

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

## I. *Source of Choline and Its Uptake by Intact Nervous Tissue*

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**ABSTRACT** Although acetylcholine is a major neurotransmitter in *Aplysia*, labeling studies with methionine and serine showed that little choline was synthesized by nervous tissue and indicated that the choline required for the synthesis of acetylcholine must be derived exogenously. Ganglia in the central nervous system (abdominal, cerebral, and pleuropedals) all took up about 0.5 nmol of choline per hour at 9  $\mu\text{M}$ , the concentration of choline we found in hemolymph. This rate was more than two orders of magnitude greater than that of synthesis from the labeled precursors. Ganglia accumulated choline by a process which has two kinetic components, one with a Michaelis constant between 2–8  $\mu\text{M}$ . The other component was not saturated at 420  $\mu\text{M}$ . Presumably the process with the high affinity functions to supply choline for synthesis of transmitter, since the efficiency of conversion to acetylcholine was maximal in the range of external concentrations found in hemolymph.

### INTRODUCTION

There is considerable neurophysiological and pharmacological evidence that acetylcholine is a major neurotransmitter substance in *Aplysia*. By bioassay using the rectus muscle of the frog, Bacq (1935) found 0.35  $\mu\text{g}$  acetylcholine per gram of *Aplysia* ganglia. More recently it has been shown that acetylcholine applied iontophoretically simulates the action of the naturally occurring transmitter on neurons in the central nervous system (Gerschenfeld, 1973 for review). Effector functions of several motor neurons on gill (Carew et al., 1974), heart, and blood vessels (Liebeswar et al., 1973) are

also simulated by acetylcholine, and specific cholinergic blocking agents abolish the response of these organs to nerve stimulation.

We chose to work with the nervous system of *Aplysia* (see Bullock and Horridge, 1965) because it offered the chance of studying identified cholinergic cell bodies, their axons and terminals. During the past decade, neurons in the abdominal ganglion have been under intensive electrophysiological investigation; consequently much is known about their function. Over 30 neurons in the ganglion are large and can be reliably recognized from animal to animal (Frazier et al., 1967); their size permits isolation of individual cell bodies for biochemical analysis (see Peterson, 1972 for review). All of the identified cells have been assayed for choline acetyltransferase (Giller and Schwartz, 1971 *a*) and for acetylcholinesterase (Giller and Schwartz, 1971 *b*). While every cell contained the esterase, only four, R2, L10, L11 (Giller and Schwartz, 1971 *a*; McCaman and Dewhurst, 1970), and LDG (Carew et al., 1972) were found to contain the transferase. [<sup>3</sup>H]-choline injected into cell bodies of these neurons was efficiently converted to acetylcholine (Koike et al., 1972; Eisenstadt et al., 1973). In R2, this [<sup>3</sup>H]-acetylcholine was shown to be transported in the axon. In addition, Koike et al. (1972) have provided evidence that the transmitter synthesized from [<sup>3</sup>H]choline injected intrasomatically can be released from L10's terminals by synaptic activity.

In order to obtain a further understanding of dynamic aspects of transmitter biosynthesis, we now have studied the source of the choline used for synthesis. As with higher organisms, we have found that the choline synthesized in *Aplysia* neurons is insufficient for synthesizing acetylcholine. Choline, however, is readily derived from hemolymph through uptake by two processes, one with an affinity constant approximately the same as the mean concentration of choline we have measured in the blood; the other process has a much lower affinity. As in other animals, synthesis of transmitter in *Aplysia* depends critically on the choline taken up by the high affinity process.

#### MATERIALS AND METHODS

*Aplysia* (Pacific Bio-Marine Supply Co., Venice, Calif.) were maintained in well-aerated, filtered Instant Ocean (Aquarium Systems, Inc., Eastlake, Ohio) at 15°C and were fed seaweed daily. Ganglia and nerves were removed through an incision in the foot and kept in Instant Ocean containing 50 mM Tris-HCl (pH 7.6), 200 U/ml penicillin G, and 0.1 mg/ml streptomycin for 30–60 min before use.

#### *Labeling with Precursors*

**INCUBATIONS** Ganglia were incubated at 15°C in disposable culture tubes (Falcon Plastics, Oxnard, Calif.) kept almost horizontal in order to maximize the surface-to-volume ratio of the 1 ml of sterile-filtered (Nalgene Filter Unit, 0.20- $\mu$ m

pore size, Sybron Corp., Rochester, N. Y.) Instant Ocean containing 10 mM Tris-HCl (pH 7.6), antibiotics, 0.1 % glucose, and [ $^3\text{H}$ ]methylmethionine (4 Ci/mmol) or [ $^3\text{H}$ ]serine (2.2 Ci/mmol), both from New England Nuclear Corp., Boston, Mass.

**INJECTIONS** [ $^3\text{H}$ ]methionine (6.4 Ci/mmol) and [ $^3\text{H}$ ]serine (6.3 Ci/mmol), both from Amersham/Searle Corp., Arlington Heights, Ill., were concentrated and injected with pressure into the cell body of R2 as previously described (Koike et al., 1972; Eisenstadt et al., 1973). After injection, ganglia were maintained for various lengths of time in 100-ml petri dishes containing 20 ml of the supplemented Instant Ocean.

#### *Synthesis of Total Choline*

After the tissue was washed for 10 min in Instant Ocean, it was homogenized in N KOH in glass tissue grinders (Micrometric Instrument Corp., Cleveland, Ohio) and hydrolyzed at 37°C for 18 h. An equal volume of 2 N perchloric acid was added to precipitate unhydrolyzed macromolecules. Perchlorate was then removed after neutralization with KOH. In addition to choline, alkaline hydrolysates would be expected to contain phosphorylcholine (which is not precipitated as reineckate). To achieve complete hydrolysis of phosphorylcholine, a portion of the alkaline hydrolysate was subjected to acid hydrolysis in 2 N HCl at 110°C in sealed tubes (Ansell and Hawthorne, 1964). Radioactivity in choline in both hydrolysates was precipitated as the reineckate. The difference between the amounts of choline in the two hydrolysates was phosphorylcholine.

#### *Synthesis of Choline, Acetylcholine, and Lipid Choline*

**LIPID CHOLINE** Ganglia after uptake or injection of precursors were homogenized in 5 % TCA in ground glass tissue grinders. The resulting acid-insoluble material was extracted in cold chloroform-methanol (2:1) (Folch et al., 1957). The aqueous phase was discarded and the radioactivity in the organic phase was characterized by chromatography or after alkaline hydrolysis (see below). The acid-insoluble residue, after chloroform-methanol extraction, was counted by scintillation after solubilization in 0.5 ml of NCS (Amersham/Searle).

**CHOLINE AND ACETYLCHOLINE** After 0.5 mg of choline and acetylcholine were added, TCA was extracted with water-saturated ether. Choline and acetylcholine were precipitated at pH 4.5 by adding an equal volume of saturated ammonium reineckate (Shaw, 1938). After standing for 1 h at room temperature, these precipitates were washed twice with supernatant obtained after the addition of saturated reineckate to unlabeled choline and acetylcholine. Washed precipitates were dissolved in 90 % aqueous acetone, and reineckate removed by the addition of  $\text{AgNO}_3$ . Recovery of labeled choline or acetylcholine was 83 %.

#### *Kinetics of Choline Uptake*

Ganglia, pinned through connective tissue to silicone plastic (Sylgard, Dow Chemical Company, Midland, Mich.) in petri dishes, were blotted dry and covered at 15°C

with 50- or 100- $\mu$ l droplets containing various concentrations of radioactive choline ( $[^{14}\text{C}]$ choline, 40 mCi/mmol, New England Nuclear;  $[^3\text{H}]$ choline, 16 Ci/mmol, Amersham/Searle). Uptake was measured in two types of experiments.

**CHOLINE ACCUMULATION** Droplets were changed at 15-min intervals. Frequent change was necessary to maintain the concentration of choline, especially at the lower concentrations. After they were incubated for a total of 45 min, ganglia were washed for 10 min in buffered Instant Ocean and then homogenized at 0°C in 0.5 ml acetone:formic acid (85:15) (Toru and Aprison, 1966) and sampled for counting.

**CUMULATIVE UPTAKE** Ganglia were incubated for up to 7 h. At 30-min intervals, the droplet was removed and sampled. Ganglia were gently blotted, fresh droplets added, and the incubation continued. The samples were dried on Whatman 3 MM paper disks (for carbon-14) or Whatman GF/C glass fiber pads (for tritium) and counted by liquid scintillation. Uptake was the difference between the amount of choline originally applied and the amount remaining in the droplet after the 30-min interval. Cumulative uptake was the sum of the amounts taken up during 30-min intervals. SAAM 25 (National Institutes of Health, Bethesda, Md.) was the computer program used to analyze the kinetic data.

To measure *efflux*, after incubation in  $[^{14}\text{C}]$ choline as already described for a total period of 80 min, we transferred ganglia to scintillation vials, each containing 1.2 ml of buffered Instant Ocean. Unlabeled choline was included at the same concentration present during the incubation period. Ganglia were transferred to fresh vials at various intervals, and the radioactivity that appeared was measured by scintillation. Data were analyzed graphically (Hertz, 1968).

#### *Analytical Methods*

Choline was assayed using a modification of the method of Horowitz and Beadle (1943) in basal medium (Luck, 1967). *Neurospora crassa*, chol-1 (34486) was provided by Dr. David Luck, The Rockefeller University, New York. We used two procedures to measure growth, gravimetric and radiochemical. The gravimetric assay was capable of detecting about 1  $\mu$ g, the radiochemical, about 5 ng. We assayed choline in hemolymph and in pooled ganglia by the gravimetric procedure, and in individual abdominal ganglia by the radiochemical method. Lipid choline was removed from samples by extraction with chloroform-methanol. Samples were assayed directly, and, in order to hydrolyze acetylcholine, after boiling in 0.1 N ammonium hydroxide at pH 10 for 10 min (phosphorylcholine is not hydrolyzed under these conditions).

We centrifuged hemolymph at 105,000  $g$  for 1 h to remove hemocyanin and other particulate material (Giller and Schwartz, 1971 *b*). The resulting supernatant was boiled for 30 min to remove protein and associated lipid, and then filtered through Nalgene units. Choline and acetylcholine were extracted from ganglia by grinding in glass homogenizers at 0°C in 95% ethanol containing 0.2% acetic acid (Jenden and Campbell, 1971).

**GRAVIMETRIC** After 3 days of shaking at 28°C, mycelial mats were collected, dried, and weighed on cellulose acetate filters (45-mm diameter, 8- $\mu$ m pore size, Millipore Corp., Bedford, Mass.). Blanks weighed less than 1 mg with an inoculum

of 500–750 conidia and maximal growth, obtained with 30  $\mu\text{g}$  of choline, amounted to 50 mg. Triplicate determinations differed maximally by  $\pm 6\%$ .

**RADIOCHEMICAL** Samples were air-dried in a sterile room under ultraviolet light in the centers of 25-mm glass cover slips. Dried samples were surrounded with a ring of petroleum jelly, and then 50  $\mu\text{l}$  of medium containing 50–75 conidia were added. The cover slip was inverted and mounted on a concavity slide. Hanging drops were incubated at 28°C for 48–60 h. Mycelial mats were transferred to 1 ml of the medium containing excess choline (1  $\mu\text{g}$ ) and 75  $\mu\text{M}$  [ $^3\text{H}$ ]leucine (New England Nuclear, 60 mCi/mmol). After a further incubation for 3 h, radioactivity incorporated into protein was precipitated for counting by the addition of TCA (Schwartz et al., 1971). Protein synthesis, which depended on the amount of growth achieved during incubation in the hanging drop, was proportional to the choline concentration from 5 to 100 ng. Growth curves were similar in form to those obtained with the gravimetric analysis.

#### *Separation of Choline-Labeled Compounds*

Aqueous soluble substances were separated by high voltage electrophoresis at pH 4.7 (Giller and Schwartz, 1971 *a*), phosphorylcholine and betaine by electrophoresis at pH 7.8 in 0.05 M ammonium bicarbonate for 1.5 h at 15 V/cm. Mobilities at pH 7.8 relative to choline (1.0) were acetylcholine, 0.81, betaine, 0.14, and phosphorylcholine,  $-0.33$ . After choline and acetylcholine were removed either by electrophoresis at pH 4.7 or by chromatography on columns of Dowex 50 (200–400 mesh, acid form, Bio-Rad Laboratories, Richmond, Calif.) (Collier and Lang, 1969), phosphorylcholine and betaine were also separated by thin layer chromatography on silica gel plates (Analtech, Inc., Newark, Del.; Brinkmann Instruments Inc., Westbury, N. Y.) used without heat activation. In acetone:water:HCl (100:20:1) (Collier and Lang, 1969) the  $R_f$  of phosphorylcholine was 0.41; that of betaine was 0.63. With chloroform:methanol:10% ammonium hydroxide::60:35:8 (Müldner et al., 1962), phosphorylcholine did not move; the  $R_f$  of betaine was 0.34.

Lipid choline, extracted with chloroform-methanol, was chromatographed on silicic acid impregnated paper (Marinetti et al., 1957). Radioactivity soluble in chloroform-methanol was treated with N KOH at 37°C for 18 h (Hack, 1947) or with saturated barium hydroxide at 100°C for 3.5 h, which would release choline from any sphingomyelin (Entenman et al., 1944). After neutralization, choline was precipitated with ammonium reineckate. In order to characterize the lipids formed as phosphatidylcholine, some samples of the chloroform-methanol extract were subjected to milder alkaline hydrolysis in methanolic KOH (Ambron and Pieringer, 1971). Aqueous deacylation products were separated by electrophoresis at pH 4.7.

Electropherograms and chromatograms were stained with iodine vapor and radioautographed with clinical X-ray film. Radioactivity from tritium was intensified using Omnispray (New England Nuclear). Carbon-14 on paper was counted directly in a toluene-based scintillation fluid (Giller and Schwartz, 1971 *a*) (Omnifluor, New England Nuclear) with an efficiency of 90%; tritium on glass fiber pads with an efficiency of 20%. Tritium on paper was first eluted with water and counted with a

similar efficiency in Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.):toluene scintillator (1:2).

#### *Partial Purification of Choline Acetyltransferase*

Enzyme activity was assayed radiochemically (Giller and Schwartz, 1971 *a*). Nervous tissue (3 g), washed in 0.7 M sucrose containing 1 mM EDTA (pH 7.4), was ground to a powder in liquid nitrogen. After it thawed, the homogenate was extracted with 20 ml of buffer (0.02 M sodium phosphate containing 1 mM EDTA and 5 mM 2-mercaptoethanol, pH 7.4), and the extract clarified by centrifugation at 105,000 *g* for 2 h. Protein was precipitated by the addition of solid ammonium sulfate to yield a 90% saturated solution at 0°C. The precipitate was sequentially extracted with ammonium sulfate solutions, first 45 and then 20% saturated. After the 45–20% ammonium sulfate extract (which contained the transferase activity) was dialyzed against the buffer, it was adjusted to pH 5 by addition of N acetic acid at 0°C. The precipitated protein was discarded, and the enzyme solution adjusted to pH 7.6 by addition of Tris-HCl buffer. This procedure resulted in a four-fold purification with a 50% recovery of enzyme activity. Mean values are presented  $\pm$  SE of (*n*) independent determinations.

#### RESULTS

*Aplysia* nervous tissue contains both choline and acetylcholine. In animals weighing 80–160 g, we found  $1.4 \pm 0.06$  (3) nmol of acetylcholine in individual abdominal ganglia. Circumesophageal ganglia from 150- to 220-g animals contained an average of  $2.1 \pm 0.05$  (7) nmol of choline and  $1.9 \pm 0.08$  (7) nmol of acetylcholine. Using an enzymatic assay, McCaman et al. (1973) found 11 pmol of choline and 25.7 pmol of acetylcholine in the cell body of R2; other neurons contained comparable amounts of choline, but acetylcholine was found only in the cell bodies of the cholinergic neurons.

#### *Synthesis of Choline in Nervous Tissue*

Vertebrate nervous tissue is unable to synthesize choline. We tested the synthetic capabilities of *Aplysia* nervous tissue by incubating isolated abdominal and pleuropedal ganglia in the presence of  $5.3 \mu\text{M}$  [ $^3\text{H}$ ]methylmethionine or  $5.6 \mu\text{M}$  [ $^3\text{H}$ ]serine, precursors of choline. After they had been incubated for 18 h, we completely hydrolyzed the ganglia, first in base and then in acid, in order to determine how much choline was synthesized in all forms. We found that 1% of the radioactivity from either labeled amino acid had been converted to choline-containing substances (Table I). Little of this choline was derived from acetylcholine or free choline, however. The sequential hydrolysis revealed that more than 90% of the choline formed was in phosphorylcholine. No differences were observed between abdominal and pleuropedal ganglia.

In other experiments we incubated the ganglia in the presence of five

TABLE I  
 EXTENT OF [<sup>3</sup>H]CHOLINE SYNTHESIS IN PAIRS OF ABDOMINAL AND  
 PLEUROPEDAL GANGLIA\* AFTER 18 HOURS IN THE PRESENCE OF  
 5.3 μM[<sup>3</sup>H]METHYL-METHIONINE OR 5.6 μM[<sup>3</sup>H]SERINE

Precursor	Total radioactivity in tissue	[ <sup>3</sup> H]choline after complete hydrolysis
	<i>nmol/ganglion</i>	<i>% total radioactivity</i>
[ <sup>3</sup> H]methyl-methionine	0.99±0.05	1.08±0.16
[ <sup>3</sup> H]serine	0.71±0.03	1.15±0.20

\* Ganglia from animals weighing 75–160 g.

times higher concentrations of the labeled amino acid precursors, in order to characterize the distribution of the radioactivity in greater detail (Table II). Only small amounts of these amino acids were converted into choline and acetylcholine. Because of the more extensive chemical characterization possible in these experiments, we were able to observe a small amount of transfer of the methyl group of methionine and some incorporation of the ethanolamine moiety of serine into lipid<sup>1</sup> (Table II). The intracellular concentrations of both precursors were apparently sufficient for protein synthesis. The amounts of both methionine and serine incorporated into protein were substantial, and consistent with those found previously with other amino acids (Schwartz et al., 1971; Eisenstadt et al., 1973).

Because of the heterogeneity of nervous tissue, we studied choline synthesis in the single cholinergic neuron R2. Radioactive precursors were introduced directly into the cell body by pressure injection<sup>2</sup> (Table II). Although a greater proportion of the radioactivity was converted to acetylcholine, free choline and lipid,<sup>1</sup> incorporation into protein with both amino acids was also greater. Even though the injected precursors were more efficiently utilized, the amount of choline formed was still very small.

#### *Source of Choline*

As with vertebrates, choline for transmitter synthesis is probably derived from the blood in *Aplysia*. At the choline concentration present in hemolymph,

<sup>1</sup> The phospholipid labeled during incubation or after intrasomatic injection of radioactive precursors was phosphatidylcholine. Originally soluble in chloroform-methanol, it released choline on alkaline hydrolysis. When chromatographed on silicic acid-impregnated paper, it migrated with an *R<sub>f</sub>* of 0.43, and was labeled with <sup>32</sup>P when inorganic phosphate was included during incubations. These results support Komai (1973) who found only trace amounts of sphingomyelin in ganglia of *Aplysia kurodai*. We also found that phosphatidylcholine was the only choline-containing phospholipid labeled in nonnervous tissue.

<sup>2</sup> With a number of injected radioactive precursors we have found that the rates of conversion to products are proportional to the amounts injected up to a saturating amount (Koike et al., 1972; Eisenstadt et al., 1973; Ambron et al., 1974). These saturating amounts vary with the substance injected, and presumably indicate saturation of some intracellular process. The dependence of conversion of injected choline to acetylcholine has been analyzed in detail in the accompanying paper (Eisenstadt and Schwartz, 1975).

TABLE II  
DISTRIBUTION OF RADIOACTIVITY AFTER INCUBATION OR INTRASOMATIC  
INJECTION OF LABELED PRECURSORS OF CHOLINE

Precursor	Time	n	Total radioactivity	Distribution of radioactivity		
				Choline + acetylcholine	Choline released from lipid by KOH hydrolysis	Incorporation into protein
	h		nmol/ganglion	% of total radioactivity		
(A) Uptake* at 25 $\mu$ M						
[ <sup>3</sup> H]methyl-methionine	18	4	5.8±0.9	0.018±0.003	0.10±0.02	12.0±1.1
[ <sup>3</sup> H]serine	24	1	6.2	0.10	0.008	7.3
			pmol/cell			
(B) Injections of R2†						
[ <sup>3</sup> H]methyl-methionine	4	1	8.6	0.5	2.3	56.1
	24	3	2.3-4.6	0.4±0.2	2.4±0.6	63.7±4.9
	24	3	10.1-17.3	1.2±0.5	3.5±0.1	41.8±0.4
[ <sup>3</sup> H]serine	6	1	49.5	0.08	0.28	28.3
	24	1	7.3	0.7	0.07	45.0
	24	3	45.6-81.5	0.15±0.09	0.07±0.02	39.7±9.0

\* Pairs of abdominal and pleuropedal ganglia from *Aplysia* weighing 50-110 g.

† In abdominal ganglia from animals weighing 80-150 g.

we found that the rate of entry of choline<sup>3</sup> into both ganglion and cell body was greater by at least two orders of magnitude than the rate of synthesis of the choline and acetylcholine from radioactive methionine or serine. By bioassay, we found that choline was present in hemolymph at a mean concentration of  $9.1 \pm 1.4 \mu\text{M}$  (11) with a range from 2 to 16  $\mu\text{M}$  in *Aplysia* weighing between 60 and 180 g.<sup>4</sup> This value is similar to that reported for human serum (Bligh, 1952). No correlation was seen between animal weight and choline concentration. It is unlikely that choline in the blood derives directly from food, since we found no differences between the concentration of choline in hemolymph from animals fasted for 1 wk and animals fed daily with seaweed.

#### *Uptake of Choline Into Nervous Tissue*

When isolated ganglia were incubated in Instant Ocean containing labeled choline, radioactive acetylcholine was readily formed. Since we presume

<sup>3</sup> In three experiments we added trace amounts of [<sup>3</sup>H]choline to whole hemolymph, and studied cumulative uptake into pairs of ganglia. The actual choline content in the blood samples was determined by bioassay. We found that the rate of uptake was half that obtained with the same concentrations of choline in Instant Ocean. This difference has not been investigated further but may result from some binding of choline to hemolymph protein. We have no indication for binding, however; the method of processing the samples for bioassay should release any bound choline from protein.

<sup>4</sup> A much higher mean value of 200  $\mu\text{M}$  was found using the procedure of Appleton et al. (1953), even after the recommended extraction with butanol. Presumably, there are substances other than choline in *Aplysia* blood which are precipitated with iodide.



that all of the choline acetyltransferase in ganglia is neuronal, this indicated that exogenous choline is taken up into neurons. We found that choline was taken up at a constant rate over a wide range of external concentrations from 1 to 400  $\mu\text{M}$  for periods at least as long as 7 h.

The rate of uptake depended on external concentration (Fig. 1). The constant of proportionality seemed to change at concentrations above 30  $\mu\text{M}$ , suggesting that there might be two uptake processes. Measurements of biochemical parameters in ganglia of *Aplysia* are quite variable, however, (see for example, Schwartz et al., 1971), and normalizations by weight or protein content are not satisfactory since ganglia contain large and variable amounts of nonnervous components. In order to obtain data suitable for kinetic

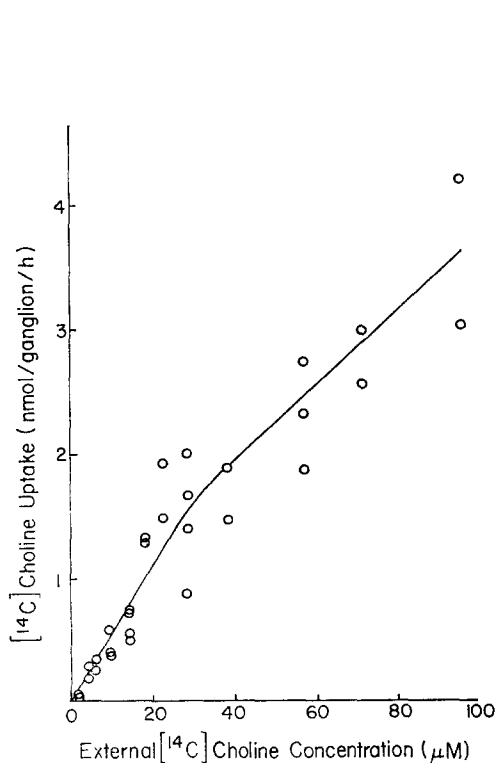


FIGURE 1

FIGURE 1. Dependence of choline accumulation on external choline concentration. Circumesophageal ganglia from *Aplysia* weighing 50–115 g, covered with a volume of 100  $\mu\text{l}$  containing [ $^3\text{H}$ ]choline or [ $^{14}\text{C}$ ]choline, were incubated for a total of 45 min at 15°C. Each point represents uptake into three ganglia.

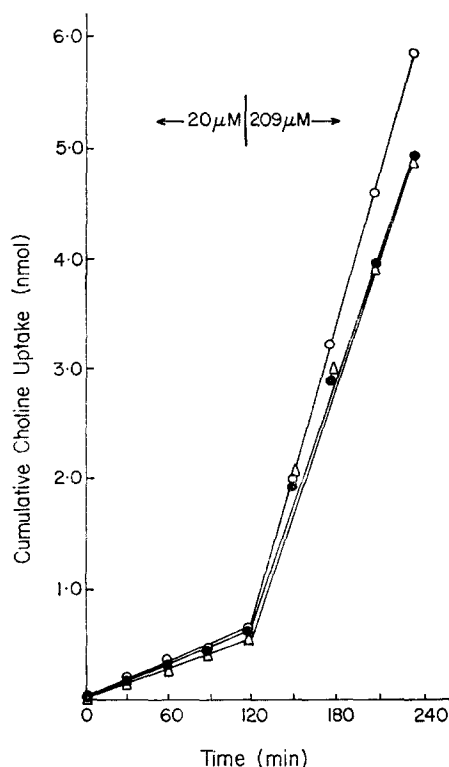


FIGURE 2

FIGURE 2. Cumulative uptake of choline. Three pairs of circumesophageal or abdominal ganglia from *Aplysia* weighing 140–180 g were covered with a 50- $\mu\text{l}$  droplet containing 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]choline, and sampled for cumulative uptake as described in Methods and Materials. After 120 min at 20  $\mu\text{M}$  the concentration of [ $^{14}\text{C}$ ]choline was increased to 209  $\mu\text{M}$ , and the experiment was continued for another 2 h.

analysis, we needed an experimental design in which a single specimen of nervous tissue would provide its own normalization. A comparison of cumulative rates of uptake by the same ganglia at two concentrations of external choline was found to yield data with little variation. Even though we have found that rates of choline uptake into abdominal and circumesophageal ganglia from the same animal were approximately equivalent, this experimental design normalizes differences among ganglia from different animals. An example of an experiment using this design is shown in Fig. 2. Coefficients of variation for the ratios of uptake at two concentrations were 11% (less than half that found for the means of unnormalized rates).

A double-reciprocal plot of the results of experiments using external choline concentrations from 17 to 420  $\mu\text{M}$  showed the two kinetic components clearly (Fig. 3). The data points can be readily fit by two straight lines indicating that at low substrate concentrations choline is taken up by a process with high affinity. At higher substrate concentrations, choline is taken up by a process which did not appear to be saturated by the highest concentration used (420  $\mu\text{M}$ ).

Once the ratios of the rates at various substrate concentrations ( $V_s$ ) to

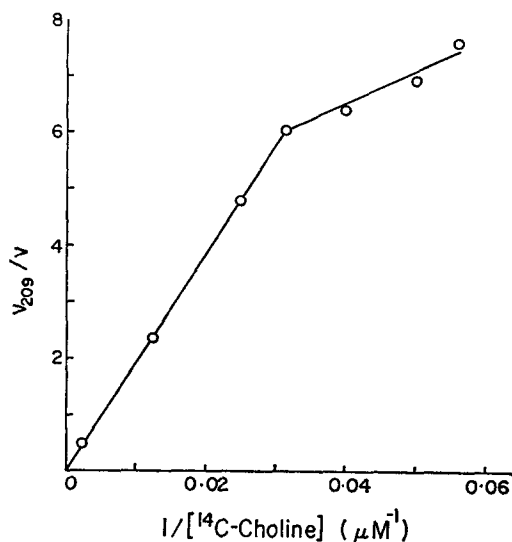


FIGURE 3. Double-reciprocal plot showing the dependence of the normalized rates of choline uptake on external concentration of choline. Groups of three abdominal and circumesophageal ganglia from *Aplysia* weighing 75–220 g were pinned together. Ratios were obtained by determining cumulative uptake at two choline concentrations, one of which was 209  $\mu\text{M}$  (see Fig. 2). In each experiment the second concentration was higher. The rates of uptake were determined graphically and the ratio of uptake velocities was determined for each concentration. Each point represents the mean of the ratios from three experiments.

the standard velocity at 209  $\mu\text{M}$  ( $V_{209}$ )<sup>5</sup> were obtained, we applied a modification of the Lineweaver-Burke equation:

$$\frac{v_{209}}{v_s} = \frac{\frac{209V_{\max}}{209 + K_m}}{\frac{V_{\max}S}{S + K_m}}, \quad (1)$$

to each of the components individually, and calculated the Michaelis constant of the high affinity process to be 16  $\mu\text{M}$  and that of the low affinity process to be 2 mM. Using Neal's graphic method (1972), in which the constants are obtained from the limiting slopes of the two distinct kinetic components on the double-reciprocal plot, we found the Michaelis constant of the high affinity process to be 11.2  $\mu\text{M}$ . These methods are essentially similar; both obtain the kinetic constants by extrapolation, and both minimize data in the region where the two components intersect.

A more complete description of the system, which presupposes two independent, saturable uptake processes, however, is given by:

$$\frac{v_s}{v_{209}} = \frac{\frac{V_1S}{K_1 + S} + \frac{V_2S}{K_2 + S}}{\frac{209V_1}{K_1 + 209} + \frac{209V_2}{K_2 + 209}}, \quad (2)$$

where  $K_1$  and  $K_2$  are the two Michaelis constants and  $V_1$  and  $V_2$  the two maximal velocities. We used a computer to find the values for the kinetic constants which fit our data best over the entire range of substrate concentration used. Best fit to the data was obtained when  $K_1$  is 2–8  $\mu\text{M}$ ,  $V_1$ , 0.09–0.11 nmol/ganglion/h and  $K_2$  is 2–10 mM, and  $V_2$ , 30–110 nmol/ganglion/h. Within the range of values given for these constants, calculated  $v_s/v_{209}$  ratios did not deviate from the observed values by more than 10% at any concentration of substrate. Similar values with a somewhat narrower range could have been obtained for the kinetic constants of the high affinity process if we had assumed that low affinity uptake occurred by diffusion. Since our experiments do not permit decision about the mechanism of the low affinity process, we chose the kinetic model of two independent saturable uptake components.

#### *Efflux of Choline*

The technique of cumulative uptake would measure choline entry accurately if efflux of choline from tissue were negligible. In order to estimate the con-

<sup>5</sup> The velocity obtained at any substrate concentration might have been used as a standard; nevertheless, because one concentration was chosen the numerator of Eq. 1 is a constant. As a result, when  $v_{209}/v_s$  was plotted as a function of  $1/s$  the Michaelis constant should be equal to the slope divided by the intercept on the ordinate.

tribution of internal choline, we incubated paired abdominal and circum-esophageal ganglia from *Aplysia* weighing 80–120 g in labeled choline for 1 h and then determined escape of radioactivity from the tissue for 4 h. The time-course of efflux from ganglia previously incubated at either 20 or 200  $\mu\text{M}$  choline was similar: rates of efflux were dependent on the amounts of choline in the tissue and were biphasic, with two exponential components. At both extracellular choline concentrations, the more rapid efflux occurred with a half-time of about 11 min, and the slower efflux with a half-time of from 7 to 9 h.

The choline rapidly lost from the ganglion was most probably extracellular. By extrapolation from the semilogarithmic efflux curves (Hertz, 1968), we estimated that at both 20 and 200  $\mu\text{M}$  about 20% of the total labeled choline in the ganglion was contained in the compartment we presumed to be extracellular. With [ $^3\text{H}$ ]inulin, the volume of the extracellular space in pleuropedal ganglia from 150-g animals was independently estimated to be about 8  $\mu\text{l}$  (E. Giller, unpublished experiments). The two measurements are in approximate agreement since an 8- $\mu\text{l}$  volume of the original bathing solutions would contain amounts of radioactivity equivalent to 20% of the [ $^{14}\text{C}$ ]choline associated with ganglion. This agreement is an indication that the inulin space corresponds to the presumed extracellular efflux compartment.

Slow escape of [ $^{14}\text{C}$ ]choline is probably efflux from an intracellular space. This slow component could be demonstrated in the absence of the rapid component when we used another experimental approach. Ganglia were incubated in the presence of 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]choline for 1 h. A large amount of unlabeled choline was then added to give a final choline concentration of 50 mM while maintaining the amount of radioactivity unchanged. Net efflux of *radioactivity* was observed with a half-time of 7 h, in agreement with the values for the rate of slow efflux described above.

Whereas the experimental procedure used for determining cumulative uptake of choline is not affected by choline in the extracellular space, efflux of intracellular choline would cause the uptake actually measured to be less than the amount truly taken up. We have shown that this efflux was relatively slow; its contribution to the rates measured was negligible since the kinetic determinations were made over short intervals, and we always progressed from a lower to a higher external concentration of choline.

#### *Concentration Dependence of Conversion of Choline to Acetylcholine and Other Compounds*

There are several indications that the uptake of choline into the nervous system at low external concentrations plays a determining role in synthesis of the transmitter. We observed that the affinity constant of the process was precisely in the range of choline concentrations normally found in the hemo-

lymph. We also found that the fate of the choline which enters the tissue at low external concentrations differs from that at high concentrations. Acetylcholine was synthesized considerably less efficiently from the excess choline which accumulated in nervous tissue at external concentrations greater than about  $30 \mu\text{M}$  (Fig. 4). At low concentrations, the fraction of the choline converted to acetylcholine approached 60–75%; this fraction decreased as the external concentrations were increased. Between 40 and  $209 \mu\text{M}$ , the proportion of acetylcholine remained at a constant low value of about 15%. Thus the excess choline which enters the tissue by the low affinity uptake component is not readily available for synthesis of acetylcholine.

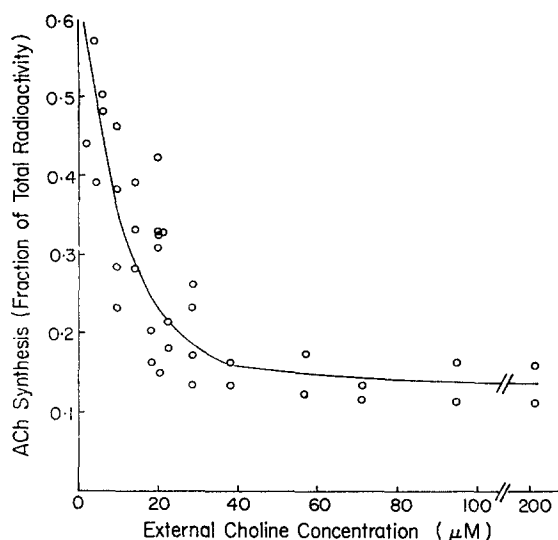


FIGURE 4. Dependence of acetylcholine synthesis on the external choline concentration. Acetylcholine was determined in extracts of ganglia labeled in the experiments described in Fig. 1 by electrophoresis at pH 4.7. Additional data were obtained for the rate at  $209 \mu\text{M}$ .

In nervous tissue incubated in the presence of labeled choline, radioactivity was also found in phosphorylcholine, betaine, and phosphatidylcholine. Conversion of choline to phosphorylcholine did not appear to depend on the concentration of external choline in the same manner as did formation of acetylcholine, since the proportion of the radioactivity in this fraction was about 10% of the total and showed little variation over a wide range of external choline concentrations from 4 to  $209 \mu\text{M}$ . The proportion of radioactivity in acetylcholine and phosphorylcholine did not change during periods of incubation up to 4 h. Because choline was taken up into nervous tissue at a constant rate, the amounts of these substances also increased at a constant

rate. The proportion of radioactivity in betaine and phosphatidylcholine increased with time: at 45 min both substances were less than 1% of the total; at 4 h betaine was 4.5% and lipid, 1%.

The proportion of the total radioactivity present in the form of unchanged choline showed a relationship reciprocal to that of acetylcholine seen in Fig. 4. At low external concentrations of choline, only about 5% of the radioactivity in nervous tissue remained unconverted. With increasing external concentrations, the fraction of unconverted choline approached 75%. Thus this excess choline appeared to be largely unavailable for synthesis. An alternative possibility is that this material, although it migrated during high voltage electrophoresis at pH 4.7 with choline and chromatographed with choline on columns of Dowex 50, was changed to an unidentified substance which cannot be converted to acetylcholine. In order to show that radioactivity which constituted most of the material in nervous tissue incubated at choline concentrations greater than 20  $\mu\text{M}$ , was unchanged choline, we isolated the radioactivity migrating with choline on electropherograms from extracts of tissue incubated 4 h in the presence of 30  $\mu\text{M}$  [ $^3\text{H}$ ]choline. This isolated material was added to reaction mixtures containing unlabeled choline, [ $^{14}\text{C}$ ]acetyl CoA and purified choline acetyltransferase. Radioactivity from the isolated choline and authentic choline served equally well as substrates of the transferase (Table III).

TABLE III  
ENZYMATIC ANALYSIS OF [ $^3\text{H}$ ]CHOLINE REISOLATED FROM *APLYSIA*  
NERVOUS TISSUE

Incubation time	Reisolated radioactivity	Choline standard
<i>min at 35°C</i>		<i>% converted</i>
(A) Conversion to [ $^3\text{H}$ ]acetylcholine		
22	4.3	4.3
45	7.3	7.2
		<i>Ratio of <math>^3\text{H}/^{14}\text{C}</math></i>
(B) Internal standardization of choline acetyltransferase		
22	0.21	0.27
45	0.21	0.26

Reisolated radioactivity or standard [ $^3\text{H}$ ]choline (23,000 cpm) were added in a reaction mixture of 50  $\mu\text{l}$  containing 1 mM unlabeled choline chloride, 0.45 mM [ $^{14}\text{C}$ ]acetyl CoA, 1 mM EDTA, 0.02 M sodium phosphate (pH 7.2), and  $10^5$  units (Giller and Schwartz, 1971 *a*) of choline acetyltransferase partially purified from *Aplysia* nervous tissue. Incubations were carried out in the absence of high salt in order to increase the enzyme's affinity for choline, which was present at a final concentration below the Michaelis constant. (A) The fraction of the total [ $^3\text{H}$ ]choline (standard or reisolated) converted to acetylcholine was determined by electrophoresis at pH 4.7 (B) The ratio of acetylcholine formed from [ $^3\text{H}$ ]choline (standard or reisolated) to the acetylcholine formed from [ $^{14}\text{C}$ ]acetyl CoA in the same reaction mixture was calculated to provide an internal standardization of the enzymatic reaction.

## DISCUSSION

*Uptake of Choline in Cholinergic Neurons*

Synthesis of acetylcholine in *Aplysia* ganglia appeared to be limited by a process with a Michaelis constant precisely in the range of the external choline concentrations found in the hemolymph; these concentrations are equivalent to the affinity constant we calculated for the high affinity component of choline uptake. We therefore presume that the high affinity process, which we have shown here in intact ganglia, is localized to cholinergic neurons. In vertebrates, high affinity uptake processes have been described for a variety of neurotransmitter substances (Iversen, 1967; Shaskan and Snyder, 1970; Bennet et al., 1973). High affinity uptake of choline into subcellular fractions of rat brain (Yamamura and Snyder, 1972; Haga and Noda, 1973) and of squid ganglia (Dowdall and Simon, 1973) is thought to be a characteristic property of cholinergic neurons because the choline taken up is primarily utilized for acetylcholine synthesis. Kuhar et al. (1973) presented additional evidence that the high affinity process is associated with cholinergic neurons. They found that synaptosomes prepared from regions of the brain containing cholinergic innervation possessed the greatest capacity for high affinity uptake; this capacity was diminished in synaptosomes prepared from these regions in brains of animals in which lesions had destroyed cholinergic innervation.

The physiological role of the low affinity process is unknown. Similar uptake processes for choline have been described previously in nervous (Hodgkin and Martin, 1965; Diamond and Kennedy, 1969; Potter, 1968) and other tissues (Martin, 1968; Sanford and Smyth, 1971; Sung and Johnstone, 1965). Although present in neural structures known not to be cholinergic (Hodgkin and Martin, 1965), the low affinity process is probably present in both cholinergic as well as noncholinergic neurons in *Aplysia* since a small and constant fraction (about 15%, see Fig. 4) of the choline taken up at high external choline concentrations was converted to acetylcholine. The constancy of conversion suggests that the distribution of choline between cholinergic neurons and other constituents of nervous tissue does not change in this range of concentrations; it also indicates that the synthetic enzyme is not saturated at the highest external choline concentrations used.

A substantial amount of choline was taken up into ganglia by the low affinity process. At 9  $\mu\text{M}$ , the average choline concentration present in the hemolymph, we can estimate (using Eq. 2) that 60% of the choline is taken up by the low affinity component. This estimate is uncertain, however, because of the wide range of kinetic constants obtained, and may be as high as 90% or as low as 25%. We favor the lower values, since much of the choline taken up was converted to acetylcholine. These estimates most

critically depend on the values of the constants chosen for the low affinity component. Using values at the extremes of the range, at 9  $\mu\text{M}$  external choline we calculated an 18-fold variation in the velocity term for the low affinity component, but only a 20% variation in the term for the high affinity process. If the choline which is taken up at high external choline concentrations enters by a nonsaturable process with an essentially infinite affinity constant, a narrower estimate would have been obtained. Diffusion alone, however, is not likely to account for the low affinity process (see Eisenstadt et al., 1975).

Although our experiments do not permit decision on the mechanism of the low affinity process, it is clear that its maximal velocity or total capacity is much greater than that of the high affinity process. This again suggests an extensive distribution with a more general function, perhaps of supplying choline to some metabolic pathway present in all neurons. Thus, although most of the choline remained unchanged, some was converted to phosphorylcholine, betaine, and lipid. Alternatively, the low affinity process might serve to take up with greater avidity some ion (inorganic or organic), of which choline would be a poor structural analogue. For example, choline has molecular dimensions similar to the sodium ion, and is widely used to replace  $\text{Na}^+$  since it does not penetrate readily. The amount of choline which does penetrate, although quite small relative to  $\text{Na}^+$  (Hille, 1971), might nevertheless be transported by a  $\text{Na}^+$  channel.

#### *Source of Choline*

In higher organisms, choline is synthesized by stepwise methylation of phosphatidylethanolamine to form phosphatidylcholine (Bremner and Greenberg, 1960; Wilson et al., 1960). In vertebrates, this reaction is present in liver but negligible in nervous tissue (Marshall et al., 1965; Ansell and Spanner, 1967, 1968). We also found that little choline was formed from methionine or serine in nervous tissue. Because we have not determined the specific radioactivities of precursors and products, we cannot compare our results directly to those obtained in vertebrates. Poor uptake of the precursors into nervous tissue in *Aplysia* is not likely to explain their low rates of conversion to choline, however, since similar results were obtained after the precursors were injected intrasomatically.

It is possible that the synthesis of choline occurs by some other pathway and that serine and methionine are not suitable precursors in *Aplysia*; but this is not likely. We have shown that transmethylation does occur within neurons for the synthesis of phosphatidylcholine. The rate of this reaction appears to be insufficient to supply the choline needed for transmitter synthesis, however. Ansell and Spanner (1971) have suggested that choline in brain is derived from a lipid carrier (phosphatidylcholine or lysophosphatidylcholine) taken up from the blood. Once in the brain, this carrier could be



hydrolyzed to provide free choline (Illingworth and Portman, 1972). We have found no indication for this pathway in *Aplysia*. Since the rate at which choline is taken up into nervous tissue is much greater than that of its endogenous synthesis from methionine or serine, the choline for synthesis of acetylcholine is probably almost entirely derived from the blood.

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