Purification and Properties of Hydroxyindole-O-methyl Transferase

JULIUS AXELROD AND HERBERT WEISSBACH

From the Laboratory of Clinical Science, National Institute of Mental Health and Laboratory of Clinical Biochemistry, National Heart Institute, United States Public Health Service, Bethesda, Maryland

(Received for publication, July 18, 1960)

The demonstration of the hormone melatonin (N-acetyl 5 methoxytryptamine) in bovine pineal gland (1) prompted a study of the enzymes involved in its formation and metabolism. In preliminary studies (2, 3) we described an enzyme, hydroxyindole-O-methyl transferase, in the pineal gland of cows, that catalyzes the O-methylation of N-acetyl-5-tryptamine to melatonin. This paper describes the purification of the enzyme from cow pineal gland, its properties, assay, and distribution.

EXPERIMENTAL PROCEDURE

Materials

We wish to thank the following for their generous donations: Dr. A. B. Lerner for 5-methoxyindoleacetic acid and bovine pineal glands; Dr. I. J. Kopin for N-acetyl 2-C14-serotonin; Sandoz Company for N-acetyl-4-hydroxytryptamine and 4-hydroxydimethyltryptamine; The Upjohn Company for N-methylserotonin, bufotenine, 5-methoxytryptamine, and melatonin. AMe1 and C14-methyl AMe were prepared enzymatically (4); N-acetylserotonin was prepared by a modification of the procedure of McIsaac and Page (5); and frozen pineal glands were obtained commercially from Canadian Packers, Toronto, Ontario.

Methods

Estimation of Melatonin—Melatonin can be separated from N-acetylserotonin and other hydroxyindoles by extraction into chloroform. Biological material in a 15-ml glass-stoppered centrifuge tube was shaken with 8 ml of chloroform for 10 minutes. After centrifugation the aqueous layer was removed and the chloroform extract washed twice with 3-ml portions of water. A 5-ml aliquot of the chloroform phase was transferred to a 20-ml beaker and evaporated to dryness in a stream of warm air. The residue was dissolved in 0.5 ml of ethanol and counted in Tri-Carb liquid scintillation counter after the addition of 10 ml of phosphor.

Method-Cl4-indoleamine derivatives formed enzymatically were extracted into 6 ml of isoamyl alcohol after adjustment of the aqueous phase to pH 10. A 4-ml aliquot of the isoamyl alcohol extract was transferred to a glass vial containing 3 ml of ethanol and 10 ml of phosphor and the radioactivity counted as described above. Although many of the methoxyindoleamine derivatives presumably formed were not available, the conditions and validity of the extraction procedure were tested with 5-methoxytryptamine as a model. Methoxy-C14-indoleacetic acid was extracted into chloroform from an acidic solution, and an aliquot of the chloroform extract was transferred to a glass vial and treated as described above.

Enzyme Assay—Hydroxyindole-O-methyl transferase was determined by measuring melatonin formed from N-acetylserotonin and AMe. If increased sensitivity was desired, either N-acetyl-2-C14-serotonin or Cl4-methyl AMe could be used. The incubation mixture in a 15-ml glass-stoppered centrifuge tube contained 100 mmoles of phosphate buffer pH 7.9, 0.2 mmole of AMe, 0.1 mmole of N-acetylserotonin, enzyme obtained from 5 to 50 mg of cow pineal gland, and water to make a final volume of 1 ml. After 30 minutes of incubation at 37°, 8 ml of chloroform were added to the tube, and melatonin was extracted and determined as described above. For radioactive experiments, 10 mmoles of C14-methyl AMe (4770 c.p.m.) or 12 mmoles of N-acetyl-2-C14-serotonin (3510 c.p.m.) were used.

Purification of Hydroxyindole-O-methyl Transferase from Beef Pineal Gland—Unless specified, all purification procedures were carried out at 4°. A quantity of 16 g of frozen pineal glands was thawed and homogenized in 80 ml of isotonic KCl. The resulting suspension was centrifuged at 78,000 X g for 1 hour, and 52 ml of the supernatant fluid were adjusted to pH 5.2 by the dropwise addition of 1 N acetic acid. The slightly turbid solution was placed on a water bath maintained between 47°-49° for 3 minutes, immediately adjusted to pH 6.5, and centrifuged at 8000 X g for 10 minutes. To 50 ml of the supernatant fluid were added 40 ml of saturated ammonium sulfate solution (adjusted to about pH 8.0) to 45% saturation. After centrifugation the supernatant fraction was removed and brought to 68% saturation by the addition of 60 ml of saturated ammonium sulfate. The precipitate obtained after centrifugation was dissolved in 12 ml of water and the solution adjusted to pH 5.3 with acetic acid. A total of 2 ml of alumina gel C14 (35 mg) was added to the resulting

1 The abbreviation used is: AMe, S-adenosylmethionine.
2 The sensitivity may be increased at least 30-fold by dissolving the residue with water instead of 3 N HCl, assaying the melatonin by activation at 290 mμ, and measuring the fluorescence at 360 mμ.
solution, and after centrifugation the gel was washed with 5 ml of water. The enzyme was eluted from the gel with three 5-ml portions of 0.1 M potassium phosphate buffer pH 6.5. An overall purification of the enzyme of about 25-fold was obtained by this procedure (Table I). The purified enzyme lost little activity when stored in a frozen state for several weeks.

**RESULTS**

**O-Methylation of N-Acetylserotonin to Melatonin by Pineal Gland**—It was previously found that incubation of N-acetylserotonin with the soluble fraction of cow pineal gland and AMe resulted in the formation of melatonin (cf. reference (2), Table I). In the absence of AMe, no detectable methylation occurred. However, when a soluble fraction obtained from large quantities (200 mg) of cow pineal gland was incubated with N-acetyl-2-CY serotonin at pH 7.9, without AMe, the chloroform extract had a significant amount of radioactivity. The radioactive material had the same RF values (ascending) as authentic melatonin when chromatographed on Whatman No. 1 filter paper in isopropanol-ammonia (5%), 8:2 (RF 0.89) and butanol-acetic acid-water, 8:2:2 (RF 0.91). No melatonin was formed when purified hydroxyindole-O-methyl transferase was incubated in the absence of AMe. These observations demonstrated that AMe and/or an AMe-forming enzyme is present in the pineal gland.

**Properties of the Purified Hydroxyindole-O-methyl Transferase**—The purified hydroxyindole-O-methyl transferase showed an absolute requirement for AMe (Table II). The Michaelis-Menten constants, Kₘ, for N-acetylserotonin and AMe were 5.4 × 10⁻⁵ M and 4.6 × 10⁻⁶ M, respectively.

Unlike catechol-O-methyl transferase (6), there was no requirement for a metal. The presence of an essential sulfhydryl group was indicated by complete inhibition of the enzyme by 10⁻⁴ M p-chloromercuribenzoate and slight activation with high concentrations of glutathione and cysteine.

Optimal enzyme activity occurred between pH 7.5 and 8.3. Figs. 1 and 2 show the effect of enzyme concentration and time on the formation of melatonin.

**Substrate Specificity**—Of all the substrates examined (Table III), N-acetylserotonin was by far the best. Other 5-hydroxyindoleamines, serotonin and its N-methylated derivatives, as well as 3-hydroxyindoleacetic acid, were also O-methylated, but at a much reduced rate. N-Acetyl-4-hydroxytryptamine was O-methylated at a slower rate than its 5-hydroxycongener. Negligible O-methylation was observed with other hydroxylated indoleamines.

**Organ and Species Distribution**—Hydroxyl-O-methyl transferase was found to be present in the pineal glands of monkey (Macaca riludetta), cow, and cat (Table IV). Pineal glands of other species, and AMe present in extracts of the pineal gland and AMe present in extracts of the pineal gland and AMe present in extracts of the pineal gland.
Purified enzyme (0.9 μg of protein) was incubated at 37° with 30 μmoles of pH 7.9 phosphate buffer, 10 μmoles of C4-methyl AMe, 0.2 μmole of substrate in a final volume of 0.5 ml. After 2 hours of incubation, the reaction mixture was examined for C14-labeled methoxy derivatives after extraction into organic solvent.

TABLE III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylsertotonin</td>
<td>100</td>
</tr>
<tr>
<td>Bufotene</td>
<td>14</td>
</tr>
<tr>
<td>5-Hydroxyindoleacetic acid*</td>
<td>12</td>
</tr>
<tr>
<td>N-Methylserotonin</td>
<td>9</td>
</tr>
<tr>
<td>Serotonin*</td>
<td>7</td>
</tr>
<tr>
<td>4-Hydroxy-N-acetyltryptamine</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4-Hydroxydimethyltryptamine</td>
<td>&lt;5</td>
</tr>
<tr>
<td>2-Hydroxytryptamine</td>
<td>&lt;5</td>
</tr>
<tr>
<td>6-Hydroxydiethyltryptamine</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* The methoxy derivatives formed were identified chromatographically.

TABLE IV

Purified enzyme (9.2 μg of protein) was incubated at 37° with 30 μmoles of pH 7.9 phosphate buffer, 10 μmoles of C4-methyl AMe, 0.2 μmole of substrate in a final volume of 0.5 ml. After 2 hours of incubation, the reaction mixture was examined for C14-labeled methoxy derivatives after extraction into organic solvent.

<table>
<thead>
<tr>
<th>Species</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey</td>
<td>5.8</td>
</tr>
<tr>
<td>Cow</td>
<td>2.1</td>
</tr>
<tr>
<td>Cat</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The purification, properties, distribution, and specificity of hydroxyindole-O-methyl transferase are described. The enzyme is highly localized in the pineal gland and catalyzes the O-methylation of N-acetylsertotonin to form the hormone melatonin. Although N-acetylsertotonin is by far the best substrate for the enzyme, other hydroxyindoles are also methylated.

REFERENCES


1 Unpublished observation (Weissbach, H., and Axelrod, J.)
Purification and Properties of Hydroxyindole-\(O\)-methyl Transferase
Julius Axelrod and Herbert Weissbach


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