Localization of Hydroxyindole-O-Methyltransferase in the Mammalian Pineal Gland and Retina

Allan F. Wiechmann,* Dean Bok,*† and Joseph Horwitz†

The pineal hormone, melatonin, has been reported to be synthesized in the retina by the enzyme, hydroxyindole-O-methyltransferase (HIOMT). Several laboratories have suggested that melatonin may be involved in photoreceptor outer segment disc shedding, photomechanical movements, and neuromodulation, but the cellular location of the retinal synthesizing enzymes has not been determined yet. Antiserum to HIOMT was obtained from rabbits immunized with bovine pineal extract. The monospecific immunoglobulins to HIOMT were isolated by positive-negative selection using pineal extract-sepharose and brain extract-sepharose affinity chromatography. The purity and specificity of the antibody to HIOMT was confirmed by immunodiffusion, electroblot immunolabeling, SDS-PAGE, and immunoprecipitin titration. Using the peroxidase-antiperoxidase (PAP) technique, HIOMT was localized in the pinealocytes of bovine and human pineal glands obtained during the light period. Rat pineal glands obtained during the dark period exhibited HIOMT immunoreactivity, whereas rat pineal glands obtained during the light period did not. Some pinealocytes of the bovine pineal did not exhibit HIOMT immunoreactivity, suggesting that not all pinealocytes are actively involved in melatonin synthesis. HIOMT was localized in the photoreceptors of bovine, rat, and human retinas, and some labeling also was observed in the inner retina, although the latter showed some species variation. This observation supports the hypothesis that photoreceptors are capable of melatonin synthesis. Invest Ophthalmol Vis Sci 26:253-265, 1985

Investigations by several laboratories have indicated that the indolamine hormone, melatonin, is synthesized in the vertebrate pineal gland and retina. N-acetyltransferase (NAT), the rate-limiting enzyme in melatonin biosynthesis, converts serotonin to N-acetylserotonin.1-3 N-acetylserotonin then is converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT).4-6 Melatonin-like immunoreactivity has been detected in the pinealocytes of the pineal gland, and in the outer nuclear layer of the retina,7,8 but this evidence for the sites of melatonin synthesis is not conclusive, since cellular elements of the pineal and retina not involved in the synthesis of melatonin could bind and take it up from the extracellular space.9,10 Recently, HIOMT has been localized immunocytochemically in bovine pineal gland.11

Since melatonin is synthesized on a diurnal rhythm in both the pineal gland and retina, with peak levels during the dark period2,12,13 several investigators have suggested that melatonin may be involved in diurnal events that normally occur in the retina. Melatonin involvement has been implicated in photoreceptor outer segment disc shedding and phagocytosis,14,15 photomechanical movements,16-18 and modulation of neurotransmitter release.19

Because of the morphologic, biochemical, and embryologic similarities of pinealocytes and retinal photoreceptors,20,21 many investigators think that the photoreceptors are the site of retinal melatonin synthesis. Some reports, however, do not fully support this hypothesis22,23 and suggest that retinal cells other than the photoreceptors may be capable of melatonin synthesis. Also, it has not been determined if all pinealocytes are involved in melatonin synthesis or whether some pinealocytes are inactive or involved in different functions. In order to better interpret the many studies involving the role of melatonin in the
retina, we thought it was necessary to localize the cellular site of retinal melatonin synthesis. In this report we describe the immunocytochemical localization of the melatonin synthesizing enzyme, HIOMT, in the mammalian retina and pineal gland.

**Materials and Methods**

**Preparation of Tissue Extracts**

The methods used to prepare and isolate the antibodies to HIOMT were the same as described by Kuwano and Takahashi, with some modifications. Bovine pineal glands (Pel Freez; Rogers, AR) were homogenized with a Waring blender in five volumes of 0.2 M sucrose, 0.02 M potassium phosphate pH 7.3, with or without proteolytic inhibitors (2 µg/ml PMSF, 5 µM pepstatin, 5 µM leupeptin, 5 µg/ml aprotinin, and 5 µg/ml chymostatin; Sigma; St. Louis, MO). The homogenate was centrifuged 105,000 g for 1 hr at 4°C, then the pellet was discarded. The supernatant (pineal extract) was used to immunize rabbits. Bovine brains and retinas were obtained from a local slaughterhouse, and extracts were prepared in the same manner as described for the pineal.

**Preparation of Antiserum**

Antiserum was obtained from New Zealand white rabbits immunized with the bovine pineal extract. The pineal extract (containing 5 mg protein) was emulsified with an equal volume of Freund’s complete adjuvant (Difco; Detroit, MI), and was injected subcutaneously into each of four rabbits. Injections were repeated every 2 weeks. After the second injection, all immunizations were done with incomplete adjuvant. Rabbits were bled from the ear vein every 2 weeks. The blood was allowed to stand at room temperature for 2 hr, then refrigerated overnight. The clotted blood was centrifuged next at 35,000 g for 30 min at 4°C, and the antiserum was aliquoted and stored at −20°C. These investigations adhered to the ARVO Resolution on the Use of Animals in Research.

**Preparation of Affinity Columns**

CNBr-activated Sepharose 4B (Pharmacia; Piscataway, NJ) was swollen with 1 raM HC1, then suspended in two volumes (40 ml) of 0.02 M sodium bicarbonate pH 9.5. Pineal extract (30 ml) dialyzed against 0.1 M sodium bicarbonate, 0.5 M NaCl pH 8.5, was added to the gel suspension. Coupling was performed for 24 hr at 4°C. The gel was filtered and resuspended in 0.38 M glyline, 100 mM Tris pH 8.3, to bind unreacted groups. After coupling for 4 hr at 4°C, it was washed successively with 1 liter each of 0.1 M sodium acetate, 0.5 M NaCl pH 4.0, 2 M Urea, 0.5 M NaCl pH 8.9, 0.1 M sodium bicarbonate, 0.5 M NaCl pH 10.0, and 0.2 M sodium acetate, 0.5 M NaCl pH 2.4. The gel was resuspended in 0.15 M potassium phosphate, 0.5 M NaCl pH 7.3. The pineal-Sepharose then was packed into a 1.5 × 20 cm Bio-Rad Econocolumn (Richmond, CA).

Brain-Sepharose was prepared in the same manner as described above. Dialyzed brain extract (200 ml) was coupled to 70 ml swollen gel suspended in 130 ml 0.2 M sodium bicarbonate pH 9.5. The washed gel was packed into a 2.5 × 30 cm column.

**Purification of Antiserum**

Antiserum was dialyzed against 0.1 M Tris-HCl pH 7.4, then applied to a QAE-A50 Sephadex (Pharmacia) column (1.5 × 11 cm), which had been equilibrated with the same buffer. Fractions were collected at a flow rate of 10 ml/hr. The eluted immunoglobulin fraction was concentrated and dialyzed against 0.02 M potassium phosphate, 0.15 M NaCl pH 7.2 (PBS) on a MicroProDiCon apparatus (Bio-Molecular Dynamics; Beaverton, OR). This immunoglobulin fraction then was applied to the pineal-Sepharose column at a flow rate of 20 ml/hr, and washed with 0.15 M potassium phosphate, 0.5 M NaCl pH 7.3 until all the unbound protein had washed through the column. The bound anti-pineal antibodies were eluted with 0.2 M glycine-HCl, 0.5 M NaCl pH 2.4 buffer, and were concentrated and dialyzed against PBS on a MicroProDiCon apparatus. The anti-pineal antibodies then were applied to the brain-Sepharose column at a flow rate of 40 ml/hr. The first immunoglobulin peak was collected and concentrated as described above, then aliquoted and stored at −50°C.

**Immunodiffusion Analysis of Antibody**

Ouchterlony immunodiffusion analysis was performed at room temperature on 1% agarose plates, containing 0.05% polyethylene glycol.

**Electroblot Immunolabeling**

The proteins of bovine pineal and retina extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were incubated in 1% SDS, 5% beta-mercaptoethanol, 10% glycerol, and 0.01% Bromophenol blue in 0.01 M Tris-HCl (pH 6.8) for 5 min at 100°C. The samples then were loaded onto a 1-mm-thick, 12.5% polyacrylamide slab gel and subjected to electrophoresis for several hours. Next, the proteins were electrophoretically transferred to a nitrocellulose sheet (Schleicher and Schuell; Keene, NH). The nitrocellulose sheet was incubated with the purified antibody (first peak from the brain-Sepharose column), then labeled with goat...
anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad). After reaction with the HRP color development reagent (Bio-Rad), the antigen–antibody complexes became visible. Nonimmune rabbit IgG was used as a control to eliminate the possibility of nonspecific interactions.

Immunoprecipitin Titration

To demonstrate that the purified antibody was directed against HIOMT, immunotitration was performed with the pure antibody. Constant amounts of the bovine pineal extract (20 μl) were incubated with increasing amounts of purified antibody (0–35 μl, 0.7 mg/ml). Appropriate amounts of normal rabbit IgG (Cappel; Malvern, PA) were added to the tubes to result in a constant amount of gamma globulin in each tube. This mixture was incubated for 45 min at 37°C, then for 7 hr at 4°C. To precipitate the antigen–antibody complex, 20 μl of goat anti-rabbit gamma globulin (GARGG, 1.35 mg/ml, E-Y Laboratories; San Mateo, CA) were added to each tube, and incubated overnight at 4°C. The samples were centrifuged at 4,000 g at 4°C for 30 min to pellet the antigen–antibody complex, and the supernatants were measured for HIOMT activity by radioenzymatic assay.

Preparation of Antibody Immunoadsorbant Column

Purified HIOMT antibodies were coupled to CNBr-activated Sepharose 4B according to the method described by Kuwano et al. A coupling efficiency of 90% was determined by absorption spectroscopy.

SDS-Polyacrylamide Gel Electrophoresis of Purified HIOMT

Pineal and retinal extracts were applied to the antibody-Sepharose immunoadsorbant column, washed with PBS, then the bound proteins were eluted with 0.2 M glycine-HCl, 0.5 M NaCl pH 2.4. The eluted proteins were collected into tubes containing 3 ml PBS, and were concentrated and dialyzed on a MicroProDiCon apparatus. SDS-PAGE was performed on the proteins as described previously. The gels were silver stained to visualize the protein bands.

Radioenzymatic Assay of HIOMT

HIOMT activity was measured by a modification of the method of Axelrod et al. Samples were incubated in 15-ml culture tubes containing 50 μg of N-acetylserylserotonin (Sigma) and 2 nmols of 3H-S-adenosylmethionine (73 mCi/mmole, Amersham; Arlington Heights, IL) in a final volume of 0.3 ml. After 30 min of incubation at 37°C, 1 ml of 0.2 M borate buffer pH 10, followed by 8 ml of chloroform, were added to the reaction mixture. The tubes were shaken and the aqueous phase then was removed by aspiration. The organic phase was washed with 1 ml of buffer, then shaken. The aqueous phase was removed, then 4 ml of chloroform were transferred to a scintillation vial and evaporated to dryness with warm air. The residue was dissolved in 1 ml of ethanol; 10 ml of Aqueous Counting Scintillant (Amersham) were subsequently added, and the radioactivity was measured.

One Dimensional Peptide Mapping

Peptide mapping was performed on the purified pineal 39,000 mol wt subunit and the retinal 25,000 mol wt subunit by the limited proteolysis method of Cleveland et al. Briefly, 100 ng of the purified subunits were incubated with 2 μg of Staphylococcus aureus V8 protease (Miles; Elkhart, IN) in 20 mM Tris-HCl pH 7.9 for 1 hr at room temperature. The reaction was stopped by addition of SDS and betamercaptoethanol to final concentrations of 2% and 10%, respectively, and boiling of the samples for 5 min. The samples were loaded onto a 12.5% polyacrylamide gel and the peptide fragments were separated by SDS-PAGE, and silver stained.

Immunocytochemistry

Tissues were immersion fixed overnight at 4°C in Perfix (Fisher; Pittsburgh, PA), then rinsed three times in 0.1 M sodium phosphate pH 7.4 prior to paraffin embedding. The fixed tissues were dehydrated in a graded ethanol series and xylene, then embedded in paraffin (Paraplast, Ted Pella; Tustin, CA). Paraffin sections (10 μm) were cut and placed on a 40°C water bath, then picked up on gelatin-subbed slides, and dried on a 40°C hot plate for 24 hr.

Paraffin was removed with xylene. The sections were incubated in 0.3% H2O2 for 5 min to remove endogenous catalase activity. The sections were rinsed, equilibrated with 0.05 M Tris-HCl pH 7.6, then incubated for 20 min in a blocking solution containing 2.5% bovine serum albumin (BSA, Sigma), 5% ovalbumin (Sigma), 0.05% sodium azide, and 0.1% Triton X-100 (Sigma) in 0.05 M Tris saline (TBS) pH 7.4. The protein solution was blotted from the slide, and the sections were incubated in either the HIOMT antibody (74 μg/ml) or nonimmune rabbit gamma globulin (74 μg/ml), diluted in the BSA solution, for 2 hr at room temperature. After the primary incubation, the sections were rinsed in TBS, and incubated in GARGG (68 μg/ml) for 0.5 hr at room temperature. The sections were rinsed again in TBS, then incubated...
Fig. 1. Immunodiffusion analysis. A, 30 µl of antiserum (Ab) before immunoadsorption in center well. B, 30 µl of antibody (Ab) after immunoadsorption in center well. The peripheral wells contain 30 µl bovine pineal (P) and brain (B) extracts. One specific precipitin line is formed against the pineal extract.

Fig. 2. The center well contains 30 µl of 0.7 mg/ml purified HIOMT antibody (Ab), and the peripheral wells contain 30 µl of bovine pineal (P), brain (B), and retina (R) extracts. The partial fusion of the precipitin lines between the retina and pineal wells indicates some immunologic identity.

Results

Analysis of Purified Antibody

Purified antibody from the brain-Sepharose column was analyzed by SDS-PAGE. Only light and heavy chain bands of the immunoglobulins were observed in the gel (data not shown), indicating that the antibody was free of contaminating proteins.

Immunoglobulins of the antiserum and pure HIOMT antibody were analyzed by immunodiffusion, to determine the presence of antibodies directed against proteins in pineal or brain extracts. Antiserum was placed in the center well, and brain and pineal extracts were placed in alternating peripheral wells (Fig. 1A). The resulting lines of precipitation indicate that the antiserum contains antibodies that recognize both brain and pineal proteins. Fusion of the immunoprecipitin lines indicate that these proteins of the brain and pineal are identical. The precipitin line of the pineal that does not fuse with any brain protein precipitin lines, indicates that the antiserum contains antibodies against proteins unique to the pineal gland.

Immunodiffusion analysis of the pure antibody (Fig. 1B) shows that all antibodies common to both the brain and pineal have been removed, with only one precipitin line against the pineal extract observed.

To determine the immunologic identity of the retinal and pineal enzymes, purified antibody was placed in the center well, and retina, pineal, and brain extracts were placed in adjacent peripheral wells (Fig. 2). The resulting precipitin pattern of partial

in peroxidase-antiperoxidase complex (40 µg/ml, Sternberger-Meyer; Jarrettsville, MD) for 0.5 hr at room temperature. After rinsing in TBS, the sections were incubated in 0.06% diaminobenzidine (Sigma) and 0.03% H2O2 in 0.05 M Tris-HCl pH 7.4, for 1-5 min, depending on the development of the color reaction. The reaction was stopped by rinsing the sections in water, then the tissues were counter stained with Mayers-hematoxylin, and examined by light microscopy.
fusion with a spur between the retina and pineal wells indicated partial immunologic identity of retinal and pineal HIOMT.

In an attempt to identify the protein to which the antibody was made, protein electroblot-immunolabeling was performed. When bovine pineal and retina extracts were electroblotted, the only protein bands recognized by the antibody were 39,000 and 25,000 mol wt species for the pineal, and a 25,000 mol wt species for the retina (Fig. 3). When the tissues were homogenized in buffers containing several proteolytic inhibitors, the identical transblot pattern was observed. The methods used to determine the molecular weights of these protein bands will be discussed later. The subunit molecular weight of bovine pineal HIOMT generally is considered to be 39,000,\(^{24,31,32}\) although a lower molecular weight subunit of about 25,000 also has been reported.\(^ {33}\)

![Fig. 3. Transblot immunolabeling of bovine pineal (P) and retina (R) extracts with the HIOMT antibody demonstrates the purity of the isolated antibody. The antibody labels two peptide bands of the pineal extract (39,000 and 25,000 mol wt) and only one of the retina extract (25,000 mol wt).](image)

To determine if the HIOMT antibody was monospecific and cross-reacting with two similar polypeptides (the 25,000 and 39,000 mol wt bands), we isolated the antibodies to the 39,000 mol wt band according to the method described by Talian et al.\(^ {34}\) Antibody eluted from the nitrocellulose strip containing the 39,000 mol wt band was used to label electroblotted bovine pineal and retina extracts. The same labeling pattern as seen on Figure 3 was observed, indicating that both proteins are recognized by a single population of the antibodies suggesting that they may be related structurally.

To further determine whether the isolated antibody characterized by the immunodiffusion and electroblot was directed against HIOMT, immunoprecipitin titration was performed with bovine pineal extract. Increasing amounts of pure antibody reacted with the HIOMT in the extract, resulting in decreasing HIOMT activity in the supernatant (Fig. 4). Analysis of the antigen–antibody pellet (data not shown) indicated that some HIOMT activity was retained, demonstrating that the antibody did not completely inactivate the enzyme. This observation was identical to that made by Kuwano and Takahashi.\(^ {24}\) Nonetheless, this experiment demonstrated that the isolated antibody was indeed directed against HIOMT.

**Analysis of Purified HIOMT**

The purified pineal HIOMT obtained from the antibody immunoadsorbant column, when analyzed by SDS-PAGE, was shown to be composed of subunit bands of 39,000 and 25,000 mol wt, while the retinal enzyme had a subunit molecular weight of 25,000 (Fig. 5). SDS low molecular weight standards (Bio-Rad) were used to determine these molecular weights, as well as those observed on the electroblots (Fig. 3).
Fig. 6. Radioenzymatic assay of purified bovine pineal HIOMT. Increasing amounts of enzyme (5 μg/ml) results in increasing HIOMT activity.

Fig. 7. Peptide map of purified bovine pineal and retinal HIOMT. The 39,000 mol wt HIOMT peptide is in lane 1, while the 25,000 mol wt HIOMT peptide is in lane 2. Protease (2 μg) is in lane 3. Lane 4 contains the incubation mixture of the 39,000 mol wt pineal HIOMT and protease, and lane 5 contains the incubation mixture of the 25,000 mol wt retinal HIOMT and protease.

The purified pineal HIOMT from the immunoabsorbant column was measured for activity by radioenzymatic assay. Increasing amounts of the pure enzyme resulted in increasing HIOMT activity (Fig. 6).

In an attempt to determine sequence homology between the 39,000 mol wt subunit and the 25,000 mol wt subunits, one-dimensional peptide mapping was performed following limited proteolysis of purified pineal and retinal HIOMT. *Staphylococcus aureus* V8 protease specifically cleaves peptide bonds on the carboxy-terminal side of glutamic and aspartic acid. Similar peptide profiles are an indication of sequence homology between two proteins. In the peptide map (Fig. 7), the resulting peptide profile shows that there are identical peptide fragments in the two HIOMT enzymes (small arrows). The upper band (large arrow) in lane 5 is obviously undigested enzyme. There are a few small peptide bands present in the pineal profile (approximately 22,000 mol wt, lane 4), which are not present in the retinal profile; but this is expected, since the pineal HIOMT protein band is of a higher
molecular weight than the retina HIOMT protein band, and would contain peptides not found in the 25,000 mol wt protein band. The sequence homology indicated in the experiment suggests that the 25,000 mol wt enzyme peptide is identical to a portion of the 39,000 mol wt enzyme peptide.

Immunocytochemistry

Tissues treated with nonimmune antibody showed no specific peroxidase labeling. Pinealocytes in bovine pineal gland treated with the HIOMT antibody were labeled heavily with the brown reaction product of the antibody-PAP complex (Fig. 8B). The interstitial cells and blood vessels were not labeled. There appeared to be many gradations of antibody labeling of the bovine pinealocytes. Some were very densely labeled, while others were not labeled at all. Fisher rat pineal gland obtained during the light period, and treated with the HIOMT antibody, did not exhibit HIOMT immunoreactivity (Fig. 9A), whereas rat
Fig. 9. Immunocytochemistry of rat pineal gland. A, Rat pineal gland obtained during the light period. B, Rat pineal gland obtained during the dark period. Both tissues were incubated with the HIOMT antibody and labeled with PAP. Only the dark-adapted pineal demonstrated HIOMT immunoreactivity (X370).

Rat pineal gland obtained during the dark period did exhibit HIOMT immunoreactivity (Fig. 9B). The labeling in the rat appeared rather diffuse and not as intense as seen in the bovine or human pineal. No peroxidase labeling was observed in dark- or light-adapted rat pineal glands treated with nonimmune antibody.

Rat retina treated with the HIOMT antibody showed labeling of photoreceptor inner segments, the outer nuclear layer, and the outer plexiform layer, indicating that the photoreceptors contain the HIOMT enzyme (Fig. 10B). There was no labeling of the photoreceptor outer segments. There also appeared to be some labeling of cells in the inner nuclear layer and ganglion cell layer.

To further demonstrate that HIOMT is localized in the photoreceptors, we analyzed the retinas of RCS rats at day 66. At this stage, most of the photoreceptors have degenerated (Fig. 10C, D). Very little HIOMT labeling was observed in the RCS retina, suggesting that photoreceptor cell loss results in loss of HIOMT in the tissue. Studies on the adult rd/le mouse model for retinal degeneration showed the same lack of HIOMT labeling. HIOMT labeling was present in the photoreceptors of both animal strains during the postnatal period before photoreceptor degeneration occurred (data not shown).

HIOMT labeling in the bovine retina predominated in the photoreceptors (Fig. 11B), although there was some labeling in the inner plexiform layer as well. To determine more readily if cone photoreceptors also display HIOMT immunoreactivity, we analyzed the retinas of the lizard, Sceloporus occidentalis, which has an all cone retina. The specific label was present in the photoreceptors (Fig. 11D), indicating that HIOMT is expressed in both cone and rod photoreceptors. Specific HIOMT labeling in the human retina appeared to be limited to the photoreceptors (Fig. 11F). The pattern and intensity of HIOMT labeling in the neuropil (inner retina) showed some variation among species and from one experiment to the next.

Discussion

Using the immunoadsorption method of Kuwano and Takahashi, 24 we have isolated monospecific immunoglobulins to HIOMT. Since HIOMT is very antigenic, 31,32,37 and is present in high concentrations in the bovine pineal gland, 31 it is an ideal antigen for this method of antibody isolation. The method is based on the assumption that HIOMT is the only unique pineal protein present in sufficient quantity in the amount of extract injected to elicit an antigenic response.

The electroblot analysis of the HIOMT antibody and the SDS-PAGE of the purified HIOMT suggest that there may be two separate HIOMT subunits. Although both the 25,000 and 39,000 subunit molecular weights have been reported in bovine pineal gland, 24,31-33 only the 39,000 mol wt subunit has been reported in chicken pineal gland. 32 The difference in electroblot labeling of the bovine pineal and retina...
Fig. 10. Immunocytochemistry of rat retina. A, Normal rat retina incubated with nonimmune antibody. B, Normal rat retina incubated with the HIOMT antibody. The photoreceptor cell bodies demonstrate HIOMT immunoreactivity. Some labeling also occurs in the inner nuclear layer. C, RCS rat retina incubated with nonimmune antibody. D, RCS rat retina incubated with the HIOMT antibody. Very little HIOMT labeling is observed in the degenerating photoreceptors. Pigment epithelium (pe), photoreceptor outer segments (os), photoreceptor inner segments (is), outer nuclear layer (onl), outer plexiform layer (opl), inner nuclear layer (inl), inner plexiform layer (ipl), ganglion cell layer (gcl), photoreceptor debris (pd) (x370).

may perhaps be explained in three ways: (1) only the 25,000 mol wt band of the retina is recognized by the antibody, and the 39,000 mol wt retina band (if it exists) is not recognized by the antibody due to a lack of sequence homology between the 39,000 mol wt subunit of the two tissues; (2) bovine retinal HIOMT is composed only of the 25,000 mol wt subunits; or (3) all of the 39,000 mol wt retinal HIOMT subunit is degraded to a 25,000 mol wt band and to other additional bands not recognized by the antibody. Based on the immunologic identity and sequence homology indicated by the immunodiffusion analysis, electroblot immunolabeling, and peptide mapping, the 25,000 mol wt polypeptide appears to be similar or identical to a portion of the 39,000 mol wt band. The 25,000 mol wt band may be a true
subunit of HIOMT, or a degradative product of the 39,000 mol wt subunit. The use of proteolytic inhibitors in the preparation of the tissue homogenates, suggests that the observed band are true subunits. Since the tissues were obtained from a slaughterhouse, however, the freshness of the tissue could not be controlled.

The pinealocytes long have been presumed to be the site of melatonin synthesis in the mammalian pineal gland. Melatonin-like immunofluorescence is detectable in the pinealocytes of the vertebrate pineal gland,4 and ultrastructural evidence5,6 indicates that these cells have a secretory function. Our immunocytochemistry data demonstrate that the enzyme, HIOMT, and therefore melatonin synthesis is located in the pinealocytes of the bovine pineal gland. This is in agreement with the results of Kuwano et al.11 Due to the varied degree of labeling of the pinealocytes for HIOMT, we suggest that not all pinealocytes are involved actively in the synthesis of melatonin. Closer examination of this labeling at the electron microscope level may help determine if HIOMT is distributed differentially in the so-called “light pinealocytes” and “dark pinealocytes,” which are presently thought to differ in their secretory activity.41

We also have demonstrated that HIOMT immunoreactivity in the rat pineal gland is higher during the dark period than during the light period. The lighter labeling of the rat pineal, when compared with the bovine or human pineal, may be due to a lower cross-reactivity of the antibody with the pineal HIOMT of the rat. Other laboratories have reported that HIOMT activity in the rat pineal is higher during the dark period than during the light period.29,42 Their observation that puromycin blocks the dark-induced rise in HIOMT activity, suggests that the increase in HIOMT activity during the dark period may be mediated by protein synthesis.29 Our data support this hypothesis and suggest further that the reported higher HIOMT activity in the rat pineal gland during the dark period is due to higher levels of HIOMT in the tissue rather than some chemical modification in activity of the enzyme.

The observation that HIOMT is present in the retinal photoreceptors supports the hypothesis that the photoreceptors are the site of retinal melatonin synthesis. Several lines of indirect evidence have supported this idea: (1) melatonin-like immunofluorescence has been observed in the outer nuclear layer of the mammalian retina as well as in the inner nuclear layer.7,8; (2) pinealocytes of some mammalian species possess many photoreceptor-like characteristics, including a 9 × 2 + 0 cilium and synaptic ribbons53; (3) pinealocytes of many submammalian vertebrates possess photosensitive outer segments54; (4) some mammalian pinealocytes undergo a transient photoreceptor-like differentiation during development55,56; and (5) both the retina and the pineal develop as evaginations of the developing diencephalon.57 Since photoreceptors and pinealocytes have these many similarities, and pinealocytes are capable of melatonin synthesis, many investigators have predicted that the photoreceptors would be the most likely site of retinal melatonin synthesis.

The presence of HIOMT labeling in the entire photoreceptor, with the exception of the outer segment, suggests that HIOMT is located in the cytosol of the cell. Reports from other laboratories have shown that HIOMT activity is confined mainly to the cytoplasmic fraction of the retina and pineal.48

Our observation of HIOMT labeling in the inner nuclear layer of the rat retina is consistent with reports of an indolamine-generating system within the inner retina.49-51 Indole-accumulating neurons are present in the inner nuclear layer and inner plexiform layer of several species,49,50 and serotonin immunoreactivity is located in amacrine cell bodies and inner plexiform layer in vertebrate retinas.52 HIOMT labeling in bovine inner retina appears to be in three sublayers of the inner plexiform layer, while there appears to be no HIOMT labeling in the human inner retina. This difference in labeling pattern may be due to antibody specificity or species variation, the latter of which has been reported for the indolamine-accumulating neurons in the inner retina.49 Further analysis at the electron microscope level is necessary, however, to determine the precise cellular location of HIOMT in the inner retina.

Since HIOMT appears to be present in retinal neurons of the mammalian inner retina, indolamines such as melatonin may be synthesized there and could, perhaps, be involved locally in retinal neurotransmission, or affect other distant parts of the retina. This is supported by reports that melatonin inhibits the calcium-dependent release of dopamine.
from rabbit retina,\textsuperscript{19} and that melatonin and its precursors have been identified in the inner retina.\textsuperscript{7,8,52}

Although there appear to be retinal neurons capable of melatonin synthesis, the photoreceptors appear to be the major source of melatonin in the retina. Some investigators think that, because of its diurnal rhythm, with peak levels during the dark period, melatonin may be involved in other diurnal events, which normally occur in the retina.\textsuperscript{2,14,53} Melatonin supports photoreceptor outer segment disc shedding in \textit{Xenopus} eyecups\textsuperscript{14} but inhibits phagocytosis in cultured chick retinal pigment epithelium (RPE).\textsuperscript{15} These effects may be due to melatonin action within the photoreceptors themselves or to melatonin released from the photoreceptors or retinal neurons, thereby affecting other cells of the retina. Melatonin-induced pigment granule aggregation in frogs,\textsuperscript{16} trout,\textsuperscript{17} and guinea pigs,\textsuperscript{18} is thought to be equivalent to the pigment granule aggregation that normally occurs during the dark period in some species.\textsuperscript{54,55} Melatonin synthesized by HIOMT in the photoreceptors during the dark period may be released by those cells, bind to cellular elements within the RPE, and induce pigment granule aggregation. The demonstration of HIOMT localization in the photoreceptors and other retinal neurons may provide for more meaningful interpretation of studies on the role of melatonin in the retina. Our observation that HIOMT is present in both the pinealocytes and the retinal photoreceptors is consistent with the hypothesis that the mammalian pinealocytes belong to a sensory cell line that has evolved from the sensory pineal photoreceptors of lower vertebrates.

Key words: hydroxyindole-O-methyltransferase, immunocytochemistry, pineal gland, retina, melatonin

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