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Isolation of Melatonin and 5-Methoxyindole-3-acetic Acid from Bovine Pineal Glands*

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Melatonin is a substance that can lighten the color of frog melanocytes by causing aggregation of melanin granules about the nuclei of the cells (1). It is present in the nervous system in very small amounts, the pineal gland being the richest source of this substance.[1] Melatonin is approximately 100,000 times more potent than noradrenaline which previously was considered to be the most active lightening agent. In the isolation of melatonin from bovine pineal glands a chemically similar, but biologically inactive, compound was also found (2–4). Melatonin was shown to be *N*-acetyl-5-methoxytryptamine (3) and the other compound, 5-methoxyindole-3-acetic acid (4). These experiments demonstrated that *O*-methylation of hydroxyindoles occurs in biological reactions.

In this report details of the isolation and determination of structure of melatonin and 5-methoxyindole-3-acetic acid are given.

EXPERIMENTAL PROCEDURE

Materials—Drs. Joseph Fisher, Eldon Nielson, and Sanford Steelman of the Armour Laboratories supplied us with more than 100 kg of bovine pineal glands (approximately 200,000 glands) in the fresh frozen or lyophilized state. Many indole derivatives were made available to us by Drs. Richard V. Heinzelman of The Upjohn Company, Albert Hofmann of the Sandoz Chemical Works, Inc., Aleck Borman of The Squibb Institute for Medical Research, and D. W. Woolley of The Rockefeller Institute.

Bioassay—A detailed account of the bioassay method for detecting melatonin will be published elsewhere[2] (5). In essence, lightening in color of isolated pieces of frog skin was observed quantitatively with a photoelectric photometer.

Skin from *Rana pipiens* was removed, soaked 1 hour in frog Ringer's solution, stretched on plastic rings, and placed in transparent plastic dishes containing 30 ml of Ringer's solution. The skin specimens were darkened by adding 30 mg of caffeine to each dish and allowing 1 hour for the reaction to go to completion. In this process pigment granules within the melanocytes became dispersed throughout the cells. Microscopically, areas with melanocyte densities of 50 to 60 cells per low power field were selected. The amount of transmitted light falling on a photocell attached to a microscope was recorded. The skin lightened within 10 to 20 minutes after addition of melatonin extracts. Photometer readings were taken after 30 minutes. Increased amounts of light striking the photocell signified lightening of skin color due to aggregation of pigment granules within the melanocyte. At the end of the assay maximal possible lightening was checked by addition of 0.1 mg of norepinephrine. One unit of melatonin was defined as that quantity per milliliter in frog Ringer's solution that produced 20 to 30% of the maximal possible lightening. This type of change usually was apparent to the naked eye.

Organic Solvents—In the isolation and identification experiments all organic solvents used (methanol, ethanol, propanol, ethyl acetate, benzene, and heptane) were of analytical reagent grade quality; and all were distilled before use. Analytical grade petroleum ether used in the Soxhlet extracter was not distilled first.

Isolation Procedure—Starting material was usually lyophilized bovine pineal glands. Adherent to the glands obtained from the supplier were bits of bone, brain, blood, and connective tissue. After manual removal of adventitious material, white glands weighing about one-third of the starting weight were obtained. The average weight of a trimmed, lyophilized bovine pineal gland was approximately 40 mg. The fresh weight was approximately 200 mg. About 500 g of the clean, dried glands were reduced at a time to a powder in 1 to 2 minutes at medium speed in a 4-liter Waring Blendor. The resulting white powder was defatted batchwise for 2 hours with petroleum ether in a Soxhlet extracter. A 15 to 17% decrease in weight of pineal powder resulted from petroleum ether extraction.

We mixed 85 g of defatted powder for 1 minute with 1700 ml of water at high speed in the large Blendor. Then the mixture was centrifuged at 16,000 × *g* for 30 minutes. Clear supernatant fluid was filtered through glass wool to remove a surface scum. The 1600-ml solution was extracted twice with equal volumes of ethyl acetate, and the ethyl acetate layers were taken to dryness in a rotary flash evaporator under reduced pressure at 40°. The residue was subjected to 29 transfers in a 30-tube Post countercurrent distribution apparatus with the system ethyl acetate-heptane-water in a 1:1:2 ratio. Fluorescence of the upper phase was determined for all 30 tubes with a Farrand model 104244 recording spectrophotofluorometer with activating light of 300 mμ wave length. The fluorescence spectrum was recorded, and the peak at 333 mμ was expressed in arbitrary units of photocell output in amperes × 10^{-8}. Fig. 1 shows that two major fluorescence peaks were separated by

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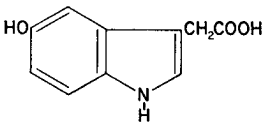
[1] J. Barchas, W. Mori, and A. B. Lerner, unpublished data.

[2] W. Mori and A. B. Lerner, to be published.

countercurrent distribution. However, on bioassay the active lightening agent was concentrated between the two fluorescence peaks. Because of these results the contents of tubes from the countercurrent distribution were pooled into three groups as follows: tubes 0 to 7, 8 to 16 and 16 to 25. Each group was worked up separately but in the same way. The organic liquid layer was separated and the aqueous layer extracted twice with equal volumes of ethyl acetate. The organic liquid layers from each group were combined and evaporated to dryness at 40° under reduced pressure. To dissolve the residue, 5 ml of benzene were added and the solution was placed on a silicic acid column. The latter was made by mixing 8 g of silicic acid powder (Mallinckrodt analytical reagent grade) of 80- to 120-mesh size with 2 g of Hyflo Super-Cel (Celite, Johns-Manville) and by putting the mixture in a glass tube 12 mm in diameter. Activation of silicic acid before use was accomplished by successive washing with 100-ml portions of methanol, acetone, and benzene. After one of the three pooled fractions from countercurrent distribution was placed on the column, benzene with increasing quantities of methanol (Fig. 2) was passed through the column. Positive pressure of 10 cm mercury was applied with nitrogen gas. Flow rate was 1.5 ml per minute at 22°. Then 15-ml fractions were collected. Fluorescence measurements, as described earlier, were made directly on the collected material. After elution of the contents of countercurrent tubes 8 to 16 from the column, three distinct fluorescence Peaks, I, II, and III, were obtained as shown in Fig. 2. When material from Tubes 17 to 25 was eluted, only a single large peak corresponding in location to I and an attenuated Peak II were obtained. After elution of the contents of Tubes 0 to 7, only a single large peak corresponding in location to III and a small Peak II were obtained. All biological activity appeared in Fraction II.

Material collected separately from Peaks I, II, and III was rerun through silicic acid columns. The activating and emitted fluorescence maxima were determined for these purified products in both benzene and ethanol. The activating maximum for Peaks I, II, and III was 304 mμ; and the fluorescence maxima were 338, 333, and 334 mμ, respectively. Since the fluorescence properties were similar to those of 5-hydroxyindoles, spot tests of the three products were made with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) on paper. All gave identical blue violet spots characteristic of indole compounds. Once the general class to which these compounds belonged became known it was possible to proceed with work on their structure.

Identification of III as 5-Hydroxyindole-3-acetic Acid—



The ultraviolet absorption curve of III in ethanol, obtained with a Cary model 14 spectrophotometer, was identical with that of 5-hydroxyindole-3-acetic acid (Fig. 3). Furthermore, both gave the same acid-base shifts. Activation and fluores-

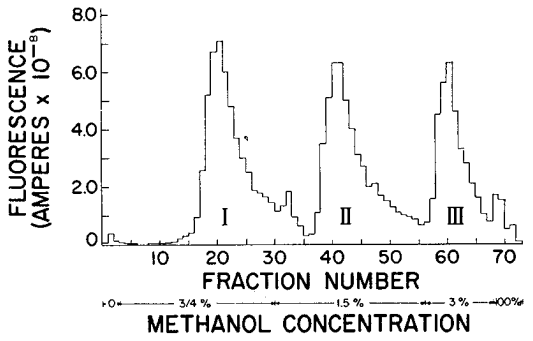


FIG. 2. Silicic acid chromatography of countercurrent pooled Fractions 8 to 16 gave three distinct peaks as measured by fluorescence. The material had been put on the column with benzene and eluted with benzene to which methanol was added in increasing quantity. Biological activity was found only in Peak II.

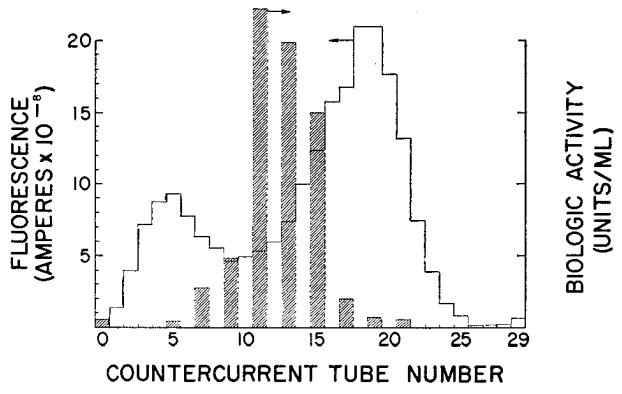


FIG. 1. Countercurrent distribution in ethyl acetate-heptane-water in a 1:1:2 ratio was carried out on an extract made from defatted, trimmed, lyophilized, bovine pineal glands which had been mixed in water and then extracted with ethyl acetate. Fluorescence and biological activity were measured for the upper phase only. The values in the chart refer to the upper phase alone. Contents of Tubes 0 to 7, 8 to 16, and 16 to 25 were pooled, and each group was put on a separate silicic acid column.

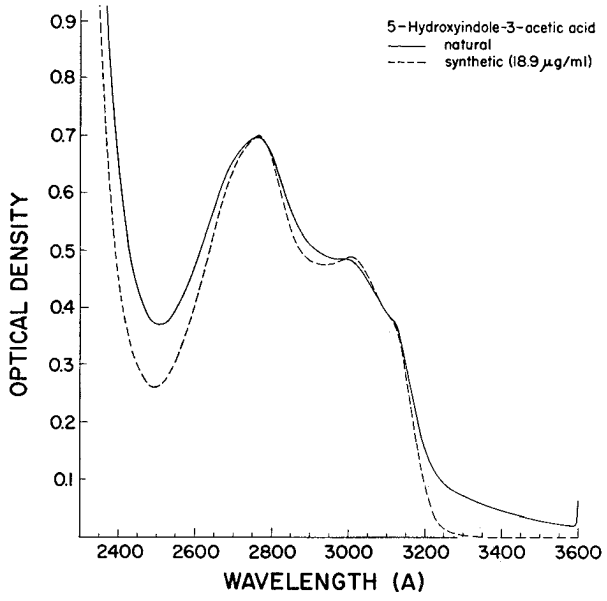
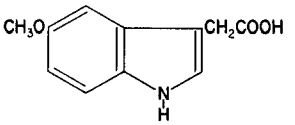


FIG. 3. The ultraviolet absorption curve of 5-hydroxyindole-3-acetic acid was almost identical with that of Fraction III from the silicic acid column. The concentration of synthetic 5-hydroxyindole acetic acid was adjusted so that at its optical density maximum both natural and synthetic compounds gave the same values. Both substances gave the same acid-base shifts.

cence maxima for III and 5-hydroxyindole-3-acetic acid were the same, and these values were different from those of I and II. Additional evidence for the identification of III as 5-hydroxyindole-3-acetic acid were the following findings: identical distribution in a 29-tube transfer countercurrent distribution run in a solvent system of ethyl acetate-heptane-water in a 1:1:2 ratio; the same elution curves from silicic acid columns; equal R_F values on paper chromatography with the system propanol-concentrated ammonia-water in a 16:1:3 ratio, and identical electrophoretic mobility at pH 4.5 and pH 8.9.

It is estimated that 100 g of fresh pineal tissue contain 200 μg 5-hydroxyindole-3-acetic acid.

Identification of I as 5-Methoxyindole-3-acetic Acid—



All three compounds, I, II, and 5-hydroxyindole-acetic acid, gave exactly the same color when reacted with Ehrlich's reagent. I and II showed similar ultraviolet absorption curves and the same fluorescence characteristics. However, these properties were significantly different from those of 5-hydroxyindole acetic acid (Fig. 4). Furthermore, the ultraviolet absorption curves of I and II disclosed no acid-base shift. Thus, these compounds seemed to be substituted 5-hydroxyindoles. Additional evidence for a block at position 5 was given by electrophoresis at pH 11.9 which failed to reveal an ionizable phenolic group (Fig. 5). The presence of a side chain at position 3 was consistent with the ultraviolet and fluorescence characteristics of many reference indoles and I. On electrophoresis it was found that I behaved as though it had a free carboxy group. At pH 1.5 its mobility was the same as that of 5 hydroxyindole-acetic acid, and at pH 11.9 its mobility was les

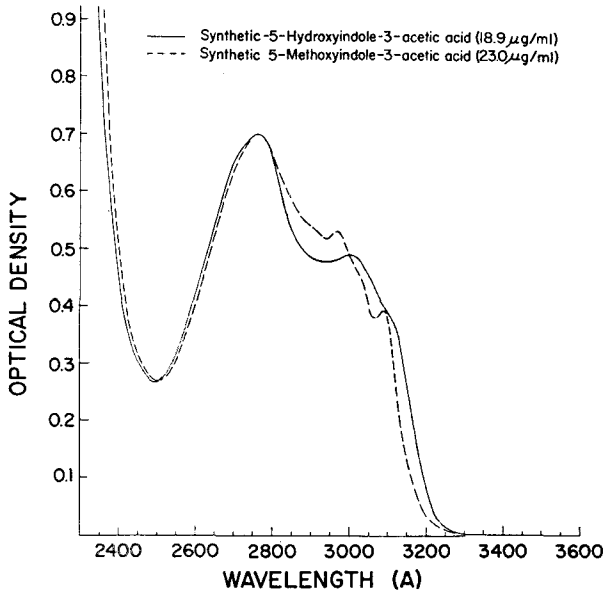


FIG. 4. The ultraviolet absorption spectra of 5-hydroxyindole-3-acetic acid and 5-methoxyindole-3-acetic acid were compared. The absorption maxima were at the same wave length, but the shoulders were different.

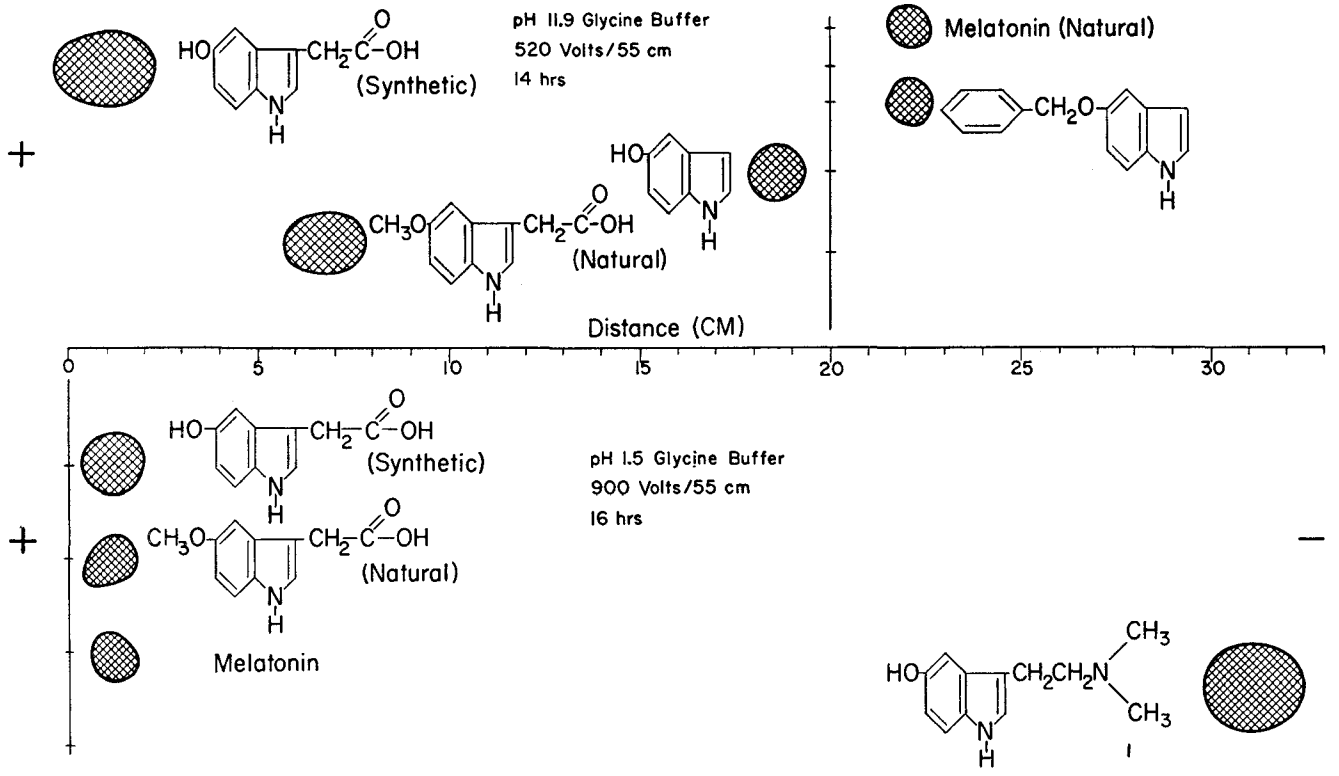
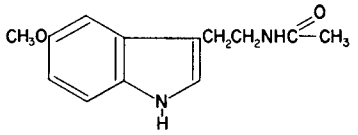


FIG. 5. Electrophoresis at pH 11.9 revealed greater mobility of synthetic 5-hydroxyindole-acetic acid toward the anode than of natural 5-methoxyindole-acid. However, at pH 1.5 their mobilities were the same. Melatonin had the mobility of an unchanged molecule at both pH 11.9 and 1.5.

than that of 5-hydroxyindole-acetic acid. No ionizable amine group could be present. One milligram of I was allowed to react with diazomethane in ether and ethanol. After removal of the organic solvents the product was subjected to a 30-tube countercurrent distribution of heptane-water in a 1:1 ratio. By fluorescence analysis the greatest concentration of the methyl ester of I was in Tube 17. Therefore, Tubes 15 to 21 were pooled. The heptane layer plus an ethyl acetate extract of the water layer were taken to dryness under reduced pressure, and the ester was sublimed at 40° under reduced pressure. When the ester was placed in a mass spectrometer, its molecular weight was found to be 219. A strong peak at mass 160 indicated the structure of a fragment to be $CH_3O—C_8H_5N—CH_2—$. These data suggested that I was 5-methoxyindole-3-acetic acid. I was synthesized from 5-methoxyindole-3-acetonitrile (6–8). The synthetic and natural compounds were found to have identical chemical and physical properties as determined by reaction with Ehrlich's reagent; infrared, ultraviolet absorption, and fluorescence curves (Fig. 6); countercurrent distribution; silicic acid column; and paper chromatography with acidic, basic, and neutral solvents. Mass spectrometry of the methyl esters of both synthetic and natural I revealed the same proportion of 219 and 160 masses.

The quantity of 5-methoxyindole-3-acetic acid in fresh bovine pineal glands is about the same as 5-hydroxyindole-3-acetic acid, being about 200 μg per 100 g of fresh gland.

Identification of II (Melatonin) as N-Acetyl-5-methoxytryptamine—



For several reasons elucidation of the structure of melatonin turned out to be a much more difficult task than identification of the other two compounds. The concentration of melatonin in the pineal gland is only one-tenth that of 5-methoxyindole-acetic acid or 5-hydroxyindole-acetic acid. At no time was it feasible to have more than 100 μg of melatonin available for analysis.

As mentioned previously, fluorescence properties and the ultraviolet absorption curves of melatonin and I were quite similar. No acid-base shift was observed. These findings suggested that melatonin also was a 5-methoxyindole. Unlike I, melatonin behaved like a neutral compound on electrophoresis at different pH values. However, a carbonyl or amide function appeared to be present in a side chain in position 3 because on the silicic acid column melatonin acted more polar than 5-methoxyindole-acetic acid but less polar than 5-hydroxyindole-acetic acid. The ultraviolet absorption and fluorescence curves revealed that if a carbonyl group were present in a side chain in position 3, it could not be conjugated with the ring (Fig. 7). Also, it was unlikely that melatonin was an ester of 5-methoxyindole-acetic acid because on hydrolysis with barium or sodium hydroxide no 5-methoxyindole-acetic acid was detected. At this point it was remembered that acetylcholine is a relatively potent frog skin-lightening agent. Perhaps melatonin was an *N*-acetylated methoxytryptamine. The recent finding of *N*-acetylserotonin as a metabolic product of serotonin (9) supported this idea. Therefore, *N*-acetyl-5-methoxytryptamine was synthesized (3, 10) and compared with natural melatonin. With a single exception all of the following properties of these two substances were identical: fluorescence, ultraviolet absorption, elution curves from silicic acid and aluminum oxide columns, paper chromatography with six different solvent systems, countercurrent distribution, and biological potencies on frog skin. The six different paper chromatography systems were: isopropanol-concentrated NH_3-water 16:1:3 (R_F 0.83); *n*-butanol-acetic acid-water 4:1:5 (R_F 0.9); *n*-butanol-acetic acid-water-pyridine 15:3:12:10 (R_F 0.8); heptane-pyridine 7:3 (R_F 0.10); heptane-pyridine 6.5:3.5 (R_F 0.80), and benzene-

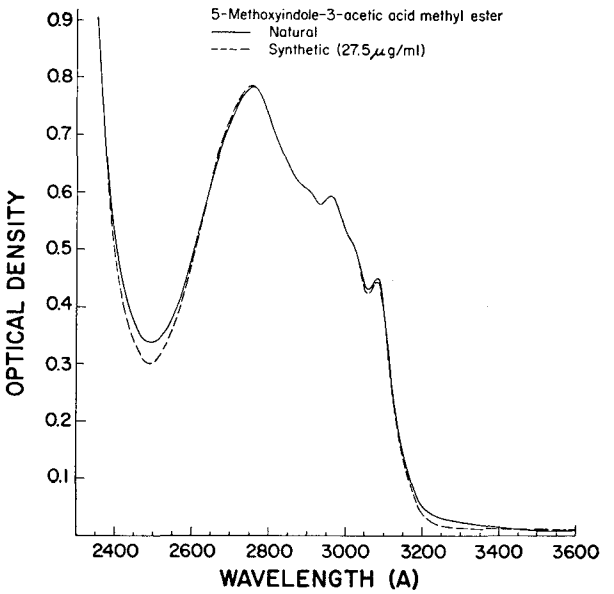


FIG. 6. The methyl esters of synthetic 5-methoxyindole-3-acetic acid and of compound I from the silicic acid column gave identical ultraviolet light absorption curves. The concentration of the synthetic ester was adjusted so that at its optical density maximum both natural and synthetic compounds gave the same values.

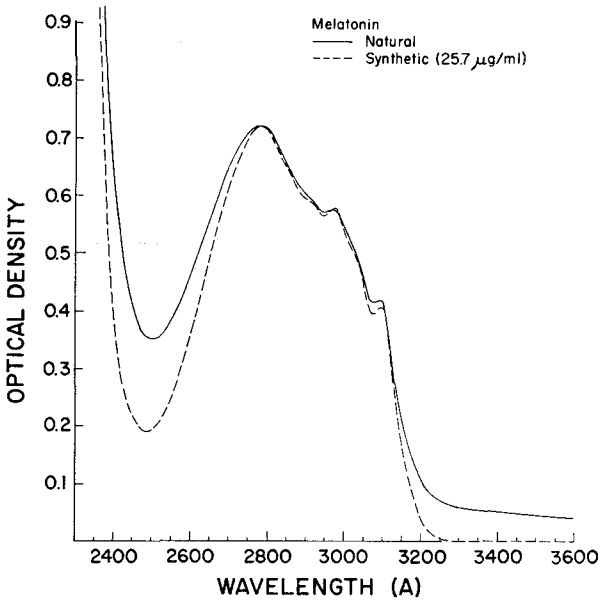


FIG. 7. Synthetic and natural melatonin (compound II from the silicic acid column) showed almost identical ultraviolet light absorption spectra.

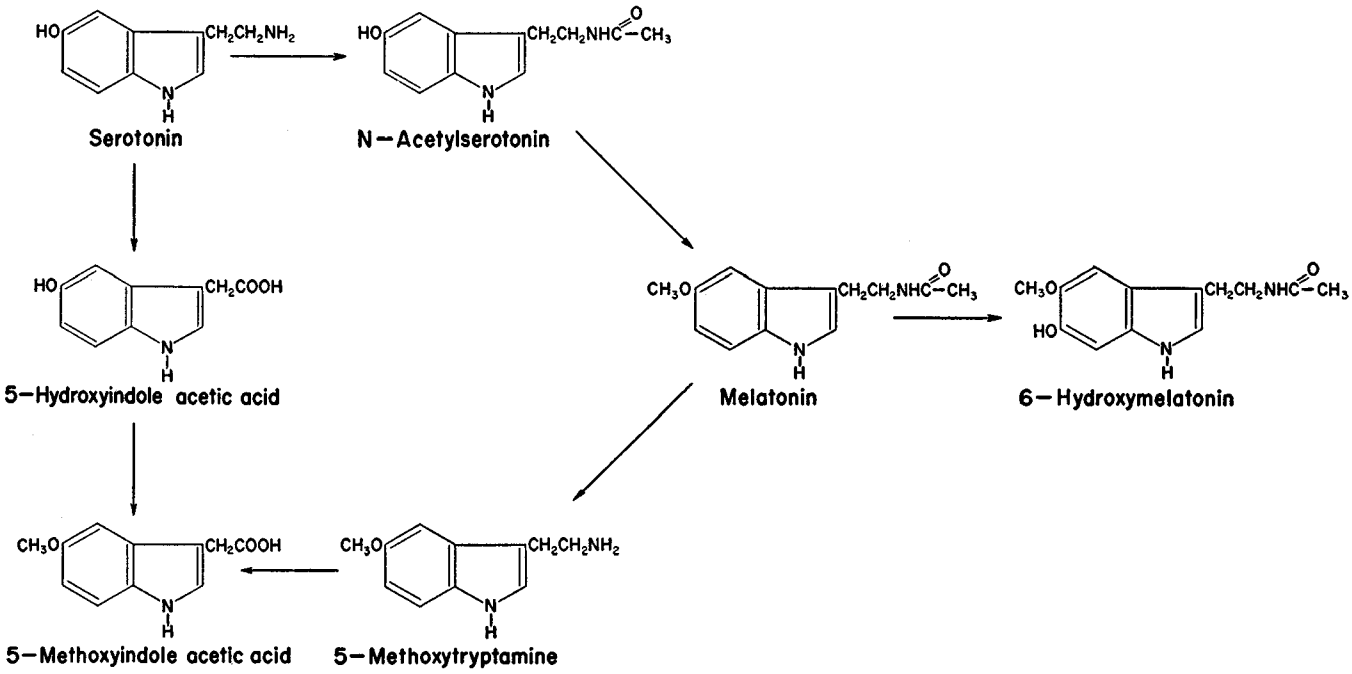


FIG. 8

ethyl acetate-water 20:1:20 (R_F 0.39). The single exception was infrared analysis. It has not been possible to purify natural melatonin to the point where it gives a good infrared curve. This difficulty may be due to the fact that melatonin does not have a readily ionizable group. Nevertheless, the other chemical, physical, and biological data indicate that melatonin is *N*-acetyl-5-methoxytryptamine.

DISCUSSION

During the past two years it has been generally assumed that in mammals *O*-methylation of catechol amines occurs but not of hydroxyindoles (9, 11, 12). The following findings point to the occurrence of *O*-methylation of hydroxyindoles: isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands and the demonstration of melatonin activity in extracts of human pineal glands and urine,[3] in human, monkey, and bovine peripheral nerves (13) and in some regions of the nervous system of monkeys and cattle.[4] The extremely small quantity of melatonin in biological tissue probably was responsible for the slow progress in this field.

Melatonin in concentrations as low as 10^{-12} g per ml can prevent and partially reverse the darkening actions of melanocyte-stimulating hormone, adrenocorticotrophic hormone, and caffeine on frog (*Rana pipiens*) skin (14). It is of interest that the most potent frog skin-darkening substance, α-melanocyte-stimulating hormone, is effective in a concentration of 10^{-12} g per ml and that, like melatonin, it also is an *N*-acetyl compound. These two *N*-acetyl compounds may compete with each other to darken or lighten melanocytes.

The physiological role of melatonin in animals is not known at the present time.

Since a great proportion of injected serotonin is metabolized to *N*-acetylserotonin, it is likely that *O*-methylation occurs after and not before *N*-acetylation. Furthermore, a hydroxyindole-*O*-methyl transferase that utilized *N*-acetylserotonin as a substrate much more effectively than serotonin recently was found in pineal tissue (15). 5-Hydroxyindole acetic acid also was utilized as a substrate for this enzyme but at a slow rate. Feeding 5-methoxytryptamine to rats resulted in the excretion of 5-methoxyindole acetic acid in the urine (16). Recently Kopin *et al.* (17) showed that intraperitoneal injection of labeled melatonin into rats resulted in the urinary excretion of a high yield of 6-hydroxymelatonin as the sulfate and glucuronide conjugates. These findings suggest that melatonin is formed and metabolized in the following manner. Serotonin is acetylated to *N*-acetylserotonin which in turn is *O*-methylated to melatonin. Melatonin can undergo hydroxylation to form 6-hydroxymelatonin, or it can be deacetylated to 5-methoxytryptamine which in turn can be oxidized to 5-methoxyindole acetic acid. The 5-methoxyindole acetic acid also may come from serotonin via 5-hydroxyindole acetic acid. These reactions are summarized in Fig. 8.

[3] Y. Takahashi and A. B. Lerner, unpublished data.

[4] J. Barchas, W. Mori, and A. B. Lerner, unpublished data.

SUMMARY

Two methoxyindoles, *N*-acetyl-5-methoxytryptamine (melatonin) and 5-methoxyindole-3-acetic acid, as well as 5-hydroxyindole-3-acetic acid, were isolated from bovine pineal glands. Melatonin is the most potent agent known to lighten the color of frog skin.

Acknowledgments—We wish to thank Drs. Teh H. Lee, M. R. Wright, W. Mori, and Richard V. Heinzelman for their help in many different phases of this research project.

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