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Hydroxyindole O-Methyltransferase: An Immunochemical Study of the Neuronal Regulation of the Pineal Enzyme

H.-Y. T. YANG AND N. H. NEFF

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D. C. 20032

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SUMMARY

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Hydroxyindole O-methyltransferase was purified from bovine pineal, and antiserum to the enzyme was produced in rabbits. Rabbit antiserum precipitated the enzyme from bovine, chicken, rat, and human pineal. The consequences of sympathetic denervation and environmental lighting on hydroxyindole O-methyltransferase were studied in rats. Enzyme activity in the pineals of rats kept in continuous darkness was 2-3 times higher than in rats kept in continuous light. Bilateral superior cervical ganglionectomy reduced the enzyme activity in pineals of rats housed under circadian lighting by about half when compared to normal rats kept under identical lighting conditions. Immunochemical titration indicated that the increase in enzyme activity induced by continuous darkness could be accounted for by an increase in enzyme molecules rather than by a change in enzyme kinetics. These results indicate that the quantity of hydroxyindole O-methyltransferase enzyme protein present in the pineal may be modulated by sympathetic nerve activity, which in turn is modulated by environmental illumination.

INTRODUCTION

Hydroxyindole O-methyltransferase (S-adenosyl-L-methionine:N-acetylserotonin O-methyltransferase, EC 2.1.1.4) catalyzes the formation of melatonin from N-acetylserotonin in pineal gland (1). The enzyme activity of rat pineal follows a circadian rhythm, with timing cues correlated with illumination. Activity is at its peak during the daily dark period; moreover, it is higher in rats kept in continuous darkness than in rats kept in continuous light. Apparently the eye participates in the regulation of hydroxyindole O-methyltransferase via the superior cervical ganglia, as enzyme modulation is abolished by blinding or by superior cervical ganglionectomy (2-4). An increase in enzyme protein or a change in the state of the existing enzyme protein has been proposed to explain the increase in activity elicited by changes of the photoperiod (3, 5). By using immunochemical titration with an antiserum for hydroxyindole O-methyltransferase, we show that the increase in enzyme activity during darkness is due to the accumulation of enzyme protein.

MATERIALS AND METHODS

Studies of hydroxyindole O-methyltransferase protein were performed in female Sprague-Dawley rats (Zivic-Miller Laboratories, Allison Park, Pa.) weighing 150-170 g. Rats were kept in continuous light or continuous dark for 14-20 days before experimentation. Superior cervical ganglionectomized (Zivic-Miller) rats were kept under diurnal lighting, from 6:00

a.m. to 6:00 p.m., for 13–14 days before experimentation. They were killed at 3:00 pm. Frozen bovine and chicken pineal glands were obtained from Pel-Freez Biologicals. Human pineal glands were dissected post mortem and obtained through the courtesy of the Neuropathology Department, Saint Elizabeths Hospital.

Enzyme assay. Hydroxyindole *O*-methyltransferase was assayed by the method of Axelrod *et al.* (3). During the enzyme purification, the various fractions were assayed as follows. The enzyme source was incubated for 10 min at 37° with 0.1 mM *N*-acetylserotonin and 7 μM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (54–57 mCi/mmole, New England Nuclear Corporation) in a total volume of 500 μl of 0.1 M potassium phosphate buffer (pH 7.9). The reaction was terminated by adding 0.5 ml of 0.5 M borate buffer (pH 10) and 6 ml of toluene-isoamyl alcohol (97:3). The radioactive melatonin was extracted into the organic phase by placing the samples on a mechanical mixer for 30 sec and then separating the two phases by centrifugation. A 5-ml portion of the organic phase was transferred to a counting vial and evaporated to dryness at room temperature under a stream of air. Aquasol (New England Nuclear), 5 ml, was added to the residue, and radioactivity was determined in a Beckman LS 250 liquid scintillation spectrometer. When rat pineal enzyme was assayed, the reaction mixture contained 28 μM *S*-adenosyl-L-methionine in a total volume of 250 μl and the reaction was terminated after 60 min of incubation at 37°.

Purification of hydroxyindole O-methyltransferase. Frozen bovine pineal, 97 g, was homogenized with 5 volumes of isotonic KCl in a Waring Blendor for 1 min and centrifuged at 100,000 × *g* for 60 min. The pellet was discarded, and the supernatant was subjected to ammonium sulfate fractionation. Enzymatically active protein was precipitated between 35% and 55% saturation with ammonium sulfate, dissolved in 5 ml of 0.02 M sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer overnight. The enzyme was placed on a DEAE-Sephadex A-50 column (2.5 × 27 cm) that had been equilibrated with 0.02 M sodium phosphate buffer (pH 7.0). The column was washed with buffer until the effluent was free of protein, and then the enzyme was eluted with an NaCl gradient formed from 300 ml each of 0.02 M sodium phosphate buffer (pH 7.0) and 0.8 M NaCl in the same buffer. Three protein peaks were detected; enzyme activity was associated with the second protein peak. The fractions with enzyme activity of 4.0–4.6 nmoles of melatonin per milligram of protein per minute were concentrated by precipitation with ammonium sulfate (75% saturation). The precipitate was dissolved in a small volume of 0.02 M sodium phosphate buffer (pH 7.0), dialyzed for 12 hr against the buffer, and placed on a Sephadex G-200 column (2.5 × 80 cm) equilibrated with the same buffer. Four protein peaks were observed following elution with the phosphate buffer, with major enzyme activity appearing at the latter part of the third protein peak. Fractions with specific activities of 8–8.5 nmoles of melatonin per milligram of protein per minute were concentrated by ultrafiltration and used for the preparation of antiserum. Acrylamide gel electrophoresis (7) of the protein used for immunization showed one major and two minor protein bands. No attempt was made to identify hydroxyindole *O*-methyltransferase activity in the gel. Protein was measured by the method of Lowry *et al.* (6) with bovine serum albumin as standard.

Immunization of rabbits. Purified enzyme (100–500 μg of protein) in 0.5 ml of 0.02 M sodium phosphate buffer (pH 7.0) was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into the back of a rabbit. Immunization was repeated at 10-day intervals, and 5 days after the fourth injection the rabbit was bled. Fifteen rabbits were immunized.

Immunochemical titration. Pineal glands were homogenized with 0.001 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and 0.01 M dithiothreitol and centrifuged at 22,000 × *g* for 20 min. To estimate the quantity of hydroxyindole *O*-methyltransferase enzyme protein present

in samples of pineal, increasing amounts of the antiserum were added to a series of fixed quantities of supernatant from pineal. Serum obtained from a normal rabbit was added to each sample so that the total quantity of serum was the same in every sample. The antiserum-enzyme mixture was left at room temperature for 30 min and then at 4° overnight. The precipitate was removed by centrifugation at 22,000 × *g* for 30 min, and the enzyme activity remaining in the supernatant was assayed.

RESULTS

Cross-reactivity of rabbit antiserum. All the rabbits immunized with purified hydroxyindole *O*-methyltransferase produced antibody against bovine pineal enzyme when examined by immunochemical titration. Nearly all of the bovine enzyme could be precipitated by the antiserum (Fig. 1). If, however, the antibody-enzyme complex was not removed by centrifugation before the enzyme assay, about 40% of the original enzyme activity remained, indicating that the antibody-enzyme complex was partially active (Fig. 1). Rabbit antiserum also cross-reacted with the pineal enzyme from humans and chickens (Fig. 2). However, only three of the rabbits immunized produced antiserum which cross-reacted with the enzyme of rat pineal.

To evaluate whether the various proteins of the pineal gland would interfere with formation of the antibody-enzyme complex, we performed an immunochemical titration of purified bovine pineal hydroxyindole *O*-methyltransferase in the absence and presence of bovine pineal protein fractions that were devoid of this enzyme activity. Apparently pineal gland proteins did not significantly influence the outcome of the immunochemical titration (Fig. 3).

Hydroxyindole O-methyltransferase of rats kept in continuous darkness or light. The hydroxyindole *O*-methyltransferase activity of rats kept in continuous darkness was about 2–3 times higher than in animals kept in continuous light (Table 1 and Fig. 4). More antiserum was required to precipitate the enzyme activity of pineal

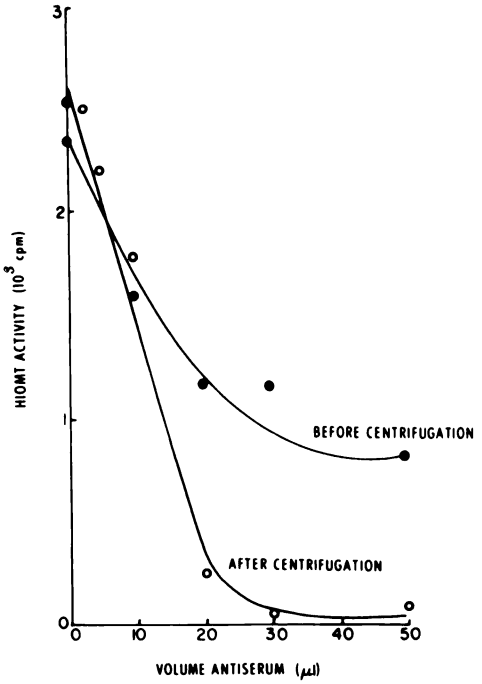

FIG. 1. *Immunochemical titration of bovine pineal hydroxyindole O-methyltransferase (HIOMT) with enzyme-specific antiserum*

Frozen bovine pineal was homogenized in 50 volume of 1 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and centrifuged at 22,000 × *g* for 30 min. Increasing quantities of antiserum were added to the supernatant, which was kept constant at 0.5 ml, and the mixture was incubated at 4° overnight. Then 10 μl were removed before and after centrifugation at 22,000 × *g* for 30 min and assayed for enzyme activity.

glands from animals kept in continuous darkness in comparison with animals kept in continuous light (Fig. 4).

To compare accurately the quantity of antiserum required to precipitate enzyme activity, the enzyme source from the animals kept in continuous darkness was diluted to one-third its original volume to equal the enzyme activity of the animals kept in continuous light, and the immunochemical titration was repeated. The titration curves were equivalent following dilution (Fig. 4). Apparently pineal glands from animals kept in continuous darkness contained 2–3 times more enzyme protein than those from animals kept in continuous light. The apparent kinetic constants

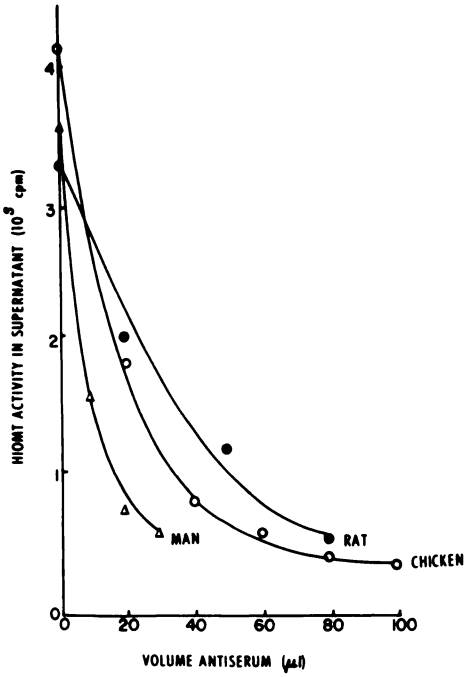

FIG. 2. *Cross-reactivity of rabbit antiserum to bovine hydroxyindole O-methyltransferase (HIOMT) with enzyme from rat, chicken, and human*

Pineal glands were homogenized with 1 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and 10 mM dithiothreitol (100 μl/chicken pineal, 60 μl/rat pineal, and 10 μl/mg of human tissue) and centrifuged at 22,000 × *g* for 30 min. Increasing quantities of antiserum were added to fixed quantities of supernatant from pineal (100 μl for chicken, 80 μl for rat, and 30 μl for human), and the samples were incubated overnight at 4°. Following centrifugation at 22,000 × *g* for 30 min, the supernatants (20 μl for chicken, 45 μl for rat, and 20 μl for human) were removed and assayed for enzyme activity.

for *N*-acetylserotonin were determined for both groups of animals from Lineweaver-Burk plots with a fixed concentration of *S*-adenosyl-L-methionine, 28 μM. A similar K_m value, about 20 μM, was obtained for both groups of animals (Fig. 5), while the apparent V_{max} increased from about 0.66 to 2.0 nmoles of melatonin per milligram of protein per minute in the animals kept in continuous darkness.

Effect of superior cervical ganglionectomy on hydroxyindole O-methyltransferase. Superior cervical ganglionectomy decreased the rat pineal hydroxyindole *O*-methyltransferase activity to about half the value for control animals (Table 1), and about twice as much antiserum was required to precipitate all of the enzyme activity from control animals (Fig. 6). When the enzyme source from the control animals was diluted by half, identical titration curves were obtained for both groups of animals (Fig. 6). These results indicate that the differences in enzyme activity are due to differences in specific enzyme protein.

DISCUSSION

Environmental lighting and sympathetic nerve activity influence the hydroxyindole *O*-methyltransferase activity of

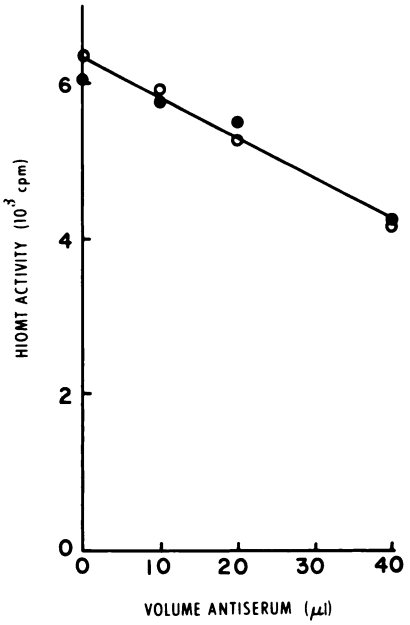

FIG. 3. *Inability of bovine pineal proteins to interfere with immunochemical titration of purified bovine hydroxyindole O-methyltransferase (HIOMT) with rabbit antiserum*

Increasing quantities of antiserum were added to 0.5 ml of purified bovine enzyme (○) (93 μg of protein with a specific activity of 7.5 nmoles/min/mg of protein) or purified bovine enzyme mixed with bovine pineal protein (330 μg of protein) devoid of hydroxyindole *O*-methyltransferase activity (●). Inactive protein was obtained during the enzyme purification procedure. The samples were incubated at 20° for 30 min and then at 4° for 1 hr. Following centrifugation at 22,000 × *g* for 30 min, 10 μl of supernatant were assayed for enzyme activity as described in MATERIALS AND METHODS.

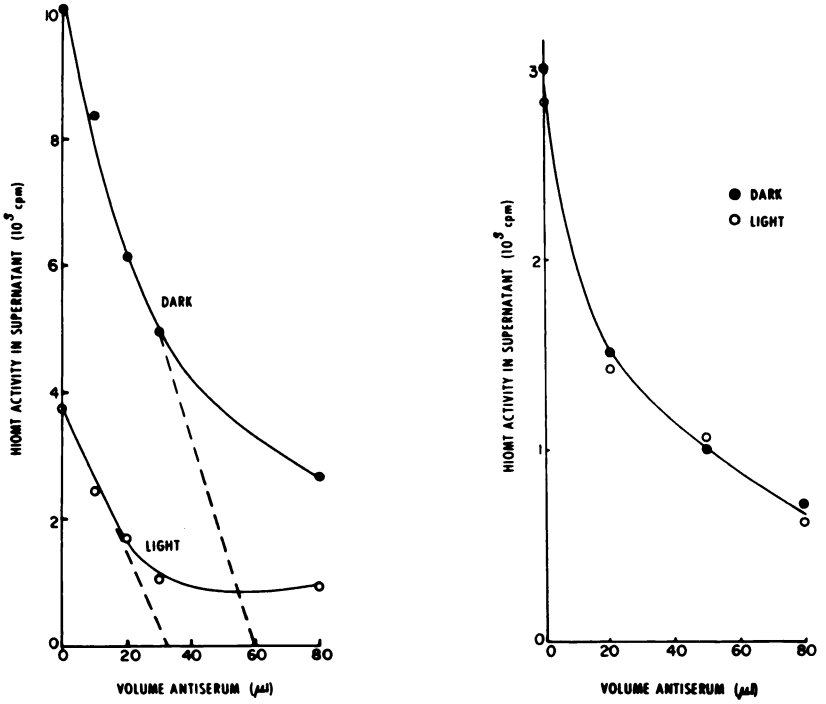

FIG. 4. *Immunochemical titration of pineal hydroxyindole O-methyltransferase (HIOMT) from rats kept in continuous darkness or light*

Pineal glands were homogenized with 1 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and 10 mM dithiothreitol (50 μl/pineal) and centrifuged at 22,000 × *g* for 30 min. In the left-hand figure, increasing quantities of antiserum were added to 80 μl of supernatant and the mixture was incubated overnight and then centrifuged at 22,000 × *g* for 30 min. Supernatant, 60 μl, was removed and assayed for enzyme as described in MATERIALS AND METHODS. In the right-hand figure, pineal glands were homogenized with 1 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and 1 mM dithiothreitol so that the final homogenates from both groups of animals contained equivalent enzyme activities (60 μl/pineal for continuous light and 180 μl/pineal for continuous darkness). The samples were then centrifuged at 22,000 × *g* for 30 min. The immunochemical titration and enzyme assay were performed as described above. Different groups of animals were used in the experiments shown.

rat pineal. Rats kept in continuous darkness for 14–20 days have about 2–3 times more enzyme activity than those kept in continuous light. By immunochemical titration with an antiserum for hydroxyindole *O*-methyltransferase, we have demonstrated that the increase in enzyme activity in continuous darkness is due to increased enzyme protein. When the samples from the two groups of animals were diluted to equal enzyme activity and then titrated with antiserum, identical titration curves were obtained. Apparently the enzyme proteins from both groups are antigenically identical and perhaps structurally similar. Moreover, the apparent K_m for *N*-acetylserotonin was similar for both groups of animals, supporting the notion that there was an increase in enzyme protein in animals kept in continuous darkness for 14–20 days rather than activation of existing enzyme. This conclusion is in agreement with a report by Axelrod *et al.* (3) that the effect of illumination on this enzyme is mediated by protein synthesis. Whether the changes result from altered enzyme protein synthesis or degradation remains to be investigated.

Jackson and Lovenberg (5) isolated three distinct forms of hydroxyindole *O*-methyltransferase from bovine pineal gland, which differed in molecular weight, and they postulated that diurnal variations of enzyme activity might be due to

TABLE 1

Effects of illumination and superior cervical ganglionectomy on rat pineal hydroxyindole O-methyltransferase activity

Values are the means and standard errors of five animals.

Treatment	Enzyme activity
	nmoles/mg protein/hr
Normal, continuous darkness[a]	3.7 ± 0.2[b]
Normal, continuous light	1.7 ± 0.1
Normal, circadian lighting[c]	2.3 ± 0.1[b]
Ganglionectomized, circadian lighting	1.3 ± 0.1

[a] Rats were kept in continuous light or dark for 14 days before analysis.

[b] $p < 0.001$ when compared with contrast treatment.

[c] Superior cervical ganglionectomy was performed 13 days before analysis. Lights were on for 12 hr (6:00 a.m. to 6 p.m.), and the rats were killed at 3:00 p.m.

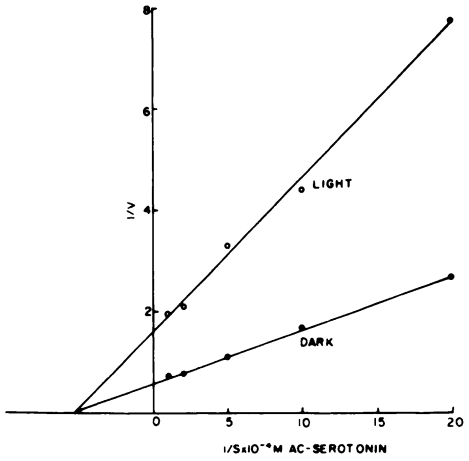

FIG. 5. *Lineweaver-Burk plot of pineal hydroxyindole O-methyltransferase activity for rats kept in continuous dark or light*

The concentration of *S*-adenosyl-L-methionine was 28 μM.

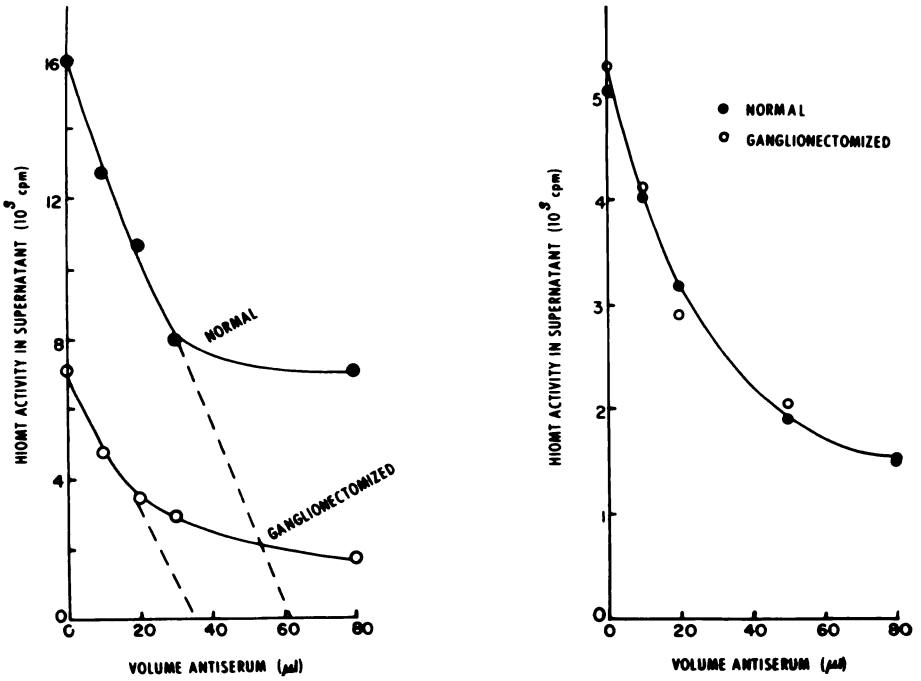

FIG. 6. *Immunochemical titration of pineal hydroxyindole O-methyltransferase (HIOMT) from ganglionectomized or normal rats kept in circadian light and killed after 7 hr of exposure to light*

The experiment in the left-hand figure was performed as described in Fig. 4. In the right-hand figure, pineal glands were homogenized with 1 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and 10 mM dithiothreitol so that homogenates from both groups of animals contained equivalent enzyme activity (100 μl/pineal for normal rats and 50 μl/pineal for ganglionectomized rats) and assayed as described in Fig. 4. Different groups of animals were used in the experiments shown.

polymerization-depolymerization of the enzyme molecule. The possibility that this mechanism may be involved in the short-term regulation of rat pineal enzyme diurnal rhythm is not excluded by our study, because of the difficulty in obtaining enough rat pineal glands for purification and subsequent identification of possible multiple forms of the enzyme. It is also possible that an inactive precursor of the enzyme, which does not cross-react with the antiserum, can be readily activated by sympathetic nerve activity.

Superior cervical ganglionectomy decreases hydroxyindole *O*-methyltransferase activity in pineals of rats kept in circadian illumination and killed after 7 hr of exposure to light (8). By immunochemical titration we have shown that reduced enzyme activity is consistent with decreased enzyme protein. The results also suggest that sympathetic nerve activity is involved in the accumulation of active protein in rat pineal gland. This hypothesis is consistent with a report that the sympathetic nerves that innervate the rat pineal form norepinephrine at a faster rate in darkness than in light (9). Increased sympathetic nerve activity may also be responsible, in part, for the presence of more hydroxyindole *O*-methyltransferase protein in rats kept in continuous darkness.

Avian hydroxyindole *O*-methyltransferase is different from the mammalian enzyme in substrate specificity, electrophoretic mobility, and kinetic properties (10–12). Surprisingly, the antibody against the bovine enzyme cross-reacted with the enzyme of chicken pineal (Fig. 2). There may be two *O*-methyltransferases in avian pineal, one with a preference for serotonin and one with a preference for *N*-acetylserotonin (10). Possibly our antiserum precipitated avian acetylserotonin *O*-methyltransferase and not serotonin *O*-methyltransferase.

Hydroxyindole *O*-methyltransferase has been detected in human metastatic parenchymatous pinealoma (13). The detection of this enzyme in blood may be a useful tool for the diagnosis of pineal tumors. The cross-reactivity of the antibody against the enzyme from bovine pineal and the enzyme from human pineal, as we have demonstrated, suggests that this enzyme-specific antibody may be an additional diagnostic aid for pineal tumors.

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