Studies on the Distribution of Factor I and Acetylcholine in Crustacean Peripheral Nerve

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ABSTRACT Extracts of whole nerve (chelipeds of *Cancer magister*) cause inhibition of impulse generation of the crayfish stretch receptor preparation, similar to that produced by gamma-aminobutyric acid (GABA). This is not found with extracts containing only sensory or sensory and motor fibers. Extracts of inhibitory fibers inhibit the stretch receptor discharge—indicating an inhibitory action equivalent to that of up to 30,000 micrograms of GABA per gm. wet weight of inhibitor fiber. This high value is taken as an indication that the inhibitory substance in crab inhibitory fibers is not identical with gamma-aminobutyric acid.

Whole nerves were found to contain 1.7 to 6.7 μg. acetylcholine per gm. nerve tissue (clam ventricle and frog rectus abdominis muscle). No acetylcholine could be detected in extracts of motor and inhibitory fibers. The acetylcholine content of sensory fibers can account for the acetylcholine activity of whole nerve extract.

It is concluded that the factor I of crustacean nerve is an exclusive property of the inhibitory fibers. The results support the assumption that factor I is the transmitter substance of inhibitory neurons in these animals.

The absence of acetylcholine in motor fibers indicates that this substance does not function as a transmitter of motor impulses in Crustacea, and explains the previously observed failure of the substance to elicit motor responses in these animals. The function of acetylcholine in sensory fibers is not yet clarified.

INTRODUCTION

At present we know that factor I extracted from mammalian brain imitates the action of inhibitory neurons in Crustacea (Florey (11–13)); and that it is present in the crustacean central and peripheral nervous system (11, 13–17). We also know that the action of factor I on crayfish stretch receptors and on the crayfish heart is blocked by low concentrations of the alkaloid

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picrotoxin (8, 13) and that this drug also prevents the action of inhibitory fibers on the ganglion (13) and somatic muscle (31). The results have been interpreted as evidence for the transmitter function of factor I in Crustacea (11–13).

In vertebrates it was shown that factor I blocks synaptic transmission (16, 17) and that its action could be prevented by strychnine (17).

Since the preliminary identification of factor I with gamma-aminobutyric acid (Bazemore, Elliott, and Florey (2)) the latter compound has been considered as inhibitory transmitter in mammals (Iwama and Jasper (21)) and Crustacea (Van der Kloot, *et al.* (31), Van der Kloot and Robbins (32), Robbins (29)). See also Edwards and Kuffler (7). Studies of McLennan (24, 25) and Florey and McLennan (18) had, however, indicated that the chief component of factor I is different from gamma-aminobutyric acid. The studies of Purpura, Girado, and Grundfest (26, 28) and of Curtis and Phillis (5) indicated that gamma-aminobutyric acid is not the inhibitory transmitter substance in vertebrates and Kuffler and Edwards (23) now feel that it is not the transmitter substance of inhibitory neurons in Crustacea. Recent studies of Chapman and Florey (4) and our own studies show that factor I of Crustacea is not identical with gamma-aminobutyric acid and that the latter does not even occur in crustacean nerve tissue.

Studies on the distribution of factor I in mammalian central nervous system have shown that it is restricted to certain cell groups and fiber tracts, and the result indicated the presence in mammalian brain of neurons which are characterized by their factor I content. They were named I-neurons (Florey and Florey (15)). In view of the fact that it appeared possible to isolate single inhibitory fibers from crustacean peripheral nerve it seemed very important to investigate the problem of localization of factor I still further by attempting to find out whether it is an exclusive property of inhibitory neurons or whether it occurs also in other types of nerve fibers.

In spite of the fact that Crustacea are ideal experimental animals for studies of nerve and muscle physiology, the problem of the chemical transmission of nerve impulses in these organisms is still very little understood. It seemed very important to take up this question anew, not only because most of the evidence for the actions of factor I was obtained with crustacean preparations, but also because the earlier findings of the presence of acetylcholine and cholinacetylase in peripheral nerve (6, 34) and of the complete insensitivity of crustacean muscle to acetylcholine offer a challenge to our concepts of chemical transmission in general and of cholinergic transmission in particular.

It is the purpose of the present study to establish the distribution of factor I and of acetylcholine in peripheral nerve of Crustacea and to find indications as to their function.

Methods

Most nerve tissue was obtained from *Cancer magister* Dana, but in a few experiments in which tissue of *C. productus* was used similar results were obtained. The animals used a carapace width of 5 to 7 inches. They were dredged in Bellingham Bay, near Bellingham, Washington, and kept in the laboratory in large tanks with running, refrigerated, filtered, and aerated sea water.

Bioassays were carried out on stretch receptor preparations of crayfish (*Astacus trowbridgii* Stimpson, *Cambarus virilis* Hagen), on the isolated ventricle of the heart of the clam (*Protothaca staminea* Conrad), and on the rectus abdominis muscle of the frog (*Rana pipiens* Schreber). *Astacus* were obtained from the Columbia River near Portland, Oregon; *Cambarus*, from Oshkosh, Wisconsin. These animals were kept in a concrete tank in shallow running and aerated tap water. The clams were obtained from the Pacific Coast north of Bellingham. They were kept in a wooden tank in running, refrigerated, filtered, and aerated sea water. Frogs were obtained from Oshkosh, Wisconsin, and stored at +2°C. The experimental animals were exposed to room temperature 24 hours prior to the experiment.

Assays on the crayfish stretch receptor for factor I were carried out in the manner described by Elliott and Florey (8). The isolated ventricle of *Protothaca* was used for bioassay of acetylcholine according to Welsh and Taub (36) and Welsh (35). The heart was mounted in a small muscle bath (capacity 2 ml.) and connected to a balanced light heart leved for recording on a kymograph. The bath was aerated and maintained at 17°C. in a constant temperature water bath. Filtered sea water equilibrated at 17° was used as a bathing medium. To the 1.8 ml. of sea water in the bath 0.2 ml. of the sample to be tested was added with the aid of a 1 ml. tuberculin syringe. The method of Chang and Gaddum (3) was also used for acetylcholine assays on the frog rectus abdominis, the extracts being added to a bath of eserinized Ringer solution (final volume 4 ml.) in a manner similar to that used with the clam heart.

In certain tests the following drugs were used: gamma-aminobutyric acid (Merck), atropine sulfate (Norwich), mytolon chloride (Sterling Winthrop), picrotoxin (Fisher), and LSD-25 (Sandoz). Acetylcholine was used in the form of acetylcholine chloride (Merck), and acetylcholine values given in this paper are expressed in terms of micrograms of acetylcholine chloride.

The identification and extraction of nerves were carried out in the following manner: chelipeds were removed from the live crabs and the nerves were exposed by cutting through the shell on both sides of the anterior surface of the meropodite, carpopodite, and proximal propodite and by removing the anterior portion of the shell together with the attached tendons and their muscles. At the level of the carpopodite the nerve splits, some of its bundles continuing to the right and some to the left of the larger closer muscle of the claw. The course of the motor and inhibitory fibers was first followed by observing the results of electrical stimulation through fine platinum wire electrodes. When the position of the nerve fibers was ascertained, staining revealed more precisely the anatomical arrangement. This was done by removing as much of the connective tissue as possible from the area and immersing

the claw for 30 minutes in a 0.05 per cent solution of methylene blue. The claw was then removed from this solution and rinsed in sea water. After the claw had been allowed to rest in moist air for about half an hour, the fibers could be clearly seen.

The two motor fibers of the closer muscle are found in one bundle, the inhibitor in another which is widely separated from the first up to the first branch point of the fibers in the carpopodite (see Fig. 1). Each of these two bundles also contains a number of sensory fibers. The nerve bundles are held together by rather tough connective

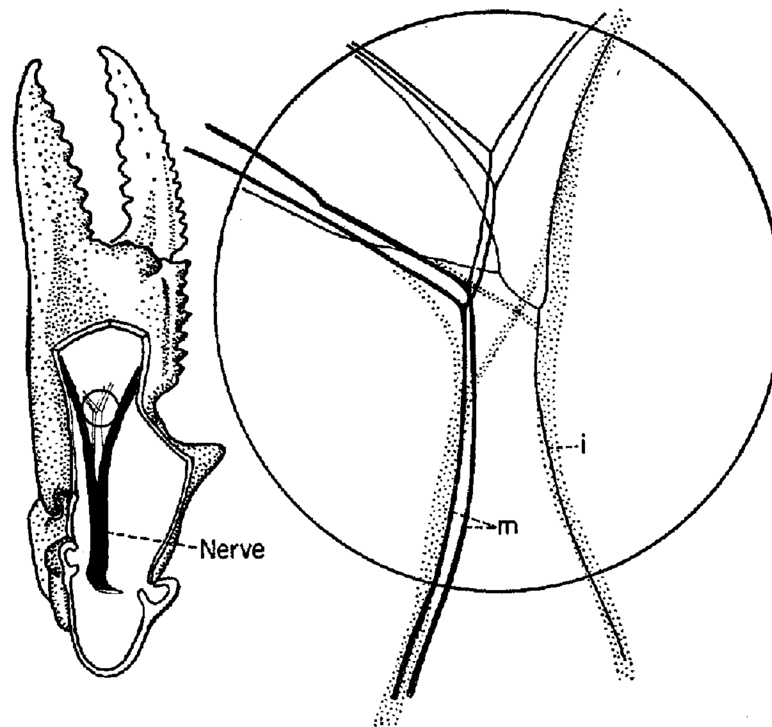

FIGURE 1. *Cancer magister*. Right cheliped, opened to show the position of nerve fibers and nerve bundles *in situ*. The encircled area is enlarged to show the course of motor fibers and of the inhibitory fiber used for isolation and extraction. Sensory bundles are indicated in stipple.

tissue. This was, however, considerably softened by the application of a 1 per cent solution of pancreatin (Merck) over a period of 10 to 15 minutes. After such "digestion" it was possible to separate and isolate thin nerve bundles containing only sensory and motor fibers, a bundle containing the inhibitory fiber of the closer muscle, and bundles containing only sensory fibers. It should be stated at this point, that we consider all those fibers as sensory fibers which are not motor or inhibitory fibers.

Once the anatomical arrangement was established it was not difficult to isolate small nerve bundles as described above, without having to resort to staining with

methylene blue. After their removal the nerve bundles were quickly weighed on an analytical balance and dropped into small volumes of boiling distilled water in homogenizing tubes. One minute later the tubes were removed from the boiling water bath and the samples were carefully homogenized. They were then made up to a known volume by the addition of distilled water. The homogenates were kept in the frozen state until used if they were not used for bioassay at once.

Before they could be tested on the stretch receptor preparation the extracts had to be made isotonic with the saline medium used (Elliott & Florey (8)). This was simply done by adding to 9 volumes of extract (e.g., 0.45 ml.) 1 volume of ten times concentrated saline medium. If the extract was more concentrated than 1 part (weight) of tissue in 19 parts (volume) of water, potassium-free saline was used, and extracts of 1:20 to 1:40 were made up with concentrated saline medium containing half the normal amount of potassium. For bioassay on the clam heart the extracts were made isotonic by adding 1 volume of five times concentrated artificial sea water to 4 volumes of extract. The concentrated artificial sea water had the following composition: NaCl 133.27 gm., KCl 3.36 gm., $CaCl_2$ 8.09 gm., $MgCl_2 \cdot 6H_2O$ 52.87 gm., H_2O to 1 liter. In the proper dilution this solution could maintain a regular heart beat for several hours.

In a number of experiments we exposed the nerve bundles of the left and right chelipeds of the same crabs and stained one of them with methylene blue, leaving the other unstained. Extracts were prepared from the stained and unstained nerves and assayed for acetylcholine and factor I. In no case did we find differences in the acetylcholine or factor I content of stained and unstained nerve bundles. This result made it possible to use stained nerves for extraction. With the aid of methylene blue staining we were able to dissect pieces of single motor and inhibitory fibers free from most of the accompanying sensory fibers and connective tissue. In some cases there were a few sensory axons removed with the motor fibers. The proportion of sensory structures to motor axons varied from an estimated 5 to 30 per cent. The weights of the dissected fibers were estimated from optical measurements of length and diameter. The estimates were based on the assumption of a cylindrical shape of the axons and of a density of 1.2. The diameter of the inhibitory fibers was consistently less than that of the motor fibers. The former had diameters ranging from 10 to 20 micra while the diameters of the latter were 35 to 50 micra before the first branching point. The quantities of nerve fibers used for extraction ranged from 0.8 to 30 micrograms.

RESULTS

A. WHOLE NERVE In preliminary experiments we extracted whole peripheral nerves. Assays on the crayfish stretch receptor preparation indicated a factor I activity equivalent to that of 90 to 166 micrograms of gamma-aminobutyric acid (GABA) per gm. of fresh tissue. The assays were complicated by the presence of excitatory material in the extracts. If these were diluted beyond a concentration which caused inhibition of impulse generation, marked excitation was noted. Impulse generation in most of our

stretch receptor preparations was blocked for 10 seconds by a concentration of 3 micrograms of GABA per ml. Peripheral nerve extract caused similar inhibition up to a dilution of 20 to 33 mg. per ml. If the stretch receptor preparations are treated with picrotoxin 10^{-4} gm./ml. the inhibitory action of peripheral nerve extract disappears and the effect is excitatory in nature. This excitation is so strong that blockade of impulse generation (the "overstretch" phenomenon of Wiersma *et al.* (39) occurs after the initial burst of high frequency spikes. Excitation was caused by extract concentrations as low as 0.5 mg. of tissue per ml. of saline medium.

Excitation was much more pronounced after the stretch receptor muscles had been stretched and receptors had been allowed to adapt to this state than when the preparation was in a more relaxed condition. For example, an extract which on an unstretched preparation did not affect the impulse frequency when applied in a concentration of 1:200, would raise the frequency from 15 to 65 impulses per second when applied in the same concentration to a preparation which had been stretched and allowed to adapt. This change in responsiveness to the excitatory principle in the extract was readily reversible by returning the muscle to a relaxed condition. If the receptor muscles were cut, only negligible excitation was produced even by more concentrated extracts.

The excitation produced by nerve extracts is not due to their acetylcholine (ACh) content for the following reasons:

1. Excitations similar to those produced by nerve extracts (10 mg./ml.) can only be achieved with very high concentrations of ACh (*e.g.*, 0.2 to 1.0 mg./ml.). According to our assays the ACh content of peripheral nerve of *Cancer magister* is equivalent to 1.7 to 5 micrograms per gm. The amount of ACh in the applied extracts could therefore have been only one-hundredth or less of that needed to produce appreciable excitation.
2. Atropine, which effectively blocks the action of ACh on the crayfish stretch receptor (Wiersma *et al.* (39), is ineffective in preventing the excitation produced by nerve extracts.
3. In contrast to the nerve extracts, ACh is very effective in "relaxed" preparations.

Bioassay on the clam heart indicated that the ACh content of whole peripheral nerve was equal to 1.7 to 5 micrograms of ACh per gm. Since Welsh and Taub (37) and Welsh (35) found that mytolon effectively blocks the action of ACh on the hearts of various clams, we have applied mytolon to the isolated ventricle preparation of *Protothaca* in order to prevent the action of ACh and in order to find out whether or not the inhibitory action of extracts on this organ is due to ACh. Mytolon was applied in a concentration of 10^{-5} gm./ml. The results obtained in a typical experiment are shown in

Fig. 2. If maintained at this concentration this drug did reduce the sensitivity of the preparation to ACh about 1000-fold. In the presence of mytolon the

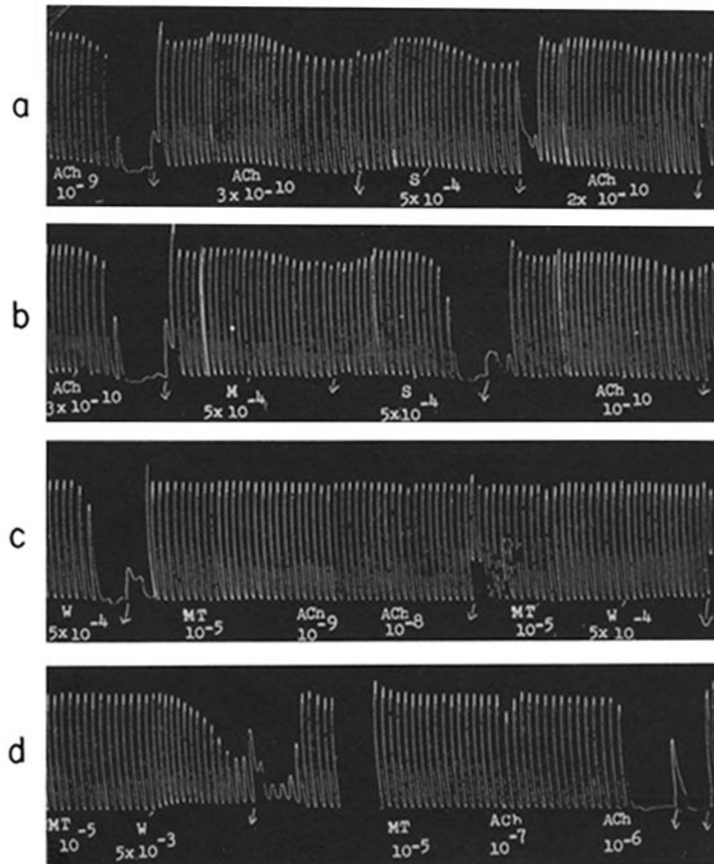

FIGURE 2. Action of nerve extracts and drugs on the isolated ventricle of *Protothaca staminea*. The records (*a*) and (*b*) were taken about 1 hour apart. Note the threefold increase in sensitivity to acetylcholine (ACh) between (*a*) and (*b*). In both records the effect of an extract of sensory bundles (*S*) 5×10^{-4} gm./ml. is similar to that of 3×10^{-10} gm. ACh/ml. Note the difference between the actions of equal concentrations of extracts of motor bundles (*M*) and of sensory bundles in (*b*).

Record (*c*) shows that the action of a dilute extract of whole nerve (*W*) as well as that of higher concentrations of ACh is blocked by the presence in the bath of Mytolon (*MT*) (10^{-5} gm./ml). Record (*d*) shows that *MT* blocks the action of as much as 10^{-7} gm. ACh/ml. but does not prevent the inhibitory action of a more concentrated nerve extract (*W*).

action of nerve extracts was diminished to 1 to 10 per cent of the original action. Extract concentrations which previously had completely stopped the heart beat became entirely ineffective; but ten to one hundred times stronger

concentrations caused a gradual decrease in amplitude and frequency; or, in some cases, caused an immediate diastolic arrest. This mytolon-resistant effect could not have been due to ACh, since the action of the amount of ACh present even in the most concentrated extracts would have been blocked by mytolon. We did not attempt any quantitative evaluation of this effect.

We have repeatedly assayed the same extracts on the clam ventricle and on the frog rectus abdominis muscle. The values obtained were always similar and fell within the limits of experimental error. We have furthermore observed that when the sensitivity of either test preparation to ACh changed, the sensitivity to the extract changed equally. Tenfold increases in sensitivity to ACh during an experiment were not infrequent. The fact that mytolon

TABLE I

ACH CONTENT OF WHOLE NERVE AND OF SENSORY BUNDLES OF CHELIPEDS OF CANCER MAGISTER IN MICROGRAMS/GRAM WET WEIGHT OF NERVE TISSUE

Whole nerve	Sensory nerve	Remarks
4.0	—	
2.5	2.5	Heterolateral
5.0	5.0	Homolateral
3.0	—	
2.1	2.2	Homolateral
5.2	—	
5.5	—	
1.7	—	
3.3	3.6	Heterolateral
6.7	6.9	Homolateral
2.0	1.9	Homolateral

blocks most of the inhibitory activity of extracts on the clam heart, and that the relative effectiveness of ACh and nerve extract on the clam ventricle and on the frog rectus abdominis muscle is the same, shows that crustacean peripheral nerves do indeed contain ACh and that the amounts present are adequately measured by the bioassay methods used.

B. NERVE BUNDLES We have repeatedly compared the ACh content of sensory bundles with that of the whole nerve of the contralateral cheliped, or we compared sensory bundles from the distal portion of one nerve with proximal portion of the whole nerve of the same cheliped. In all cases the amount of ACh per unit weight was higher in the sensory bundles than in whole nerve. The data, as given in Table I, show relatively wide variation. This is probably due to occasional incomplete removal of all nerve bundles and to the consequent alteration of the proportion of motor and inhibitory fibers to sensory fibers. No attempt was, however, made to determine the number of sensory, motor, and inhibitory fibers present in the whole nerve samples.

When the ACh content of sensory bundles was compared with that of small bundles containing sensory fibers and motor fibers, or sensory fibers and an inhibitory fiber, the differences were always conspicuous, particularly with the "motor bundles" (see Figs. 2 and 3). From the observations it appeared that the ACh content of nerve bundles varied with the proportion of sensory fibers in the extracted bundles. This assumption was confirmed in experiments in which extracts from single fibers were analyzed, as will be described in the next section.

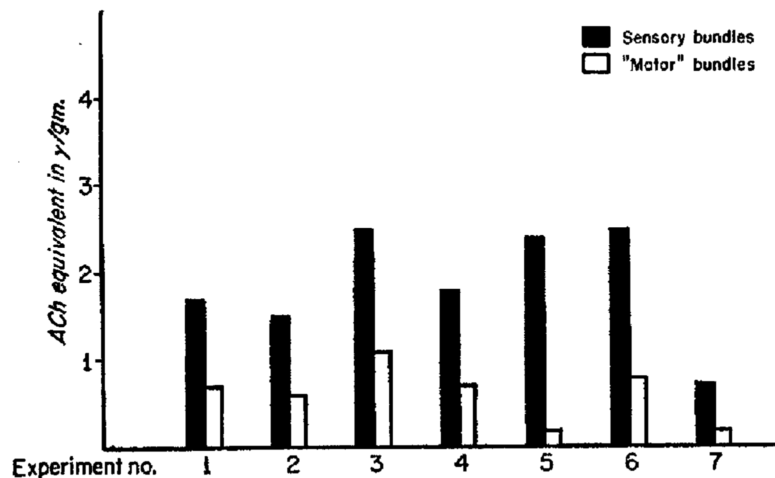

FIGURE 3. Acetylcholine content of sensory and "motor" bundles of peripheral nerve of *Cancer magister*, as determined in bioassays on isolated ventricles of *Protothaca staminea*. In each experiment the two types of bundles were dissected from the same cheliped.

Tests on the crayfish stretch receptor preparation had the following result: sensory bundles and bundles containing sensory and motor fibers never caused inhibition of impulse generation indicative of the presence of factor I. They did, however, cause marked excitation in dilutions as low as 0.2 to 1.0 mg./ml. The excitatory action was much more pronounced with extracts of pure sensory bundles.

Nerve bundles containing the inhibitory fiber to the closer muscle of the claw always caused inhibition of impulse generation in concentrations of 1 to 10 mg./ml. The inhibition was more pronounced if the extracts were tested on "relaxed" preparations. This indicates the presence of some excitatory material similar to that responsible for the excitation caused by whole nerve extract, and by extracts of sensory and sensory-motor bundles.

The inhibitory action of extracts containing the inhibitory fiber was blocked by picrotoxin. The drug was applied in a concentration of 5×10^{-4} gm./ml. for a few seconds. The blocking action persisted through repeated washing of the preparation with saline medium.

C. SINGLE FIBERS As mentioned before, we succeeded in isolating single motor and inhibitory fibers. Fibers of several preparations had to be pooled in order to amount to enough material for extraction. Bioassays on the clam heart for acetylcholine indicated that these fibers contain no acetylcholine or less than was detectable by our method. We can state that motor fibers and inhibitory fibers contain less than 0.1 microgram of acetylcholine per gm. fresh weight.

Tests on the crayfish stretch receptor for the presence of factor I have shown that extracts of the inhibitory fiber in concentrations as low as 1:10,000 could inhibit impulse generation. This effect is blocked by picrotoxin. If expressed in terms of the activity of gamma-aminobutyric acid, the tests indicate an inhibitory activity equivalent to that of 10,000 to 30,000 micrograms of GABA gm. wet weight of inhibitory fiber.

Extracts of motor fibers failed to show any effect on the stretch receptors. Due to the fact that these extracts were of relatively low concentration, we can only conclude that motor fibers contain less than 5 per cent of the inhibitory activity found in a corresponding amount of inhibitory nerve fiber; and that the excitatory material present is less than 10 per cent of that found in an equal amount of pure sensory fibers. It is very well possible that motor fibers contain no material inhibitory to the stretch receptor organ.

One would, however, expect that motor fibers contain a material capable of exciting the stretch receptor organ by stimulating the muscle, provided they normally operate by means of chemical transmission. If such material was present in our extracts, it was certainly not concentrated enough to induce an excitatory effect.

DISCUSSION

A. FACTOR I The main mass of the peripheral nerves studied consists of sensory fibers (see also van Harreveld and Wiersma (20). The distal portion of the nerve of the crab chelipeds which we have analyzed contains only three motor fibers and three inhibitory fibers (two of which are branches of one axon (see Wiersma and Ripley (40). On the basis of optical measurements and weight determinations we estimate the weight of the motor fibers and inhibitory fibers to be less than 20 per cent of that of the whole nerve. The three inhibitory fibers thus constitute less than 10 per cent of the weight of the nerve. Repeated assays for the factor I content of whole peripheral nerve (distal portion) indicated maximum values equivalent to the activity of 166 micrograms of GABA per gm. of nerve. Assays of single inhibitory fibers indicated activities equivalent to as much as 30,000 micrograms of GABA per

gm. of nerve fiber. The factor I content of the inhibitory fibers, therefore, more than accounts for the factor I content of whole peripheral nerve.

From the assays of extracts of pure sensory bundles we can conclude that the factor I content of sensory fibers is less than 1 per cent of that of the inhibitory fibers.

The quantities of motor fibers available for extraction were not large enough to allow application of extracts more concentrated than 1:2000. Consequently, we can only state that motor fibers contain per unit weight less than 20 per cent of the factor I content of inhibitory fibers.

It appears likely that factor I is an exclusive property of inhibitory neurons, and that it is absent in sensory and motor fibers. This means that factor I is not a general constituent of nerve cells or of cells accompanying nerve cells; thus it is likely to have a very specific function in those neurons in which it occurs. Since this factor I imitates the action of inhibitory fibers and since the release from inhibitory fibers of an inhibitory substance which, like factor I, is blocked by picrotoxin could be demonstrated (13), our new findings add further weight to the assumption that factor I is indeed the transmitter substance of inhibitory neurons, at least in Crustacea.

Factor I cannot be identical with GABA. If the inhibitory action of extracts of inhibitory fibers would have to be accounted for by the occurrence of GABA in these fibers, this compound would have to be present in quantities of up to 30,000 micrograms per gm. nerve fiber. Thus 3 per cent of the wet weight of these neurons or from 9 to 15 per cent of the dry weight of these neurons would have to be GABA. It is more reasonable to assume that the inhibitory substance of Crustacea is more potent than GABA. Chapman and Florey (4) have recently been able to isolate the crustacean factor I chromatographically and found it to be a single substance, different from gamma-aminobutyric acid.

B. ACETYLCHOLINE The fact that all the acetylcholine of peripheral nerve is localized in sensory fibers is somewhat surprising. The findings fit, however, very well the fact that ACh does not cause contraction, and that such drugs as eserine, atropine, or curare are without effect on neuromuscular transmission, in crustacean leg muscles (9). Earlier reports (Florey and Florey (14)) that injection under pressure of ACh into crayfish claws causes rapid and transient closure, can possibly be explained by assuming that this substance stimulates sensory cells and that their axons ephaptically transmit their excitation to motor axons (see Florey (10)). The presence of ACh in sensory fibers, does not, however, establish the cholinergic nature of these neurons. As Bacq (1) says, "C'est une grave erreur de croire démontrée l'existence de nerfs cholinergiques, quand on a mis en évidence la présence d'acétylcholine et de cholinestérase dans un organe." Until other criteria for

a transmitter function of acetylcholine of sensory fibers are demonstrated, the function of the acetylcholine of sensory neurons of Crustacea remains unknown.

It should be pointed out that the reported failure of ACh to affect central synaptic transmission in Crustacea (Schallek and Wiersma (30, 31)) may be due to the fact that only the action on synapses between giant fibers and motor axons was studied. These synapses are simply points of contact between giant and motor axons (Johnson (22); Wiersma (38)) and as Furshpan and Potter (19) have recently shown, chemical transmission is unlikely to occur at these specialized synapses. The problem of a possible cholinergic mechanism for the transmission of sensory impulses in the central nervous system deserves new investigation.

The results of our study demonstrate that functionally different nerve fibers (*e.g.*, motor, sensory, and inhibitory fibers) differ in their neurochemistry.

Our studies provide furthermore the answer to the riddle of the presence of acetylcholine in peripheral nerve of crustacea and end the long discussion of whether or not neuromuscular transmission in Crustacea is a cholinergic phenomenon. It is not.

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