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

III. *Studies of an Identified Cholinergic Neuron*

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ABSTRACT [^{3}H]choline and [^{3}H]acetyl CoA were injected into the cell body of an identified cholinergic neuron, the giant R2 of the *Aplysia* abdominal ganglion, and the fate and distribution of the radioactivity studied. Direct evidence was obtained that the availability of choline to the enzymatic machinery limits synthesis. [^{3}H]choline injected intrasomatically was converted to acetylcholine far more efficiently than choline taken up into the cell body from the bath. Synthesis from injected [^{3}H]acetyl CoA was increased more than an order of magnitude when the cosubstrate was injected together with a saturating amount of unlabeled choline. In order to study the kinetics of acetylcholine synthesis in the living neuron, we injected [^{3}H]choline in amounts resulting in a range of intracellular concentrations of about four orders of magnitude. The maximal velocity was 300 pmol of acetylcholine/cell/h and the Michaelis constant was 5.9 mM [^{3}H]choline; these values agreed well with those previously reported for choline acetyltransferase assayed in extracts of *Aplysia* nervous tissue. [^{3}H]acetylcholine turned over within the injected neuron with a half-life of about 9 h. The ultimate product formed was betaine. Subcellular distribution of [^{3}H]acetylcholine was studied using differential and gradient centrifugation, gel filtration, and passage through cellulose acetate filters. A small portion of acetylcholine was contained in particulates the size and density expected of cholinergic vesicles.

INTRODUCTION

The amount of transmitter in the cell body of a neuron is relatively small compared with that in its terminals. In cortex, for example, Whittaker (1965) found that about two-thirds of the total acetylcholine could be isolated in pinched-off nerve endings. Uptake, turnover, and release of acetylcholine can occur at neuromuscular junctions independently of the nerve cell body, at

least for short periods of time (Potter, 1970). It is therefore not surprising that the cell body and its proximal axon have been somewhat neglected in current views of the cholinergic mechanism.

Evidence was presented (Schwartz et al., 1975; Eisenstadt et al., 1975) that synthesis of acetylcholine in neurons of *Aplysia* is limited by the uptake of exogenous choline. Introduction of precursors directly into neurons by injection circumvents uptake, permitting the analysis of other cellular processes. In order to investigate the role of the cell body in the cholinergic process, Koike et al. (1972) developed a technique for injecting [^{3}H]choline intrasomatically. They found that [^{3}H]choline injected into the cell body of an identified neuron, R2, was converted to acetylcholine and was transported down the axon. Koike et al. (1974) provided evidence that the transmitter, newly synthesized in the cell body of another cholinergic neuron, L10, can be transported to nerve terminals and be released by nerve impulses within a few hours. Here we present further studies on the synthesis, distribution, and turnover of acetylcholine in R2, the giant cholinergic neuron of the abdominal ganglion.

MATERIALS AND METHODS

Aplysia weighing 75–150 g (Pacific BioMarine Corp., Venice, Calif.) were maintained at 15°C in Instant Ocean (Aquarium System Inc., Eastlake, Ohio); the central nervous system with all ganglia interconnected and with peripheral nerves 2–5 cm long attached was dissected from the animal through the foot, and pinned for intracellular recording and intrasomatic injection. All experiments were carried out at 15°C.

Injections

Identified nerve cell bodies were impaled with double-barreled micropipets and injected with up to 3-nl volumes as previously described (Koike et al., 1972; Eisenstadt et al., 1973). After the injection, nervous tissue was kept for various periods in 20 ml sterile filtered Instant Ocean (Aquarium Systems) containing 0.1% glucose, 50 μM choline chloride, 200 U/ml penicillin G, 0.1 mg/ml streptomycin, and 10 mM Tris-HCl (pH 7.6).

Preparation of Solutions for Injection

1 mCi of [^{3}H]choline (16 Ci/nmol; 0.25 Ci/mmol, Amersham/Searle, Arlington Heights, Ill.), and [^{3}H]acetyl-coenzyme A, 0.25 mCi (0.9 Ci/mmol, New England Nuclear, Boston, Mass.) were dried under a stream of nitrogen and dissolved in 1 μl of water in small depressions (formed by heat) at the bottom of plastic petri dishes. The droplets were covered with *200* Dielectric Fluid (350 cs, Dow Corning Corp., Midland, Mich.). More dilute solutions of [^{3}H]choline were also used. The solution of [^{3}H]acetyl CoA and unlabeled choline was prepared by drying 1 μl of 0.1 M choline chloride in a depression and then adding about 0.5 μl of the concentrated [^{3}H] acetyl CoA (approximately 1 pmol choline per picomole of [^{3}H]acetyl CoA). Solutions were stored at −20°C.

Subcellular Fractionation

Nervous tissue containing injected neurons was treated with 50 μM eserine in buffered Instant Ocean for 10 min. The tissue was washed twice at 0°C with 0.2 M sucrose, 0.3 M sodium chloride (Israel et al., 1970) containing eserine, and then homogenized in a tissue-to-volume ratio of 1–5% in order to prepare a 1,000-*g* supernatant (S_1) as already described (Eisenstadt et al., 1975). Differential centrifugation was carried out at 10,000 *g* for 10 min and (after adding as carrier an S_1 resulting from homogenization of six unlabeled, eserinized ganglia) at 300,000 *g* for 30 min. Lipid choline was removed from pellets by extraction with chloroform-methanol (2:1). In some experiments, S_1 supernatants layered on top of 1 ml of a 2 M sucrose density cushion were centrifuged at 300,000 *g* for 30 min in order to concentrate particulate material.

Gradient Sedimentation and Flotation

One aliquot of S_1 was mixed with 0.5 vol of 55% (wt/vol) Ficoll (Pharmacia, Uppsala, Sweden) in 0.8 M sucrose, and transferred to a cellulose nitrate tube. Above this layer (equivalent in density to 1.2 M sucrose) we layered 4% Ficoll in 0.8 M sucrose (equivalent to 0.9 M sucrose). Layers containing 0.7, 0.38, and 0.2 M sucrose were placed above, each adjusted to 0.8 osM by the addition of sodium chloride. The other aliquot of the S_1 was layered onto a similar discontinuous gradient with density steps of 0.38, 0.7, 0.9, and 1.2 M (15% Ficoll, 0.8 M sucrose). Both gradients were centrifuged together for 90 min at 27,000 rpm in an SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions were pumped from the top with an Autodensiflow (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N. J.) and a peristaltic pump (Extracorporeal Medical Specialties, Inc., King of Prussia, Pa.) into 50 μl of 0.01 N ammonium acetate (pH 4.5); fractions were boiled for 4 min to inactivate cholinesterase.

Gel Filtration

Samples of S_1 were applied at 4°C to a column (9 × 0.9 cm) of 4% agarose (Bio-Gel A-15 m, 100–200 mesh, Bio-Rad, Richmond, Calif.) in 0.2 M sucrose containing 0.3 M NaCl and 10 mM Tris-HCl (pH 7.6). The flow rate was 15 ml/h and 1-ml fractions were collected.

Analysis of Radioactive Compounds

Choline and acetylcholine in gradient fractions and in effluents from 4% agarose columns were analyzed after precipitation as reineckates; radioactivity was separated and counted by liquid scintillation (Schwartz et al., 1975). Under our conditions of counting 1 pmol of [^{3}H]choline corresponded to 102 cpm (0.25 Ci/mmol) or 6,600 cpm (16 Ci/mmol) and 1 pmol of [^{3}H]acetyl CoA, to 400 cpm.

RESULTS

Fate of Injected Choline

Intrasomatic injection of [^{3}H]choline permitted us to show directly that synthesis of acetylcholine in a cholinergic nerve cell body is limited by the

accessibility of substrate. In 100 g *Aplysia* the cell body of R2 contains about 8% of the total choline acetyltransferase of the abdominal ganglion (Giller and Schwartz, 1971), but the rate of conversion actually obtained from the [^{3}H]choline taken up from the bath was only 0.02% of its enzymatic capacity (Eisenstadt et al., 1975). Injected choline was converted to acetylcholine much more efficiently than was the choline taken up from the bath. In 26 neurons, choline was injected in amounts that resulted in a range of intracellular concentration of about four orders of magnitude (Table I A). When we injected relatively small amounts of choline, equivalent to amounts associated with the cell body after a 1-h incubation in the presence of 65 μM [^{3}H]choline, we found that 20 times more acetylcholine was formed from the injected choline. With these amounts of injected choline the transferase was functioning at about 0.4% of its total estimated capacity. Greater amounts of choline were found to be associated with the cell body after incubation at higher external choline concentrations (Table I B). Even so, an equivalent amount of [^{3}H]choline introduced by injection resulted in synthesis of 50 times more acetylcholine (Table I A). Moreover, much more synthesis was achieved with larger injections, approaching 25% of the enzymatic capacity.

By injecting [^{3}H]acetyl CoA we obtained compelling evidence that the normal endogenous concentration of choline limits synthesis of acetylcholine (Table II). When [^{3}H]acetyl CoA was injected together with 200 pmol of unlabeled choline, most of the radioactivity within the neuron after 1 h was almost quantitatively converted to acetylcholine. When [^{3}H]acetyl CoA was injected in the absence of the choline, radioactivity escaped from the neuron. It was probably in the form of acetate, since it was volatilized when acidified. The portion which remained within the neuron was shown by electrophoresis to be mostly unchanged coenzyme with less than 20% converted to acetylcholine. These results suggest that the amount of choline normally present within the neuron that is *available for synthesis* is probably an order of magnitude less than 200 pmol (equivalent to about 3 mM) (see Table II). This observation is in agreement with the values that McCaman et al. (1973) have measured in the cell body of R2 (11 pmol). Both when choline was injected and when it was not, the nervous tissue was perfused in 50 μM unlabeled choline. Thus, these results again indicate that choline taken up from the bath is relatively unavailable for synthesis in the cell body.

Acetylcholine synthesized in the cell body of R2 from [^{3}H]acetyl CoA was transported into the neuron's major axon. After intrasomatic injections of [^{3}H]acetyl CoA together with unlabeled choline (see Table II), the proportion of acetylcholine in the right connective increased at a constant rate, and at 20 h was about 30% of the total neuronal acetylcholine (Fig. 1). Similar results have been obtained with transport of acetylcholine labeled after intrasomatic injection of [^{3}H]choline (Koike et al., 1972; and unpublished ex-

TABLE I

EFFICIENCY OF ACETYLCHOLINE SYNTHESIS FROM [^{3}H]CHOLINE BY R2

	Content of [^{3}H]choline	Estimated intracellular [^{3}H]choline concentration	Acetylcholine synthesis
	pmol	*mM*	*pmol/cell/h*
(A) Intrasomatic injection			
	1.1	0.017	0.9
	2.8	0.042	1.9
	3.0	0.046	2.6
	6.3	0.095	4.8
	16.0	0.24	12.6
	22.3	0.34	15.2
	23.2	0.35	14.5
	28.2	0.43	22.4
	40.6	0.62	29.8
	52.9	0.80	43.6
	76.2	1.2	29.0
	80.1	1.2	45.0
	104	1.6	66
	129	1.9	86
	155	2.4	102
	178	2.7	95
	203	3.1	114
	244	3.7	162
	326	4.9	153
	482	7.3	164
	482	7.3	166
	647	9.8	191
	1,081	16.4	207
	2,174	32.9	176
	2,230	33.8	212
	4,906	74.3	118
(B) Uptake at 65 μM*	7.9	0.12	0.21
(C) Uptake at 650 μM (4)	69±11	1.1	0.71±0.25

* Data from Eisenstadt et al. (1975).

Various volumes and dilutions of [^{3}H]choline (0.25 or 16 Ci/mmol) were injected into R2 intrasomatically. After 1 h at 15°C, abdominal ganglia were homogenized in acetone-formic acid. Samples were counted for total radioactivity injected. Ganglia were incubated for 1 h for the uptake studies (Schwartz et al., 1975). Cell bodies of R2 were removed by dissection. Intracellular concentration of [^{3}H]choline was calculated using a value of 66 nl as the volume for R2's cell body (Giller and Schwartz, 1971).

periments). After injection of [^{3}H]acetyl CoA, essentially all of the radioactivity in the nerve was in the form of acetylcholine. The increasing proportion of acetylcholine synthesized from [^{3}H]acetyl CoA found in the right connective strongly suggests that it is transmitter synthesized in the cell

TABLE II

INTRASOMATIC INJECTION OF [^{3}H]ACETYL CoA ALONE OR TOGETHER WITH UNLABELED CHOLINE

	Time after injection	Total radioactivity initially injected	Radioactivity remaining within neuron	Content of [^{3}H]ACh
	h	*pmol/neuron*		*% total radioactivity injected*
(A) No choline injected with [^{3}H]acetyl CoA	1	101	55	10
		103	29	6.4
	6	195	22	1.3
(B) Equimolar unlabeled choline injected with [^{3}H]acetyl CoA	1	134	120	84
		164	155	90
	6	258	184	66
		252	189	70
	20	330	138	35
		125	39	29

After injection, nervous systems were incubated in buffered Instant Ocean containing 50 μM unlabeled choline. Ganglia were homogenized in acetone-formic acid, and the radioactivity analyzed by electrophoresis at pH 4.7. Total radioactivity injected was the sum of the radioactivity in the homogenates and that which escaped into the bath.

body which is transported along the axon. Most of the [^{3}H]acetyl CoA which remains in the neuron is almost completely converted to acetylcholine within the first hour after the injection (Table II). Since [^{3}H]acetate, lost from acetylcholine, escapes mainly from the neuron, and that which remains probably cannot be efficiently incorporated into acetylcholine again, the transmitter found in the nerve must have originated in the cell body.

Kinetic Analysis of Acetylcholine Synthesis in the Living Cell

With injections resulting in intracellular [^{3}H]choline concentrations lower than about 4 mM, a constant proportion of the total radioactivity in R2, about 75%, was converted to acetylcholine (Eisenstadt et al., 1973). With intracellular concentrations greater than about 15 mM, synthesis reached a plateau. These results suggested that the Michaelis-Menten model might describe the kinetics of acetylcholine synthesis. We therefore analyzed the data in Table I A using a computer to obtain the double-reciprocal plot shown in Fig. 2. The curve fits all the data points but one with an error of less than 10% over the entire range of choline concentrations. The kinetic constants obtained were an apparent Michaelis constant for choline of 5.9 mM and a maximal velocity of 300 pmol/cell/h. This rate is 33% of the

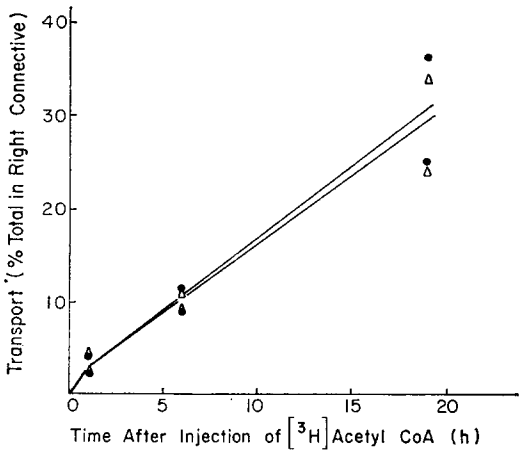

FIGURE 1. Distribution of [^{3}H]acetylcholine between cell body and axon of R2 after intrasomatic injection of [^{3}H]acetyl CoA. 100–300 pmol of [^{3}H]acetyl CoA were injected together with equimolar amounts of unlabeled choline. At various periods after injection abdominal ganglia containing the cell body, and right connective containing the major axon of R2, were separated and analyzed for their contents of radioactivity and [^{3}H]acetylcholine. *Transport* is expressed as the proportion of the total neuronal content of either [^{3}H]acetylcholine (●) or radioactivity (△) in the right connective. Other data from these experiments appear in Table II B.

capacity at 15°C expected from assays of the transferase in homogenates of the cell body.

Synthesis of Other Choline-Containing Substances

When R2 was injected with [^{3}H]choline, the major product formed in 1 h was acetylcholine. Other labeled substances were phosphorylcholine, betaine, and lipid. After 1 h, incorporation into lipid was less than 1%, but increased with time. After 4 h, about 7% of the total radioactivity was extracted by chloroform-methanol, and after 24 h, about 11%. Most of the lipid choline was associated with neuronal membranes. After 4 h about 4% of the total radioactivity in the homogenate was extracted by chloroform-methanol from particulate material isolated by differential centrifugation or by filtration through cellulose acetate filters. The lipid labeled was found to be phosphatidylcholine, not sphingolipid (Schwartz et al., 1975). One hour after injecting R2, the fractions in phosphorylcholine and betaine were each 10%.

There was also some indication that CDP-choline was formed from [^{3}H]-choline injected into R2. A small amount of the total radioactivity migrated toward the anode during electrophoresis at pH 4.7. After 24 h this material represented 3% of the total radioactivity. When [^{32}P]inorganic phosphate was injected together with [^{3}H]choline, this material contained both labels.

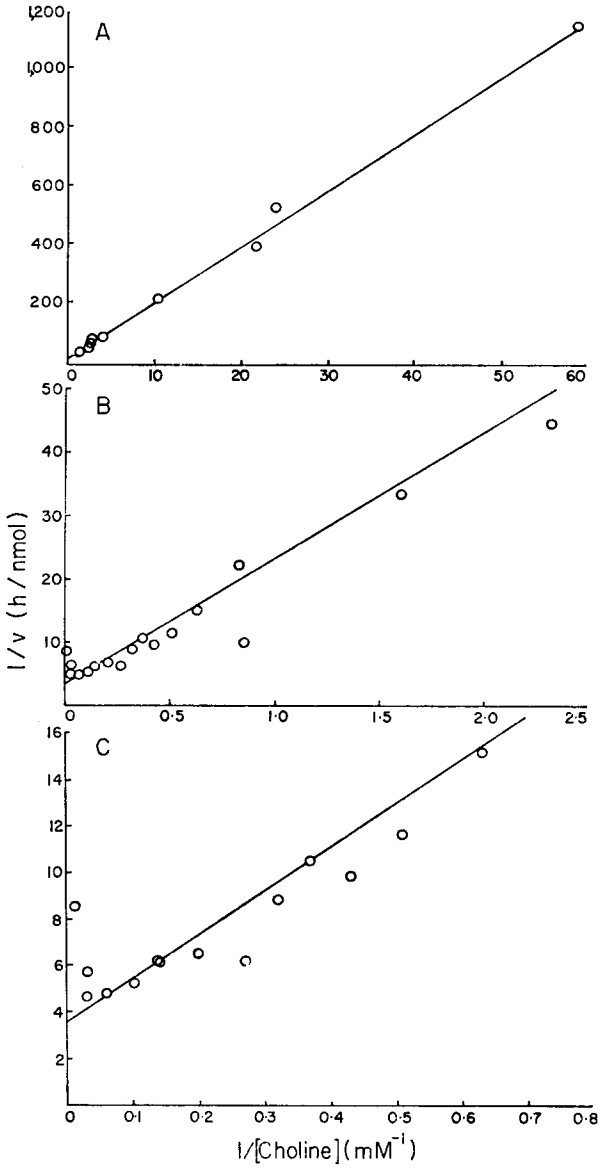

FIGURE 2. Double-reciprocal plot showing the dependence of acetylcholine synthesis in the living cell on the estimated intracellular choline concentration. Data, from the determinations presented in Table I A, were plotted using SAAM 25, a computer program (Schwartz et al., 1975). Some of the data points are presented in more than one of the three panels in order to allow resolution over a very wide range of choline concentrations.

Turnover of Acetylcholine

Newly synthesized [^{3}H]acetylcholine appeared to be relatively stable within the injected neuron. Estimated from data obtained within the first 6 h after injection, the half-life of the [^{3}H]acetylcholine was about 9 h (Fig. 3). Data

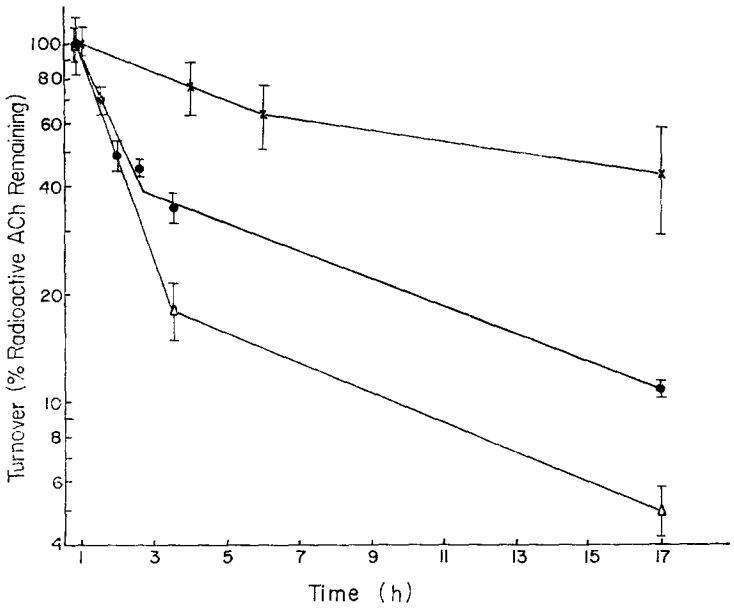

FIGURE 3. Turnover of acetylcholine synthesized in R2 from [^{3}H]choline injected intrasomatically and in ganglia from [^{14}C]choline taken up from the bath. *Injection* (×): R2 cell bodies were injected with approximately 20 pmol of [^{3}H]choline (16 Ci/mmol). Nervous tissue was maintained in buffered Instant Ocean containing 50 μM unlabeled choline for 1–24 h. *Uptake:* Pairs of circumesophageal ganglia were incubated at 15°C in 100-μl droplets containing 20 (●) or 200 μM (△) [^{14}C]choline. The droplets were changed every 15 min. After a total incubation of 45 min, tissue was washed for 10 min and then transferred to 20 ml of buffered Instant Ocean containing 20- or 200-μm labeled choline. The proportion of acetylcholine, determined by electrophoresis at the indicated times, was calculated using the total radioactivity initially injected or taken up. Only negligible amounts of radioactivity escaped from injected neurons. Total radioactivity taken up was the sum of radioactivity in the tissue at the time of homogenization and the total efflux after the initial washing. The data are the means and SE of at least four determinations.

obtained after 16 h were considerably more variable: in some experiments there was no change between 6 and 16 h and in others there was much less [^{3}H]acetylcholine. The proportion of acetylcholine decreased with time after the injection, with a concomitant rise in betaine. Thus the ultimate product of the turnover of acetylcholine is probably betaine (data not shown). Since

the decline in acetylcholine was quite variable, conversion to betaine was difficult to quantitate reliably.

Experiments with [^{3}H]acetyl CoA (Table II) also show a similar rate of acetylcholine turnover. One hour after injecting [^{3}H]acetyl CoA together with unlabeled choline, less than 10% of the radioactivity escaped from the cell, and about 95% of that remaining in the cell was converted to acetylcholine. With time, increasing proportions of radioactivity escaped from the neuron; at 20 h, about 60% had been lost. Since the radioactivity remaining within the neuron was almost entirely in the form of acetylcholine, and since there are few if any presynaptic terminals of R2 in the central nervous system (see Koike et al., 1974), the escaped radioactivity is probably a measure of the intraneuronal hydrolysis of transmitter with subsequent loss of acetate from the neuron.

Acetylcholine formed from choline taken up exogenously is evidently more labile than is acetylcholine synthesized from injected precursors (Fig. 3). The data plotted in Fig. 3 suggest, however, that there are two kinetic populations of acetylcholine with different rates of turnover, one with a half-life of about 2.5 h, and the other with a half-life close to that of transmitter synthesized from injected precursors. The proportion of acetylcholine in these two populations seems to depend on the external concentrations of [^{14}C]choline at which the nervous tissue was originally incubated. A greater proportion of the labeled acetylcholine formed at the higher external choline concentration turns over at the faster rate. Acetylcholine formed at the lower external choline concentration behaves more like the transmitter synthesized from the injected precursors.

Subcellular Distribution of Acetylcholine

A substantial amount of the total acetylcholine in the neuron was found to be sedimented during high speed centrifugation (Koike et al., 1972). The proportion of acetylcholine which sedimented increased with the time after intrasomatic injection of [^{3}H]choline. After 24 h, up to 46% of the labeled acetylcholine in the right connective was sedimented; considerably less was found in this fraction in the cell body (Koike et al., 1972). Since structures with the characteristic morphology of synaptic vesicles have been seen in electron micrographs of both cell bodies and axons of cholinergic neurons in *Aplysia* (Coggeshall, 1967; Thompson et al., 1973), these results suggested the possibility that vesicular structures might be the vehicle for axonal transport.

We have further characterized the particulate acetylcholine by gel filtration on agarose, differential and density gradient centrifugation, and passage through cellulose-acetate filters of various pore sizes. From these studies we have concluded that, while some of the newly synthesized acetylcholine

was isolated in structures the size of synaptic vesicles, this is only a small fraction of the total. Possibly because of the instability of cholinergic vesicles, most of the [^{3}H]acetylcholine in the neuron was in the supernatant. This fraction was completely hydrolyzed in the absence of 50 μM eserine. Moreover, much of the sedimented transmitter was in membranous structures larger than synaptic vesicles described in other animals.

Differential centrifugation of an S_1 from nervous tissue containing an R2 kept 4 h after injection showed that most of the particulate [^{3}H]acetylcholine sedimented at the relatively low force of 10,000 *g* for 10 min (Table III A).

TABLE III

SUBCELLULAR DISTRIBUTION OF [^{3}H]ACETYLCHOLINE 4 H AFTER INTRASOMATIC INJECTION OF [^{3}H]CHOLINE INTO R2

Fraction	Amount of radioactivity in fraction	Acetylcholine content	Enrichment ratio	Distribution of particulate ACh
	% of total cpm in homogenate	*% of total ACh in homogenate*	*% ACh in fraction/% ACh in total homogenate*	*% of total particulate ACh*
(A) Differential centrifugation				
Force (*g*)				
10,000	4.1	2.4	1.2	88
300,000	2.2	0.3	1.7	12
(B) Gradient centrifugation*				
Sucrose (*M*), density interface				
0.2–0.38	0.6	0.2	0.5	10
0.38–0.7	0.7	1.4	1.1	62
0.7–0.9	0.7	0.4	1.4	20
0.9–1.2	0.2	0.2	1.0	8
(C) Gel filtration*				
Excluded volume	3.1	1.6	1.3	—
(D) Filtration through cellulose acetate membranes				
Pore size (μm)				
8	2.8	1.1	1.2	22
1.2	2.4	1.3	1.8	28
0.45	1.5	1.9	1.9	39
0.05	0.6	0.6	1.8	11

Subcellular fractionation was carried out as described in the text on ganglia containing R2 injected with 50–200 pmol of [^{3}H]choline (16 Ci/mmol). Aliquots of S_1 were analyzed both by sedimentation and by flotation (Fig. 4).

* Because of the large volumes containing salts and sucrose in fractions from the gradients and from the agarose column, radioactivity was precipitated with reineckate for analysis. In these experiments, phosphorylcholine, betaine, and lipid were not recovered. Entries for the experiments therefore represent values only for radioactivity precipitated as reineckate. Values are single determinations representative of at least three independent experiments.

In this fraction, which would not be expected to contain free synaptic vesicles, half of the radioactivity was lipid. Compared to the original homogenate, a greater proportion of the nonlipid radioactivity in the high speed (vesicle) fraction was in acetylcholine.

When S_1 was sedimented in discontinuous gradients, radioactivity was found at all density interfaces; the large amount of supernatant radioactivity (free choline and acetylcholine) contaminated the lighter boundaries. When aliquots of S_1 were floated from the bottom of the tube into a similar step-gradient, the lighter fractions were uncontaminated, but the heavier interfaces were obscured. The separations obtained from both sedimentation and flotation are shown in Fig. 4. Radioactivity at the interfaces was analyzed for acetylcholine. The results presented in Table III B show that one particulate fraction is enriched with labeled transmitter; this enrichment would have been greater if the particulate fractions were uncontaminated by soluble radioactivity. We therefore attempted to decrease contamination from the supernatant by first concentrating particulates by sedimentation for 30 min at 300,000 *g* either with or without a dense cushion of sucrose. The resuspended particulate material was then subjected to gradient centrifugation. Although sharp separations were obtained showing little contamination, the distribution of particulate radioactivity was considerably altered. In contrast to the distribution shown in Fig. 4, we found all of the radioactivity at the two densest interfaces, almost none at lighter densities. This result indicates that membranous structures in *Aplysia* aggregate readily; once pelleted, they did not disperse completely.

When samples of an S_1 prepared from nervous tissue containing an R2 maintained for 4 h after injection were subjected to gel filtration, 3% of the total radioactivity emerged in the excluded volume. The excluded fraction was enriched with acetylcholine (Table III C). The included fraction was almost identical in composition to a sample of the 105,000-*g* supernatant from the same homogenate. Consistent with the membranous character of the excluded material was the presence of a substantial amount of radioactivity in lipid (26% of the total radioactivity in this fraction); no lipid choline was found in the included material.

Centrifugation separates according to both size and density of particles. Filtration on a column of agarose separates according to size, but does not permit graded discrimination between particles of various sizes. Filtration through cellulose acetate filters with various pore sizes should allow fractionation of particulate material primarily according to size. Filtration has been used previously for rapid isolation of subcellular organelles, including synaptosomes (Diamond and Milfay, 1972; Dowdall and Simon, 1973) and synaptic vesicles (Israel and Gautron, 1969). When an S_1 from a nervous system containing an injected R2 was passed through a series of filters with pores from

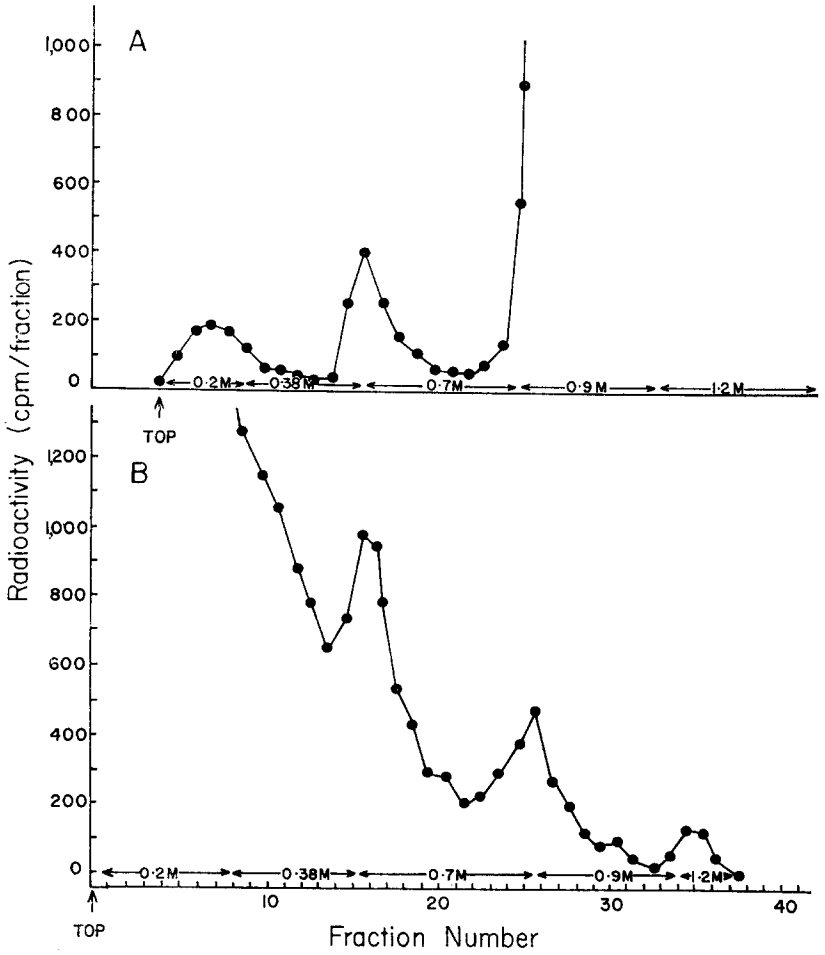

FIGURE 4. Subcellular fractionation of radioactivity in R2 4 hr after intrasomatic injection of [^{3}H]choline by centrifugation in density gradients. Three nervous systems, each containing an R2 injected with about 100 pmol of [^{3}H]choline (16 Ci/mmol), were combined for homogenization. Aliquots of the S_1 were centrifuged either for flotation (A) or for sedimentation (B). Other data from these experiments are presented in Table III B.

0.05 to 8 μm in diameter, we found that particulate radioactivity was distributed on all filters (Table III C). Half of the material was retained on filters with pores 8 and 1.2 μm in diameter. Although all of the particulate fractions contained radioactivity in lipid form, the two with largest particles contained more.

Pinched-off fragments of axon, formed during homogenization from nerve terminals or other regions, would probably be retained by 1.2 μm filters. When the particulate material was osmotically shocked by rapid dilution of

an S_1 with an equal volume of water, more than half of the radioactivity was lost from the larger particles, but only a two- to three-fold increase was observed in radioactivity on the 0.45- and 0.05-μm filters, which might be expected to retain synaptic vesicles. Thus most of the radioactivity released by osmotic shock also was soluble.

In experiments in which we carried out filtration and density gradient centrifugation sequentially in order to determine the correspondence between the two fractionation procedures, we found that the densest material, sedimenting at 0.9 and 1.2 M sucrose in gradient centrifugation, was completely retained by filters with pores 0.45 μm in diameter. Conversely, radioactivity from these dense fractions was distributed in particles retained by 8-, 1.2-, and 0.45-μm filters.

Although only a small fraction, some of the particulate acetylcholine had the characteristics expected of synaptic vesicles. During gradient centrifugation, most of the particulate acetylcholine sedimented at the interface between 0.38 and 0.7 M sucrose steps, the position occupied by cholinergic vesicles of *Torpedo* (Israel et al., 1970). Most of the particulate radioactivity which passed through the 0.45-μm filters also was found at this interface when subsequently analyzed by gradient centrifugation. This radioactivity might be contained in synaptic vesicles, which have been observed in *Aplysia* cholinergic neurons to have a mean diameter of 0.09 μm (Thompson et al., 1973).

DISCUSSION

Efficiency of Transmitter Synthesis

At low external concentrations of choline, a high affinity process takes up choline into the neuron where it is used efficiently for synthesis of transmitter. At higher external choline concentrations, the choline taken up seems to be largely unavailable for synthesis. In the cell body, even at low external concentrations, conversion is poor, and we have suggested that the high affinity uptake process is deficient or totally absent from that region of the neuron. Intrasomatic injection of [^{3}H]choline circumvents uptake, and the injected choline is utilized efficiently for synthesis even when injected in extraordinarily large amounts (Table I A).

The most obvious explanation for poor utilization of the external choline is that it is merely associated with the outside of the cell body. Nonspecific sites of adsorption would be expected to have low capacity and to equilibrate rapidly. Simple contamination is therefore unlikely since the amount of radioactivity associated with the cell body increases at a constant rate (Eisenstadt et al., 1975). Borys (personal communication) obtained evidence that some of the radioactivity associated with R2 after incubation in 100 μM

choline is located *within* the cell. Using frozen sections and microdissection, he isolated nucleus, cytoplasm, and external membrane regions of R2, and found radioactivity distributed in them all. Greatest amounts were located in the external membrane fraction.

R. E. McCaman (personal communication) suggested the possibility that the injection itself might stimulate the conversion of choline to acetylcholine, possibly because of changes in membrane resulting from simultaneous injection of Cl^- ion together with choline. In order to test this suggestion, we injected 200 pmol of unlabeled choline chloride intrasomatically, and then incubated the ganglion containing the injected R2 in the presence of [^{3}H]choline. After an hour, the cell body was dissected out. Neither uptake of [^{3}H]-choline nor its conversion to acetylcholine was significantly influenced by the injection of unlabeled choline chloride.

Although most probably within neurons, the choline taken up would seem to be in compartments unavailable to the transferase, which is free in the cytoplasm (Giller and Schwartz, 1971). There was substantial efflux of the choline taken up from the bath (Eisenstadt et al., 1975). In contrast, essentially none of the injected radioactivity escaped from the neuron, even when present as unconverted choline in large amounts. These observations can be explained if the injected choline is set apart from the choline taken up from the bath. One possible compartment might be mitochondria, which presumably take up choline for oxidation to betaine aldehyde, with further oxidation to betaine occurring in the cytoplasm (see Greenberg, 1969). We suggest that the choline taken up by the low affinity process is sequestered, perhaps by being bound electrostatically at sites usually occupied by some common cation, for example, Na^+ or K^+. These sites would be quite numerous since the amount of the common cation within the cell would be enormous compared to choline. Some of the choline in this compartment would be in equilibrium with free choline and serve as precursor of the small fraction of acetylcholine found to be synthesized from the choline taken up from the bath. The route of entry of choline into the cell seems to determine the compartmentalization. The choline injected directly into the cytoplasm apparently remains free and available to the transferase.

Acetylcholine Synthesis and Turnover

The kinetic constants that we obtained for choline acetyltransferase using intrasomatic injection were remarkably similar to those previously determined for the extracted enzyme. This correspondence indicates that the extracted enzyme is the one responsible for synthesis of the transmitter in the living cell. Values for the total amount of transferase in injected cell bodies were similar to those previously obtained by assay in extracts of isolated cell bodies.

The maximal velocity extrapolated from the kinetic data, 300 pmol/h (Fig. 2) was about half that found by assay at 15°C in extracts with choline and acetyl CoA, both present in saturating concentrations.

The intracellular concentration of acetyl CoA is unknown. It is likely, therefore, that the Michaelis constant for choline which we have determined by intrasomatic injection is only apparent, since the concentration of the cosubstrate may not be saturating. We also neglected the concentration of unlabeled choline normally present in the cell (0.17 mM, McCaman et al., 1973). The resulting error in the Michaelis constant would not be very great, since the concentration of choline normally available to the enzyme is low relative to the Michaelis constant. We found that the Michaelis constant for choline was 5 mM, which was quite similar to the 7.5 mM obtained for the extracted transferase in the presence of high salt (Giller and Schwartz, 1971). A decrease in synthesis of acetylcholine was observed when large amounts of choline were injected. This inhibition possibly resulted from the extraordinarily high intracellular concentrations of choline (approaching 100 mM). It is unlikely that the acetylcholine formed (approaching an intracellular concentration of 6 mM) inhibited the transferase directly. Under standard assay conditions (20 mM choline) we found that the addition of acetylcholine at 100 mM decreased the enzymatic rate of synthesis by only 20%, and at 400 mM, by only 33%. We therefore have no evidence that product inhibition is important in controlling synthesis of the transmitter in the neuron, as suggested by Kaita and Goldberg, 1969.

Synthesis of acetylcholine is a characteristic biochemical property of cholinergic neurons and occurs only in cells containing choline acetyltransferase; intrasomatic injection of [^{3}H]choline therefore provides a convenient microassay for identifying cholinergic neurons (Eisenstadt et al., 1973). High affinity uptake of choline seems also to be specific to cholinergic neurons (Yamamura and Snyder, 1972; Kuhar et al., 1973; Schwartz et al., 1975; Eisenstadt et al., 1975). Both cholinergic and noncholinergic cells use choline for two other pathways: synthesis of phospholipid and oxidation to betaine. Phosphorylcholine, presumably an intermediate in the synthesis of lipid, was the major product formed in noncholinergic neurons (Eisenstadt et al., 1973). In cholinergic cells, however, acetylcholine was the major product, and only a small fraction of the injected choline was converted to phosphorylcholine. This selective utilization of choline is difficult to explain, since the Michaelis constant for the kinase was reported to be an order of magnitude lower than that of the transferase and the total activities of the two enzymes in R2 are comparable. Dewhurst (1972) studied the kinase in extracts at pH 9, and the properties determined under these conditions might not reflect its intracellular characteristics. Alternatively, the injected choline might not be as available to the kinase as it is to the transferase. Both enzymes are sol-

uble in homogenates of *Aplysia* nervous tissue, however, and there is no indication that either exists in a bound form.

The proportion of radioactivity in the form of betaine in injected neurons increased with time after injection. Oxidation to betaine appears to be the fate of the choline not used for lipid synthesis and of the choline released by turnover of acetylcholine. Since most of the choline injected into cholinergic neurons was initially converted into acetylcholine, the proportion of betaine in the neuron at later times largely indicates the extent of acetylcholine turnover. The average rate of turnover in an injected cell was about 60% in 24 h, but was extremely variable (Fig. 3). Since choline oxidase is an intracellular enzyme (in other animals, localized to the inner membrane of the mitochondrion) and since betaine is quantitatively retained by the neuron, it is likely that turnover of acetylcholine occurs within the neuron. The pathway by which acetylcholine breaks down is not known. There is some evidence for an intracellular cholinesterase (Collier and Katz, 1971); and the transferase reaction might be reversible (Schuberth, 1966). The great variability in turnover of acetylcholine and oxidation of choline may depend on the store of oxidizable carbohydrate present in the neuron when the ganglion was isolated. This may vary greatly from animal to animal. Turnover does not reflect an inability to utilize choline for acetylcholine synthesis, however. Treistman and Schwartz (unpublished experiments) found the synthesis of acetylcholine in R2 from [^{3}H]choline injected intrasomatically to be undiminished in ganglia kept in culture for 24 h.

Role of the Cell Body in the Metabolism of Acetylcholine

Acetylcholine can be synthesized under physiological conditions in all parts of a cholinergic neuron (see Hebb, 1972 and Treistman and Schwartz, 1974). Synthesis is limited by the uptake of choline from hemolymph, and the uptake process seems to be localized primarily in axons and in the neuropile, presumably at nerve terminals. Even though cell bodies are deficient in this uptake process, transmitter made in the cell body can be transported down the axon to nerve terminals, some of it probably in vesicles, and there is evidence that this acetylcholine can be released synaptically by nerve impulses (Koike et al., 1974).

Since relatively little choline is taken up by the cell body, it is likely that somatic synthesis of transmitter is trivial compared to the amounts formed in axons and terminals. Thus, while the cell body probably plays no immediate role in the metabolism of the transmitter, all of the macromolecular constituents required for synthesis, transport, and release of acetylcholine are presumably formed there. Subcellular analysis of the [^{3}H]acetylcholine labeled after intrasomatic injection might, therefore, be useful for identifying

and characterizing the apparatus involved in axonal transport of the transmitter.

An injected neuron should be singular experimental material for studying subcellular distribution quantitatively. In the cells we used for these studies about 75% of the radioactivity in the neuron was initially in the form of acetylcholine; not more than 10% was present as unconverted choline. Koike et al. (1972) found that the proportion of particulate [^{3}H]acetylcholine increased with time after injection. The greatest proportions were particulate in the right connective, which contains the major axon of the injected neuron, and this suggested that acetylcholine might be transported in synaptic vesicles. Because the amount of turnover of [^{3}H]acetylcholine varied so greatly at longer periods (Fig. 3), we primarily studied neurons 4 h after injection. At this time, only relatively small amounts of the total neuronal [^{3}H]acetylcholine had entered the right connective (Koike et al., 1972; see also Fig. 1).

We have used a variety of techniques to characterize the particulate radioactivity. More than half the acetylcholine was lost upon homogenization even in the presence of eserine. Since this acetylcholine was not protected, it was presumably free. As already found (Koike et al., 1972) we rarely obtained more than 5% of the labeled transmitter in particulate form shortly after the injection. While some acetylcholine was in particles the size of synaptic vesicles, most was in much larger material. We have presented evidence that the large particles contain free acetylcholine and acetylcholine in smaller, possibly synaptic vesicles. The external membrane of R2, both of cell body and axon, is extensively infolded (Coggeshall, 1967; Ambron et al., 1974). It is therefore possible that most of the particulate radioactivity might be contained within bits of membrane artifactually pinched off during homogenization. Since the tissue taken for homogenization contained no labeled presynaptic terminals of the injected neuron, however, the particulate acetylcholine cannot be synaptosomal.

Even though we were unable to isolate a substantial fraction of the newly synthesized transmitter in synaptic vesicles, it may nevertheless exist in that form within the neuron. Wilson et al. (1973) isolated much less of the total acetylcholine from bovine sympathetic ganglia in vesicles than could be isolated from brain. Moreover, vertebrate cholinergic vesicles containing newly synthesized acetylcholine seem to be extremely labile (see for example Marchbanks and Israel, 1971; Richter and Marchbanks, 1971). It is also possible that vesicles from cell body and axon may be immature and lack the stability of vesicles isolated from synaptic regions of other animals. In any case, we may not have used appropriate conditions for their isolation. In addition to isosmotic sucrose-sodium chloride at 0 and 15°, we have tried many other solutions with identical results, however. We have used artificial

seawater; a solution with ion concentrations presumed to be like that within cells (Florey and Winesdorfer, 1968); we have included albumin and Ficoll; we have used Cs^+ in place of Na^+, and have even extracted with cell sap (S_1 of *Aplysia* nervous tissue frozen and powdered in liquid nitrogen). Cholinergic vesicles in *Aplysia* seem to be more fragile than serotonergic granules, since about 60% of the labeled serotonin has been isolated in the high-speed vesicle fraction after homogenizing in isotonic sucrose-sodium chloride a ganglion containing a serotonergic neuron injected intrasomatically with either [^{3}H]5-HTP or [^{3}H]serotonin (Goldman and Schwartz, 1974).

The radioactivity in our homogenates was contained in the cell body and proximal axon of R2, where the [^{3}H]acetylcholine may actually have existed free. Greater amounts of particulate transmitter were found in the right connective, where more of the radioactivity was in the form of acetylcholine (Koike et al., 1972). This enrichment of the transmitter in the nerve suggested that it was being transported selectively, or, alternatively, that its turnover might be more rapid in cell body than in axon. Both explanations for the enrichment observed are consistent with the idea that more of the acetylcholine in the connective might be in a protected, vesicular form. Although turnover studies (Fig. 3) indicated that [^{3}H]acetylcholine might be contained in two subcellular compartments, both in injected neurons and in ganglia incubated in the presence of choline, they do not provide information about regional differences in the rates of turnover between cell body and axon.

Regional differences in the activity of choline acetyltransferase relative to the capacity of vesicles might also explain why so little labeled transmitter was recovered in synaptic vesicles from the cell body. Somatic vesicles would probably be loaded with the endogenous, unlabeled acetylcholine which McCaman et al. (1973) have shown to be present. Moreover, they do not function in the cell body, and therefore their content of transmitter might be quite stable, and exchange only slowly. The amount of acetylcholine synthesized from injected [^{3}H]choline in these experiments was always in excess of the endogenous transmitter by 1.5 to 3 times. Thus the capacity of these vesicles might have been exceeded. If this explanation is correct, saturation of somatic vesicles would occur at intracellular concentrations of choline far below those which saturate the synthetic enzyme (Table I).

There is of course no reason to expect that choline acetyltransferase activity should match the storage capacity of synaptic vesicles *in the cell body*. Both of these macromolecular components are present in the cell body because they are formed there to be transported to terminals. It is likely that their relative capacities do not reflect any somatic role, but rather are appropriate to the activities ultimately required for synaptic function, and are determined by their individual rates of synthesis and degradation. Terminals are rich in synaptic vesicles. New transmitter is required because vesicles

in the terminal region of the neuron are depleted by synaptic release. Presumably transferase activity and the capacity of synaptic vesicles are more closely matched at terminals.

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