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

## II. *Regional Localization and Characterization of Choline Uptake*

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ABSTRACT The choline required for synthesis of acetylcholine is derived exogenously by *Aplysia* ganglia. Under physiological conditions choline was taken up primarily by neuropile and nerves and not by cholinergic cell bodies. In addition, compared with their contents of choline acetyltransferase, those components of nervous tissue which contain nerve terminals and axons synthesized acetylcholine far more efficiently. Choline was accumulated by high and low affinity uptake processes; the high affinity process appeared to be characteristic of cholinergic neurons (Schwartz, J. H., M. L. Eisenstadt, and H. Cedar. 1975. *J. Gen. Physiol.* **65**:255). The two uptake processes were similarly affected by temperature with a $Q_{10}$ of 2.8. Both were dependent on a variety of ions in a complicated manner. High affinity uptake seemed to be more dependent on $Na^+$, showed greater inhibition by ouabain, and was selectively inhibited by oxotremorine. We found that the functional state of neurons did not alter uptake of radioactive choline by either process, nor did it change the conversion to radioactive acetylcholine.

### INTRODUCTION

Information about dynamic aspects of acetylcholine metabolism is largely derived from studies on tissues containing cholinergic nerve terminals (Birks and MacIntosh, 1961; Collier, 1969; Israël et al., 1970; Marchbanks, 1969; Potter, 1970; and Whittaker, 1965). It has been established that acetylcholine turns over at cholinergic terminals rapidly, and that a major proportion of the transmitter substance actually released is made from choline taken up by terminals. Nevertheless, cholinergic cell bodies and their axons contain

choline acetyltransferase, and therefore these regions also have the potential of synthesizing acetylcholine (Giller and Schwartz, 1971 *a*; Treistman and Schwartz, 1974). In the accompanying paper, Schwartz et al. (1975) present evidence that in *Aplysia* the choline used for synthesis is derived exogenously, and that at physiological external concentrations, choline is taken up into cholinergic neurons by a high affinity process.

Because the nervous system of *Aplysia* presents the opportunity for studying various regions of individual neurons, we have attempted to determine where the uptake processes are located within the cell. In addition, we have studied the effects of various conditions and the actions of pharmacological agents on uptake.

## MATERIALS AND METHODS

*Aplysia*, weighing 75–150 g (Pacific Bio-Marine Corp., Venice, Calif.) were maintained at 15°C in Instant Ocean (Aquarium Systems, Inc., Eastlake, Ohio); ganglia were removed from animals and maintained as already described (Schwartz et al., 1975).

### *Choline Uptake*

Ganglia were incubated usually at 15°C in various concentrations of [$^{14}$C]choline (40 mCi/mmol, New England Nuclear Corp., Boston, Mass.) or [$^{3}$H]choline (16 Ci/mmol, Amersham/Searle, Arlington Heights, Ill.) in sterile Instant Ocean containing 50 mM Tris-HCl (pH 7.6), 0.1% glucose, 200 U/ml penicillin G and 0.1 mg/ml streptomycin. Removal of R2 cell bodies by free-hand dissection and assay of choline acetyltransferase was described previously (Giller and Schwartz, 1971 *a*).

Subcellular fractionation was carried out at 0–4°C. Ganglia were homogenized in an isotonic solution containing 0.2 M sucrose and 0.3 M sodium chloride (Israël et al., 1970) in a Potter-Elvejhem tissue grinder with a Teflon pestle and a clearance of 0.25 mm (Kontes Glass Co., Vineland, N. J.) using 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 was again centrifuged at 1,000 *g*. The two 1,000-*g* supernatants were combined for analysis either by differential centrifugation (10,000 *g* for 10 min, followed by 105,000 *g* for 90 min) or by passage with line vacuum through a stack of cellulose acetate filters (25 mm in diameter, Millipore Corp., Bedford, Mass.) with a 10-mm diameter dacron mesh (Millipore) separating each filter from the next. Filters were washed with isotonic sucrose-salt solution, and the uppermost removed and transferred to 0.01 M ammonium acetate (pH 4.2). The reduced filter series was again washed, and the next disk removed. Filters were boiled at pH 4.2 for 5 min to inactivate acetylcholinesterase and to extract water-soluble radioactivity. Extracts were lyophilized, and choline-containing compounds separated by high voltage electrophoresis at pH 4.7 (Giller and Schwartz, 1971 *a*). The extracted filters were counted as a measure of radioactivity in lipid.

### *Analysis of Radioactivity*

Ganglia and pellet fractions were ground at 0°C in glass homogenizers (Micrometric Instrument Co., Cleveland, Ohio) containing 0.2 ml–0.5 ml of acetone: 1 N formic acid: (85:15). Substances on paper electropherograms were located by staining with $I_2$ vapor and radioactivity was eluted with water and counted by liquid scintillation.

### *Electrical Stimulation*

Abdominal or pleuropedal ganglia with 2–5-cm lengths of nerves attached were pinned through connective tissue to paraffin. Six nerves were each wrapped around a silver-silver chloride electrode pair in a chamber which held a 7-ml volume of buffered Instant Ocean containing radioactive choline. Cell bodies were penetrated with glass microelectrodes to monitor postsynaptic activity when nerves were stimulated and when we studied the effects of altering ionic conditions. Nerves were stimulated individually with 0.5-ms pulses at frequencies of 5–20 Hz. When response to stimulation of one nerve declined, another nerve was stimulated. High $Mg^{2+}$ Instant Ocean contained 220 mM $Mg^{2+}$ (4 ×) and 1 mM $Ca^{2+}$ (0.1 ×); high $K^+$ contained 40 mM $K^+$ (4 ×). Values are presented as means ±SE of ($n$) independent determinations. Significance was assessed with Student's $t$ test.

## RESULTS

### *Regional Localization of Choline Uptake*

At first we measured the amount of [$^3$H]choline in the cell body of R2, the giant neuron of the abdominal ganglion, after incubations in 20 μM [$^3$H]choline. We found that 0.95 ± 0.1 (2) pmol were taken up after 30 min and 7.6 (1) pmol after 4 h. (At both time periods only 6% of the radioactivity was converted to acetylcholine.) The cell body of R2 constitutes at least 5% of the volume of the neuronal components of an abdominal ganglion and contains 8% of the total choline acetyltransferase (Giller and Schwartz, 1971 *a*). Thus the amount of choline taken up was surprisingly small considering that the ganglion accumulates 1 nmol/h at 20 μM (Schwartz et al., 1975). This suggests that most of the choline is taken up into regions of the neuron other than the cell body.

Since the amount of radioactivity taken up by the cell body was so low, we attempted to determine which regions of the neuron most actively take up choline. After incubating isolated ganglia, with connectives[1] attached, in a higher concentration of [$^3$H]choline (65 μM) for 1 h, we measured the amount of radioactivity associated with cell body, ganglion, and connectives (Table I). We found [$^3$H]choline in all components, but most was taken up into

[1] Connectives are nerves running between ganglia; they contain no nerve cell bodies, but consist of numerous axons in a muscular connective tissue sheath (see Coggeshall, 1967).

TABLE I

REGIONAL DISTRIBUTION OF CHOLINE UPTAKE IN THE ABDOMINAL GANGLION

| Component | $n$ | Uptake of choline: Rate | Uptake of choline: Estimated intracellular choline concentration | Acetylcholine synthesis: Rate | Acetylcholine synthesis: Percent efficiency |
|---|---|---|---|---|---|
| | | *pmol/h* | *mM* | *pmol/h* | *nmol/U of choline acetyltransferase in component,* × 100 |
| Cell body of R2 | 7 | 7.9±1.3 | 0.12 | 0.21±0.02 | 0.007 |
| Residual ganglion* | 7 | 481±44 | 0.36 | 49±1.7 | 0.19 |
| Nerves (per cm length) | 4 | 390±33 | 0.86 | 63±9.5 | 0.80 |

Individual abdominal ganglia with nerves attached were incubated in 65 μM [$^3$H]choline for a total of 1 h. The 100-μl droplet was changed at 30 min. During the last 5 min, we added eserine to a final concentration of 50 μM. The tissue was washed for 10 min, and the cell body and nerves dissected for homogenization in acetone: formic acid. Concentrations of choline were estimated using 66 nl for the volume of R2's cell body (Giller and Schwartz, 1971 a); we estimated that this was 5% of the volume occupied by the ganglion's neuronal components (Giller and Schwartz, 1971 *a*). The volume of a 1-cm length of nerve was calculated assuming a cylinder with a radius of 0.12 mm (Ambron et al. 1974). Efficiency of acetylcholine synthesis was calculated using the activities of the transferase at 15°C.

* Dissection to remove the cell body resulted in loss of tissue.

connectives. The amount in connectives is expressed per unit length; since these nerves are usually a total of 4–8 cm long, they took up considerably more choline than did the ganglion. Moreover, when this value was normalized by dividing by the estimated volume of the structure we found that radioactivity was also most highly concentrated in the connectives.

The efficiency of conversion to acetylcholine was also greatest in the connectives. When radioactivity in each component was analyzed by high voltage electrophoresis, the proportion acetylated appeared to parallel the uptake of choline. We found about 16% of the radioactivity in the nerves was acetylcholine; in the ganglion this fraction was 10%; and the cell body of R2 had only 2.6%. Regional synthesis of acetylcholine was also compared to the amount of synthetic enzyme contained in the component. Values for choline acetyltransferase were obtained by assay in extracts of the various components. This normalization shows how efficiently the [$^3$H]choline taken up was utilized within the component. We found that the efficiency of in vivo acetylation was low in the cell body; in the remainder of the ganglion (presumably in neuropile) and the connectives (axons) the efficiency was considerably greater (Table I).

*Subcellular Fractionation of Ganglia after Incubation in the Presence of 2 μM [³H]Choline*

*Aplysia* ganglia contain a cortex of nerve cell bodies around a core of neuropile rich in nerve endings (see Coggeshall, 1967). With the idea of determining whether a substantial amount of the choline taken up in nervous tissue might be in terminals, we fractionated ganglia which had been incubated in a low concentration of [³H]choline for 45 mm. In homogenates of vertebrate brain, most of the newly synthesized acetylcholine was present in particulate form (see for example Schuberth et al., 1970). Less was isolated in particulate form from some vertebrate peripheral structures (sympathetic ganglion: Giacobini et al., 1971; Wilson and Cooper, 1972; diaphragm: Potter, 1970). In *Aplysia*, almost all of the [³H]acetylcholine was soluble; less than 5% was in a particulate form. Using differential centrifugation, we found that about 1% of the total radioactivity sedimented at 1,000 *g*; 2% at 10,000 *g*, and 1% at 105,000 *g*. To estimate the size of particles that contained the [³H]acetylcholine, we passed the 1,000-*g* supernatant through membrane filters with various pore sizes. About 5% of the total radioactivity of the homogenate was retained by a stack of filters with pore sizes ranging from 0.8 to 0.05 μm in diameter (Table II). This value corresponded well with the amount of radioactivity sedimented by differential centrifugation. Most of the radioactivity was contained in large particles since about 84% was retained above the 1.2-μm filters. When compared to the total homogenate, the proportion of acetylcholine within particles was not increased. Only a small amount of

TABLE II

SUBCELLULAR FRACTIONATION OF GANGLIA AFTER INCUBATION IN [³H]CHOLINE

| Pore diameter of filter | Amount of radioactivity in fraction | Acetylcholine content | Enrichment ratio | Distribution of ACh in fractions |
|---|---|---|---|---|
| *μm* | *% of total in homogenate* | *% of total ACh in homogenate* | *ACh in fraction/ACh in homogenate* | *% of total particulate ACh* |
| 8 | 3.0 | 2.3 | 0.9 | 58 |
| 1.2 | 1.1 | 1.1 | 1.1 | 28 |
| 0.45 | 0.5 | 0.4 | 1.1 | 10 |
| 0.05 | 0.3 | 0.2 | 0.8 | 4.3 |

Pairs of circumesophageal ganglia were incubated at 15°C in 100 μl containing 2 μM [³H]choline. The droplet was changed every 15 min; after 45 min, the ganglia were washed in buffered Instant Ocean containing 50 μM eserine for 10 min. Homogenates were prepared and filtered through stacks of cellulose acetate disks as described in Materials and Methods. Values are means of four experiments.

the particulate radioactivity had been incorporated into lipid (7.2% was extracted by chloroform-methanol).

*Conditions Affecting Uptake of Choline*

In the preceding paper, Schwartz et al. (1975) found that choline was taken up into *Aplysia* nervous tissue by both a high and a low affinity process. To learn more about the nature of the two uptake processes, we tried to find conditions which would distinguish them. Using the cumulative uptake technique, we first measured the rate of uptake into nervous tissue in buffered Instant Ocean at 15°C (standard condition). We then determined uptake under the experimental condition, and calculated the ratio of the experimental rate to the control rate ($v_e/v_c$). The effects of each condition were tested at a relatively low concentration of choline, 20 $\mu$M, and also at a high concentration, 209 $\mu$M. At 209 $\mu$M, almost all of the choline would be taken up by the low affinity process. From the kinetic model already presented (Schwartz et al., 1975), we estimate the contribution of the high affinity process to be a third of the total at a choline concentration of 20 $\mu$M. Because of the range of kinetic constants obtained this estimate is uncertain. For these experiments, we did not use concentrations lower than 20 $\mu$M, although proportionally they would have reflected more of the high affinity process. The technique of cumulative uptake cannot be used at much lower concentrations conveniently. In order to obtain initial rates at lower concentrations, we would have had to make measurements at shorter intervals of time; consequently the amounts of choline taken up into ganglia become too small to assess reliably.

Despite the calculated overlap of the two processes at 20 $\mu$M choline, we found a variety of conditions which affected the two uptake processes selectively (Table III). Dependence on ions, sensitivity to ouabain, and inhibition by oxotremorine were found to distinguish the high and low affinity processes. Several other conditions affected both processes equally.

*Effect of Ions on the Uptake of Choline*

High affinity uptake of choline was completely abolished when all the ions in Instant Ocean were replaced by isotonic sucrose. Uptake at a choline concentration of 209 $\mu$M was inhibited to a lesser extent (Table III). Inhibition of uptake in sucrose was almost completely reversible; when the tissue was placed in Instant Ocean again, uptake was 49% of control after 40 min; after 1 h, it was 91%. These results suggested that the high affinity uptake process depended on some ion or group of ions. Although also affected, the low affinity process was less dependent.

TABLE III

CONDITIONS AFFECTING UPTAKE AT HIGH AND LOW CONCENTRATIONS OF [14C]CHOLINE

| Condition | $n$ | Effect on rates of uptake | |
|---|---|---|---|
| | | 20 $\mu$M | 209 $\mu$M |
| | | $v_e/v_c$ | |
| (A) Control (Instant Ocean) | 4 | 0.96±0.07 | 1.03±0.09 |
| (B) Replacement of Ions | | | |
| Replacement of $Na^+$ | | | |
| Guanidinium$^+$ | 3 | −0.19±0.03* | −0.36±0.04 |
| Tris$^+$ | 3 | −0.14±0.03 | −0.04±0.04 |
| $Li^+$ | 3 | −0.10±0.05 | 0.07±0.06 |
| Sucrose | 3 | 0.04±0.01* | 0.33±0.08 |
| All ions removed | | | |
| Sucrose (0.8 M)‡ | 8 | 0.26±0.06* | 0.57±0.07 |
| (C) Pharmacological Agents | | | |
| Ouabain (0.2 mM) | 5 | 0.44±0.05* | 0.91±0.16 |
| HC-3 (0.1 mM) | 3 | 0.70±0.11 | — |
| (1.0 mM) | 3 | 0.54±0.06 | 0.74* |
| Eserine (50 $\mu$M) | 3 | 0.99±0.17 | 0.89±0.11 |
| Oxotremorine (30 mM) | 3 | 0.53±0.07* | 0.92±0.02 |
| (D) Temperature (°C) | | | |
| 28 | 3 | 1.13±0.04 | 1.30±0.09 |
| 22 | 3 | 1.23±0.03 | 1.66±0.23 |
| 15 | — | 1 (set) | 1 (set) |
| 7 | 3 | 0.62±0.19 | 0.55±0.01 |
| 2 | 3 | 0.27±0.02 | 0.29±0.03 |
| (E) Metabolic Inhibitors | | | |
| Sodium cyanide (1.3 mM) | 3 | 1.02±0.09 | 0.91±0.16 |
| Dinitrophenol (0.2 mM) | 1 | 0.88 | 0.83 |
| Sodium azide (10 mM) | 3 | 0.52±0.04 | 0.70±0.18 |
| N-ethylmaleimide (0.25 mM) | 2 | 0.35±0.12 | 0.58±0.18 |
| (2.5 mM) | 3 | −0.36±0.05 | −0.27±0.09 |

Pairs of circumesophageal ganglia were incubated at 15°C in 50-$\mu$l droplets using procedures described for cumulative uptake (Schwartz et al. 1975). Control rates were determined for a total period of 100 min at 15°C in buffered Instant Ocean containing either 20 or 209 $\mu$M [14C]choline. After a 30-min period of equilibration in the experimental solution or experimental temperature, rates were measured during a second period of 100 min in 50-$\mu$l droplets of the experimental solutions or in Instant Ocean at the experimental temperature. The concentration of [14C]choline was the same throughout both phases of the experiments. The ratio of experimental uptake velocity ($v_e$) to control ($v_c$) was determined for each pair of ganglia. A negative ratio indicates that radioactivity was lost from ganglia. An asterisk denotes a significant difference ($P < 0.03$) between the extent of inhibition at the two concentrations of [14C]choline.

* Single determination only.

‡ Buffered with 10 $\mu$M Tris (pH 7.6).

Since transport of a variety of substances has been shown to be dependent on $Na^+$ (see for example Stein, 1967), we suspected that the critical ion was $Na^+$. To determine which ions are necessary, we studied uptake of choline in artificial salt solutions lacking $Na^+$ and containing a variety of replacements for $Na^+$, and in several isotonic sucrose solutions, each containing a single salt. These experiments also indicated that $Na^+$ is critical for the high affinity process. For example, replacement of normal NaCl with sucrose in the presence of all of the other ions in seawater completely inhibited uptake at a choline concentration of 20 $\mu$M; this condition depressed uptake at 209 $\mu$M choline by 67% (Table III). Substitution of $Li^+$ and organic monovalent cations for $Na^+$ caused complete inhibition of uptake or even net loss of [$^{14}$C]choline from nervous tissue at both 20 and 209 $\mu$M choline (Table III). In addition to suggesting that uptake of choline depends on $Na^+$, these experiments also revealed that other ions play a complicated role in the uptake processes. Uptake was restored after a period in salt-free isotonic sucrose if all of the ions in seawater were replaced in proper combination. Addition of any single salt to the sucrose was insufficient, and with some salts greater inhibition was produced than occurred in isotonic sucrose alone. Addition of NaCl alone in the same concentration as in seawater did not reverse the inhibition (data not shown). When $K^+$, $Ca^{2+}$, and $Mg^{2+}$ were added to sucrose individually or $Ca^{2+}$ and $Mg^{2+}$ together as the chlorides in their normal concentration in seawater, each produced greater inhibition than sucrose alone.

*Effect of Specific Pharmacologic Agents*

The electrogenic Na pump might play a role in high affinity choline uptake since 0.1 mM ouabain profoundly inhibited uptake at the low concentration of choline, but was only slightly effective at the higher concentration (Table III). Treatment of *Aplysia* ganglia with this concentration of ouabain rapidly depolarizes most, and perhaps all, neurons, and causes them to fire repeatedly (Carpenter and Alving, 1968; Pinsker and Kandel, 1969). Oxotremorine (Nutritional Biochemical Co., St. Louis, Mo.) selectively inhibited uptake at the low concentration of choline (Table III). This drug also inhibited choline uptake into synaptosomes from guinea pig brain (Diamond and Kennedy, 1969). Its biochemical mechanism of action is unknown, however. Hemicholinium-3 (Aldrich Chemical Co., Inc., Milwaukee, Wis., HC-3) which has been shown in many other animals to inhibit the uptake of choline competitively, blocked cholinergic transmission in *Aplysia* (Stinnakre, 1970). Since HC-3 acts competitively, we did not determine the extent of inhibition at the high concentration of choline. The inhibition constant was 0.67 mM, determined in experiments using concentrations of choline between 20 and 120 $\mu$M. This value seems rather high. Haga and Noda (1973) have shown that the inhibition constant for HC-3 decreases (HC-3 becomes a relatively

more potent inhibitor) at lower concentrations of choline. The constant we have determined using the cumulative uptake technique may therefore not reflect the actual potency of the drug against uptake of the small amounts of choline present in the synaptic cleft after synaptic activity. Eserine at 50 $\mu$M, a concentration which inhibited *Aplysia* acetylcholinesterase by more than 95% (Giller and Schwartz, 1971 *b*), did not inhibit uptake. We did find however that the conversion into acetylcholine of the choline taken up in the presence of eserine was doubled. This increase may be analogous to surplus acetylcholine in other animals (Birks and MacIntosh, 1961). Slight inhibition was observed at 1 mM eserine.

### *Effects of Temperature*

Both high and low affinity uptake of choline appeared to involve metabolic reactions, since both components were quite dependent on temperature. In the range from 2 to 15°C, the rates of uptake increased with a $Q_{10}$ of 2.8 (Table III).

### *Metabolic Inhibitors*

Inhibitors of oxidative phosphorylation affected the rates of uptake at both the high and the low concentration of choline. Although sodium cyanide caused no inhibition, dinitrophenol and sodium azide decreased uptake. Since both uptake processes were highly dependent on temperature, the varying degrees of inhibition observed probably indicate that these agents do not penetrate well or that *Aplysia* nervous tissue contains reserves of ATP which are sufficiently large to be unaffected during the course of the experiments. The alkylating agent, *N*-ethylmaleimide, was found to decrease high and low affinity choline uptake equally. At 0.25 mM uptake by both processes was inhibited, and at 2.5 mM radioactivity was lost from the ganglia.

### *Effect of Electrical Activity*

We were unable to detect any changes in the amounts of choline taken up into ganglia at either a low or a high external concentration during electrical stimulation at frequencies of 5–20 Hz (Table IV). We stimulated the nerves leading into the ganglia, making certain that stimulation was effective by monitoring several cell bodies intracellularly. Since these experiments had to be carried out in relatively large volumes, we did not use the technique of cumulative uptake. Instead, we measured the total amount of radioactivity taken up into a ganglion at the end of an hour's incubation. An advantage of this procedure is that it allowed us to use lower external concentrations of [$^3$H]choline. We chose 0.1 $\mu$M, since at this concentration more than 90% of the choline was taken up by the high affinity process; at the same time, the amount of radioactivity associated with single ganglia was ample for

TABLE IV

UPTAKE OF CHOLINE AND CONVERSION TO ACETYLCHOLINE IN STIMULATED GANGLIA

| External choline concentration | n | Condition | Uptake | Conversion to ACh |
|---|---|---|---|---|
| *μM* | | | | *% of total radioactivity* |
| (A) Electrical activity (23°C) | | | *pmol/ganglion/h* | |
| 0.1 | 6 | Control | 15.16±6.08 | 63.30±5.44 |
| | 4 | Electrical stimulation | 15.62±4.19 | 62.43±1.92 |
| | 2 | High $Mg^{2+}$ | 13.35±4.67 | 60.85±14.5 |
| | | | *nmol/ganglion/h* | |
| 65 | 4 | Control | 4.42±0.54 | 16.45±5.59 |
| | 2 | Electrical stimulation | 3.74±1.16 | 16.54±1.65 |
| | 2 | High $Mg^{2+}$ | 3.78±0.39 | 24.70±3.82 |
| | | | *pmol/ganglion/h* | |
| (B) Ionic depolarization (15°C) | | | | |
| 0.1 | 5 | Control | 7.09±3.20 | 38.84±9.83 |
| | 5 | High $K^+$ | 5.81±2.39 | 33.36±7.40 |
| | | | *nmol/ganglion/h* | |
| 65 | 4 | Control | 2.37±0.54 | 18.53±4.48 |
| | 5 | High $K^+$ | 2.53±0.43 | 16.31±5.09 |

detection. At this low concentration any change in the rate of uptake should have reflected a modification in the activity of the high affinity process.

We chose 65 μM as the high concentration to detect a possible change in the efficiency with which choline, taken up by the low-affinity process, would be utilized for synthesis of acetylcholine. Much more of the choline taken up at 209 μM is unavailable for transmitter synthesis. Although most of the choline taken up at 65 μM enters by the low affinity process, it is still a low enough concentration that a change in the efficiency of conversion might be detected. Nevertheless, we found that electrical stimulation did not result in increased formation of [$^3$H]acetylcholine (Table IV).

Many neurons of *Aplysia* are spontaneously active. Endogenous electrical activity might make it difficult to detect differences between stimulated ganglia and unstimulated controls. We therefore bathed ganglia in Instant Ocean supplemented with high $Mg^{2+}$. This condition blocks synaptic activity in *Aplysia* neurons effectively (see for example Koike et al., 1974). Although we found that postsynaptic potentials were absent and that there was a

sharp reduction in total spiking activity throughout the ganglion, uptake and conversion of [$^3$H]choline were unchanged (Table IV).

Chemical depolarization by incubation in Instant Ocean supplemented with high $K^+$ was also without effect at choline concentrations of 0.1 or 65 $\mu$M. Neither the total amount of [$^3$H]choline in the ganglion nor its conversion was affected by high $K^+$. These experiments were carried out at room temperature as were most previous electrophysiological studies (see for example Koike et al., 1974).

*Effect of Maintaining Ganglia in the Absence of Exogenous Choline*

To test whether stores of transmitter might become depleted, we perfused ganglia with Instant Ocean in the absence of exogenous choline for 23 h, at a rate of 1 ml/min. Control ganglia were perfused with Instant Ocean supplemented with 20 $\mu$M unlabeled choline. After they were washed the ganglia were incubated in either 0.1 or 65 $\mu$M radioactive choline for a final hour. The amount of choline taken up and converted into acetylcholine was similar in ganglia which had been perfused for the 23-h period in the presence or in the absence of unlabeled choline. Compared to ganglia freshly isolated from the animal, however, both experimental and control ganglia after prolonged incubation took up only half the amount of radioactive choline at either the high or the low concentration. Schwartz et al. (1971) noted that neuronal protein and RNA synthesis also diminished after overnight maintenance of isolated ganglia in Instant Ocean.

During the experiment we monitored activity in L10, a cholinergic neuron which fires spontaneously. Its firing rate varied little from a mean of 2.3 Hz. The inhibitory postsynaptic potential in L5, a left upper quadrant follower cell of L10, was still intact and only slightly diminished in amplitude after 23 h in the absence of exogenous choline (Fig. 1). Unimpaired transmission has

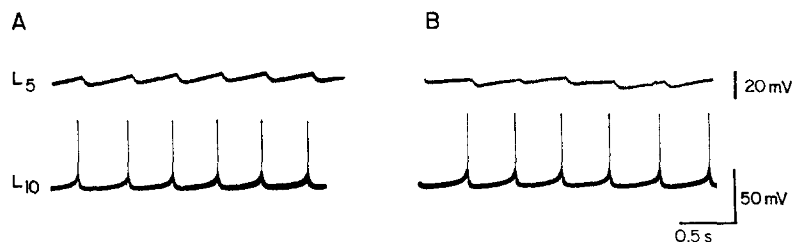


FIGURE 1. Cholinergic transmission in isolated ganglia maintained in the absence of choline. (A) Spontaneous activity in L10 (lower trace) and corresponding IPSP's in L5 during the first hour after removing the ganglion from the animal. (B) The same cells were impaled again after 23 h. The resulting membrane potential of the follower cell was unchanged.

also been observed at other cholinergic synapses in the ganglion after periods as long as 31 h in the absence of choline (Schwartz et al. 1971).

## DISCUSSION

Evidence was presented that *Aplysia* nervous tissue accumulates choline by two uptake processes (Schwartz et al. 1975). One of these operated at low external choline concentrations, and the choline taken up by this process is used efficiently for the synthesis of acetylcholine. This is strong evidence that the process is localized in cholinergic neurons, rather than in glial cells or in other components of nervous tissue. The high affinity process is capable of taking up far less choline than the low affinity process, consistent with localization to a small proportion of the neurons in the ganglion. The greater uptake capacity of the low affinity process suggests that it is more generally distributed. It can be argued that some of the choline taken up by the low affinity process was in cholinergic neurons, since some was converted to acetylcholine, but we cannot be certain that the unconverted choline was entirely within neurons. The fate of the choline can only be used for localization of the uptake process if it is characteristic of a particular cell type. Although most of the choline taken up by the low affinity process remained unchanged, some was converted to phosphorylcholine and betaine. Unlike the synthesis of acetylcholine, however, formation of these derivatives occurs in most, if not all, cells of the body. We have no evidence against the possibility that some of the choline taken up by the low affinity process was in glial cells.

We conclude that choline enters a cholinergic neuron by both uptake processes. Since the synthesis of the transmitter is controlled by availability of exogenous choline, it is important to determine the anatomical localization *within the neuron* of the uptake processes. The cell body is most available for study in *Aplysia*, and we have presented evidence that the high affinity uptake process is deficient in this region of the cholinergic neuron. Although the cell body is the site of synthesis of choline acetyltransferase and of the membrane constituents involved in storage and release of the transmitter, it is generally believed that acetylcholine itself serves no purpose in the cell body and is required only at terminals (but see Nachmansohn, 1970). Absence of choline uptake would restrict synthesis of acetylcholine in the cell body, however rich it is in the other components involved in the metabolism of the transmitter.

Since it was lacking in the cell body, the high affinity uptake must be localized to other parts of a cholinergic neuron. By cumulative uptake measurements similar to those used in ganglia we have shown that isolated lengths of connective take up choline by both high and low affinity processes. In isolated connectives a large proportion of the choline was converted to acetyl-

choline. We can therefore again conclude that at least some of the choline was taken up into cholinergic axons.

These regional differences in uptake might result from variation in the number of uptake sites in the neuronal membrane of the particular region. Two other possible explanations cannot be eliminated, however. Uptake per unit area of neuronal membrane might actually be uniform throughout the neuron, but the cell body might have less membrane area than does the axon. The external membranes of both cell body and axon in *Aplysia* are complicated with many areas of infolding and invagination (Coggeshall, 1967; Thompson et al., 1973); it would therefore be difficult to compare the amount of membrane covering the two regions. An alternative explanation involves the numerous small glial cells which invest the entire perimeter of *Aplysia* neurons. Coggeshall (1967) noted that the glial cells covering cell bodies differed in appearance from those over axons and neuropile. Glial cells covering cell bodies might compete more effectively for external choline than do glial cells in the other regions of the nerve cell.

It has been suggested that choline in other animals is predominantly taken up into nerve terminals, mainly because of studies with subcellular (synaptosome) fractions. Choline is probably taken up at terminals of *Aplysia* also. We found that little newly synthesized acetylcholine was in particulate material, however. We have no evidence in *Aplysia* that nerve terminals can pinch off during homogenization of nervous tissue to form synaptosomes (see Eisenstadt and Schwartz, 1975).

We have found both high and low affinity uptake components in subcellular fractions prepared from *Aplysia* nervous tissue by differential centrifugation (unpublished experiments) confirming work in other animals (Yamamura and Snyder, 1972; Haga and Noda, 1973; Dowdall and Simon, 1973). At 3 $\mu$M choline, uptake into the particulates in a 1,000-*g* supernatant prepared from a ganglion was as much as 40% of the uptake measured in the intact ganglion. When normalized to the content of choline acetyltransferase, conversion to acetylcholine of the choline taken up was about equivalent to that we have observed in nerves. Since it is unlikely that much of the particulate material arises from presynaptic terminal regions, this approach is not as useful for localizing the regional distribution of the uptake process as in other animals (Cotman et al., 1971).

### *Mechanism of Choline Uptake*

The mechanisms of both processes of choline uptake are unknown. Both are dependent on temperature, and both require the presence of $Na^+$ and are affected in a complicated way by other ions. The high affinity component in *Aplysia*, as in other animals (Yamamura and Snyder, 1972; Haga and Noda,

1973; Dowdall and Simon, 1973) showed a more pronounced dependence on $Na^+$ and was inhibited more profoundly by ouabain. Since the uptake processes differed only in their degree of dependence, this property is difficult to use for comparing uptake mechanisms between species. In addition, transport of many substances other than choline is dependent on $Na^+$ and inhibited by ouabain, indicating that these properties are general to a class of transport mechanisms.

Whatever the mechanisms of choline transport in *Aplysia*, it is likely that specific binding or permeation sites are involved since uptake was inhibited by HC-3 competitively. In addition, choline appears to be accumulated against a concentration gradient by both processes. We have measured cumulative uptake for as long as 7 h, and found that uptake at both 20 and 209 $\mu$M occurred at a constant rate. At 20 $\mu$M, unconverted [$^{14}$C]choline was concentrated over 15-fold, if distributed in the estimated total ganglion volume (including extracellular space), and over 100-fold if distributed in the estimated neuronal volume. Uptake of choline by either mechanism might result from an increase in the intracellular concentration of free choline or from a binding of choline to some intracellular constituent. Since we have shown that the choline taken up remained largely unchanged, covalent binding within the tissue cannot account for the accumulation observed in *Aplysia*.

### *Effects of Physiological Activity on Uptake and Turnover*

Cholinergic transmission fails after brief periods of activity when the eserinized superior cervical ganglion of the cat is perfused in the absence of choline (Birks and MacIntosh, 1961). We were unable to do similar experiments since in *Aplysia* eserine rapidly blocked transmission postsynaptically.[2] In the absence of eserine we found that L10, an identified cholinergic interneuron, was capable of producing normal responses in follower cells for at least 24 h when ganglia were perfused without choline. This observation suggests that *Aplysia* neurons efficiently recapture choline from released acetylcholine. Alternatively they may have a more ample supply of endogenous choline or acetylcholine relative to the amounts of transmitter released during stimulation than do vertebrate neurons. These explanations are not mutually exclusive; and we have some evidence for both.

We presume that it is the high affinity process which serves to recapture choline from released acetylcholine. Evidence for this idea has been presented

[2] Postsynaptic response in a follower cell of L10 was abolished within 15 min after adding 50 $\mu$M eserine sulfate. This resulted from a postsynaptic action of the drug, since the PSP was abolished just as quickly when L10 was hyperpolarized and did not fire, and since the inhibition was unaffected by addition of choline. There is evidence that eserine affects various cholinergic synapses differently and acts in ways other than as an anticholinesterase (Dennis et al., 1971; Koike et al., 1974).

in this and the preceding paper. It is difficult to produce compelling evidence that *Aplysia* neurons contain more ample reserves of transmitter than do vertebrate neurons. Nevertheless the concentrations of both acetylcholine (about 10 $\times$) and of choline acetyltransferase (about 3 $\times$) in the abdominal ganglion of *Aplysia* are higher than those in the cat sympathetic ganglion (Hebb, 1963; MacIntosh, 1963). The significance of these values is difficult to evaluate because of the heterogeneity of nervous tissue.

Electrical stimulation has been shown to increase both the amount of choline taken up into mammalian nervous tissue and its conversion to acetylcholine (Collier and MacIntosh, 1969; Potter, 1970). These effects were observed in the absence of eserine. Consistent with the idea that *Aplysia* neurons contain substantial reserves of the transmitter, we found that neither electrical stimulation nor chemical depolarization with a high concentration of $K^+$ were effective (Table IV). Thus Collier and MacIntosh (1969) showed that, although the total ganglionic acetylcholine was unchanged during stimulation, [$^3$H]choline was incorporated into the transmitter at five to six times the resting rate. After 1 h, they found an amount of acetylcholine equivalent to 85% of the total content was labeled. Under similar experimental conditions we found no change in the rate of incorporation. We have not studied the total (unlabeled) choline and acetylcholine in *Aplysia,* and therefore changes in the total neuronal concentrations of these substances might not be detected by our measurements. This is unlikely, however, since the fraction of transmitter most responsive to physiological activity in other animals is the acetylcholine most recently synthesized (see for example Collier and MacIntosh, 1969; Richter and Marchbanks, 1971). Moreover, if changes of the degree observed by Collier and MacIntosh had occurred in *Aplysia,* we should have been able to detect them.

Although we have shown that synthesis of acetylcholine in *Aplysia* is dependent on exogenous choline, we have not been able to demonstrate that the uptake process changes during neuronal functioning. This presumably indicates that the store of transmitter is greatly in excess of the amounts needed for release. It also suggests that uptake functions maximally whether or not the neuron is firing. Thus in *Aplysia,* in contrast to vertebrates, no short-term, fine control appears to be exerted. An abundant supply of releasable transmitter substance may be an important feature of neurons in simple nervous systems. The small number of individual neurons which control entire behaviors (Kandel and Kupfermann, 1970) can therefore function continuously although there may be great variations in precursor concentrations in the blood.

The authors thank M. H. Kumarasiri for excellent technical assistance and Drs. J. Koester and I. Kupfermann for their critical reading of the manuscript.
This work was supported by Research Grant NS 09955 from the National Institutes of Health.

Dr. Schwartz was supported by U. S. Public Health Service Career Award GM-28, 125, Dr. Eisenstadt by Medical Scientist Training Grant GMO-1688, and Dr. Treistman by a grant from the Scottish Rite Schizophrenia Program.
Some of the data in this paper are taken from a thesis submitted by Dr. Eisenstadt for the degree of M.D.-Ph.D. in the Graduate School of Arts and Sciences and the School of Medicine of New York University.

*Received for publication 10 June 1974.*

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