GAMMA-AMINOBUTYRIC ACID AND OTHER BLOCKING COMPOUNDS IN CRUSTACEA

III. THEIR RELATIVE CONCENTRATIONS IN SEPARATED MOTOR AND INHIBITORY AXONS

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INTRODUCTION

In the two preceding papers it was reported that gamma-aminobutyric acid (GABA) was the most active of ten blocking substances extracted from the nervous systems of lobsters and crabs. The concentrations of GABA in several peripheral nerves were measured and found to be highest in a nerve that contained only one motor and one inhibitory axon. It was natural to wonder if GABA was specifically concentrated in the inhibitory axon. In the present study, therefore, we have isolated individual axons and found, within the limits of sensitivity of our enzymic assay, that motor fibers contained no GABA while inhibitory neurons contained surprisingly high concentrations. Other blocking compounds were also extracted from separated motor and inhibitory axons, but in contrast to GABA, these are present in both neuron types, as is the precursor of GABA, glutamic acid.

The current studies strongly suggest that GABA has a specific physiological role confined to inhibitory neurons.

METHODS

Two types of experiments were performed. The first was to compare enzymically the GABA contents of isolated motor and inhibitory axons and the second was to compare chromatographically the concentrations of other blocking compounds.

Isolation of single motor and inhibitory axons. Long stretches of isolated fibers were most easily obtained from a small nerve bundle in the meropodite (Fig. 1A in ref. 18). This bundle contains two prominent axons, one motor and one inhibitory, running side by side unbranched for 90–110 mm. (in 8- to 12-lb. lobsters) with a group of smaller sensory fibers. The motor fiber innervates the stretcher of the carpopodite and the opener (abductor) of the dactyl in the walking leg. The inhibitory axon also innervates the stretcher of the carpopodite but beyond that muscle it separates from the motor axon to supply the closer (adductor) of the dactyl (28). The axons are of similar and uniform diameter (50–60 μ) during their course in the meropodite. The tissue around them and the accompanying smaller nerve fibers were removed and the two axons separated and identified by electrical stimulation through fluid electrodes, noting excitation or inhibition of the innervated...
muscles (8). During dissection and separation most of the adhering connective tissue was removed (Fig. 1). Each dissection took about 3 hours and was performed at 6°-12° C. in unbuffered saline containing (mM/liter) NaCl 462, KCl 15.6, and CaCl2 25.9. Several lengths, each up to 120 mm., of the two fiber types were pooled to obtain sufficient material for the analyses (Table 1). Our emphasis lies in comparing the relative amounts of certain materials contained in fibers of similar diameter and of similar length. As far as possible the handling and chemical treatment of fibers were identical.

For GABA assay, the separated fibers were homogenized in ice-cold 5% trichloroacetic acid or 0.5 M acetic acid and assayed as described below. For blocking compounds other than GABA the axons were homogenized in 0.5 M acetic acid, and after centrifugation the supernatant fluid was applied to a continuous flow electrophoresis apparatus as described in the preceding paper (18). The resulting physiologically active regions, B1, B2, and B3 (18) were fractionated further by ascending paper chromatography in the solvent butanol-acetic acid-water (60-15-25). Region E was chromatographed in the solvent ethanol-acetic acid-water (160-15-25) at 4° and two strips, one containing betaine, the other aspartic acid plus glutamine, were eluted, the first for physiological assay, the second for further fractionation (paper chromatography in butanol-acetic acid-water, and phenol-water (70-30); for further details on the methods, see first paper, 7).

GABA assays were also done on the pairs of motor and inhibitory fibers which innervate the opener muscle of the dactyl of the walking leg of lobsters (Fig. 1B, ref. 18). No other fibers are known to accompany these two axons in their course over the inner surface of the muscle. The axons were separated for a few millimeters and then stimulated with a fluid electrode. In some experiments recordings were made from a muscle fiber with an intracellular electrode to observe the resulting excitatory or inhibitory junctional potentials. In other experiments the excitatory nerve alone was identified simply by observing muscular contractions. The two fibers were then pulled apart with whatever connective tissue adhered to them, extracted, and treated in the same way as the fibers from the meropodite. There were a number of uncertainties in these dissections. There was a possibility of false identification through escape of the stimulus from the fluid electrode to the fiber lying nearby. Furthermore, when many short lengths of axon had to be pooled for the spectrophotometric enzymic assay (Table 2, exp. 1-4), the chances were increased that segments of inhibitory axon were mistakenly placed in the excitatory pool. Moreover, branches of the two fibers sometimes ran in the main connective tissue sheath for a distance (Fig. 2, ref. 18), and there was a chance that they did not separate properly with the parent stems. Separation was also more difficult than in the meropodite due to the relatively small size and tapering diameters towards the periphery; the range of diameters of isolated fibers in various sizes of lobsters was 40-15 μ. The method was improved by increasing the sensitivity of the enzymic assay until single axon segments of 30-40 mm. could be used. But, in contrast to the meropodite dissection, the possibility of contamination of one fiber with fragments of the other remained.

Enzyme assays for GABA. When sufficient material was available, assays were performed spectrophotometrically as described in the preceding paper (18). If less than 10⁻⁹ moles of GABA was expected, a fluorimetric procedure was used. The enzyme preparation was the same as that in the spectrophotometric method, but the reduced nicotinamide adenine dinucleotide phosphate (NADPH) was measured by chemical conversion to its oxidized form (NADP) which was measured fluorometrically. The techniques were based on the procedures of Lowry and co-workers (22, 23) and were similar to the GABA assay described by Hirsch and Robins (17). Assay tubes contained enzyme, NADP (9 X 10⁻⁴ μmoles), β-mercaptoethanol (0.09 μmole), α-ketoglutarate (9 X 10⁻⁴ μmoles), tris buffer, pH 7.9 (1.8 μmoles), and experimental samples in 12-μl volumes. Zero-time controls (to which α-ketoglutarate had not been added) were run with experimental samples to establish tissue blank levels of fluorescence, and five GABA standards (within the range 2.5 X 10⁻¹⁰ moles to 1.25 X 10⁻⁹ moles) were run with each assay. Incubations were for 45 min. at room temperature except for zero-time controls. The reactions were ended, and excess NADP destroyed, by pipetting 10 μl of the samples into 50 μl of 0.25 M NaHPO₄·0.35 M K₂HPO₄ buffer, and heating at 60° for 15 min. One hundred microliters of 10 n NaOH containing 0.03% H₂O₂ was added and the tubes were heated at 60° for 10 min. Finally, 1 ml of water was added and the fluorescence measured in a Farrand fluorometer, model A, with a Corning 5860 primary filter (365 μm) and a Baird-Atomic B-1 type interference filter (460 μm peak transmission) as the secondary filter.
GABA IN ISOLATED AXONS

RESULTS

GABA content of motor and inhibitory fibers. The results of the analyses of isolated axons from the meropodite region are presented in Table 1. The total material was over 5 m. of axon length. In each case we have compared the GABA content of paired inhibitory and excitatory neurons; these were of similar diameter and were treated in the same way.

No GABA was found in the meropodite excitatory axons; if it was present at all, it was in amounts too small to be detected by the enzymic assay.

Table 1. GABA content of isolated motor (M) and inhibitory (I) axons from the meropodite

<table>
<thead>
<tr>
<th>Nerve</th>
<th>Length, mm.</th>
<th>Separation Technique</th>
<th>GABA, µg/100 mm.</th>
<th>Total GABA Moles</th>
<th>Threshold, † moles</th>
<th>I/M ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-I</td>
<td>200</td>
<td>Columns</td>
<td>1.2</td>
<td>2.35×10⁻⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>370</td>
<td>Columns</td>
<td>1.0</td>
<td>3.61×10⁻⁵</td>
<td>4×10⁻¹⁰</td>
<td>90</td>
</tr>
<tr>
<td>M</td>
<td>350</td>
<td>Column</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>500</td>
<td>Columns</td>
<td>2.4</td>
<td>1.2×10⁻⁷</td>
<td>4×10⁻¹⁰</td>
<td>300</td>
</tr>
<tr>
<td>M</td>
<td>500</td>
<td>Column</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>500</td>
<td>Electrophoresis</td>
<td>1.1</td>
<td>5.3×10⁻⁵</td>
<td>4×10⁻¹⁰</td>
<td>130</td>
</tr>
<tr>
<td>M</td>
<td>500</td>
<td>Electrophoresis</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1,085</td>
<td>Electrophoresis</td>
<td>1.25</td>
<td>1.32×10⁻⁷</td>
<td>1.1×10⁻¹⁰</td>
<td>1,200</td>
</tr>
<tr>
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<td>Electrophoresis</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Separation techniques: columns refers to the Dowex-50-II⁺ techniques described in preceding paper (18); electrophoresis means crude extracts were separated by electrophoresis and GABA regions were pooled and assayed for GABA. † The minimum amounts of GABA which could be detected were 10⁻¹⁰ moles spectrophotometrically and 8×10⁻¹² moles fluorimetrically. The threshold values in the table are higher than this because only part of the sample was used for the assay. ‡ No GABA was detected in the M fiber. This calculation uses the threshold value for GABA (M_T) and represents a minimum ratio of GABA contents.

Therefore, no reliable value can be given to the ratio of GABA concentrations in the two types of fibers. However, it was possible to calculate how much GABA could have been present in the motor fibers and yet have remained undetected by the assay ("Threshold" in Table 1); this amount was dependent on the sensitivity of the assay and the quantity of material used. The threshold value was then compared to the measured GABA content of the corresponding inhibitory fiber to give the minimum value of the ratio of GABA concentrations in the two types of fibers (I/M_T of Table 1). In our most striking experiment, in which the sensitivity of the assay was highest, if GABA was present at all in the motor axons it could not have been more than 1 part in 1,200 of that in the inhibitory fibers. In the other tests, smaller amounts of tissue were used, the assay was less sensitive and therefore the I/M_T values were lower.
In one experiment of this series we also assayed combined motor and inhibitory fibers (no. 1). These contained 1.2 μg. of GABA/100 mm., an amount per unit length similar to the average of inhibitory axons alone.

The GABA contents of isolated axons from the surface of the opener muscle were compared in seven of the eight experiments of Table 2. Assays in the first four experiments were done spectrophotometrically and to obtain

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**Table 2. GABA content of isolated M and I axons from the opener muscle surface**

<table>
<thead>
<tr>
<th>Nerve</th>
<th>Length, mm.</th>
<th>Separation Technique*</th>
<th>GABA&lt;sub&gt;μg&lt;/sub&gt;/100 mm.</th>
<th>Total GABA</th>
<th>Moles</th>
<th>I/M</th>
<th>Threshold&lt;sub&gt;+&lt;/sub&gt; moles</th>
<th>I/M&lt;sub&gt;+&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-I</td>
<td>320</td>
<td>Columns</td>
<td>0.11</td>
<td>3.4 × 10⁻⁹</td>
<td></td>
<td></td>
<td>10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>350</td>
<td>Columns</td>
<td>0.14</td>
<td>4.6 × 10⁻⁹</td>
<td>11.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>340</td>
<td></td>
<td>0.012</td>
<td>4 × 10⁻¹⁰</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1,010</td>
<td>Columns</td>
<td>0.19</td>
<td>1.91 × 10⁻⁸</td>
<td>9.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>1,010</td>
<td></td>
<td>0.02</td>
<td>1.96 × 10⁻⁹</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>300</td>
<td>Columns</td>
<td>0.37</td>
<td>1.07 × 10⁻⁸</td>
<td></td>
<td></td>
<td>10⁻⁹</td>
<td>10</td>
</tr>
<tr>
<td>M</td>
<td>275</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Connective tissue</td>
<td>$</td>
<td>Columns</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>10⁻⁹</td>
<td>10</td>
</tr>
</tbody>
</table>

* No separation, GABA assays were performed directly on crude extracts; columns refers to the Dowex-50-H⁺ techniques described in preceding paper (18). † See Table 1 for explanation of threshold. ‡ See Table 1 for explanation of I/M<sub>+</sub>. § The volume of this tissue was greater than that of the axons.

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sufficient material many short fiber segments (8–20 mm. each from 2-lb. lobsters) were pooled. In the last four experiments with single pieces of individual axons from large lobsters, the fluorometric assay was used. In three cases no GABA was found in motor fibers and the I/M<sub>+</sub> values were 10 and 24. On the other hand, traces of GABA were found in four experiments, including two (no. 7 and no. 8) in which single fibers were assayed. In experiment no. 4 the connective tissue that was removed from the axons was also analyzed but no GABA could be detected. It should also be noted that while
the GABA content of the inhibitory axon of the opener was only 0.1–0.2 μg/100 mm length, approximately one-tenth that of the meropodite axons, the fiber diameter was smaller; in addition, as mentioned earlier (METHODS), a different inhibitory neuron was involved while the motor axon to the opener was the same as the one analyzed in the meropodite.

The results on the opener fibers raise the unexpected possibility that while GABA is absent in a motor axon along its course, it appears toward the periphery of the same cell. However, such a change in the chemistry of a motor axon in respect to GABA should not be considered seriously on the basis of our results because the experimental conditions were not satisfactory.

![Fig. 1. Cross sections of isolated motor (A) and inhibitory (B) axons from the meropodite of the walking leg of a large (10 lb.) lobster. The axons were fixed in Millonig's solution after isolation, embedded in Araldite and stained with toluidine blue. In this sample the motor axon is somewhat better cleaned of connective tissue. The scale is the same for the two fibers. Note that diameter of the motor axon is greater in the meropodite than in its peripheral portion (Fig. 2, ref. 18). The sections were kindly provided by Dr. W. Fahrenbach.](image)

There was a good chance of contamination of the excitatory axons by sections of the inhibitory axons, as already discussed (METHODS). It is hoped that with improved techniques of separation and even more sensitive assay methods the opener nerves may be studied more profitably.

One should note that the isolated fibers we analyzed were still a complex tissue because the dissections left varying amounts of connective tissue and the Schwann cell layer around the neuron membrane as indicated in the histological section of isolated axons in Fig. 1. It seems quite unlikely that connective tissue around motor fibers is different from that surrounding inhibitory axons. As far as Schwann cells are concerned there is no evidence at present that their chemical composition differs around various axon types. We therefore attribute the differences in GABA content to the axoplasm. From
the data of Table 1 one obtains a GABA content of about 0.5 g. of GABA/100 g. of wet wt. of inhibitory axon. The following figures were used for this calculation: average fiber diameter 60 μ; GABA content 1.4 μg/100 mm.; density of axoplasm, 1.0.

Distribution of other blocking compounds in motor and inhibitory fibers. We did not have specific sensitive enzymic assays for the blocking compounds other than GABA (18). Therefore the relative amounts of these compounds were estimated by comparing the density of Ninhydrin-stained spots on paper chromatograms, or, in the case of betaine, by elution from the paper chromatograms and bio-assay. While these techniques are not quantitative, they easily detected marked differences between motor and inhibitory fibers. The results of an experiment with 1.085 mm. of each type of axon are presented in Figs. 2 and 3. The Ninhydrin-stained spots in the principal physiologically active regions (B1, B3, E) are shown for inhibitory (I) and motor (M) fibers along with standard compounds run for comparison. The greatest difference between the two types of fibers occurs in the region B1 to which GABA runs. There is no GABA spot in the motor axon chromatogram. The absence of GABA was confirmed by fluorometric assay of one-tenth of each B1 fraction set aside at the time the remainder was chromatographed (see last experiment, Table 1). A spot-absorbing ultraviolet light was present in the GABA regions of I as well as M fibers (lightly outlined) but the material was physiologically inactive. β-Alanine occurred in both fibers, but in greater concentration in inhibitory than in motor axons (dotted circle). Since there was far less β-alanine than GABA and it is only about 1/50 as active as GABA in our physiological assay, this unequal distribution has not been studied further. There is a prominent spot high on the chromatogram (RF = 0.7) in the inhibitory fiber only. We believe this compound to be the cyclic amide (lactam) of GABA, for it has the RF value and staining characteristics (yellow, slowly turning blue) reported for GABA lactam (27). Presumably it was formed during spotting for chromatography. If this is the case, it is not surprising that we found it only in the inhibitor. The experiment illustrated in Fig. 2 was the only one in which we have seen this fast-moving spot, a fact which strengthens our suspicion that it was an artifact. One of the lower spots in region B1 is the blocking substance "unknown A" (18), a very weakly active guanido compound which is present equally in the two fibers.

The blocking region, B3, was subdivided; the bulk of the taurine was in one fraction, (on right in Fig. 2) the remaining taurine and homarine in the other (left). These blocking compounds were present in about equal concentration in the two types of fibers. The position and relative amounts of homarine on the chromatogram were determined with ultraviolet light (254 mμ). The excitatory regions, E, of M and I fibers were not spotted on the same chromatogram, but streaked along the base lines of two chromatograms for 25 cm. Narrow side strips of these chromatograms were stained with Ninhydrin and are shown in Fig. 3. Glutamate was present in both fibers but more prominent in the motor axon. Aspartate and glutamine (no standard
FIG. 2. Comparison of the Ninhydrin-positive components of regions B1 and B3 (see also Fig. 3, ref. 18) from electrophoretic fractionation of isolated inhibitory (I) and motor (M) axons; ascending paper chromatography in butanol-acetic acid-water (60-15-25). Standard components were not labeled at the base line but were marked on the spots as follows: G, GABA; bA, beta-alanine; H, homarine; T, taurine; UV, absorption of ultraviolet light. Taurine was present in both fractions of B2, homarine in one (left). Areas of ultraviolet light absorption and homarine are outlined. In B1, the outline of the beta-alanine spot in the motor fiber was faint and was dotted in. Solvent fronts and origins are retouched. Note that the large GABA spot appearing in the inhibitor is absent in the motor axon. Both neurons had an UV-absorbing region in GABA area (lightly outlined). The fast-moving spot in the inhibitory fibers is believed to be the cyclic amide of GABA (see text).

illustrated) run together in this solvent, but after elution from the chromatograms they were separated in another solvent and found to be uniformly distributed between the two fibers (not shown). Betaine is not revealed by Ninhydrin but was eluted from the chromatograms and bio-assayed; weak activity was found in both types of fibers. The weak blocking substance
alanine (dotted in) may have been present in higher concentration in the motor than in the inhibitory fiber. A large part of the alanine was in region B2 and is not shown. Ninhydrin-staining of the other regions of the electrophoretically separated extracts failed to reveal any other differences between motor and inhibitory axons.

Therefore, of the ten blocking compounds found in peripheral nerve (see 18) GABA may be the only one which is present exclusively in inhibitory fibers. The other nine are more or less evenly distributed between motor and inhibitory axons, with the possible exception of β-alanine, whose distribution has not been settled with the present material.

DISCUSSION

Widespread interest in GABA was stimulated by the discovery that it occurred in the mammalian CNS in surprisingly high concentrations (for a review see ref. 2). It has been suggested that GABA functions as a key metabolic intermediate in a pathway of glutamate (or α-ketoglutarate) metabolism, bypassing the oxidative decarboxylation of α-ketoglutarate to succinyl coenzyme A. The sequence of enzyme reactions in this pathway is shown in Fig. 4. The enzymes catalyzing these reactions have been demonstrated in extracts prepared from brains of various species (2).

The possible reasons for GABA accumulation in cells have been extensively discussed in the literature (e.g., 17, 25). The relative rates of enzymic synthesis and destruction of GABA and factors which influence these enzymes are considered to be explanations for differences in GABA content of cells. The mechanisms of GABA accumulation are of special interest in the case of crustacean motor and inhibitory axons. At present, the distribution of glutamic decarboxylase, the enzyme which synthesizes GABA, is under study in separated axons.

Physiological interest in GABA was largely sparked by the important findings of Bazemore, Elliott, and Florey (1) that GABA was the principle compound in an extract of the mammalian CNS that blocked the discharges in crustacean stretch receptors. There followed studies by many workers on the pharmacology of GABA and of allied compounds on the vertebrate and invertebrate nervous systems (for reviews of the extensive literature see 4, 9, 14, 21). The physiological aspects of GABA action at synapses could be...
analyzed most thoroughly in invertebrates. In the CNS of crayfish (13) and in peripheral tissues of Crustacea such as the stretch receptor (20, 16) and the neuromuscular junction (14, 15, 19) GABA imitates the postsynaptic action of the inhibitory transmitter. Recently this analogy was extended to presynaptic inhibition in the crayfish (8) and an effect on the impulse spread in motor nerve terminals has been demonstrated (6). The results of experiments with GABA in the mammalian CNS have been less favorable for an assumption that this compound has a specific synaptic action. For instance,

Curtis and his colleagues (3, 4), using electrophoretic application from micropipettes on spinal motoneurons, suggested that GABA as well as many of its analogues acted only as general depressants. In recent studies, however, the effect of GABA on the Mauthner cell, a large neuron in the goldfish medulla, was more striking and apparently more specific. Its action was similar to that of the neural transmitter substance and was confined to certain regions of the Mauthner neuron (5).

It has been proposed by many that GABA is an inhibitory transmitter. This hypothesis had the following weakness: the basic requirement that it be present in crustacean tissues, where it had been extensively studied and where

![Diagram of enzymic formation and further metabolism of GABA.](http://jn.physiology.org/)

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**Fig. 4.** Enzymic formation and further metabolism of GABA.
its mechanism was best known, had not been met. In fact, it was emphatically stated (12) that there was no GABA in the crustacean nervous system. In the mammalian nervous system, on the other hand, where the presence of GABA had been known for a long time, the evidence was far from convincing that GABA imitated the action of the neurally released transmitter. It was natural, therefore, that in several recent symposia and reviews (e.g., 24, 10, 26, 4) the concensus developed that GABA was not a candidate for a role as an inhibitory transmitter.

Some studies that in several respects are similar to ours were made by Florey and co-workers (11, 12). From motor, sensory, and inhibitory axons they obtained extracts which they tested by physiological assay for blocking activity. It is interesting that in inhibitory neuron extracts they found strong blocking activity but none in sensory and motor fibers. One of the major discrepancies between our findings and theirs is that they could not detect any GABA. Further, their inhibitory material (11) was believed to be more active than GABA. They were led to this conclusion because, in order to explain the great activity of their extract, they would have had to assume that “up to” 3% of wet wt. of their inhibitory neurons was GABA. So far we have not been able to find substances more effective than GABA and in fact there may be no need for them to explain the calculated physiological blocking activity in extracts from crabs (11). Our measured GABA content in lobster inhibitory fibers was about 0.5% of the wet wt. GABA actually accounted for only 30–50% of the total blocking activity in our tissues, the rest being contributed largely by taurine and betaine. If these latter substances, as well as GABA, were present in Florey and Biederman’s (11) extracts, much of their blocking activity would be accounted for. In view of the inherent inaccuracies in calculation of fiber weight and in the quantitation by bio-assays, and the fact that different species were used, it is uncertain whether any substantial discrepancies remain. The absence of blocking effects in their motor and sensory extracts (11) was presumably due to the small amount of tissue that was used for the assays and the admixture of excitatory substances.

The present series of studies have a direct bearing on the possible physiological role of GABA in Crustacea only. The following conclusions can be drawn: 1) GABA is a constituent of the peripheral as well as the central nervous system (7, 18). 2) GABA is present in surprisingly high concentration in isolated inhibitory axons. It makes up about 0.5% of the wet wt. along the unbranched course of this neuron. We have no way at present to determine the GABA concentration in the nerve terminal regions. 3) GABA may be confined to inhibitory neurons. On this important point the results are not complete. Principally, we know little about sensory neurons in which a small amount of GABA was found (18). Further, because of technical difficulties, we cannot exclude the presence of GABA in the peripheral portions of motor fibers.

The above conclusions indicate that GABA has a specific role linked with the function of inhibitory neurons. The crucial test that could establish it as
a transmitter has not been made. It would have to be shown that it is released from the inhibitory terminals by the inhibitory nerve impulse in adequate amounts at the appropriate time during the process of transmission. In respect to the other blocking compounds all we can conclude is that their function is not uniquely related to inhibitory neurons since they are present in motor and inhibitory fibers.

As far as the vertebrate nervous system is concerned, an extension of single cell analyses, such as those of Hirsch and Robins (17), to known inhibitory and excitatory neurons would be very interesting.

SUMMARY

1. Two efferent axons of similar diameter, one inhibitory, the other excitatory, run side by side in the leg of the lobster (Homarus americanus). Long unbranched stretches of these neurons were removed and separated; the isolated axons were analyzed for their content of gamma-aminobutyric acid (GABA) and nine other synaptic blocking compounds that had been previously found in the crustacean nervous system (18). The GABA contents were compared enzymically; the contents of the other blocking substances were compared chromatographically or by physiological assay.

2. The GABA content along the course of the inhibitory axon was about 0.5% of its wet wt., while no GABA was detected in the accompanying excitatory fiber. GABA may therefore be confined to inhibitory nerves. The other blocking compounds, with the possible exception of $\beta$-alanine, were found in both neuron types. The distribution of $\beta$-alanine (a much weaker blocking substance than GABA) cannot be stated with confidence.

3. The findings indicate that GABA has a function specifically related to inhibitory neurons.

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