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O-METHYLATION IN THE CONVERSION OF TYROSINE TO MELANIN

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SUMMARY

Catechol-*O*-methyl transferase catalyzes methylation of 5,6-dihydroxyindole and 5,6-dihydroxydihydroindole primarily at position 6. Hydroxyindole-*O*-methyl transferase catalyzes methylation of 5,6-dihydroxyindole mainly at position 5. 5,6-Dihydroxydihydroindole is not a substrate for this enzyme.

O-methylation probably plays a role in melanin formation *in vivo*.

INTRODUCTION

In the oxidation of tyrosine to melanin as catalyzed *in vitro* by tyrosinase the following compounds occur in succession: tyrosine, DOPA, DOPA quinone, 5,6-dihydroxydihydroindole-2-carboxylic acid, DOPA-chrome, 5,6-dihydroxyindole, indole-5,6-quinone and melanin[1]. It was generally assumed that the same steps occur *in vivo*.

In the past few years it has been shown that ortho-dihydroxyphenyl compounds and hydroxyindoles are enzymically *O*-methylated *in vitro* and *in vivo*[2,3]. *O*-Methylation of ortho-dihydroxyphenyl compounds is catalyzed by catechol-*O*-methyl transferase, an enzyme present in most tissue. *O*-methylation of hydroxyindoles is catalyzed by hydroxyindole-*O*-methyl transferase, which is found only in the pineal gland. Since some of the intermediates in melanin formation, such as 5,6-dihydroxyindole, are structurally similar to ortho-dihydroxyphenyl compounds and hydroxyindoles, it seemed reasonable that *O*-methylation could play an important role *in vivo* in the conversion of tyrosine to melanin. To investigate this possibility 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid were tested as substrates for catechol- and hydroxyindole-*O*-methyl transferase.

EXPERIMENTAL

5,6-Dihydroxyindole was prepared by a modification of the method of CROMARTIE AND HARLEY-MASON[4]. 2 ml of an aqueous solution containing 100 mg DOPA and 35 mg

Abbreviations: COMT, catechol-*O*-methyl transferase; HIOMT, hydroxyindole-*O*-methyl transferase.

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sodium bicarbonate was mixed with 3 ml water containing 300 mg potassium ferricyanide and 140 mg sodium bicarbonate. A deep red color formed immediately and gradually darkened. After 15 min 1.2 g zinc sulfate in 5 ml water was added and the mixture allowed to stand another 15 min. 5–10 mg of sodium hydrosulfite was added and the mixture was extracted with twice the volume of ethyl acetate and centrifuged. The clear, light pink ethyl acetate layer was taken to dryness *in vacuo*, and the dark brown residue was sublimed *in vacuo* at 130–150°. Approx. 3 mg 5,6-dihydroxyindole was obtained from 100 mg DOPA. 5,6-Dihydroxyindole was stored under vacuum at — 10°.

5,6-Dimethoxyindole was kindly supplied to us by Dr. H. V. HEINZELMAN of the Upjohn Company and Dr. A. HOFMANN of Sandoz, Ltd. Small samples of 5-hydroxy-6-methoxyindole, 5-methoxy-6-acetoxyindole and 5,6-diacetoxyindole-2-carboxylic acid were a gift from Dr. R. J. S. BEER of the University of Liverpool. The acetoxy derivatives were hydrolyzed to their free hydroxy forms in the following manner[5]. 2 mg was dissolved in 50 μl methanol and cooled to 5°. 50 μl of 10 % sodium hydrosulfite in 10 % aq. sodium hydroxide was added. During the next 15 min the mixture was shaken periodically and alternately warmed by hand and cooled again. At the end of this time 1 ml of 0.1 N acetic acid was added. The mixture was extracted twice with 1-ml portions of ethyl acetate. The combined ethyl acetate fractions were concentrated *in vacuo* and then identified by chromatography.

The enzymic *O*-methylation of 5,6-dihydroxyindoles was determined by incubating these substances with *S*-adenosyl[*Me*-^{14}C]methionine. The [^{14}C]methoxyindoles formed were extracted into organic solvents and measured in a liquid scintillation counter after the addition of phosphor. The *O*-methylating enzymes employed were the soluble supernatant fractions from one of the following: 1 mg rat liver (COMT), 1 mg bovine pineal body (HIOMT), or 3 mg dried hamster melanoma. The enzyme was incubated in a 15-ml glass-stoppered centrifuged tube containing 0.1 μmole substrate, 4.5 mμmoles of *S*-adenosyl[*Me*-^{14}C]methionine (10000 counts/min), 10 μmoles of $MgCl_2$ and 100 μmoles of phosphate buffer (pH 7.9). After 1 h of incubation in an atmosphere of nitrogen, the mixture was assayed for [^{14}C]methoxy derivatives. [^{14}C]Methoxyacetylserotonin (melatonin) was determined as described previously[3]. [^{14}C]Methoxy catecholamines were extracted into 6 ml of isoamyl alcohol at pH 10. [^{14}C]Methoxyindoles were extracted at pH 7.9 into 6 ml of isoamyl alcohol, and [^{14}C]methoxyindole-2-carboxylic acid was extracted at an acid pH into a mixture of toluene containing 20 % isoamyl alcohol. Under these conditions from 90 to 100 per cent of the methoxyindoles was extracted. A 4-ml aliquot of the extract was transferred to a vial containing 3 ml of ethanol and 10 ml of phosphor, and the radioactivity was measured in a liquid scintillation spectrometer. A control incubation without substrate was run concurrently to correct for the small amount of *S*-adenosylmethionine-$^{14}CH_3$ extracted into the solvent.

Catechol-*O*-methyl transferase from rat liver was found to *O*-methylate both 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid as well or better than the normal substrate of this enzyme, noradrenaline (Table I). Hydroxyindole-*O*-methyl transferase from bovine pineal gland *O*-methylated 5,6-dihydroxyindole but not the corresponding carboxylic acid. The rate of *O*-methylation of 5,6-dihydroxyindole was about one-third that of *N*-acetylserotonin, the normal substrate for this enzyme. Enzymes in hamster melanoma catalyzed the *O*-methylation of the catechol-

amines—noradrenaline, adrenaline and DOPAamine—as well as 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid but not *N*-acetylserotonin. The substrate specificity would suggest that catechol-*O*-methyl transferase but not hydroxyindole-*O*-methyl transferase was present in the hamster melanoma tissue.

TABLE I

ENZYMIC *O*-METHYLATION OF 5,6-DIHYDROXYINDOLES

Enzyme	*Substrate*	*O-Methylated product formed (μmole/g tissue)*
Liver (COMT)	L-Noradrenaline	3.8
Liver (COMT)	5,6-Dihydroxyindole	4.5
Liver (COMT)	5,6-Dihydroxyindole-2-carboxylic acid	4.8
Pineal body (HIOMT)	*N*-Acetylserotonin	3.8
Pineal body (HIOMT)	5,6-Dihydroxyindole	1.3
Pineal body (HIOMT)	5,6-Dihydroxyindole-2-carboxylic acid	0.0
Hamster melanoma	L-Noradrenaline	0.22
Hamster melanoma	L-Adrenaline	0.25
Hamster melanoma	DOPAmine	0.26
Hamster melanoma	5,6-Dihydroxyindole	0.37
Hamster melanoma	5,6-Dihydroxyindole-2-carboxylic acid	0.40
Hamster melanoma	*N*-Acetylserotonin	0.00

To determine the identity of the *O*-methylated products of the 5,6-dihydroxyindoles, larger quantities of these compounds were prepared enzymically and their R_F values and color reactions compared with authentic samples of 5-methoxy-6-hydroxyindole and 5-hydroxy-6-methoxyindole. 5,6-Dihydroxyindole (1 mg) was incubated with 5 μmoles of non-radioactive *S*-adenosylmethionine and either COMT obtained from 1 g of rat liver or HIOMT from 1 g of bovine pineal gland. After a 1-h incubation, the *O*-methylated metabolites were extracted with isoamyl alcohol as described above. The solvent was evaporated *in vacuo*, and the residue was subjected to thin-layer chromatography on silica gel using carbon tetrachloride–pyridine (7:2) as the solvent system. After 90 min the plates were dried and sprayed with Ehrlich's reagent. Two spots appeared after incubation with each enzyme, one having the same R_F value (0.67) and color reaction (olive green) as synthetic 5-methoxy-6-hydroxyindole and the other having the same R_F (0.50) and color reaction (red purple) as authentic 6-methoxy-5-hydroxyindole. The relative amount of *O*-methylation on each position was determined by preparing [^{14}C]methoxyhydroxyindoles with the liver and pineal gland enzymes. Extracts of the reaction mixtures were co-chromatographed with synthetic 5-methoxy-6-hydroxyindole and 5-hydroxy-6-methoxyindole as described above. After 90 min the plate was dried and sprayed with Ehrlich's reagent. 1-cm sections of the silica gel were scraped from the plate and transferred to vials containing 1 ml of ethanol. After each vial was shaken for a few seconds, 10 ml of phosphor was added and radioactivity measured. Two areas of radioactivity were found corresponding in R_F to 5-hydroxy-6-methoxyindole and 5-methoxy-6-hydroxyindole. From the ratio of radioactivity of each metabolite (Table II), it appears that catechol-*O*-methyl transferase from rat liver *O*-methylates 5,6-dihydroxyindole predominantly on position 6 while hydroxyindole-*O*-methyl transferase methylates mainly on position 5. 5,6-Di-

hydroxyindole-2-carboxylic acid was incubated with S-adenosyl[*Me*-^{14}C]methionine and rat liver, and the [^{14}C]methoxy derivatives were extracted into a toluene–isoamyl mixture at an acid pH. The extracts were evaporated to dryness under a stream of nitrogen, and the [^{14}C]methoxyindole-2-carboxylic acid derivatives were decarboxylated by heating *in vacuo* at 150°. The resulting methoxyindoles were chromatographed as described above. Two areas of radioactivity were found which corresponded to 5-methoxy-6-hydroxyindole and 5-hydroxy-6-methoxyindole. *O*-Methylation of 5,6-dihydroxyindole-2-carboxylic acid was mainly on the 6 position.

TABLE II

O-METHYLATED METABOLITES OF 5,6-DIHYDROXYINDOLES

Enzyme	*Substrate*	*5-Hydroxy-6-methoxy-indole (% formed)*	*5-Methoxy-6-hydroxy-indole (% formed)*
Rat liver (COMT)	5,6-Dihydroxyindole	69	31
Pineal body (HIOMT)	5,6-Dihydroxyindole	33	67
Rat liver (COMT)	5,6-Dihydroxydihydroindole-2-carboxylic acid	64	36

DISCUSSION

Our experiments demonstrate that in the presence of catechol-*O*-methyl transferase 5,6-dihydroxyindole is *O*-methylated on either hydroxy group but predominantly at position 6. This indole is as good a substrate as noradrenaline, which is considered the natural one. 5,6-Dihydroxyindole-2-carboxylic acid also is readily *O*-methylated at position 6. On the other hand, 5,6-dihydroxyindole is *O*-methylated predominantly at position 5 with hydroxyindole-*O*-methyl transferase. The dihydroxyindole is about one-third as effective in this reaction as the accepted natural substrate, *N*-acetylserotonin. 5,6-Dihydroxyindole-2-carboxylic acid is not *O*-methylated with this enzyme.

O-Methylation of 5,6-dihydroxyindole or 5,6-dihydroxydihydroindole-2-carboxylic acid at the 6 position results in the formation of methoxyhydroxyindoles that oxidize spontaneously in the test tube to melanin more slowly than the parent compounds. Nevertheless, these derivatives could go on to form methylated melanins. The significance of *O*-methylation of indoles as they are formed during melanin synthesis is still unknown.

Hydroxyindole-*O*-methyl transferase has been found only in the pineal gland. That this enzyme catalyzes the *O*-methylation of 5,6-dihydroxyindole mainly at position 5 may not be important in melanin formation. However, other normally occurring dihydroxyindoles may be natural substrates.

Earlier experiments have shown that DOPA can be *O*-methylated in the presence of liver catechol-*O*-methyl transferase[2]. DOPA presumably also is a substrate for this enzyme from melanomas and other tissue. Consequently, as tyrosine is oxidized to melanin, the intermediates—DOPA, 5,6-dihydroxyindole and 5,6-dihydroxydihydroindole-2-carboxylic acid—could be *O*-methylated in the presence of catechol-*O*-methyl transferase. Since the enzyme is present in many tissues including melanomas,

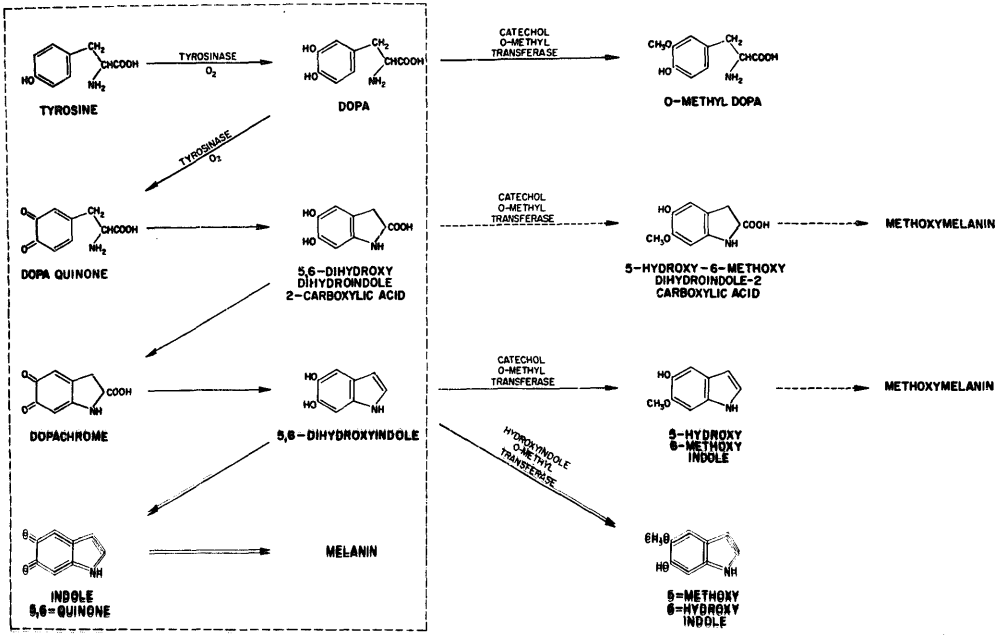

Fig. 1. The sites of action of catechol and hydroxyindole-*O*-methyl transferases in the conversion of tyrosine to melanin are shown. Each enzyme catalyzes *O*-methylation to either the 5 or 6 position. However, *O*-methylation is mainly at position 6 with COMT and at position 5 wth HIOMT.

it is probably present in normal melanocytes as well. In the special situation of a melanoma with metastasis to the liver there would be an abundance of catechol-O-methyl transferase to act on the oxidation products of tyrosine.

DOPA is a unique intermediate in the conversion of tyrosine to melanin. Its continued presence is necessary to keep tyrosinase in an active reduced state, and it is reformed as the reaction proceeds by the interaction between DOPA quinone and 5,6-dihydroxydihydroindole-2-carboxylic acid[6]. The accumulation of DOPA makes it a likely target for O-methylation. VON STUDNITZ[7] found that some patients with neuromas excrete O-methyl-DOPA in the urine. Apparently the increased conversion of tyrosine to noradrenaline in these tumors leads to an excess of DOPA which is then partially O-methylated. This same process should occur when melanin is formed by either normal or malignant melanocytes. A portion of the DOPA that accumulates, as tyrosine is oxidized to melanin, might be O-methylated. This O-methyl-DOPA could not be utilized further to make melanin, and its formation would act as a brake in the tyrosine to melanin conversion because less DOPA would be available to keep tyrosinase active. The physiologic significance of O-methylation of DOPA as a control step in the regulation of melanin formation must be investigated further. It was recently found that patients with melanomas excrete increased amounts of homovanillic acid[8]—the O-methylated metabolite of DOPA-mine. The possibility exists that patients with melanomas excrete increased amounts of O-methyl-DOPA and the methoxyhydroxyindoles. If this is so then the excretion of these compounds might be used as a guide to the patient's clinical course.

It was of particular interest to find that 5,6-dihydroxyindole could serve as an effective substrate for two distinct O-methyl transferases. In one case the compound behaves as a catechol derivative and in the other as an hydroxyindole. We are left with the challenge to find out how one enzymic process yields chiefly a 6-methoxyindole while another produces a 5-methoxyindole.

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