Investigations into whether 5-hydroxytryptamine is a neurotransmitter in the retina of rabbit and chicken
Ingrid Floren and H. Christer Hansson

A system of indoleamine-accumulating neurons exists in the retina of several species, but the exact transmitter of these neurons is not known. 5-Hydroxytryptamine (5-HT) is a likely candidate, but there are conflicting reports on its amount in the retina. We have determined the amounts of 5-HT with two sensitive, specific, and independent methods. Because the content was found to be lower than what was expected for a neurotransmitter, the rate-limiting enzyme in the 5-HT synthesis was also estimated, as was the effect of 5-HT on cyclic AMP content of the retina. In the rabbit the 5-HT content was found to be 25 to 35 ng/gm wet weight, with no difference between light- and dark-adapted animals. There was no detectable activity of the rate-limiting enzyme tryptophan hydroxylase. It was not possible to raise the 5-HT concentration by treating the rabbits with a monoamine oxidase inhibitor and L-tryptophan. 5-HT induced no change in the cyclic AMP content of the rabbit retina. In both newly hatched and older chicken retina, the 5-HT concentration was higher than in rabbit. The 5-HT concentrations in all retinas were lower than would be expected for a monoamine neurotransmitter, and these results argue against 5-HT being the neurotransmitter of the indoleamine-accumulating neurons.

Key words: 5-hydroxytryptamine, tryptophan hydroxylase, dopamine, cyclic AMP, retina, rabbit, chicken

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ported that a special indole-containing retinal cell was present during a short embryonic phase; they suggested that it contained 5-hydroxytryptamine (5-HT). There are conflicting reports of the presence of 5-HT in the retina of several species, including rabbit and chicken.\textsuperscript{10–13} Although 5-HT seemed to be the most plausible transmitter candidate of the indoleamine-accumulating neurons, the difficulty of demonstrating these neurons in untreated retinas with the histofluorescence method of Falck and Hillarp makes it probable that their 5-HT content is low. It therefore seemed important to measure the retinal concentration of 5-HT by the more sensitive and specific enzymatic isotopic microassay\textsuperscript{14} and high-performance liquid chromatography (HPLC) methods.\textsuperscript{15} Rabbits were chosen because of the easily demonstrable indoleamine-accumulating neurons in their retina, and chickens because of their earlier reported indole-containing retinal cells.

By demonstration of the existence of a proposed transmitter, only one of several criteria is fulfilled. In addition, enzymes that can synthesize and degrade the transmitter must be located at the place of its action. 5-Hydroxytryptophan decarboxylase, one of the two enzymes that synthesize 5-HT, has been reported to be present in the retina,\textsuperscript{16} as has monoamine oxidase (MAO), which is the degrading enzyme of 5-HT.\textsuperscript{16–18} Both enzymes are, however, nonspecific because they are also required in the metabolism of dopamine (DA), an established retinal transmitter. Since tryptophan hydroxylase is the rate-limiting enzyme for the synthesis of 5-HT from its precursor L-tryptophan, it was considered necessary to determine the existence of this enzyme, particularly since it has not previously been identified in the retina.\textsuperscript{19}

Much attention recently has been focused on cyclic AMP as a "second messenger" in the nervous system. Several neurotransmitters, including 5-HT, have been shown to increase cyclic AMP levels in the brain.\textsuperscript{20, 21} DA stimulates the production of cyclic AMP in the retina,\textsuperscript{22, 23} but 5-HT lacks a similar effect on the level of cyclic AMP in the retina of calf and rat.\textsuperscript{22} Because indoleamine-accumulating neurons have not been demonstrated in these species, we investigated whether 5-HT could cause an increase of cyclic AMP in rabbit retina.

**Materials and methods**

*Experimental animals* were rabbits weighing 1 to 2 kg, newly hatched chicks, and chickens of 11 to 14 weeks (Derko, a hybrid between Rhode Island Red and White Plymouth Rock). The rabbits were sacrificed by air embolism, and the newly hatched chicks and chickens by decapitation, the latter under ether anesthesia.

*Tryptophan hydroxylase activity* was measured as described by Kuhar et al.\textsuperscript{24} with the method of trapping $^{14}$CO$_2$ released from L-[1-$^{14}$C]tryptophan (39.8 mCi/mmol; The Radiochemical Center, Amersham, England). The method requires a sufficiently low concentration of the substrate to eliminate its direct decarboxylation. To avoid this a final L-tryptophan concentration around $6 \times 10^{-6}$M is necessary because the apparent KM for the decarboxylating enzyme is $3 \times 10^{-3}$M for tryptophan and thus much higher than that for 5-hydroxytryptophan (2 $\times 10^{-3}$M).\textsuperscript{25}

Rabbit retinas were pooled (200 to 250 mg) and homogenized in 10 vol of oxygenated 0.1M Tris-acetate buffer, pH 8.1, with 0.2 mg/ml ascorbic acid. All samples were assayed in duplicate. Buffer without tissue was used for blanks. L-[1-$^{14}$C]tryptophan was added to 0.9 ml of the homogenate, and this was then incubated for 15 min at 21° C in flasks containing 95% O$_2$ and 5% CO$_2$. The produced $^{14}$CO$_2$ was captured in center wells containing a mixture of ethylene glycol monomethyl ether–monoethanolamine. The reaction was terminated by adding perchloric acid, and the flasks were then incubated for 1 hr more at 37° C to capture all CO$_2$. The produced $^{14}$CO$_2$ was captured in center wells containing a mixture of ethylene glycol monomethyl ether–monoethanolamine. The reaction was terminated by adding perchloric acid, and the flasks were then incubated for 1 hr more at 37° C to capture all CO$_2$. The ethylene glycol monomethyl ether–monoethanolamine mixture was transferred to scintillation vials, and the radioactivity was determined with quenching corrected by the external standard channels ratio method. Conditions as those described above were adopted as standard with rabbit retina as well as rabbit midbrain. Various additional requirements for optimal assay of tryptophan hydroxylase have been proposed,\textsuperscript{26} and the following modifications in the basic procedures were made.\textsuperscript{27, 28} In one experiment 0.54 $\mu$mol 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (DMPH$_4$), 180 nmol pyridoxal phosphate (both from Sigma Chemical Co., St. Louis, Mo.), and 18 nmol ferrous sulfate were...
added. The same co-factors were also tested after a change of pH from 8.1 to 7.5. Boiled tissue was used for the blanks on one occasion.

Aromatic amino acid decarboxylase activity with DOPA as substrate was assayed by trapping [l-14C]alanin (DL-DOPA-[14C]carboxyl) (53 mCi/mmol; Radiochemical Center), which was added to a final concentration of $6 \times 10^{-6}$M. Assay conditions were identical with the standard assay system for tryptophan hydroxylase activity except for the addition of 60 or 540 mmol pyridoxal phosphate.

5-HT was measured by an enzymatic-isotopic microassay described by Saavedra et al. In short, tissue was homogenized in 10 vol of 0.1M HCl and centrifuged. To a portion of the supernatant, partially purified rat liver N-acetyl-transferase (a gift from Dr. A. Bjorklund), which is present in the pineal gland, was then added to the supernatant. This enzyme transfers the [3H]methyl group from N-acetyl-5-HT and thus forms [3H]melatonin. This final product was extracted into toluene, and the radioactivity was measured. The amount of 5-HT was calculated from a standard curve derived from samples with known amounts of 5-HT (obtained as creatinine sulfate from Sigma).

5-HT was also measured with HPLC by a method described by Hansson and Rosengren. The two retinas of one animal were homogenized in 2 ml of 0.4M HClO$_4$ together with $\alpha$-methyl-5-HT (a gift from F. Hoffmann-La Roche, Basel, Switzerland) as an internal standard. After centrifugation and adjustment of pH to 6.0, the sample was purified and concentrated on a weakly acidic cation exchange resin (Amberlite IRP-64). 5-HT as well as other adsorbed amines and the internal standard were then eluted with 2 x 0.5 ml of 1.2M HCl. One hundred microliters of the eluted volume were introduced with a Rheodyne Model 7120 sampling valve injector and chromatographed on microparticulate reverse-phase packing material with chemically bonded octadecyl groups on 5 /m silica (Nucleosil C18) using a Waters Model 6000-A high-pressure pump. Elution was carried out by using 12% methanol with 2.9 gm/L phosphoric acid and 10.0 gm/L methanol sulfonic acid at pH 2. The flow rate was 1.0 ml/ min. Detection was performed by a thin-layer amperometric detector (Model LC-10; Bioanalytical Systems, Inc., West Lafayette, Ind.). The ratio between the height of the 5-HT peak and that of the internal standard $\alpha$-methyl-5-HT was calculated. The 5-HT amount was then obtained from a standard curve. In addition to normal animals, three experimental groups were studied. All animals were sacrificed at 9 A.M. One group was given reserpine (Serpatil; CIBA-Geigy, Basel, Switzerland), 2 mg/kg intraperitoneally, 20 hr before sacrifice in order to deplete the monoamine stores. Another group was given a 50 mg/kg intraperitoneal dose of a MAO inhibitor (Pargyline; Sigma), 30 min before 50 $\mu$g of the 5-HT precursor L-tryptophan (Sigma) was injected into the vitreous humor. The retinas were processed 4 hr later. A third group was kept in darkness for 24 hr. The dark-adapted retinas were dissected under infrared illumination with the aid of darkroom image conversion goggles (N. V. Optische Industri "De Oude Delft," Delft, Holland). Light-adapted control retinas were similarly dissected to exclude the influence of otherwise unavoidable variation in the dissection technique in dark and light. The dissections under infrared illumination took only a few minutes, so that the light-adapted retinas did not dark-adapt to any significant extent. There

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<th>Table 1. 5-HT concentration in retina (ng/gm wet weight)</th>
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| All values are means ± S.E.M.; number of determinations within parentheses; n.d. = not determined. Values A vs. B, C vs. D, C vs. F, and D vs. E show no statistical difference (p > 0.05), G vs. H is statistically different (p < 0.001).
Fig. 1. Dopamine concentration in rabbit retina determined by HPLC. Rabbits were dark-adapted for 24 hr or given reserpine (2 mg/kg i.p.) 20 hr before sacrifice. Each bar is shown as mean ± S.E.M. Statistical calculations were made with Student’s t test. N.S., No significance (p > 0.05).

was no significant difference between the light-adapted retinas that were dissected in dark and those dissected in light (Table I). The retinas of the dark-adapted and light-control animals were dissected submerged in ice-cold 0.9% NaCl in order to avoid contamination with choroidal blood. After removal the tissue was stored in liquid nitrogen (−196°C) for less than 1 hr before it was analyzed.

DA was measured by HPLC as described for 5-HT but with the following modifications. Two retinas were homogenized in 2 ml of 0.4M HC1O4, and the sample was centrifuged. To 1.2 ml of the supernatant was added 1 ml of concentrated acetic acid and 0.4M HC1O4 to a final volume of 20 ml. The sample was then adsorbed on A12O3, pH 8.6, and eluted with 3 ml of 0.1M HC1. A 100 μl amount of eluate was analyzed on the chromatographic system using an aqueous mobile phase with 2.9 gm/L phosphoric acid and 6.0 gm/L methane sulfonic acid. The pH was adjusted to 1.75 with 5M sodium hydroxide. The flow rate was 1.5 ml/min. Known amounts of DA (obtained as the hydrochloride from Sigma) were analyzed by HPLC immediately before and after each sample. The DA content of the sample was then calculated by comparing the height of the peaks of the standards with that of the sample. Other rabbits were treated with reserpine or dark-adapted as described above.

Cyclic AMP was measured in intact rabbit retina as described by Bucher and Schorderet29 and Schorderet.30, 31 Retinas were dissected submerged in ice-cold Krebs-Binger bicarbonate buffer, divided into halves along the vertical axis, and then preincubated in the same buffer, pH 7.4, at 35°C under continuous gassing with 95% O2 and 5% CO2. After the 40 min incubation period, one half retina was transferred to a glass homogenizer containing fresh buffer, 5 mM theophylline (Sigma), 4 mM EDTA, and the pharmacological agent to be tested in a total volume of 0.5 ml. The incubation was terminated after 10 min by transferring the homogenizers into boiling water for 10 min. The tissue was then homogenized at 4°C and centrifuged at 12,000 x g for 10 min at 4°C, and the protein in the pellet was determined according to the method of Lowry et al.32 Duplicate 50 μl samples of the supernatant were analyzed for cyclic AMP with an assay kit (Radiochemical Centre).

Results

Tryptophan hydroxylase activity in rabbit retina. Equal amounts of retina and brain tissue were run simultaneously in each experiment. Enzyme activity was easily measured in the standard assay system in brain (22.5 nmol 14CO2/gm wet weight/hr; S.E.M. = 4.2, n = 5) but could not be detected in retina. The limit of detectability was found to be about 1 nmol 14CO2/gm wet weight/hr, indicating that the retina has a concentration of tryptophan hydroxylase at least 20 times less than that in the brain. Changing the pH from 8.1 to 7.5 either with or without adding DMPH4, pyridoxal phosphate, and ferrus sulfate to the standard assay system did not increase the enzyme activity in either retina or brain.

Aromatic amino acid decarboxylase activity in rabbit retina. Decarboxylase activity was measured in retina and brain with 1-[14C]DOPA as substrate but otherwise under the same conditions as the standard assay system for tryptophan hydroxylase. The decarboxylase activity was also measured with two different concentrations of pyridoxal phosphate.
added. All three systems yielded virtually the same results. The enzyme activity was determined to be 5.2 µmol \( ^{14}\)CO\(_2\)/gm wet weight/hr in brain (S.E.M. = 0.6, n = 6) and 0.26 in retina (S.E.M. = 0.05, n = 4). The figures are the averages for all the analyses with or without pyridoxal phosphate. The enzyme activity in the retina is thus less than that in the brain but is sufficient to decarboxylate 5-hydroxytryptophan formed by the tryptophan hydroxylase.

**5-HT concentration in rabbit and chicken retina.** The concentration of 5-HT was measured by the enzymatic-isotopic method of Saavedra et al., and as can be seen in Table I, the concentration in rabbit was less than in the 11 to 14 week chicken, which in turn was less than in newly hatched chick. Midbrain from rabbit and chicken always yielded at least a 10-fold higher concentration of 5-HT. The corrected recovery for each run was about 90%, with a maximum sensitivity of 50 pg of 5-HT.

When the concentration of 5-HT was determined by HPLC, this method gave a somewhat lower concentration than the enzymatic-isotopic method, but the same relationships were apparent (Table I). No 5-HT could be detected in the retina of reserpine-treated rabbits. Since the level of detectability was 25 pg and the retinas usually weighed around 120 mg, after reserpine the 5-HT level was less than 2 ng/gm. Neither dark-adaptation nor pretreatment with Pargyline and L-tryptophan caused any statistically significant change of the 5-HT content of the rabbit retina. Corrected recovery for each run was about 80%.

**DA concentration in rabbit retina.** The normal content was found to be 445 ng/gm wet weight (S.E.M. ± 34; n = 7). As can be seen in Fig. 1 reserpine caused a decrease of retinal DA content by approximately 90% as previously reported by Häggendal and Malmfors. Dark adaptation did not significantly change the DA content (p > 0.05). Corrected recovery for each run was approximately 70%.

**Cyclic AMP concentration in intact rabbit retina.** The level of cyclic AMP was found to be 17 pmol/mg of protein in normal retina, in good agreement with the report of Bucher and Schorderet (15 pmol/mg of protein). As shown in Fig. 2, 10\(^{-4}\)M DA increased the concentration almost twofold (p < 0.001), but 10\(^{-4}\)M 5-HT failed to induce any increase (p > 0.05).

**Discussion**

Retinal indoleamine-accumulating neurons have now been found in rabbit, cat and goldfish, river lamprey, chicken, and Cebus monkey. 5-HT has been considered to be the main transmitter candidate because (1) there are reports of 5-HT in the retina of rabbit and chicken; (2) in the retina of chick embryos Hauschild and Laties described an indole-containing cell in which
several pharmacological and histochemical criteria argued for 5-HT as the indole; (3) 5-HT has been shown to affect the ganglion cells in cat and rabbit; and (4) there is a high affinity uptake of 5-HT in the retina of chicken and rabbit, comparable to that in CNS, where 5-HT is an accepted transmitter. In the brain the assumption is generally made that the transmitter content of serotonergic and catecholamine-containing neurons is in the same range, which explains the demonstration of both types by the histochemical procedure of Falck and Hillarp (refs. 7 and 8). However, the indoleamine-accumulating neurons cannot be seen in the normal retina prepared for fluorescence microscopy according to the method of Falck and Hillarp as can the serotoninergic neurons of the brain. One possible reason for this might be that they have a low content but rapid turnover of their transmitter. The observation that retinal indoleamine-accumulating neurons retain exogenously applied indoleamines for several days makes this explanation less probable. If retinal serotoninergic neurons have the same characteristics as those in the brain, one would expect a higher content of 5-HT than of DA in the rabbit retina because there are more indoleamine than DA neurons. The present determination of retinal 5-HT and DA content, however, contradicts this expectation. The very low 5-HT content amounts to only 25 to 35 ng/gm wet weight and is in sharp contrast to the DA content of more than 400 ng/gm. These data all argue against 5-HT being the transmitter of the indoleamine neurons in the retina.

The 5-HT content we report is considerably lower than earlier reported values except for those of Haggendal and Malmfors, who could find no 5-HT in rabbit retina. Using various less specific fluorometric methods, Levene and Welsh reported in rabbit retina plus choroid 0.85 and 0.3 µg of 5-HT per gram wet weight, respectively. Suzuki et al. in chicken retina found 0.176 µg/gm 5-HT.

The enzymatic-isotopic method of Saavedra et al. used in this study is based on the ability of the enzyme hydroxy-indole-O-methyl-transferase specifically to methylate N-acetylserotonin formed by the enzymatic acetylation of serotonin. Because of the selectivity of the substrate affinities of the enzymes involved, this method is more specific than most fluorometric assays used previously. Both the enzymatic-isotopic and HPLC methods have sensitivities in the picogram range and are thus more sensitive than the previously used fluorometric methods, which provide analysis down to the nanogram range. A great advantage of HPLC is its ability to detect the native substance without prior conversion into other compounds, and in this respect it is even better than the enzymatic-isotopic assay. This advantage is the reason why the chromatographic method gives slightly lower figures for the 5-HT content, although both methods agree concerning the relative levels of 5-HT in different types of retinas.

Our finding of some 5-HT in rabbit retina can be explained by the presence of thrombocytes known to be rich in 5-HT. On the basis of an amount of 185 µg of 5-HT in 10^10 rabbit platelets and 2 × 10^9 platelets/µl of blood, 30 ng of 5-HT would be found in 0.005 gm of blood, which is only 0.5% of the total retina weight. Although low, this figure is reasonable because only a part of the rabbit retina is vascularized. We therefore suggest that the major part of the 5-HT detected in our assays is in the thrombocytes present either in the few vessels of the rabbit retina or in blood contaminating the retina during the dissection, and it is significant that the 5-HT content is lower when the retina is dissected submersed in saline (see Table I). Reserpine is known to empty both neuronal and thrombocyte monoamine stores, so that the lack of 5-HT in the retina of reserpine-treated rabbits would support its localization in the thrombocytes.

Further arguments against 5-HT being a retinal transmitter is that tryptophan hydroxylase, the rate-limiting enzyme in 5-HT synthesis from L-tryptophan, is lacking or at
least below the detectable limit in the CO$_2$-trapping method we used, similar to results found in whole mouse eye.\textsuperscript{19} One reason for not detecting any tryptophan hydroxylase in retina might be inactivity in our assay system of the 5-hydroxytryptophan decarboxylating system in retina. Its positive activity in identical assays of brain tissue and the satisfactory activity of retinal DOPA decarboxylase found in an assay system identical with that for tryptophan hydroxylase make this possibility unlikely, since DOPA and 5-hydroxytryptophan are generally considered to use the same L-aromatic amino acid decarboxylase.\textsuperscript{44} The DOPA decarboxylase activity has not previously been measured in the rabbit retina as far as we know, but Schwarz and Coyle\textsuperscript{45} found a formation of 32 nmol $^{14}$CO$_2$/mg of protein per hour in chicken retina. Finally, the increased availability of the precursor L-tryptophan and the concomitant inhibition of MAO activity did not increase the 5-HT content in rabbit retina (See Table I), whereas the same conditions cause a sevenfold increase in 5-HT content of rat midbrain.\textsuperscript{46}

The present study on retinal DA content determined with HPLC was undertaken to re-examine with this new technique the previous reports of DA content in the retina and to compare our DA data and 5-HT data. Our findings of 445 ng of DA per gram wet weight (2.9 nmol/gm) in rabbit retina is somewhat higher than but still close to the values found by most authors,\textsuperscript{13,47-49} who report DA concentrations in the range 0.7 to 1.5 nmol/gm wet weight. Drujan et al.\textsuperscript{50} report 8.3 nmol/gm wet weight. The striking difference between the relatively high DA concentration and very low 5-HT concentrations is very conspicuous and hardly compatible with a neurotransmitter function for 5-HT.

Nichols et al.\textsuperscript{48} reported a decrease in the retinal DA with dark adaptation, whereas Drujan et al.\textsuperscript{50} reported an increase. Using HPLC and comparing light- and dark-adapted retinas, we found no difference for either DA or 5-HT. Since there is a high risk of contaminating the dark-adapted retinas by dissection under infrared or dim red illumination, our controls of dissecting both light- and dark-adapted retinas under saline using infrared illumination eliminated the possibility of this differential contamination.

Cyclic AMP is presently given much attention as a "second messenger" in synaptic neurotransmission. In brain, DA has a well-documented stimulatory effect on cyclic AMP. In the retina it activates adenylate cyclase (the enzyme forming cyclic AMP from ATP)$^{22,23,45,51}$ and increases cyclic AMP in intact retina$^{22,29,51}$; our rabbit data are in agreement with a DA stimulation on cyclic AMP. In brain, 5-HT significantly increases cyclic AMP in cortical slices of squirrel monkey,\textsuperscript{52} and a close correlation was found between the regional distribution of 5-HT nerve terminals and the distribution of 5-HT-sensitive adenylate cyclase.\textsuperscript{53} Although the rabbit retina is richly supplied with indoleamine-accumulating neurons, we could find no influence of 5-HT on the cyclic AMP content, again diminishing the likelihood that 5-HT is a retinal transmitter in rabbit. A similar failure of 5-HT stimulation of cyclic AMP was found in homogenates of calf and rat retina and intact calf retina,\textsuperscript{22} but indoleamine-accumulating neurons have not been demonstrated in these retinas.

Unlike the rabbit, the higher content of 5-HT that we find in chicken retina suggests that it is localized in neurons. Since the retina of birds is avascular,\textsuperscript{54} contamination by thrombocytes is reduced. We therefore suggest that the 5-HT found in the chicken retina is of the special type of neurons observed by Hauschild and Laties.\textsuperscript{9} Although previous workers\textsuperscript{12} found a threefold to fivefold higher content in chicken retina, their report and the present one agree that there is a higher indoleamine level in chicken retina than in rabbit retina and that the indoleamine content of the newly hatched chick is higher than that of older chickens. This 5-HT might be localized in the indole-containing cells which are observable for only a short period during development,\textsuperscript{9} although its concentration is still too low for a transmitter. Rather, this embryonic 5-HT might reflect
the accumulation of an intermediate substance in an as yet immature indole metabolism system which is different from the known 5-HT pathways. This idea is supported by several reports16, 18, 19 of rapid and profound changes in the activity of enzymes involved in indole metabolism as well as of the metabolites formed in the eye of embryonic and newborn animals.

Our previous morphological1, 4 and quantitative6 studies strongly suggest the presence in rabbit and chicken retina of a special system of neurons utilizing a transmitter related to influence the results of the various fluorometric procedures. Melatonin is unlikely because it is not accumulated by the indoleamine-accumulating neurons. Like melatonin, bufotenine is substituted on the nitrogen of the side chain and has little influence on the indoleamine-accumulating system,6 suggesting that it is not taken up significantly and that the presumed, unknown transmitter of the indoleamine-accumulating neurons is unlikely to be so substituted. 5,6-Dihydroxytryptamine, 5,7-dihydroxytryptamine, or 5-methoxytryptamine is not able to produce fluorophores in the histological method of Falck and Hillarp (refs 7 and 8). Further experiments will be necessary to test the large number of possible transmitter substances for these neurons.

REFERENCES