GAMMA-AMINOBUTYRIC ACID DISTRIBUTION IN THE LOBSTER NERVOUS SYSTEM: CNS, PERIPHERAL NERVES AND ISOLATED MOTOR AND INHIBITORY AXONS[1]

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Received March 6, 1962

Gamma-aminobutyric acid (GABA) closely mimics the physiological effect of the inhibitory transmitter compound released at synapses of the crustacean central nervous system (CNS) and peripheral nervous system (PNS) (Boistel and Fatt, 1958; Kuffler and Edwards, 1958; Furshpan and Potter, 1959; Grundfest et al., 1959; Hagiwara et al., 1960; Dudel and Kuffler, 1961). GABA has been isolated from both the PNS and CNS of lobsters and crabs (Kravitz, Potter and van Gelder, 1962) and particulate enzyme fractions prepared from these tissues (from lobsters) catalyzed the formation of GABA from glutamic acid (Kravitz, 1962). The present work was undertaken to compare the amounts of GABA in the various parts of the crustacean nervous system, and to determine whether GABA found in the PNS was present in equal amounts in all types of nerve fibers or occurred in larger amounts in those fibers having an inhibitory action on muscle.

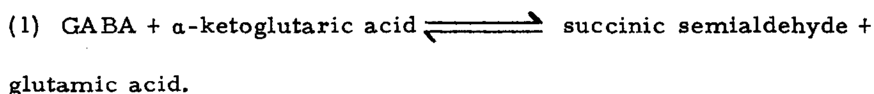
Nerve tissues were taken from lobsters (Homarus americanus); central ganglia from the abdomens, and nerve bundles (containing motor, inhibitory and sensory) from the meropodites of the first 2 pairs of walking legs. Nerve, which was presumed to be only sensory, was obtained along with its associated

1. Supported by grants from the U. S. Public Health Service (B-2252 and B-2253).
2. Special Research Fellow of the NINDB (BF-5899).

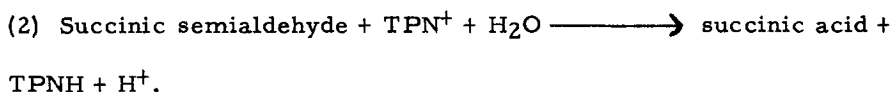
connective tissue(which probably constituted about 80% of the tissue weight) from the dactyls of these legs. The pairs of motor and inhibitory axons which innerva the "opener" muscles of the dactyls were removed together from the surfaces of these muscles. All of these tissues were washed in lobster saline before furthe treatment. In two other experiments the motor and inhibitory fibers were separ from each other by fine dissection; before completing the separation, the two nerves, which were visually indistinguishable from each other, were identified b electrically stimulating first one and then the other while recording the characte istic changes in the membrane potential of a muscle fiber with a microelectrode. After their identities were established, the dissection was completed, and each nerve was removed separately from the muscles and frozen in saline or 10% TCA In the first of these experiments, control lengths of combined axons were remov from the same 42 muscles as the separated fibers (from 1 lb. lobsters). In the second experiment 60 muscles were used (mostly from 2 1/2 lb. lobsters). The separated fibers were stored frozen until sufficient material had been gathered for the assay. An equal volume of 10% TCA was added to the tubes of material frozen in saline, prior to thawing the solutions. The fibers in all the tubes were crushed with a glass stirring rod and the entire solutions were then applied to Dowex-50-H^+ columns as described below. The lengths and diameters of motor and inhibitory fibers were measured for calculation of their volumes and weights. All other nerve tissues were lyophilized, ground to powders and weighed directly The dried tissues were extracted by grinding in cold 5% TCA in Kontes duall glass homogenizer tubes. The resulting suspensions were centrifuged, the supernatant fluids collected, and the precipitates washed with second portions of cold 5% TCA. The two supernatant fluids were pooled.

The TCA extracts from all tissues were applied to Dowex-50-H^+ columns (6.5 cm x 0.503 cm^2). The columns were then washed with 15 ml of

water to ensure the complete removal of acids (such as α-ketoglutaric acid) which interfere with the running of proper controls in the GABA assay, after which the adsorbed compounds were removed with 15 - 20 ml of 1M-NH_4OH. The NH_4OH solutions were taken to dryness at 60°-70° under reduced pressure in a Rotary Evapo-mix (Buchler Instrument Corp.). The residues were dissolved in suitable volumes of water (40 μl to 0.2 ml), centrifuged if necessary, and used directly in the GABA assay. GABA standards carried through similar separation procedures yielded at least 90% recovery with as little as 0.0035 μmoles GABA on a column. The amount of GABA present was determined using a micro adaptation of the enzyme assay of Jakoby and Scott (1959). The enzymes used catalyze the following reactions:



(1) GABA + α-ketoglutaric acid $\rightleftharpoons$ succinic semialdehyde + glutamic acid.



(2) Succinic semialdehyde + TPN^+ + H_2O $\longrightarrow$ succinic acid + TPNH + H^+.

The optical density increase at 340 mμ is directly proportional to the GABA content of a sample when all of the other reactants are present in excess. The two enzymes were extracted from lyophilized cultures of <u>Pseudomonas fluorescens</u> ATCC 13430 (Worthington) and purified through step 4 (transaminase) and step 5 (dehydrogenase) of the procedure outlined by Jakoby (1962). Incubations were carried out in three cuvettes, each containing the unknown sample, TPN (0.1 mg), Tris, pH 8 (5 μmoles), β-mercaptoethanol (0.5 μmole), and transaminase and dehydrogenase enzymes. α-ketoglutarate (0.16 μmole) was added to cuvettes 2 and 3 and a GABA standard (0.002 μmole) was added to cuvette 3. The final volume was 0.1 to 0.12 ml and a micro cell adapter (Pyrocell Mfg. Co.) in a Beckman spectrophotometer was used to measure the TPNH production. The enzymes were added in amounts such that the formation of TPNH was complete

in 20 to 30 minutes and readings were taken for an additional 10 to 15 minutes to check for reoxidation of TPNH or any other, slower, TPNH forming reactions. This method allowed the very accurate measurements of 0.001 μmole (± 5%) of GABA in each cuvette.

As far as is known, β-hydroxy-GABA (GABOB) is the only other compoun which will lead to an α-ketoglutaric-dependent production of TPNH with the enzym used in this assay. GABOB reacts, however, at 1/7 to 1/8 the rate of GABA. Since we saw no further TPNH production after 30 minutes incubation (the time for complete GABA conversion to succinate) in most of our assays, it is unlikely that GABOB is present. The presence of large amounts of GABOB in crustacean nervous tissue can also be excluded on the basis of chromatographic evidence (Kravitz, Potter and van Gelder, 1962). It is possible that the use of the Dowex-50-H^+ columns leads to the formation of GABA through the cleavage of a labile compound containing GABA as part of the molecule. This possibility has not yet been ruled out.

The results of the assays on the first series of experiments, in which motor and inhibitory fibers were not separated, are shown in Table 1. Central ganglia contain about 4 times the amount of GABA present in mixed peripheral nerve (on a dry wt. basis) and about 40 times the amount in sensory nerve. The mixed nerve used in these experiments contains at least 3 inhibitory fibers and about 5 motor fibers, all of which innervate muscles of the leg, and hundreds of sensory fibers per bundle. The bundles containing only the motor and inhibitory fibers from the "opener" muscle (each bundle contained 1 motor and 1 inhibitory axon) had 200 times the concentration of GABA of this mixed nerve and 55 times as much GABA as is present in the central ganglia. In mixed nerves, sensory nerve and motor and inhibitory fibers from crab legs (Cancer borealis) comparab amounts of GABA have been found.

Table 1.

GABA content in various parts of the nervous system of lobsters.

	Dry wt. of sample (mg)	GABA content (μg)	GABA (μg/gm dry wt.)
Central ganglia (abdominal section)	81.4	38.8	480
Mixed nerve	80.1	10.4	130
Sensory	*14.0	*0.12 - 0.2	*9 - 14
Motor & inhibitory	†0.034	0.89	26,200

*GABA was definitely detectable in sensory nerve, but the amount present was below the limit for an accurate assay. The dry wt. of the sample was not corrected for connective tissue.

†Wt. estimated by calculation of the volume of both fibers assuming a density of 1 for axoplasm and dry wt. = 1/6 wet wt.

The results of the experiments in which the motor and inhibitory axons were identified and separated are shown in Table 2. There was a striking difference between the amounts of GABA present in the two types of axons. In the second experiment, where sufficient motor fibers had been gathered to allow an accurate assay, the GABA content of these axons was only 22% that of the inhibitory ones.

Florey and Biederman (1960) separated motor and inhibitory axons in crabs and made the observation that large amounts of biological inhibitory activity were found only in the inhibitory fibers. Florey and Chapman (1961), however, stated that the active compound was not GABA. The most effective (on a molar basis) inhibitory compound that we have been able to extract from crustacean nervous tissue is GABA (Kravitz, Potter and van Gelder, 1962). The physiological effects of the neurally released inhibitory transmitter compound of Crustacea are duplicated by GABA (see references above). The present experiments show that GABA is found in high concentration in peripheral nerves of Crustacea, and that peripheral inhibitory axons contain considerally more GABA than motor axons.

These findings are consistent with the hypothesis that GABA is the synaptic inhibitory transmitter compound of Crustacea.

Table 2.

GABA content of separated motor and inhibitory axons.

	Length of sample (mm)	[†]Calculated dry wt. (mg)	GABA content (μg)	GABA (μg/gm dry wt.)
Motor and inhibitory	320	0.0180	0.34	18,900
Motor	344	0.0065	*0.04	*6,150
Inhibitory	352	0.014	0.46	32,800
Motor	1012	0.052	0.202	3,900
Inhibitory	1011	0.113	1.97	17,400

*The minimal amount of GABA which was definitely detectable was 0.1 μg. Since the motor nerve extract contained less than this amount, the figure cited is not accurate.

†Wt. estimated by calculation of the volume of the fibers assuming a density of 1 for axoplasm and dry wt. = 1/6 wet wt.

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