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# Identification of 5-Hydroxy-6-indolyl-O-sulfate in Urine of Patients with Malignant Melanoma*

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**It has previously been shown that enzymatically hydrolyzed urine of patients with malignant melanoma contains 5,6-dihydroxyindole (5,6DHI). In this study we describe the elucidation of the entire structure of urinary 5,6DHI-conjugate. Differential hydrolysis of melanotic urine revealed that, in contrast to β-glucuronidase, sulfatase can liberate 5,6DHI from its conjugated form. 5,6DHI-sulfate was synthetized by reacting 5,6DHI with sulfur trioxide trimethylamine complex. Thin-layer chromatography (TLC) documented its close similarity to the Thormählen-positive compound usually entitled "C." Gas chromatographic-mass spectrometric (GC-MS) analysis of methylated and subsequently hydrolyzed synthetic 5,6DHI-sulfate showed that the synthetic product consisted of a mixture of 5-hydroxy-6-indolyl-O-sulfate and 6-hydroxy-5-indolyl-O-sulfate (with a certain amount of 5,6DHI-disulfate). 5,6DHI-sulfate was purified with use of DEAE-cellulose column chromatography from melanotic urine. Methylation of this conjugate with deuterated dimethylsulfate and subsequent GC-MS analysis of the hydrolyzed product provided evidence that 5,6DHI from melanotic urine was almost exclusively sulfated in position 6. It was concluded (1) that 5,6DHI is excreted as a 6-O-sulfate, and (2) that this compound is consistent with Thormählen-positive compound "C."**

The formation of pigment melanin belongs to the characteristic features of melanocytes. It is generally known that the process of melanogenesis involves the enzymatic hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (dopa) which is then converted to indolic metabolites (eumelanin precursors) with a high ability to polymerize.

5,6DHI is known to play a rather important role in melanogenesis, being considered as a basic monomer unit in melanin polymer. Our recent GC-MS analyses showed that urine from patients with malignant melanoma contained 5,6DHI [1]. To better understand the metabolism of eumelanin precursors, we turned our attention to the elucidation of the entire structure of the urinary 5,6DHI-conjugate.

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Abbreviations:
5,6DHI: 5,6-dihydroxyindole
DMS: dimethylsulfate
dopa: 3,4-dihydroxyphenylalanine
dopamine: 3,4-dihydroxyphenylethylamine
GC-MS: gas chromatography-mass spectrometry
PFPA: pentafluoropropionic anhydride
TLC: thin-layer chromatography

## MATERIALS AND METHODS

### *Chemicals*

3,4-Dihydroxyphenylethylamine (dopamine) and arylsulfatase [E.C. 3.1.6.1.] (from *Aerobacter aerogenes*, activity 18.5 U/ml) were purchased from Sigma Chemical Co., St. Louis, Missouri; pentafluoropropionic anhydride (PFPA) was from Pierce Chemical Co., Rockford, Illinois; β-glucuronidase [E.C. 3.2.1.31.] (from *Escherichia coli*, activity 200 U/ml) was from Boehringer, Mannheim, F.R.G.; *Helix pomatia* juice (β-glucuronidase activity $10^5$ Fishman units/ml, arylsulfatase activity $10^6$ units [Roy]/ml) was from l'Industrie Biologique Française, Gennevilliers, France; DEAE-cellulose was obtained from Fluka AG, Buchs, Switzerland; and sulfur trioxide trimethylamine complex from Aldrich-Europe, Beerse, Belgium. Affi-Gel 601 (boronate gel) was purchased from Bio-Rad Laboratories, Richmond, California. All other chemicals were from Merck, Darmstadt, F.R.G.

### *Enzymatic Hydrolysis of Melanotic Urine*

Twenty milliliters of melanotic urine was divided into 8 tubes. Each hydrolysis was performed in duplicate. To 2.5 ml of urine 0.5 ml of 2.5 mol/liter sodium acetate buffer (pH 6.2) was added (tubes 1–4, 7, 8). In case of sulfatase hydrolysis (tubes 5, 6), the acetate buffer of the same concentration but with pH 7.1 was used. The urine samples were hydrolyzed with 50 μl of β-glucuronidase (tubes 1, 2), 50 μl of *Helix pomatia* juice (tubes 3, 4), and 50 μl of arylsulfatase (tubes 5, 6). The urine samples without enzyme served as a control (tubes 7, 8). The contents of each tube were shortly bubbled with nitrogen, the tubes tightly closed and placed in a shaking water-bath at 37°C for 120 min.

### *Extraction*

The samples were extracted with 2 × 4 ml of diethyl ether, pooled extracts dried over anhydrous sodium sulfate, and evaporated to dryness at 40°C under a stream of nitrogen.

### *Derivatization*

Dry samples were derivatized by adding 150 μl of PFPA and heating at 60°C for 10 min. The remaining PFPA was then evaporated and the residue dissolved in freshly prepared 5% solution of PFPA (v/v) in ethyl acetate.

### *Synthesis of 5,6DHI-Sulfate*

5,6DHI was prepared from dopamine as described elsewhere [2]. About 30 mg of freshly prepared 5,6DHI was dissolved in dry pyridine (300 μl), and solid sulfur trioxide trimethylamine complex (200 mg) was added. The mixture was then allowed to stay at room temperature overnight. The reaction product was dissolved in 5 ml of water, centrifuged, and the supernatant passed through a 0.8 × 5 cm Affi-Gel 601 column to remove unreacted 5,6DHI. The presence of 5,6DHI-sulfate in water eluate was checked by Thormählen reaction. The fractions containing Thormählen-positive substances were pooled and applied to a 0.8 × 5 cm column of Amberlite GC. The elution was carried out with water and the presence of Thormählen-positive compounds was tested again by Thormählen reaction. Positive fractions were pooled and freeze-dried. The residue was then dissolved in 2 ml of water and examined by TLC.

### *Methylation of the Synthetic Product*

One hundred microliters of the above-mentioned solution were mixed with 1 ml of 4% NaOH, and 100 μl of dimethylsulfate (DMS) were added in several portions under thorough mixing. The unreacted DMS was then removed by extraction with 2 × 4 ml of diethyl ether. The contents of the tube were mixed with 0.5 ml of 2.5 mol/liter sodium acetate buffer (pH 6.2) and 100 μl of *Helix pomatia* juice. Hydrolysis,

extraction, and derivatization were performed as described above. The sample was then analyzed by GC and GC-MS.

*Partial Isolation of the Thormählen-Positive Compound "C" from Melanotic Urine*

Melanotic urine with a high content of Thormählen-positive compounds was used. Ten milliliters of urine was applied to a 1 × 50 cm column with DEAE-cellulose. The column was washed with 100 ml of water and the Thormählen-positive substances eluted by applying a continuous nonlinear gradient of ammonium acetate (solution I–300 ml, 0.4 mol/liter; solution II—300 ml, 0.8 mol/liter; flow 0.2 ml/min). The content of Thormählen-positive compounds in 5-ml fractions was examined using Thormählen reaction and TLC. The fractions containing compound "C" (No 42–45) were pooled and freeze-dried. The residue was dissolved in 1 ml of water and used for structural studies.

*Methylation and Hydrolysis of the Partially Isolated Thormählen-Positive Compound "C"*

The methylation and hydrolysis were performed as described above for the synthetic 5,6DHI-sulfate. Deuterium-labeled dimethylsulfate (DMS-$d_6$) was used for methylation in this case.

*Thormahlen Reaction*

One milliliter of sample was mixed with 250 μl of freshly prepared 1% sodium nitroprusside and 500 μl of 10% KOH. After about 2 min, the solution was acidified with 50 μl of concentrated acetic acid. If necessary, smaller parts were used in the same ratios. Absorbance was measured at 620 nm.

*TLC*

TLC was carried out on cellulose plates (PSC-Fertigplatten Cellulose, 20 × 20 cm, layer thickness 0.5 mm; Merck, Darmstadt, F.R.G.) in a solvent system containing n-butanol:pyridine:water (1:1:1). The spots were made visible by Thormählen [3] and Ehrlich [4] reaction.

*GC and GC-MS*

GC and GC-MS were performed as described elsewhere [5].

## RESULTS AND DISCUSSION

Differential hydrolysis of urine samples from melanoma patients (see *Materials and Methods*) revealed that 5,6DHI could be liberated from the conjugated form only when samples were treated with sulfatase or *Helix pomatia* juice (containing also sulfatase activity). This finding led us to an assumption that 5,6DHI was present as a sulfate in melanotic urine.

It is generally believed that all so-called Thormählen-positive compounds detected in melanotic urine are conjugates of melanin-related indoles with glucuronate or sulfate [6,7]. Leonhardi was the first to separate 3 Thormählen-positive compounds from melanotic urine using paper chromatography, and he labeled them "A", "B," and "C." The structures of the substances "A" and "B" have recently been resolved [5,9].

When the 5,6DHI-sulfate was synthetically prepared, TLC showed this compound to be very similar to Thormählen-positive compound "C." The methylation of the synthetic 5,6DHI-sulfate with subsequent hydrolysis documented that the synthetic product comprised 5-O- and 6-O-sulfated 5,6DHI probably with a certain amount of 5,6DHI-disulfate. In our hands, the disulfate appeared to be unstable, forming monosulfates spontaneously.

For partial isolation of Thormählen-positive substance "C" from melanotic urine, DEAE-cellulose chromatography was employed. The baseline separation of all 3 main urinary Thormählen-positive substances is illustrated in Fig 1 (*lower*). TLC provided the possibility of labeling the compounds as "A," "B," and "C" according to their chromatographic mobility and color reactions (Fig 1, *upper*). GC-MS analysis showed that the fractions containing compound "C" afforded free 5,6DHI when subjected to sulfatase hydrolysis (Fig 2, *upper*).

To elucidate the exact position of the linked sulfate, the partly isolated compound "C" was methylated. Because of possible small contamination of samples by traces of 5-hydroxy-6-methoxy and/or 6-hydroxy-5-methoxyindole from compound "B" [5], the deuterated dimethylsulfate was used for methylation. The GC and GC-MS analyses of the methylated and subsequently hydrolyzed substance "C" brought the evidence that only the 5-O- position of 5,6DHI was available for methylation. It was therefore concluded that the 6-O- position was protected by linked sulfate. A mass spectrum of the PFP derivative of 6-hydroxy-5-trideuteromethoxyindole is shown in Fig 2 (*lower*). The whole analytical procedure is diagramatically summarized in Fig 3.

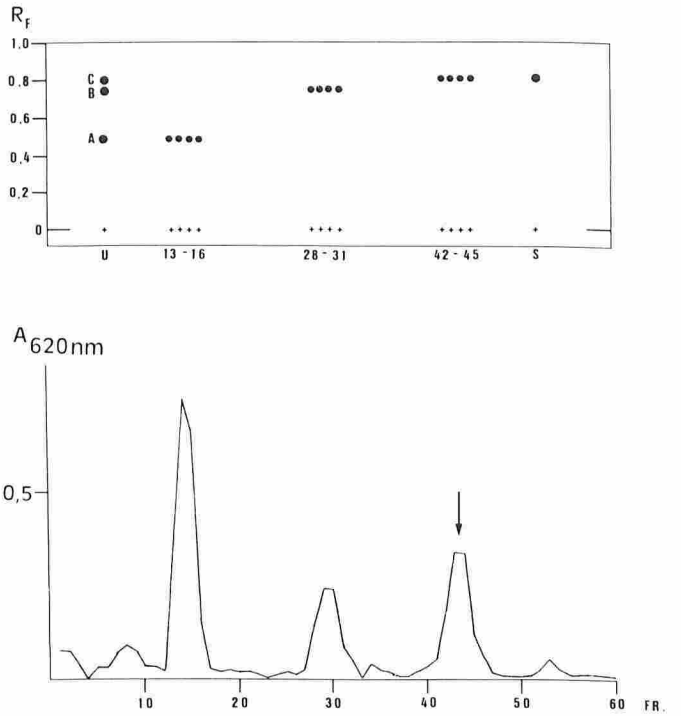


FIG 1. Chromatography of Thormählen-positive compounds from melanotic urine. *Upper*, A diagram of TLC of eluted fractions containing Thormählen-positive compounds "A" (No 13–16), "B" (No 28–31), and "C" (No 42–45). For comparison, an untreated urine (*U*) and synthetically prepared 5,6DHI-sulfate (*S*) applied as well. Detection: Thormählen reaction [3]. *Lower*, An elution profile of Thormählen-positive substances from melanotic urine after DEAE-cellulose column chromatography. The *arrow* indicates the position of fractions with 5,6DHI-sulfate.

It is well known that conjugation with sulfate plays an important part in catecholamine metabolism. Our results show that sulfation is also a very important method for biotransformation of melanin precursors excreted in higher amounts in urine of melanoma patients. Although it has been described that methoxyhydroxyindoles are conjugated with both glucuronate and sulfate [5,9], the present work indicates that the 5,6DHI is almost exclusively sulfated. As documented above, this sulfation takes place at the 6-O- position. Interestingly, 6-hydroxy-5-methoxyindole is preferably sulfated [5] while isomeric 5-hydroxy-6-methoxyindole is known to be predominantly glucuronated [9]. In any event, the conjugation of 5,6DHI and its O-methylated derivatives represents in all cases an efficient method to facilitate the elimination of these relatively apolar compounds from organisms.

We suggest that the known phenomenon of generalized (diffuse) melanosis secondary to malignant melanoma may result from the extreme production of indolic eumelanin-related compounds by melanotic tumor and reduced conjugation capacity caused by tumor infiltration of the liver. Such a situation may lead to the accumulation of the unconjugated indolic compounds in the body, with their diffusion into many organs including skin, and subsequent polymerization.

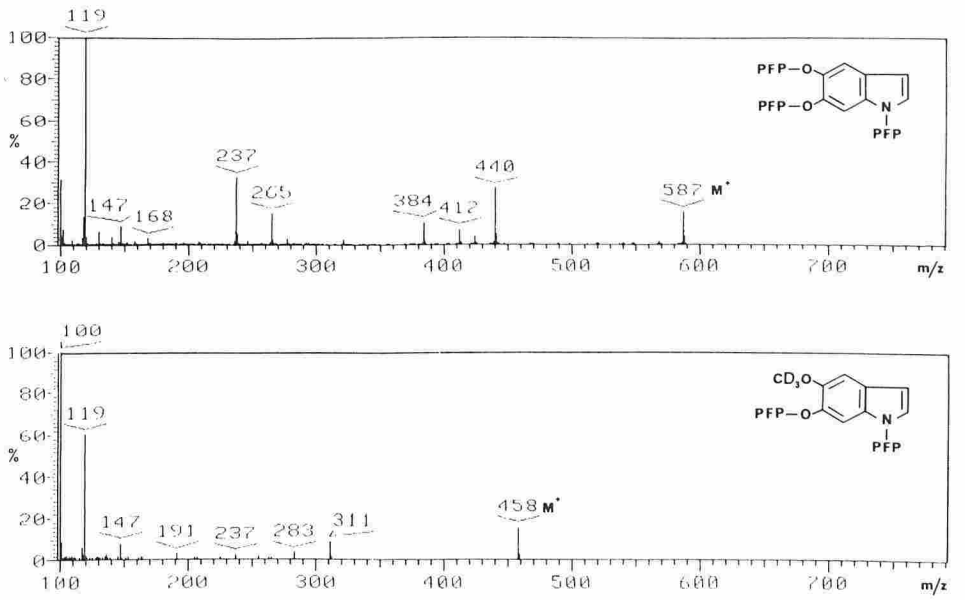


FIG 2. Mass spectra of indolic derivatives. *Upper*, The mass spectrum of the PFP derivative of 5,6DHI from fractions No 42–45. The pooled fractions were hydrolyzed with arylsulfatase, compounds extracted into ethyl acetate, derivatized and analyzed by GC-MS; m/z 587 = molecular ion $[M]^+$, m/z 440 = $[M\text{-PFP}]^+$. *Lower*, The mass spectrum of the PFP derivative of 6-hydroxy-5-trideuteromethoxyindole obtained by methylation and subsequent hydrolysis of the urinary 5,6DHI-sulfate (see *Materials and Methods*); m/z 458 = $[M]^+$, m/z 311 = $[M\text{-PFP}]^+$.

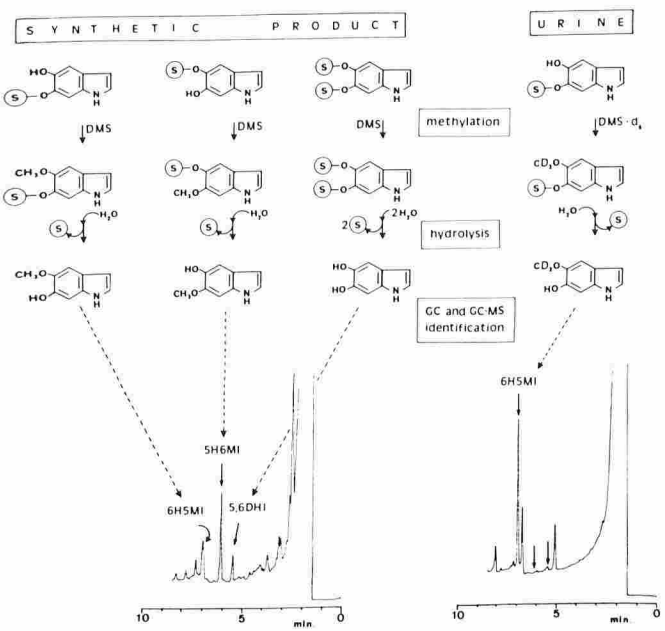


FIG 3. A summarizing scheme of analytical procedures used for resolving of the structure of the urinary 5,6DHI-sulfate. The indoles were identified on the basis of their retention time and mass spectra.

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