

# THE METABOLISM OF GAMMA AMINOBUTYRIC ACID IN THE LOBSTER NERVOUS SYSTEM

## Enzymes in Single Excitatory and Inhibitory Axons

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### ABSTRACT

$\gamma$ -aminobutyric acid (GABA) is the inhibitory transmitter compound at the lobster neuromuscular junction. This paper presents a comparison of the enzymes of GABA metabolism in single identified inhibitory and excitatory axons from lobster walking legs. Inhibitory axons contain more than 100 times as much glutamic decarboxylase activity as do excitatory axons. GABA-glutamic transaminase is found in both excitatory and inhibitory axons, but about 50% more enzyme is present in inhibitory axons. The kinetic and electrophoretic behavior of the transaminase activity in excitatory and inhibitory axons is similar. Succinic semialdehyde dehydrogenase is found in both axon types, as is an unknown enzyme which converts a contaminant in radioactive glutamic acid to GABA. In lobster inhibitory neurons, therefore, the ability to accumulate GABA ultimately rests on the ability of the neuron to accumulate the enzyme glutamic decarboxylase.

### INTRODUCTION

There is considerable heterogeneity among nerve cells, which can be recognized in several ways. The most obvious is the variation in geometrical shape. Neurons can also differ in the chemical transmitter compound which they secrete. There are more subtle differences which are shown up by the ability of neurons to recognize and contact other cells specifically. This heterogeneity will depend upon protein differences between the individual cells. To deduce the type of information specified in determining neuronal function it therefore seems desirable to search for and catalog specific protein differences between neurons.

The lobster nervous system is a useful preparation to begin studies in this direction. Biochemical

analyses can be performed on single large nerve cells which can be recognized, physiologically identified, and readily isolated. Moreover, cells serving precisely the same function can be isolated repeatedly from different animals.

Our studies have been concerned with the biochemistry of transmitter compounds. Lobster exoskeletal muscles are innervated by both excitatory (E)<sup>1</sup> and inhibitory (I) axons.  $\gamma$ -amino-

<sup>1</sup> *Abbreviations used in this paper:* CNS, central nervous system; DPN, diphosphopyridine nucleotide; E, excitatory axons; GABA,  $\gamma$ -aminobutyric acid; I, inhibitory axons; NAD, nicotinamide adenine dinucleotide; TCA, trichloroacetic acid.

butyric acid (GABA) is the transmitter compound released by inhibitory nerves (1) and the leading candidate for the excitatory transmitter substance is glutamate (2, 3). We previously reported that I axons contain about 0.1 M GABA, while E axons contain less than 1% as much, and that the glutamic decarboxylase activity is about 10 times higher in I-axon extracts (4). Other components of the pathway of GABA metabolism (Fig. 1),  $\alpha$ -ketoglutarate, glutamate, and GABA-glutamic transaminase, show little or no difference in extracts of E- and I-axons.

This study presents further data on enzymes isolated from single axons and will begin a comparison of the properties of enzymes in functionally different axons.

## MATERIALS AND METHODS

### Tissues

Live lobsters (*Homarus americanus*) weighing 0.5–2.5 kg were obtained from a local dealer and stored in a cold room at 4°C or in a circulating sea-water tank at 12°C until used. Dissections from the meropodite segment of the walking legs of lobsters were carried out as described previously (5) to obtain 5–6-cm lengths of the I- and E-axons (Fig. 2), which respectively innervate the closer and opener muscles of the dactyl. Occasionally, other identified axons were used (6). Axons were 40–50  $\mu$  in diameter and were surrounded by a glial sheath about 1  $\mu$  in thickness and a variable small amount of connective tissue. Contaminating tissues represented less than 10% of the total volume of each axon. The approximate volume of axoplasm per cm of axon was 0.01  $\mu$ l.

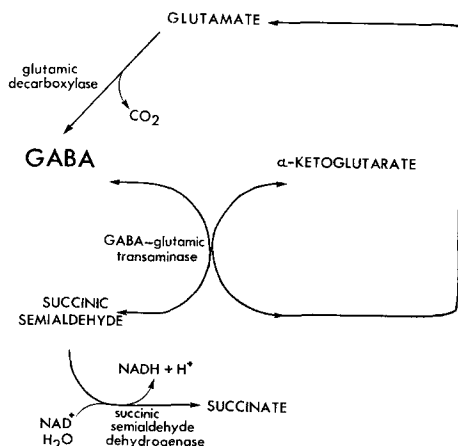


FIGURE 1 The pathway of GABA metabolism in the lobster nervous system.

### Enzyme Preparations and Assays

After dissection, single axons were picked up on the tip of a ground-glass microhomogenizer plunger, rinsed gently in isotonic KCl, or lobster physiological salt solution (6) for enzyme assays, or water (for gel electrophoresis), and then homogenized in 5  $\mu$ l of buffer (see below) in microhomogenizer tubes. The homogenizers were constructed by cutting off the top half of a conical 0.1 ml test tube (Misco Scientific Corp., Berkeley, Calif.) and grinding the inside of the bottom half with an approximately fitting glass-rod in a graded series of abrasives until the rod fit snugly into the tube.

For the decarboxylase assay, axons were homogenized in 5  $\mu$ l of 0.1 M potassium phosphate (pH 7.2–7.4), 0.025–0.032 M  $\beta$ -mercaptoethanol and 0.1 mM pyridoxal phosphate (I axon), or in the same medium with 0.003 M GABA (E axon), except where noted in the text. The volume of fluid in the microhomogenizer tubes was measured by weighing the tubes. Suitable dilutions were made and samples were removed for assay. Reactions were started by adding 2  $\mu$ l of substrate (0.25–0.5  $\mu$ moles of glutamate-U-<sup>14</sup>C, approximate specific activity; 30 mCi/mmmole) to 3  $\mu$ l of enzyme. For <sup>14</sup>CO<sub>2</sub> collection, reactions were carried out at 25°C in closed tubes and experiments were ended at appropriate times by injection of 50  $\mu$ l of 5% trichloroacetic acid (TCA). The <sup>14</sup>CO<sub>2</sub> evolved was collected overnight in 50  $\mu$ l of Hyamine hydroxide (1 M solution in methanol) in a 0.2 ml test tube suspended above the reaction mixture. The Hyamine solution was transferred to a toluene scintillation fluid and the radioactivity measured in a liquid scintillation spectrometer (see reference 7). In certain experiments acidic amino acids were removed from reaction mixtures with ion-exchange columns. In these cases, the reaction was stopped by transferring the incubation mixtures to small (0.5  $\times$  5 cm) Dowex 1-acetate (AG-1-x2, 100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) ion-exchange columns. Reaction products were eluted with 5 ml of H<sub>2</sub>O, taken to dryness in a Rotary Evapo-Mix (Buchler Instruments, Inc., Fort Lee, N.J.) and a portion counted. The remainder was chromatographed by ascending paper-chromatography at room temperature to identify the compounds formed. The solvents used were: phenol:water, 160 gm:40 ml (solvent 1) and butanol:acetic acid:water, 12:3:5 (solvent 2) using water-washed Whatman No. 1 paper. Strips from chromatograms were scanned for radioactivity in a Savant strip counter (Savant Instruments, Inc., Hicksville, N.Y.). Since no radioactive basic amino acids were detected, this fraction is subsequently called *neutral amino acids*.

For the transaminase assay, axons were homogenized in 5  $\mu$ l of 0.4 M glycylglycine buffer, pH 8.5, 30 mM  $\beta$ -mercaptoethanol, and 2 mM pyridoxal phosphate. After homogenization, the volume was meas-

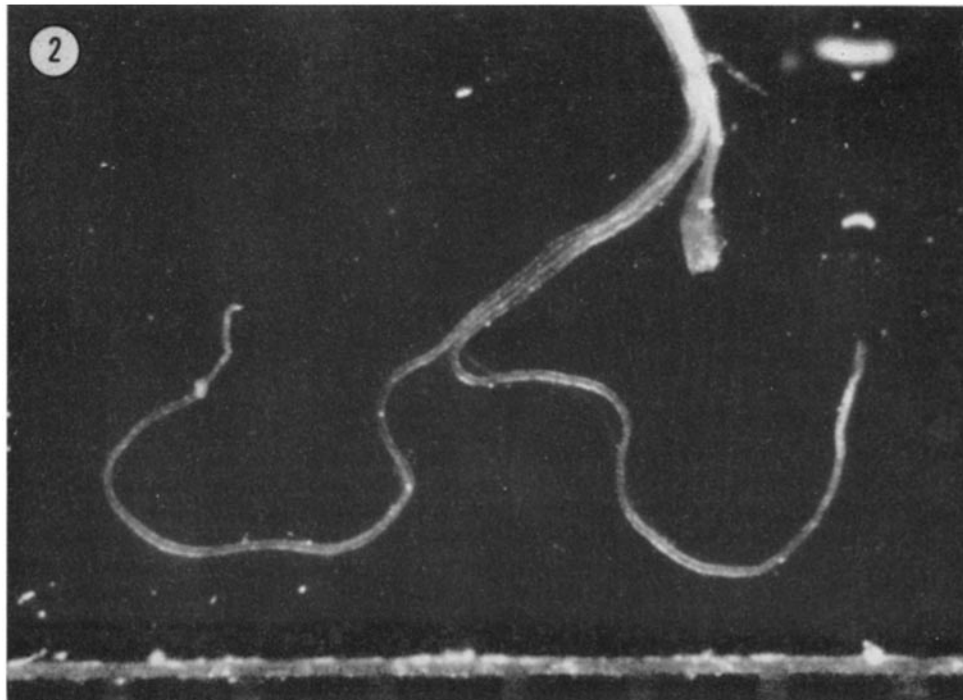


FIGURE 2 Single axons dissected from the meropodite segment of the walking leg of the lobster, as viewed through a dissecting microscope. The ruler divisions are 1 mm and individual axons are 40–50  $\mu$  in diameter. Axons are identified by stimulation. A knot is tied in one axon (the E in this case) to facilitate identification in case of axon damage during the dissection. About 5–6 cm lengths of axon are routinely obtained (see reference 5 and Methods for details).

ured as above, a suitable dilution was made, and samples were placed in 0.2 ml test tubes for assay. Substrates and cofactors were added so that final incubation concentrations were 2 mM  $\alpha$ -ketoglutarate, 3 mM diphosphopyridine nucleotide (DPN), 1 mM succinate, and 3 mM GABA-U- $^{14}\text{C}$  (20–140 mCi/mmmole). The final incubation volume was 20  $\mu\text{l}$ . After 1–3 hr at 25°C, 5  $\mu\text{l}$  of 40% trichloroacetic acid was added and the incubation mixture applied to a 0.5  $\times$  5 cm column of Dowex 50-H $^{+}$  (Dowex 50-W-x2, 100–200 mesh, J. T. Baker Chemical Co., Phillipsburg, N.J.). The column was washed with 5 ml of water and the radioactivity of a portion of the fluid passing through the column was measured. The remainder was freeze-dried and the products were separated by either paper chromatography or high voltage electrophoresis. The paper chromatographic solvent used was *n*-butanol:acetic acid:pyridine:water, 120:20:25:35 (solvent 3). High-voltage electrophoreses were performed at pH 6.4 in pyridine:acetic acid:water (10:0.8:90) at 50 V/cm as described by Naughton and Hagopian (15).

### *Gel Electrophoresis*

Separations of proteins from single-axon homogenates were carried out by electrophoresis in 1 mm-diameter columns of polyacrylamide gel. A single buffer system was used, Tris-glycine (pH 9.3). Gel solutions and buffers were prepared as described by Matson (8). Unpolymerized gel solution was drawn into capillary tubes 30 mm long (made from 100  $\mu\text{l}$  Drummond microcaps, Drummond Scientific Co., Inc., Plymouth, Mich.). The tubes were placed upright in soft paraffin as a support and 5  $\mu\text{l}$  of gel solution removed and replaced with 5  $\mu\text{l}$  of water. After polymerization of the gel (30 min), the water was removed and the tubes placed in an electrophoresis apparatus at 4°C. Unreacted persulfate and other toxic by-products of the polymerization were removed by applying a potential of 10 V/cm for 1 hr. Enzyme solutions were prepared as above (in 5–10  $\mu\text{l}$  of fluid, see section on enzyme preparation) in 0.02 M potassium-phosphate buffer containing 2% sucrose and centrifuged at 20,000 *g* in a Misco microcentrifuge (Misco Corp.)

for 20 min to remove particulate matter. 5- $\mu$ l samples were applied to the top of the pretreated gels and electrophoresis was run at 10 V/cm for 30 min, followed by approximately 30 min at 20 V/cm. Bromophenol blue was used as a marker dye to determine the end of the electrophoresis run. Gels were extruded from the glass capillaries with a metal plunger and sliced lengthwise in half. One-half was placed in 10% trichloroacetic acid to precipitate the proteins in the gel and the other half was sliced by hand into 20–25 1-mm portions. Each slice was placed into 5  $\mu$ l of incubation medium and assayed as above for transaminase or, with the  $^{14}\text{C}$  assay, for decarboxylase. The half gel in TCA was stained with fluorescent dansyl reagent (9) to visualize the proteins present as follows: The gel was rinsed in 0.5 ml 0.1 N  $\text{NaHCO}_3$  for 15 min and placed in a second portion (0.5 ml) of 0.1 N  $\text{NaHCO}_3$ . Dansyl chloride (0.5 ml of a 1 mg/ml solution in acetone) was added and the gel left for 3 hr at 23°C. Excess dansyl reagent was removed by rinsing overnight in 0.1 N  $\text{NaHCO}_3$  and the protein bands were observed using an ultraviolet hand lamp (Mineralight Inc., San Gabriel, Calif., model SL 3660) and graphed by visual inspection.

### Substrates

Glutamate- $\text{U-}^{14}\text{C}$  (approximately 200 mCi/mmmole) was obtained from New England Nuclear Corp., Boston, Mass. It was diluted to an appropriate specific activity and purified shortly before use. The isotope in 0.01 N HCl was taken to dryness in a Rotary Evapo-Mix, dissolved in a small volume of 1 N  $\text{NH}_4\text{OH}$ , and taken to dryness again. The residue was dissolved in water and applied to a column of Dowex 1-acetate (0.6  $\times$  3 cm). The column was washed with water and the glutamate was eluted with about 10 ml of 1 N acetic acid. The eluate was evaporated to dryness, a small volume of 1 N formic acid was added, and the solution again evaporated to dryness. The residue was dissolved in water and applied to an 0.6  $\times$  1.5 cm column of Dowex 50-Tris. Glutamate passed through the column with the water wash, was evaporated to dryness, and made up to a standard volume. Purified isotope was used for about a month before it was re-purified. GABA- $\text{U-}^{14}\text{C}$  was prepared from glutamic acid- $\text{U-}^{14}\text{C}$ , using glutamic decarboxylase partially purified from an acetone powder of *Escherichia coli* (Worthington Biochemical Corp., Freehold, N.J.) by modification of the procedure of Shukuya and Schwert (10). Glutamate- $\text{U-}^{14}\text{C}$  (0.2 mCi) was incubated with 300  $\mu$ moles of sodium acetate, pH 4.4, and 0.8 units of decarboxylase in a volume of 2.6 ml for 2 hr at 23°C. The extent of the reaction was followed by collection of  $^{14}\text{CO}_2$  in Hyamine hydroxide. When the reaction was complete, the incubation mixture was applied to a 0.5  $\times$  5 cm column of Dowex 50- $\text{H}^+$ .

GABA was eluted with 1 N  $\text{NH}_4\text{OH}$ , evaporated to dryness, dissolved in water, and passed over a 0.5  $\times$  3 cm column of Dowex 1-acetate. The radioactive GABA was collected in the first 5 ml of water washed through the column.

Pyridoxal phosphate, nicotinamide adenine dinucleotide (NAD),  $\alpha$ -ketoglutarate, and dansyl chloride were purchased from Sigma Chemical Co., St. Louis, Mo. Guinea pig succinic semialdehyde dehydrogenase was prepared by the method of Pitts, Quick, and Robins (11).

## RESULTS

### Decarboxylase in Single-Axon Extracts

We previously reported that inhibitory axon extracts could synthesize about 100  $\mu$ moles of GABA/cm axon/hour while the value for excitatory extracts was about 9 (4). These values were determined using an assay in which we measured the production of radioactive GABA from high specific-activity glutamate- $\text{U-}^{14}\text{C}$  using an ion-exchange procedure to separate glutamate and GABA. The identification of the reaction product as GABA was verified in two ways: (a) by paper chromatography in two solvent systems (Fig. 3 illustrates a radioactive scan of a chromatogram using solvent 1); and (b) by the enzymic conversion of the product to an acidic substance (presumably succinate) upon reaction with a bacterial enzyme system containing GABA-glutamic transaminase and succinic semialdehyde dehydrogenase (12). Recently we found that a highly purified lobster glutamic-decarboxylase preparation contained a contaminating enzyme which converted a contaminant in radioactive glutamic acid to GABA without the simultaneous evolution of  $^{14}\text{CO}_2$  (7). The contaminating enzyme does not require  $\beta$ -mercaptoethanol for activity, while the glutamic decarboxylase does.

In the present experiments, glutamic decarboxylase activity was measured by the production of  $^{14}\text{CO}_2$  from glutamate- $\text{U-}^{14}\text{C}$ , and we observed a serious discrepancy with the previous results. In the I-axon extracts there was a linear evolution of  $^{14}\text{CO}_2$  for at least 3 hr, while there was essentially no  $^{14}\text{CO}_2$  produced in E-axon extracts. The average decarboxylase activity using the  $^{14}\text{CO}_2$  assay was 90  $\mu$ moles per cm of axon per hr (six I-axons pooled; total length 40 cm), a value comparable to that previously reported with the neutral amino acid assay. In the E axon, on the

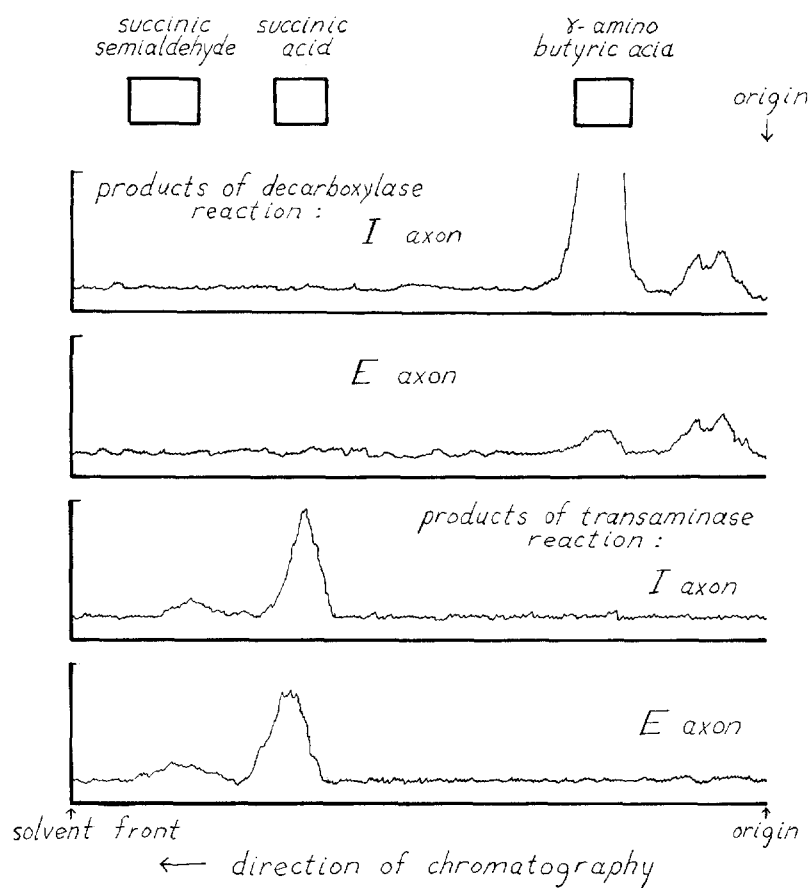


FIGURE 3 Scans of radioactivity in chromatograms of reaction products of decarboxylase and transaminase reactions. The double peak near the origin in decarboxylase incubations is also seen in boiled enzyme controls and is a contaminant. The principal product formed in both E- and I-axon decarboxylase incubations corresponds to GABA. There are no radioactive contaminants seen in controls of transaminase incubations, and the products formed correspond to succinate and succinic semialdehyde. Paper chromatography was ascending at room temperature and solvent 2 was used for decarboxylase products and solvent 3 for transaminase products. See Methods for experimental details. Full scale (indicated by bar) was 500 cpm.

other hand, there was no detectable  $^{14}\text{CO}_2$  (five E axons pooled; total length 40 cm). Thus, E axons seemed to have less than 0.3% of the decarboxylase activity of I axons with the  $^{14}\text{CO}_2$  assay.

To help verify this we next measured the production of  $^{14}\text{CO}_2$  and radioactive neutral-amino acids in single-axon extracts in the presence and absence of  $\beta$ -mercaptoethanol (Table I) In order to emphasize any contribution by the radioactive substrate contaminant in this experiment, a relatively impure substrate was used. This should have the effect of drastically reducing the apparent I/E decarboxylase ratio as measured by the

production of neutral amino acids.  $^{14}\text{CO}_2$  was formed in I-axon extracts and this required  $\beta$ -mercaptoethanol, while neutral amino acid production was reduced by about 40% in the I-axon extracts in the absence of  $\beta$ -mercaptoethanol. In contrast, E-axon extracts produced a negligible amount of  $^{14}\text{CO}_2$  and the amounts of neutral amino acids were the same with or without added  $\beta$ -mercaptoethanol. Thus the enzyme forming GABA from the contaminant in the substrate was present in both E- and I-axon extracts and accounted for the previous report of decarboxylase activity in E-axon extracts (4). In

TABLE I  
Neutral Amino Acids and  $^{14}\text{CO}_2$  Production from Glutamate- $U\text{-}^{14}\text{C}$  in the Presence and Absence of  $\beta$ -Mercaptoethanol

	Neutral amino acids/4			$^{14}\text{CO}_2$	
	dpm/hr/cm	$\Delta$ dpm	$\Delta$ $\mu\text{moles/hr/cm}$	dpm/hr/cm	$\mu\text{moles/hr/cm}$
Excitor					
(+) $\beta$ -mercaptoethanol	13500	300 <sup>+</sup>	—	35 <sup>+</sup>	—
(-) $\beta$ -mercaptoethanol	13200			25 <sup>+</sup>	—
Inhibitor					
(+) $\beta$ -mercaptoethanol	19500	8100	72	5230	47
(-) $\beta$ -mercaptoethanol	11400			0	—

The experimental details were as in the methods except that axons were homogenized in buffer without  $\beta$ -mercaptoethanol. The substrate used was not highly purified.  $\beta$ -mercaptoethanol was added to specified experimental vessels to give a final concentration of 0.03 M. Since glutamate- $U\text{-}^{14}\text{C}$  was used as substrate the neutral amino acid figure (GABA) is divided by four to make it equivalent to the  $\text{CO}_2$  figure. +, these values are not significant. The lower limits of detection in this experiment were about 1  $\mu\text{mole/hr/cm}$  for the  $^{14}\text{CO}_2$  assay and about 5  $\mu\text{moles/hr/cm}$  for the neutral amino acids.

I-axon extracts, even when the correction for the contaminant-metabolizing enzyme is subtracted, we still observe a discrepancy in  $^{14}\text{CO}_2$  collected and neutral amino acids produced. This discrepancy could result from some binding of  $^{14}\text{CO}_2$  or from yet another source of neutral amino acid production, and could significantly affect the absolute I-axon decarboxylase values. Therefore, further experimentation is required before assigning an absolute figure to the I-axon decarboxylase level.

The absence of decarboxylase from E axons could be due to the presence of a specific decarboxylase inhibitor, to the presence of an inactivated enzyme, or to the absence of the enzyme. E- and I-axon extracts were mixed and there was no depression of the decarboxylase activity of the I-axon extracts. If an inhibitor was present, therefore, it was exactly titrated to the amount of enzyme in E axons. The failure to demonstrate decarboxylase activity after gel electrophoresis of E-axon extracts (see below) is a further argument against the existence of an enzyme inhibitor. It is possible that enzyme was present but inactive in E-axon extracts. However, agents known to increase the activity of purified lobster glutamic-decarboxylase ( $\text{K}^+$ ,  $\beta$ -mercaptoethanol, pyridoxal phosphate) did not restore E-axon activity (7).

#### GABA-Glutamic Transaminase Activity in Single-Axon Homogenates

Prior to measurements of single-axon activities, homogenates of pooled E and I axons were used

to show that the transaminase-reaction velocity was constant for at least 3.5 hr and was proportional to the amount of homogenate used. The pH optimum of the reaction determined with this preparation was approximately 8.0.

Assays were then carried out on single E- and I-axon extracts and paper chromatograms of the reaction products are shown in Fig. 3. Similar results were obtained upon high-voltage electrophoresis of the products. In the standard assay, with DPN present, succinate- $^{14}\text{C}$  was the principal product (61–88% in eight experiments). This suggested that succinic semialdehyde dehydrogenase activity in both E- and I-homogenates was in excess of transaminase activity. To test this, portions were assayed with and without added guinea pig kidney succinic-semialdehyde dehydrogenase. No increase in the production of radioactive acids from GABA- $U\text{-}^{14}\text{C}$  was observed with exogenous dehydrogenase. Therefore, transaminase activity in both axons is rate-limiting for the metabolism of GABA, and succinic semialdehyde dehydrogenase-activity was not studied further.

When GABA-glutamic transaminase activity was assayed in 12 pairs of I axons, an average value of 32  $\mu\text{moles/cm axon/hr}$  was obtained. In E-axon homogenates the average value was 23  $\mu\text{moles/cm/hr}$ . The values obtained for both kinds of axons were quite variable. Much of the variability was apparently due to differences between animals, since when pairs of axons from opposite legs of the same animal were compared

(2 and 3, 8 and 9, 11 and 12 in Table II) the values were more consistent. Transaminase activity was about 1.5 times higher in I than in E axons (Table II).

### Single-Axon Enzyme Comparisons

The presence of transaminase activity in both axons offered an opportunity to compare the kinetic properties and electrophoretic behavior of the enzyme in each homogenate, to see if the presence of different isozymes in functionally different axons could be detected.

Kinetic constants for both GABA and  $\alpha$ -ketoglutarate at a fixed concentration of the other substrate were determined. The lobster GABA-glutamic transaminase has been previously shown (13) to have kinetic behavior consistent with a reaction mechanism in which each substrate reacts alternately with the enzyme ("ping-pong bi-bi"). Determination of true kinetic constants requires variations of both substrates. Because of limitation of material, this was not possible. The simpler procedure of varying one substrate at a fixed concentration of the other gives an apparent Michaelis constant adequate for comparison.

The determination of an apparent  $K_m$  for

GABA was complicated by the presence of large amounts of endogenous GABA in I-axon extracts. This difficulty was overcome by using a high concentration (40 mM) of  $\alpha$ -ketoglutarate, which is a competitive inhibitor for GABA as well as a substrate in the reaction. The high  $\alpha$ -ketoglutarate concentration raised the apparent  $K_m$  for GABA to a value high enough to be accurately measured. Under these conditions, in two experiments the apparent  $K_m$  for GABA for the enzyme in E-axon extracts was 1.8 and 1.5 mM, while in I-axon extracts, the values were 2.9 and 2.5 mM. A kinetic plot of the data from one of these experiments is shown in Fig. 4. The observed apparent  $K_m$  differences between E- and I-axons were within experimental error.

For  $\alpha$ -ketoglutarate there was considerable

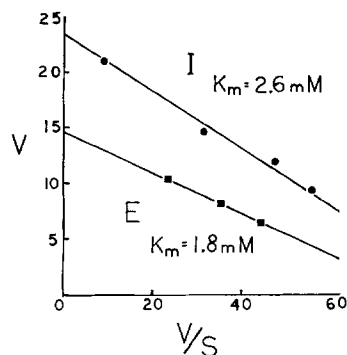


FIGURE 4 Kinetic plot of the effect of GABA concentration on transaminase activity in single E- and I-axon homogenates. The velocity ( $V$ ) is expressed as moles  $\times 10^{11}$ /hr. For experimental details see text, Methods, and reference 13. The apparent  $K_m$ 's in the E- and I-axon homogenates are not significantly different.

TABLE II  
GABA-Glutamic Transaminase Activity in Separated E- and I-Axons

Experiment No.	Activity		I/E
	E	I	
	<i>μmoles/cm/hr</i>		
1	15	25	1.7
2	11	19	1.7
3	12	18	1.6
4	23	28	1.2
5	29	32	1.1
6	35	43	1.3
7	26	40	1.5
8	23	33	1.4
9	24	32	1.4
10	27	41	1.5
11	22	33	1.5
12	24	37	1.5
Average	23	32	1.5 $\pm$ 0.1*

Experiments 2 and 3, 8 and 9, and 11 and 12 were pairs carried out on opposite legs of the same animal. The experimental details were as in Methods. \* Standard error of the mean.

TABLE III  
Apparent  $K_m$  ( $K_m'$ ) for  $\alpha$ -Ketoglutarate of the Transaminase in E- and I-Axons

Experiment No.	$K_m'$ (mM)	
	E	I
1	2.7	2.0
2	1.2	1.1
3	0.7	0.8

Prior to homogenization, axons were rinsed in calcium-free lobster saline in Experiment Nos. 1 and 2 and in 1 M sucrose in Experiment No. 3. The other experimental details are as in Methods.

variation of the apparent  $K_m$  from experiment to experiment but E- and I-axon values in any single experiment were similar (Table III). All of the measured values exceeded the previous reported value with the partially purified lobster central nervous system (CNS) enzyme. The reasons for this discrepancy and the variation between experiments were not found, but the consistency within experiments allowed the tentative conclusion that the kinetic properties of the E- and I-enzymes were the same.

As with the partially purified enzyme, transaminase activity in both E- and I-homogenates was inhibited by high concentrations of  $\alpha$ -ketoglutarate (Table IV).

#### *Polyacrylamide Gel Electrophoresis of Single-Axon Extracts*

The electrophoresis of the decarboxylase and transaminase enzymes from single axons is shown in Fig. 5. Approximately 90% of the activity applied to the gel was recovered in the single bands of activity observed. The resolution of fluorescent protein bands and enzyme activities was not good enough for the identification of enzyme activities with single protein bands. However, the method was sufficient to demonstrate that the transaminase enzymes from E- and I-axons have similar mobilities, slightly higher than the mobility of the I-axon decarboxylase. There was no measurable decarboxylase activity in the E-axon extract. Glutamic decarboxylase and GABA-glutamic transaminase prepared from homogenates of the lobster central nervous system and electrophoresed on standard gels (5 mm

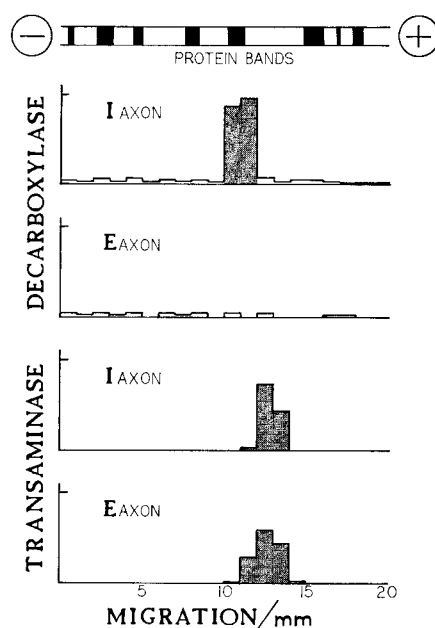


FIGURE 5 Decarboxylase and transaminase enzyme activities measured in slices of micropolyacrylamide gel columns. The experimental procedure is described in Methods. The gel pattern illustrated at the top is a typical distribution of protein bands seen on inspection after conjugation of proteins with fluorescent dansyl reagent. No significant differences were noted between E- and I-axons, and it was not possible to associate particular bands with specific enzymes. Full scale (indicated by bar) was 400 cpm.

diameter) moved to the same relative positions on gels as the single-axon enzymes.

#### DISCUSSION

One goal of these studies is to explain how inhibitory and excitatory nerve cells control their transmitter levels. The axon terminals of these cells, the regions directly involved in transmitter release, cannot be studied because techniques are not yet available for physically separating them from the muscle tissue they innervate. However, transmitter accumulation along the length of the nerve cells can be studied, and may provide a model for what occurs in the nerve endings.

An intensive study of the synthesis and metabolism of the inhibitory transmitter, GABA, in single lobster E- and I-axons has been carried out in this laboratory (4). The enzymes involved have been isolated and purified from the lobster nervous system and their properties studied (7, 13).

TABLE IV  
*Comparison of  $\alpha$ -Ketoglutarate Inhibition of Transaminase Activity in E- and I-Axons*

	$\mu$ moles/ hr/cm	% In- hibition
<b>Excitor</b>		
4 mM $\alpha$ -ketoglutarate	91	
40 mM $\alpha$ -ketoglutarate	59	35
<b>Inhibitor</b>		
4 mM $\alpha$ -ketoglutarate	128	
40 mM $\alpha$ -ketoglutarate	94	27

The experimental details are as in Methods. The  $\alpha$ -ketoglutarate concentrations used are listed in the table.



Such studies revealed, among other things, that glutamic decarboxylase is inhibited by its product, GABA. The levels of GABA, glutamate, and  $\alpha$ -ketoglutarate, and the activities of glutamic decarboxylase and GABA-glutamic transaminase were measured in single E- and I-axon extracts. The measurements allowed us to postulate a mechanism for the selective accumulation of GABA in I axons. The I axon can synthesize more GABA than it can destroy, and therefore GABA will accumulate. The increasing GABA concentration progressively produces a greater inhibition of the decarboxylase until synthesis is balanced by destruction. Of course, our inference of in vivo enzyme activities from in vitro measurements of optimum enzyme activities and steady-state levels of substrates is necessarily indirect. Clearly, a direct demonstration that the GABA level in I neurons is maintained by a steady-state system of the type proposed would be most desirable.

What still remains unexplained is the presence of GABA in E axons, where we are unable to demonstrate decarboxylase activity. A small, as yet undetected, amount of decarboxylase could account for the GABA found. However, it is also possible that GABA is not present in the axon at all but in the small, variable amount of connective tissue surrounding the preparations. Lobster nerve-muscle preparations contain a transport system for GABA (14). The principal site of GABA uptake has recently been found to be the connective tissue and Schwann cells which surround lobster nerve and muscle (P. Orkand, unpublished). Whether this accounts for the GABA in E-axon extracts remains an unresolved problem.

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A selective comparison of the protein composition of individual, identified nerve cells can begin with the results presented in this paper. For enzymes concerned with GABA metabolism, the glutamic decarboxylase activity is found only in I-axon extracts, while transaminase, dehydrogenase, and the unknown enzyme converting the glutamate contaminant to GABA are found in both axon types. As is the case with the enzymes that degrade acetylcholine and norepinephrine, therefore, the GABA-glutamic transaminase and succinic semialdehyde dehydrogenase are not confined to neurons that secrete GABA as a neurotransmitter compound. Moreover, the kinetic and electrophoretic data, although preliminary in characterizing an enzyme, suggest that the same protein catalyzes the transaminase reaction in E- and I-axons. Thus, the ability to accumulate GABA ultimately depends on the ability of I cells to accumulate the enzyme glutamic decarboxylase.

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