

# GLUTAMATE UPTAKE BY A STIMULATED INSECT NERVE MUSCLE PREPARATION

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## ABSTRACT

Recent reports suggest that glutamate may be the excitatory neuromuscular transmitter in insects. In this study, glutamate uptake by isolated cockroach nerve muscle preparations was investigated by means of chemical and electron microscope radioautographic techniques. We found that the preparation had a high affinity for glutamate and that nerve stimulation enhanced glutamate uptake. Chemical studies showed that the average tissue concentration of glutamate bound during a 1 hr incubation period in  $10^{-5}$  M glutamate- $^3\text{H}$  after nerve stimulation was  $2.8 \times 10^{-5}$  M. Less than 1% of the radioactivity was present in the perchloric acid-precipitated protein fraction. Using electron microscope radioautography, we observed that sheath cells showed the highest glutamate concentration of all cellular compartments. Uptake was greater at neuromuscular junctions than in other regions of the tissue. The data suggest a possible mechanism for transmitter inactivation and protection of synapses from high blood glutamate.

## INTRODUCTION

Transmission at insect neuromuscular junctions is noncholinergic (see Hill and Usherwood, 1961; Faeder et al., 1970). Several studies have suggested that glutamate may be the excitatory transmitter at such junctions. Application of glutamate to nerve muscle preparations gives effects consistent with this hypothesis (Usherwood and Machili, 1966, 1968; Beranek and Miller, 1968; Faeder and O'Brien, 1970). Glutamate release has also been reported during motor stimulation (Kerkut et al., 1965; Usherwood et al., 1968).

One difficulty with accepting glutamate as a transmitter stems from the fact that many investigators have found a high glutamate concentration ( $10^{-8}$  M) in insect hemolymph (Benassi et al., 1959, 1961; Stevens, 1961; Corrigan and Kearns, 1963), although this has recently been disputed (Usherwood and Machili, 1968). Further-

more, no inactivation mechanism for glutamate has yet been demonstrated at insect excitatory neuromuscular junctions. An inactivation mechanism could involve enzymatic degradation, as is the case with acetylcholine at vertebrate neuromuscular junctions, suggested by Usherwood and Machili (1968). It could also operate via uptake by nerve or surrounding tissue as is the case with norepinephrine in the vertebrate sympathetic system (for review, see Iversen, 1967) or with GABA at crustacean inhibitory synapses (Iversen and Kravitz, 1968; Morin and Atwood, 1969). Glutamate uptake has indeed been demonstrated in nerve muscle preparations in Crustacea (Iversen and Kravitz, 1968), where there is strong evidence that it functions as an excitatory neuromuscular transmitter.

In the present study, we have investigated the

uptake of glutamate by isolated nerve muscle preparations. Chemical analysis and electron microscope radioautography were employed to provide information on both the localization and the quantity of the radioactivity taken up. A mechanism which could act as a barrier against hemolymph glutamate as well as provide for its inactivation as a transmitter is also described.

## MATERIALS AND METHODS

### *Nerve Muscle Preparation*

A metathoracic coxal adductor (analogous to muscles 177a and c in *Periplaneta*) with nerve 4 attached (Dresden and Nijenhuis, 1955, 1958) was dissected from adult male cockroaches (*Gromphadorhina portentosa*) under light CO<sub>2</sub> anesthesia.

### *Preincubation*

A. (NONSTIMULATED): The preparation was placed in a beaker containing 5 ml of phosphate-buffered saline (pH 7.2; see Yamasaki and Narahashi, 1959) at room temperature (23°C) for 1 hr before being transferred to the radioactive incubation medium.

B. (STIMULATED): The preparation was placed in a chamber containing 5 ml of saline at room temperature. The nerve was stimulated electrically at 0.5/sec (duration 0.05 msec) for 1 hr before the preparation was transferred to the radioactive incubation medium.

### *Incubation Conditions*

The preparations were immersed in 5 ml of saline containing either glutamate-<sup>3</sup>H or leucine-<sup>3</sup>H and placed on a metabolic shaker at room temperature for 1 hr.

DL-Glutamic-<sup>3</sup>H-acid (SA 5 × 10<sup>3</sup> mCi/mmole) at 2 × 10<sup>-5</sup> M was used for the radioautographic samples. L-Glutamic 3, 4-<sup>3</sup>H-acid (SA 5 × 10<sup>2</sup> mCi/mmole) at 1 × 10<sup>-5</sup> M and L-leucine 4, 5-<sup>3</sup>H (SA 5 × 10<sup>2</sup> mCi/mmole) at 1 × 10<sup>-5</sup> M were used in the chemical studies.

### *Chemical Analysis*

Paired preparations from five animals were used for each of the amino acids studied. One preparation from each animal was stimulated (preincubation condition B), while the other served as a nonstimulated control (preincubation condition A). Both preparations were then incubated identically in either glutamate-<sup>3</sup>H or leucine-<sup>3</sup>H under the conditions described above. Each preparation was then rinsed briefly in saline, blotted, weighed, and homogenized in 0.4 M HClO<sub>4</sub>. After centrifugation, an aliquot of the supernatant was removed and the

radioactivity was determined by liquid-scintillation counting in toluene cocktail plus Beckman Biosolv-3 (nonprotein amino acid and small molecule metabolites; Beckman Instruments Inc., Fullerton, Calif.). The precipitate obtained after centrifugation was washed three times with 70% ethanol, digested in 0.5 ml 30% H<sub>2</sub>O<sub>2</sub> at 70°C, and the radioactivity was determined by liquid-scintillation counting (protein-bound amino acid).

### *Radioautographic Analysis*

(1) FIXATION AND EMBEDDING: Following incubation in glutamate-<sup>3</sup>H, two preparations from each preincubation group were rinsed briefly in saline, and pinned out in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min. The muscle was then diced and placed in two changes of fresh fixation for a total of an additional 1½ hr, rinsed three times and overnight in 0.1 M phosphate buffer containing 0.2 M sucrose, and postfixed in phosphate-buffered 1% OsO<sub>4</sub> (Hagopian, 1967) for 1 hr (all at 0°C). After block-staining in 2% uranyl acetate for 1 hr, the tissues were then dehydrated in graded alcohols and embedded in Epon 812 (Luft, 1961).

Retention of radioactivity during tissue processing was studied in three paired nonstimulated preparations. One preparation from each pair was analyzed chemically for uptake as described above. The second preparation was fixed in glutaraldehyde and processed through the overnight buffer rinse. Preliminary experiments had indicated that very little radioactivity is lost during postfixation and dehydration. The total radioactivity lost was determined from aliquots of fixative and rinse solutions. The fixed tissue was digested in H<sub>2</sub>O<sub>2</sub> (see above) and analyzed for radioactivity. The amount of radioactivity remaining in the fixed tissue can be compared to that of the unfixed paired preparation as well as to the radioactivity lost in the histological solutions. (In each pair, the total radioactivity of the fixed tissue plus processing solutions was within 15% of the total radioactivity in its unfixed mate.) By both of these criteria, 50–60% of the initial radioactivity was retained in the fixed tissue of each pair.

(2) SPECIMEN PREPARATION FOR RADIO-AUTOGRAPHY: Thick sections (0.5 μ) coated with purple layers of Ilford L4 emulsion were used for light microscope radioautography. Light-gold sections (1000 ± 100 Å, checked interferometrically) were prepared for electron microscope radioautography by means of the "flat substrate" method of Salpeter and Bachmann (1964). Ilford L4 emulsion, diluted to form purple layers, was coated by dipping. Special care was taken to reproduce the conditions calibrated for quantitation (Bachmann and Salpeter, 1967). After exposure periods ranging from 29 to

130 days, radioautograms were developed with Microdol X (Eastman Kodak Co., Rochester, N.Y.) (3 min at 23°C). Under these conditions, the sensitivity is  $1/10 \pm 20\%$  (i.e. 10 radioactive decays give, on the average, one developed grain) (Bachmann and Salpeter, 1967). Controlled experiments have indicated that uranyl staining under these conditions has no effect on emulsion sensitivity or background, and that there is no latent image fading during the exposure periods used (Salpeter and Szabo, in preparation).

(3) QUANTITATIVE ANALYSIS: The radioautograms were viewed with a Phillips 300 electron microscope, and all areas containing nerve were photographed at constant magnification ( $\times 7500$ ). Grain densities (grains/ $\mu^2$  per exposure day) were determined for the entire tissue and separately for several cellular compartments (e.g. axon, sheath; see Table II) as follows: Developed grains were assigned to different compartments (see Fig. 1) by the location of the grain center (i.e., the center of the smallest circle which would circumscribe the grain). The area per compartment was determined by placing a grid with uniformly spaced points (one point per  $2.96 \mu^2$ ) over the photographic plate and tabulating the number of points over each compartment. The density (grains/ $\mu^2$ ) was then divided by the exposure time in days. The sampling error (standard error of the densities) was calculated, assuming the ratio of two independent Poisson distributions (i.e. grains and points), from the formula

$$\frac{N_1}{N_2} \pm \frac{N_1}{N_2} \sqrt{\left(\frac{SE_1}{N_1}\right)^2 + \left(\frac{SE_2}{N_2}\right)^2}$$

The standard error of a Poisson distribution of mean  $N$  is  $\sqrt{N}$ .

All densities were corrected for background and converted to molar concentrations by use of the sensitivity value given above and the specific activity of the glutamate- $^3\text{H}$ .<sup>1</sup>

When all random sources of error are considered (i.e., standard error of Poisson distributions, fluctuation in section thickness of 10%, fluctuation in sensitivity of 20%), the values given in Table II are estimated to be accurate within a factor of 2. One systematic source of error stems from our direct

$$^1M = \frac{(d)(10^{12})}{(s)(t)(SA)(3.2 \times 10^{15})}$$

where  $d$  = density = grains/ $\mu^2$ /day  
 $1 \text{ ml} = 1 \text{ cm}^3 = 10^{12} \mu^3$

$s$  = sensitivity = 0.1 grains/decay

$t$  = section thickness =  $0.1 \mu$

$SA$  = specific activity of glutamate- $^3\text{H}$  in incubation medium = 5 Ci/mmmole  
 (1 Ci =  $3.2 \times 10^{15}$  decays/day)

conversion of grain densities to molar concentrations without correcting for radiation spread. Such spread causes a certain fraction of grains to lie in the emulsion outside the radioactive area. The extent of spread depends on the resolution of the preparation and on the size of the radioactive structure (Salpeter et al., 1969, Figs. 13 and 15). With the resolution in the present study ( $HD = 1600 \text{ \AA}$ ; see Salpeter et al., 1969), the percentage of total grains falling outside a structure is negligible for circular structures  $> 1 \mu$  in radius and is less than 50% in structures  $> 0.5 \mu$  in radius. This percentage decreases further as the structure elongates (compare Fig. 13 with Fig. 15 in Salpeter et al., 1969). The structures tabulated in Table II are  $0.5 \mu$  or larger with the exception of sheath cells at the neuromuscular junctions. The molar concentration in this compartment thus represents a lower limit relative to values elsewhere (e.g., nonjunctional sheath). A correction for radiation spread would raise the ratio reported in Table III by about a factor of 2.

## RESULTS

The results of the chemical analyses showed that the preparations had a high affinity for glutamate relative to leucine and that glutamate uptake was enhanced by nerve stimulation (see Table I A). Less than 1% of the glutamate taken up by the tissue was incorporated into protein under these conditions (see Table I B). The amount of amino acid incorporated into protein is similar for both glutamate and leucine. Stimulation appears to enhance protein synthesis significantly (Table I B).

Light microscope radioautography of stimulated preparations incubated in glutamate- $^3\text{H}$  showed a considerable label over the muscle. The grain density, however, varied appreciably from fiber to fiber. Between muscle fibers one could see many small intensely labeled "hot" spots which appeared to coincide with nerve and tracheole cells. In the unstimulated preparations, the grain density over muscle was lower and more uniform than in the stimulated preparations, and no "hot" spots were apparent.

To determine the nature of the "hot" spots, we performed quantitative analysis of a stimulated preparation, using electron microscope radioautographs. The results are summarized in Table II. The highest concentration of label was found in the sheath (see Figs. 2 and 3). This concentration was significantly higher than in the axon (sheath/axon ratio =  $2.8 \pm 0.5$ ). It must be remembered that with two adjacent structures the radiation spread always favors the less radioactive neighbor. Without making a slight mathe-

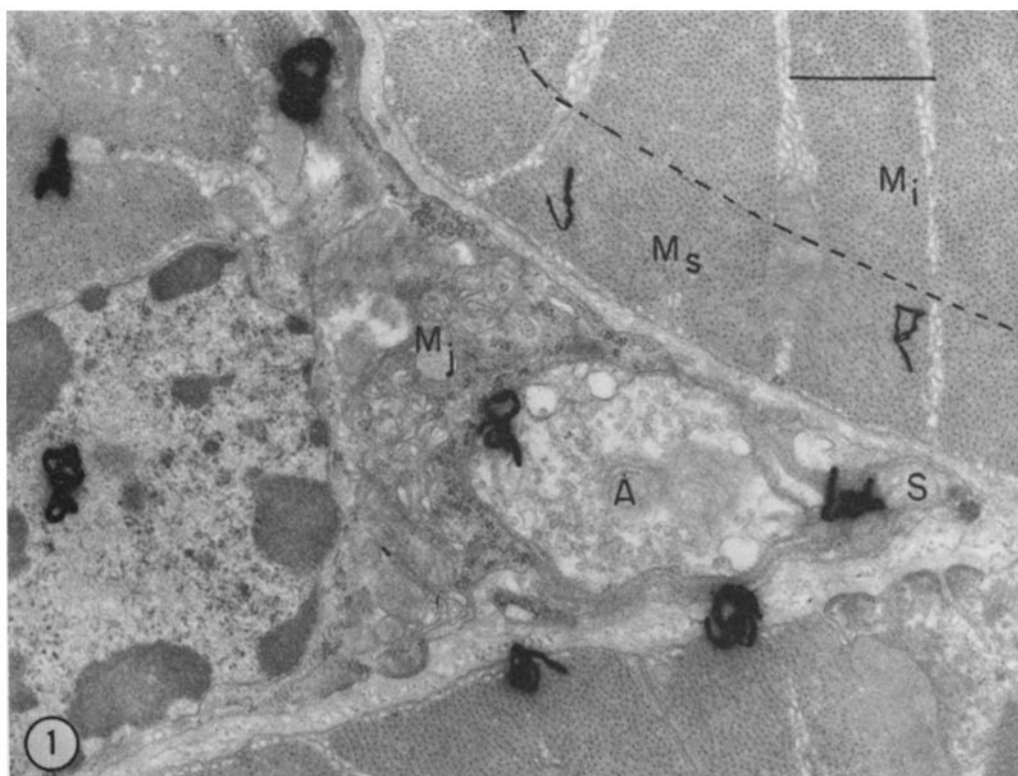


FIGURE 1 Typical radioautograph of a neuromuscular junction in tissue exposed to glutamate-<sup>3</sup>H after nerve stimulation. Tissue compartments used in analysis are indicated: *A*, axon; *S*, sheath; *M<sub>j</sub>*, post-synaptic region of muscle fiber; *M<sub>s</sub>*, muscle surface to 1  $\mu$ ; *M<sub>i</sub>*, muscle interior.  $\times 18,750$ .

TABLE I  
A. Nonprotein Amino Acid (and Small Molecule Metabolites)

Pretreatment	Uptake* ( $\times 10^{-5}$ mmoles/g)	
	Glutamate $\ddagger$	Leucine
Nonstimulated	1.25 $\pm$ 0.43	0.21 $\pm$ 0.07
Stimulated	2.78 $\pm$ 0.84	0.16 $\pm$ 0.07

Pretreatment	Uptake ( $\times 10^{-6}$ mmoles/g)	
	Glutamate $\S$	Leucine $\S$
Nonstimulated	0.004 $\pm$ 0.001	0.005 $\pm$ 0.003
Stimulated	0.011 $\pm$ 0.003	0.011 $\pm$ 0.003

\* An extracellular space of 20% of tissue wet weight has been estimated on the basis of results obtained by Iversen and Kravitz (1968). Uptake values have been corrected for extracellular space, assuming equilibration with the incubating medium.

$\ddagger$  Difference between glutamate uptake by stimulated and nonstimulated preparations significant (by *t* test) at 1% level.

$\S$  Difference in incorporation into protein of both glutamate and leucine between stimulated and nonstimulated preparations significant (by *t* test) at 1% level.

TABLE II  
Radioautographic Localization of Glutamate Uptake in Stimulated Preparation ( $\times 10^{-5}M$ )\*

Axon	Sheath	Tracheae	Muscle interior†	Muscle surface‡	Postsynaptic region of muscle§
$2.1 \pm 0.4$	$5.9 \pm 0.5$	$4.5 \pm 0.5$	$0.97 \pm 0.04$	$1.2 \pm 0.1$	$2.9 \pm 0.5$

\* In obtaining the tissue concentrations given above, it should be recalled that grain densities were converted to molar concentrations using the specific activity of administered glutamate. Therefore, the concentrations given describe the total amount of exogenously obtained glutamate (both labeled and carrier molecules) retained by the tissue after processing for electron microscopy.

† Muscle surface represents a zone of  $1 \mu$  inside the sarcolemma. The remaining muscle fiber volume is called muscle interior.

§ The postsynaptic region was delineated by its fine structural specialization (see text).

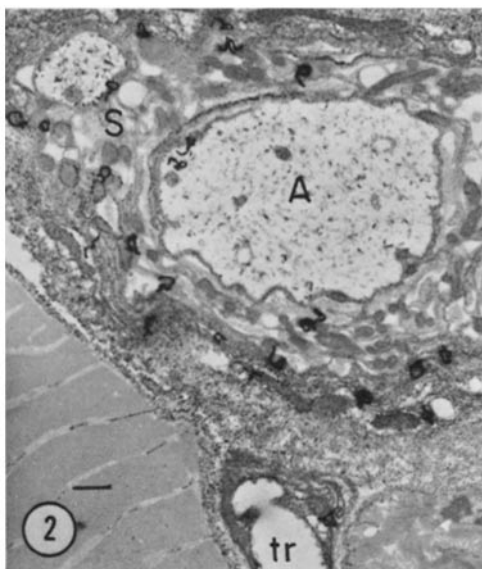


FIGURE 2 Radioautograph of nerve branch running between muscle fibers showing selective labeling of sheath in tissue exposed to glutamate- $^3H$  after nerve stimulation. *tr*, tracheole.  $\times 5300$ .

matical correction this ratio represents a lower limit. The sheath did not differ significantly from the tracheole cells (sheath/tracheole ratio =  $1.3 \pm 0.2$ ). There was considerable label in both the surface and interior portions of the muscle fibers, and a marked inhomogeneity in the distribution of label, some muscle fibers being much more heavily labeled than others. Thus, even though the density of radioactivity in the muscle

is approximately a factor of 6 lower than in the sheath and tracheole cells (Table II), the large volume of the muscle relative to the nerve plus tracheole cells ( $>80x$ ) effectively causes the muscle uptake to dominate the results in the chemical studies. It is primarily from the radioautography, therefore, that the role of the nerve can be seen.

The concentration of label in the nerve was greater at neuromuscular junctions than in the nerve branches running between muscle fibers (see Table III). The concentration in the sheath at the junction ( $13 \pm 2 \times 10^{-5} M$ ) was the highest of any compartment tabulated. Owing to radiation spread from such a small structure, this value represents only about 60% of the actual concentration (see Methods, Radioautographic Analysis (3)). The postsynaptic region of the muscle fiber surface, characterized by electron-opaque aposynaptic granules, abundant mitochondria, and the "rete synapticum" (see Edwards et al., 1958), was more heavily labeled than the nonsynaptic portion of the muscle fiber surface (see Tables II and III, and Fig. 3). There is also a significant grain density over the axon terminal at the neuromuscular junction ( $\sim 30\%$  of that in the surrounding sheath). Since the axon terminals are often small ( $\sim 1 \mu$  diameter), and are almost surrounded by a very radioactive sheath, radiation spread from the sheath constitutes a considerable fraction of the axonal grain density. A detailed analysis is now being conducted to determine the extent of this contribution and thus an accurate estimate of axonal radioactivity.

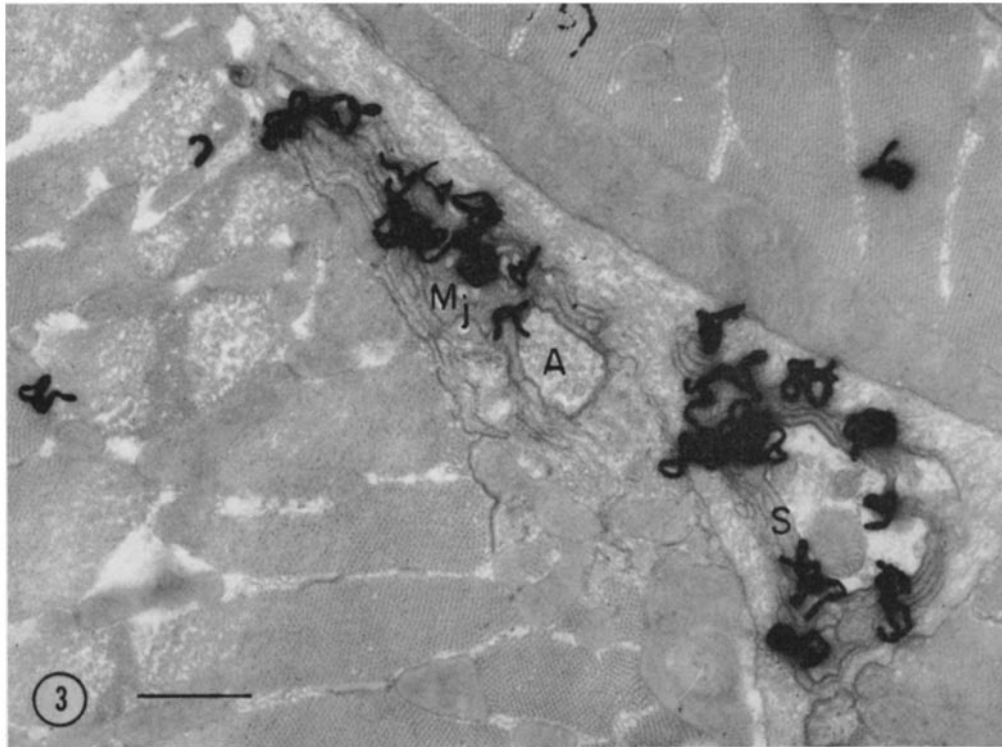


FIGURE 3 Overexposed radioautograph of nerve branch and neuromuscular junction in tissue exposed to glutamate-<sup>3</sup>H after nerve stimulation. High grain density over sheath cells and postsynaptic region of muscle fiber is demonstrated.  $\times 15,000$ .

TABLE III  
Ratio of Glutamate Uptake at Junctional (*j*) vs. Non-junctional (*n-j*) Regions after Stimulation

Sheath <sub>j</sub> /Sheath <sub>n-j</sub>	Postsynaptic region of muscle / Nonsynaptic muscle surface
3.2 ± 0.6	2.4 ± 0.5
*p < 0.01	p < 0.01

\* By  $\chi^2$  test comparing junctional and nonjunctional concentrations. The hypothesis rejected was that the junctional and nonjunctional densities came from the same population, i.e. the sampling errors in both grain counts and area corrections could account for the difference in concentrations observed.

#### DISCUSSION

The results of the chemical experiments demonstrate that glutamate is readily taken up and bound by insect nerve muscle preparations and

that this uptake is enhanced by nerve stimulation. Leucine uptake is significantly lower than glutamate uptake, and is not affected by nerve stimulation. However, nerve stimulation does appear to increase the amount of both glutamate and leucine incorporated into protein.

Peters and Ashley (1967) have emphasized that glutaraldehyde is capable of binding free amino acids to tissue, and that this effect must be considered as a potential source of artifact in electron microscope radioautography. Their study does not, however, present data on glutaraldehyde-bound amino acids in absolute terms, but only relative to the amount incorporated as protein. They, thus, demonstrate a potential problem in studying the very early stages of protein synthesis. However, they ignore the possibility that nonprotein amino acids can be bound to tissues in a physiologically meaningful way, which one might want to visualize.

The radioautographic data demonstrate the importance of sheath cells in glutamate uptake. The concept that sheath cells play a more active role in nerve function than just providing insulation and protection has been gaining strength in recent years (see Singer and Salpeter, 1966). The marked glutamate uptake by insect sheath cells reported here indicates this to be true in insects as well as in vertebrates.

The high glutamate uptake of sheath and tracheole cells provides a large glutamate sink which could protect neuromuscular junctions from high concentrations of blood glutamate, previously considered by many to be a major obstacle to acceptance of glutamate as a transmitter in this system. At first inspection, it may seem surprising that both sheath and tracheole cells show high concentrations of label (see Table II). However, it should be noted that the fine structure of these two types of cells is extremely similar. Furthermore, where tracheoles enter the sheaths of nerve branches, tracheole cells are closely associated with the axon. If the sheath provides an effective barrier against hemolymph glutamate, the tracheole cells must be expected to participate in this function or they would introduce sizeable holes in such a barrier.

If glutamate is acting as a transmitter in this system, one may view the results of the present experiments along the lines of the following working hypothesis. By providing a glutamate sink, the

sheath cells can act as a barrier to blood glutamate and control the supply of glutamate to the axon. During nerve stimulation, the sheath glutamate pool may contribute an input into the axon. (A mechanism for the transport of amino acids from sheath to axon has been demonstrated in vertebrates by Singer and Salpeter [1966], and could easily be present in insects.) In the junctional region, the sheath cells could inactivate released glutamate by re-uptake. Transmitter inactivation could also be effected by uptake of released glutamate by adjacent muscle tissue. The high glutamate uptake in the junctional region after stimulation indicates a functional specialization which could be associated with transmitter activity at this synapse.<sup>2</sup> Further radioautographic and chemical studies are currently underway to determine the chemical state of the label observed, as well as further characterization of the properties of the uptake mechanism.

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<sup>2</sup> It should be pointed out that muscles 177 a and c are innervated by four fast axons; slow and inhibitory innervation appear to be absent (Dresden and Nijenhuis, 1955, 1958; Usherwood, 1962).

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