

Metabolism of Acetylcholine in the Nervous System of *Aplysia californica*

IV. *Studies of an Identified Cholinergic Axon*

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ABSTRACT [^3H]Choline, injected directly into the major axon of the identified cholinergic neuron R2, was readily incorporated into [^3H]acetylcholine. Its metabolic fate was similar to that of [^3H]choline injected into the cell body of R2. Over the range injected, we found that the amounts of acetylcholine formed were proportional to the amounts injected; the synthetic capability was not exceeded even when 88 pmol of [^3H]choline were injected into the axon. Newly synthesized acetylcholine moved within the axon with the kinetics expected of diffusion. We could not detect any selective orthograde or retrograde transport from the site of the injection. In contrast, as indicated by experiments with colchicine, 30% of the [^3H]acetylcholine formed after intrasomatic injection was selectively exported from the cell body and transported along the axon. Most of the [^3H]acetylcholine was recovered in the soluble fraction after both intra-axonal and intrasomatic injection of [^3H]choline; only a small fraction was particulate. The significance of large amounts of soluble acetylcholine in R2 is uncertain, and some may occur physiologically. The concentrations of choline introduced by intraneuronal injection into both cell body and axon were, however, greater than those normally available to choline acetyltransferase in the cholinergic neuron; nevertheless, these large concentrations were efficiently converted into the transmitter. The synthetic capacity of the neuron supplied with injected choline may exceed the capacity of storage vesicles and of the axonal transport process.

INTRODUCTION

Because the enzymes which catalyze formation of neurotransmitter substances are synthesized in the perikaryon and move down the axon (4, 10, 37), it is not surprising that choline acetyltransferase is found in cholinergic cell bodies and axons (14). In neurons which contain norepinephrine and other biogenic amines, histochemical techniques indicate that significant amounts of the transmitters are present in cell body and axon (3); direct measurement of acetylcholine (17, 23) as well as biogenic amines (25, 27, 38) and GABA in identified invertebrate nerve cell bodies (26) and axons (21); also shows that these transmitters are present in substantial concentrations. In these studies, however, it was not possible to compare the capacity for neurotransmitter synthesis in vari-

ous regions of the same neuron, or to determine whether the region in which the transmitter is formed can affect its movement along the axon. By using intracellular injection of precursors, it has been shown directly that acetylcholine can be synthesized efficiently in the cell bodies of cholinergic neurons in the isolated central nervous system of *Aplysia* (6, 7). Similarly, serotonin can be formed in identified serotonergic cell bodies (6). We have also noted briefly that acetylcholine could be synthesized in the major axon of R2, the giant cholinergic neuron of *Aplysia's* abdominal ganglion after intra-axonal injection of [³H]choline (36).

We undertook the present studies in order to determine whether the acetylcholine synthesized in the axon is metabolized differently from that synthesized in the cell body. We also thought that a comparison of the movement of axonal transmitter with that of somatic acetylcholine might provide insight into the mechanisms of orthograde and retrograde transport. We found that although axonal acetylcholine was metabolized in the same manner as somatic transmitter, we were unable to detect any active transport along the axon in either direction. Most of the axonal acetylcholine remained free, possibly because it was produced in amounts which exceeded the capacity of the components of the transport system. Although not conclusive, these results can be explained if acetylcholine is actively transported only in vesicular form, and if the capacity or loading mechanism of vesicles is limited in the axon relative to the synthetic potential of the choline acetyltransferase in this region.

MATERIALS AND METHODS

Animals

Aplysia californica weighing 80–160 g were supplied by Pacific Bio-Marine Laboratories, Inc., Venice, Calif., and were maintained at 15°C in well aerated aquaria of Instant Ocean (Aquarium Systems, Inc., Eastlake, Ohio).

Preparation of Radioactive Materials for Injection

[³H]Choline chloride (16 Ci/mmol), [G-³H]serotonin creatinine sulfate (10.7 Ci/mmol) (Amersham/Searle Corp., Arlington Heights, Ill.), and L-[1,5,6-³H]fucose (4.8 Ci/mmol) (New England Nuclear, Boston, Mass.) were each concentrated under a stream of nitrogen to a volume of about 1 μ l, and stored in water under silicone oil (200 Dielectric Fluid, 350 cSt, Dow Corning Corp., Midland, Mich.). [³H]Serotonin was kept at -70°C, the others at -20°C. (Under our conditions of counting, 1 pmol of [³H]choline corresponded to 6,600 cpm.) 0.25 mCi of [³H]acetyl CoA (0.9 Ci/mmol, New England Nuclear, Boston, Mass.) was lyophilized in a conical tube, and the dried material was taken up in 0.1 ml of water and then concentrated under nitrogen as already described.

Intra-Axonal Injection

The central nervous system was removed from animals through an incision in the foot. As described previously in greater detail (36), a region of the right connective, from 5 to 10 mm long, and usually midway between the attached abdominal and right pleuropedal ganglia (ranging from 14 to 26 mm from the cell body of R2), was placed into a well bordered by petroleum jelly; the well contained 50 μ l of 0.05% fresh bovine trypsin (diphenyl carbamyl chloride [DCC] treated, crystalline, Miles-Seravac Ltd., Maidenhead, Berks., England) in Instant Ocean buffered with 10 mM Tris-HCl (pH 7.6). After 20 min at room temperature, the solution containing trypsin was replaced for 1–2 min with In-

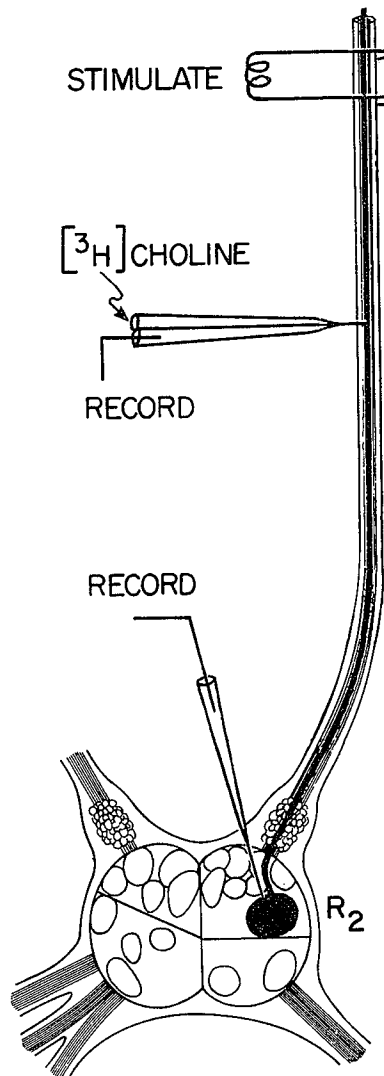


FIGURE 1. Experimental arrangement. The abdominal ganglion and the right connective were pinned through the connective tissue sheath. The injection site ranged from 14 to 26 mm from the cell body. A single-barreled glass microelectrode was used to impale R2's cell body, while the double-barreled micropipette for recording and injection was advanced into the region of the right connective softened by exposure to trypsin. Impalement of R2's axon was signaled by a sudden negative shift in recorded potential, usually accompanied by a few action potentials recorded in both cell body and axon. Stimulation of the distal right connective through chlorided silver electrodes caused a spike to appear first at the recording electrode in the axon and then at the electrode in the cell body.

stant Ocean containing 0.1% soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.). The nervous system was then pinned through the connective tissue of the abdominal and circumesophageal ganglia and along the right connective nerve at either end of the trypsinized region in a dish lined with Sylgard (Dow Corning) (Fig. 1). For both re-

ording and stimulating, the cell body of R2 was impaled with a glass micropipette filled with 2 M potassium citrate. A double-barreled micropipette, one barrel filled with 2 M potassium citrate and connected to an amplifier with a bridge circuit for recording and stimulating, and the other filled with about 1 nl of radioactive solution drawn into the tip by suction (19) was advanced into the trypsinized region of the connective. Impalement of an axon was signaled by a negative change in potential of 45–60 mV; an impaled axon was identified as R2's if we observed: (a) synchronous somatic and axonal action potentials immediately after impalement; and (b) an action potential first in the axon and then, with delay, at R2's cell body after stimulation with extracellular electrodes distal to the intra-axonal electrode; and (c) that direct depolarization of R2's cell body resulted in transmission of an action potential to the axon or that depolarization of the axon resulted in transmission to the cell body.

After impalement, radioactive solution was injected by positive pressure. Only those cells with a resting potential after injection greater than -45 mV and an action potential greater than 70 mV at both axon and cell body were further studied. After maintaining the isolated nervous system containing an injected axon for various periods of time (see below) the cell body was penetrated again and the electrophysiological condition of the neuron assessed by testing for an antidromic response in the cell body to stimulation of the connective nerve distal to the site of the injection.

Intrasomatic Injections

The cell body of R2 in the isolated central nervous system was injected as previously described (6, 7, 19). As with intra-axonal injection, the resting potential and antidromically evoked action potential were measured after the injection and after maintenance for the period of the experiment.

Conditions of Maintenance after Injections

After injection of [^3H]choline or [^3H]acetyl CoA, nerve tissue was maintained at 15°C in 10 ml sterile Instant Ocean containing 50 mM Tris-HCl (pH 7.6), penicillin G (200 U/ml), streptomycin (0.1 mg/ml), 0.1% glucose, and 20 μM choline chloride. After injection of L-[^3H]fucose and [^3H]serotonin, the tissue was maintained in an artificial seawater supplemented with amino acids, vitamins, and 0.1% glucose (for composition see reference 6). In some experiments nerve tissue was exposed to freshly prepared 10 mM colchicine sulfate (Fisher Scientific Co., Springfield, N. J.) at 15°C in the dark for 4 h before injection. After injection, nervous systems were maintained in the dark at 15°C in the presence of fresh colchicine. Antidromic responses obtained in the cell body appeared normal after exposure to colchicine for up to 24 h.

Measurement of Transport

The surface of the connective at the site of an intra-axonal injection was marked by Procion Scarlet (ICI, Stamford, Conn.) which was released onto the sheath by pressure from the tip of a micropipette broken to a diameter of approximately 25 μm . The mark formed remained visible during all subsequent procedures. The injected nervous system was frozen quickly with solid CO_2 on a brass block, with the right connective stretched to the point where coiling was no longer visible, and cut sequentially into 1-mm segments with a Mickle gel slicer (Brinkmann Instruments, Inc., Westbury, N. Y.). When total radioactivity was to be measured, each segment was extracted in 1.3 ml of water in a scintillation vial by freezing and thawing three times. Radioactivity was counted after addition of 10 ml of scintillation fluid containing Triton-X 100 (Rohm and Haas Co., Philadelphia, Pa.) (6). This procedure released as much radioactivity as did digestion of the segments for 24 h with the alkaline solubilizer, NCS (New England Nuclear, Boston,

Mass.); homogenization of segments after repeated freezing and thawing also released no additional radioactivity. When radioactivity in segments was to be characterized biochemically, each segment was homogenized separately in a glass tissue grinder (Micrometric Instruments, Cleveland, Ohio). When total radioactivity in the cell body was to be counted, the nervous system was not frozen but the cell body was dissected from the ganglion and homogenized. In these experiments the remainder of the abdominal ganglion contained less than 1% of the radioactivity in the isolated cell body, indicating that radioactivity injected into the axon remained within R2.

Analytic Procedures

CHOLINE AND DERIVATIVES. Samples were homogenized at 0°C in acetone-formic acid 1 M (85:15) (35). [³H]Acetylcholine was separated from other derivatives by high-voltage electrophoresis at pH 4.7 for 2 h (11). Phosphorylcholine was separated from betaine by electrophoresis at pH 7.8 in 0.05 M ammonium bicarbonate for 1.5 h in a potential gradient of 15 V/cm (29).

After injection of [³H]acetyl CoA, nerve tissue was extracted with acetone-formic acid, and particulate material was removed by brief centrifugation at low speed. The extract was dried under nitrogen. The residues were dissolved in 10 mM ammonium acetate buffer (pH 4.2), and [³H]acetylcholine was precipitated as the reineckate (29). After washing, reineckate was removed as the silver salt, leaving choline derivatives in solution in aqueous acetone. This solution was dried and then assayed for [³H]acetylcholine by electrophoresis at pH 4.7.

After injection of [³H]fucose, tissue samples were homogenized at 0°C in 10% TCA. Precipitate was collected on glass fiber pads (Whatman GF/C), and radioactivity incorporated into glycoprotein measured by scintillation (28). Acid-soluble radioactivity in the filtrate was also sampled.

After injection of [³H]serotonin, tissue samples were homogenized in 0.3 M PCA. Macromolecules were precipitated, and serotonin was extracted from the resulting supernate by liquid cation exchange, modified from McCaman et al. (22).

Subcellular Fractionation

Nervous systems containing an R2 injected with [³H]choline were maintained in Instant Ocean for 20 h at 15°C, placed in Instant Ocean containing 50 μM eserine sulfate for 10 min, and then washed for 10 min with three changes of 0.2 M sucrose containing 0.3 M NaCl (18) and 50 μM eserine. The right connective was cut from the abdominal ganglion, and ganglion and connective were minced separately in 0.8 ml of 0.2 M sucrose containing 0.3 M NaCl at 0°C. An eserinated abdominal ganglion from an uninjected nervous system was minced together with the right connective as carrier. The minced tissue was transferred to a loose-fitting Potter-Elvehjem grinder with a Teflon pestle having a clearance of 0.25 mm (Kontes Glass Co., Vineland, N. J.) and homogenized by three up and down strokes, each with five revolutions. The homogenate was centrifuged at 1,000 g for 10 min. The resulting pellet was homogenized again in a tissue grinder with a clearance of 0.13 mm (Kontes) and again centrifuged at 1,000 g. The combined supernates were centrifuged at 105,000 g for 90 min. Negligible trapping of low molecular weight substances by particulate fractions occurred under these conditions. This was determined by adding [³H]choline to a 1,000 g supernate: essentially none of the radioactivity was sedimented during the subsequent centrifugation at 105,000 g. In addition, less than 0.5% of radioactivity was sedimented when these fractionation procedures were carried out with nervous tissue containing an identified serotonergic giant cerebral neuron injected with [³H]choline (12). Pellets were extracted in acetone-formic acid, and

analyzed by electrophoresis at pH 4.7. Supernates were treated with reinecke salt, and the precipitated choline derivatives analyzed by electrophoresis.

RESULTS

Synthesis of Acetylcholine in the Axon of R2

The fate of [³H]choline after injection into R2's axon was similar to that already described in the cell body after intrasomatic injection (7, 19). Within 1 h most of the radioactivity in the axon was converted to acetylcholine (Table I). After 4 h the proportion of the radioactivity in the form of the transmitter appeared to decrease. Synthesis of acetylcholine was proportional to the amount of [³H]choline injected, indicating that the intra-axonal concentration of choline did not saturate the synthetic mechanism, even when as much as 88 pmol of [³H]choline were injected. Due to extensive folding of the axonal membrane

TABLE I
FATE OF RADIOACTIVITY AFTER INJECTION OF [³H]CHOLINE INTO THE AXON OF R2

Time after injection h	No. of experiments	[³ H]Choline injected* pmol	Composition of radioactivity	
			Acetylcholine % of total‡	Choline
1	4	4-15	79±7	5±1
4	2	56, 88	78, 78	4, 5
10	1	28	65	8
22	2	12, 30	29, 58	9, 3

* Between 1% and 5% of the [³H]choline escaped from the neuron after injection.

‡ Remainder of radioactivity was present as phosphorylcholine and betaine. Means ± SE.

(1, 34), it is difficult to estimate the volume of axoplasm involved in synthesis. A lower limit of the [³H]choline concentration in the axon 4 h after injection when most of the radioactivity was limited to a 10-mm stretch of axon would be between 1 and 10 mM, if one assumes this region to be a cylinder 40 μm in diameter. Eisenstadt and Schwartz (7) found that saturation in the cell body occurred after injection of 650 pmol, which corresponded to an estimated somatic concentration of 10 mM.

[³H]Choline was also converted into other substances in the cholinergic axon. During the first 4 h after injection, most of the radioactivity not in acetylcholine was found in phosphorylcholine. As in the cell body (18), an increasing proportion of betaine, a choline metabolite, was formed at the longer time periods (data not shown). At all times after injection, only a small amount of the [³H]choline remained unconverted (Table I).

In contrast to the efficient conversion of [³H]choline into [³H]acetylcholine, little [³H]acetylcholine was formed 1 h after [³H]acetyl CoA was injected into the axon. After three injections which ranged from 22 to 30 pmol, only 0.18-0.21 pmol of [³H]acetylcholine were formed. This amount was less than 10% of the acetylcholine synthesized from even the smallest injection of [³H]choline (Table I). Injections into the cell body of R2 also showed that little acetylcholine was synthesized from acetyl CoA; synthesis increased, however, when choline was

injected together with the coenzyme (7). Choline concentrations in the range of 0.2–0.4 mM have been reported for lobster axons (17) and *Aplysia* cell bodies (23). Similar concentrations of choline were found in cholinergic and noncholinergic neurons. It is possible that only a portion of the measured choline is used for synthesis of the transmitter. Our results suggest that the amount of endogenous choline normally available for reaction with the [³H]acetyl group of the coenzyme is quite small in both regions of the neuron.

Movement of Acetylcholine along the Axon

Movement of radioactivity occurred in both orthograde and retrograde directions from the site of the injection. One measure of movement along the axon is the average displacement of total radioactivity. This we defined as the distance along the axon from the injection site beyond which one-half of the radioactivity had passed in either direction. Average displacements in both directions were

TABLE II
MOVEMENT OF RADIOACTIVITY AFTER INTRA-AXONAL INJECTION OF
[³H]CHOLINE

Time	No. of experiments	[³ H]Choline injected	Average displacement (mm)		Distribution	
			Retrograde	Orthograde	Retrograde	Orthograde
<i>h</i>		<i>pmol</i>	<i>mm</i>		<i>% of total radioactivity in neuron</i>	
4	4	9–72	2.5±0.5	3.2±1.2	43±8	57±8
5	3	3–7	2.9±0.2	3.1±0.4	48±3	52±3
6	3	6–32	2.8±0.2	2.9±0.4	47±1	53±1
16	1	1	5.2	6.3	41	59
24	3	6–20	5.6±0.4	5.0±1.5	45±1	55±1

similar (Table II). The distribution of total radioactivity on the orthograde and retrograde sides of the injection site is also shown in Table II. A somewhat larger proportion of the radioactivity consistently moved in the orthograde direction. With increasing time, a significant amount of the radioactivity moving retrograde arrived at the cell body. At 20 h, for example, 6.4 ± 2.4% (*n* = 5) was found there.

Most of the [³H]acetylcholine in the axon appeared to be moving in both directions by diffusion. The values of average displacements of radioactivity after intra-axonal injection of [³H]choline were within the range of those which might be expected of a molecule of acetylcholine diffusing in water through a cylinder with the diameter of R2's axon (19) (Fig. 2). Average displacement is only a measure of the general distribution of total radioactivity along the axon, however. By plotting the amount of radioactivity in sequential 1-mm segments of the right connective at various times after intra-axonal injection, we obtained more detailed kinetics of transport (Fig. 3). First, to determine the extent of spread which might have resulted from the pressure of the injection itself, we sectioned the right connective 15 min after injecting 110 pmol of [³H]choline. Even this large amount of [³H]choline was confined almost entirely to the site of the injection (Fig. 3 A). At longer time periods, radioactivity was distributed in both directions in small accumulations along the axon (Figs. 3,

4 B). Neither the number nor the position of these accumulations of radioactivity was constant from neuron to neuron.

These transport patterns differed from those obtained after injection of [^3H]choline into the cell body. Koike et al. (19) consistently found a crest of [^3H]acetylcholine moving along the axon which presumably represented a component being actively transported. They also observed that after injection of [^3H]choline into R2's cell body the proportion of the radioactivity in the form of acetylcholine was much greater distally than in the cell body, suggesting selective transport of the transmitter. In contrast, after intra-axonal injection we found no enrichment with distance from the site of injection. In eight experiments, analysis of the radioactivity in segments of injected axons showed that the ratio of [^3H]acetylcholine to the other [^3H]choline-containing compounds (phosphorylcholine and betaine) did not differ at any point along the length of the axon. An example of this analysis is shown in Fig. 4. It is therefore unlikely that a large

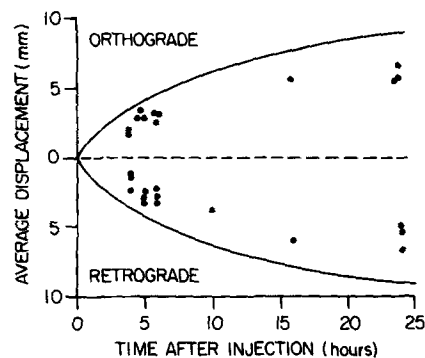


FIGURE 2. The rate of movement of radioactivity along the axon of R2 after injection of [^3H]choline. The average displacements observed (closed circles) in both orthograde and retrograde directions are compared with the displacements (solid curves) calculated with the equation $X^2 = 2Dt$ for a particle with a diffusion constant of $5 \times 10^{-6} \text{ cm}^2/\text{s}$.

proportion of the [^3H]acetylcholine was being selectively transported after axonal injection.

Effects of Colchicine on the Movement of [^3H]Acetylcholine

Even though most of the radioactivity in the axon after intra-axonal injection appeared to move by diffusion, some moved rapidly. We used colchicine to test whether any portion of the acetylcholine might be moving by fast axonal transport. We compared the effects of 10 mM colchicine on movement of the transmitter after both intrasomatic and intra-axonal injection of [^3H]choline. To show that the drug effectively blocked macromolecular transport in this *Aplysia* neuron, we injected [^3H]fucose into the cell body and observed that the movement of [^3H]glycoproteins into the axon was completely inhibited (Table III A). Colchicine applied locally to a small segment of a connective isolated in a chamber constructed of petroleum jelly was also effective in blocking the orthograde transport of [^3H]glycoproteins synthesized from [^3H]fucose injected intrasomatically; unincorporated [^3H]fucose, however, moved through the segment

(data not shown). After intrasomatic injection of [^3H]choline, however, colchicine reduced the amount of the transmitter appearing in the axon by 30%. Thus, the movement of some somatic [^3H]acetylcholine was blocked by colchicine, and this fraction might have been exported by fast transport. In contrast, we

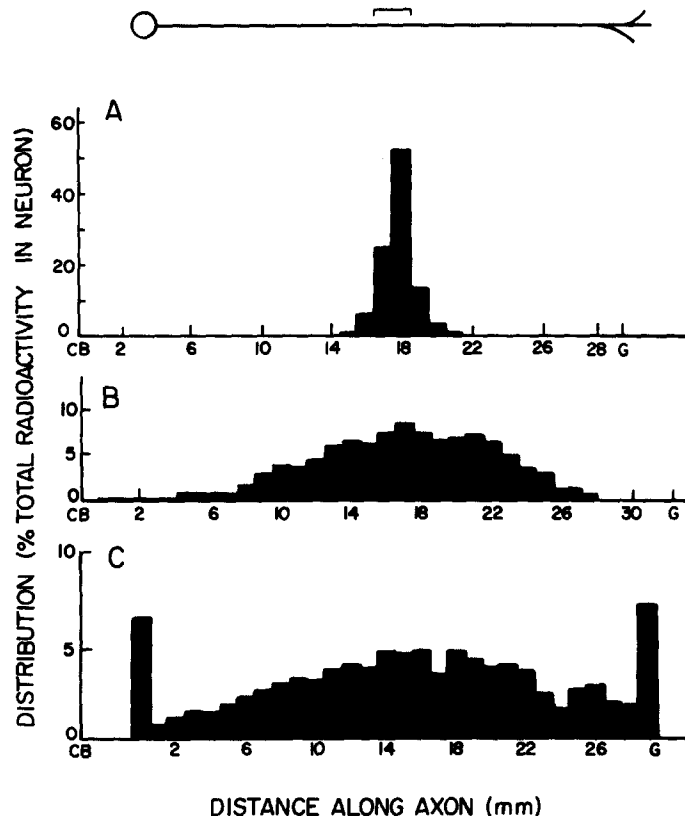


FIGURE 3. Distribution of radioactivity along axon of R2. A, 15 m; B, 6 h; and C, 24 h after intra-axonal injection of [^3H]choline. The histograms are aligned so that the injection sites are indicated by the bar above the diagram of R2, showing cell body (CB), axon, and circumesophageal ganglia (G). Radioactivity was measured in sequential 1-mm segments of the right connective. To correct for the general uptake of [^3H]choline which escaped into the bath, we determined the total amount of radioactivity in the left connective, which is the same length as the right, but contains no process of R2. This value, which was consistently found to be less than 1% of the radioactivity in the right connective, was divided by the length of the connective; this estimate of counts per minute taken up per millimeter was subtracted from the radioactivity in 1-mm segments of the right connective. 1% of total radioactivity corresponds to: A, 800 cpm; B, 1,595 cpm; C, 7,260 cpm.

presume that the 70% which appeared in the axon in the presence of the drug had moved there by diffusion.

After intra-axonal injection of [^3H]choline in the presence of colchicine, we found that the average displacement in both the orthograde and retrograde directions was unchanged (Table III B). It is unlikely that even the

[³H]acetylcholine moving most rapidly from the site of the injection was being transported actively, since application of colchicine did not greatly diminish the distance traveled in 4 h by the leading edge of radioactivity (arbitrarily taken as 0.3% of the total radioactivity in the connective: this amount of radioactivity always exceeded 100 cpm). In the orthograde direction, that distance (in mm) was 10.3 ± 0.6 in four untreated nervous systems, and 8.5 and 9.5 in two experiments with colchicine; in the retrograde direction, 9.6 ± 0.6 (four) and 7.5 and 9.8 in the presence of colchicine. This radioactivity most probably represents the leading edge of diffusion.

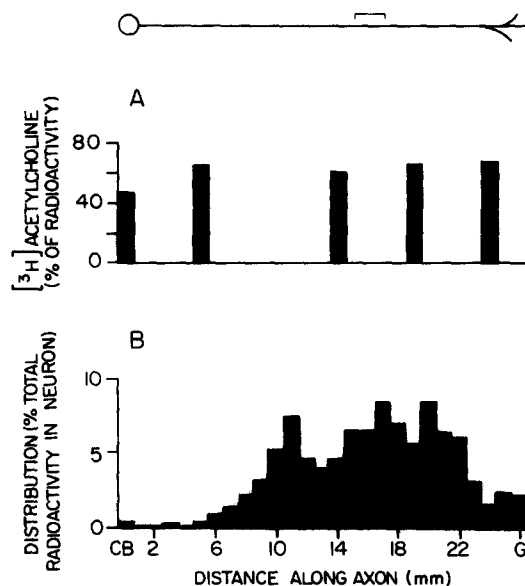


FIGURE 4. Distribution of [³H]acetylcholine 8 h after intra-axonal injection of [³H]choline. Bar on diagram of cell body and axon indicates site of injection. The proportion of the radioactivity in selected segments in [³H]acetylcholine is shown in A; the distribution of total radioactivity in B. 1% of the total radioactivity represents 5,580 cpm. CB, cell body of R2; G, circumesophageal ganglion.

Axonal Movement of [³H]Serotonin, an Alien Transmitter Substance in R2

Much of the [³H]acetylcholine formed in R2 appears to be free to diffuse along the axon. It is difficult, however, to predict the form of the kinetics for the diffusion of acetylcholine. Experimentally, this may be approached by injecting [³H]serotonin into the cell body of R2. Molecular models show that serotonin is similar in size to acetylcholine. Both molecules are positively charged at physiological values of pH, and both are transmitter substances. Goldman and Schwartz have shown that serotonin is incorporated into particulate form in serotonergic neurons of *Aplysia* (13), and is rapidly transported in accumulations along the axon (12). In contrast, serotonin synthesized from [³H]5-hydroxytryptophan injected intrasomatically in R2 was not exported actively into the cholinergic axon (13). After intrasomatic injection of [³H]serotonin itself we also found only a small amount in the right connective; its distribution along

TABLE III
EFFECT OF COLCHICINE ON MOVEMENT OF RADIOACTIVITY AFTER
INJECTION OF [³H]CHOLINE OR [³H]FUCOSE INTO THE CELL BODY AND
AXON OF R2

	Addition	Export		Inhibition	
		% of total neuronal radioactivity appearing in connective		%	
A, Intramuscular injection (20 h after injection)					
(1) [³ H]Acetylcholine	none (10)	32 ± 4		30	
	colchicine (7)	21 ± 2*			
(2) [³ H]Glycoproteins	none (6)	40 ± 4		100	
	colchicine (3)	0 ‡			
		Average displacement (mm)		Distribution % of total radioactivity in neuron	
Addition		Retrograde	Orthograde	Retrograde	Orthograde
B, Intra-axonal injection of [³ H]choline					
(1) 4 h after injection	colchicine	2.7, 2.9	2.6, 3.3	43, 47	57, 53
	(2) none (4)	2.5 ± 0.5	3.2 ± 1.2	43 ± 6	57 ± 6
(2) 20 h after injection	colchicine	5.2, 6.3	4.7, 6.2	42, 45	58, 55
	(2) none (2)	5.2, 5.4	5.2, 5.4	44, 46	56, 54

* Significantly different ($P < 0.05$) using Student's *t*-test.

‡ Data obtained in collaboration with Dr. R. T. Ambron where injected nervous systems were maintained for 15 h after injection; in two subsequent experiments we obtained 36% and 40% exported 20 h after injection. These values refer to export of particulate glycoprotein.

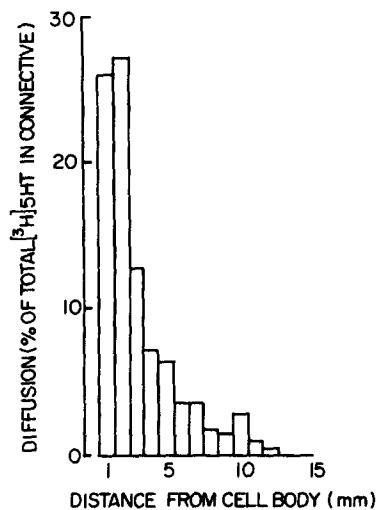


FIGURE 5. Movement of [³H]serotonin along the axon of R2 6 h after its injection into the cell body. 6.8% of the total [³H]serotonin in the neuron appeared in the right connective. 1.0% of total radioactivity in connective represents 1,600 cpm. For a comparison with the distribution of [³H]acetylcholine in the right connective 6 h after intramuscular injection of [³H]choline, see Fig. 2 C of reference 19.

the axon was consistent with the idea that the alien transmitter was moving by diffusion (Fig. 5). This distribution was similar to the exponential decline of radioactivity either proximal or distal to the site of intra-axonal injection of [³H]choline. It differed from those obtained after intrasomatic injection of [³H]choline because it lacked the peaks or crests which were characteristic of the transport of [³H]acetylcholine (19).

Subcellular Distribution of [³H]Acetylcholine

In previous studies with *Aplysia* neurons it was not possible to recover a significant fraction of the newly synthesized acetylcholine in vesicular form (7, 8). In addition, the results of subcellular fractionation experiments were difficult to interpret, since the crude particulate fractions obtained by differential centrifugation were heterogeneous, and substantial hydrolysis of [³H]acetylcholine, even in the presence of eserine, occurred during the procedure (see reference 7 for

TABLE IV
SUBCELLULAR DISTRIBUTION OF [³H]ACETYLCHOLINE 20 H AFTER
INJECTION OF [³H]CHOLINE* INTO EITHER THE AXON OR THE
CELL BODY OF R2

	Region of neuron analyzed	Site of Injection	
		Cell Body	Axon
% of total [³ H]ACh in region which was sedimented			
A, Distribution of [³ H]acetylcholine	cell body	19, 15	27, 18
	axon	30, 30	28, 21
% of total radioactivity in region as [³ H]ACh‡			
B, Composition of radioactivity			
(1) Sedimented	cell body	8, 10	48, 40
	axon	44, 56	75, 58
(2) Supernate	cell body	15, 11	20, 15
	axon	18, 18	29, 23

* In these experiments, 18, 35 pmol of [³H]choline were injected into the cell body, and 10, 16 pmol into the axon.

‡ The radioactivity not in acetylcholine was predominantly betaine, but also contained phosphorylcholine and a small amount of choline (see Table I).

discussion). Nevertheless, with the availability of the technique of axon injection, we have again attempted to examine the subcellular distribution of [³H]acetylcholine. In these experiments we introduced [³H]choline either into R2's axon or into its cell body, and therefore were able to compare the distributions obtained of the transmitter substance formed in the two regions of the neuron. Moreover, since 20 h after injection much of the radioactivity had moved away from the region injected into the other region (i.e., from axon to cell body or from cell body to axon) we can also compare the subcellular distributions of the [³H]acetylcholine which had moved away from the injection site (Table IV). We found that most of the [³H]acetylcholine throughout the neuron synthesized after intra-axonal injection was recovered in the supernatant fraction (Table IV A), but the composition of the radioactivity in the axon in sedimentable form was enriched with respect to acetylcholine (Table IV B). In agreement with Koike et al. (19), we found that after intrasomatic injection the

proportion of [^3H]acetylcholine sedimented in the axon was considerably greater than that in the cell body. In contrast, after intra-axonal injection, we had no indication that the [^3H]acetylcholine which reached the cell body was selectively transported in particulate form since the transmitter in the cell body was no more sedimentable than that in the axon, and a smaller proportion of the somatic radioactivity was recovered in the form of acetylcholine.

DISCUSSION

Cell bodies and nerve fibers, as well as cholinergic nerve terminals, have been shown to contain choline acetyltransferase, the synthetic enzyme for acetylcholine (see for example, references 11 and 14). Eisenstadt and Schwartz (7) showed that acetylcholine could be synthesized in *Aplysia* in the nerve cell body at a rate which was approximately equal to that found under optimal in vitro conditions if choline were made available to the transferase by intrasomatic injection. Similarly, using intra-axonal injection, we have found that the enzyme can also function in the intact axon of the cholinergic neuron R2 (36), its efficiency again being determined by the availability of choline. Eisenstadt et al. (8) showed that isolated segments of nerve (which contain no nerve cell bodies or terminals) take up choline with high affinity; there is evidence in *Aplysia* (8) and in the chick parasympathetic nervous system (31, 32) that high-affinity uptake mechanisms are present in axons and terminals but are lacking in cell bodies. Under physiological conditions, these processes presumably supply some choline from the blood to the axonal enzyme, permitting synthesis of acetylcholine within the axon.

The fate of choline in the cholinergic axon appears to be similar to that in the cell body. Even though the amounts of choline which can be taken up by the axon are greater than those taken up by the cell bodies, availability of choline appears to limit production of the transmitter in both regions of the neuron. As in the cell body, much more [^3H]choline can be injected into the axon and retained than is physiologically taken up from hemolymph. Consequently, relatively huge amounts of [^3H]acetylcholine can be formed. Thus, synthesis of acetylcholine in both somatic and axonal regions of a cholinergic neuron (6, 30) appears to be controlled by uptake of choline.

Movement of [^3H]Acetylcholine in the Axon

While not firmly established, it is likely that fast axonal transport of transmitter substances occurs by virtue of their being incorporated into vesicular structures (4, 5, 12, 37). Although we have had some indication that a portion of the acetylcholine exported from the cell body may occur by fast transport (19), we found no evidence for active transport of the transmitter synthesized within the axon. Colchicine did not block any of the movement (Table III). Furthermore, [^3H]acetylcholine synthesized after intra-axonal injection was found to move at the same rate as did the other choline-containing compounds, phosphorylcholine and betaine. In addition, differential centrifugation revealed that only a small proportion of the transmitter could be sedimented, but there was no difference in the proportion in particulate form of transmitter which had reached the cell body compared to the transmitter remaining near the site of the injection. These results indicate that, under the conditions of our experiments,

most of the [^3H]acetylcholine is free and, as expected of a small, soluble molecule, the [^3H]acetylcholine formed after intra-axonal injection moved along the axon with the kinetics of diffusion. Using a more indirect approach, Evans and Saunders (9) found that acetylcholine was recovered from the outflow of cut and stretched mammalian ventral root axons only in the presence of an anticholinesterase. This observation led them to suggest that most of the transmitter in mammalian axons exists in an unbound state.

Although the subcellular fractionation experiments (Table IV) are difficult to interpret, we did find some enrichment of [^3H]acetylcholine in particulate axonal fractions after intra-axonal injection, and this is consistent with a specific association of some of the transmitter with vesicles. Our experiments with colchicine, and the movement of radioactive transmitter along the axon in waves previously observed by Koike et al. (19) indicate that some of the [^3H]acetylcholine synthesized after injecting the cell body of R2 was being transported actively. Furthermore, in contrast to our observations after intra-axonal injection (Fig. 4), [^3H]acetylcholine was selectively exported from the cell body (19): phosphorylcholine and betaine remained close to the cell body after intrasomatic injection, presumably moving into the axon by diffusion. Thus, even though most of the [^3H]acetylcholine in the axon appears to be free, it is uncertain whether transmitter in this form plays any specific role in the release of acetylcholine from the neuron, as has been often suggested (see, for example, references 2, 24, and 33).

Although not detected, a small fraction of the transmitter formed in the axon may be moving in vesicles. We could have failed to observe its active transport against the background of the great amounts of free acetylcholine formed near the site of the injection. Since [^3H]choline itself presumably can be acetylated at any point within the axon, its diffusion presents an additional technical problem. Diffusion of low molecular weight precursors could complicate any study of rapid axonal transport, since the displacements expected for small molecules by diffusion can approach those which have actually been measured for the large particulate materials which move by fast axonal transport.

Function of Axonal Synthesis and Bidirectional Migration of Acetylcholine

It is not known whether the transmitter substance itself plays a physiological or regulatory role in the cell body or axon and, therefore, whether the extent to which vesicles are loaded in these regions of the neuron has any effect on neuronal function. An axonal reservoir of diffusible acetylcholine moving toward the terminals could be important to synaptic transmission for replenishment of releasable transmitter (2, 15, 16, 20, 24). The functional role of retrograde transport is more in doubt. Relatively high concentrations of acetylcholine have been reported in R2's cell body (23). Much of this acetylcholine may reach the cell body by retrograde flow, since the somatic membrane appears not to possess an efficient high-affinity uptake mechanism for choline (8). Whatever the functional role of extrasynaptic transmitter substance, it seems clear that, under the conditions of our experiments, the synthetic capabilities of both the cell body and axon for acetylcholine exceed the capacity of vesicles to package the transmitter in either of these regions. Presumably, under physiological con-

ditions, where choline is supplied by uptake from hemolymph, synthesis and packaging of acetylcholine are more closely matched.

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