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Pineal Gland in Organ Culture

I. Inhibition by Harmine of Serotonin-^{14}C Oxidation, Accompanied by Stimulation of Melatonin-^{14}C Production

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SUMMARY

The effect of harmine on serotonin-^{14}C metabolism by pineal glands in organ culture was studied to examine the possibility that inhibition of monoamine oxidase activity would result in stimulation of melatonin-^{14}C formation. Glands treated with harmine (0.1–10 μM) produced 2–5 times more radiolabeled melatonin than did untreated controls. Thin layer chromatographic analysis of culture media revealed that harmine treatment increased *N*-acetylserotonin-^{14}C as well as melatonin-^{14}C levels, and decreased the production of hydroxyindoleacetic acid-^{14}C, hydroxytryptophol-^{14}C, and methoxytryptophol-^{14}C. The activity of hydroxyindole *O*-methyltransferase in harmine-treated glands, or in homogenates of untreated glands to which harmine had been added, was no different from that of untreated controls.

These observations suggest that inhibition of oxidation of serotonin resulted in enhanced *N*-acetylation and that the resulting high levels of *N*-acetylserotonin caused increased melatonin production by a mechanism not dependent upon increased production of hydroxyindole *O*-methyltransferase. This effect can be explained on the basis of mass action.

INTRODUCTION

Melatonin (5-methoxy-*N*-acetyltryptamine) is synthesized enzymatically from serotonin (5-hydroxytryptamine) by *O*-methylation of the intermediate *N*-acetylserotonin (5-hydroxy-*N*-acetyltryptamine) in a reaction which requires *S*-adenosylmethionine as a methyl donor (1–3) (Fig. 1). Axelrod and colleagues have reported that the enzyme necessary for this *O*-methylation, hydroxyindole *O*-methyltransferase, is found among mammals only in the pineal gland (4). Although it is known that production of melatonin *in vitro* can be stimulated by norepinephrine and other amines (5, 6), the precise mechanisms involved in the biochemical regulation of melatonin production are not well defined.

One possible factor influencing melatonin production is the rate of serotonin destruction by monoamine oxidase (5). The recent studies of Tyce, Flock, and Owen (7, 8) have demonstrated that inhibition of monoamine oxidase increases the formation of *N*-acetylserotonin in the perfused rat liver. This suggests an inverse relationship between the oxidation and *N*-acetylation pathways of

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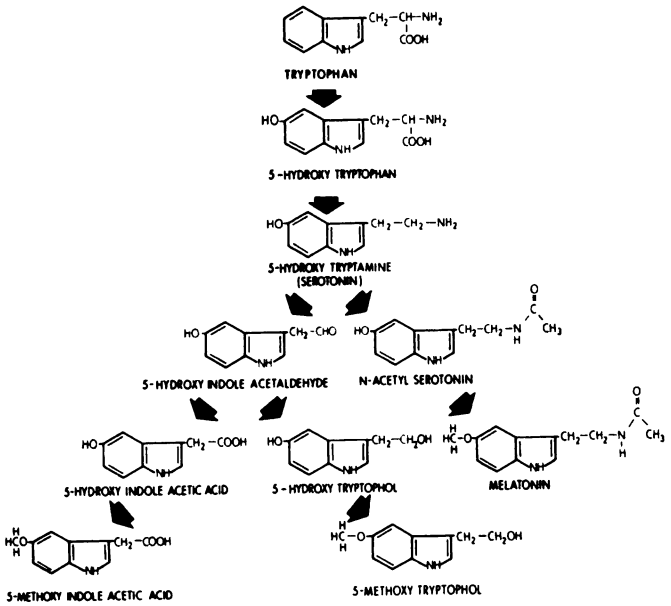

FIG. 1. *Outline of pertinent aspects of indole metabolism in the pineal gland*

Oxidation of serotonin by monoamine oxidase results in the eventual formation of hydroxyindoleacetic acid and hydroxytryptophol. Acetylation of serotonin results in the formation of *N*-acetylserotonin. *O*-Methylation of these compounds by hydroxyindole *O*-methyltransferase produces methoxyindoleacetic acid, methoxytryptophol, and melatonin, respectively (1–3).

serotonin metabolism. If an analogous situation is present in the pineal gland, and decreased monoamine oxidase activity results in elevated *N*-acetylserotonin formation, it seems likely that melatonin synthesis would also increase.

In the study presented here, the effects of a potent monoamine oxidase inhibitor, harmine, on the conversion of serotonin-^{14}C, tryptophan-^{3}H, and methionine-methyl-^{14}C to radioactive melatonin and other radiolabeled serotonin derivatives was studied using organ cultures of pineal glands in a synthetic medium.

MATERIALS AND METHODS

Technique of organ culture. Pineal glands are obtained by sterile dissection from 180–200 g, male Sprague-Dawley rats following decapitation. Organ cultures are prepared by the method of Trowell (9), as modified by Raisz for parathyroid glands and embryonic bones (10, 11). One or two rat pineal glands are incubated in 0.5 ml of medium at 37° and continually gassed with a mixture of oxygen (95%) and carbon dioxide (5%). Chemically defined culture medium (modified BGJ, Grand Island Biological Corporation) supplemented with 1 mg/ml of bovine serum albumin (Fraction V, Pentex), as described by Raisz and Niemann (12) is used. Under the conditions described, pineal glands are maintained for at least 6 days with the ability to synthesize melatonin-^{3}H from tryptophan-^{3}H continuously (6).

Harmine was purchased from Aldrich Chemical Company. The following precursors were used in this study: serotonin-3′-^{14}C–creatinine sulfate (Nuclear-Chicago; 40 μCi/μmole), L-tryptophan-^{3}H (uniformly labeled; Schwarz BioResearch; 2.3 mCi/μmole), and L-methionine-methyl-^{14}C (Nuclear-Chicago; 46 μCi/μmole).

Assay of radioactive melatonin and hydroxyindoleacetic acid. The radiolabeled melatonin in 0.1 ml of culture medium or in 0.2 ml of the 0.5-ml homogenate of a single rat pineal gland is extracted into 8 ml of chloroform from 2 ml of 0.5 M sodium borate buffer (pH 10) by shaking. After phase separation,

this aqueous layer is removed. The chloroform extract is washed with an additional 2 ml of sodium borate buffer (pH 10), and twice with 2 ml of 1.0 N HCl. Acid washes are necessary to remove small amounts of serotonin-^{14}C extracted into the chloroform. The aqueous layer is removed after each wash. An aliquot of the chloroform extract is evaporated to dryness in a counting vial, 10 ml of scintillation counting fluid are added, and the sample is counted. This procedure is a modification of the method of Axelrod and Weissbach for extraction of radioactive melatonin formed in the hydroxyindole *O*-methyltransferase assay (2).

Labeled hydroxyindoleacetic acid is extracted from 1 ml of the first sodium borate buffer aqueous layer obtained from the melatonin extraction described above. A modification of the method of Udenfriend *et al.* (13) is used. The sodium borate buffer is acidified and saturated with salt. Hydroxyindoleacetic acid is then extracted into ether (10 ml). The labeled product is finally extracted from ether into 2 ml of sodium phosphate buffer (pH 7, 0.1 M). An aliquot of the aqueous extract is evaporated to dryness in a counting vial, dissolved in 0.1 ml of HCl, 0.6 ml of Nuclear-Chicago solubilizer (NCS; Amersham Searle), and 10 ml of scintillation counting fluid, and counted.

The identity of the radioactive compounds extracted by the above procedures is routinely verified by thin layer chromatography (see below and ref. 14). Pooled extracts of medium and glands from individual treatment groups are evaporated to dryness and redissolved in a solution of ethanol and HCl (1:1) containing authentic standard compounds. The pooled extracts are then analyzed separately. Between 70 and 90% of the chloroform-extracted radioactivity chromatographed with authentic melatonin, and 75–85% of the radioactivity extracted into 0.1 M sodium phosphate buffer, pH 7, from ether chromatographed with authentic hydroxyindoleacetic acid. In both cases 5–10% of the radioactivity in the extracts remained at the origin.

Medium blank values are obtained from medium incubated with no gland under normal experimental conditions. Gland blanks for the melatonin extraction are obtained from glands dipped in medium for 1 sec. There is no difference between this value and the tissue blank obtained from a 24-hr, 0° incubation of pineal glands.

Radioactivity is measured with a liquid scintillation spectrometer. Single-labeled samples containing ^{14}C are counted at an efficiency of either 54% or 74%. For dual-labeled samples, ^{14}C efficiency is 54% and ^{3}H efficiency is 28%. The efficiency for ^{14}C in the ^{3}H channel is 14%, and appropriate corrections are made. ^{3}H is counted in the ^{14}C channel at an efficiency of less than 0.01%.

Thin layer chromatography. Whole media and concentrated pools of chloroform and ether extracts are analyzed by thin layer chromatography (14). Ten-microliter aliquots are mixed with an equal volume of standard carrier solution containing hydroxyindoleacetic acid, hydroxytryptophol, methoxytryptophol, methoxyindoleacetic acid, melatonin, *N*-acetylserotonin, serotonin, and tryptophan (Regis Chemical Company). Standards are dissolved in a solution of ethanol (50%), 0.1 N HCl (50%), and ascorbic acid (0.5%). A mixture of standards at a final concentration of 1 mM is used in chromatography. This solution is stored at −4°.

Twenty-microliter samples containing standards and the unknown samples are applied to activated, coated, thin layer plates (10 × 20 cm, Brinkmann Instruments, silica gel) and dried under a stream of nitrogen. Each plate is run first in chloroform–methanol–acetic acid (93:7:1 by volume) along the long axis. Development in this solvent is immediately repeated in the same direction. The chromatogram is finally developed in the second direction in ethyl acetate (7). Spots are localized by fluorescence under short-wave ultraviolet light after spraying with a solution of methanol and 12.5 N HCl (1:1 by volume). R_F values appear in Table 1. Specific areas are scraped off, pulverized between flat glass surfaces, and placed in a scintillation vial. This powder is treated with 0.6 ml of NCS; 10 ml of scintillation solution are added, and counting is performed by routine liquid scintillation methods. Between 98 and 100% of the radioactivity applied is recovered.

TABLE 1

R_F values of serotonin metabolites separated by thin layer chromatography

Solvent A is chloroform–methanol–acetic acid, 93:71:1 by volume. The total front movement in this system is 36 cm. Solvent B is ethyl acetate. The R_F values in this system are valid only when solvent A precedes solvent B (14). Total front movement in solvent B is 8 cm. All spots are less than 1 cm in diameter after development in both solvents.

Metabolite	Solvent A	Solvent B
	$R_F \times 100$	
5-Methoxytryptophol	24	50
Melatonin	23	23
5-Methoxyindoleacetic acid	17	54
5-Hydroxytryptophol	10	47
N-Acetylserotonin	10	21
5-Hydroxyindoleacetic acid	5	47
Serotonin (5-hydroxytryptamine)	0	0

Hydroxyindole O-methyltransferase. Hydroxyindole *O*-methyltransferase activity is determined by a modification of the method of Axelrod and Weissbach (2), using a final *S*-adenosylmethionine concentration in the assay medium of 10^{-5} M.

RESULTS

Stimulation of formation of melatonin-^{14}C from serotonin-^{14}C by harmine. Rat pineal glands incubated for 24 hr with serotonin-^{14}C (5 μCi/ml, 0.125 mM) synthesized melatonin-^{14}C, as evidenced by the presence in culture media and gland homogenates of chloroform-extractable radioactivity identified as melatonin by thin layer chromatography. Approximately 0.2% of the serotonin-^{14}C was converted into melatonin-^{14}C by one pineal gland during a 24-hr incubation period. Treatment of pineal glands with harmine at a final medium concentration of 0.1–10 μM resulted in a 2–5-fold increase in total (medium plus gland) melatonin-^{14}C (Fig. 2). Comparison of the effect of harmine on the medium and the gland contents of melatonin-^{14}C indicated that more than 95% of the labeled melatonin synthesized as a result of harmine treatment (1–10 μM) was released into the medium.

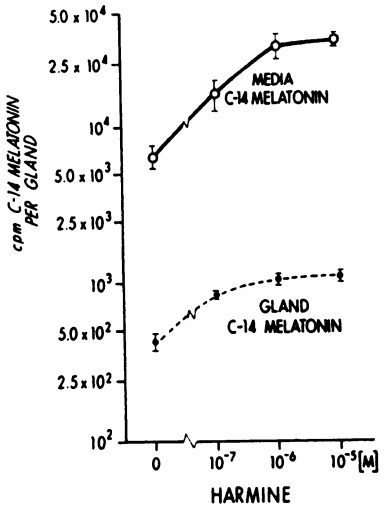

FIG. 2. *Effects of harmine on ^{14}C-melatonin metabolism*

The total medium and gland content of ^{14}C-melatonin after 24 hr of incubation with serotonin-^{14}C (40 μCi/μmole, 0.125 mM) are given as counts per minute per gland. Harmine was present where indicated throughout the incubation period. A maximally stimulated gland converts approximately 1% of the available serotonin to melatonin in 24 hr. Each point is the mean of four determinations. Vertical lines indicate standard errors.

Stimulation of labeled melatonin synthesis from methionine-methyl-^{14}C and tryptophan-^{3}H by harmine. To examine the effect of harmine on labeled melatonin production in the absence of exogenous serotonin, pineal glands were incubated with methionine-methyl-^{14}C and tryptophan-^{3}H. Under these conditions, dual-labeled melatonin, identified by thin layer chromatography, was synthesized. This demonstrated that pineal glands in organ culture will utilize tryptophan-^{3}H or the methyl-^{14}C group of methionine via *S*-adenosylmethionine to form melatonin. Harmine (10^{-5} M) treatment of pineal glands incubated with the above isotopes resulted in a 4–6-fold increase ($p < 0.001$) in ^{14}C- and ^{3}H-labeled melatonin in the medium (Table 2).

Inhibition of hydroxyindoleacetic acid-^{14}C formation by harmine (Table 3). Pineal glands incubated with serotonin-^{14}C (5 μCi/ml, 0.125 mM) formed hydroxyindoleacetic acid-^{14}C. The addition of harmine (0.1–10 μM) caused a 40–60% reduction ($p < 0.001$) in the medium levels of this oxidation product.

TABLE 2

Harmine-stimulated production of isotopically labeled melatonin from methionine-methyl-^{14}C and tryptophan-^{3}H

Each organ culture contained two pineal glands and 0.5 ml of medium, in which the specific activity of tryptophan (0.2 mM) was 50 μCi/μmole and that of methionine (0.3 mM) was 2.8 μCi/μmole. Results are expressed per gland for a 24-hr incubation period (mean ± standard error).

Treatment	No. of organ cultures	Conversion of labeled precursor into radioactive melatonin	
		^{14}C	^{3}H
		%	%
Control	4	0.13 ± 0.02	0.35 ± 0.05
Harmine (10^{-5} M)	4	0.87 ± 0.08[a]	1.42 ± 0.11[a]

[a] Statistically greater than control ($p \leq 0.001$ by Student's *t*-test).

Effects of harmine on formation of N-acetylserotonin-^{14}C, hydroxytryptophol-^{14}C, methoxytryptophol-^{14}C, and methoxyindoleacetic acid-^{14}C (Fig. 3). A thin layer chromatographic system which separated four serotonin oxidation products and two *N*-acetylated derivatives (see MATERIALS AND METHODS) was used to determine the relative effects of harmine on these six metabolites. The analysis of culture media by this technique substantiated the stimulatory effects of harmine on melatonin production presented above, and also indicated that production of *N*-acetylserotonin-^{14}C was stimulated ($p < 0.001$). No *N*-acetylserotonin-^{14}C was identified in the untreated pineal gland organ culture medium, and harmine treatment resulted in a medium concentration of *N*-acetylserotonin-^{14}C that exceeded that of melatonin-^{14}C.

Harmine treatment significantly decreased ($p < 0.001$) the levels of hydroxyindoleacetic acid-^{14}C, hydroxytryptophol-^{14}C, and methoxytryptophol-^{14}C. No significant effect of harmine on methoxyindoleacetic acid-^{14}C in the medium was observed in these experiments.

Investigations with hydroxyindole O-methyltransferase. The possibility that harmine influences melatonin production by altering the activity of hydroxyindole *O*-methyl-

TABLE 3

Hydroxyindoleacetic acid-^{14}C in pineal gland organ culture media treated with harmine

Each value is the mean ± standard error of the hydroxyindoleacetic acid-^{14}C extracted from 0.5 ml of the medium of an organ culture of two pineal glands incubated for 24 hr. Results are expressed per gland. Each control gland converts approximately 4% of the available serotonin-^{14}C to hydroxyindoleacetic acid-^{14}C in 24 hr.

Treatment	No. of organ cultures	Hydroxyindole acetic-^{14}C in medium	
		Activity	Inhibition
		(*cpm/gland*) × 10^4	%
Control	4	4.8 ± 0.73	0
Harmine, 10^{-7} M	4	2.9 ± 0.30[a]	39
Harmine, 10^{-6} M	4	1.8 ± 0.25[a]	62
Harmine, 10^{-5} M	4	2.0 ± 0.44[a]	58

[a] Statistically lower than control ($p \leq 0.001$ by Student's *t*-test).

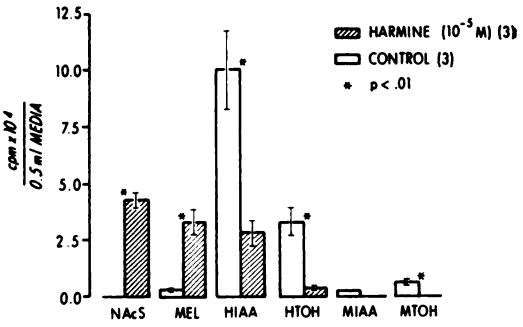

FIG. 3. *Thin layer chromatographic analysis of culture media*

Harmine (10^{-5} M) was added to the medium of one group of pineal organ cultures at the beginning of a 24-hr incubation with serotonin (40 μCi/μmole, 0.125 mM). Results are expressed per gland. A total of 2.5 × 10^6 cpm was contained in 0.5 ml of medium at the start of incubation. Aliquots (10 μl) of the medium were applied to a thin layer of silica gel; 98–100% of the radioactivity applied was recovered at the origin and in the six areas corresponding to added carriers. NAcS, *N*-acetylserotonin; MEL, melatonin; HIAA, hydroxyindoleacetic acid; HTOH, hydroxytryptophol; MIAA, methoxyindoleacetic acid; MTOH, methoxytryptophol. The numbers in parentheses indicate the number of organ cultures. Vertical lines indicate standard errors. Statistical analysis was done by Student's *t*-test.

TABLE 4

Hydroxyindole O-methyltransferase activity of rat pineal glands

In experiment 1, the effect of harmine on the hydroxyindole *O*-methyltransferase activity in unincubated glands was measured. Harmine was added directly to the enzyme assay mixture, which contained the following, in a volume of 0.3 ml: *N*-acetylserotonin, 50 μg; *S*-adenosylmethionine-methyl-^{14}C, 50 mμCi (48 μCi/mmole); *S*-adenosylmethionine, 3 mμmoles; pineal gland homogenate; and sodium phosphate buffer, 0.05 M, pH 7.9. In experiment 2, the enzyme activity was measured after incubation with harmine in the organ culture medium. The results of this experiment are expressed per gland (mean ± standard error of five glands).

Experiment	Harmine	Hydroxyindole *O*-methyltransferase activity			
		15 min	35 min	45 min	60 min
	μM	*μμmoles ^{14}C-melatonin formed*			
1	500				44
	100				45
	50				45
	10	12	27	31	44
	5				44
	1				43
	0	11	26	30	44
2	0				57 ± 9.3
	10				61 ± 11.5

transferase was examined. Pineal glands incubated with harmine for 24 hr had the same activity of this enzyme as untreated glands (Table 4). In addition, no effect of harmine on the hydroxyindole *O*-methyltransferase activity of unincubated gland homogenates was observed when harmine was added directly to the enzyme assays.

DISCUSSION

The technique of organ culture was used to investigate whether melatonin production by the pineal gland is altered when serotonin oxidation is inhibited. Sensitive and specific determination of the relative levels of serotonin-^{14}C metabolites in the culture media was made possible by using radiolabeled precursors and separating the products by thin layer chromatography.

Harmine was used as an inhibitor of monoamine oxidase because of the high potency of this compound at low concentrations, reported in brain and other tissues (15). In this experimental system, harmine-treated glands incubated with serotonin-^{14}C released substantially less hydroxyindoleacetic acid-^{14}C, hydroxytryptophol-^{14}C, and methoxytryptophol-^{14}C. This reduction in the concentration of three oxidation products of serotonin-^{14}C in the medium indicated that harmine inhibited monoamine oxidase activity.

Axelrod *et al.* (5) have reported that isopropylphenylhydrazine, another monoamine oxidase inhibitor, stimulated the production of melatonin by pineal glands in organ culture. The present investigations, using harmine, provide some understanding of the molecular mechanisms involved. No evidence that harmine stimulated only the release of melatonin-^{14}C without increasing its production was found. Rather, it was observed that total production of melatonin-^{14}C was increased, with the majority of the product being released into the culture medium. No evidence suggesting a causative role of hydroxyindole *O*-methyltransferase was provided; harmine had no apparent effect on this enzyme.

The use of three different radiolabeled precursors of melatonin eliminated the possibility that harmine caused an increase in labeled melatonin production without actually increasing unlabeled melatonin production. This could have arisen as result of alterations in the relative availability for *N*-acetylation of the following precursor pools: (a) unlabeled serotonin stores present at the start of the experiment, (b) labeled serotonin synthesized from tryptophan-^{3}H, and (c) labeled serotonin added to the cultures. A decrease in the availability of unlabeled serotonin (a) could have resulted in increased use of labeled serotonin (b, c) as a substrate for *N*-acetyltransferase without altering the total rate of *N*-acetylserotonin production. However, this did not seem to be the case, because the synthesis of melatonin-*O*-methyl-^{14}C was also stimulated by harmine, an effect presumably independent of the source of serotonin.

It seems most likely that harmine stimulated melatonin production by causing a shift in the cellular mode of degradation of

serotonin from oxidation to *N*-acetylation, which resulted in elevated cellular concentrations of *N*-acetylserotonin. This was supported by our findings of high levels of *N*-acetylserotonin-^{14}C in the medium containing harmine-treated pineal glands, assumed to be caused by elevated cellular concentrations of this compound. Elevated cellular *N*-acetylserotonin apparently raised melatonin production by simple mass action in an enzymatic reaction that was substrate-limited in the untreated gland. It was not clear from these studies what caused the increase in *N*-acetylserotonin. Inhibition of monoamine oxidase by harmala alkaloids is known to raise serotonin concentrations in brain (15, 16). High levels of serotonin in these studies could increase the production of *N*-acetylserotonin by making more unbound substrate available, as suggested by Axelrod *et al.* (5), or by enhancing the activity of *N*-acetyltransferase (17).

The studies of Snyder *et al.* (18) have demonstrated that administration of a monoamine oxidase inhibitor to rats will prevent the fall in serotonin concentrations in the pineal gland that occurs during the hours of darkness. This observation, together with our findings, raises the possibility that, in studies *in vivo* in which monoamine oxidase inhibitors are used, these compounds may cause increased production and release of *N*-acetylserotonin and melatonin.

Although monoamine oxidase activity as estimated in dilute broken cell preparations of pineal glands is consistently quite high (19), the possibility that this enzyme is involved in the daily regulation of melatonin synthesis should be considered. Little is known about the mechanisms controlling the activity of this enzyme on a cellular level; intracellular inhibitors and activators may exist. One possible regulatory factor working through monoamine oxidase is norepinephrine. Norepinephrine could alter serotonin oxidation as a competitive inhibitor, because both these compounds are substrates for monoamine oxidase. Consistent with this suggestion are the findings of Axelrod *et al.* (5) demonstrating that norepinephrine added to organ cultures of pineal glands inhibited the production of hydroxyindoleacetic acid-^{14}C from tryptophan-^{14}C and stimulated the production of melatonin-^{14}C. Wurtman and colleagues have observed that norepinephrine concentrations in the pineal gland exhibit a 24-hr rhythm (20, 21). Such a rhythm might indirectly produce a similar rhythm in melatonin production through the mechanisms discussed above.

In their physiological studies, Axelrod and Wurtman have inferred that melatonin synthesis can be approximated by the measurement of hydroxyindole *O*-methyltransferase (1, 22). The studies presented here emphasize the necessity of considering the relative importance of the levels of serotonin, monoamine oxidase, the *N*-acetylating enzyme, *N*-acetylserotonin, and hydroxyindole *O*-methyltransferase in attempting to estimate melatonin production in studies *in vivo*, in which melatonin cannot be measured directly.

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