

# AUTORADIOGRAPHIC IDENTIFICATION OF ACETYLCHOLINE IN THE RABBIT RETINA

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## ABSTRACT

Rabbit retinas were studied *in vitro* under conditions known to maintain their physiological function. Retinas incubated in the presence of [<sup>3</sup>H]choline synthesized substantial amounts of both [<sup>3</sup>H]phosphorylcholine and [<sup>3</sup>H]acetylcholine. With time, [<sup>3</sup>H]phosphorylcholine proceeded into phospholipids, primarily phosphatidylcholine.

Retinas pulse-labeled by a 15-min exposure to 0.3  $\mu$ M [<sup>3</sup>H]choline were incubated for a subsequent hour under chase conditions designed either to retain newly synthesized acetylcholine within synapses or to promote its release. At the end of this time the two groups of retinas were found to contain equal amounts of radioactivity in the phospholipid pathway, but only the retinas incubated under the acetylcholine-protecting conditions contained [<sup>3</sup>H]acetylcholine. Freeze-dried, vacuum-embedded tissue from each retina was autoradiographed on dry emulsion. All retinas showed silver grains over the photoreceptor cells and faint labeling of all ganglion cells. In the retinas that contained [<sup>3</sup>H]acetylcholine, silver grains also accumulated densely over a few cells with the position of amacrine cells, over a subset of the cells of the ganglion cell layer, and in two bands over the inner plexiform layer.

Fixation of the retina with aqueous osmium tetroxide retained only the radioactive compounds located in the photoreceptor and ganglion cells. Sections from freeze-dried tissue lost their water-soluble choline metabolites when exposed to water, and autoradiography of such sections again revealed radioactivity primarily in the photoreceptor and ganglion cells. Radioactive compounds extracted from the sections were found to faithfully reflect those present in the tissue before processing; analysis of the compounds eluted from sections microdissected along the outer plexiform layer showed [<sup>3</sup>H]acetylcholine to have been synthesized only by cells of the inner retina.

Taken together, these results indicate that the photoreceptors and ganglion cells are distinguished by a rapid synthesis of choline-containing phospholipids, while acetylcholine synthesis is restricted to a few cells at both margins of the inner plexiform layer. They imply that the only neurons to release acetylcholine within the rabbit retina are a small group of probable amacrine cells.

KEY WORDS retina · acetylcholine · autoradiography

That most vertebrate retinas contain cholinergic synapses is indicated by many biochemical (13, 34, 37, 55, 70), enzymological (37, 68, 69), and histochemical (56, 57, 64, 65, 76) observations. In the rabbit such evidence has been further supported by the electrophysiological demonstration of acetylcholine's selective effect on neuronal activity, and by measurement of light-stimulated acetylcholine release (50, 51).

The experiments that demonstrate the presence of cholinergic synapses in the rabbit retina also provide an indication of their site: (a) choline acetyltransferase and acetylcholinesterase are concentrated in the inner plexiform layer (57, 64, 69). (b) Retinas increase their rate of acetylcholine release in response to illumination, a stimulus that hyperpolarizes the cells synapsing in the outer plexiform layer but that depolarizes many of the cells synapsing in the inner plexiform layer (50). Because the signal for neurotransmitter release is depolarization, this suggests the inner plexiform layer as a site of acetylcholine release. (c) Low concentrations of acetylcholine applied to the isolated, superfused retina excite many of the ganglion cells. This is true even in retinas in which synaptic transmission has been blocked by a combination of high  $Mg^{++}$  and low  $Ca^{++}$ , indicating that the ganglion cells themselves possess acetylcholine receptors (51).

These findings strongly suggest that most of the rabbit retina's cholinergic synapses are in the inner plexiform layer, but they do not directly identify the cells involved. The question seemed particularly interesting because acetylcholine affects ganglion cells of differing functional classes selectively. Most of the acetylcholine-sensitive ganglion cells have on-center or directionally selective receptive fields. Others, usually those with off-center fields, are unaffected by high concentrations of acetylcholine, by anticholinesterase, or by acetylcholine's competitive antagonists (51). This implies that acetylcholine plays a quite selective role in the retina's physiology—one presumably mediated by a functionally defined subset of the amacrine or bipolar cells.

The goal of the experiments reported here was to identify, morphologically, the cells that secrete acetylcholine. Various indirect methods have previously been used as indicators of cholinergic neu-

rons, including histochemical localization of acetylcholinesterase (57, 65); introduction of substances shown in other tissues to bind acetylcholine receptors (63, 76, 82); and autoradiographic identification of cells with high-affinity choline uptake (13), which is correlated in brain subareas with the presence of acetylcholine (42). Of these methods, the latter seemed the most promising. For our purposes, however, it appeared to have an important limitation; it depends on most of the choline taken up being destined for acetylcholine. Even at low concentrations of extracellular choline, some tissues—including the rabbit retina—incorporate choline more rapidly into phospholipids and their precursors than into acetylcholine (10, 50, 78, 81).

We therefore sought to identify [ $^3H$ ]acetylcholine directly in autoradiographs of retinas exposed to [ $^3H$ ]choline. This required experiments to distinguish silver grains caused by [ $^3H$ ]acetylcholine from those caused by [ $^3H$ ]compounds of the phospholipid pathway. Retinas were incubated *in vitro* under conditions known to maintain their physiological function. After a brief pulse-labeling, they were incubated under chase conditions designed either to retain acetylcholine within synapses (high  $Mg^{++}$ ) or to promote its release (flashing light). This allowed a subtractive identification of acetylcholine, in which retinas that contained radioactivity only in the phospholipid pathway were compared autoradiographically with retinas that contained the same amount of radioactivity in the phospholipid pathway but also contained [ $^3H$ ]acetylcholine.

Because acetylcholine is very water-soluble, and is not immobilized by known fixatives, the comparison described above was made on tissue that was quickly frozen, freeze-dried, and autoradiographed on dry emulsion (13, 53). The choline-containing phospholipids, in contrast to acetylcholine, are water-insoluble and are immobilized during treatment with osmium tetroxide; this allowed an independent verification of the sites of [ $^3H$ ]phospholipid synthesis, by autoradiography of retinal tissue in which phospholipids were selectively retained. We found in addition that the water-soluble choline metabolites could be recovered for analysis from sections of freeze-dried tissue. Microdissection of thick sections then permitted direct chemical confirmation of the radiochemical contents of cells located within some of the retina's layers.

## MATERIALS AND METHODS

New Zealand White rabbits were dark-adapted for at least 0.5 h, sedated with sodium pentobarbital (15–20 mg/kg), and brought to surgical anesthesia with ether. The eye was removed under dim red light. It was hemisected, the vitreous body was lifted away, and the posterior eyecup was immersed in oxygenated incubation medium. The eyecup was everted over a Teflon rod, and the retina was teased free from the pigment epithelium. The retina was cut free and transferred to a 20-ml "boat" (31). The control incubation medium contained (mM): Na<sup>+</sup>, 143.0; K<sup>+</sup>, 3.6; Ca<sup>++</sup>, 1.15; Mg<sup>++</sup>, 1.2; Cl<sup>-</sup>, 125.4; HCO<sub>3</sub><sup>-</sup>, 22.6; H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 0.1; HPO<sub>4</sub><sup>-</sup>, 0.4; SO<sub>4</sub><sup>-</sup>, 1.2; glucose, 10. It was equilibrated with 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The boats were incubated at 37°C in a flowing water bath, and were gently rocked. These techniques, which were developed by Ames and his colleagues (2, 3, 51), have been described in detail in previous publications. Extensive precautions to prevent contamination of the medium with possibly toxic substances were taken. Previous work has shown that if these precautions are taken, retinas maintain protein synthesis (4, 5), morphological integrity (77), photoreceptor sensitivity (59), and electrophysiological function as reflected by ganglion cell receptive fields (51) for at least 8 h.

### Incubations

Every retina was exposed to [<sup>3</sup>H]choline for the same time and under the same conditions. After exposure to [<sup>3</sup>H]choline, retinas were incubated in one of two ways, to favor acetylcholine retention or acetylcholine release (Fig. 1).

Labeling was done by incubation of the retina for 15 min in the presence of 0.3 μM [*methyl*-<sup>3</sup>H]choline chloride (69.5 Ci/mmol, New England Nuclear, Boston, Mass.) and 30 μM physostigmine salicylate (Sigma Chemical Co., St. Louis, Mo.). (It was preceded by 10-min preincubation in control medium, in order for the tissue to stabilize after isolation from the eye.) During labeling the retina was stimulated with light, using parameters previously (50) found effective in promoting

acetylcholine turnover in this preparation (xenon strobe flashes at 3 Hz, peak intensity ~334 lumen/m<sup>2</sup>). Physostigmine was used because it promotes labeling of the neuronal acetylcholine pool (15, 61).

After labeling, retinas were transferred into one of two incubation media. The first was designed to retain acetylcholine within synapses. It contained 20 mM Mg<sup>++</sup> and 0.2 mM Ca<sup>++</sup> (to prevent acetylcholine release) and 30 μM physostigmine (to prevent the hydrolysis of acetylcholine by intracellular esterases [61]). It also contained 1 mM unlabeled choline, to prevent the continued synthesis of [<sup>3</sup>H]choline-containing compounds and promote clearing of [<sup>3</sup>H]choline from the cells.

A second group of retinas was incubated, after the initial labeling, under conditions designed to clear the retina of [<sup>3</sup>H]acetylcholine. The medium contained no anticholinesterase and contained 1 mM unlabeled choline. During this period the retinas were again stimulated by flashing light.

Both postlabeling chases lasted 1 h. At the end of this time, each retina was rinsed for 4 min in fresh medium identical to that of the chase. Previous work has demonstrated that >99% of the labeling choline is cleared from the interstitial space by this procedure (3). Roughly half of the retina was analyzed chemically, a small strip was fixed with osmium tetroxide, and the remainder was frozen for autoradiography (Fig. 2).

### Chemical Analysis

The half-retina was touched three times to glass and was dropped into a tared Kontes glass homogenizer (Kontes Co., Vineland, N. J.) containing 0.5 ml of electrophoresis buffer (0.47 M formic acid, 1.4 M acetic acid, pH 1.9) to which physostigmine (100 μM) had been added. The tube was quickly weighed and the retina was homogenized. A 100-μl aliquot of the homogenate was counted in Aquasol (New England Nuclear). Counting efficiency was established by internal toluene standards. (Homogenates solubilized by strong base ["NCS," Amersham Corp., Arlington Heights, Ill.] before counting gave results identical to those obtained by counting the crude

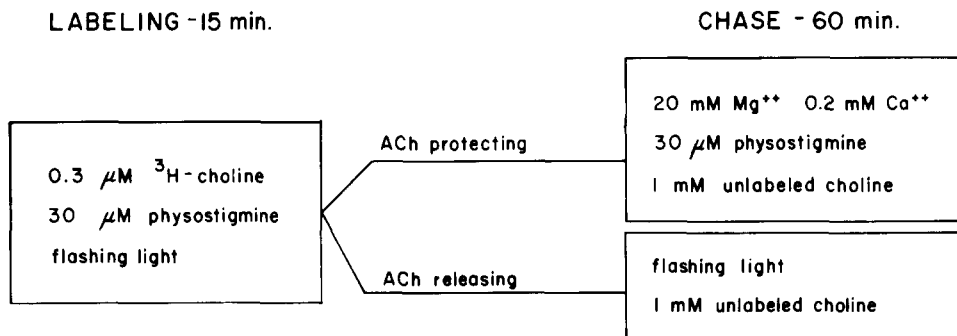


FIGURE 1 Conditions under which retinas were incubated. Retinas were harvested immediately after the chase. For details see text.

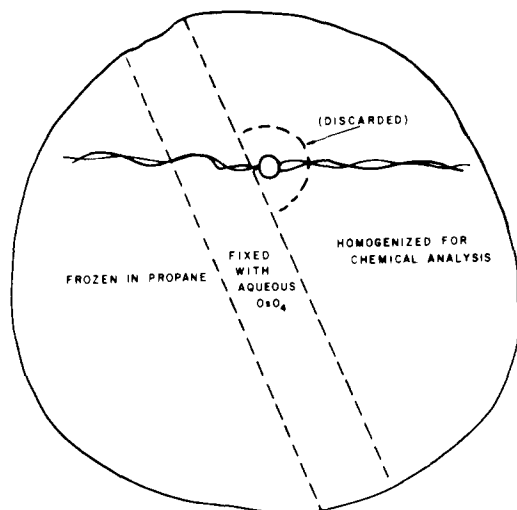


FIGURE 2 Front view of the intact rabbit retina. At the end of incubation the retina was divided into three pieces, with the myelinated fiber bundles and accompanying blood vessels as a guide. Five to eight small squares of tissue were cut from the sector indicated and individually frozen. Because the region of high ganglion cell density (visual streak) is an elongated region that extends horizontally across the retina (39), each of the three sectors samples both the central and peripheral retina. Aside from the variation consequent to differing cell density, autoradiographic results were identical at all retinal locations.

homogenate.) The remaining homogenate was centrifuged at 50,000 g for 20 min. 50  $\mu$ l of the supernate was spotted on Whatman 3 MM paper and subjected to electrophoresis for 2 h 20 min at 3,000 V, as previously described (38, 50, 62). The identity of peaks of radioactivity corresponding to phosphorylcholine, acetylcholine, and choline was established by staining adjacent lanes, spotted with authentic compounds, with iodine vapor. In some cases this was confirmed by analysis of samples to which the authentic  $^{14}$ C compounds had been added. The remaining supernate was carefully taken off and the pellet was twice resuspended in the acid buffer and centrifuged. Negligible radioactivity was found in the supernate of the third extraction. Most of the final pellet could be readily solubilized by 2:1 chloroform-methanol, leaving a fine white precipitate that was solubilized by NCS and contained negligible radioactivity. When the chloroform-methanol extract was subjected to electrophoresis as described above, radioactivity remained at the origin but none ran as phosphorylcholine, choline, or acetylcholine. The radioactivity recovered from the supernate as phosphorylcholine, choline, or acetylcholine plus that solubilized from the pellet by chloroform-methanol accounted for 92% of the total radioactivity present in the homogenate. Previous data in a variety of neural

tissues indicate that most of the choline present in the phospholipid pathway after brief exposure to [ $^3$ H]choline is in the form of phosphorylcholine or phosphatidylcholine (10, 20, 46, 48). That this was the case for the rabbit retina was confirmed in retinas where a chloroform-methanol extract of the pellet was prepared according to Folch et al. (28) and analyzed by thin-layer chromatography on silica gel in 95:36:6 chloroform:methanol:water according to Flower and Blackwell (27). More than 90% of the extracted radioactivity ran as phosphatidylcholine.

### Autoradiography

Pieces of retina were placed on thin tin foil that had been closely perforated with a needle. Excess fluid was drawn off by suction through the tip of a Pasteur pipet placed against a piece of lens paper pressed to the opposite side of the foil. The tissue was then plunged into a propane slurry maintained at liquid nitrogen temperature. Tissue samples were slowly freeze-dried by a method previously described (53). The sample chamber was brought to atmospheric pressure with dry  $N_2$ ,  $OsO_4$  crystals were added, and the specimen chamber was again evacuated. After vapor fixation for 3 h, the cham-

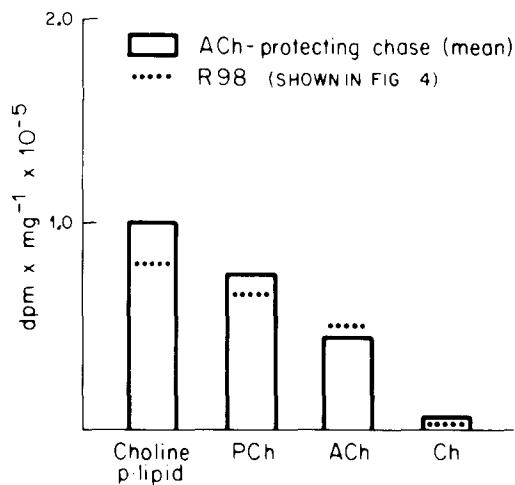


FIGURE 3 Radioactive choline metabolites contained in the retina at the end of an incubation designed to clear free [ $^3$ H]choline but retain [ $^3$ H]acetylcholine (acetylcholine-protecting chase). Abbreviations in this and all other figures: *Choline p-lipid*, choline containing phospholipids; *PCh*, phosphorylcholine; *ACh*, acetylcholine; *Ch*, choline. Bars show the mean values obtained for the six retinas incubated under these conditions and also studied autoradiographically. Standard errors of the means were <15% for these retinas and for all other groups of retinas presented here. Rows of dots indicate the distribution of radioactive choline metabolites contained in the homogenized half of retina No. R98, an autoradiograph of which is shown in Fig. 4.

ber was brought to atmospheric pressure again with  $N_2$  and the tissue infiltrated for 3 h with Spurr (73) resin that had previously been degassed and dried in a vacuum oven. The resin was polymerized overnight at  $70^\circ C$ . Other samples of retina were fixed after incubation for 1 h by immersion in 1%  $OsO_4$  in bicarbonate buffer, dehydrated through a graded series of alcohols, and infiltrated and embedded in Spurr resin. (We used low-viscosity Spurr rather than the somewhat more polar, but more viscous, Epon plastic primarily to ensure adequate and rapid infiltration. A few retinas embedded in Epon gave the same autoradiographic results as those embedded in Spurr.)

For autoradiography, two different sectioning and coating techniques were employed. Tissue that had undergone immersion fixation in 1%  $OsO_4$  was sectioned at  $1\ \mu M$ , collected on a water drop, dried on acid-cleaned slides, and dipped in NTB-2 emulsion (Kodak) by conventional techniques (66). Freeze-dried tissue was sectioned at 1 or  $2\ \mu m$  with a dry glass knife. The sections were transferred with a 27-gauge syringe needle to a glass slide. To reduce hydration of sections by atmospheric moisture, the slides were kept covered in the presence of a desiccant, and a clear plastic shield was arranged between the operator and the cutting stage to prevent breathing on the cut sections. Five sections were placed on each slide, and at the end of each cutting session the slides were gently pressed against slides that had been dipped in NTB-2 emulsion and dried in an upright position. When the slides were separated, the tissue sections adhered to the emulsion-coated one. We found that the best results were obtained when the sections were pressed against the thinnest part of the dry emulsion, near the upper end of the slide. Slides were stored in light-tight boxes at  $4^\circ C$  for periods of up to 7 wk. Autoradiographs were developed by standard techniques (53). Appropriate controls were run to determine chemographic effects.

As will be shown, this autoradiographic technique effectively localizes acetylcholine, a highly water-soluble compound. Certain precautions are, however, necessary for reliable results. Extreme care must be taken to ensure that sections are protected against moisture. As the sections are dry, they are difficult to flatten. The contact between sections and emulsion, after pressing, is thus subject to variability, and many sections from each block should be examined to ensure that an observed grain pattern is reproducible. Pressing very hard, to ensure contact, produces a prohibitively high background and prevents the photographic fixing solution from clearing the emulsion under the section. Finally, the presence of emulsion under the section is an impediment to successful staining, because stain precipitate collects in this area. The technique should perhaps be used for the localization of compounds as hygroscopic as acetylcholine only when corroborative information, as presented here, is available.

### *Sample of Retinas*

A total of 28 retinas was studied both chemically and autoradiographically. An additional 10 were studied only chemically. 39 others were studied using the experimental protocols described above, but with the concentration of choline during incubation  $0.1$  or  $0.2\ \mu M$  instead of  $0.3\ \mu M$ , or with the postlabeling chase 2 h instead of 1 h. These experiments gave results qualitatively identical to those obtained with the procedure described above, but for simplicity the text will describe only the results of experiments in which  $0.3\ \mu M$  extracellular choline and a 1-h chase were used.

## RESULTS

As expected, cells of the rabbit retina took up extracellular choline rapidly and incorporated it into acetylcholine. A substantial amount of [ $^3H$ ]-phosphorylcholine was also synthesized, and with time was incorporated into [ $^3H$ ]phospholipid. Because several radioactive choline metabolites were formed, meaningful autoradiography required experimental manipulations by which silver grains seem over individual retinal elements could be attributed either to [ $^3H$ ]acetylcholine or to  $^3H$  compounds of the phospholipid pathway.

### *Comparison of Retinas Containing or Depleted of [ $^3H$ ]Acetylcholine*

Retinas analyzed soon after a 15-min exposure to  $0.3\ \mu M$  [ $^3H$ ]choline contained radioactivity in the form of free choline (8%), acetylcholine (30%), phosphorylcholine (54%), and phospholipid (8%). In order that we could control the tissue's distribution of radioactive choline metabolites, separate retinas were incubated after labeling under conditions designed either to retain acetylcholine within the tissue or promote its release (Fig. 1). In both cases the postlabeling incubation also cleared free [ $^3H$ ]choline from the tissue.

One group of retinas was incubated after labeling in a medium containing  $20\ mM\ Mg^{++}$  and  $0.2\ mM\ Ca^{++}$ , to prevent acetylcholine release;  $30\ \mu M$  physostigmine, to prevent the hydrolysis of acetylcholine by the presynaptic intracellular esterase; and  $1\ mM$  unlabeled choline, to clear free [ $^3H$ ]choline from the tissue. Under these conditions a substantial fraction of the [ $^3H$ ]acetylcholine originally present in the tissue was retained, free [ $^3H$ ]choline was essentially eliminated from the tissue, and roughly half of the [ $^3H$ ]phosphorylcholine originally present was incorporated into phospholipid (Fig. 3).

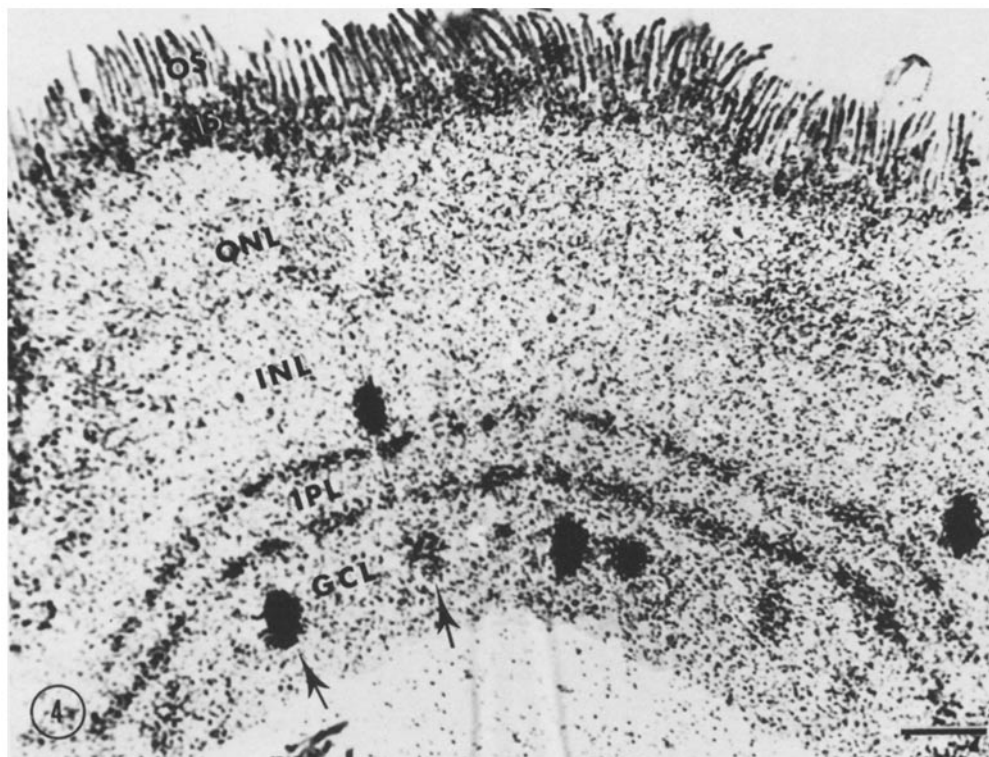


FIGURE 4 Autoradiograph of freeze-dried tissue from a retina (R98, Fig. 3) incubated under acetylcholine-protecting conditions. Silver grains are seen over the photoreceptor cells, especially in the region of the inner segment (*IS*). Two cells at the inner margin of the inner nuclear layer (*INL*) are densely labeled, and grains have accumulated in two bands over the inner plexiform layer (*IPL*). Two densities of grains are seen over cells of the ganglion cell layer (*GCL*, arrows; see also Figs. 12, 13, and 15). *OS*, outer segments; *ONL*, outer nuclear layer. Section is 2  $\mu\text{m}$  thick, as are all of the autoradiographed sections shown here. Exposure, 6 wk. Bar, 20  $\mu\text{m}$ .

An autoradiograph of a retina incubated under this protocol is shown in Fig. 4. Silver grains were seen over the photoreceptor cells, over some of the cell bodies at the inner margin of the inner nuclear layer, and in two bands over the inner plexiform layer. A few cells of the ganglion cell layer were densely labeled, and the other cells of the ganglion cell layer were faintly labeled.

A second group of retinas was exposed to [ $^3\text{H}$ ]choline in the same way, but was incubated after labeling under conditions designed to promote the elimination of [ $^3\text{H}$ ]acetylcholine from the retina. The medium contained 1 mM unlabeled choline, which cleared free [ $^3\text{H}$ ]choline and prevented its further incorporation into acetylcholine, and the retinas were stimulated with flashing light at a frequency (3 Hz) previously found optimal for the release of retinal acetylcholine (50). The distribu-

tion of radioactivity found in the phospholipid pathway of these retinas was essentially identical to that observed in retinas incubated under acetylcholine-protecting conditions. Free [ $^3\text{H}$ ]choline was again cleared from the tissue. These retinas, however, contained negligible amounts of [ $^3\text{H}$ ]acetylcholine (Fig. 5).

Autoradiography showed many silver grains over the photoreceptor cells and a few grains over the cells of the ganglion cell layer. Grains were no longer seen over the inner nuclear or inner plexiform layers, and no densely labeled cells were seen in the ganglion cell layer (Fig. 6).

This result implied that acetylcholine is synthesized by the densely labeled cells seen in the inner nuclear and ganglion cell layers of acetylcholine-containing retinas, while the photoreceptors and most ganglion cells synthesize significant amounts

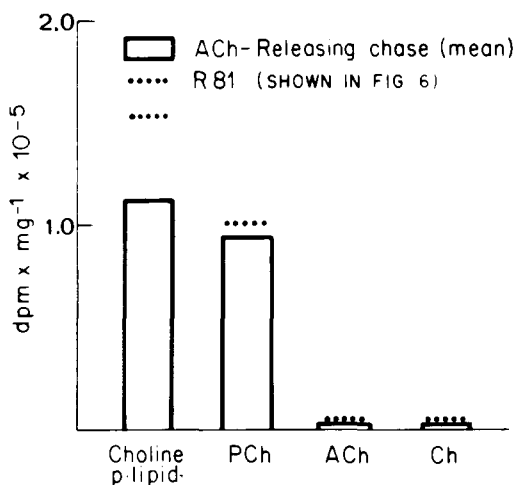


FIGURE 5 Radioactive choline metabolites contained in the retina at the end of an incubation designed to clear free [<sup>3</sup>H]choline and [<sup>3</sup>H]acetylcholine from the tissue (acetylcholine-releasing chase). Conventions as in Fig. 3. Bars show means for six retinas. An autoradiograph of freeze-dried tissue from retina R81 (rows of dots) is shown in Fig. 6.

of choline-containing phospholipids but no acetylcholine. The three following observations corroborated this conclusion.

#### *Retinas Initially Fixed with Osmium Tetroxide*

Phosphatidylcholine (and the minor choline-containing phospholipids) are retained after fixation by osmium tetroxide (74), while phosphorylcholine and acetylcholine are not. The samples from acetylcholine-containing retinas that were directly fixed with 1% aqueous osmium tetroxide showed few silver grains over the inner plexiform or inner nuclear layers, and had no densely labeled cells in the ganglion cell layer. Such tissue retained the radioactivity responsible for dense grains over the photoreceptors and sparse grains over the cells of the ganglion cell layer (Fig. 7).

#### *Wetted Sections*

Acetylcholine, phosphorylcholine, and choline are very soluble in water, while the choline-containing phospholipids are insoluble. When sections from acetylcholine-containing retinas were exposed to water before application of the emulsion, the pattern of silver grains seen in autoradiographs became that of retinas incubated under conditions

that deplete acetylcholine: many grains were seen over the photoreceptor cells and some were seen over the ganglion cells, but few were seen over the inner nuclear and plexiform layers, and the dense labeling of a subset of cells in the ganglion cell layer was no longer observed (Fig. 8).

#### *Analysis of Microdissected Retinal Subareas*

The severe loss of radioactivity seen autoradiographically when sections were exposed to water suggested that we might be able to systematically extract the water-soluble compounds for chemical analysis. This proved to be the case.

When 10- $\mu$ m sections containing 1-3 linear mm of retina were immersed in formate-acetate buffer, radioactivity was eluted into the buffer. If the amount of tissue contained in the section was invariant, as in adjacent sections from a single block, the amount of radioactivity recovered was remarkably constant: analysis of the radioactivity from 20 serial sections typically yielded radioactivity with standard errors of <2% of the mean. The recovery of radioactivity probably varied so little because the extraction of radioactivity was nearly complete. After sections had been immersed in the acid buffer for 48 h, no further radioactivity could be extracted. When the total radioactivity eluted into the buffer was compared with that recovered from adjacent sections completely solubilized by 5% ethanolic KOH or by NCS, we found that >85% of the retina's total water-soluble radioactivity had been eluted from the section into the buffer. Apparently, a 10- $\mu$ m section of Spurr plastic is penetrated almost completely by the buffer.

The radioactive compounds eluted into acid buffer could be separated by high-voltage paper electrophoresis. The results showed that eluted phosphorylcholine, acetylcholine, and choline were present in proportions essentially identical to those found when the freshly homogenized half of the same retina was analyzed. That is, retinas known to have varying distributions of the radioactive choline metabolites before freezing yielded the same relative amounts from the washed sections (Fig. 9). This indicated that the compounds survive fixation with osmium vapor and embedding in Spurr resin, and that none are preferentially retained by the sections.

Knowing that the water-soluble choline metabolites could be reliably extracted from the sections, we undertook to compare the radioactive compounds of the photoreceptors with those of the

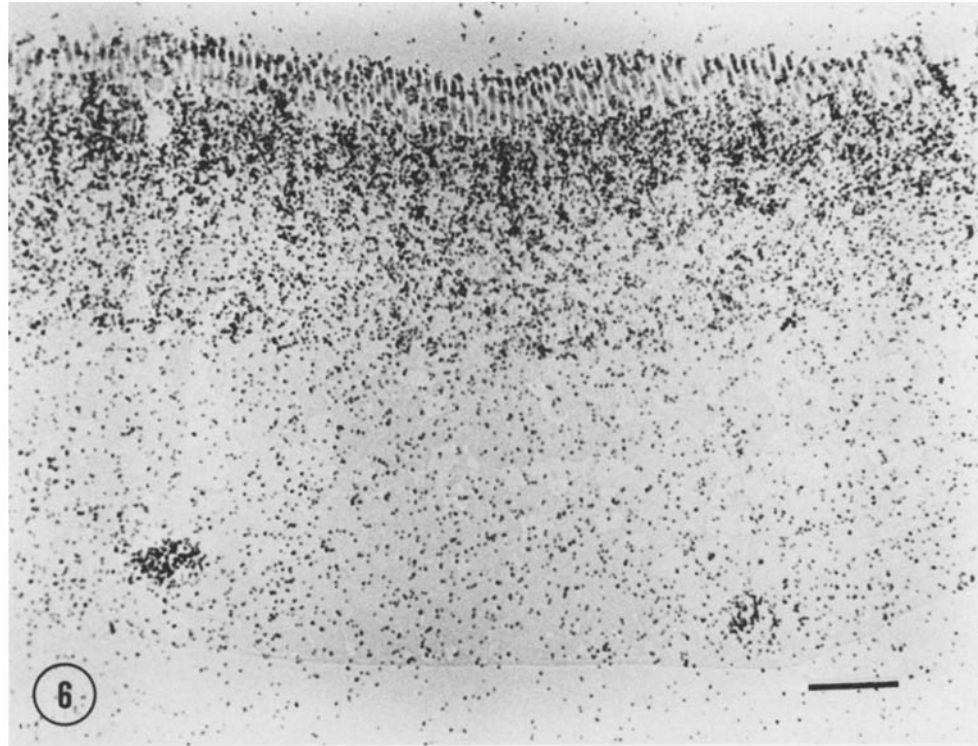


FIGURE 6 Autoradiograph of freeze-dried tissue from a retina (R81, Fig. 5) incubated under acetylcholine-releasing conditions. Silver grains are seen over the photoreceptor cells, and two cells located in the ganglion cell layer are faintly labeled. In contrast to autoradiographs of retinas that contain a significant amount of [ $^3\text{H}$ ]acetylcholine, no densely labeled cells are seen in the ganglion cell or inner nuclear layers, and the bands of grains previously seen over the inner plexiform layer are absent. Exposure, 6 wk. Bar, 20  $\mu\text{m}$ .

inner retina. A razor blade chip was attached to the objective of a microscope, so that its cutting edge was just above the focal plane of the objective but was still visible within the field. Sections 10  $\mu\text{m}$  thick, selected to provide the longest possible run of straight retina, were placed on a glass slide and visualized under 200 $\times$ . The slide was manipulated on a rotating stage until the blade was positioned above the desired line of cutting, which lay along the outer plexiform layer. The objective was then lowered until the blade divided the section (Fig. 10). The slide was transferred to the stage of a dissecting microscope, where the cut halves of the section were trimmed to exclude areas of retina that were not divided at the desired level (usually where the section began to curve).

It was possible to make the cut with considerable accuracy. The greatest source of error was folding or dimpling of the embedded tissue, causing local deformation of the retinal layers. Although we

often could find an area of retina that was free of these defects (they probably arose when the isolated retina was lifted onto foil for freezing), the cut occasionally invaded the outer edge of the horizontal cell bodies or the inner edge of the outer nuclear layer. Inspection of the divided sections under high magnification showed that the volume of tissue inappropriately included with the outer or inner half-sections was at most a few percent of the total volume of tissue analyzed. For the present purpose this amount of error is insignificant, as we assume only that we succeeded in separating the photoreceptors from the rest of the retina.

The outer and inner half-sections were collected and 20 of each were pooled and extracted together. Electrophoresis showed that the inner retina contained radioactive acetylcholine, choline, and phosphorylcholine, as would be predicted from our autoradiographic evidence that the inner ret-



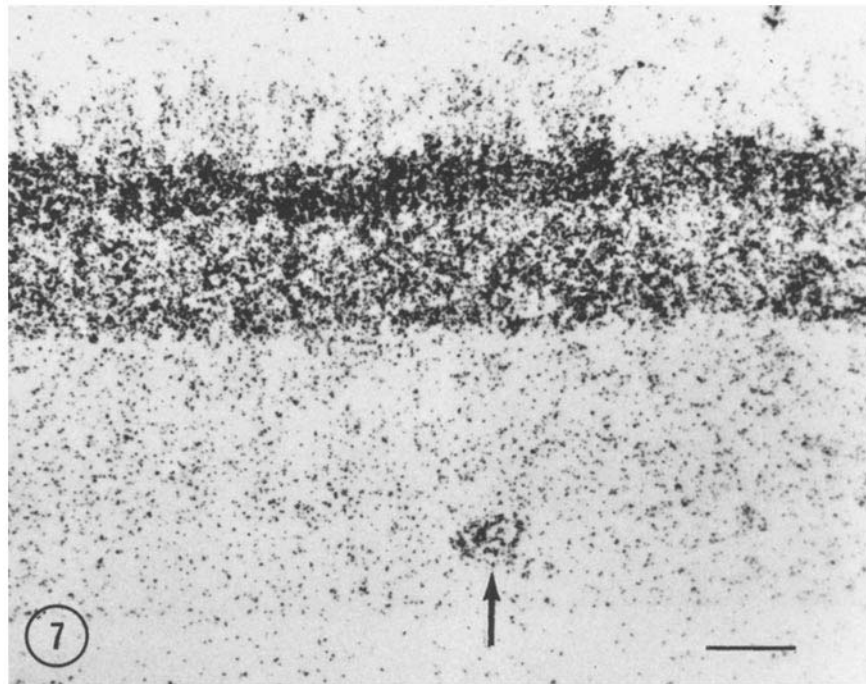


FIGURE 7 Autoradiograph of osmium-fixed tissue from a retina incubated under the acetylcholine-protecting condition. Radioactivity is present in the photoreceptor cells and a ganglion cell (arrow), but the radioactivity associated with acetylcholine in freeze-dried tissue is absent. Exposure, 7 wk. Bar, 20  $\mu$ m.

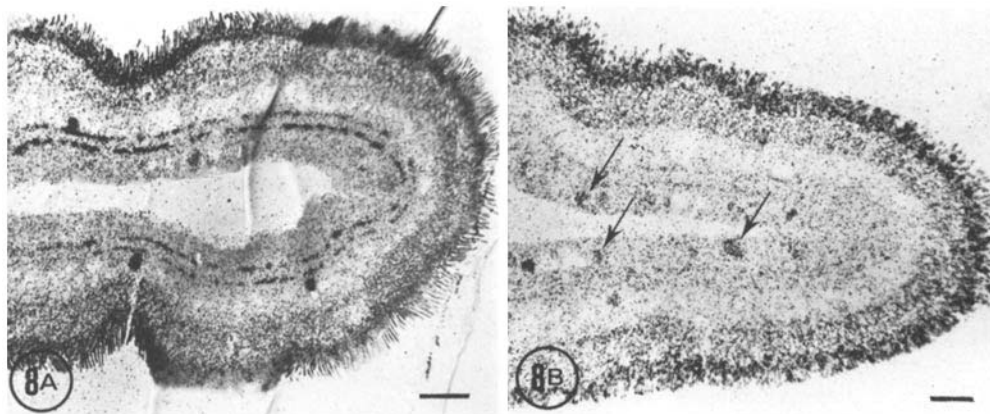


FIGURE 8 Effects of wetting on the retention of water-soluble choline metabolites. (A) Autoradiograph of a section from a retina incubated under the acetylcholine-protecting condition. Section was cut dry. (B) Autoradiograph of a section cut from the same block as the one shown in Fig. 8A, but sectioned and collected on a water drop. Faintly labeled ganglion cells (arrows) were seen in sections exposed to water, but the radioactivity responsible for the densely labeled cells of the inner nuclear and ganglion cell layers, and for the bands of grains over the inner plexiform layer, is greatly reduced. The density of labeling of the photoreceptors is much less affected. Exposure, 6 wk. Bar, 40  $\mu$ m.

ina contains both cells that synthesize acetylcholine and cells that synthesize choline-containing phospholipids. Most of the radioactivity eluted

from the outer retina was in the form of phosphorylcholine (Fig. 11). Acetylcholine detected in the outer half-retina represented <4% of the total

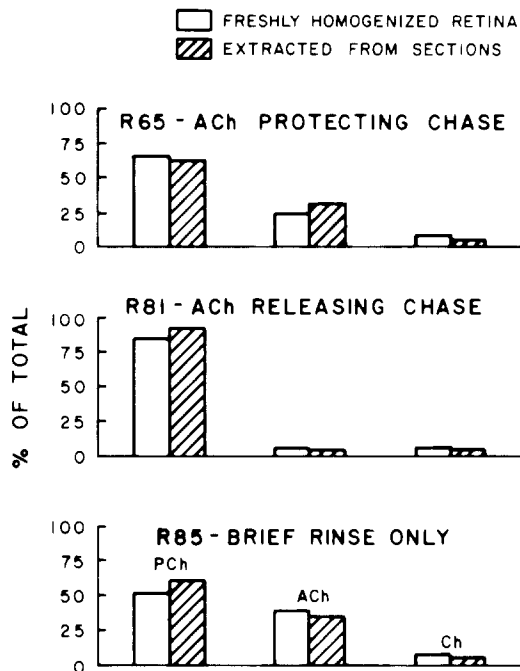


FIGURE 9 Comparison of the radioactive compounds eluted from sections with those present in the retina before freeze-drying and embedding. Fresh homogenates were compared with tissue from the same retina that was freeze-dried, embedded, and sectioned, as for autoradiography. In each case 20 sections, 10  $\mu\text{m}$  thick, were pooled for elution of radioactive compounds. These results show that the water-soluble choline metabolites eluted from the sections faithfully reflect those present in the retina before processing.

$^3\text{H}$ acetylcholine found in the sections. That small amount probably diffused from the inner retina during infiltration and embedding—a faint halo of grains was seen at the margins of the tissue in many of the autoradiographs.

One additional retina was divided by a different method. Fifteen 10- $\mu\text{m}$  sections were re-embedded flat and sectioned serially at 5  $\mu\text{m}$ , starting from the photoreceptor side. Occasional 1- $\mu\text{m}$  sections were cut and stained. When a 1- $\mu\text{m}$  section showed that the outer edge of the inner nuclear layer had been reached, the thicker sections were pooled and analyzed as above. Radiochemical analysis of eluate from the inner and outer retina gave results identical to those obtained from sections microdissected under direct visual control.

#### Controls

Retinas autoradiographed soon after labeling—

when radioactivity in the phospholipid pathway was mostly in the form of phosphorylcholine, and when  $^3\text{H}$ choline was present—showed essentially the same distribution of grains as acetylcholine-containing retinas studied after the postlabeling incubation that was used to control the retina's  $^3\text{H}$ acetylcholine content (Fig. 12). The localization thus is not an artifact of events that might occur during the postlabeling chase.

Nor was the localization peculiar to that after *in vitro* exposure of the retina to  $^3\text{H}$ choline. A qualitatively identical autoradiographic result was obtained when 50  $\mu\text{Ci}$  of  $^3\text{H}$ choline chloride was injected into the posterior chamber *in vivo* and the retina isolated and frozen 3 h later (Fig. 13). Relatively fewer silver grains were seen over the outer retina than when exposure to  $^3\text{H}$ choline was carried out *in vitro*, presumably because  $^3\text{H}$ choline diffusing from the vitreous was diluted as it met unlabeled choline diffusing from the choroidal circulation. The retina's acetylcholine pool is smaller and turns over more rapidly than the pool of choline-containing phospholipids. If 6 h or more elapsed between injection of  $^3\text{H}$ choline into the eye and analysis of the retina,  $^3\text{H}$ phospholipid accounted for most of the tissue's radioactivity. It obscured the retina's  $^3\text{H}$ acetylcholine in autoradiographs.

When the retina was exposed to 0.3  $\mu\text{M}$   $^3\text{H}$ choline in the presence of 50  $\mu\text{M}$  hemicholinium-3—an agent that blocks choline transport (15)— $^3\text{H}$ acetylcholine synthesis and  $^3\text{H}$ phosphorylcholine synthesis were depressed to 0.5 and 15%

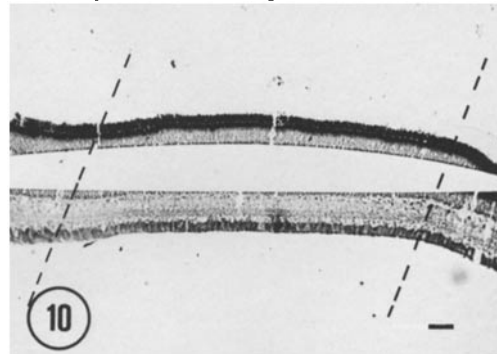


FIGURE 10 Microdissection of a 10- $\mu\text{m}$  section from a freeze-dried, vapor-fixed, and plastic-embedded retina. A region of retina has been divided along the outer plexiform layer. The section was then trimmed as indicated, yielding an outer half-retina containing the photoreceptors and an inner region containing the rest of the retina. The two pieces were teased apart for analysis. Bar, 40  $\mu\text{m}$ .

of their control values, respectively, and few silver grains were seen over the tissue (Fig. 14).

#### Characteristics of the Acetylcholine-Synthesizing Cells

The majority of cells of the ganglion cell layer had the faint labeling that was associated with [ $^3\text{H}$ ]phospholipid synthesis. These cells were clearly distinct from a smaller population of cells in the ganglion cell layer that had the dense accumulation of silver grains associated with the presence of [ $^3\text{H}$ ]acetylcholine. Because of the variability of ganglion cell position and size, our material does not allow us to determine whether the position or size of the densely labeled cells within the layer differs from those of the faintly labeled cells.

The labeled cells of the inner nuclear layer were without exception located immediately adjacent to the inner plexiform layer. Their size was not noticeably different from that of the other cells found in that location. In all of our autoradiographic material (over 1,300 sections from 25 [ $^3\text{H}$ ]acetylcholine-containing retinas), three densely labeled cells were seen to lie unequivocally within the inner plexiform layer (Fig. 15).

The frequency of occurrence of acetylcholine-containing cells was estimated by counting them in unstained, autoradiographed sections and comparing the result with the total number of cells observed in adjacent stained sections. All sections were 2  $\mu\text{m}$  thick. They were selected to sample both the central and peripheral retina. A total of 24 stained sections, and their autoradiographed neighbors, were studied: three pairs of sections per block for two blocks from each of four retinas. In this sample the ganglion cell layer contained an average of  $24 \pm 6$  cells per linear mm of retina. An average of  $7 \pm 1$  cells per mm were densely labeled, indicating that roughly 29% of the ganglion cell layer cells synthesized acetylcholine.

The same sections contained  $101 \pm 15$  cells per linear mm in the inner cell row of the inner nuclear layer. The autoradiographed sections contained an average of  $5.5 \pm 0.5$  labeled cells per mm, or 5% of the total cells present.

An independent measure of the frequencies of acetylcholine-synthesizing cells relative to each other was obtained in a second group of 30 autoradiographed sections from seven different blocks. 96 densely labeled cells were encountered in the ganglion cell layer and 75 in the inner nuclear

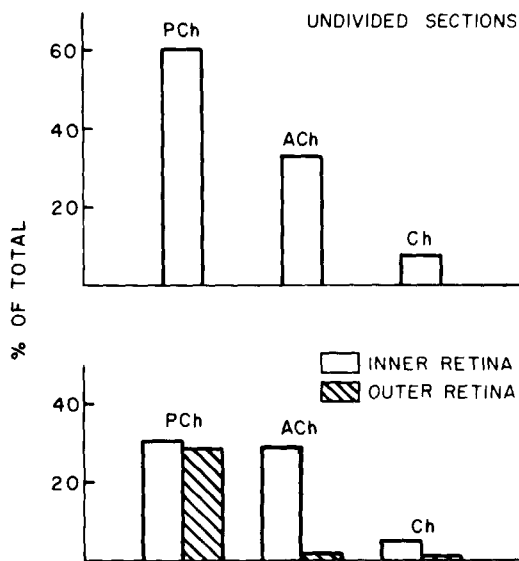


FIGURE 11 Radiochemical analysis of the inner and outer half-retinas. Top shows analysis of 20 undivided sections. Bottom shows the compounds eluted from 20 sections divided at the level of the outer plexiform layer. The sections that were divided were cut in a continuous run with those analyzed undivided. For the divided sections, the percentage of radioactivity recovered as the individual choline metabolites is expressed relative to the total recovered from both half-sections. Data are mean values obtained from four retinas.

layer. This again suggests that the absolute number of acetylcholine-synthesizing cells in the ganglion cell layer is somewhat greater than that of acetylcholine-synthesizing cells in the inner nuclear layer.

These estimates should be considered as preliminary ones, for two reasons: First, we have not made a size correction; because the ganglion cells are on the average larger than the cells of the inner nuclear layer, this leads to an overestimate of the relative number of cells of the ganglion cell layer. Second, cells identified by an accumulation of silver grains in an autoradiograph are not strictly comparable to cells identified in stained sections. Retinal ganglion cells are stained rather palely by toluidine blue, so that in grazing sections they are occasionally hard to detect. A grazing section of an autoradiographed acetylcholine-containing cell, however, may be made visible by autoradiography. The comparison thus could tend to increase the apparent frequency of acetylcholine-synthesizing cells located in the ganglion cell layer. Our subjective impression is that the problem is not

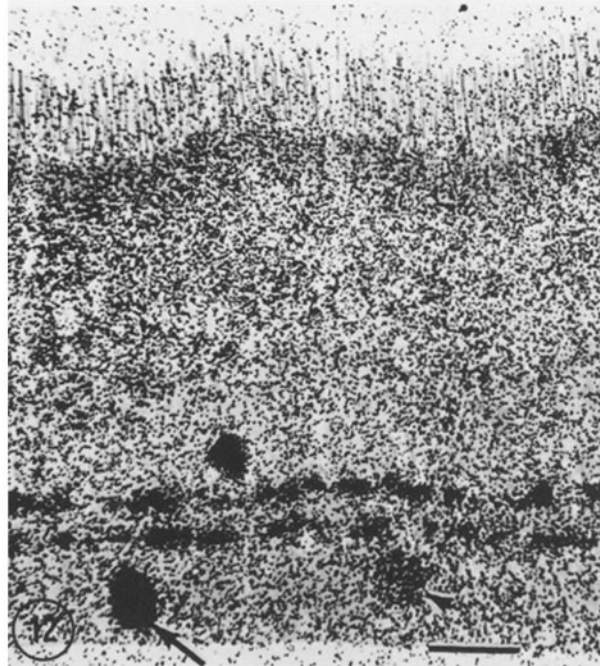
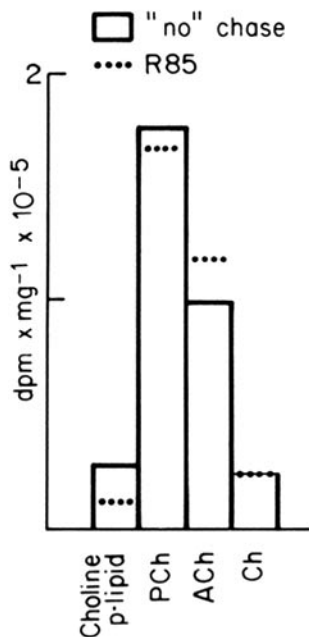


FIGURE 12 Chemical analysis and autoradiograph of a retina harvested immediately after labeling. Such retinas contained a substantial amount of [<sup>3</sup>H]acetylcholine, and the autoradiographic results are very similar to those seen in retinas subjected to the acetylcholine-protecting chase. Retina was rinsed for 4 min in medium containing 20 mM Mg<sup>++</sup>, 0.2 mM Ca<sup>++</sup>, and 30 μM physostigmine, to clear extracellular [<sup>3</sup>H]choline. These retinas nonetheless showed a large background radioactivity throughout the tissue. (During the 4-min rinse some of the intracellular [<sup>3</sup>H]choline was also eluted, so that the radiochemical results should not be taken as representing the amount of free [<sup>3</sup>H]choline present in the cells at the end of the 15-min labeling period.) Bars show means for four retinas. Note the difference in silver grain density between the heavily labeled ganglion cell (arrow) and the more weakly labeled cell to its right (arrowhead). Exposure, 6 wk. Bar, 20 μm.

very serious; few cells of the ganglion cell layer were ambiguous in the stained sections. But we prefer to conclude simply that somewhat more than 20% of the cells in the ganglion cell layer synthesized acetylcholine. These problems seem insignificant in the case of the inner nuclear layer cells, which are more uniform in size and stain more darkly than the cells of the ganglion cell layer.

#### *Phospholipid Synthesis in the Photoreceptors*

The autoradiographic results (Figs. 6 and 7) and analysis of radioactivity eluted from sections (Fig. 11) both indicate that about two-thirds of the retina's total synthesis of choline-containing phospholipid occurs in the photoreceptor cells. Because of the pulse-chase procedure used in these experiments, the progressive synthesis of photoreceptor

phospholipid from phosphorylcholine could be followed both chemically and autoradiographically. Fig. 16 shows that after pulse labeling with [<sup>3</sup>H]choline, continued incubation in the presence of a saturating concentration of unlabeled choline led to a progressive fall in the retina's content of [<sup>3</sup>H]phosphorylcholine and a reciprocal rise in [<sup>3</sup>H]phospholipid. Autoradiography of retinas fixed with aqueous osmium tetroxide showed that immediately after labeling, the density of grains over the photoreceptors was low (Fig. 17). 2 h later, when significant [<sup>3</sup>H]phospholipid had been formed, the total density of grains over the photoreceptors was higher; many grains were seen over the inner segments, and a significant number of grains was seen over the outer segments (Fig. 17, see also Figs. 6 and 7). At the times studied, grains appeared uniformly distributed over the outer segment. Fixable radioactivity accumulated

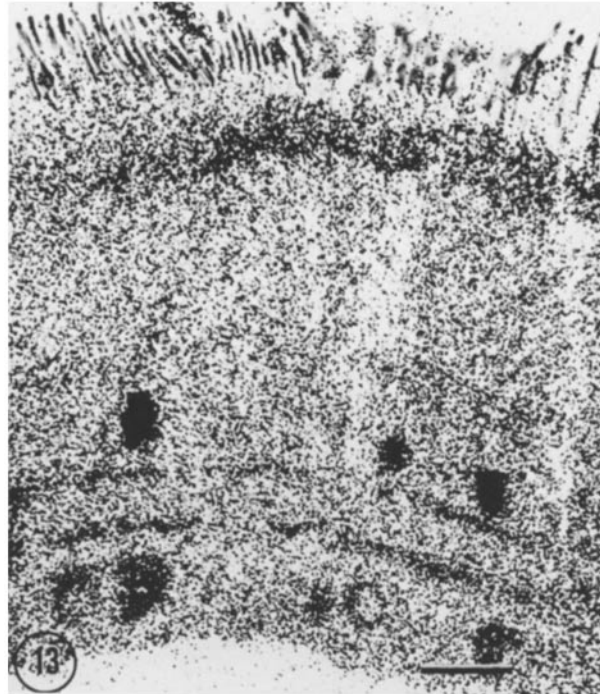
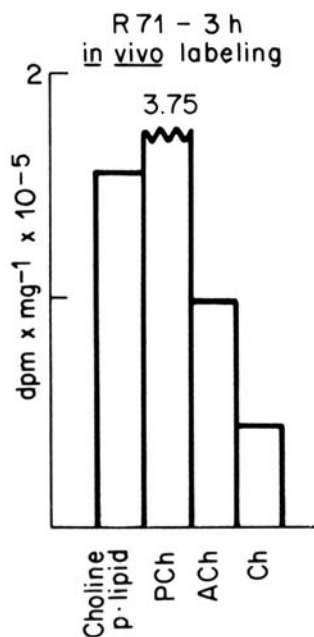


FIGURE 13 Chemical analysis and autoradiograph of a retina exposed to [<sup>3</sup>H]choline by injection into the posterior chamber. 3 h after injection, the retina was isolated and processed as usual. The autoradiographic results are qualitatively identical to those obtained from retinas labeled in vitro. Results obtained by injection of [<sup>3</sup>H]choline into the posterior chamber were more variable than for retinas labeled in vitro, presumably because intravitreal injection exposes the retina to [<sup>3</sup>H]choline inhomogeneously. Exposure, 6 wk. Bar, 20  $\mu$ m.

more rapidly in the inner segments and cell bodies of cones (17) than in rods.

## DISCUSSION

### *Localization of Acetylcholine*

Acetylcholine synthesized from extracellular choline by the rabbit retina was found in two bands within the inner plexiform layer and in a subset of the cell bodies at both of its margins. This localization was initially indicated by the fact that the marked cells, and the bands in the inner plexiform layer, were present or absent as [<sup>3</sup>H]-acetylcholine was retained in or cleared from the retina. It was confirmed by the insusceptibility of the compounds found in these locations to fixation with osmium, by their water solubility, and by direct analysis of the radioactive compounds eluted from the inner retina.

That acetylcholine is released in the inner plexiform layer is consistent with the physiological studies of this preparation that were reviewed

earlier (50, 51). Since the inner plexiform layer of the rabbit retina has been shown independently to contain high levels of acetylcholinesterase and choline acetyltransferase (56, 64, 69) the conclusion that this layer contains cholinergic synapses seems quite certain. A variety of evidence suggests that this is also true for the chicken, pigeon, and mouse retinas (13, 63, 68, 69, 76, 82).

The presence of cholinergic photoreceptor cells in the retinas of the goldfish (71) and turtle (45, 70) has been suggested, but the present findings—as well as those of others—indicate that such cells do not exist in mammals. (a) Rabbit photoreceptors seem not to synthesize acetylcholine; when acetylcholine eluted from sections was measured chemically, none was found distal to the outer nuclear layer. One might argue that the neurotransmitter released by the photoreceptors would be limited to their synaptic terminals, so that our cut, which approximately divided the outer plexiform layer, would have partitioned some of the photoreceptors' hypothetical acetylcholine into the

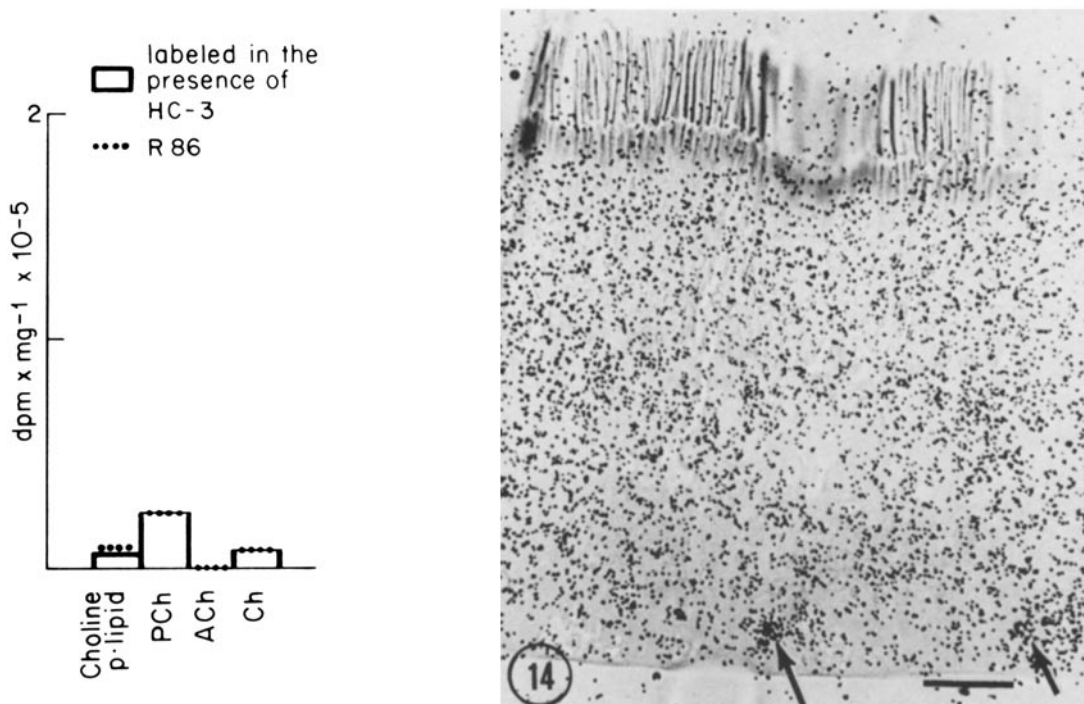


FIGURE 14 Chemical analysis and autoradiograph of a retina incubated with [<sup>3</sup>H]choline in the usual way, but with 50  $\mu$ M hemicholinium-3 also added to the medium. Retina was harvested immediately after labeling. The synthesis of [<sup>3</sup>H]acetylcholine was reduced essentially to zero, and the grain pattern associated with acetylcholine is not seen. [<sup>3</sup>H]phosphorylcholine synthesis was reduced to 15% of that seen in the absence of the inhibitor, and the density of grains seen over the photoreceptors and over the ganglion cells (arrows) is correspondingly very low. Bars show the mean for four retinas. Exposure, 6 wk. Bar, 20  $\mu$ m.

inner half-retina. However, much evidence (see below) indicates that neurotransmitters synthesized by small neurons exist in significant concentration throughout the cell, so that the outer nuclear layer should contain a substantial amount of acetylcholine if it were used as a neurotransmitter by photoreceptor synapses. (b) The outer retina of mechanically or biologically fractionated rabbit and mouse retinas has been shown to contain little choline acetyltransferase or acetylcholinesterase (68, 69). In the rabbit, <0.5% of the retina's total choline acetyltransferase activity was found distal to the inner nuclear layer. (c) Recording from the perfused cat eye (58) has shown the activity of horizontal cells to be unaffected by mecamylamine or atropine, even at concentrations known to affect the activity of ganglion cells. (d) Both the rabbit (51) and cat (58) retinas contain a population of ganglion cells whose activity is unaffected by high concentrations of acetylcholine or related drugs; the photoreceptors afferent to such ganglion cells

are presumably not cholinergic. While one can perhaps imagine an unorthodox cholinergic synapse that would not be revealed in any of these experiments, it seems far more likely that the photoreceptors of mammalian retinas do not release acetylcholine.

A small group of the cell bodies at either margin of the inner plexiform layer accumulated [<sup>3</sup>H]acetylcholine. Those of the inner nuclear layer made up about 5% of the total number of cells seen adjacent to the plexiform layer. The proportion of acetylcholine-synthesizing cells in the ganglion cell layer is harder to estimate because of the lower and more variable ganglion cell density, but it is probably 20–30% of the total.

The number of acetylcholine-synthesizing cells is of some importance for attempts to understand their role in retinal function, and it is therefore worthwhile to consider factors that might restrict the labeling of retinal neurons. The most important such possibility is that acetylcholine formed

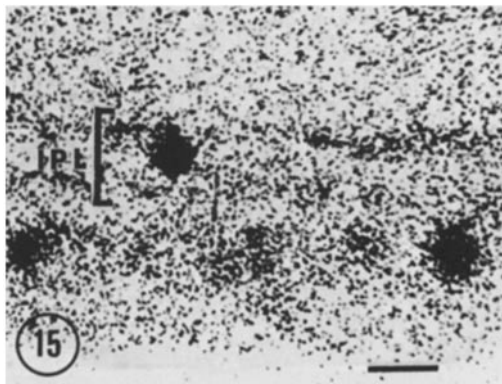


FIGURE 15 Autoradiograph of freeze-dried tissue from a retina incubated under acetylcholine-protecting conditions. A labeled cell lies near the center of the inner plexiform layer (IPL), between the bands of grains associated with acetylcholine.

at synapses is retained there, so that the acetylcholine formed in the inner plexiform layer does not diffuse into all of the parent cell somas. However, several facts suggest that this is not the case. First, the available evidence indicates that the enzymes of neurotransmitter synthesis are in general present throughout neurons, so that acetylcholine synthesis is not restricted to the presynaptic terminal (12, 22, 33, 41). Furthermore, diffusion within small neurons is rapid and relatively complete (as shown by the filling of fine neuronal processes by injected dyes or retrogradely transported horseradish peroxidase) so that a compound synthesized in the cytoplasm (29, 32, 40) should not long remain inhomogeneously distributed within the cell. In fact, previous studies using morphological methods that provide high resolution of neurotransmitters in the retina have revealed dopamine (25, 26), gamma-aminobutyric acid (49, 54), and glycine (54) to be present throughout their respective neurons. Finally, we do not see partially labeled cells in the inner nuclear layer: the cells are either densely labeled, like those shown in Figs. 4, 8a, 12, and 13, or contain negligible amounts of radioactivity. A diffusion restriction would thus have to be absent in some cells and perfect in others.

The low frequency of labeled cells is not an artifact of the 1-h *in vitro* chase period used in most of our experiments, because it was also found in retinas harvested immediately after *in vitro* labeling and in retinas labeled *in vivo*. Evidence against a failure of the autoradiographic methods comes from the fact that the bands of grains located over the inner plexiform layer continued

through stretches of retina where no labeled cells were observed in the adjacent inner nuclear or ganglion cell layers. Finally, electron microscope studies of  $\alpha$ -bungarotoxin binding in the inner plexiform layer of the mouse retina (63) reveal only a small population of synapses that bind the toxin. For all of these reasons, then, it seems probable that the fraction of cholinergic cells in the rabbit retina is in fact very small.

#### Identity of the Acetylcholine-Containing Cells

Acetylcholine-containing cell bodies lined both margins of the inner plexiform layer. Those of the inner nuclear layer had the position of amacrine cells; all were located immediately adjacent to the plexiform layer. Identification of the acetylcho-

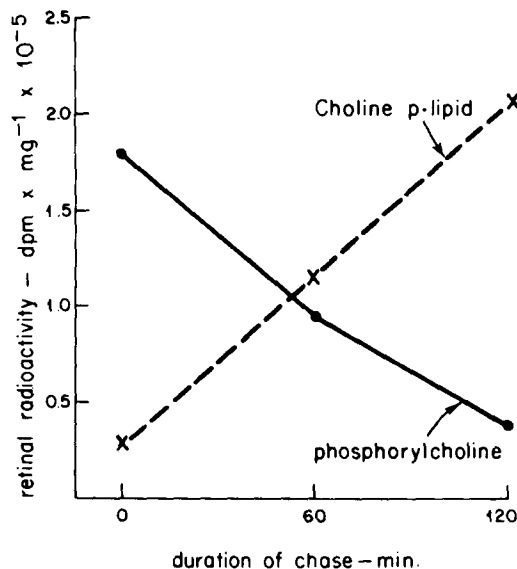


FIGURE 16 Changing retinal contents of  $[^3\text{H}]$ phosphorylcholine and  $[^3\text{H}]$ phospholipid during the postlabeling chase. These retinas were labeled in the standard way, and then incubated under acetylcholine-releasing conditions, i.e., in the presence of 1 mM unlabeled choline. (Identical results were obtained in retinas treated by the acetylcholine-protecting protocol.) That the unlabeled choline in fact prevented further incorporation of  $[^3\text{H}]$ -choline into phosphorylcholine is confirmed by the near constancy of the total radioactivity contained in phosphorylcholine and phospholipid. Thus, the retinal phosphorylcholine pool was rapidly labeled during exposure of the retina to extracellular  $[^3\text{H}]$ choline, after which  $[^3\text{H}]$ phosphorylcholine was more slowly incorporated into phospholipids. The increase in  $[^3\text{H}]$ phospholipid is shown autoradiographically in Fig. 17. Mean values for six retinas.

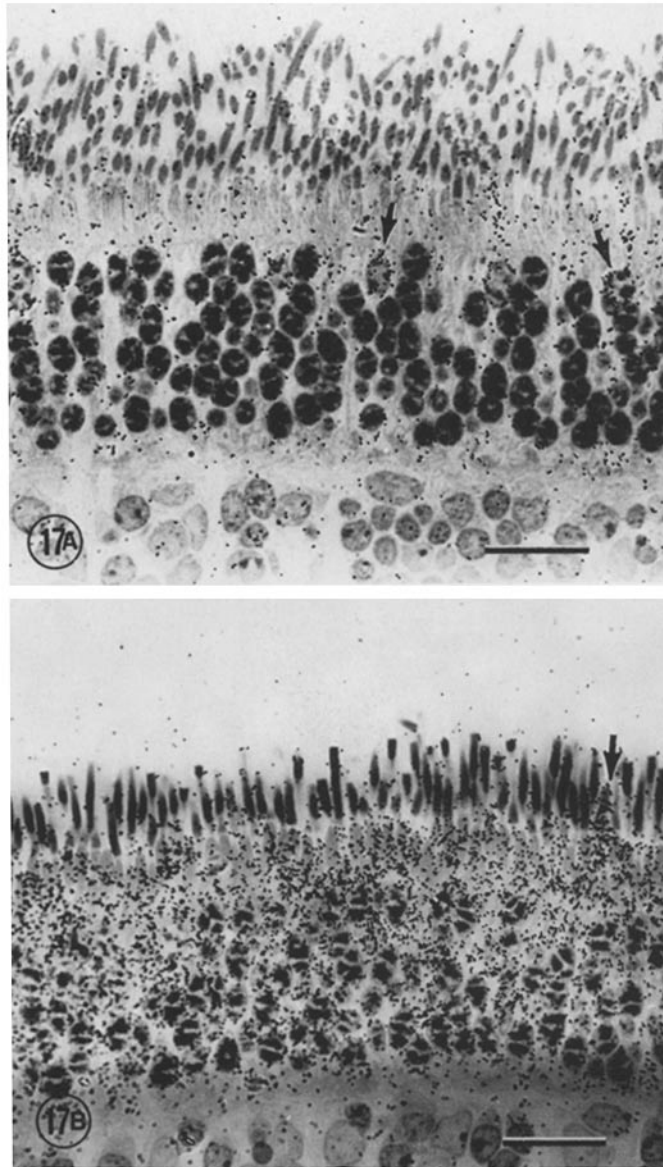


FIGURE 17 Increase in osmium-fixed radioactivity in the photoreceptors with time of incubation. (A) Retina fixed immediately after labeling. (B) Retina labeled in the same way and subsequently incubated for 1 h in the presence of 1 mM unlabeled choline. At the end of the chase (B) a significant number of grains is seen over the photoreceptor outer segments. Even then, the majority of grains are over the outer nuclear layer or inner segments. Cones (arrows) were more heavily labeled than rods. Note (B) that much more osmium-fixed radioactivity is contained in the photoreceptors than in the outer plexiform or outer nuclear layers. Exposure, 4 wk. Bar, 20  $\mu$ m.

line-synthesizing cells as amacrine would be consistent with the reported synthesis of acetylcholinesterase by amacrine and ganglion cells of the rabbit (56). Their size was not strikingly different

from that of the unlabeled cells seen at the inner margin of the inner nuclear layer. Although the inner nuclear layer of many vertebrate retinas is known to contain a few displaced ganglion cells,



they are thought to be substantially larger than amacrine cells (16–19).

The acetylcholine-containing cells seen in the ganglion cell layer could be either ganglion cells or displaced amacrine cells. (They are too large to be glia.) In favor of their being ganglion cells is their relatively high frequency (one would expect anatomists and perhaps electrophysiologists to have seen many displaced amacrines if they make up >20% of the cells of the ganglion cell layer) and evidence suggesting that the retinal input to the tectum of the toad may be cholinergic (30). Against their being ganglion cells is the fact that cholinergic agents introduced iontophoretically into the lateral geniculate body of the cat had equivocal effects (21, 23, 24, 60, 67), which is not the result expected if a substantial input to the lateral geniculate body were cholinergic. The question thus cannot be resolved from the available evidence. Its answer is of course irrelevant to our main conclusion, which is that the only neurons to release acetylcholine within the rabbit retina are a small group that are probably amacrine cells.

These results support conclusions reached from earlier studies in which Baughman and Bader carried out dry autoradiography of chicken (13) and turtle (personal communication) retinas exposed to a low concentration of choline. Acetylcholine accounted for about four-fifths of these retinas' total choline metabolism, so that the cells that accumulated the most radioactivity after exposure to low concentrations of choline were almost certainly those that synthesize acetylcholine. In both cases radioactivity accumulated in two bands within the inner plexiform layer and in cells at both of its margins. Although a few cells deep in the inner nuclear layer of the chicken retina also accumulated radioactivity, the overall pattern of labeling was very similar to the localization of acetylcholine seen in the rabbit retina. It is striking that the three species, differing widely in phylogenetic position, show a distinctive and nearly identical arrangement of acetylcholine-synthesizing cells.

#### *Functional Role of the Cholinergic Cells*

Cholinergic input contributes to the response to light of many retinal ganglion cells, but such input is not essential for a response: in electrophysiological experiments we found that cholinergic antagonists depressed, but never absolutely prevented, the cells' light-evoked activity. This was true despite the fact that the same antagonists could

entirely block the response to a test dose of exogenous acetylcholine, showing that the antagonists were appropriate to the retina's acetylcholine receptor (51). It thus appears that the acetylcholine-sensitive ganglion cell also receives input via neurotransmitters other than acetylcholine, and that the other neurotransmitters can sustain a substantial part of the cell's response to light. When one remembers that the retina also contains a group of ganglion cells that are not sensitive to acetylcholine at all, the evidence seems clear that the few acetylcholine-releasing cells identified in the present experiments are not part of an obligatory through-pathway from photoreceptor to ganglion cell.

The precise role of the acetylcholine-synthesizing cells remains to be learned. They must ramify widely, because the bands of label representing acetylcholine-containing processes were continuous throughout the inner plexiform layer. An arrangement in which a few widely ramifying cells have control—but an incomplete control—over many others evokes the possibility that the ramifying cells perform a rather global role, such as regulation of the excitability of the elements on which they synapse. Alternatively, the cholinergic cells could be part of the microcircuitry that shapes some highly restricted element of ganglion cell receptive fields. Such a function would have to be exquisitely specific, both because of the paucity of cholinergic cells and because of our experience that the grosser characteristics of receptive fields, such as center-surround organization, are not selectively affected by application of cholinergic agents to the retina (51). To understand the role of the cholinergic cells will require both detailed physiological experiments and higher resolution in anatomical ones, and the latter is particularly important because the designation "amacrine" (if indeed these are amacrine cells) is less informative than might once have been thought. It is now clear that there is a variety of amacrine cell subtypes, and a subpopulation as small as that constituted by the cholinergic cells could well have escaped either anatomical or physiological description. Because of this diversity, an exact definition of the acetylcholine-synthesizing cells will require methods by which their connections may be identified.

#### *Phospholipid Localization and Overall Choline Metabolism*

In our material the cells that label for acetylcho-

line stand out strongly from the others, and in autoradiographs exposed for short times one might have concluded that the [<sup>3</sup>H]choline incorporated into phospholipid formed a diffuse background throughout the retina. However, on closer examination we found that the photoreceptor cells and ganglion cells selectively accumulated [<sup>3</sup>H]choline compounds that were relatively water-insoluble, were fixed by osmium tetroxide, and were present when the retina was known to contain much [<sup>3</sup>H]phosphorylcholine and [<sup>3</sup>H]phospholipid but a negligible amount of [<sup>3</sup>H]acetylcholine. By microdissection we isolated the photoreceptors, and found that most of their water-soluble radioactivity was in the form of phosphorylcholine. As [<sup>3</sup>H]phosphorylcholine was progressively incorporated into [<sup>3</sup>H]phosphatidylcholine during a chase with unlabeled choline, the amount of fixable label in the photoreceptors and ganglion cells increased. Clearly, the photoreceptors and ganglion cells of these retinas synthesized a disproportionate amount of the retina's choline-containing phospholipid.

One expects photoreceptor cells to synthesize phospholipid rapidly because of the large amount of disk membrane required for outer segment renewal; in the rat, each rod must apparently replace something between 200 and 600  $\mu\text{m}^2$  of disk membrane per day (47). Phosphatidylcholine comprises ~45% of the total outer segment phospholipid (6–9). We found radioactive phospholipid in the outer nuclear layer, inner segments, and outer segments of the rabbit retina, with the greatest accumulation in the inner segments. These findings agree with conclusions reached by Young and his colleagues from experiments in which frog retinas were exposed to tritiated glycerol or choline (14, 83). The outer segments are apparently unable to synthesize phospholipids (14, 72), and the photoreceptors seem accordingly to synthesize phosphatidylcholine in the inner segments for export to the outer segments.

Similarly, the ganglion cells may require phospholipid for the maintenance of their axons: phosphatidylcholine is known to be transported down a variety of sensory and motor nerves (1, 11, 36, 75, 79). It is worth noting, however, that a greater fraction of the retina's total phosphatidylcholine synthesis occurs in the photoreceptors than in the ganglion cells (see Figs. 6 and 7), so that the photoreceptors dominate the retina's total metabolism of choline-containing phospholipid. The fact

that the photoreceptor cells synthesized a large amount of [<sup>3</sup>H]phospholipid at even a very low concentration of extracellular choline suggests that they may possess a specialized choline uptake system (35, 43, 44, 80), as a concomitant to their need for outer segment membrane renewal. Further evidence on this possibility will be presented elsewhere (52).

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