AXONAL TRANSPORT OF [^{3}H]SEROTONIN IN AN IDENTIFIED NEURON OF *APLYSIA CALIFORNICA*

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ABSTRACT

A population of characteristic ellipsoidal dense-core vesicles was identified in axons of the giant cerebral neuron of the mollusc *Aplysia*. We injected [^{3}H]serotonin into th cell body of this identified serotonergic neuron in the isolated central nervous system in order to study the subcellular components associated with the neurotransmitter. Subcellular fractionation by differential centrifugation indicated that injected serotonin was rapidly taken up into particulate form. [^{3}H]Serotonin appeared in the axons within 2 h after injection, and export continued at a constant rate of 6% of the total in the neuron/h thereafter. The dependence of the total amounts of [^{3}H]serotonin which appeared in the axons in 6 h (export from the cell body) on the amounts injected was consistent with the idea that export is a saturable process, possibly depending on the capacity of somatic vesicles or of some unidentified carrier for serotonin. [^{3}H]Serotonin moved into both major branches of the axon, where it was translocated rapidly. The transmitter, which was shown by autoradiography to be restricted to the axons of the injected cell, was distributed along axons in accumulations of radioactivity; in contrast, its precursor, [5-^{3}H]hydroxytryptophan, moved slowly along axons in a smooth, declining curve, its kinetics consistent with diffusion. Quantitative electron microscope autoradiography revealed that the dense-core vesicles and the cytosol of axons fixed with glutaraldehyde were labeled with [^{3}H]serotonin.

A variety of processes can be approached dynamically in the living neuron by direct injection of labeled substances (1, 10, 41, 47). Metabolism of transmitters, their incorporation into vesicles, formation of organelles, and the process of axonal transport can thus be examined in a single identified cell. We have undertaken the present study in order to initiate the characterization of components involved in transport of a single transmitter substance, serotonin, in the identified giant serotonergic cerebral neuron (GCN) of the mollusc *Aplysia*. We have injected [^{3}H]serotonin into GCNs in an attempt to label structures associated with the transmitter during axonal transport. Serotonin, like other transmitter molecules that contain an amino group, can be retained in tissue by aldehyde fixation (20).

In vertebrates, numerous studies with adrenergic fibers have indicated that norepinephrine is transported along axons within specific storage granules (7, 17, 18, 29). Dense-core vesicles, revealed by electron microscopy, and the transmit-

ter, detected by light microscope histofluorescence, were found to accumulate in the nerves behind ligatures; these accumulations were prevented by the administration of reserpine. Dahlstrom and Fuxe (8) first presented evidence that the dense-core vesicles originated in the cell body. In *Aplysia*, we found that some of the serotonin being transported in the axon is contained in dense-core vesicles. This study should provide the foundation for future work on the formation of the vesicles and on their role in axonal transport. A preliminary account of these studies has been published (22).

MATERIALS AND METHODS

Distribution of GCN's Axons

The GCNs are a pair of pigmented neurons, about 300 μm in diameter, which lie symmetrically at the rostral edge of the cerebral ganglion's dorsal surface (Fig. 1). We have studied the distribution of their processes with the light microscope after intrasomatic injection of $CoCl_2$ (43) and by autoradiography after injection of [^{3}H]*N*-acetylgalactosamine (2) and [^{3}H]serotonin. The single axon of the GCN enters the neuropil of the cerebral ganglion where it sends out a number of fine processes. Similar fine branches of proximal axons have been seen in other *Aplysia* neurons (49, 53), and they probably function to receive synaptic input. The axon bifurcates about 1 mm from the cell body, sending one branch into the ipsilateral cerebrobuccal connective and the other into the ipsilateral posterior lip nerve. Once the axon in the connective has entered the buccal ganglion, it makes synaptic contacts with several neurons (34, 39) and also sends branches out through several buccal nerves into the buccal mass (51). The lip nerve terminates in the posterior region of the lip musculature.

Preparation of Animals for Intrasomatic Injection

Specimens of the mollusc, *Aplysia californica*, weighing 50–280 g, were supplied by Pacific Biomarine Supply Corp., Venice, Calif., or reared in laboratory culture (33), and kept at 15°C in aerated aquaria of Instant Ocean (Aquarium Systems, Inc., Eastlake, Ohio). Seaweed was included as food. Animals were dissected by a longitudinal incision through the foot, and the cerebral ganglion was separated from right and left pleural and pedal ganglia. Lip nerves were cut as they entered lip musculature, a distance of 9–27 mm from the ganglion. The right and left cerebrobuccal connectives were left intact; other cerebral nerves were cut within 2 mm of the ganglion. Buccal nerves were cut as they entered the buccal mass. The isolated ganglia were then pinned in a Sylchamber designed for intracellular recording and stimulation of nerves (16). Most of the connective tissue covering the dorsal surface of the cerebral ganglion was removed by dissection to facilitate impalement with microelectrodes.

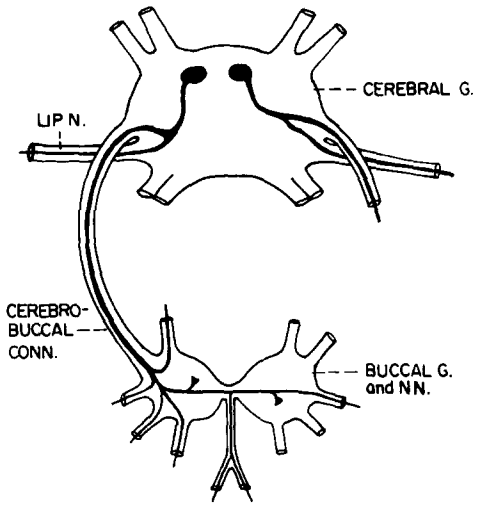

FIGURE 1 Diagram of the dorsal surface of the cerebral ganglion (G), showing the two giant cerebral neurons and their axon distributions. The distributions of proximal axons were drawn after specimens injected with $CoCl_2$ (43). These neurons have also been named Cl (50) and MCC (metacerebral cell) (34), the latter designation arising from the original description of presumably homologous neurons in the metacerebral ganglion of the snail, *Helix pomatia* (27). These neurons contain serotonin (5); the electrophysiological characteristics of the cells in *Aplysia* are similar to those in the metacerebral neurons of *Helix* (28, 34).

Intrasomatic Injection

D,L-[G-^{3}H]hydroxytryptophan (5HTP) (5.8 Ci/mmol) and [G-^{3}H]serotonin (10.7 Ci/mmol), both from Amersham-Searle Corp., Arlington Heights, Ill., and [*methyl*-^{3}H]choline (2.3 Ci/mmol, New England Nuclear Corp., Boston, Mass.) were injected into cell bodies in concentrated aqueous solutions of less than 2 nl, exactly as previously described (10, 23). Under our conditions of scintillation counting, 1 pmol of [^{3}H]serotonin corresponded to 6,650 cpm. After injection, ganglia were maintained in supplemented artificial sea water (for composition, see reference 10) at 22°C and perfused continuously at about 15 ml per hour.

Analysis of Axonal Transport

EXPORT: In order to evaluate how much serotonin had been exported from the cell body, we determined the distribution of radioactivity between cell body and axons. Cell bodies of injected neurons were dissected from ganglia under cold 70% ethylene glycol in sea water (21). The ipsilateral lip nerves and cerebrobuccal connectives were cut as they emerged from the ganglia.

Radioactivity was extracted from cell bodies and nervous tissue with 0.2 M PCA (10); serotonin was separated from its precursor, 5HTP, and from a metabolic product by high-voltage electrophoresis at pH 4.7 (10) and pH 1.9 (38). The mobility of 5HTP (relative to serotonin) at pH 1.9 was 0.54; the metabolite remained at the origin at both pH 4.7 and pH 1.9.

TRANSLOCATION: In order to study the distribution of radioactive compounds along the axons and the kinetics of their transport, we sectioned nerves sequentially into 1-mm segments. The accuracy of cutting has a coefficient of variation of 5% with a maximum range of ±15% (1). The cerebral ganglion and attached nerves were placed on the stage of a Mickle Gel Slicer (Brinkman Instruments, Inc., Westbury, N. Y.), the nerves extended until coiling disappeared, and the tissue was rapidly frozen with solid CO_2. The connective was cut as it emerged from the cerebral ganglion and was moved to another place on the stage so that it could be sectioned independently of the lip nerve. The position of the injected cell body was marked with ink, and the cerebral ganglion and nerves were then cut into 1-mm pieces beginning at the cell body. The cell body was not dissected from the ganglion in these experiments; light microscope sections through most of the cerebral ganglion after [^{3}H]serotonin injection have revealed no other labeled neurons (L. Shkolnik and J. H. Schwartz, unpublished observations). After injections of ^{3}H[5HTP], we extracted each millimeter segment by the liquid cation exchange method of McCaman, McCaman, and Lees (36) to separate serotonin from its precursor: the plh of the sample was raised to 6.8 by addition of 2.7 vol of 0.25 M Na_2HPO_4, 7 mM Na_2EDTA; 0.4 vol of bis-[diethylhexyl] phosphoric acid (Eastman Kodak Co., Rochester, N. Y.) in chloroform was shaken with the neutralized sample at room temperature. The aqueous phase was sampled for counting in Triton X-100 scintillation fluid (10), and the extracted radioactivity calculated by difference. In our hands, 85% of [^{3}H]serotonin and 4% of [^{3}H]5HTP were extracted; the metabolite formed in GCNs remained in the aqueous phase. After injections of [^{3}H]serotonin, radioactivity in the cell body, cerebral ganglion, and nerves was analyzed either by cation exchange or by high-voltage electrophoresis at pH 1.9 or 4.7.

In some experiments, one nerve containing a GCN axon was exposed to 10 mM colchicine (Fisher Scientific Co., Pittsburgh, Pa.) in sea water in a chamber 2-mm long constructed of petroleum jelly. In these experiments, the rest of the nervous system was bathed in the supplemented artificial sea water in the absence of the drug. Exposure to colchicine began at the time of injection and was continued throughout the experiment.

About 35% of the injected radioactivity escaped into the bath by 6 h after injection of [^{3}H]5HTP (23). 1 h after injection of [^{3}H]serotonin, about 35% of the injected radioactivity was found in the bath; an additional 15% appeared during the next 5 h. At the end of several experiments in which one GCN had been injected, we analyzed the contralateral lip nerve and cerebrobuccal connective which contained the axons of the other (noninjected) GCN but none of the injected GCN and found that uptake of radioactivity from the bath was negligible: only 0.3 ± 0.1% (n = 8) of the radioactivity remaining in the tissue was present in the two contralateral nerves.

Subcellular Fractionation

Cerebral ganglia, containing injected cell bodies, and nerves, containing axons of injected cells, were fractionated by differential centrifugation (23). Nervous tissue was homogenized with 10 strokes of a Potter-Elvejhem tissue grinder with a loose-fitting teflon pestle (clearance 0.25 mm), motor-driven at 1,000 rpm, and centrifuged at 1,000 g for 10 min. The resulting supernate was combined with a 1,000 g supernate from homogenized nervous tissue (equivalent to one ganglion) and centrifuged again at 9,000 g and at 105,000 g. Before the 105,000 g centrifugation, additional carrier homogenate (again equivalent to one ganglion) was added. Thus, each pellet contained particulate material from at least one *whole* ganglion. Particulate radioactivity, however, presumably originated only from the injected GCN. No radioactivity was found to be associated with particulate material in control experiments in which [^{3}H]serotonin was added to 1,000 g supernates and subsequently centrifuged at the higher forces. Serotonin in fractions was extracted by liquid cation exchange.

Microscopy and Autoradiography

Ganglia and nerves were fixed for 2 h in 5% glutaraldehyde and 1 h in 1% osmium tetroxide, washed for 1.5 h in collidine buffer, and dehydrated with an ethyl alcohol series. Samples were then infiltrated and embedded in Epon (49). Sections, 2 μm thick, were mounted on glass slides and coated with L4 emulsion (Ilford Ltd., Essex, England). These were later developed in Kodak D-19 at 68°C for 4 min. Nerves fixed 6 h after intrasomatic injection of [^{3}H]serotonin lost 17% of the total radioactivity during glutaraldehyde fixation. An additional 5% was lost during buffer washes and dehydration. Gershon and Ross (20) reported similar kinetics of washout from vertebrate tissues. Washout during osmium tetroxide fixation was not determined.

For electron microscopy, sections with pale gold interference patterns of Epon-embedded tissue on parlodion-coated grids were stained with 5% (vol/vol) uranyl acetate and lead citrate, as described by Frasca and Parks (15). Sections were examined in a Siemens Elmiskop 1 equipped with a decontamination device and operated at 80 kV.

Quantitative Autoradiography

The distribution of silver grains on autoradiographs was analyzed by the method of Williams (52). The circles used for scoring grains and for determining the areas

occupied by various tissue components had a radius of 1.5 times the half distance (the distance from a radioactive line within which half of the developed silver grains will fall); for the conditions used, Salpeter et al. (46) have determined a half distance of 165 nm. We scored 319 silver grains. Most of the grains covered at least 20% of the area of the circle, although this was not determined precisely; 10 additional grains were not scored since these grains covered more than 75% of their circle's area.

The distributions of silver grains and of dense-core vesicles relative to the axolemma were measured in micrographs enlarged 30,000 times, using the method of Salpeter et al. (46). Distance to the nearest point on the axolemma from grain center or from the center of a dense-core vesicle was obtained in units of half distance. Since the shape of the axon is quite irregular, for normalization we obtained in the same micrographs the distribution of distances from 1,561 random points to the axolemma, using a regular grid of points, each separated by six half distances.

RESULTS

Fate of Injected Serotonin

[^{3}H]serotonin injected into the GCN was rapidly converted to a single compound, which disappeared from the neuron over several hours. We have evidence that this material is a hexuronide of serotonin.[1] 1 h after injection, only half of the radioactivity in cell bodies was in the form of [^{3}H]serotonin; by 2 h, labeled transmitter constituted about 75% of the total radioactivity, and by 6 h, 85%. This suggests that transmitter gradually became protected from metabolism.

About 60–70% of the serotonin synthesized from [^{3}H]5HTP injected into serotonergic neurons was contained in the particulate fraction obtained by differential centrifugation (23). 1 h after [^{3}H]serotonin was injected, in three experiments, we found that 40% sedimented at 105,000 *g* and 23% at 9,000 *g*. Radioactivity in these fractions was recovered almost entirely as serotonin, the metabolic product remaining free in the supernate. By 6 h after injection, when about a third of the transmitter had moved into the axons (see below), 40% of the [^{3}H]serotonin in the axon was sedimented at 105,000 *g*, and 10% at 9,000 *g*. All of the radioactivity in the axons, both particulate and supernate, was present in the form of serotonin. Cytoplasmic trapping of soluble radioactivity was no greater than 3%: this proportion of the [^{3}H]5HTP and metabolite was recovered in the particulate fractions. In other experiments in which [^{3}H]choline was injected intrasomatically into GCN, only 2% of the radioactivity in homogenates of either cell bodies or axons was found in the particulate fractions.

Export

In the isolated nervous system, [^{3}H]serotonin injected into cell bodies, or synthesized from injected [^{3}H]5HTP, moved into the nerves that contain the axons of the injected GCN. Radioactive transmitter first appeared in the nerves 2 h after injection (Fig. 2). This initial lag period must result from the mobilization of serotonin into an exportable form, and its subsequent translocation along that portion of the axon lying within the cerebral ganglion (L. Shkolnik and J. H. Schwartz, unpublished autoradiographic observations). After the lag period, about 6% of the total neuronal serotonin appeared in the nerves each

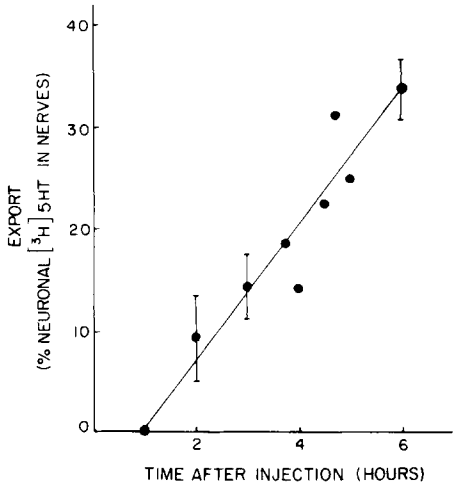

FIGURE 2 Export of [^{3}H]serotonin (5HT, ●–●) into nerves. Points were obtained by adding all of the [^{3}H]serotonin in lip nerve and cerebrobuccal connective together and expressing the sum as a percentage of the total [^{3}H]serotonin in the neuron (axonal and somatic). Mean values ± SE shown for 2 h (n = 3), 3 h (n = 3), and 6 h (n = 11).

[1] Goldman, J. E., and J. H. Schwartz. Manuscript in preparation. This compound, labeled in the serotonin moiety, was identified as an *o*-hexuronide by its mobility during electrophoresis and chromatography, by its sensitivity to acid and alkaline hydrolysis, and by its digestibility with β-glucuronidase. Although most likely serotonin-*o*-glucuronide, the sugar moiety has not yet been established.

hour. By 6 h, 33% of the [^{3}H]serotonin in the neuron had entered the nerves.

The process of export appeared to depend on the intracellular content of [^{3}H]serotonin (Fig. 3). With smaller injections, the amounts exported after 6 h were found to be proportional to the amounts present in the neuron. When more than 4 pmol were present, export did not increase proportionately, suggesting that some component of the export process had become saturated. The proportionately lower export observed with larger injections did not occur because larger volumes were injected. Solutions of various concentrations of [^{3}H]serotonin were used. With the range of volumes injected (about 0.05–2 nl), we did not observe effects either on the amounts of [^{3}H]serotonin exported or on the electrophysiological properties of the cells. Weinreich et al. (50) have reported that the average endogenous concentration of serotonin in the GCN is 6 pmol.

The axon of the GCN bifurcates within the cerebral ganglion to send branches into the cerebrobuccal connective and the lip nerve. 6 h after intrasomatic injection, the ratio of [^{3}H]serotonin in the connective to that in the nerve was 1.94 ± 0.31 SE (n = 11) with a range of 0.81–4.07.

Autoradiographic Localization of [^{3}H]Serotonin in Axons after Intrasomatic Injection

In order to show that radioactivity was confined to the axons of the injected neuron, we examined transverse sections of the cerebrobuccal connective by autoradiography after injecting [^{3}H]serotonin into the cell body. With the light microscope, silver grains were found over the axon of the GCN, the largest process in the nerve (Fig. 4). Axons of other neurons were not labeled. Labeling, only slightly greater than background, was observed over a narrow band of tissue just adjacent to the axon; this may have resulted artifactually from some movement of the transmitter during fixation. Alternatively, a small amount may have escaped from the axon during transport.

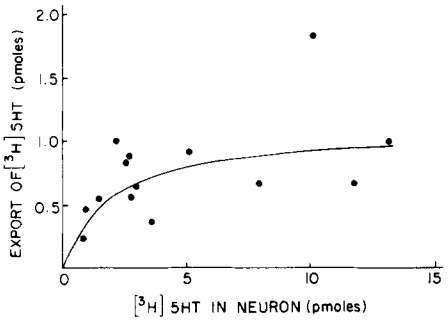

FIGURE 3 Dependence of export on the amount of [^{3}H]serotonin in GCN 6 h after injection. The data were fit by a least-squares computer program to a double-reciprocal plot. The curve in the figure was drawn by replotting the fitted points.

Localization of [^{3}H]serotonin was also studied by autoradiography with the electron microscope (Fig. 5). Silver grains were seen over one axon in transverse sections of the connective. Only a few were found outside the axon, and these were located close to the axolemma of the labeled neuron (Fig. 6). In addition to containing the organelles observed in cholinergic *Aplysia* neurons (mitochondria, smooth endoplasmic reticulum, multivesicular bodies, dense bodies, and a variety of vesicle profiles), the axon labeled with serotonin contained a population of dense-core vesicles, which while not abundant (Table I) were absent in cholinergic neurons (49). This organelle appeared to be composed of an outer membrane separated from a dense core by a clear ring (Fig. 7). Intensity of staining of the core varied considerably, however.

Unlike the larger, densely staining profiles in cholinergic neurons, which were irregular in shape and which varied greatly in size,[2] the dense-core vesicles in the serotonergic axon were quite regular and were fairly uniform (Fig. 8). These vesicles appear to be ellipsoids: the two maxima (66 and 79 nm) obtained when 219 profiles were measured probably are an index of the major and minor axes. The profiles are similar to the dense-core granules which Rosenbluth (44) first described in *Aplysia* and Gerschenfeld (19) found in synapses of several pulmonate molluscs. Both Rosenbluth and Gerschenfeld postulated that these granules were aminergic vesicles.

We observed significant labeling only over dense-core vesicles and over cytosol (which included microtubules and neurofilaments); no other components were labeled (Table I). Because of their similarity to dense-core aminergic vesicles in other animals, we presume that these profiles,

[2] Dense-core vesicles in the axon of the cholinergic neuron, L10, were larger than those in GCN's axon (average diameter 108 nm compared to 75 nm, with the largest 173 nm compared to 96 nm) (49).

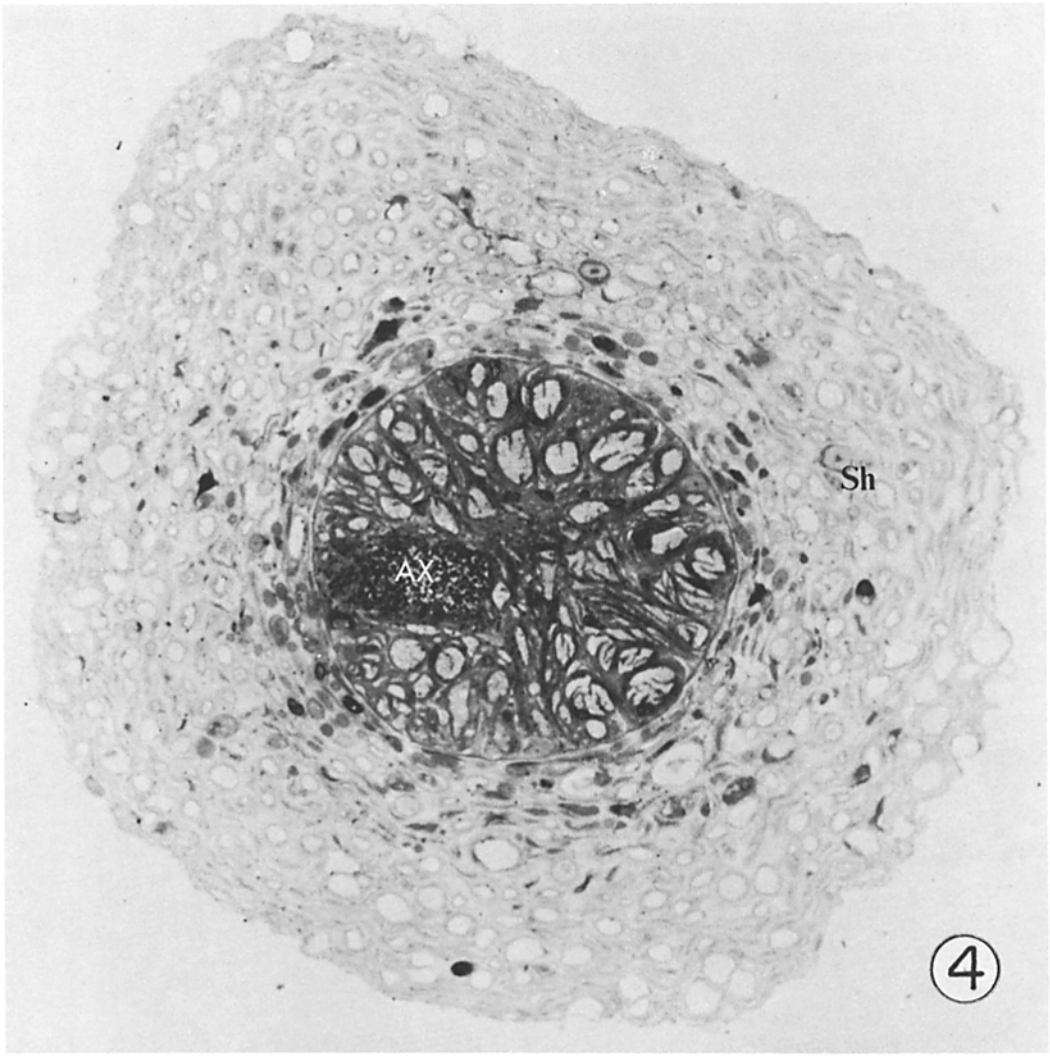

FIGURE 4 Light microscope autoradiography of a transverse section through the cerebrobuccal connective 6 h after intrasomatic injection of [^{3}H]serotonin. Tissue was stained with methylene blue. Silver grains retained by glutaraldehyde fixation were found almost entirely over a single axon *(Ax)*. Other axons and connective tissue sheath *(Sh)* were not labeled. The autoradiograph was exposed for 31 days. × 630.

which contained the radioactive transmitter, are serotonergic vesicles.

In anumber of autoradiographic studies, membranous organelles, labeled with various glycoprotein precursors, have been found not to be distributed uniformly throughout axonal cross sections: labeled membranes have usually been most heavily concentrated in regions close to the axolemma (see; for example, reference 3). We analyzed distributions both of dense-core vesicles and of silver grains in the axon of the GCN. The normalized histograms presented in Fig. 6 A show that the vesicles were concentrated close to the axolemma. It was our impression that they tended to occur in clusters, but this was not examined systematically. The distributions of vesicles and silver grains were not precisely congruent; the grains were less concentrated peripherally (Fig. 6 B).

Distribution of [^{3}H]Serotonin Along Axons

Injected [^{3}H]serotonin or the serotonin synthesized from injected [^{3}H]5HTP was distributed along axons in discrete accumulations of radioac-

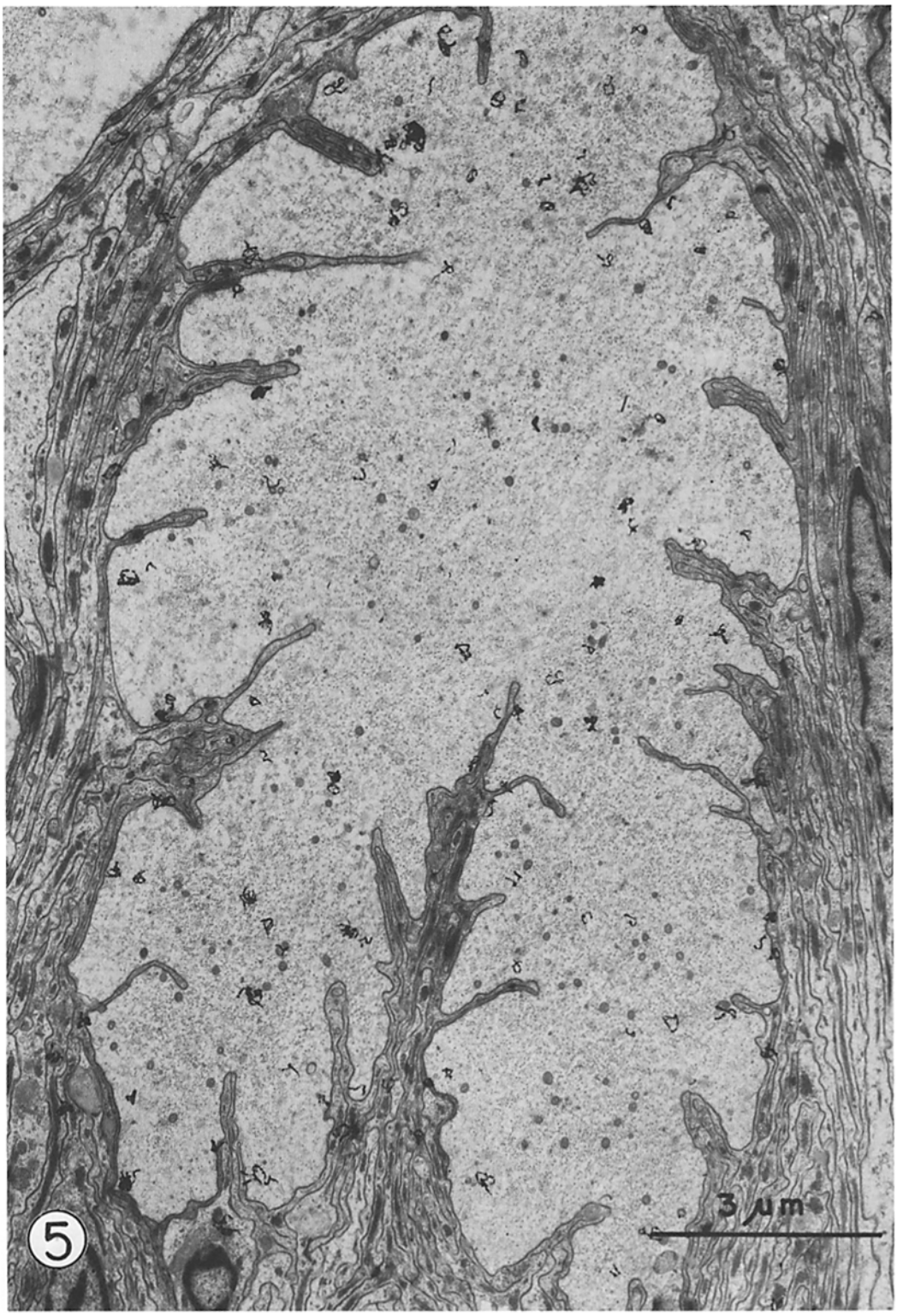

FIGURE 5 Electron microscope autoradiograph of the axon of a GCN 6 h after injection of [^{3}H]serotonin in the cerebrobuccal connective cut in transverse section about 6 mm from the cell body. The autoradiograph was exposed for 2 mo. × 12,000.

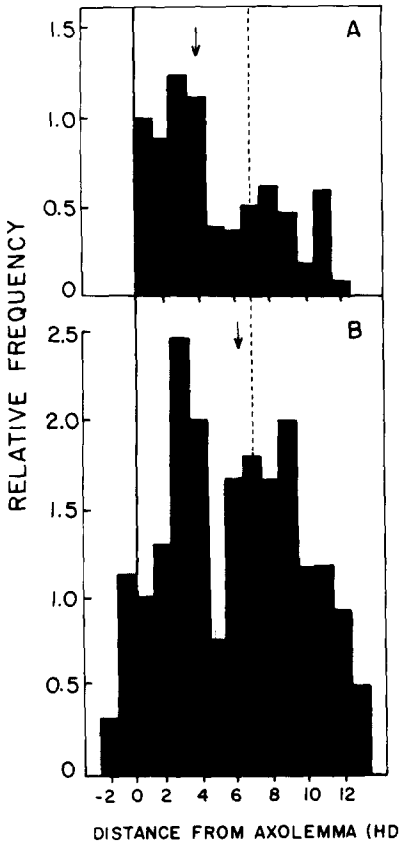

FIGURE 6 Histogram showing the distribution of (A) dense-core vesicles and (B) silver grains in autoradiographs of the cerebrobuccal connective containing an axon of a GCN 6 h after injection of [^{3}H]serotonin. The shortest distance from each vesicle or silver grain to the axolemma was measured in units of half distance (HD, 165 nm) on the autoradiographs used to obtain the data presented in Fig. 7 and Table I. The actual proportion of the 219 vesicles or 329 grains at each distance was normalized by dividing by the proportion of the 1,561 random points at that distance. The 329 grains used for this histogram include the 10 grains covering 75% of the area circle and thus were not scored for morphometry (see Materials and Methods). In order to obtain the *relative* frequency at each distance, we divided these normalized values by the normalized value obtained for vesicles or grains one-half distance within the axon from the axolemma, which is indicated in the figure by a solid vertical line at zero distance. The arrows indicate the distance from the axolemma which contains half of all the vesicles or grains, and the dashed vertical line indicates the distance from the axolemma which contains 50% of the random points.

tivity, rather than in a smooth curve or single moving front (Fig. 9). The profiles shown in the figures are typical, but in 12 experiments the positions and numbers of these accumulations varied from cell to cell. The longer the period of time after the injection, the farther the most distal accumulation of serotonin had moved from the cell body. In a few experiments, however, a distinct distal boundary of radioactivity was not observed. Calculation of a single rate of transport was difficult, since it necessitates an arbitrary judgment about the location of the most distal moving boundary. Nevertheless, in all experiments significant amounts of radioactivity (0.5% of the total [^{3}H]serotonin in the neuron) had moved at a rate of at least 50–60 mm per day.

When injected intrasomatically, [^{3}H]5HTP did not become associated with particulate fractions (23), and, thus, presumably was not taken up into vesicles. The precursor might therefore be expected to move out of the cell body and along the axons by diffusion alone. As anticipated, [^{3}H]5HTP was distributed along axons in a smooth, declining curve; no accumulations of radioactivity were seen (Fig. 9). Furthermore, as the distance along the axon from the cell body increased, a greater proportion of the radioactivity in each millimeter segment was recovered in the form of [^{3}H]serotonin (Fig. 11). Enrichment of transmitter in the axon with distance from the cell body was also characteristic of acetylcholine transport after intrasomatic injection of [^{3}H]choline (11).

Effect of Colchicine

Colchicine has been shown to block axonal transport in a number of animals (6, 26, 30, 32). To provide additional evidence that serotonin moves by fast transport, we examined the effect of colchicine on transport of serotonin in the lip nerve. The transmitter accumulated at the site where the drug had been applied (Fig. 10). In this experiment, translocation in the cerebrobuccal connective, which was maintained in the absence of the drug, was not impeded. Conversely, in another experiment in which colchicine was applied only to the connective, translocation was blocked in that nerve, but not in the lip nerve.

Transport in a Semi-Intact Animal

In most of our experiments, we cut the lip nerve at the lip musculature and buccal nerves as they entered the buccal mass, so that injected cell bodies were severed from their synaptic terminals. To rule out the possibility that this injury had a marked effect on the rate or amount of serotonin

TABLE I

Distribution of Silver Grains Over the Axon of a GCN after Intrasomatic Injection of ^{3}H-Serotonin

Structures	Effective area	Silver grain distribution	Relative specific activity	Observed no. of grains	Expected no. of grains	χ^2
	%	%				
A. Single						
Cytosol	52.6	70.0	1.3	222	168.0	46.2*
Outside of axon	27.6	7.5	0.3	23	87.0	17.2*
B. Junctional						
Cytosol-outside	8.3	6.9	0.8	22	26.0	0.6
C. Compound						
Dense-core vesicles	0.36	5.3	14.7	17	1.1	210.0*
Mitochondria	2.6	1.6	0.6	5	8.3	0.3
Dense bodies	1.32	2.8	2.1	9	4.2	4.4
Smooth membranous structure	5.4	3.1	0.6	10	17.2	2.6
Smooth membranes and outside	1.74	0.9	0.5	3	5.6	0.8
Dense-core vesicles and others*	0.06	0.6	–	3	–	–
Dense bodies and others*	0.12	0.6	–	3	–	–
Mitochondria and others*	0.06	0.3	–	2	–	–
					χ^2 (8df) =	40.4

The distribution was analyzed by the method of Williams (52). Structures possibly containing radioactivity within circles (see Materials and Methods) were scored as single (structures which completely filled circle), junctional (two or more structures within circle), or compound (structures too small to fill circle). All of the circles containing compound structures also included cytosol. A regular grid of circles identical in size to that used for scoring grains was used to measure the relative areas occupied by each structure. The frequency of occurrence of a structure within the grid circle was the effective area. Relative specific activity is the ratio of the frequency of grains to the effective area. The χ^2 test indicated that the asterisked entries were either greater (cytosol, dense-core vesicles) or less (outside of the axon) than expected ($P < 0.01$, two tailed).

* These items were not included in the statistical evaluation since the data associated with them were too few to be reliable.

transported, we injected a GCN in an animal from which the cerebral and buccal ganglia had not been removed from areas of GCN innervation; the buccal nerves were left attached to the buccal mass and the lip nerve was not cut. The cerebral ganglion was then separated from the pleural and pedal ganglia, pinned to a translucent substage, and illuminated from below. The rates of translocation and the proportion of total [^{3}H]serotonin exported were not different from those found in neurons separated from their terminals (data not shown).

DISCUSSION

Dynamics of Export and Translocation of Serotonin

Axonal transport is a complex process which consists of at least two steps: export from the cell body and translocation along axons (2). It is uncertain whether vesicles are involved in either of these processes. The observed saturation of the export process (Fig. 3) might be explained if the transmitter could be exported only within vesicles, and if the reaction that resulted in their loading were concentration dependent. Thus, vesicles in the cell body might be incompletely filled. Export of vesicles from the cell body and their translocation along axons may therefore be independent of their content of transmitter. Alternatively, some soluble factor might act as the carrier.

A few experimental studies have indicated that serotonin, like other neurotransmitters (7, 13, 18), can be transported along axons in the orthograde direction. In rat spinal cord, serotonin accumulated proximal to a transection at 0.4–0.5 mm/h (9); in molluscs, transport along axons of the metacerebral cells of the snail, *Helix pomatia* (41), and along axons within nerves of *Anodonta cygnea* has been shown autoradiographically (24). In *Aplysia*, we found that serotonin moved at rates similar to those of fast transport found in other poikilotherms (12, 14, 37). Moving at 50–60 mm/day, material synthesized in the cell body would

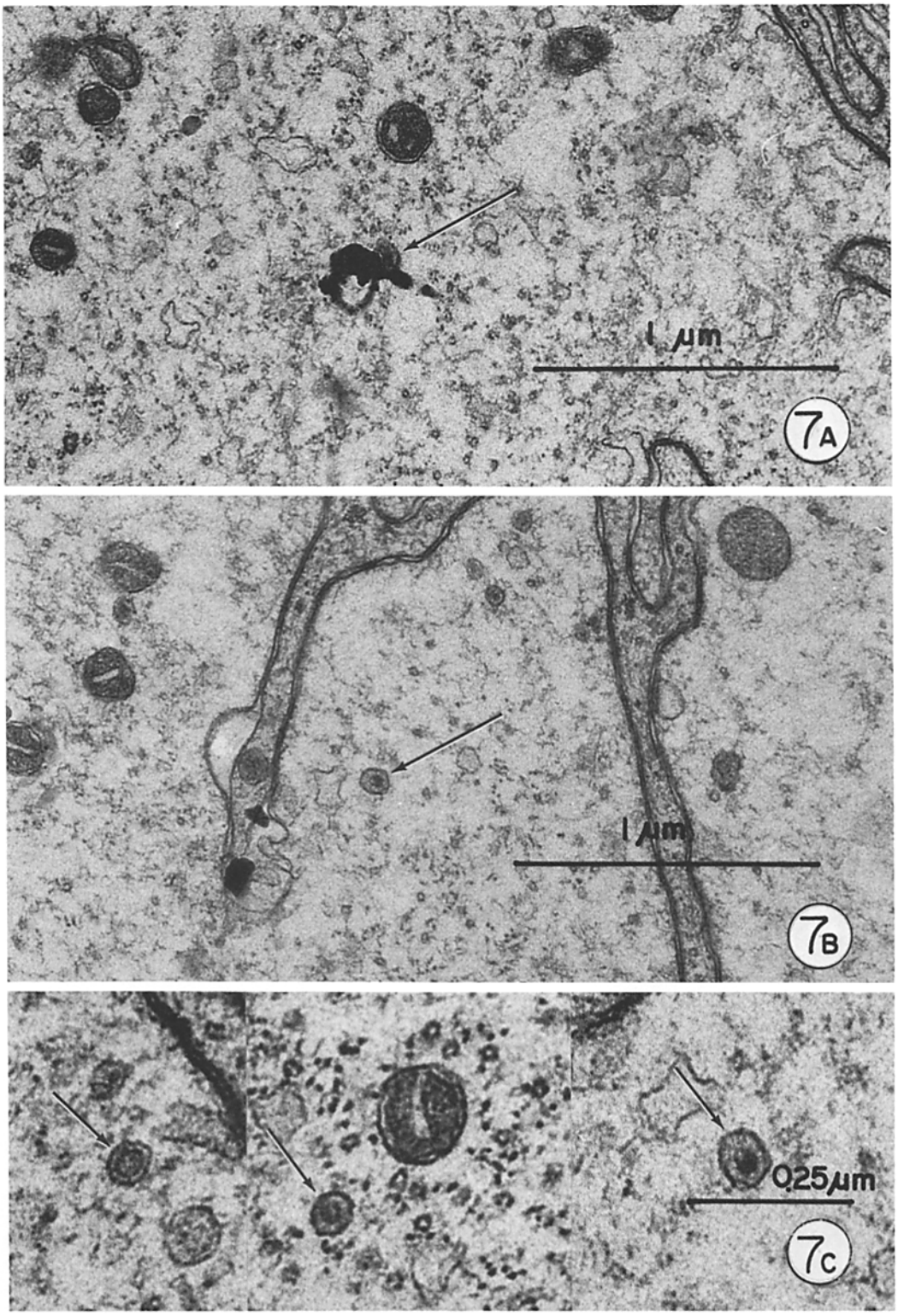

FIGURE 7 Electron micrographs of various fields within the axon of the GCN shown in Fig. 4. Arrows point to dense-core vesicles. In A, the vesicle is partially covered by a silver grain. The variability in the intensity of staining is best seen in the vesicles shown in C. (A, B) × 47,000; (C) × 90,000.

reach synaptic terminalsin the buccal ganglion in about 4.5 h and terminals at the end of an 18-mm lip nerve in about 8 h. Thus, changes in biosynthetic activity in the cell body would not be felt in synaptic regions for several hours.

It is unlikely that the relatively small amounts of transmitter synthesized in the cell body contribute substantially to the store of transmitter at synapses. It is not known whether the transmitter substance itself plays a physiological or regulatory role in the cell body or axon. We rather presume that movement of [^{3}H]serotonin reflects the transport of serotonergic vesicles, synthesized in the cell body, for long-term replenishment of the macromolecular components of synaptic vesicles.

Association of Serotonin with Dense-Core Vesicles

Indirect evidence that axonal transport of serotonin might involve vesicles was provided by our earlier study of the fate of serotonin in serotonergic and cholinergic neurons (23). The fast rates of translocation observed (Fig. 9) also suggest that the transmitter is moving within vesicles, although serotonin bound to a soluble carrier might also move rapidly. In other animals, however, most of the material being transported rapidly has been shown to be particulate (see references 3, 12, 14, 37). Labeled membrane glycoproteins in *Aplysia* neurons are also rapidly translocated along axons (1). If the serotonin were *free* in the axon, its movement might be expected to have kinetics of diffusion. We have observed smooth, rapidly declining distributions along the axon with small average displacements with several small molecules: [^{3}H]5HTP in GCN (see Fig. 9); [^{3}H]serotonin in R2,[3] a cholinergic neuron which

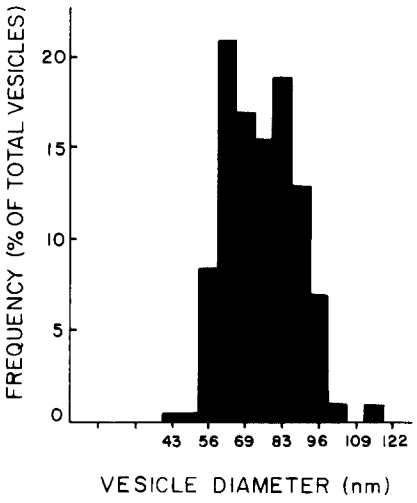

FIGURE 8 Histogram showing dimensions of 219 dense-core vesicles in the axon of the GCN in the cerebrobuccal connective. The largest diameter of each dense-core profile was measured in micrographs enlarged 30,000 times. The same micrographs were used to obtain the data in Fig. 6 and Table I.

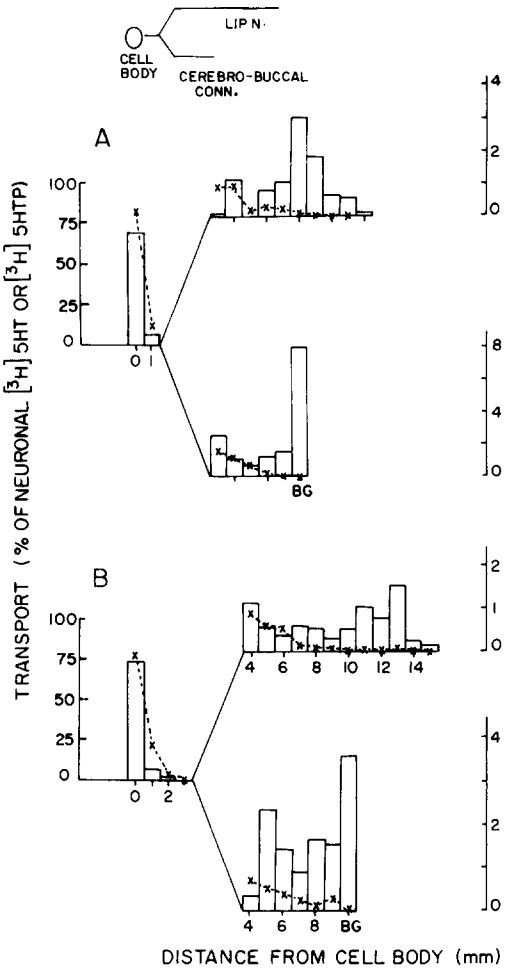

FIGURE 9 Distribution of [^{3}H]serotonin (□) and [^{3}H]5HTP (×——×), (A) 4.5 h after injection. 1% of total [^{3}H]serotonin corresponds to 279 cpm, 1% of [^{3}H]5HTP to 408 cpm. (B) 6 h after injection. 1% of total [^{3}H]serotonin corresponds to 117 cpm, 1% of [^{3}H]5HTP to 403 cpm. Figures are constructed in the format of the neuron's axon distribution, as diagrammed (upper left).

[3] Treistman, S. N., and J. H. Schwartz. Manuscript in preparation.

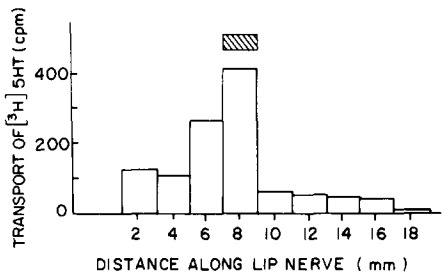

FIGURE 10 Effect of 10 mM colchicine on translocation of [^{3}H]serotonin along lip nerve 6 h after intrasomatic injection. Colchicine was applied to the nerve in supplemented artificial sea water in the position indicated by the hatched bar.

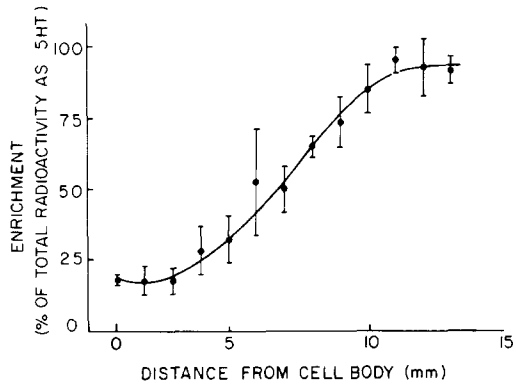

FIGURE 11 Enrichment of [^{3}H]serotonin with distance from cell body along the lip nerve. [^{3}H]Serotonin was expressed as a percentage of total cpm contained in each mm segment. Points are mean values ± SE of three experiments; the curve was drawn by eye.

did not package the transmitter (23); Pi in R2 (31); and both [^{3}H]fucose and [^{3}H]*N*-acetylgalactosamine in both GCNs and R2 (Ambron, Goldman, and Schwartz, unpublished observations). Moreover, movement of these low molecular weight substances along axons was not blocked by colchicine.

Direct support for the idea that transport of some of the transmitter occurs in association with dense-core vesicles was obtained by electron microscope autoradiography. [^{3}H]Serotonin, introduced into the cell body by pressure injection, labeled small vesicles with characteristic dense cores; the distribution of silver grains over other membranous organelles was not different from random. Weinreich et al. (50) noted similar profiles in the cell body of the GCN. In general appearance, these profiles were similar to aminergic vesicles described in other animals (17). Similar vesicles have been seen in the cell body (42) and axons (41) of the giant serotonergic neuron of *Helix* as well as in unidentified molluscan neurons (see, for example, references 19, 44, 54). Dense-core vesicles with a wider size range were observed in a dopamine-containing neuron of the snail, *Planorbis corneus* (40). In the heart of *Aplysia,* which receives serotonergic innervation (35), [^{3}H]serotonin is taken up into nerve terminals containing dense-core vesicles (48). The vesicles in GCN also resemble the smaller dense-core vesicles found in vertebrate sympathetic (17) and central serotonergic (4) axons. Because of these similarities, and because a population of vesicles of this type was not seen in R2 or L10, cholinergic *Aplysia* neurons (49), we conclude that the dense-core profile is the serotonergic vesicle as it appears in the axon.

Even though dense-core vesicles from various animals have features in common, they nevertheless are difficult to compare precisely, since descriptions in the literature show considerable variation. For example, Rude et al. (45) have found dense-core vesicles in the cell bodies of the giant serotonergic Retzius cells of the leech, and Howes et al. (24) have injected [^{3}H]5HTP into ganglia of the freshwater clam, *Anodonta cygnea,* and localized silver grains to dense-core vesicles of unidentified neurons. The morphology of vesicles in the Retzius cell and in *Anodonta* is different from that described in the GCN. To some extent, these differences probably result from differences in conditions of fixation. Thus, the dimensions of the particle are possibly sensitive to osmotic environment, and intensity of staining appears to vary with the protocols of fixation (25).

Although we have shown that dense-core vesicles were the only organelles labeled with [^{3}H]serotonin, our autoradiographic studies do not permit us to conclude that all of the transmitter in the axon is vesicular. Most of the grains were located over cytosol. It could be argued that serotonin moves along axons in some soluble form. Nevertheless, we believe that quantitative autoradiography underestimates the fraction of free transmitter in vesicles. Some of the silver grains apparently over cytosol might have masked underlying vesicles, which are quite small relative to the size of the grains. Even so, the relative specific radioactivity associated with the vesicles was 11 times greater than that in cytosol (Table I).

A critical indication supporting the hypothesis that a large proportion of the axonal serotonin was

in vesicles in the living cell is that at least half of the radioactive transmitter could be recovered in particulate form by differential centrifugation. Electron microscopy of particulate fractions revealed a heterogeneous population of membranes and vesicles. This might be expected, since they largely represented material from the carrier nervous tissue added during the fractionation procedure, rather than only the contents of the injected neuron. Because of this great heterogeneity, we have not examined these fractions by electron microscope autoradiography. Control experiments argue against nonspecific trapping of cytoplasmic [^{3}H]serotonin in these fractions, however. Thus, it is reasonable to infer from our results that translocation of at least some of the serotonin reflects the movement of dense-core vesicles.

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