The Fate of Melatonin in Animals

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Melatonin (N-acetyl-5-methoxytryptamine), the most potent melanocyte-contracting substance known, has been demonstrated in bovine pineal glands and peripheral nerve of man, monkey, and cattle (1, 2). Its formation from serotonin, by N-acetylation (3) and subsequent O-methylation (4), has been indicated. In a preliminary communication, we provided evidence for 6-hydroxylation as the major metabolic pathway for melatonin (5).

This report describes the synthesis of melatonin labeled in three different positions with C14 or H3, the physiological disposition of administered hormone, and its metabolism in vivo and in vitro.

**EXPERIMENTAL PROCEDURE**

**Melatonin-2-C14—Serotonin-2-C14** (5 mg) having a specific activity of 3.75 με per μmole (obtained as the creatinine sulfate complex from Nuclear-Chicago Corporation), was dissolved in 1 ml of water in a 15-ml glass-stoppered centrifuge tube. To this solution, 0.1 ml of triethylamine and 0.2 ml of acetic anhydride were added and the contents shaken. After the solution cooled, it was gently warmed on the steam bath for 15 minutes. Paper chromatography of the solution in 7% NaCl in 1% acetic acid showed the presence of a single compound having the same Rf as N-O-diacetylsertotonin. Hydrolysis of the O-acetyl group was effected by the addition of excess NaHCO3 and warming the solution on a steam bath for 15 minutes. Unhydrolyzed N,O-diacetylsertotonin was removed by extraction into chloroform. The aqueous residue was then made acid with 6 N HCl and the N-acetylsertotonin-2-C14 extracted several times with ethyl acetate. The organic phase was evaporated in a vacuum and the residue taken up in two 0.1 ml portions of ethanol and distributed along a 12-inch starting line on Whatman No. 3MM filter paper. After chromatography in 8% NaCl in 1% acetic acid, the radioactive area corresponding to N-acetylsertotonin (Rf, 0.40) was eluted with water. Conversion of the N-acetylsertotonin-2-C14 to melatonin-2-C14 by O-methylation was accomplished with the use of 5-hydroxyindole-O-methyl transferase from bovine pineal gland (4) and S-adenosylmethionine as follows. Frozen bovine pineal glands (Canada Packers Ltd., Toronto, Canada), 12 g., were homogenized in 3 volumes of ice-cold isotonic KC1 solution and centrifuged at 75,000 × g. The clear supematant solution was incubated for 2 hours at 37° with 2 ml of 0.5 M phosphate buffer (pH 7.9), N-acetylsertotonin-2-C14 obtained above, and 20 μmole of S-adenosylmethionine. The melatonin-2-C14 formed was extracted twice into 5 volumes of chloroform, the combined organic phases evaporated in a vacuum, and the residue taken up in ethanol and applied to a starting line on Whatman No. 3MM filter paper. After chromatography in benzene-ethyl acetate-water (20:1:20), the single radioactive area (Rf, 0.35 to 0.50) corresponding to melatonin was eluted with water. By the use of these procedures, 30 to 40% of the serotonin-1-C14 could be converted to melatonin-2-C14.

**Melatonin-methoxy-C14—N-Acetylserotonin (5 mg) was incubated with 5-hydroxyindole-O-methyl transferase as described above, with the use of 10 μmole of S-adenosylmethionine-methyl-C14 prepared (6) from L-methionine-methyl-C14 (2.29 mc per mmole obtained from New England Nuclear Corporation). The enzymatically formed melatonin methoxy-C14 was extracted and purified as described above.

**Melatonin-acetyl-H3—5-Methoxytryptamine (5 mg) was dissolved in 1 ml of ethyl acetate and 0.1 ml of triethylamine was added. Two μmole of acetic anhydride H3 (100 μg per μmole) in 0.02 ml of benzene were added to this mixture. After standing for 30 minutes, the solvents were evaporated in a vacuum, the residue taken up in 15 ml of chloroform, and the solution washed successively with 5 ml of 5% Na2CO3, 5 ml of water, 5 ml of 0.1 N HCl, and twice with 5 ml of water. The chloroform was then evaporated in a vacuum, the residue taken up in ethanol, and the melatonin isolated by chromatography as described above.

**Preparation of Tissues for Estimation of Total Radioactivity and Radioactive Melatonin—Tissues were immediately removed and homogenized with 3 volumes of 0.1 N HCl. An aliquot was taken and radioactive melatonin was measured as described below. Another aliquot was freeze-dried and a portion of the dried residue was dissolved in 2 ml of Hyamine and 10 ml of phosphor. Radioactivity measurements were made in a scintillation spectrometer and internal standards were used for correction of quenching.

**Estimation of Radioactive Melatonin—Melatonin present in the tissue homogenate was extracted into 3 volumes of chloroform. After centrifugation and removal of the aqueous phase by aspiration, the chloroform extract was washed once with one-third volume of 0.5 M phosphate buffer, at pH 7, and an aliquot of the organic phase transferred to a glass vial. The solvent was evaporated in a current of warm air and the residue dissolved in 4 ml of ethanol. Phosphor was added and the solution counted in a scintillation spectrometer. About 80% of the methylnicotin-acetyl-H3 added to tissues was recovered by this procedure. The radioactive material present in biological material and extracted by this procedure showed a single peak having the same Rf values as authentic melatonin after chromatography in butanol-acetic acid-water (4:1:1) and benzene-ethyl acetate-water (20:1:20).

**RESULTS**

**Disappearance of Melatonin in Whole Mouse—Melatonin-acetyl-H3 (40 μmc) was injected into the tail veins of mice (21 to
After 40 minutes, the rate of disappearance of the melatonin was much slower, corresponding to a half-life of about 35 minutes.

Physiological Disposition of Administered Melatonin—Melatonin-acetyl-H³ was injected intravenously into the tail veins of ten adult, male Sprague-Dawley rats. Five rats were given 200 µg (401 mpc) of melatonin-acetyl-H³ and killed after 1 minute; five were given 400 µg (812 mpc) of melatonin-acetyl-H³ and killed after 30 minutes. Tissues were removed immediately and assayed for total radioactivity and unchanged melatonin. (Table I).

Within 1 minute, melatonin was found to be present in all tissues examined. The highest concentrations were found in the liver and kidney, and the lowest in fat and skin. Melatonin was also found in the brain, indicating that there is little hindrance to its crossing the blood brain barrier. After 1 minute, unchanged melatonin accounted for 70 to 80% of the radioactivity present in each tissue, except for the liver and plasma. After 30 minutes, melatonin accounted for only 35 to 50% of the total radioactivity in most tissues. In the adrenal gland and brain, about two-thirds of the radioactivity was present as melatonin, whereas in the liver and plasma it accounted for only 10 to 20%. The total amount of melatonin was decreased in all tissues after 30 minutes, and total radioactivity was increased in kidney, intestines, and skin.

Excretion of Melatonin Metabolites—Melatonin, labeled in one of several positions, was injected into the tail veins of male Sprague-Dawley rats and the urine and feces were examined for radioactive products. Four rats were given melatonin-acetyl-H³ (10 µc each), two rats melatonin-2-C¹⁴ (1 µc each), two rats melatonin-methoxy-C¹⁴ (1 µc each), and one rat a mixture of acetyl-H³ and methoxy-C¹⁴ melatonin. Urine and feces were collected for a period of 48 hours. The total radioactivity found in the urine during this time accounted for 60 to 70% of the administered melatonin, and about 15% of the administered radioactivity was found in the feces. No significant differences were found in the total radioactivity of the urine and feces after the administration of the various types of labeled melatonin.

The urinary metabolites were separated and estimated with paper chromatography as follows. An aliquot of the urine containing about 15,000 c.p.m. was applied to a starting line on Whatman No. 3MM filter paper. After chromatography in isopropanol-5% ammonia (4:2), three radioactive peaks were found. The first two radioactive compounds have been identified as the glucuronic acid (Rₜ, 0.22) and the sulfate (Rₜ, 0.55) conjugates of 6-hydroxymelatonin (N-acetyl-5-methoxy-6-hydroxytryptamine) (5). The third radioactive compound (Rₜ, 0.8) has only been partially characterized. Unchanged melatonin could not be detected in the urine or feces.

Strip sections of the chromatograms containing the various radioactive compounds were eluted with water and the eluate evaporated in a current of warm air in glass vials. The residues were dissolved in 0.2 ml of water and the radioactivity determined after the addition of 4 ml of ethanol and 10 ml of phosphoric acid, and the animals were killed at various time intervals. The whole mouse, including excreta, was homogenized in 100 ml of 0.4 N perchloric acid in a Waring blender, and aliquots of the homogenate were assayed for melatonin as described above. The results shown in Fig. 1 indicate a multiphasic disappearance curve for melatonin. In the first 10 minutes, there is a rapid decrease in the melatonin (approximating a half-life of 2 minutes). After 40 minutes, the rate of disappearance of the melatonin was much slower, corresponding to a half-life of about 35 minutes.

**Table I**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 minute after 401 mpc of melatonin-acetyl-H³</th>
<th>30 minutes after 802 mpc of melatonin-acetyl-H³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total radioactivity (mµc/g)</td>
<td>Melatonin (mµc/g)</td>
</tr>
<tr>
<td>Liver</td>
<td>7.81 ± 0.66 (3.62 ± 0.54)</td>
<td>6.88 ± 0.40 (0.80 ± 0.04)</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.70 ± 0.30 (3.07 ± 0.46)</td>
<td>18.21 ± 7.5 (9.96 ± 0.06)</td>
</tr>
<tr>
<td>Lung</td>
<td>1.90 ± 0.14 (1.29 ± 0.18)</td>
<td>1.68 ± 0.17 (0.29 ± 0.07)</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.71 ± 0.16 (1.84 ± 0.31)</td>
<td>1.10 ± 0.09 (0.34 ± 0.05)</td>
</tr>
<tr>
<td>Pelvic fat</td>
<td>0.45 ± 0.07 (0.27 ± 0.02)</td>
<td>0.57 ± 0.07 (0.24 ± 0.04)</td>
</tr>
<tr>
<td>Mesenteric fat</td>
<td>1.97 ± 0.29 (1.44 ± 0.31)</td>
<td>1.21 ± 0.33 (0.42 ± 0.07)</td>
</tr>
<tr>
<td>Adrenals</td>
<td>3.18 ± 0.56 (1.90 ± 0.18)</td>
<td>1.98 ± 0.42 (1.33 ± 0.42)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.80 ± 0.05 (0.60 ± 0.14)</td>
<td>0.42 ± 0.01 (0.26 ± 0.06)</td>
</tr>
<tr>
<td>Heart</td>
<td>2.80 ± 0.16 (1.85 ± 0.28)</td>
<td>1.33 ± 0.16 (0.37 ± 0.05)</td>
</tr>
<tr>
<td>Abdominal muscle</td>
<td>0.70 ± 0.10 (0.52 ± 0.05)</td>
<td>1.40 ± 0.36 (0.32 ± 0.09)</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2.23 ± 0.15 (1.65 ± 0.26)</td>
<td>6.30 ± 3.4 (1.28 ± 0.50)</td>
</tr>
<tr>
<td>Skin</td>
<td>0.29 ± 0.10 (0.17 ± 0.01)</td>
<td>1.54 ± 0.62 (0.30 ± 0.10)</td>
</tr>
<tr>
<td>Plasma</td>
<td>2.00 ± 0.16 (0.99 ± 0.07)</td>
<td>2.16 ± 0.62 (0.46 ± 0.05)</td>
</tr>
</tbody>
</table>

**Fig. 1. Disappearance of melatonin-acetyl-H³ in the mouse.** Each mouse was given (40 mpc) melatonin-acetyl-H³ intravenously. Each point represents the mean (± standard deviation) of five mice.
FIG. 2. Chromatogram of the radioactive metabolites of melatonin-acetyl-H in urine. Percentages of excreted radioactivity of various metabolites are shown at tops of bars. See text for identity of radioactive peaks.

Fig. 2 shows the distribution of radioactivity among the various metabolites. Essentially, the same results were obtained after the administration of melatonin-2-C, melatonin-methoxy-C, and melatonin-acetyl-H. The major metabolite, accounting for 70 to 80% of the radioactivity, was the sulfate conjugate of 6-hydroxymelatonin, whereas the glucuronic acid conjugate of this compound represented only 5% of the administered radioactivity. The unidentified metabolite (R, 0.8) accounted for about 12% of the radioactivity.

After the administration of melatonin-2-C, the urine was examined for the presence of 5-methoxyindoleacetic acid, a compound normally present in the pineal gland (7), by acidifying and extracting with 5 volumes of ethyl acetate. The organic phase was evaporated in a vacuum, and the residue dissolved in ethanol and subjected to chromatography on Whatman No. 1 paper with n-butanol-acetic acid-water (4:1:1) and isopropanol-5% ammonia (4:2). A radioactive compound corresponding in R, values to authentic 5-methoxyindoleacetic acid was found, accounting for less than 0.5% of administered radioactivity.

Metabolism of Melatonin in Vitro—Previous work in this and other laboratories (8, 9) has shown that indole derivatives are hydroxylated in position 6 by an enzyme in the microsomes of the liver that requires TPNH and oxygen. Incubation of melatonin (50 μg) with microsomes and soluble supernatant fraction obtained from 50 mg of rat liver, 4 μmoles of TPN, 20 μmoles of glucose 6-phosphate, 150 μmoles of phosphate buffer (pH 7.4), 10 μmoles of MgCl₂, and 20 μmoles of nicotinamide, in a final volume of 3 ml at 37° for 1 hour, resulted in the disappearance of 50% of the melatonin. When the pyridine nucleotide and glucose 6-phosphate were omitted, no metabolism of melatonin was observed. The incubated reaction mixture contained a metabolite having the same R, value as 6-hydroxymelatonin (obtained from rat urine after the administration of melatonin and subsequent hydrolysis with β-glucuronidase).

**FIG. 3. Formation and metabolism of melatonin**

**DISCUSSION**

The absence of melatonin among the radioactive substances found in the urine after its intravenous administration demonstrated that the circulating hormone is completely metabolized. Its rapid rate of metabolism is shown by the disappearance curve of melatonin in the whole mouse (Fig. 1). The multiphasic disappearance curve indicates that a portion of the administered hormone is protected from enzymatic destruction, perhaps by binding, or increased solubility in lipid depots, or both. After 1 minute, the hormone was found in all tissues, including the brain. Certain tissues, such as adrenal gland and small intestine, retain the hormone after 30 minutes in concentrations exceeding that of the plasma.

The major pathway for metabolism of melatonin is hydroxylation on position 6 followed by conjugation, primarily with sulfate (70%) and, to a small extent, with glucuronic acid (6%). A minor transformation product of melatonin (12%) has only been partially characterized. This compound retained the 5-methoxy-C and the H-acetyl-ethylamine side chain. It did not react with Ehrlich’s reagent, which suggests that either the indole nucleus was cleaved, or that there was oxidation or substitution on position 2 of the indole ring. Since indoles have been shown to be oxidized on position 2 (10) or undergo ring cleavage (11), melatonin might be metabolized by either of these pathways.
Lerner et al. (7) have found 5-methoxyindoleacetic acid in the pineal body. We have shown that less than 0.5% of administered melatonin is converted to 5-methoxyindoleacetic acid. It was previously demonstrated that 5-hydroxyindoleacetic acid can be O-methylated by hydroxyindole-O-methyl transferase (4), an enzyme highly localized in the pineal body. From these observations, it would appear that 5-methoxyindoleacetic acid arises in the pineal body either by deacetylation of melatonin followed by deamination, or by O-methylation of 5-hydroxyindoleacetic acid.

The formation and metabolism of melatonin are shown in the accompanying scheme (Fig. 3).

The major route of formation of melatonin involves acetylation of serotonin by an enzyme in the liver and pineal body that requires the presence of acetyl coenzyme A (3). N-Acetylserotonin is then, O-methylated to form melatonin by an enzyme localized in the pineal gland that requires S-adenosylmethionine as the methyl donor (4). Melatonin is mainly metabolized by hydroxylation in position 6 by an enzyme in the microsomes of the liver that requires a TPNH-generating system. The hydroxylated melatonin is then excreted as sulfate and glucuronic acid conjugates.

SUMMARY

The synthesis of melatonin-2-3H, melatonin-methoxy-3H, and melatonin-acetyl-3H is described.

After the administration of radioactive melatonin to mice, the melatonin is rapidly metabolized and a small portion is bound and retained. Circulating melatonin is rapidly taken up by all tissues, including the brain.

Melatonin is completely metabolized in the body; the major pathway involves 6-hydroxylation, followed by conjugation mainly with sulfate. A minor metabolite (about 12%) that retains its methoxy and N-acetyl group has been found in the urine. Only traces of administered melatonin form 5-methoxyindoleacetic acid.

An enzyme that hydroxylates melatonin in position 6 has been found in the microsomes of the liver.

REFERENCES

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