Proc. Nat. Acad. Sci. USA
Vol. 69, No. 7, pp. 1987–1991, July 1972

Biosynthesis of Acetylcholine in Turtle Photoreceptors

(*Pseudemys scripta elegans*/cell dissociation/cell separation/neurotransmitters/sensory neurons)

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Contributed by David H. Hubel, March 29, 1972

ABSTRACT **For determination of possible neurotransmitters synthesized by photoreceptor cells, turtle retinas were dissociated into single cells with proteolytic enzymes. These cells were partially separated by velocity sedimentation to yield a fraction rich in photoreceptors. Individual photoreceptor cells were then sucked into a micropipette and incubated with labeled precursors of known or suspected neurotransmitters. After incubation, the radioactive products were analyzed by high-voltage electrophoresis. Of all the chemicals tested, turtle photoreceptor cells synthesized only acetylcholine, suggesting that these cells may be cholinergic.**

Identities of neurotransmitters in the central nervous system of vertebrates are known for only a few cell types (1). Thus, although we now have a fair understanding of the neuronal connections and physiological behavior of various retinal cell types (2–7), the chemistry of synapses in the retina is still unknown. A direct way of obtaining this information is by examination of the ability of single, intact, and identified cells to synthesize known or suspected neurotransmitters. The vertebrate retina is a convenient part of the central nervous system for such an analysis, because most retinal cell types can be readily identified even when they are isolated.

The presence of several known or suspected neurotransmitters, such as acetylcholine, γ-aminobutyric acid, and dopamine, has been suggested or demonstrated in some vertebrate retinas (8–11). There is, however, no direct evidence that any of these compounds are synthesized or used in a particular cell type. In order to assign possible chemical transmitters to individual cell types, single cells were dissociated by proteolytic enzymes, separated by velocity sedimentation, and analyzed by incubation of selected cells with labeled precursors of known or suspected neurotransmitters. Photoreceptors were chosen for this initial analysis because they are first-order sensory neurons of the visual pathway. Turtle photoreceptors were used because they are unusually large and contain brightly colored oil droplets that permit easy identification. In addition, their structure permits analysis of transmitter synthesis by the cell bodies in the absence of the pedicles, which may contain postsynaptic fragments from horizontal and bipolar cells. Furthermore, the anatomy (12) and physiology (13) of turtle photoreceptors have been studied in detail.

MATERIALS AND METHODS

Cell Dissociation. All media and Ringer's solutions were supplemented with 1000 units/ml of penicillin G and 0.5 mg/ml of streptomycin sulphate (Microbiological Associates, Inc., Bethesda, Md.), and sterilized (18). For cell dissociation and separation, isotonic calcium-free Ringer's solution supplemented with ethyleneglycol bis(aminoethyl) tetraacetic acid (EGTA) was used (in g/liter: 7.3. NaCl–0.5 $NaHCO_3$–0.07 NaH_2PO_4–0.25 KCl–0.1 $MgCl_2$–2 glucose; 5 mM EGTA; pH 7.2).

Fresh-water turtles (*Pseudemys scripta elegans*, with shells about 20 cm long) were adapted to darkness for at least 4 hr, decapitated, and pithed. The eyes were enucleated in very dim light. Each eye cup was cut radially into two equal pieces through the posterior pole and was incubated for 1 hr in the dark at 20° in 1 ml of calcium-free Ringer's solution containing 1 mg of purified papain* (13.5 U/mg, Worthington Biochemical Corp. Freehold, N.J.). The retinas were isolated from the eye cups, washed with Ringer's solution, incubated for another 45 min in calcium-free Ringer's solution supplemented with papain (1 mg/ml), washed with Ringer's solution, and put into a conical tube containing 1 ml of Ringer's solution supplemented with Ficoll (0.3%, Pharmacia, Uppsala, Sweden) and purified deoxyribonuclease (1 mg/ml, 1200 U/mg, Worthington Biochemical Corp.). The retinal cells were dissociated by gently stirring the tissue with a Pasteur pipette, and the cell suspension was allowed to settle for 5 min. The top portion of the cell suspension was then filtered through sterile gauze to remove cell clumps. This procedure yielded a cell suspension that was counted microscopically with a hemocytometer.

Separation of Cells by velocity sedimentation has been described in detail (14, 15); the method used here was similar to that of Lam *et al.* (15). The sedimentation chamber used was a modified 50-ml plastic syringe (Fig. 1). The Ringer's solution, cell suspension, and Ficoll gradient were loaded at a rate of 0.5 ml/min, and the cells were allowed to sediment at 20° for 2–3 hr. 1-ml Fractions were then collected at a rate of 1 ml/min, and the concentration and purity of cells in each fraction were checked with a hemocytometer. 2 ml of normal turtle Ringer's solution (containing 0.2 g/liter of $CaCl_2$ and no EGTA) were added to each ml of the fraction rich in photoreceptor cells, and the cell suspension (about 2×10^3 cells/ml) was kept at 4°.

* Several other proteolytic enzymes (trypsin, Pronase, crude or purified collagenase, and hyaluronidase, from Worthington Biochemical Corp.) were also tested for dissociating retinal cells, but papain seemed to produce least trauma. After this work had started, it was learned that similar enzymes were used by Drs. M. A. Ali and B. Drujan for dissociating cells from the amphibian and teleost retinas.

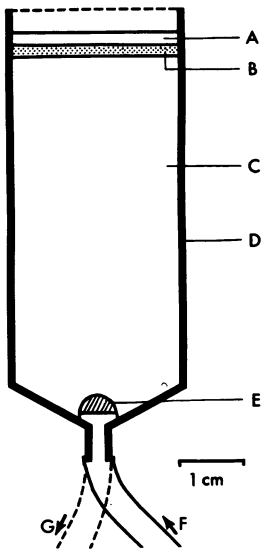


FIG. 1. Cross section of the cylindrical sedimentation chamber. (*A*) Layer of turtle Ringer's solution (1 ml); (*B*) layer of about 4×10^5 retinal cells in 0.3% Ficoll dissolved in calcium-free Ringer's solution (1 ml); (*C*) linear gradient of 0.8–3% Ficoll in Ringer's solution (30 ml); (*D*) plastic syringe; (*E*) flow deflector; (*F*) from linear gradient maker; (*G*) to fraction collector.

0.1 ml of the fraction rich in photoreceptor cells was spread on a glass slide and observed under 250× magnification. Individual photoreceptor cells were chosen and sucked into a micropipette (15-μm tip) attached to a micromanipulator (Figs. 2 and 3). In this way, different types of photoreceptor cells (such as rods or cones with red, yellow, colorless, or no oil droplets) could be selected. Suction was applied by connection of the micropipette to a 1-ml syringe fitted with a spring and micrometer.

Radioactive Precursors. The following labeled precursors were used: L-[*U*-^{14}C]glutamic acid (255 Ci/mol) and L-[*U*-^{14}C]tyrosine (54 Ci/mol) from Schwarz–Mann BioResearch, Orangeburg, N.Y.; L-[*methylene*-^{14}C]tryptophan (54.5 Ci/mol), [*methyl*-^{14}C]choline chloride (54 Ci/mol), and [*methyl*-^{3}H]choline chloride (6.7 Ci/mmol) from Amersham–Searle Corp., Arlington Heights, Ill.; L-[^{3}H]glutamic acid (generally labeled, 2.5 Ci/mmol) and L-[*3,5*-^{3}H]tyrosine (25 Ci/mmol) from New England Nuclear Corp., Boston, Mass. These compounds were purified twice by high-voltage electrophoresis (16–18) so that more than 99.999% of the radioactivity was associated with the region of the labeled precursor as determined by electrophoresis. These precursors were then added to L-15 media deficient in one or a combination of L-glutamate, L-tyrosine, L-tryptophan, and choline (all media were obtained from Grand Island Biological Co., Grand Island, N.Y. and were diluted to 270 mOsM), depending on the labeled precursors added (16, 18).

RESULTS

Neurotransmitter Synthesis in Turtle Retina. As a first step in the study of neurotransmitters synthesized by individual cells, whole turtle retinas were incubated in the appropriate ^{14}C-labeled precursors for 1 hr (16, 18). Paper electrophoresis of the retinal extracts revealed the synthesis of acetylcholine from choline, γ-aminobutyric acid from L-glutamate, and dopamine from L-tyrosine. No synthesis of noradrenalin or serotonin was detected. The identities of acetylcholine and γ-aminobutyric acid were verified by treatments with specific enzymes (16, 18); the identity of dopamine was confirmed by paper chromatography (Lam, D. M. K., in preparation).

Dissociation and Fractionation of Retinal cells. Proteolytic enzymes have been used for dissociating tissues into individual, viable cells (19–22). In the present study, single cells were obtained by incubation of turtle retina with papain in calcium-free Ringer's solution. Microscopic examination of dissociated retinal-cell suspensions showed a great variety of cell sizes and shapes, suggesting that the cells should be separable by velocity sedimentation (14, 15). Indeed, after the cells were allowed to sediment for 2–3 hr, subcellular particles were segregated from the intact cells and the different cell types were partially separated from each other. In particular, fractions containing 90–95% photoreceptor cells, which had a sedimentation velocity of 8.0 ± 1.6 mm/hr at 20°, were obtained.

The turtle retina contains rods and several types of cones (23). The photoreceptor cells are particularly easy to identify because the cones have red, yellow, or colorless oil droplets (Figs. 4–6); the rods can be recognized by their characteristic morphology (Fig. 7).

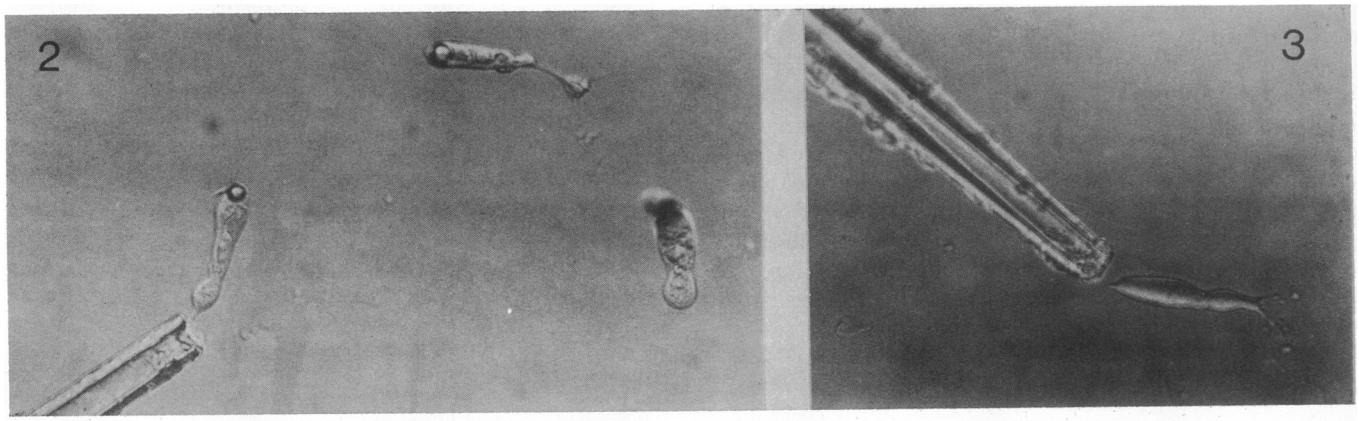


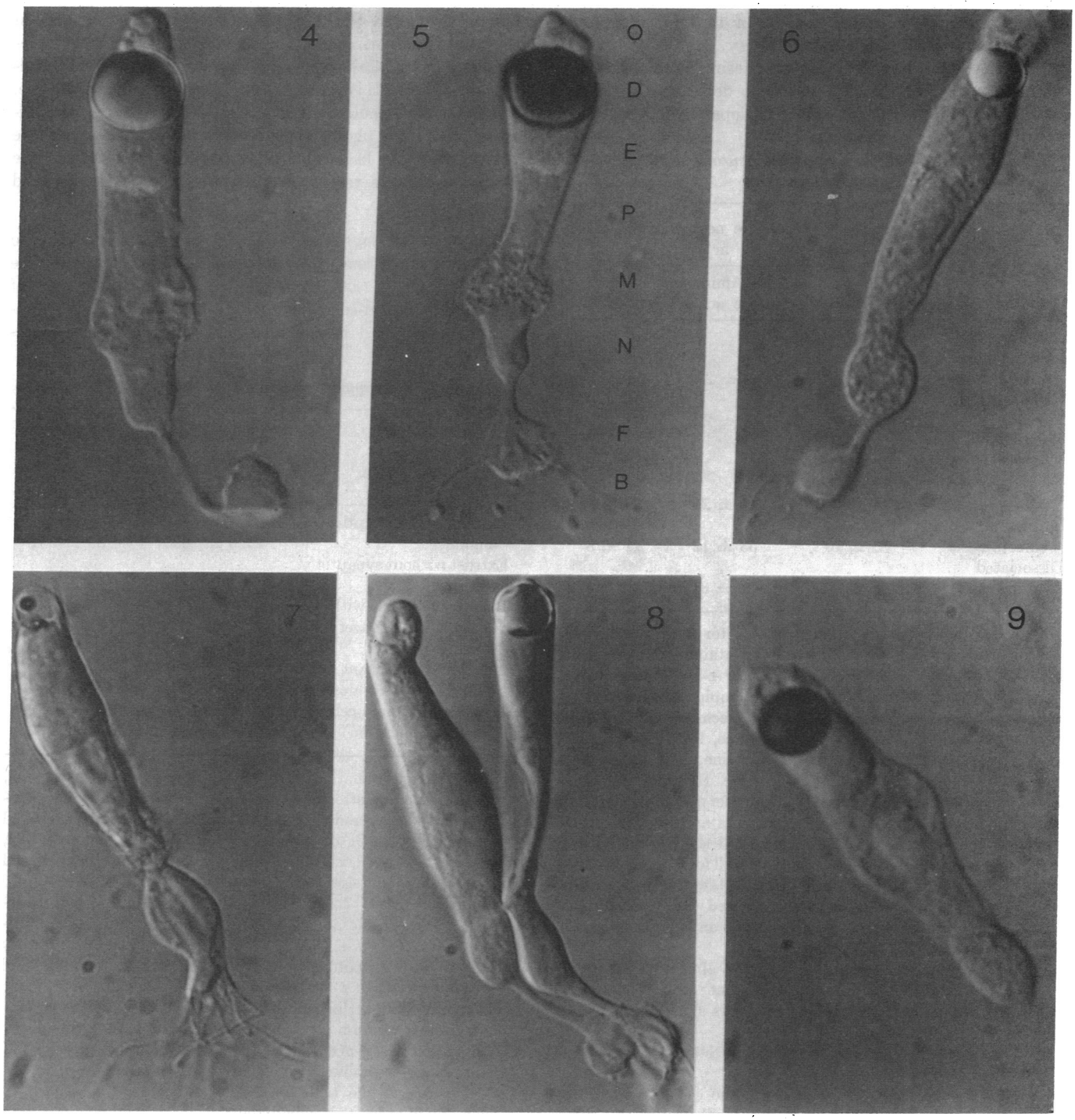
FIG. 2. A turtle cone being sucked into a micropipette, the cone pedicle is at the tip of the pipette. At the far right is a photoreceptor that has lost its pedicle. Normarski Optics. Magnification: × 300.

FIG. 3. A rod being sucked into a micropipette. Magnification: × 300.

Biosynthesis of Presumed Neurotransmitters. For assignment of a neurotransmitter to a cell type, contaminations from other cell fragments had to be kept to a minimum. The method for obtaining pure samples was to select cells under the microscope and to suck them one by one into a micropipette (Figs. 2 and 3). For each analysis, 20–100 cells in less than 0.05 μl of Ringer's solution (supernatant) were used.

Presumed neurotransmitters synthesized from labeled precursors by photoreceptors were analyzed by high-voltage paper electrophoresis (16–18). As shown in Table 1, turtle photoreceptors synthesized [^{3}H]acetylcholine from [^{3}H]-choline. In some experiments, the identity of [^{3}H]acetylcholine was verified by elution of the label from the acetylcholine region of the paper after electrophoresis, and incubation of the labeled compound with specific acetylcholine esterase (EC 3.1.1.7) (16). In addition, rods and each type of cone were incubated separately with labeled precursors, and were found to synthesize acetylcholine.

Since photoreceptors make synaptic contacts with bipolar and horizontal cells, isolated photoreceptors with their



FIGS. 4–9. Turtle photoreceptors viewed with Normarski interference optics. Cones with yellow (*4*), red (*5*), colorless (*6*) oil-droplets; rod (*7*); double cone that is partly detached (*8*); cone (with red oil droplet) that has lost its pedicle (*9*). *O*, outer segment; *D*, oil droplet; *E*, ellipsoid; *P*, paraboloid; *M*, myoid; *N*, nucleus; *F*, pedicle (foot-piece); *B*, basal process. Magnification: × 1100.

pedicles might contain postsynaptic fragments. It was important to exclude the possibility that these fragments, rather than the photoreceptors themselves, contributed to the biosynthesis of acetylcholine. Accordingly, single photoreceptors that had lost their pedicles (Figs. 2 and 9) during the dissociation procedure were collected into a micropipette and incubated with labeled precursors. As shown in Table 1, such cells were also capable of biosynthesis of acetylcholine, although they synthesized a smaller amount than the apparently intact photoreceptors. This finding suggested that either a large portion of choline acetyltransferase in the photoreceptors was present in the pedicle, or that acetylcholine synthesis was reduced because of cell injury.

Although intact turtle retinas synthesized acetylcholine, γ-aminobutyric acid, and dopamine, no detectable amounts of [^{3}H]γ-aminobutyric acid and [^{3}H]dopamine were synthesized by the photoreceptors (Table 1). Since the intracellular pool sizes of choline, L-glutamate, and L-tyrosine were not known, the detection of neurotransmitter synthesis from labeled precursors might differ in sensitivity for different transmitters. This difficulty was overcome in part by incubation of dissociated retinal cells that contained various cell types with one or a combination of 100 μCi/ml [^{3}H]-choline, 100 μCi/ml L-[^{3}H]glutamate, and 200 μCi/ml L-[^{3}H]tyrosine. The concentrations of labeled precursors were chosen so that dissociated cells synthesized significant and comparable amounts of [^{3}H]acetylcholine, [^{3}H]γ-aminobutyric acid, and [^{3}H]dopamine (Table 1). However, when the photoreceptors alone were incubated with labeled precursors, they contained at least 100 times more [^{3}H]acetylcholine than [^{3}H]γ-aminobutyric acid or [^{3}H]dopamine. Moreover, the amounts of [^{3}H]γ-aminobutyric acid or [^{3}H]-dopamine in the photoreceptors were not significantly more than those in the background (Table 1). Thus, of the possible neurotransmitters tested, turtle photoreceptors synthesized only acetylcholine.

TABLE 1. *Biosynthesis of possible neurotransmitters by turtle photoreceptors*

Incubations	Biosynthesis of possible neurotransmitters (dpm/6 hr)		
	[^{3}H]-Acetylcholine	[^{3}H]γ-Amino-butyric acid	[^{3}H]-Dopamine
Photoreceptors with synaptic endings*	1320 ± 686	88 ± 27†	55 ± 12†
Without synaptic endings*	544 ± 265	75 ± 22	52 ± 10
Acetylcholine esterase‡	90 ± 15§	—	—
0.1 μl of supernatant	130 ± 43	72 ± 24	50 ± 11
Labeled precursors alone	85 ± 10	53 ± 12	41 ± 8
Dissociated retinal cells¶	10,000 ± 2020	15,800 ± 3130	2790 ± 522

Cells and supernatants were incubated for 6 hr at 20° with 1 μl of precursor-deficient L-15 medium containing one or a combination of 1 μCi of [^{3}H]choline, 1 μCi of L-[^{3}H]glutamate, or 2 μCi of L-[^{3}H]tyrosine in the suction micropipettes. Dissociated retinal cells were incubated for 4 hr in precursor-deficient L-15 medium containing 100 μCi/ml [^{3}H]choline, 100 μCi/ml L-[^{3}H]-glutamate, and 200 μCi/ml L-[^{3}H]tyrosine. The radioactive products were extracted with 1 μl of 0.1 N HCl and were analyzed by paper electrophoresis (16, 18). After electrophoresis, the regions occupied by the labeled precursors and possible neurotransmitters were cut into 1-cm strips and the radioactivity in each strip was eluted with 4 ml of 0.01 N HCl for 6 hr. Each eluate was then mixed with 12 ml of Aquasol (New England Nuclear Corp.), and the radioactivity was measured with a scintillation counter. Each value in the table represents an average and standard deviation of at least six experiments.

* 100 Photoreceptors with less than 0.05 μl of supernatant.

† Values are not significantly different from background (0.1 μl of supernatant or labeled precursors alone) as calculated by Student's *t* test.

‡ Treatment with specific acetylcholine esterase after incubation with [^{3}H]choline.

§ After incubation with [^{3}H]choline, the cells were lysed by freezing and thawing. The extract was incubated in the micropipette for 30 min at 37° with 0.5 μl of specific acetylcholine esterase (10 mg/ml) and analyzed by electrophoresis.

¶ Before separation.

Enzymatic Assays. Acetylcholine biosynthesis was also studied by measurement of the activity of the enzyme choline acetyltransferase (EC 2.3.1.6) in the cell extracts by use of radioactive acetyl-coenzyme A. In 10 experiments, photoreceptors collected in the micropipettes were lysed by re-

TABLE 2. *Enzymatic activities from turtle photoreceptors*

Incubations	Biosynthesis (dpm/4 hr)	
	[^{3}H]Acetyl-choline	[^{3}H]γ-Amino-butyric acid
Extract* with synaptic endings	2080 ± 1030†	89 ± 23‡
Extract without synaptic endings	933 ± 436†	71 ± 20
Extract incubated with 1 μg of specific acetyl-choline esterase	82 ± 13§	—
0.1 μl of supernatant	188 ± 52†	65 ± 13
Labeled precursor alone	68 ± 12	57 ± 14
Dissociated retinal cells (before separation)	10,000 ± 3220†	3670 ± 1020

For the determination of activity of choline acetyltransferase, cells in a micropipette were lysed by repeated freezing and thawing several times and then incubated for 4 hr at 20° with 0.9 μl containing 0.1 μCi of [^{3}H]acetyl-coenzyme A (980 Ci/mol, New England Nuclear Corp.), 100 mM KCl, 0.2 mM neutralized ethylenediamine tetraacetic acid (EDTA), 5 mM choline, 10 mM KH_2PO_4 (pH 7.2), and 0.1 μl of either 5 mM physostigmine (†) or specific acetylcholine esterase (§, 10 mg/ml). Glutamate decarboxylase (EC 4.1.1.15) activity was measured by incubation of a photoreceptor lysate for 4 hr at 20° with 1 μl containing 1 μCi of L-[^{3}H]glutamate, 25 mM 2-mercaptoethanol, 0.25 mM pyridoxal phosphate, and 50 mM KH_2PO_4 (pH 7.2), as described by Lam (18). Both enzymatic assays were stopped by addition of 1 μl of 0.1 N HCl to each incubation medium, and the radioactive products were analyzed by paper electrophoresis. Each value in the table represents an average and standard deviation of at least six experiments.

* From 100 photoreceptor cells.

‡ Value not significantly different from background as calculated by Student's *t* test.

peated freezing and thawing, and the activities of either choline acetyltransferase or glutamate decarboxylase (EC 4.1.1.15) were measured. Since the intracellular concentrations of acetyl-coenzyme A and glutamate were not known, accurate calculations of enzyme activities in molar values could not be obtained. Nevertheless, as shown in Table 2, [^{3}H]acetylcholine was synthesized from [^{3}H]acetyl-coenzyme A by the photoreceptor extracts, whereas no synthesis of [^{3}H]γ-aminobutyric acid was detected. The identity of the [^{3}H]acetylcholine was again confirmed by treatment with specific acetylcholine esterase.

DISCUSSION

The present study represents an initial attempt to isolate individual cell types by treatment with enzymes and physical separation, and to establish the synthesis of neurotransmitters by these cells. In particular, these procedures were used here to demonstrate the synthesis of acetylcholine by turtle photoreceptors. Histochemical staining has already shown acetylcholine esterase in the outer plexiform layer of amphibian and teleost retinas (25, 26). In addition, electron microscopic studies have demonstrated the presence of specific acetylcholine esterase in the synaptic clefts between photoreceptors and horizontal, bipolar cells of the newt (27). Physiological studies by Val'Tsev (28) showed that atropine reduced the amplitude of the b-wave of the frog electroretinogram, perhaps by blocking the acetylcholine receptors. These histochemical and electroretinographic studies, although less direct than our studies, also suggest the existence of cholinergic synapses in the outer plexiform layer of some vertebrate retinas.

Unlike the well-established cholinergic nature of vertebrate motor neurons, little is known about the neurotransmitters used by sensory neurons. The existence and synthesis of acetylcholine in crustacean sensory neurons make it likely that these cells are cholinergic (29–31). Thus, although it remains to be shown that acetylcholine is released by photoreceptors, the synthesis of this compound and the failure to synthesize other known or suspected neurotransmitters are a first indication that it may be the transmitter substance used by turtle photoreceptors.

I thank Torsten Wiesel and Zach Hall for continual encouragement and guidance, and Lindy Ferris for preparing the manuscript. The author is a recipient of a Centennial Award from the Medical Research Council of Canada. This work is supported by NIH Grants 5T21 MH 11400-03 and 2 RO1 EYO 0606-7.

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