgical separation of axon and soma by removal of the distal third of the antenna 3 hours after labeling did not block transport. These data suggest a peripheral, self-regenerating mechanism of transport involving microtubules and are in accord with results obtained with many other vertebrate and invertebrate species and with nerves having quite different morphologies (*10*). External application of cytochalasin B (0.2 mg/ml) also resulted in the disappearance of the 6-hour peak. However, it was necessary to dissolve the cytochalasin B in 20 percent dimethylsulfoxide (DMSO), a compound which has considerable biological activity by itself (*11*).

Acetylcholine has been proposed as an excitatory transmitter in the insect central nervous system (*12*), and is probably the excitatory transmitter of the cercal nerve–giant fiber synapses (*13*). The fact that acetylcholine appears in the roach brain after peripheral uptake of choline suggests that the enzyme choline acetyltransferase is present in antennal chemoreceptor cells and provides indirect evidence that acetylcholine is the antennal sensory transmitter. The different transport rates indicated by the amino acid and choline labeling show that the two types of precursors are involved in different areas of axonal metabolism even though both undergo axoplasmic transport.

In what is so far the only other report on the axonal transport of acetylcholine, Koike *et al.* (*14*) found that acetylcholine was transported at about 1.7 and 5.5 cm/day in a giant cholinergic cell in the abdominal ganglion of *Aplysia californica*. This is considerably slower than the 12 to 13 cm/day of the antennal system. However, the estimated rates of protein and acetylcholine transport in the roach antenna fall into the range of rates reported by Smith (*1*), who studied protein transport in the giant fibers of the roach abdominal nerve cord. The range of figures given for fast axoplasmic transport of protein in the giant fibers (1.9 to 2.4, 7.2, and 20 cm/day) brackets the rate of acetylcholine transport in the antennal fibers (12 to 13 cm/day) even though the antennal fibers are nearly 200 times smaller than the giant fibers and possess a vastly different structural organization (*1–3, 15*).

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Learning: Classical and Avoidance Conditioning in the Mollusk Pleurobranchaea

Abstract. *Naive specimens of the marine gastropod* Pleurobranchaea *withdraw from tactile stimulation of the oral veil and show feeding responses to food chemicals. Experimental subjects, trained by pairing touch (conditioned stimulus) with food chemicals (unconditioned stimulus), soon acquired a classically conditioned feeding response to touch alone. Control subjects that received touch alone or unpaired touch and food chemicals showed significantly fewer feeding responses to touch than did experimentals. Classically conditioned specimens were used for avoidance conditioning. Subjects that received aversive electrical stimulation when they did not withdraw from touch rapidly learned to withdraw rather than to feed in response to touch alone. Controls that received touch alone or unpaired touch and shock continued to exhibit the feeding response to touch alone. The learned responses persisted for up to 2 weeks without reinforcement before extinction, and could be demonstrated in the isolated nervous system.*

The cellular processes underlying learning are largely unknown, in part because suitable experimental preparations have not been developed (*1*, *2*). Such preparations must be capable of learning, and in addition the learned response must be succeptible to analysis on the level of single nerve cells. Gastropod mollusks are attractive candidates for such studies because the neuronal circuitry underlying simple behavioral acts can be comprehensively analyzed in these animals (*3*) to provide the necessary background information for cellular studies of complex behavioral phenomena (*4*). Moreover, previous behavioral studies suggest that gastropods are capable of learning (*5*). We report that the feeding behavior of the carnivorous marine gastropod *Pleurobranchaea californica* can be conditioned, and that the effects of this conditioning can then be detected in the isolated nervous system. This study demonstrates the first case of associative learning involving a behavior that can be readily studied on the cellular level (*6*, *7*).

Specimens of *Pleurobranchaea* (less than 12 cm long) were maintained individually in white Rubbermaid dishpans (capacity 8 liters) which were flushed continuously with fresh seawater (2 to 3 liter/min) at ambient temperatures (11° to 18°C). Animals were acclimatized to laboratory conditions for 1 week and then randomly sorted into experimental and control groups. The containers of the two groups were intermingled so that experimental and control animals received the same exposure to light. While in the laboratory, all animals received seawater from a common source and the same quantities of general handling and food (squid twice weekly). Most specimens remained in good physiological condition and gained weight during the experiments.

In the classical conditioning paradigm, experimental animals were trained 20 times daily by stroking the leading edge of the oral veil (Fig. 1A) for 5 to 10 seconds with the tip of a glass probe coated with homogenized squid. Such simultaneous presentation of the conditioned stimulus (tactile stimulation of the oral veil) with the unconditioned stimulus (chemostimulation with natural food substances) elicited the unconditioned response (the feeding behavior) (Fig. 1) in 99 percent of more than 20,000 trials on 65 experimental animals (interstimulus interval, 30 to 60 seconds). In contrast, presentation of the conditioned stimulus alone (tactile stimulation of the oral veil with a sterile glass probe) with the same interstimulus interval caused withdrawal (Fig. 1B) within 3 seconds in 90 percent of 2280 trials on 114 naive specimens. The bite-strike response (Fig. 1F) occurred in only 4.5 percent of these trials, and 53 percent of these responses were produced by seven specimens. The three highest responders were not used in subsequent experiments. These data show that, as required for classical conditioning (*8*), (i) the unconditioned stimulus (food chemicals) reliably elicited the unconditioned response (feed-

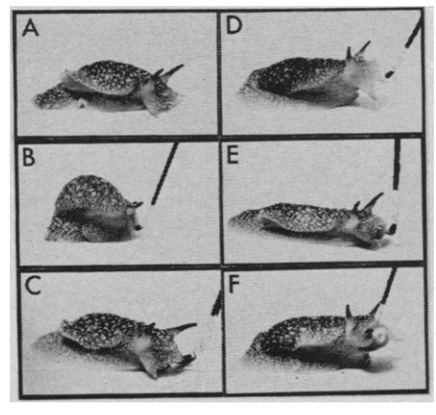

Fig. 1. Behavioral responses of *Pleurobranchaea*. (A) A specimen of *Pleurobranchaea* is shown from the animal's right side; the anterior oral veil and fused lateral tentacles are on the right side of the photograph. (B) A naive specimen withdraws in response to tactile stimulation of the oral veil with a glass probe. The probe is partially wrapped to make it visible for photography. (C to F) Components of the feeding behavior illustrated in a classically conditioned animal are (C) no withdrawal; (D) orientation and following movements; (E) extension of the proboscis; and (F) the bite-strike feeding response. The bite-strike response is easy to distinguish because it happens so fast.