

# Analysis of Pre- and Postsynaptic Factors of the Serotonin System in Rabbit Retina

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**ABSTRACT** [<sup>3</sup>H]Serotonin is accumulated by a specific set of amacrine cells in the rabbit retina. These cells also accumulate the neurotoxin, 5,7-dihydroxytryptamine, and show signs of necrosis within 4 h of *in vivo* exposure to the drug. Biochemical analysis of [<sup>3</sup>H]serotonin uptake reveal a sodium- and temperature-dependent, high affinity uptake system with a  $K_m$  of 0.94  $\mu$ M and  $V_{max}$  of 1.08 pmol/mg protein/min. [<sup>3</sup>H]Tryptophan is also accumulated in rabbit retinal homogenates by a high affinity process. Accumulated [<sup>3</sup>H]serotonin is released in response to potassium-induced depolarization of intact, isolated retinas. *In vitro* binding studies of rabbit retinal homogenate membranes demonstrate specific sets of binding sites with characteristics of the postsynaptic serotonin receptor. These data strongly suggest that rabbit retina contains virtually all of the molecular components required for a functional serotonergic neurotransmitter system. The only significant difference between the serotonin system in rabbit retina and that in the well-established serotonin transmitter systems in nonmammalian retinas and in brains of most species is the relatively low concentration of endogenous serotonin in rabbit retinas, as demonstrated by high-performance liquid chromatography, histofluorescence, or immunocytochemistry.

Serotonin has been identified as a transmitter in nervous tissue from the brain and retina of many vertebrates. Retinas from frogs and goldfish contain relatively high levels of endogenous serotonin (14, 22), while bird and lizard retinas contain somewhat lower levels (7, 15). In these species, neurons that possess endogenous stores of serotonin (demonstrated by histofluorescence or immunocytochemical techniques) and/or serotonin uptake systems (demonstrated by histofluorescence, autoradiography, or neurotoxic reactions) have neurites that are limited to the inner plexiform layer. The cell bodies of these neurons are found primarily in the amacrine cell layer of the inner nuclear layer, although some are found in the ganglion cell layer and are thought to be displaced amacrine cell bodies. One exception is the report by Tornquist et al. (22) that describes accumulation of [<sup>3</sup>H]-serotonin by cells in the outer plexiform layer of pigeon and chick retinas, tentatively designated interplexiform cells.

Although serotonin is considered a strong transmitter candidate in the retinas of the nonmammalian species mentioned above, its role in mammalian retinas has been questioned because of the relatively low concentration of endogenous serotonin. Consistent with this finding, attempts to demonstrate endogenous serotonin by histofluorescence or immu-

nocytochemistry have failed. Thus, Ehinger et al. (4) (see also reference 7) have proposed that the true indoleamine transmitter is not serotonin but some other closely related compound. By using a microdansylation procedure, Osborne et al. (10, 12) observed that the low concentrations of serotonin in the bovine retina were localized in the inner nuclear and inner plexiform layers that could not be due exclusively to contamination from blood platelets as suggested by Floren and Hansson (7). With high-performance liquid chromatography (HPLC)<sup>1</sup>, serotonin was identified in at least a thousand times higher concentration than other indoleamines in retinas of lizards, frogs, cows, and rabbits (12). Therefore, the presence of serotonin, even though in low concentrations, as well as its specific localization, suggests that serotonin may be a viable candidate for a transmitter in the bovine retina.

Because many *in vivo* experimental manipulations are not feasible with bovine retinas and because relatively little is known about its physiology, we have chosen the rabbit retina for investigation of the retinal serotonergic system in mam-

<sup>1</sup>*Abbreviations used in this paper:* HPLC, high-performance liquid chromatography; DHT, dihydroxytryptamine; LSD, lysergic acid diethylamide.

mals. In the study reported herein, we provide a comprehensive morphological and biochemical analysis of both pre- and postsynaptic markers for the serotonin system. We offer this evidence to suggest that, in spite of low endogenous levels of serotonin, the rabbit retina possesses the molecular components necessary for serotonergic transmission. Properties of these components do not differ significantly from those of other neuronal tissues containing much higher levels of endogenous serotonin.

## MATERIALS AND METHODS

### *Uptake Studies*

Retinas were isolated from New Zealand white rabbits and rod outer segments were removed as previously described by Redburn (18, 19). The tissue was then hand-homogenized with five strokes using a Teflon pestle in 0.32 M sucrose.

Tissue samples containing ~250  $\mu$ g protein in a volume of 200  $\mu$ l modified Ringer's solution [pH 7.4] (10 mM glucose, 20 mM HEPES, 150 mM NaCl, 6.2 mM KCl, 1.2 mM  $MgSO_4$ , 10  $\mu$ M pargyline, and 100  $\mu$ M ascorbic acid) were preincubated at 37°C for 4 min. After preincubation, 50  $\mu$ l radioactive ligand ( $[^3H]5-HT$  29.1 Ci/mmol or  $[^3H]$ tryptophan 9.0 Ci/mmol [New England Nuclear, Boston, MA]) was added to the samples and the incubation continued for 4 min in a shaking water bath. The tissue samples were gassed with a mixture of 95%  $O_2$  and 5%  $CO_2$  during preincubation and incubation. 4 ml of buffer were added to the sample to stop the incubation and samples were then filtered under vacuum on GF/A filters (Whatman Ltd., Springfield Mill, Maidstone, Kent, U. K.). The filters were washed twice in 4 ml of buffer. Parallel incubations were carried out at 4°C to correct for nonspecific uptake. The amount of uptake at 4°C was ~10% of that at 37°C for both  $[^3H]5-HT$  and  $[^3H]$ tryptophan. The filters were removed and placed in vials and allowed to dry. A toluene base, Triton X-100 counting solution (16), was added and the samples were counted in a Searle Mark III liquid scintillation counter (TM Analytic, Inc., Elk Grove Village, IL).

### *Release Studies*

Intact retina were dissected from New Zealand white rabbits and placed in 0.5 ml oxygenated Krebs-bicarbonate Ringer's solution (pH 7.4) (4.7 mM KCl, 1.17 mM  $KH_2PO_4$ , 6.0 mM  $CaCl_2$ , 118 mM NaCl, 25 mM  $NaHCO_3$ , 11.1 mM glucose, 1.2 mM  $MgSO_4$ ) with 10  $\mu$ M pargyline, 100  $\mu$ M ascorbic acid, and 50  $\mu$ Ci  $[^3H]5-HT$ . Pargyline (10  $\mu$ M) and ascorbate (100  $\mu$ M) were added to all buffer solutions to prevent metabolism and oxidation of serotonin. Under these conditions, >80% of the recovered radioactivity was in the form of authentic  $[^3H]5-HT$  by thin-layer chromatography analysis. Retinas were incubated at 37°C for 15 min and gassed with 95%  $O_2$  and 5%  $CO_2$ . After the incubation period, the buffer was removed and retinas were rinsed with fresh buffer before being placed on 13 mM Metricel membrane filters (Gelman Sciences, Inc., Ann Arbor, MI). Filters were placed in a 2-ml perfusion chamber connected to a continuous flow pump. 1 ml Krebs-bicarbonate Ringer's solution containing 6  $\mu$ M imipramine, 10  $\mu$ M pargyline, and 100  $\mu$ M ascorbate was added manually every 60 s. The rate of flow on the pump was adjusted to pull the buffer through the chamber completely into a collecting vial before the next milliliter was added. After 10 ml of buffer was collected in this manner, the retinas were perfused with buffer containing 56 mM K to induce the release of serotonin. After a return to buffer containing only 6.2 mM K for 5 min, retinas were exposed again to depolarizing levels of potassium and perfusates were collected for 4 min.

### *Binding Studies*

Two distinct serotonin receptor binding sites in rabbit retina homogenate can be differentiated by radioactive ligand studies. Using the method of Peroutka and Snyder (17), 5-HT<sub>1</sub> receptor sites were labeled selectively by  $[^3H]$ lysergic acid diethylamide (LSD) (56.2 Ci/mmol)(New England Nuclear), displaced with 0.1  $\mu$ M serotonin, and 5-HT<sub>2</sub> receptor sites were labeled with  $[^3H]$ spiperone (23.0 Ci/mmol)(New England Nuclear) using 1  $\mu$ M cinanserin as the displacer. The binding assays were performed as follows. Retinas were removed from New Zealand white rabbits and stored at -5°C for at least 24 h before use. Thawed tissue was homogenized in 50 mM Tris-HCl buffer (pH 7.4) at 37°C using a Polytron at setting 6 for 30 s, and centrifuged at 48,000 g for 10 min. The supernatant was removed and pellet was resuspended in Tris-HCl buffer and centrifuged again. The final pellet was resuspended in Tris-HCl buffer containing 4 mM  $CaCl_2$ , 10  $\mu$ M pargyline, and 100  $\mu$ M ascorbic acid. Each assay tube contained 0.1 ml  $[^3H]$ -ligand, 0.1 ml displacer, and 0.8 ml 0.3-

0.5 mg protein/ml. Samples were incubated at 37°C for 10 min for  $[^3H]$ LSD binding and for 15 min for  $[^3H]$ spiperone binding. The incubation was terminated by rapid filtration under vacuum on GF/B filters (Whatman Ltd.) The filters were rinsed three times in 5 ml cold Tris-HCl buffer and were then placed in vials and allowed to dry. Triton X-100 counting solution was added and the samples were counted on a Searle Mark III liquid scintillation counter.

Kinetic analysis of the receptor sites was determined by varying concentrations of the  $[^3H]$ -ligand in the absence (total binding) or presence (nonspecific binding) of a displacer. Specific binding was defined as the differences between total binding and nonspecific binding. The specific binding was analyzed on a Scatchard plot to determine the  $B_{max}$  and  $K_d$  of the receptor sites. To determine the displacement potency of receptor agonists and antagonists, various concentrations of the displacer were added during incubation in the presence of the  $K_d$  concentration of the  $[^3H]$ -ligand. Results were plotted on log-probit paper to determine the  $IC_{50}$  from which the  $K_i$  was calculated.

In binding studies using bovine retinas, eyes were obtained from a local slaughterhouse. Retinas were removed and synaptosomal fractions  $P_1$  and  $P_2$  were prepared as previously described (18). Using differential centrifugation, large photoreceptor synaptosomes originating from the outer plexiform layer ( $P_1$ ) were separated from smaller conventional-sized synaptosomes formed primarily from amacrine cell neurites in the inner plexiform layer. Synaptosomes were then frozen at -5°C for a minimum of 24 h before analysis of  $[^3H]$ LSD binding.

### *Autoradiography*

Animals were decapitated and rapidly enucleated. An eyecup preparation was made and placed into a dish of aerated buffer as previously described (8). Portions of each eyecup were incubated in a 37°C water bath under ambient light in buffer containing 100  $\mu$ Ci/ml of  $[^3H]5-HT$ , 100  $\mu$ M ascorbic acid and 10  $\mu$ M pargyline. After 15 min, eyecups were removed and placed in 3% glutaraldehyde buffered with 0.05 M sodium cacodylate (pH 7.2) for 30 min at room temperature and subsequently refrigerated overnight. The tissue was rinsed with buffer and postfixed in 1%  $OsO_4$  buffered with 0.05 M sodium cacodylate for 1½ h at 0°C. After dehydration with a graded ethanol series, the tissue was embedded in Epon 812 Epoxy resin.

Thick sections (1  $\mu$ M) were cut with glass knives and placed onto glass slides cleaned with nitric acid. Slides were coated with Kodak MTB-2 emulsion, allowed to dry, and placed in light-tight boxes containing dessicant. Sections were incubated for 2-4 wk in the cold. They were developed in 1:1 Dektol for 2 min, rinsed, fixed in Kodak Fixer for 5 min, washed, and dried at 60°C. Some sections were stained with toluidine blue for comparison with unstained sections.

### *Treatment with 5,7-dihydroxytryptamine*

New Zealand white rabbits were anesthetized with an Acepromaxine-Ketamine Rompun cocktail (0.7, 21.4, and 4.3 mg/kg, respectively) via an intramuscular injection in the hind limb. Each animal also received 50 mg/kg pargyline i.p. 30 min after injection, the orbits of the eyes were bathed with 1% lidocaine hydrochloride and a 27- × 0.5-inch gauge needle was used for intraocular injections of 50  $\mu$ g of 5,7-dihydroxytryptamine (DHT) dissolved in 0.9% NaCl with ascorbic acid (1 mg/ml) as an antioxidant. Injection volume was 50  $\mu$ l. The first group of animals was killed 4 h after the intraocular injection and retinas were prepared for fluorescence microscopy. Single injections of 5,7-DHT were previously shown by Ehinger and Floren (3) to cause indoleamine-accumulating neurons to appear fluorescent. Thus, the first group of animals was used to verify the position and number of indoleamine-accumulating cells in normal retina. A second intraocular injection was given to the remaining animals on the following day. 1 wk later, a third intraocular injection of DHT was given 4 h before killing the remaining animals. Cells that accumulated the toxin were thus allowed to degenerate during the week-long interval, and their loss was made obvious by the specific loss of cells with DHT-induced fluorescence.

### *Histofluorescence*

Localization of catecholamine and indoleamines was visualized by histofluorescence using the method of de la Torre and Surgeon (23). Animals were decapitated and quickly enucleated. The globes were hemisected and the vitreous was gently removed. Eyecups were immediately placed into O.C.T. cryostat embedding medium (Lab-Tek Div., Miles Laboratories Inc., Naperville, IL) and frozen on dry ice for histofluorescent observation. Frozen 20- $\mu$ m sections were cut in a cryostat set at -20°C. The sections were placed on clean glass slides and immediately dipped into fresh glyoxylic acid stain (pH 7.4). The slides were blotted, dried under a stream of cold air for 3 min, then heated

in an 80°C oven for 5 min. Coverslips were mounted on with mineral oil and the sections observed under a Zeiss fluorescent photomicroscope III.

## Electrochemical HPLC

**REAGENTS:** Solutions of 100 ng/ $\mu$ l of DA (HCl), 5-HT (creatine sulfate), 5-HIAA (dicyclohexylammonium salt), and dihydroxybenzylamine (all Sigma Chemical Co., St. Louis, MO) were stored at -80°C and diluted daily to 1.0 ng/ $\mu$ l. Octane sulfonate (Fisher Scientific Co., Fair Lawn, NJ) solutions were passed through a C<sub>18</sub> Sep-pak (Waters Associates, Milford, MA) to remove contaminants. The chromatographic buffer consisted of 0.67% phosphoric acid, 0.1 mM EDTA, 10.0 mM octane sulfonate, 0.8 methanol, and NaOH to pH 2.7. All organic solvents were HPLC grade (MCB Manufacturing Chemists, Inc., Cincinnati, OH). Solutions were made with distilled water and filtered (0.22  $\mu$ m) with a Millipore apparatus and degassed under vacuum in a sonicating bath.

**TISSUE PREPARATION:** Pooled retinas (2-4) were homogenized by sonication (Sonicator W-10) (Heat Systems Ultrasonics, Inc., Plainview, NY) in 200  $\mu$ l 0.1 N perchloric acid containing 1.0 mg sodium metabisulfite and 5.0 ng dihydroxybenzylamine. The supernatant was then filtered (0.2  $\mu$ m) by centrifugation using microfiltration tubes (Bioanalytical Systems, Inc., West Lafayette, IN).

**HPLC:** A model M-45 solvent delivery system pumped (3.0 ml/min) the buffer to a U6K Universal injector, a 4-cm guard column packed with C<sub>18</sub> Corasil, and the chromatographic column (all Waters Associates). A Waters Z-module applied radial compression to the reverse-phase  $\mu$ m-Bondpak C<sub>18</sub> 10- $\mu$ m Radial-Pak column. Compounds of interest in the eluent were oxidized at +0.80 V using an LC-4 amperometric detector, TL-5 glassy carbon electrode, and RE-1 Ag/AgCl reference electrode (Bioanalytical System, Inc.). Peak areas were determined with a 3390A Hewlett-Packard (Hewlett-Packard Co., Palo Alto, CA) integrator.

## RESULTS

### Morphological Analysis

[<sup>3</sup>H]Serotonin is accumulated by cell bodies in the amacrine cell layer, and by processes in the inner plexiform layer (Fig. 1). Labeled cell bodies are 3-5  $\mu$ m in diameter and they are sparsely distributed in the amacrine cell layer. Labeling of neurites is highly laminated in a single band located on the innermost border of the inner plexiform layer representing approximately one-fifth of the total thickness of the plexiform layer.

A similar set of neurons is preferentially affected by the neurotoxin, DHT, which is specific for serotonin-accumulating neurons. A solution of DHT (50  $\mu$ g) and ascorbate (50  $\mu$ g) was injected intraocularly 1 h after the animal had received an intramuscular injection of pargyline. The contralateral eye (control) received an injection of the carrier solution. Retinas were removed 4 h after the DHT injection and processed for standard light microscopy and for histofluorescence using a modified glyoxylic acid method. As shown in Fig. 2, retinal morphology appears unaffected by the injection of the carrier alone. However, in retinas harvested 4 h after a single DHT injection, specific morphological damage was observed in a set of amacrine cell bodies with a size and distribution similar to those that accumulate [<sup>3</sup>H]serotonin (Fig. 3). The most prominent and consistent effect noted is nuclear chromatolysis. Some swelling in the inner plexiform layer is also noted.

1 wk after the initial injection of DHT, a second intraocular injection was administered, again preceded 2 h by an i.m. injection of pargyline. Four hours after the intraocular injection, retinas were prepared for microscopic examination (Fig. 4). Necrotic cells were not observed in these treated retinas presumably because the cellular debris of the DHT-sensitive cells had been previously removed.

Glyoxylic acid treatment forms fluorescent derivatives with the following catecholamines: dopamine, epinephrine, and norepinephrine; and with the following indoleamines: sero-

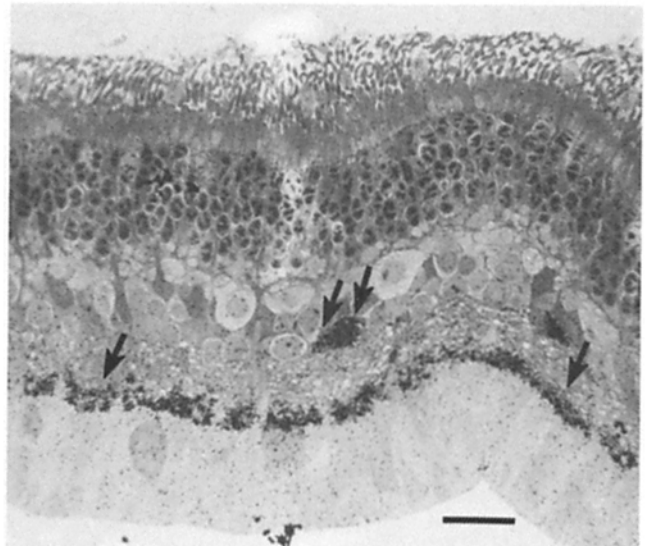


FIGURE 1 Autoradiography of [<sup>3</sup>H]serotonin accumulating cells of the rabbit retina. Isolated retinas were incubated in [<sup>3</sup>H]serotonin and processed for autoradiography. Heavy accumulations of label are seen in a sparse population of cell bodies (double arrows) in the amacrine cell layer and in a dense band (single arrows) at the innermost portion of the inner plexiform layer. Bar, 25  $\mu$ m.  $\times$  400.

tonin and dihydroxytryptamine. In control rabbit retina, glyoxylic acid-induced fluorescence is primarily confined to cells characteristic of dopaminergic amacrine (Fig. 5). Large fluorescent cell bodies are located on the innermost edge of the inner plexiform layer and three fluorescent lamellae are present in the inner plexiform layer with the outermost band being most prominent. The innermost portion of the inner plexiform layer (that coincides with the [<sup>3</sup>H]serotonin accumulating lamination) does not show fluorescence in control retinas.

In retinas treated with a single injection of DHT, an increase was observed in the number of fluorescent cell bodies, suggesting that DHT-accumulating cells as well as dopamine-containing cells show fluorescent properties. There was also an increase in the histofluorescence in the inner plexiform layer with the largest increase seen in the innermost portion, or [<sup>3</sup>H]5-HT accumulating area.

After a second intraocular injection of DHT given 1 wk later, retinal histofluorescent patterns were similar to controls and showed only the dopaminergic cell distribution. The specific set of DHT-accumulating fluorescent cells appeared to have been completely removed by the initial DHT treatment.

### Endogenous Levels of 5-HT

The endogenous content of the rabbit retina was determined using HPLC. Results are shown in Table I. The six retinas analyzed showed an average 5-HT content of 0.021 ng/mg wet weight. Dopamine content was roughly ten times that level, 0.219 ng/mg wet weight. The content of 5-HIAA, the major metabolite of 5-HT in brain, was 0.191 ng/mg wet weight.

### Uptake

The uptake of [<sup>3</sup>H]serotonin and [<sup>3</sup>H]tryptophan by rabbit retinal homogenates was analyzed by incubating homoge-

FIGURE 2 Morphology of control retinas 4 h after intraocular injection of isotonic saline solution. Cellular elements appear normal (cf. Fig. 1). Distinct nucleoli are seen within large, pale staining nuclei of the inner nuclear and ganglion cell layer with no evidence of chromatolysis. Bar, 20  $\mu\text{m}$ .  $\times 375$ .

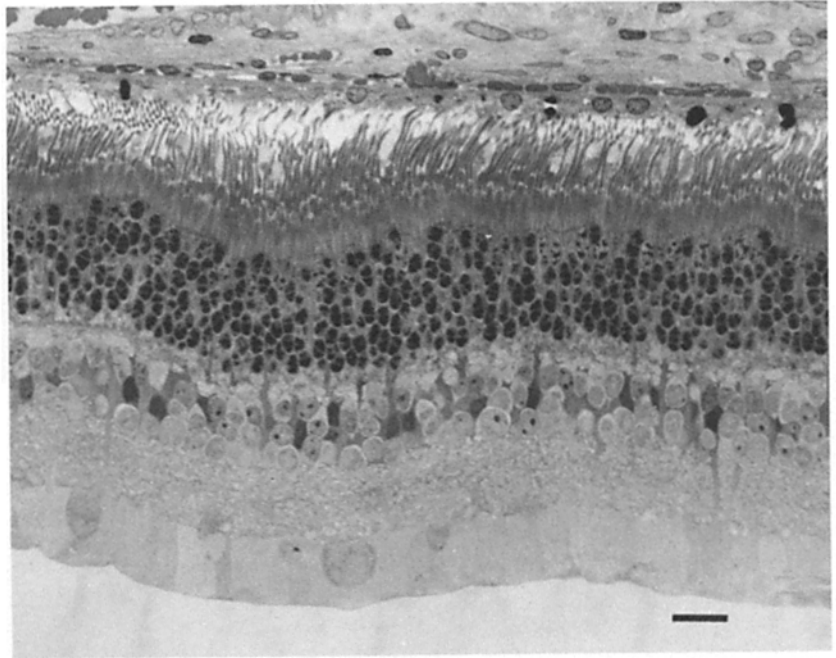
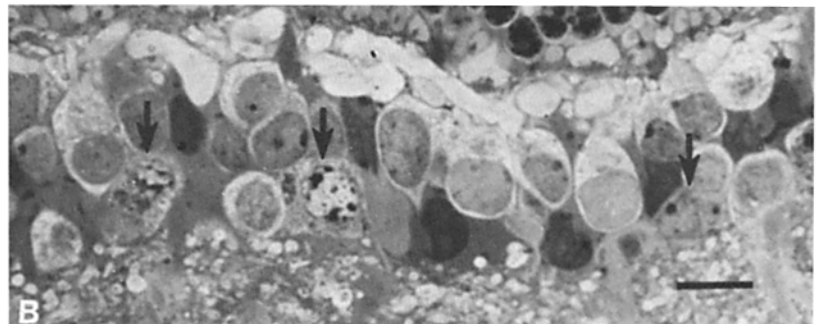
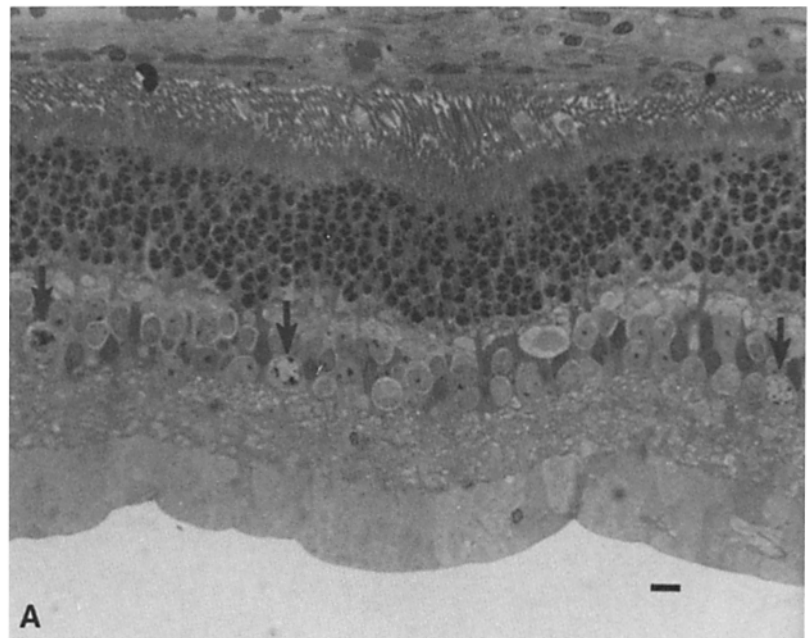


FIGURE 3 Morphology of retinas 4 h after intraocular injection of 5,7-DHT. Nuclear chromatolysis is observed in a widely scattered population of cell bodies in the amacrine cell layer (arrows). Some increased vacuolization may be present in elements of the inner plexiform layer. Other cell types appear relatively unaffected. (A) Bar, 10  $\mu\text{m}$ .  $\times 400$ . (B) Bar, 10  $\mu\text{m}$ .  $\times 1,000$ .



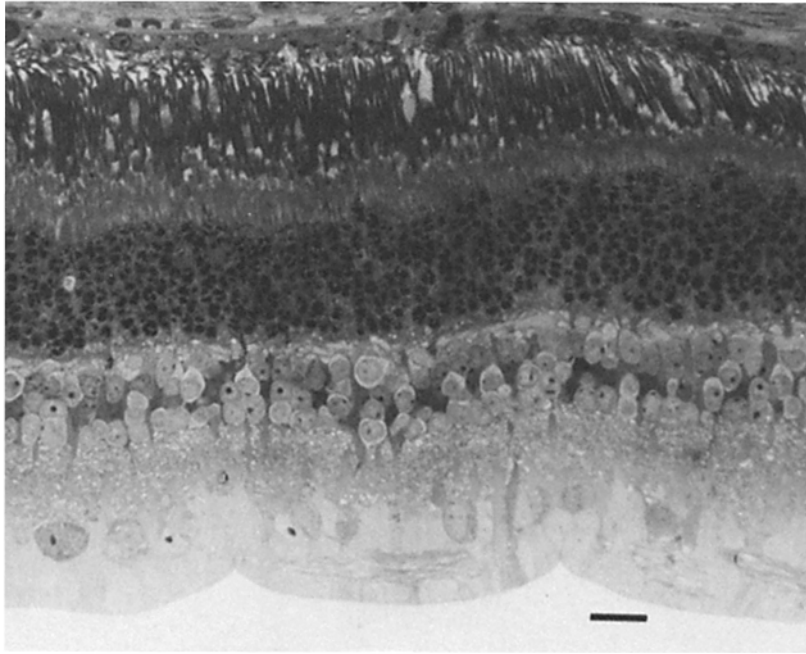


FIGURE 4 Morphology of retinas treated with two intraocular injections of 5,7-DHT given 1 wk apart. Retinas were harvested 4 h after the second injection. Necrotic cells are not observed. Bar, 20  $\mu\text{m}$ .  $\times 375$ .

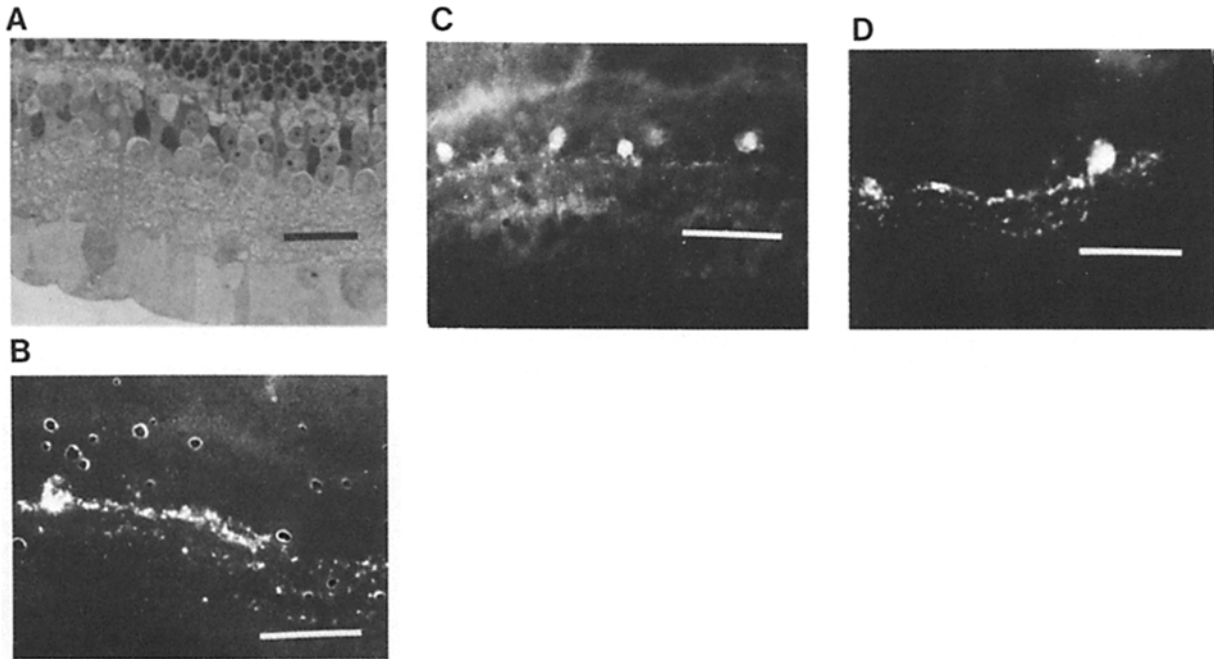


FIGURE 5 Histofluorescence in control and 5,7-DHT-treated retinas. (A) Normal morphology of the rabbit retina. (B)\* Histofluorescence of normal retinas. A sparse population of cell bodies in the amacrine cell layer exhibit fluorescence with a trilaminar banding of terminals in the inner plexiform layer. The outermost band is the most prominent. This pattern is characteristic of dopaminergic amacrine cells in this species. (C) Histofluorescence of retinas 4 h after an intraocular injection of 5,7-DHT. The number of fluorescent cell bodies in the amacrine cell layer is increased compared to control. In addition, the inner plexiform layer shows increased fluorescence particularly in the innermost region. This distribution of fluorescence corresponds to the combined distribution of dopamine-accumulating and serotonin-accumulating amacrine cells in this species. (D) Histofluorescence in rabbit retina receiving two intraocular injections of 5,7-DHT 1 wk apart and harvested 4 h after the second injection. The fluorescence pattern is indistinguishable from controls. (A) Bar, 25  $\mu\text{m}$ .  $\times 400$ . (B-D) Bar, 25  $\mu\text{m}$ .  $\times 575$ . \*(Reproduced from Redburn et al. [18], with permission of the publisher.)

nized retinas in 0.05  $\mu\text{M}$  – 1.0  $\mu\text{M}$  of radioactive ligand. The tissue was incubated at 37°C for 10 min and parallel incubations were carried out at 4°C. Accumulation of both compounds was highly temperature-dependent with uptake at 4°C equaling only 10% of that taken up at 37°C. The uptake for both ligands was saturable. For Lineweaver-Burke plot anal-

yses of these data, only temperature-dependent (uptake at 37°C minus that at 4°C) was plotted (Figs. 6 and 7). The results of the analysis suggest that both serotonin and tryptophan are taken up by a saturable, high affinity system in rabbit retina. In comparing the results of the two uptake systems, the amount of specific uptake per milligram protein

TABLE 1  
Endogenous Content of Dopamine, 5-HT, and 5-HIAA in Isolated Rabbit Retina Determined by Electrochemical HPLC Analysis

Rabbit	Dopamine	5-HT	5-HIAA
	ng/mg wet weight		
1			
Left retina	0.172	0.016	0.330
Right retina	0.187	0.047	0.227
2			
Left retina	0.176	0.013	0.126
Right retina	0.181	0.013	0.153
3			
Left retina	0.352	0.027	0.113
Right retina	0.251	0.015	0.197
Mean	0.291	0.021	0.191

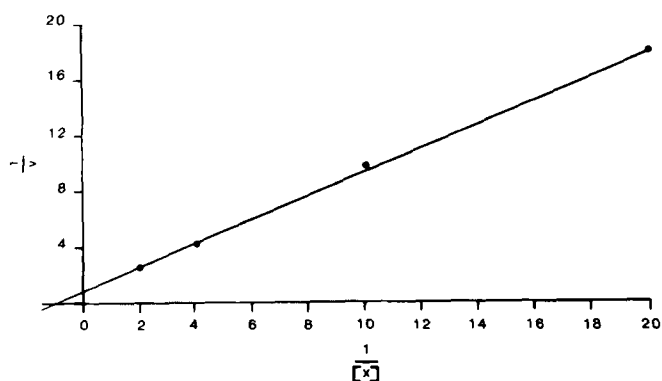


FIGURE 6 Lineweaver-Burke Plot of  $[^3\text{H}]$ serotonin accumulation by rabbit retinal homogenates. Parallel incubations were done in sodium medium at  $37^\circ\text{C}$  (total uptake) and in a sodium-free medium at  $0^\circ\text{C}$  (nonspecific uptake). Only specific uptake, the difference between the two values, is plotted. The slope of the line was determined by linear regression analysis ( $r = 0.99$ ). Apparent  $K_m = 0.94 \mu\text{M}$ ;  $V_{max} = 1.1 \text{ pmol/mg protein/min}$ .

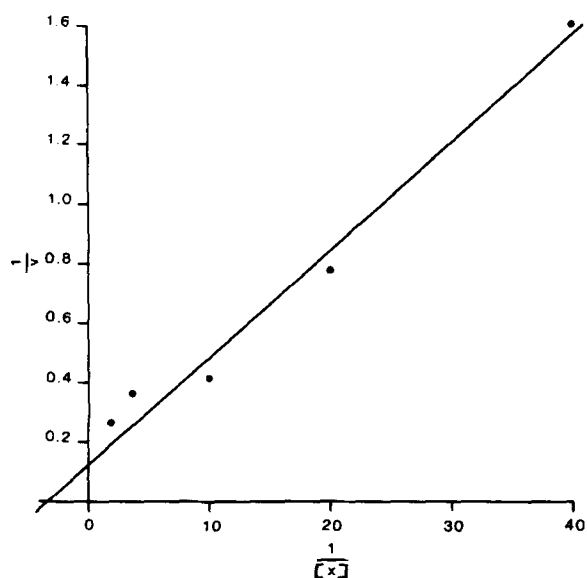


FIGURE 7 Lineweaver-Burke Plot of  $[^3\text{H}]$ tryptophan accumulation by rabbit retinal homogenates. Only specific uptake (the difference between total uptake measured in sodium-containing medium at  $37^\circ\text{C}$ , and uptake measure in sodium-free medium at  $0^\circ\text{C}$ ) is plotted. The slope of the line was determined by linear regression analysis ( $r = 0.98$ ). Apparent  $K_m = 0.26 \mu\text{M}$ ;  $V_{max} = 6.7 \text{ pmol/mg min}$ .

is approximately fivefold higher for tryptophan ( $6.7 \text{ pmol/mg protein/min}$ ) than serotonin ( $1.1 \text{ pmol/mg protein/min}$ ), and the uptake system for tryptophan also has a higher affinity ( $0.26 \mu\text{M}$ ) than the serotonin uptake system ( $0.94 \mu\text{M}$ ).

### Release

Intact, isolated rabbit retinas were first preincubated in  $[^3\text{H}]$ serotonin, then immobilized on a filter and perfused with standard buffer. Perfusate samples were monitored for  $\sim 10$  min, at which times a relatively stable rate of  $[^3\text{H}]$ 5-HT efflux was observed. The potassium concentration in the buffer was then raised to depolarizing levels ( $56 \text{ mM K}^+$ ). The resulting increase in release of  $[^3\text{H}]$ 5-HT observed in the perfusate from a typical experiment is shown in Fig. 8. In a series of nine experiments, the basal efflux rate was calculated to be  $\pm 2.0\%$  ( $n = 9$ ) of the total tissue pool of  $[^3\text{H}]$ 5-HT. The initial potassium-stimulated efflux rate was  $\pm 3.7\%$  ( $n = 9$ ), or approximately double that of the basal rate. After returning to normal buffer, a second potassium stimulation was applied, but evoked release under these conditions was either very small or nonexistent.

### Receptor Binding

In vitro binding studies indicate the presence of both  $5\text{-HT}_1$  and  $5\text{-HT}_2$  receptor sites in rabbit retina. In brain tissue, the  $5\text{-HT}_1$  site is described as agonist-preferring site based on its relatively high affinity of 5-HT and its relatively low affinity for certain antagonists such as cinanserin, mianserin, and methysergide compared with the  $5\text{-HT}_2$  antagonist site (5, 20). In rabbit retinal homogenate membranes, binding activity of the  $5\text{-HT}_1$  site was measured using  $[^3\text{H}]$ LSD as a ligand, and the amount of  $[^3\text{H}]$ LSD binding displaced by  $0.1 \mu\text{M}$  5-HT was taken as the measure of specific receptor binding (Fig. 9). The specific binding was saturable at low concentrations of the ligand. A Scatchard analysis of the specific binding

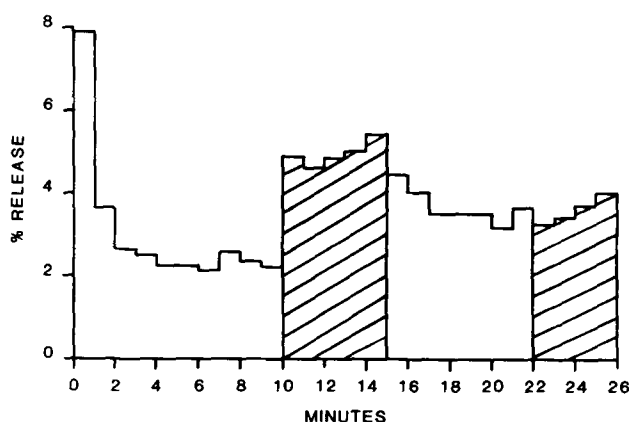


FIGURE 8 Release of  $[^3\text{H}]$ serotonin from isolated, intact rabbit retina. After preincubation in  $[^3\text{H}]$ serotonin, retinas were perfused with standard medium for 10 min, at which time a stable baseline of release had been reached. Depolarizing levels of potassium ( $56 \text{ mM}$ ) were then added to the perfusates for 5 min (striped bars). A doubling of the release rate was observed. A return to normal medium for 7 min caused a decrease in release rates. Reapplication of potassium failed to elicit the magnitude of response observed after the initial depolarization, although a slight rise in release rate was noted in the following 4-min perfusion period. Values represent results of a typical experiment. Mean values from a series of nine separate experiments are listed in the text.

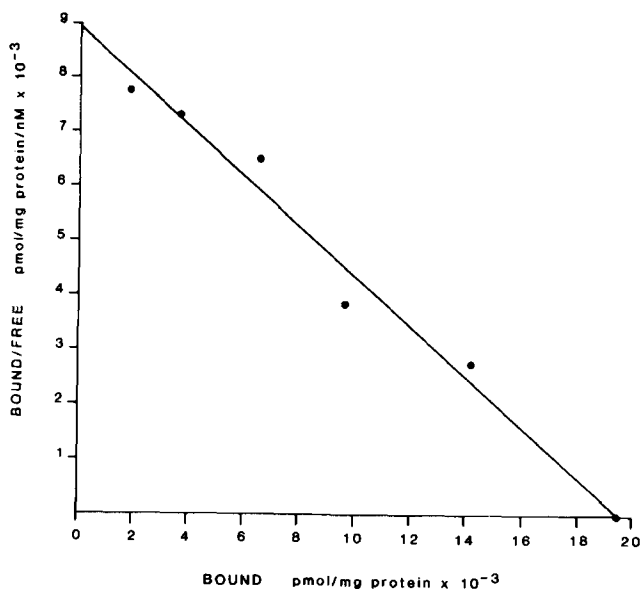


FIGURE 9 Scatchard Plot of specific binding of [<sup>3</sup>H]LSD displaced by 0.1 μM 5-HT. These conditions favor binding to the 5-HT<sub>1</sub> receptor site. As determined by linear regression, the apparent affinity of the site is 2.2 nM and the  $B_{max}$  is 0.019 pmol/mg protein.

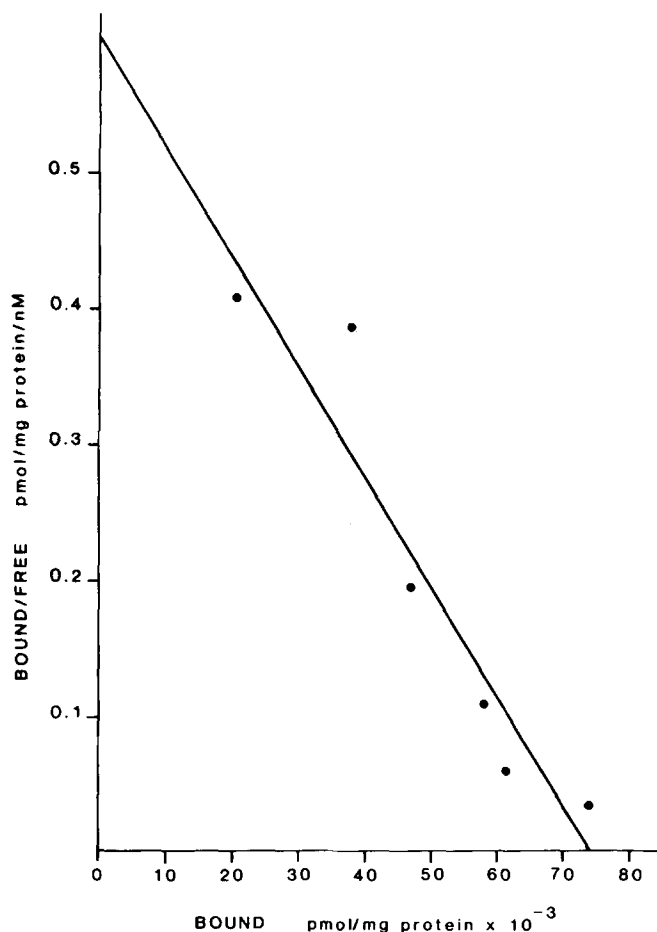


FIGURE 10 Scatchard Plot of specific binding of [<sup>3</sup>H]spiperone displaced by 1 μM cinanserin. These conditions favor binding to the 5-HT<sub>2</sub> site. As determined by linear regression, the apparent affinity of the site is 0.11 nM and the  $B_{max}$  is 0.072 pmol/mg protein.

revealed a binding site having an apparent affinity of 2.2 nM and a  $B_{max}$  of 0.019 pmol/mg protein.

Specific binding to the 5-HT<sub>2</sub> site was measured using [<sup>3</sup>H]spiperone as the ligand and determining the amount of the total binding that was displaced by 1 μM cinanserin. From Scatchard analysis, the apparent affinity of the site for the ligand was 0.11 nM and the  $B_{max}$  was 0.072 pmol/mg protein (Fig. 10). A series of six compounds were tested for their ability to displace binding at these two sites (Table II). Consistent with studies in the brain, binding to the 5-HT<sub>2</sub> site was more readily displaced by the antagonists: cinanserin, mianserin, and methysergide.

In a series of earlier experiments, we analyzed the specific binding to 5-HT<sub>1</sub> sites in bovine retinal synaptosomal fractions using [<sup>3</sup>H]LSD as the ligand and 1 mM 5-HT as the displacer. The  $P_2$  fraction, enriched in conventional-sized terminals predominantly from amacrine cell processes in the inner plexiform layer, contained significant levels of specific binding. In contrast, no specific binding was observed in the  $P_1$  synaptosomal fraction, which is enriched in photoreceptor cell terminals. The apparent affinity of the binding was 7.7 nM and the  $B_{max}$  was 0.37 pmol/mg protein. This represents only a slightly lower affinity of the site in bovine synaptosomes as compared to rabbit homogenate. However, the measure of the number of sites ( $B_{max}$ ) was over 10-fold greater in the bovine retinal synaptosomes than in rabbit retinal homogenates. In this series of experiments we also used [<sup>3</sup>H]5-HT as a ligand with 5-HT as the displacer for specific binding. Our results showed a virtually identical number of binding sites per milligram of synaptosomal protein ( $B_{max}$  = 0.31 pmol/mg protein). The affinity of the site for 5-HT was somewhat lower than for LSD (14 vs. 7.7 nM). The potency of four different compounds in displacing [<sup>3</sup>H]LSD binding in bovine

TABLE II  
5-HT Receptor Binding in Rabbit Retina Homogenates

Binding constants derived from Scatchard analysis		
5-HT <sub>1</sub> receptor sites	5-HT <sub>2</sub> receptor sites	
[ <sup>3</sup> H]LSD binding (0.1 μM 5-HT displacer)	[ <sup>3</sup> H]spiperone binding (1 μM cinanserin displacer)	
$B_{max}$ - 0.019 pmol/mg protein	$B_{max}$ - 0.072 pmol/mg protein	
$K_D$ - 2.2 nM	$K_D$ - 0.11 nM	
Pharmacological sensitivity of 5-HT binding sites		
Drug	$K_i$ (μM) for 5-HT <sub>1</sub> sites	$K_i$ (μM) for 5-HT <sub>2</sub> sites
5-HT agonists		
Serotonin (5-HT)	0.25 ± 0.03	5.5 ± 0.5
5-HT antagonists		
Cinanserin	131.5 ± 7.3	8.5 ± 0.6
Mianserin	68.5 ± 6.1	16.0 ± 0.8
Methysergide (LSD analogue)	27.9 ± 2.4	10.5 ± 1.1
Mixed agonist		
Trazadone	3.31 ± 0.9	12.0 ± 2.2
Dopamine agonist		
ADTN	>1,000	>1,000

5-HT and dopamine agonists and antagonists were tested at various concentrations to determine their potency in displacing 5-HT receptor binding. 2 nM [<sup>3</sup>H]LSD was used as the ligand for 5-HT<sub>1</sub> sites and 0.1 nM spiperone was used as the ligand for 5-HT<sub>2</sub> sites. The  $K_i$  values were calculated as follows:  $K_i = (IC_{50} [\text{concentration required to displace 50\% of the ligand}]) / ((\text{concentration of ligand used})/K_d \text{ of ligand} + 1)$ .

retinal synaptosomes was similar to that in rabbit retinal homogenate.

## DISCUSSION

### *Serotonin Content*

A great deal of effort and discussion has been directed toward the question of whether or not the mammalian retina contains sufficient amounts of serotonin to be considered a viable transmitter candidate. There is considerable species variation; however, retinas from most other species contain significantly higher levels of serotonin than found in mammals (10, 12, 13). Frog retina contains the highest concentrations of serotonin (500–1,700 ng/gm wet weight) (14, 22). Lizard (100 ng/gm wet weight), goldfish (230–1,100 ng/gm wet weight) (see references 14, 22), and bird (34–119 ng/gm wet weight) (see references 7, 15) retinas contain intermediate levels. Mammalian retinas contain the lowest levels ranging from 5 ng/gm wet weight in the pig and guinea pig to 30 ng/gm wet weight in the cow (14, 22).

Using HPLC analysis, we found the endogenous content of serotonin in rabbit retinas to be 21 ng/g wet weight, which is in good agreement with previously published figures using similar methods (12). The major metabolite of serotonin, 5-HIAA, was detected in rabbit retina at levels considerably higher than that of serotonin. The level of dopamine present in this retina is roughly 10-fold greater than that of serotonin. The autoradiographic studies discussed below are consistent with the suggestion that the dopaminergic system appears to represent a much larger neuronal population than the serotonergic system. Some investigators, (6, 7) have argued vigorously that the amount of serotonin present in mammalian retinas is not consistent with a role as a retinal transmitter. However, lower limits have never been delineated for any neurotransmitter below which transmitter function could not be carried out.

A comparison of the relative concentrations of serotonin with those of other transmitter substances is helpful in suggesting which system may be more prominent. However, it is not valid to suggest that the small amount of serotonin is not used as a transmitter. At this point a more valid approach is to turn to other criteria to further establish or disprove its transmitter role.

### *Serotonin and Tryptophan Uptake Systems*

The termination of serotonergic transmission is primarily achieved by removal of released serotonin from the synaptic cleft by passive diffusion and by active uptake into presynaptic neurons. Active uptake of serotonin requires the presence of sodium and chloride ions and it is energy- and thus temperature-dependent (20). Accumulation of [<sup>3</sup>H]5-HT by rabbit retinal homogenates was found to be both sodium- and temperature-dependent. The uptake was saturable at low concentrations, with an apparent affinity for the transport site of 0.94  $\mu$ M. Only a single affinity was observed; however, higher affinity sites were not rigorously ruled out. The values are in good agreement with those previously published (2).

[<sup>3</sup>H]Tryptophan was also accumulated by rabbit retina homogenates by a high affinity system ( $K_m = 0.26 \mu$ m) and the maximum velocity of this system (6.7 pmol/mg protein/min) was more than fivefold greater than that for serotonin (1.1 pmol/mg protein/min).

### *Serotonin Release*

The release of [<sup>3</sup>H]5-HT from isolated retinas was stimulated by depolarizing levels of potassium. The stimulated release represented a doubling of the resting release rate in the absence of potassium. It is interesting to note that a second pulse of potassium often failed to elicit a stimulation of release. The fatigability seen in this system is much more pronounced than that in other neurotransmitter release systems that have been studied in this retina and others. This may suggest that the releasable pool of serotonin is very small or alternatively that inhibitory autoreceptors located on presynaptic serotonergic terminals are very effective in inhibiting sustained release.

### *Autoradiographic Analysis of [<sup>3</sup>H]5-HT Uptake Sites in Rabbit Retina*

Serotonin uptake sites in the rabbit retina appear to be localized to specific neuronal populations as demonstrated by autoradiography of [<sup>3</sup>H]5-HT. Serotonin-accumulating neurons represent a small group of amacrine cells whose terminals are restricted to a discrete band in the innermost portion of sublamina b of the inner plexiform layer. The terminal arborization of each individual neuron appears to be very widespread. Although the distance between labeled cell bodies was usually substantial, the labeled band of terminals was, without exception, continuous throughout the section. Thus, the neuritic spread of each neuron must be extensive.

### *Treatment with DHT*

DHT has a specific toxic effect on serotonergic neurons in the brain and this has provided a tool for both morphological and physiological investigations of the serotonin system (9). DHT toxin is accumulated intracellularly by serotonergic neurons via the high affinity transport system for serotonin. Once inside the cell, DHT becomes irreversibly bound to intracellular structures of serotonergic neurons and thus causes the death of the neuron. Since DHT forms a fluorescent derivative when exposed to glyoxylic acid, its presence in specific sets of neurons can be observed directly.

Intraocular injections of DHT into rabbits produced highly specific morphological lesions. 4 h after the injection, a small group of amacrine cells showed signs of necrosis and chromatolysis. The remaining cells of the retina were unaffected. 1 wk after the injection, the necrotic cells were no longer observed, presumably because the cellular debris has been previously removed. At this time, a second injection of DHT has no visible effect on the retina, consistent with the suggestion that the DHT-sensitive cells had been previously removed by the first DHT-treatment.

Histofluorescent analysis of these sections supports this interpretation, although the results are somewhat obscured by the histofluorescence of the dopaminergic amacrine cells. In control retinas, the characteristic histofluorescence pattern of the dopamine system is seen, characterized by a trilaminar arrangement of terminals in the inner plexiform layer. 4 h after DHT treatment, there is an increase in the apparent number of fluorescent cell bodies and an enhancement of the fluorescence in the innermost portion of the inner plexiform layer. This suggests that in addition to the normally fluorescing dopamine cell population, the serotonergic cell bodies have become fluorescent due to the accumulation of DHT. 1 wk after the initial injection, retinas showed only the dopa-



mine fluorescent pattern, both before and after a second injection of DHT. Thus, the serotonergic neurons appear to be specifically destroyed by the treatment, and dopaminergic neurons appear unaffected. These data are in agreement with previous observations by Ehinger and Floren (3), except that they did not report chromatolysis after 4 h of exposure.

### Receptor Binding

Two distinct 5-HT receptors have been differentiated in mammalian brain based on electrophysiological, pharmacological, behavioral, and ligand-binding studies (for review see references 5, 21). The 5-HT<sub>1</sub> receptor has a high affinity for 5-HT and is thought to mediate inhibitory input. It appears to be regulated by guanine nucleotides and may be linked, in part, to adenylate cyclase. LSD is an agonist at this site and metergoline is a high affinity antagonist. In contrast, the 5-HT<sub>2</sub> receptor mediates excitation and is antagonized by LSD. In addition, a wide variety of neuroleptics and antidepressants, such as cinanserin, mianserin, and chlorpromazine, are potent antagonists at this site. Based on these criteria, the rabbit retina demonstrates the presence of both 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptor sites.

The 5-HT<sub>1</sub> sites in bovine retina were measured using both [<sup>3</sup>H]5-HT and [<sup>3</sup>H]LSD as ligands. [<sup>3</sup>H]LSD binding was associated mainly with fractions enriched in terminals from the inner plexiform layer. Affinities of the two ligands observed in these assays were similar to that reported in whole retinal. The number of sites in bovine retina was also significantly higher than in rabbit retina.

Osborne (11) has described a single binding site in bovine retina using [<sup>3</sup>H]serotonin as a ligand, with a  $K_d$  value of 7.6 nM and a  $B_{max}$  of 3.8 pmol/gm. Binding sites were enriched in the synaptosomal fraction from the inner plexiform layer (13 fmol/mg protein), compared with the synaptosomal fraction from the outer plexiform layer (6 pmol/mg protein) and whole retinal homogenate (8 fmol/mg protein). Serotonin agonists and antagonists were potent displacers of the binding. Since the 5-HT<sub>1</sub> site has a higher affinity for 5-HT than does the 5-HT<sub>2</sub> site, and [<sup>3</sup>H]5-HT was used as a ligand in these studies, it is reasonable to assume that most of the binding reported was preferentially associated with the 5-HT<sub>1</sub> site. The pharmacological specificity of [<sup>3</sup>H]5HT and [<sup>3</sup>H]LSD binding sites in both bovine and rabbit retina is consistent with that expected of a 5-HT<sub>1</sub> site (see Table II). The  $IC_{50}$  values for 5-HT, methyergside, and mianserin displacement of [<sup>3</sup>H]5-HT binding in the bovine and rabbit retina are in general agreement with those reported for the 5-HT<sub>1</sub> site in brain. In addition, [<sup>3</sup>H]5-HT binding in bovine retina was inhibited by guanine nucleotides which is a specific characteristic of 5-HT<sub>1</sub> sites in brain. These results raise the possibility that at least a portion of the retinal serotonergic system may use 5-HT<sub>1</sub> receptors, and furthermore that secondary messengers such as cyclic nucleotides may be used to amplify the postsynaptic signal.

### SUMMARY

We have surveyed a wide variety of serotonergic properties associated with the rabbit retina. In every case, the rabbit retina appears to possess the characteristics necessary for serotonergic transmission. However, the serotonergic system in rabbit retina appears to be less prominent than other mammals, such as in cows. Fewer cell bodies are labeled by [<sup>3</sup>H]5-HT or by the specific serotonergic neurotoxin, 5,7-

DHT. Stimulated release of [<sup>3</sup>H]5-HT can be demonstrated; however, the system appears to fatigue very quickly so that a second stimulation often fails to produce measurable release. 5-HT<sub>1</sub> receptors are present in rabbit retina in lower concentration than in bovine; however, these receptors may activate cyclic nucleotides as second messengers; the effect of activating a small number of receptors may be subsequently amplified to produce significant postsynaptic responses. In support of this suggestion, Ames and Pollen (1) report that serotonin does enhance the electrophysiological activity of ganglion cells in the perfused rabbit retina.

These findings are consistent with the suggestion that serotonin itself is a functional neurotransmitter in the rabbit retina but that it represents a very small neuronal component. There is little evidence to support the suggestion that another indolamine is the true neurotransmitter. The question of its functional significance remains to be determined. However, it is not unreasonable to assume that the small but continuous band of serotonergic influence in sublamina b in the inner plexiform layer could have an important role in information processing in the retina.

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