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ACETYLCHOLINE AND LOBSTER SENSORY NEURONES

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SUMMARY

Experiments are presented in support of the hypothesis that acetylcholine functions as a sensory transmitter in the lobster nervous system.

1. Several different peripheral sensory structures incorporate radioactive choline into acetylcholine. The preparation most enriched in sensory as opposed to other nervous elements (the antennular sense organs of the distal outer flagellum) does not incorporate significant amounts of glutamate, tyrosine or tryptophan into any of the other major transmitter candidates.

2. There is a parallel between the distribution of the enzyme choline acetyltransferase and the proportion of sensory fibres in nervous tissue from many parts of the lobster nervous system.

3. Isolated sensory axons contain at least 500 times as much choline acetyltransferase per cm of axon as do efferent excitatory and inhibitory fibres.

4. Abdominal ganglia and root stumps show a decline in the rate of incorporation of choline into acetylcholine 2 to 8 weeks after severing the first and second roots bilaterally (leaving the connectives and third roots intact). Extracts of the root stumps exhibit a significantly lower level of choline acetyltransferase 2 weeks after this operation.

5. Curare and atropine partially block an identified sensory synapse in the lobster abdominal ganglion.

INTRODUCTION

The anatomy of the crustacean nervous system permits the investigation of the physiological and biochemical properties of individual neurones. Previous work in this laboratory demonstrated the selective accumulation

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of γ-aminobutyric acid (GABA) and its biosynthetic enzyme, glutamate decarboxylase, in lobster peripheral inhibitory (I) axons, and the presence of GABA in the central somata of I neurones (for summary see Hall, Bownds & Kravitz, 1970). These data, when combined with physiological and pharmacological observations from this and other laboratories, established that GABA is the inhibitory neuromuscular transmitter compound in Crustacea (for review, see Kravitz, 1967).

While GABA and its biosynthetic enzyme, glutamate decarboxylase, are present exclusively in I cells, the degradative enzymes, GABA-glutamate transaminase and succinic semialdehyde dehydrogenase, are found in excitatory (E) cells as well (Hall *et al.* 1970). This suggests that development of the capacity to synthesize the enzyme glutamate decarboxylase is a crucial event in the differentiation of lobster GABA-containing I neurones.

We have now turned to sensory neurones in the lobster to attempt an analysis of transmitter accumulation similar to that carried out with the efferent neurones. The crustacean sensory transmitter is unknown. However, Florey & Biederman (1960) discovered that all the acetylcholine (ACh) present in crab cheliped nerves (2–6 μg/g, measured by bio-assay) was localized in sensory fibres; none was detected in efferent fibres (0·1 μg/g). Further association of ACh with crustacean sensory elements came from trial applications of a radiochemical method for detecting synthesis of possible neurotransmitter compounds (Hildebrand, Barker, Herbert & Kravitz, 1971). We found that incubation of an abdominal ganglion with [methyl-^{14}C]choline resulted in appearance of radioactive ACh in first and second roots of these ganglia, which contain both sensory and efferent fibres, but not in third roots, which contain exclusively efferent axons.

These observations motivated a comprehensive examination of sensory elements and the role of ACh in the lobster nervous system. The beginnings of that study are presented in this communication.

METHODS

Tissues

All tissues were dissected from 0·5 kg *Homarus americanus* obtained from local suppliers, and kept in circulating sea-water tanks at 12–15° C.

Central ganglionic chain. Portions or all of the ventral nerve cords of lobsters were removed by cutting the nerve roots linking central ganglia to peripheral tissues. The excised ganglionic chain was placed in normal lobster physiological salt solution (called saline; see below for composition) unless ganglia were to be incubated with radioactive precursors under organ culture conditions. In the latter cases, aseptic dissection techniques and lobster organ culture media were used (Hildebrand *et al.* 1971, and below). The central ganglia, connectives linking ganglia, and ganglionic root stumps were subdivided in various ways for the different experiments.

Cell bodies in ganglia were exposed by removing the connective tissue sheath from the ventral surface of the ganglion and washing away the loose cell layer covering the neuronal cell bodies with a stream of saline (Otsuka, Kravitz & Potter, 1967). This allowed access to the cell bodies for intracellular monitoring of physiological activity, iontophoretic application of drugs, chemical analyses, etc.

Muscle receptor organ–ganglion preparation. The lobster abdomen was pinned ventral side up in a deep dish and covered with cold saline. A mid line incision was made to expose the ganglionic chain. A cut in the exoskeleton exposed the second root of one of the ganglia, so that the root could be followed laterally across the flexor musculature and then dorsally toward the back of the abdomen. It was necessary to cut several branches of the nerve in following the main trunk around the flexor muscles. All the remaining roots and the anterior and posterior connectives were cut, freeing the ganglion from the abdomen. The flexor muscles and the ventral exoskeleton were then removed, leaving the ganglion attached, by the second root, to the extensor musculature on the dorsal exoskeleton. Next, the main mass of the fast extensor muscles was removed, and finally the superficial slow extensor muscles covering the nerve trunk to the slow extensors and receptors were carefully cut away. When the nerve bundle from the receptors to the second root was clearly visible, all other nerve branches and the ends of the receptor muscle fibres were cut, freeing the receptor-ganglion preparation.

Sensory preparations. Several sensory elements were dissected under aseptic conditions for incubation with radioactive precursors of the known or suspected transmitter compounds (see below). In general these preparations were rich in sensory neurones but were contaminated by connective tissue, glial elements, and sometimes other neuronal elements as well. Text-fig. 1 shows sites in the lobster from which the various sensory preparations were obtained.

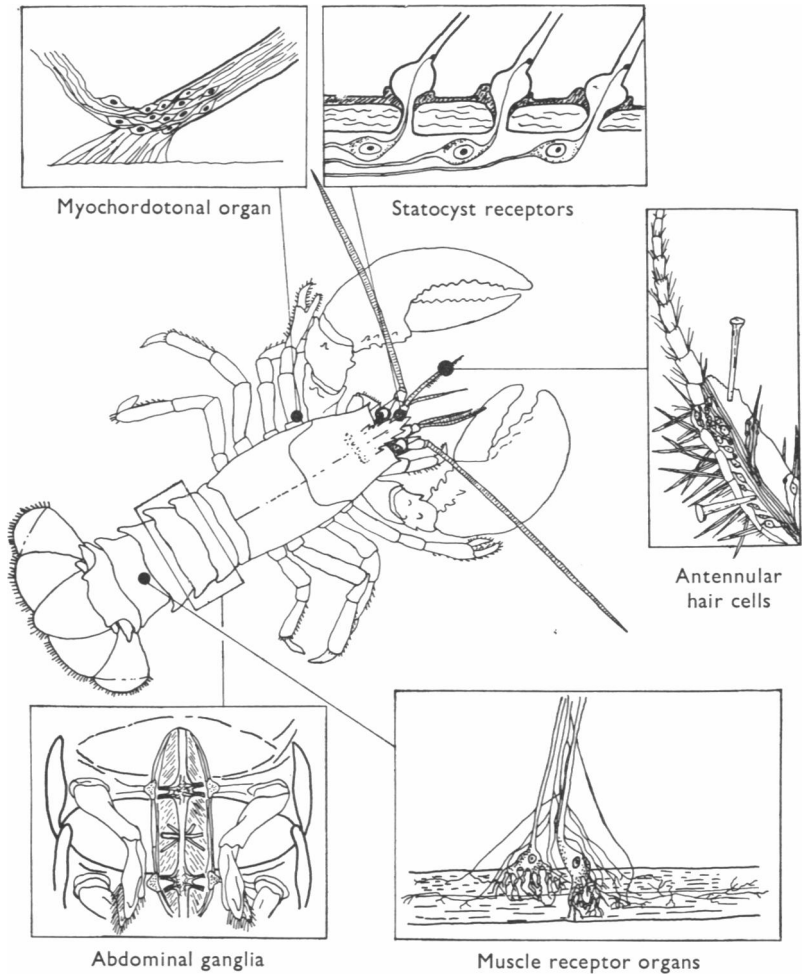
Abdominal muscle receptor organs (Alexandrowicz, 1951) used in these studies were dissected from a dorsal strip of exoskeleton containing the extensor musculature alone. Otherwise they were isolated as above. There were two sensory cell bodies, their dendrites, and 1 cm lengths of axon in each incubation. Other axons (the excitors to the receptor and extensor muscles and the inhibitors to the sensory cells and extensor musculature) contaminated the preparations. After incubation, preparations were divided into two regions which were analysed separately. One consisted of muscle free of sensory elements; the other included sensory cells and short lengths of the various axons.

Myochordotonal organs are proprioceptive systems found in the meropodite segment of decapod crustacean walking legs (Barth, 1934; Clarac & Masson, 1969). Organs from the first and second walking legs were used. The preparation consists of about thirty bipolar sensory neurones lying at the base of an elastic strand which branches into fine fibres that attach to the hypodermis. The sensory cell bodies and their initial axon lengths and the base of the elastic strand were extracted following incubation in radioactive media.

The *statocyst receptor* is located in the hollow basal segment of each antennule and provides information about spatial equilibrium (see Cohen & Dijkgraaf, 1961, for detailed description and references). The antennular segment containing the statocyst was incubated intact except that part of the ventral exoskeleton was removed to expose the sensory cell bodies and the initial segments of their axons to the radioactive medium. The cell bodies lie in a thick hypodermis adjacent to a chitinous sac in which the sensory hairs contact the statolith. The sensory neurones were removed for extraction by peeling the hypodermal layer away from the sac. The extract contained the hypodermis, the sensory cell bodies and initial lengths of axon, and probably other small fibres in this region. In one experiment, the sensory neurones

underlying the dorsal external hair tuft adjacent to the statocyst were also extracted and analysed separately for the synthesis of possible transmitter compounds.

The *antennular sense organs* of the distal region of the outer flagellum of each lobster antennule consists of chemoreceptive, mechanoreceptive and proprioceptive neurones (Laverack, 1964). In one flagellum of *Panulirus argus* there are about 500000 chemosensory neurones alone (Laverack, 1964; Laverack & Ardill, 1965).



Text-fig. 1. Lobster sensory preparations for transmitter screening experiments.

Although the *Homarus* structure is shorter, the similar internal morphology (Maynard, 1971) suggests the presence of a very large number of sensory elements. There is no known efferent innervation of the antennular flagella. For incubation, the exoskeleton of an outer flagellum was slit up the side opposite the distal hair tuft. After incubation in radioactive medium, all the soft tissue in the flagellum was scraped out of the cuticle and extracted.

One experiment made use of a system of *thoracico-coxal proprioceptors* described by Alexandrowicz & Whitear (1957).

Single axons: sensory, efferent E, efferent I. E and I axons were dissected from the meropodite segment of the walking legs as described previously (Hall *et al.* 1970). The two axons were separated from all other neural elements and from the bulk of the associated connective tissue and were used together in enzyme assays (see below). Sensory axons were dissected from the first or second abdominal segment by removing the receptor organ-ganglion preparation described above and isolating the muscle receptor organ axons from the level of their cell bodies to the ganglion. Dissections were performed with Dumont no. 5 forceps with sharpened tips and fine scissors and were begun at the receptors by removing the bundle of axons innervating the superficial extensors from the bundle (or bundles) containing the two muscle receptor organ axons. Once the two large (25–40 μ in diameter) sensory axons were located, they were freed of other axons and much connective tissue by pulling apart the connective tissue strands holding the bundles together and surrounding the axons.

Axotomy of sensory and efferent neurones. Operations were carried out in a sterile-air laminar-flow hood with sterile instruments. The ventral surface of the lobster abdomen was scrubbed with a solution of Phisohex (Winthrop Labs.), and the Phisohex was removed with a stream of 80 % ethanol. The first and second roots of the third abdominal ganglion were severed bilaterally by piercing the integument with the sharp point of a scissors just posterior to the third rib about 0·5–0·7 cm from the mid line and cutting completely across the rib. A small piece of sterile Gel-Foam (UpJohn Co.) was inserted into each wound to aid the clotting process. The animals were returned to sea water within 10 min after removal in all cases. They were kept in sea water at 12–16° C, and were fed small quantities of shrimp at 10- to 12-day intervals. Inability of the lobster to move the swimmerets of the third segment while swimming indicated that roots had been cut, and this was confirmed through visual examination of the ganglia when they were later removed for study. The animals referred to as control or unoperated animals were kept in the same sea water tanks as the operated animals for approximately the same lengths of time. The survival rate after operation was as follows: 90 % of the animals survived for at least 1 week, 60–70 % survived for at least 5 weeks, and about 50 % of the animals survived for at least 8 weeks. This was comparable to survival rates among control animals.

Neurotransmitter synthesis from radioactive precursors

Investigations of transmitter synthesis followed the procedure of Hildebrand *et al.* (1971). Briefly, nervous tissue was dissected from lobsters and incubated for 1–3 days in sterile physiological medium with radiochemical precursors of possible transmitter compounds. After washing in unlabelled medium, preparations were extracted in formate-acetate buffer, pH 1·9. The volume used depended on the size of the preparation and the amount of radioactive product anticipated; e.g. for ganglia 50 μl. buffer was used. The precursors and products in the extracts were separated by high-voltage paper electrophoresis. Small quantities of non-radioactive marker compounds (5-hydroxytryptamine (5 HT), norepinephrine, dopamine, octopamine, tyramine, GABA, ACh and choline) were co-electrophoresed with the extracts. The positions of these markers were revealed by spraying with colour reagents and/or exposure to iodine vapour, and the electropherograms were scanned in a Packard Model 7201 Radiochromatogram Scanner to localize radioisotopes. Quantitative determinations were made by cutting the electropherogram into 1 cm sections, placing each section in toluene-Liquifluor (New England Nuclear) scintillation fluid,

and determining radioactivity by scintillation counting. These data were corrected for quenching and background (called 'corrected cpm' in text and Tables).

The basic physiological medium, suitable for maintenance of lobster nervous tissue for periods of up to 8 weeks (E. A. Kravitz & R. P. Bunge, unpublished), contained 100 ml. Eagle's Minimal Essential Medium (MEM) with Earle's Balanced Salt Solution (Grand Island Biological Co., GIBCO), 10 ml. of dialysed foetal calf serum (GIBCO), and 12 ml. of a concentrated solution of salts and glucose (NaCl, 3 M; KCl, 100 mM; $CaCl_2$, 240 mM; glucose, 167 mM). In some experiments, L-15 medium (GIBCO), buffered at pH 7·4 with 5 mM-imidazole, was used in place of MEM. Media used in preparing tissue for radioactive incubation were supplemented with Penicillin-G 1000 u./ml. and streptomycin sulphate 0·5 mg/ml., and in certain experiments mycostatin 10 u./ml. was also added.

Modified media containing labelled precursors were prepared using GIBCO MEM or L-15, deficient in the compounds to be added in radioactive form. Two such media were used: Glu*-Ch* medium contained 10 μc/ml. [methyl-^{14}C]choline chloride (61 mc/m-mole, Amersham-Searle) and 10 μc/ml. [U-^{14}C]L-glutamate (255 mc/m-mole, Schwarz Bioresearch); Tyr*-Tryp* medium contained 10 μc/ml. [U-^{14}C]L-tyrosine (455 mc/m-mole, Schwarz Bioresearch) and 10 μc/ml. [methylene-^{14}C]L-tryptophan (54·5 mc/m-mole, Amersham-Searle). See Hildebrand *et al.* (1971) for details of media preparation and the high-voltage electrophoresis procedure.

In most experiments in which ACh synthesis was observed, the chemical identity of the isotope migrating with ACh was verified as follows. A portion (usually 10 μl.) of the acidic extract was evaporated to dryness under N_2 and redissolved in 10 or 15 μl. 0·05 M-K phosphate buffer, pH 7·0. To this was added 5 μl. of the same buffer containing the specific acetylcholinesterase (1 mg/ml.) from *Electrophorus electricus* (Worthington Biochemical Corp.). After 30 min at 20–22° C, the mixture was submitted to high-voltage electrophoresis, and the radioactivity in the ACh and choline regions of the electropherogram was determined quantitatively in the scintillation counter. Usually, over 95% of the isotope which migrated as ACh in untreated extracts migrated as choline after acetylcholinesterase treatment. This procedure also allowed for an accurate measurement of the amount of radioactive GABA in extracts, since large amounts of radioactive ACh would obscure small quantities of GABA.

In experiments with several different lobster nervous elements and muscles, we observed a radioactive product which migrated in the dopamine-octopamine (DA-Oct) region of electropherograms. Single precursor experiments showed this compound to be derived from glutamate, and less efficiently, from tyrosine. The radioactive material was eluted from the DA-Oct region with H_2O and spotted on two different microcrystalline-cellulose thin-layer-chromatography plates (Brinkman Instruments). One plate was developed with a solvent system containing 90 ml. ethanol, 5 ml. H_2O, and 5 ml. concentrated NH_4OH, and the other with 100 g phenol, 15 ml. 0·1 N-HCl, saturated with SO_2. Several nonradioactive amino acids including alanine were co-chromatographed with the sample on the first plate and several catecholamines and alanine were included on the second plate. In each case, over 95% of the radioactivity was observed to migrate with alanine. It was also shown that alanine migrated with the radioactive product to the DA-Oct region of electropherograms.

When protein measurements were made, the acid-insoluble fraction was used. The precipitated material was washed twice with 100 μl. portions of 7% trichloroacetic acid. 100 μl. 0·5 M-NaOH were added and the samples kept at 24° C for 18–24 hr. The samples were then heated at 90° C for 15 min. Essentially all of the precipitated material was solubilized by this procedure. 10–20 μl. samples were analysed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951). It was deter-

mined that the acid-insoluble fraction contained over 95 % of the aqueous-extractable protein of the tissues. The results in Text-figs. 2 and 3 are expressed in terms of amounts of alkali-soluble protein in the acid-insoluble fractions of the tissue samples analysed.

Choline acetyltransferase (E.C. 2.3.1.6.) assays

Enzyme characterization. The activity of choline acetyltransferase (ChAc) was determined by measuring the transfer of labelled acetyl moieties from [$^{14}C_1$]acetyl-CoA to choline. Two procedures were employed, one for assays of activity in whole ganglia, roots, and large nerve bundles ('macro assay') and the other for small nerve bundles, single axons, and cell bodies ('micro assay'). Dissections and enzyme preparatory operations were carried out at 0–4° C.

With both the macro and the micro assay procedures (described below), preliminary studies demonstrated that product formation was linear with the amount of added tissue extract over the range of enzyme activities studied. The conditions described below are those which gave maximal activity with crude supernatant enzyme. The substrates, choline and acetyl-CoA, were shown to be present at saturating and non-inhibitory concentrations. Achievement of full enzyme activity required the addition of thiol, salt, EDTA, and cholinesterase inhibitor, and all were therefore included in the incubation mixtures. Product formation was linear with time for 15 min. The crude enzyme showed a broad pH optimum from pH 7·5 to 8·2 and a temperature optimum at 37–38° C.

Macro assay. Tissue samples were homogenized in 0·02 M sodium phosphate buffer, pH 7·5, containing 0·1 mM-EDTA disodium salt and 1·0 mM dithiothreitol (DTT). The homogenate was centrifuged in a MISCO micro centrifuge at about 5000 rev/min for 10 min, after which the supernatant fluid was retained and the pellet was resuspended in buffer and centrifuged. The supernatant solutions were combined and the volume, in each case, was adjusted to 0·2 ml. with buffer. An aliquot of this extract (usually 5 or 10 μl.) was placed in an ice-cold 0·2 ml. MISCO conical tube, water was added to bring the volume to 10 μl., and then 15 μl. of ice-cold buffer-substrate was added. The buffer-substrate contained: sodium phosphate, 16·7 mM, pH 7·5; DTT, 0·15 M; EDTA disodium salt, 16·7 mM; neostigmine methyl sulphate, 16·7 mM; choline chloride, 16·7 mM; KCl, 33 mM; [$^{14}C_1$]acetyl-CoA (2×10^6–$3{\cdot}1 \times 10^6$ dmp/m-mole). Labile acetyl groups in the substrate were routinely measured by means of the hydroxamate method of Hestrin (1949). Tubes were incubated for 15 min at 37° C. 20 μl. aliquots of each sample were then spotted at the centre of a 3×34 cm strip of Whatman 3MM paper, to which 40 μg each of choline and ACh chlorides had already been supplied. Papers were moistened with buffer (1·5 M-acetic acid, 0·75 M-formic acid, pH 1·9) and the strips submitted to electrophoresis in a Beckman Durrum cell at 425 V for 1 hr at room temperature. After the strips had been dried, choline and ACh were localized with iodine vapour. The ACh region was cut from the paper and counted in a scintillation spectrometer. For each assay, a blank was subtracted which included all incubation components except enzyme. This control value was found to be the same as a boiled-enzyme blank.

The method of Lowry *et al.* (1951) was used to measure the protein content of enzyme extracts.

Micro assay. Each small tissue sample was homogenized in a micro glass homogenizer (total capacity 50 μl.) in 5 μl. of a buffer mixture, which contained sodium phosphate, 11 mM pH 7·5; DTT, 0·11 M; choline chloride, 11 mM; EDTA disodium salt, 11 mM; neostigmine methyl sulphate, 2 mM; KCl, 22 mM. To 5 μl. homogenate was added 2 μl. ice-cold buffer mixture containing 7 mM-[$^{14}C_1$]acetyl-CoA (New England Nuclear, 59·2 mc/m-mole). Following incubation at 37° C for 15 min, the

tube was returned to an ice bath, and 5 μl. of the incubation mixture was submitted to paper electrophoresis and scintillation counting as described above.

Physiological experiments

Synaptic input from muscle receptor organs to cells in the central ganglia. The receptor organ-ganglion preparation was isolated as described above except that the connective tissue sheath was removed from the dorsal as well as the ventral surface of ganglia. A ganglion was pinned, ventral side up, to a layer of Sylgard 184 (Dow Corning) in a small petri dish so that the dorsal side of the ganglion rested over the outlet of a length of fine polyethylene tubing embedded in the substrate. Saline medium continuously superfused the preparation at a rate of 1–2 ml./min with the aid of a Sage Model 220 syringe pump (Sage Instruments, Inc.). The Sylgard dish containing the preparation was supported by a metal plate mounted on thermoelectric cooling units (Cambion Corp.) in order to maintain the temperature at 7–10° C throughout the experiment. A gentle stream of oxygen served to mix the contents of the dish and supply oxygen necessary for long term survival of the preparation. The muscle receptor organs were usually trimmed to a small preparation containing the receptor cell bodies, their dendrites, and associated bits of muscle, and drawn into suction electrodes for stimulation. When the dissection permitted, the two receptor cells were drawn into separate suction electrodes for stimulation. Otherwise a single suction electrode was used and the difference in threshold stimulation was used to fire the two neurones separately. The thin nerve trunk adjoining the receptor was drawn into another suction electrode for extracellular recording of action potentials from receptors.

Micro-electrodes. Micro-electrodes for intracellular recording were filled with 2 M-potassium acetate. The electrode resistance ranged from 20 to 40 MΩ. ACh-filled micro-electrodes were prepared by first filling the electrode tips by immersion in ACh and then filling the shanks with a solution of 1 M-ACh chloride. Electrode resistance varied from 5 to 20 MΩ.

Solutions and drugs. Normal lobster physiological salt solution (saline), had the following composition: NaCl, 462 mM; KCl, 16 mM; $CaCl_2$, 26 mM; $MgCl_2$, 8 mM; glucose, 11 mM; Tris 10 mM; maleic acid 10 mM; and the pH was adjusted to 7·4 with NaOH.

ACh chloride and neostigmine methyl sulphate were obtained from Sigma Chemical Co.; D-tubocurarine chloride and hexamethonium chloride from Mann Research Labs; atropine H_2SO_4 and picrotoxin from Calbiochem; and Mecamylamine HCl was from Merck, Sharp and Dohme.

RESULTS

Distribution of ChAc. In lobsters, as in vertebrates, ACh synthesis in the nervous system is catalysed by the enzyme ChAc. A survey was made of the distribution of this enzyme in extracts of structures from different regions of the lobster nervous system (Table 1). The central ganglia and the roots bringing sensory information to the brain (optic, antennular, antennal nerves) were particularly good sources of enzyme. Of all tissues analysed, the antennular nerve had the highest specific activity (2·2 n-mole/hr.μg protein). The connectives running between ganglia had lower levels of activity. The first and second roots of abdominal ganglia which contain sensory and efferent axons had activities comparable to those of the

connectives, while third roots, which are devoid of sensory fibres, had about 1/50th of the activity of first and second roots. Finally, muscle from the walking legs had the lowest activity (less than 1/500th the activity of thoracic ganglia).

Sectioning of first and second roots of abdominal ganglia; sensory degeneration or atrophy. When abdominal ganglia are isolated with short segments of their roots attached and incubated with [methyl-^{14}C]choline, the amount

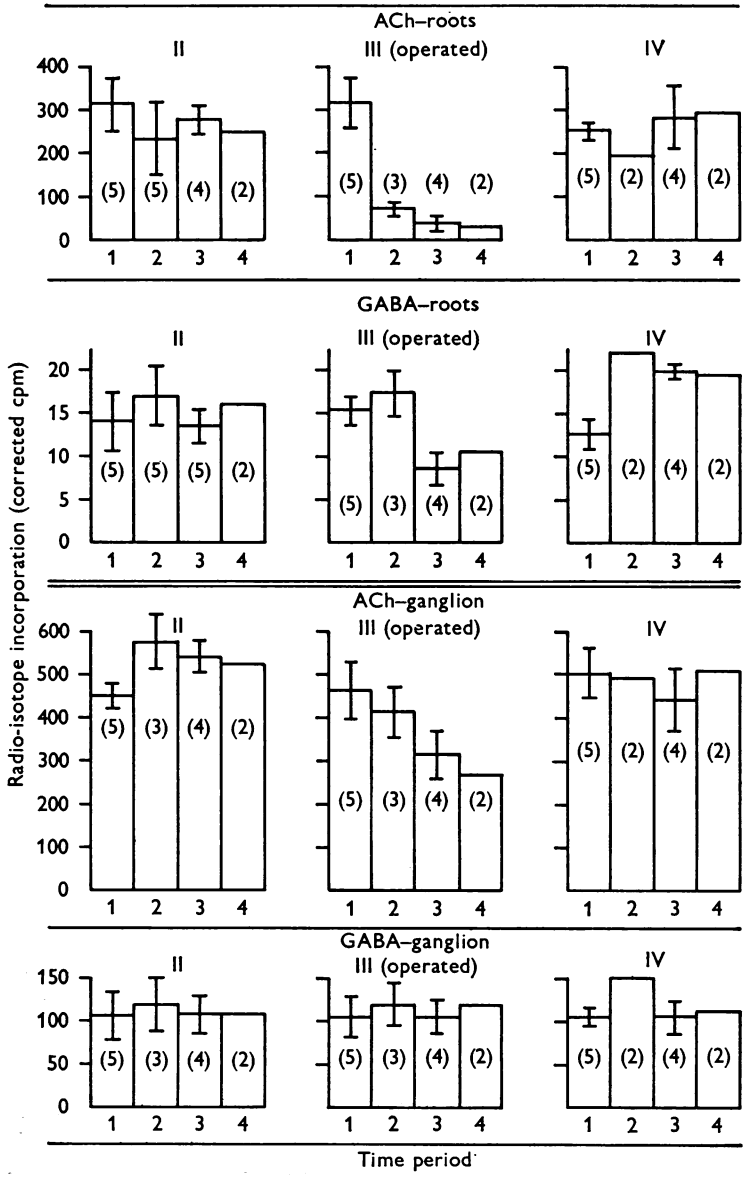
TABLE 1. Choline acetyltransferase activity in various tissues

	Activity (n-mole/hr. μg protein)
Ganglia	
Brain	1·56
Sub oesophageal	1·12
Thoracic 1	1·16
Abdominal 2, 3, 4 (av.)	0·56
Connectives	
Thoracic	0·304
Abdominal	0·156
Roots, nerves	
Optic	0·72
Antennular	2·20
Antennal	0·80
Sensory bundle (walking leg dactyl)	0·23
1st roots (abd.)	0·17
2nd roots (abd.)	0·34
3rd roots (abd.)	0·004*
Muscle	
Walking leg	0·002*

* Not significant.

of radioactive ACh appearing in first and second roots is much greater than that appearing in third roots (Hildebrand *et al.* 1971). The rate of accumulation of radioactive ACh parallels the level of the enzyme ChAc in these structures (Table 1), suggesting that differences in levels of radioactivity reflect true differences in synthetic capacity. All three roots accumulate radioactive GABA when ganglia are incubated with [U-^{14}C]glutamate.

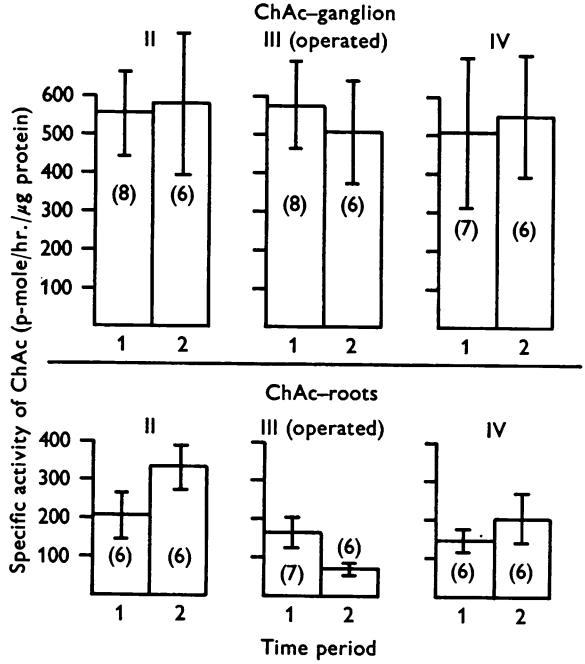
The cell bodies of the efferent neurones (including those that synthesize GABA) are found within the central ganglia, while most sensory cell bodies are found in the periphery. By sectioning first and second roots to an abdominal ganglion one might expect to cause degeneration or atrophy of afferent (but not efferent) elements central to the cut. If the sensory transmitter were ACh, then capacity to incorporate radioactive choline into ACh in operated ganglia and root stumps should fall faster than GABA production. This is precisely the effect observed. In Text-fig. 2, bar



Text-fig. 2. Labelling of ACh from [methyl-^{14}C]choline and GABA from [U-^{14}C]glutamate in roots and ganglia of abdominal segments II-IV of control (unoperated, time point 1) and experimental (operated – first and second roots of third abdominal ganglion severed bilaterally) animals in four time periods: 1, 0 days; 2, 8–18 days; 3, 55–58 days; 4, 85 days. Numbers of animals used are shown in parentheses; vertical bars are S.D.

graphs depict the synthesis of ACh in roots and central ganglia of control (unoperated) and experimental (operated) animals at varying periods of time (up to 85 days) after nerve section. In the experimental animals, only the roots of the third abdominal ganglia were cut; therefore, the second and fourth abdominal ganglia served as internal controls for the effects of the operation.

Significant changes in capacity to incorporate [methyl-^{14}C]choline into ACh were seen exclusively in third (operated) ganglia and attached root

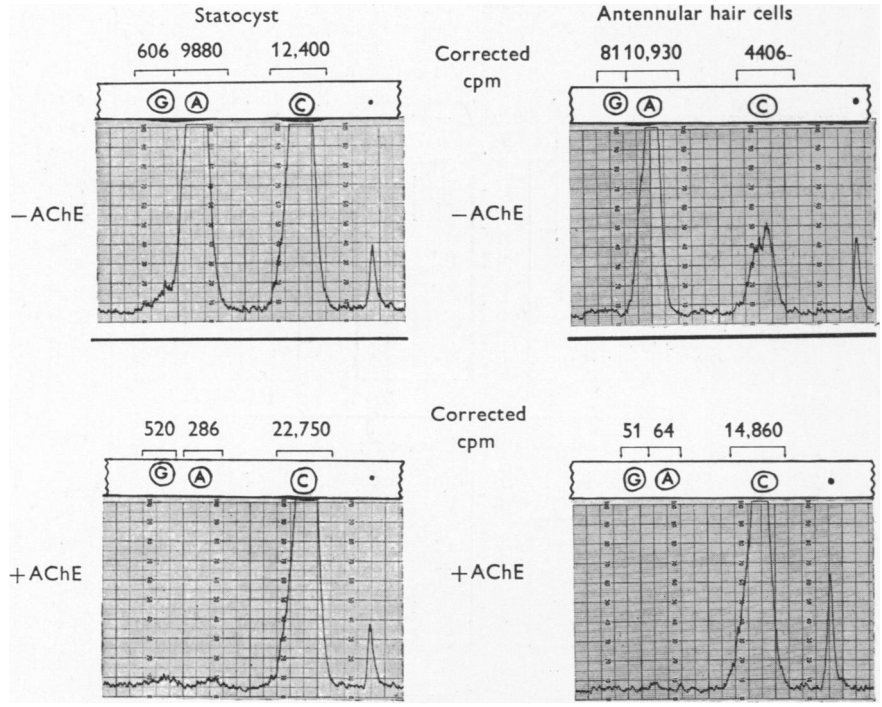


Text-fig. 3. ChAc levels in roots and ganglia of abdominal segments II–IV of control (unoperated, time point 1, 0 days) and experimental (operated, time point 2, 43–61 days) animals. Numbers of animals used are shown in parentheses; vertical bars are S.D.

stumps. In the roots, incorporation fell in 1–2 weeks (time period 2, Text-fig. 2) to 1/5th of the normal level and to about 1/10th of normal by 8 weeks (time period 3). In the ganglion proper the effect was less dramatic but a significant decrease (about 30 %) was seen after eight weeks. GABA synthesis was unchanged or slightly increased in the control or operated roots or ganglia for 2–5 weeks. Thereafter a decrease was seen in GABA synthesis in roots of operated ganglia. ChAc levels (Text-fig. 3) also decreased in roots from operated animals. These levels decreased to about

50 % of normal within 2 weeks; thereafter no further decrease took place despite the observation of a marked reduction in the numbers of axon profiles in the operated roots (Pl. 1).

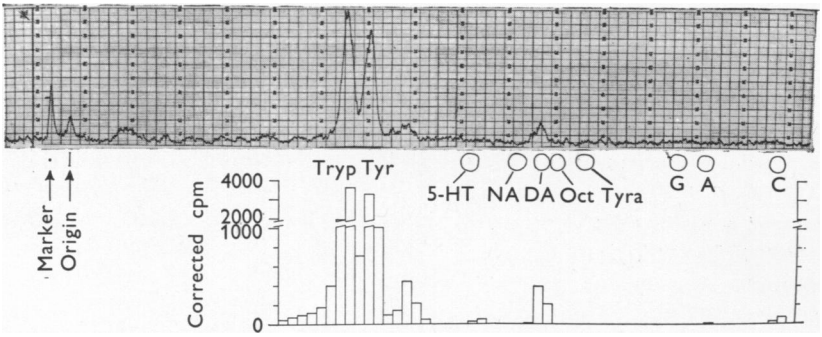
ACh synthesis in sensory preparations. For more detailed exploration of the association of ACh with sensory elements, four different sensory preparations were examined for their capacity to incorporate radioactive choline into ACh.



Text-fig. 4. GABA-choline regions of electropherograms of extracts of statocyst and distal antennular preparations incubated with Glu*-Ch* medium. Traces labelled +AChE were derived from extracts pre-treated with acetylcholinesterase (see Methods). G, A, and C refer to GABA, ACh, and choline, respectively. Peak marked by dot is a radioactive marker applied to paper strip after electrophoresis.

Abdominal muscle receptor organs (stretch receptors), myochordotonal organs, statocyst receptor cells and antennular sense organs were dissected from a number of lobsters and incubated in radioactive medium as described in Methods (Text-fig. 1). These preparations represent several different sensory modalities, and only the muscle receptor organs are known to have an efferent inhibitory innervation. Two different precursor media were used for each type of preparation. One contained radioactive

glutamate and choline (Glu*-Ch*); the other, tyrosine and tryptophan (Tyr*-Trypt*). The extracts from the Glu*-Ch* incubations were divided in half before electrophoresis; one half was treated with specific acetylcholinesterase. Electropherograms from the Glu*-Ch* experiments consistently showed labelled material in the transmitter region only at positions corresponding to DA-Oct, GABA, ACh and precursor choline. For each preparation, the peak at DA-Oct was eluted and found to be alanine (see Methods). The GABA to choline regions of the electropherograms of extracts of statocyst and distal antennular preparations incubated



Text-fig. 5. Radioscan of electropherogram of the extract of a statocyst preparation which had been incubated with Tyr*-Tryp* medium. Abbreviations not explained elsewhere in the text are: NA, noradrenaline; Tyra, tyramine.

in Glu*-Ch* medium are shown in Text-fig. 4. These data demonstrate the quantitative conversion of ACh to choline by the acetylcholinesterase treatment, supporting the identity of the radioactive product. By this test the identity of ACh was verified for each sensory preparation.

The extracts of preparations incubated in Tyr*-Trypt* medium did not demonstrate the synthesis of large amounts of transmitter candidates derived from these precursors. A complete scan of an electropherogram of a statocyst extract after incubation in Tyr*-Trypt* medium is shown in Text-fig. 5. The histogram gives the quantitative scintillation-counting data from 1 cm sections of the paper strip. In the transmitter region, radioactivity rises above background near 5 HT (1·2 % of Trypt* uptake), around DA-Oct, at ACh and at choline. As above, the isotope at DA-Oct was shown to be in alanine. A similar result was obtained with an antennular hair cell preparation, except that no radioactivity was detected near 5 HT on the electropherogram.

The radioactivity in each of the GABA, ACh and choline regions of electropherograms from Glu*-Ch* incubations was quantified by counting

sections of the paper strip in scintillation vials. The quantitative data for all four sensory preparations are recorded in Table 2. The last column in Table 2 is a calculation of the ratio of ACh to GABA synthesized. The abdominal muscle receptor organs had the lowest ratio (0·85), and the ratio varied considerably in different experiments. The purest sensory preparation (suspected of having no efferent innervation) was the antennular sense organ, and here the ratio was greater than 200 to 1.

TABLE 2. Incorporation of radioisotope into GABA and ACh in various sensory preparations

Preparation	Corrected cpm			
	GABA	ACh	Choline	ACh/GABA
Abdominal muscle receptor organ (two pairs)	*5,600	4,730	12,600	0·85
Myochordotonal organs (two)	99	180	1,070	1·7
Esterase control	107	24	1,250	—
Statocyst receptor cells	2,430	39,500	49,700	19
Esterase control	2,080	1,140	91,000	—
Antennular sense organs	324	43,700	17,600	214
Esterase control	204	256	59,400	—

All incubations were in a MEM-based medium for 60 hr at 12° C. In the calculation of ACh/GABA, the value for GABA was taken from the esterase controls. *48 hr incubation, no esterase control.

TABLE 3. Choline acetyltransferase activity in single fibres

Tissue	Total axon length (cm)	Activity (p-mole/hr.cm)	Average (p-mole/hr.cm)
Abdominal muscle receptor organ axons (both receptors)	8	14·2	17·1
	5·5	20·0	
Excitatory and inhibitory axons from walking leg (pooled)	8	0*	0·030
	9	0·084	
	34	0*	
	37	0·035	
Muscle receptor organ axons plus E and I axons	16	11·2†	

* Lower limit of detection = 0·75 p-mole/hr in entire sample.
† Only the 8 cm of sensory axon was used to calculate this value.

Two other sensory elements, each investigated in one experiment, also synthesized ACh and a smaller amount of GABA. These were the cells underlying the external hairs on the second antennular segment and the receptor organs spanning the thoracico-coxal articulation.

ChAc activity in single axon extracts. To examine more closely the

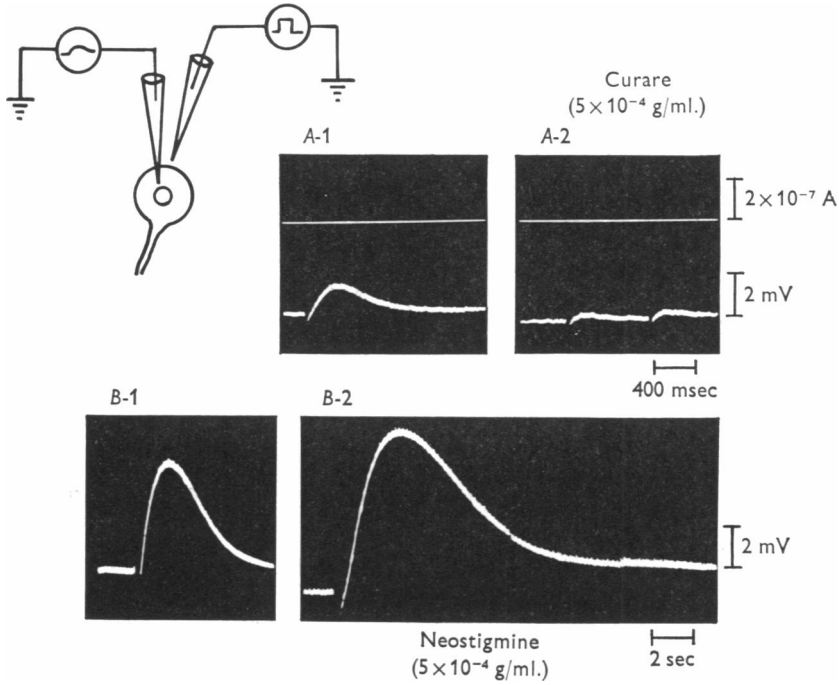
association of ACh synthesis with sensory neurones indicated by the experiments described above, the ChAc assay was modified for application to isolated axons. Enzyme activity in extracts of single, identified E, I, and sensory axons was determined. The results are summarized in Table 3, in which enzyme activity is expressed as p-mole labelled ACh formed per hr per cm of axon. Pairs of abdominal muscle receptor axons up to 4 cm in length were dissected free from other nerve fibres and muscle. In two determinations, extracts of these axons were tested alone. The average activity was 17·1 p-mole/hr.cm. The pair of E and I axons running in the meropodite segment of the walking leg was dissected free from other neural elements and assayed in four experiments. In the first two, axon pairs from single legs were used, while massed axon pairs were used in the next two experiments to provide increased amounts of tissue in the assay tube. As can be seen in the table, the average activity per unit length in E and I fibres was 0·030 p-mole/hr.cm. This value differs from the sensory activity by a factor greater than 500. In a final experiment, to test for a possible inhibitor of ChAc in E and I axon extracts, equal lengths of an E and I axon pair (8 cm total) and a sensory axon pair (8 cm total) were homogenized together, and the activity of the resultant homogenate was measured. Since only a small decrease was observed in activity in the mixed extract (11·2 p-mole/hr.cm) compared to pure sensory extract (17·1 p-mole/hr.cm) it is clear that the very low or absent enzyme activity in E and I axon extracts cannot be simply the result of inhibition by a soluble factor.

Physiological studies; ACh receptors on cell bodies. The effect of iontophoretic application of ACh on to the somata of neurones within the central ganglia was examined next. The aim of these studies was to explore the pharmacology of cholinergic receptors on easily accessible sites before an examination of the pharmacology of the sensory input in the neuropil.

Ganglia were isolated, their ventral sheaths removed, and neuronal cell bodies exposed by washing with a stream of saline (see Methods and Otsuka *et al.* 1967). A recording micro-electrode was inserted into a cell body and the surface of the cell explored for ACh sensitivity with an external ACh micro-pipette. Many cells showed a depolarizing response to applied ACh. The evoked potential was of long duration (up to several sec; see Text-fig. 6, A-1 and B-1) and required $0{\cdot}5–2 \times 10^{-7}$ A current pulses through the ACh electrode, often for time periods of 20–100 msec, to be effective. The highest sensitivity observed was on the order of 1 mV/nC.

The position of the ACh electrode was very critical. When good responses were observed, the tip of the electrode was sufficiently close to the surface of the cell that a very large current pulse caused the electrode to enter the cell. Often, rapid fluctuations in membrane potential were seen in lowering

the electrode to the cell surface. This appeared to be due to passage through another cell layer (the thin glial cell layer?) prior to having the electrode in position to obtain good ACh potentials. In some cases cell surfaces appeared exceptionally well cleared of surrounding tissues and gave evidence of differential sensitivity of regions of the soma to ACh. The polarity of the pulse applied to the ACh electrode was routinely reversed to check for electrical or other artifacts produced by the rather large

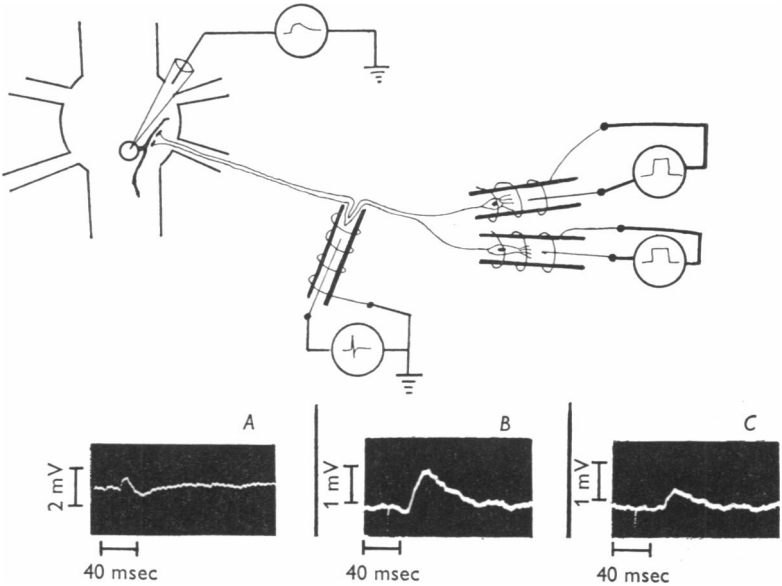


Text-fig. 6. Intracellularly recorded potentials evoked by ACh iontophoresed on to two different neuronal cell bodies (*A* and *B*) in an abdominal ganglion. *A* is a cell in the extensor group of neurons, *B* is I_2. The current pulse in *B* was 2×10^{-7} A for 40 msec. *A*-2 shows the effect of curare (5×10^{-4} g/ml.) on potential *A*-1; *B*-2 shows the effect of neostigmine (5×10^{-4} g/ml.) on potential *B*-1.

current pulses used. Other drugs applied electrophoretically (e.g. GABA) did not produce potential changes of the type seen in these experiments.

Curare at 5×10^{-4} g/ml. markedly reduced the response to applied ACh (Text-fig. 6*A*2); atropine and hexamethonium at similar concentrations were less effective. Neostigmine produced a large potentiation of the response at 5×10^{-4} g/ml. (Text-fig. 6*B*2), and was still effective at 1/10 that concentration. The effect of curare was slowly and incompletely reversible, while the effects of the other reagents were more easily reversed.

Preliminary studies on sensory contacts in the neuropil. The search for a known sensory input to a neurone in the lobster abdominal ganglion centred on the reflex activation of the superficial extensor musculature by the muscle receptor organ neurones (Fields, Evoy & Kennedy, 1967). An excitatory cell was found in the ganglion which innervated a large number of the slow extensor muscles (E. Frank, personal communication). The cell was called M-15 in keeping with the previous nomenclature of cell bodies in abdominal ganglia (Otsuka *et al.* 1967) and was readily



Text-fig. 7. Intracellularly recorded responses of abdominal ganglion cell M-15 to firing of muscle receptor organ cells: *A*, normal biphasic response; *B*, monophasic depolarizing potential in the presence of picrotoxin (0·2 mg/ml.); *C*, reduced monophasic depolarizing potential with both picrotoxin (2×10^{-4} g/ml.) and atropine (5×10^{-4} g/ml.). In this experiment the slowly adapting receptor neurone was stimulated. The experimental design allowed for the separate stimulation of either receptor neurone (see Methods).

located by its position (the most medial, posterior cell body in the ganglion) and by the existence of spontaneous inhibitory post-synaptic potentials (i.p.s.p.s). No other cell with spontaneous i.p.s.p.s has been found in the ganglion (except the contralateral M-15). In order to observe synaptic potentials when recording from cell bodies in these ganglia, it is necessary to maintain the temperature below 10° C. When either the slowly adapting or rapidly adapting muscle receptor neurones fire, a biphasic (depolarizing followed by hyperpolarizing) response is usually observed in M-15 (Text-

fig. 7*A*). The slowly adapting receptor neurone is the one usually used for these studies since the depolarizing component of its response is much larger than that produced by the rapidly adapting neurone. The hyperpolarizing component can be blocked by picrotoxin, yielding a purely depolarizing post-synaptic potential (Text-fig. 7*B*). At high concentrations (5×10^{-4} g/ml.) curare or atropine reduced the size of this potential to about one half. The two drugs were about equally effective, and took a long time to have their effects (15–30 min), which were not reversible. In the experiment illustrated (Text-fig. 7*C*), atropine was used. At these same concentrations curare and atropine are without effect on excitatory junctional potentials at lobster neuromuscular junctions. Higher concentrations of drugs were not used because they had significant effects on receptor firing and axonal conduction. Other drugs, including hexamethonium, mecamylamine, choline and succinylcholine, were either less effective than curare and atropine or produced depolarization in the cell soma. Acetylcholinesterase inhibitors, such as neostigmine and eserine, gave results that were difficult to interpret. These drugs caused a great increase in spontaneous excitatory post-synaptic potential (e.p.s.p.) activity. The depolarizing potential induced by stimulating the muscle receptor was somewhat longer than normal and usually led to firing or multiple firing of M-15 (whereas previously it did not). However, in view of the enhanced background activity of spontaneous e.p.s.p.s, these effects cannot be clearly attributed to esterase inhibition.

DISCUSSION

ACh in crustacea: central vs. peripheral actions. Piotrowski (1893) reported that curare and atropine had no effect on excitation and inhibition of crayfish walking leg muscles. When injected into intact crayfish, however, these drugs paralysed the animals. Accordingly it was suggested that curare and atropine act principally on the C.N.S. The lack of actions of cholinergic agents on peripheral tissues in Crustacea was extended and confirmed separately by Katz (1936) and Bacq (1935). ACh, cholinergic receptor blocking agents (curare and atropine again) and an acetylcholinesterase inhibitor (eserine) were found to be without physiological effects on crustacean neuromuscular junctions. At about the same time, ACh was detected in crab nervous tissue (Welsh, 1938). In subsequent years it was demonstrated that large amounts of ACh were found generally in crustacean and insect nervous tissues (for tabulations of data and historical reviews see Florey (1962) and Treherne, 1966). A particularly interesting finding was that peripheral nerve trunks also contained ACh. Florey & Biederman (1960) made the important observation that sensory nerve bundles from crab chelipeds contained ACh (measured by bio-assay),

while E and I axons did not. This led Florey (1967) to suggest that ACh might be the crustacean sensory transmitter compound. In the past, it had proved difficult to demonstrate conclusive physiological effects of bath-applied ACh on crustacean central neurones (Prosser, 1940; Schallek & Wiersma, 1948). In view of the high concentrations of the enzyme acetylcholinesterase associated with glial cells that surround cell bodies and neuropil branches of cells in these animals (Maynard, 1971), it is not surprising that physiological effects were not seen.

Synthesis of ACh in central ganglia, nerve trunks and various sensory preparations. The synthesis of ACh in extracts of crustacean nervous tissue was first demonstrated by Easton (1950). More recently, Welsch & Dettbarn (1970) examined the subcellular distribution of ACh, ChAc, and acetylcholinesterase in extracts prepared from lobster walking leg nerve bundles. Dettbarn and his colleagues have been concerned with the possible involvement of the ACh system in axonal conduction (see Dettbarn, 1967) and have cited both a spontaneous release of ACh with changes in the ionic environment bathing nerve trunks (Dettbarn & Rosenberg, 1966) and the symmetrical distribution of the enzyme in E and I axons (Dettbarn, De Lorenzo & Brzin, 1971) as support for this idea. Our results conflict with theirs in that we can detect little or no ChAc activity in E and I axon extracts while sensory axon extracts have high levels of activity (see below). The results of Dettbarn *et al.* were presented in an abstract, so that we cannot compare our findings with theirs. However, W.-D. Dettbarn (personal communication) now reports a significant difference (sixtyfold) in ChAc activity in sensory axons when these are compared to E and I axons.

Our survey of the distribution of ACh biosynthetic activity yielded the following results. The nerve trunks that bring sensory information to the brain have high levels of ChAc. The brain and suboesophageal ganglia containing the nerve terminals of many sensory elements are among the richest sources of enzyme. Other ganglia in the central chain also have high levels of the synthetic enzyme, perhaps owing mainly to their sensory input from the periphery. The first and second roots of abdominal ganglia, with incoming sensory and outgoing efferent axons, contain ChAc and accumulate radioactivity in ACh when ganglia with root segments are incubated with [methyl-^{14}C]choline (Hildebrand *et al.* 1971). Third roots, which have no sensory fibres, do not accumulate radioactivity in ACh under these conditions and have little or no synthetic enzyme. Exoskeletal muscles are essentially devoid of ACh biosynthetic activity. At the level of single fibres (discussed below), sensory axons contain the enzyme ChAc, while efferent E and I axons do not (or have very small amounts). Finally, ACh is synthesized from radioactive choline in the four different

peripheral sensory preparations analysed by a transmitter screening procedure. These sensory preparations comprise different types of sensory neurones including mechanoreceptors, proprioceptors and chemoreceptors, yet all make ACh. Of the major known or suspected transmitter compounds, only ACh is formed in the preparation (the antennular sense organ) which is most enriched in sensory elements compared to extraneous nerve tissue.

In general, our results demonstrate that the ability of an intact tissue to incorporate radioactive choline into ACh is roughly paralleled by the level of the enzyme ChAc in extracts of the tissue. The one exception was the degeneration experiment where first and second roots to abdominal ganglia were sectioned. The marked decrease in ability of central root stumps to incorporate radioactive choline into ACh (reduced to about 15%) was considerably greater than the decrease in ChAc activity (to about 50%). Perhaps this reflects an increase in acetylcholinesterase, a greater accessibility of the esterase to its substrate in degenerating fibres, or a consequence of neural inactivity in the operated roots.

A more quantitative evaluation of the ability of lobster neurones to accumulate ACh must await a detailed enzymic analysis of the synthetic and degradative capacity of cholinergic cells, a knowledge of the kinetic and other properties of the enzymes involved, and of the intracellular levels of substrates.

Physiological and pharmacological studies. There is little doubt from the iontophoretic studies that ACh receptor sites are widely distributed on lobster neurones. This result was anticipated by earlier findings that ACh and drugs which interact with cholinergic systems affect the firing of muscle receptor organ neurones in crustacean preparations (Wiersma, Furshpan & Florey, 1953). Moreover a recent report by Denburg, Eldefrawi & O'Brien (1972) demonstrates that lobster peripheral nerve preparations are an excellent source of material for binding cholinergic ligands and in particular, α-bungarotoxin. Pharmacologically, the receptors on the cell bodies are somewhat like those of the vertebrate neuromuscular junction, in that they are blocked more effectively by curare than by atropine. However, detailed studies of drug effects and of other properties (induced ionic conductances, etc.) of the receptor sites have not yet been carried out. Furthermore, the present experiments do not deal with the possibility that the receptors might actually be at some distance from the soma (e.g. on the initial portion of the cell process that enters the neuropil). The effects of acetylcholinesterase inhibitors are obvious in all preparations tested. ACh action is significantly potentiated, probably owing to inhibition of the acetylcholinesterase which is associated, in high concentrations, with the glial cells that completely invest these neurones (Maynard, 1971).

The preliminary experiments on a synapse between identified peripheral sensory neurones (the muscle receptor organ neurones) and an identified central neurone (M-15) are consistent with cholinergic pharmacology but do not yet permit firm conclusions. Curare or atropine reduced the size of a depolarizing synaptic potential, but the effect was irreversible and rather high drug concentrations (5×10^{-4} g/ml.) present for over 30 min did not suppress activity fully. By contrast, picrotoxin (0·2 mg/ml.) completely blocked a hyperpolarizing, presumably inhibitory, synaptic activity in the same post-synaptic cell within 15 min, and its effect was reversible. It is possible that some of the input from the sensory receptors to M-15 might be through electrical contacts rather than chemical synapses. Another possibility is that the 'synaptic' receptors in the neuropil are different pharmacologically from 'non-synaptic' receptors on the soma, or that M-15 has more than one type of receptor on its branches within the neuropil. Furthermore, invertebrate, as contrasted to vertebrate, preparations are penetrated rather slowly by exogenous agents. Whatever barriers are present may retard the diffusion of some drugs more than others.

Acetylcholinesterase inhibitors seemed to lengthen the depolarizing potential from the muscle receptor organ neurone, but meaningful comparisons were prevented by the greatly increased frequency of spontaneous e.p.s.p.s which also resulted from application of these drugs. Experiments with other cholinergic blocking and activating agents are now in progress.

Degeneration or atrophy of lobster sensory axon trunks severed from their cell bodies. The results obtained when first and second roots were severed merit separate comment. The studies presented in this paper demonstrate a marked reduction in ACh synthesis in the central segments of first and second roots and some reduction in the ganglion as well. Because each ganglion receives considerable sensory input from anterior and posterior ganglia, and there may be non-sensory cholinergic synapses as well, one would not expect that all ACh synthesis in the ganglion would be abolished. Whether these observations result from a marked atrophy or a true degeneration of the sensory fibres, we cannot unequivocally state from this study. The anatomical observations suggest that fibres are no longer present, but electron microscopic observations are necessary to confirm this. Moreover, a detailed study of the time course of anatomical and associated physiological changes following root section should be carried out to complement the present biochemical observations. In a light microscopic study of degenerative changes in the spiny lobster (*Panulirus argus*) following eyestalk or antennule removal, it was reported that after three weeks nerve fibres are not seen along the degenerating tract (Maynard, 1967). If lobster sensory neurones do degenerate, this is in marked

contrast to the behaviour of efferent E and I axons. The distal stumps of these axons apparently do not degenerate when severed from their cell bodies in central ganglia (Hoy, Bittner & Kennedy, 1967) for periods of time up to several months after nerve section. It remains an interesting possibility that the failure of severed efferent axons to degenerate may be a property unique to these axon types and not a general property of crustacean axons.

Accumulation of neurotransmitter compounds. Two related, but different mechanisms appear to regulate transmitter accumulation by neurones. One operates at the enzymic level and derives from the kinetic properties and cytological localization of the enzymes catalysing transmitter synthesis and degradation, as well as the amounts of substrates present in the cells. The second is genetic control of accumulation of the enzymes themselves. At present, the genetic regulation, while of the greatest interest, can only be inferred from studies on the detection of particular enzymes in cells and from attempts to alter amounts of enzyme by physiological manipulations. With invertebrate preparations, it is possible to isolate large, physiologically specified neurones repeatedly from a series of animals. Thus, one can carry out detailed biochemical analyses on single neurones of known function and begin to list the activities of transmitter-related enzymes found within single neurones.

Our previous studies have shown that efferent E axons (whose transmitter may be glutamate) and I axons (in which GABA is the transmitter compound) differed in their activities of the GABA biosynthetic enzyme, but not the degradative enzymes (Hall *et al.* 1970). Glutamic decarboxylase (the synthetic enzyme) has a specific activity in I axons at least 300 times that in E axons, while GABA-glutamic transaminase and succinic semialdehyde dehydrogenase (the degradative enzymes) are found in roughly equal activities in the two axon types.

The present studies focus on a third type of nerve cell: lobster sensory cells. These neurones show ChAc activity about 500 times greater than that of efferent I and E axons. Thus for these presumably cholinergic sensory cells, the transmitter-synthesizing enzyme again has a markedly asymmetric localization. Studies on acetylcholinesterase in all three neurone types are in progress, as are analyses in sensory cells of the enzymes of the GABA pathway.

It is of interest to note that in *Aplysia* neurones the enzyme ChAc is found only in neurones thought to be cholinergic, while acetylcholinesterase has been found in all cells examined (Giller & Schwartz, 1971*a*, *b*). In cloned mouse neuroblastoma cells one can obtain cell lines high either in tyrosine hydroxylase or ChAc, but not both, while all cell lines have high levels of acetylcholinesterase (Amano, Richelson & Nirenberg, 1972).

Thus observations made in this laboratory and by others suggest that, whereas the transmitter-degrading enzymes appear to be made and retained by many (or all) types of neurones, and therefore may be constitutive gene products, each biosynthetic enzyme may accumulate only in those cells destined to employ its product as transmitter. To examine this possibility, studies of the regulation of the production of the transmitter-synthesizing enzymes (at the level of gene duplication, transcription, or translation) will be needed. Those studies, together with others on factors that influence the activity of the synthetic enzymes, should be of great importance in elucidation of neuronal differentiation.

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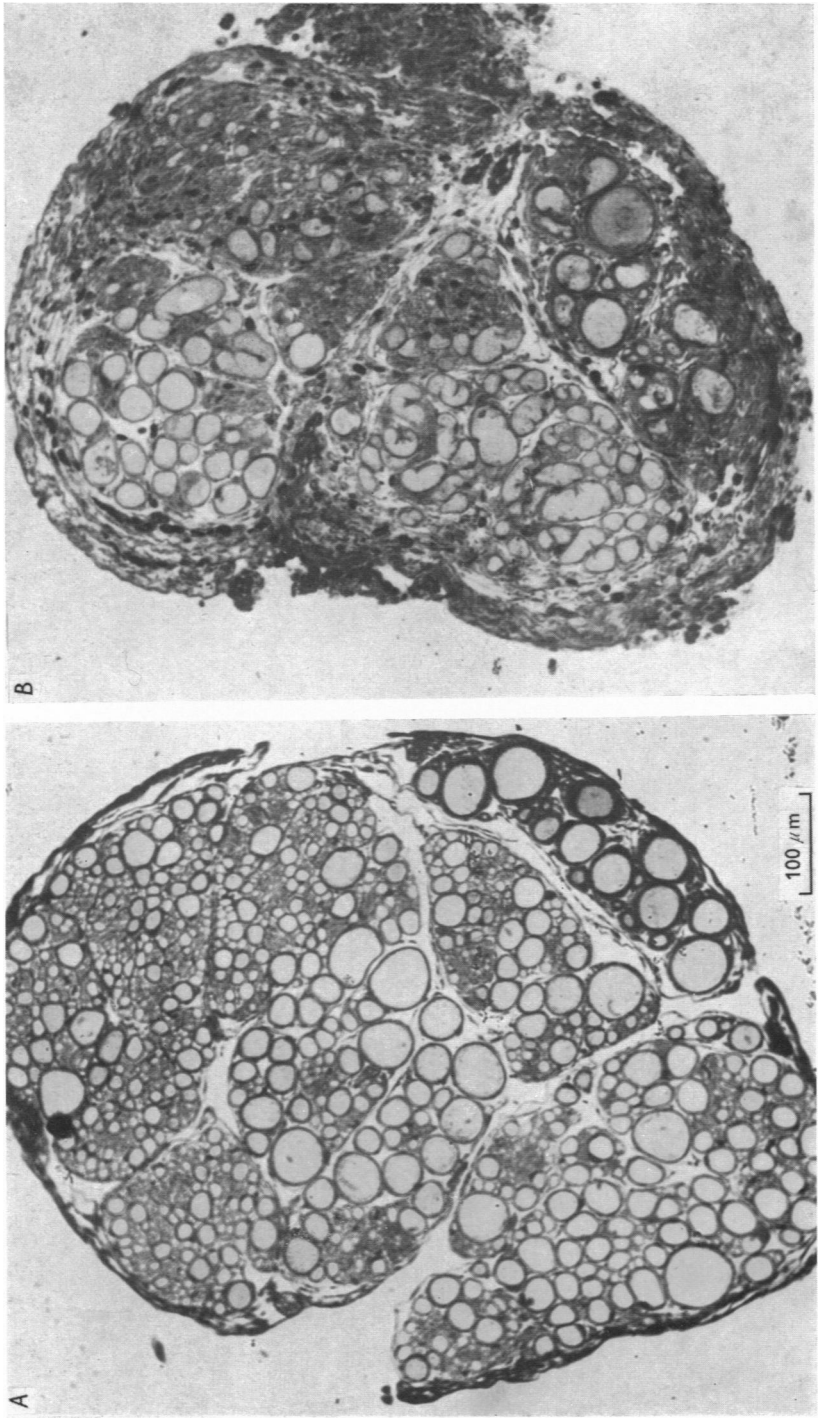

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EXPLANATION OF PLATE

Transverse sections through second roots of control (unoperated) ganglia (*A*) and central second root stumps of experimental (operated) ganglia (*B*) 35 days after axotomy. Histological procedure is described in Orkand & Kravitz (1971).