Identification of p-Hydroxy-α-(methylaminomethyl)benzyl Alcohol (Synephrine) in Human Urine

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During the development of an assay for the metanephrines (m-O-methylepinephrine and m-O-methylnorepinephrine) in human urine (1), another compound was detected which had similar chemical and chromatographic properties. Like the metanephrines, it was adsorbed onto a weak cation exchange resin, and upon treatment with periodate it yielded a product which absorbed ultraviolet light. The identification of the periodate cleavage product as p-hydroxybenzaldehyde suggested that the unknown base in urine was a derivative of p-hydroxy-(m-O-methylepinephrine and m-O-methylnorepinephrine) in human urine (1), another compound was detected which had similar chemical and chromatographic properties. Like the metanephrines, it was adsorbed onto a weak cation exchange resin, and upon treatment with periodate it yielded a product which absorbed ultraviolet light. The identification of the periodate cleavage product as p-hydroxybenzaldehyde suggested that the unknown base in urine was a derivative of p-hydroxy-

\[
\text{HO-} \quad \text{-CHOHCH₂NHCH₃}
\]

a compound which heretofore had not been implicated in mammalian metabolism.

EXPERIMENTAL PROCEDURE

Isolation of Synephrine from Human Urine

Collection and Hydrolysis of Urine—Because of the known increase in excretion of many amines during treatment with monoamine oxidase inhibitors (2), isolation and identification of synephrine was carried out on urine from subjects who had received 25 mg per day of /I-phenylisopropylhydrazine (JB-516).1 One-half of a 24-hour urine sample (collected in 10 ml of 6 N HCl and stored at 3°) was adjusted to pH 1.0, placed in a boiling water bath, and kept there for 20 minutes after the temperature of the urine had reached 90°. The urine was then cooled and adjusted to pH 6.4 with 1 N NaOH.

Ion Exchange Chromatography—The neutralized urine was allowed to pass through a column (10 × 7 cm) of the weak cation exchange resin, Amberlite IRC-50 buffered at pH 6.4 with ammonium acetate. After washing the column with 1 liter of water, the amines were eluted with 4 N NH₄OH. When the NH₄OH front reached the bottom of the column, 250 ml were collected. The front was visible as a brown ring on the column. The eluate was evaporated to dryness at 40° in a rotatory evaporator under reduced pressure, and the residue was dissolved in a mixture of equal volumes of ethanol and water.

Preparative Paper Chromatography and Paper Electrophoresis—The ethanolic solution of the urinary amines was streaked on 5 sheets of Whatman No. 3 paper and chromatographed with the solvent system, n-butanol-concentrated acetic acid-water, 50:12:50 (butanol layer). The substances in the areas having the same migration as authentic synephrine (Rf 0.61) were eluted from the paper with water, combined, and assayed. The eluted amines were then applied to each of 5 sheets of Whatman No. 3 paper as 5-inch bands for electrophoresis in 0.1 M sodium borate buffer, pH 8.5, at 550 volts (15 volts per cm) for 3 hours. Authentic synephrine migrated 10 cm toward the cathode, and only the material in the center portion of this area of the papers, containing the urinary amines, was eluted with water. The eluates were combined and used for the identification of synephrine.

Identification of Synephrine

Paper Chromatography—A sample of the isolated material was examined by two-dimensional paper chromatography, first in n-butanol-concentrated acetic acid-water, 120:30:50, and then in isopropyl alcohol-concentrated ammonium hydroxide water, 200:10:20.

Spectrophotofluorometry—Excitation and fluorescence maxima of aqueous solutions of the unknown compound and of authentic synephrine were determined on an Aminco-Bowman spectrofluorometer.

Oxidation with Periodate, Analysis of Products—Synephrine is oxidized by periodate to form equivalent amounts of p-hydroxybenzaldehyde, formaldehyde, and methylamine as shown in the equation below:

\[
\text{HO-} \quad \text{-CHOHCH₂NHCH₃} + \text{NaIO₄} \rightarrow \text{HO-} \quad \text{-CHO} + \text{CH₃NH₂} + \text{HCHO} + \text{NaIO₃}
\]

Identification of p-hydroxybenzaldehyde as a periodate degradation product of the unknown compound was effected by gas chromatography followed by spectrophotometric analysis of the material recovered from the column. A portion of the isolated material was dissolved in 5 ml of 2 N NH₄OH in a 40-ml extraction tube; then 0.5 ml of a 2% solution of NaIO₄ was added, followed after two minutes by 0.5 ml of 10% Na₂S₂O₅. The tube was immersed in an ice bath, the solution was acidified with ice-cold 6 N HCl, and the p-hydroxybenzaldehyde was extracted with 20 ml of benzene by shaking the mixture for 5 minutes. The benzene was transferred to another tube and the extraction was...
repeated five times. The benzene extracts were combined, evaporated to dryness, and the residue was taken up in benzene and brought to a final volume of 0.1 ml by evaporation under a stream of nitrogen. Of this, 20 μl were analyzed by gas chromatography in a six-foot U-shaped column filled with Chromasorb W (Johns Manville) on which ethylene glycol adipate polyester was adsorbed (10% by weight). An argon-ionization detector containing 100 μe radium source was used (3). The temperature of the column was 215° and the gas flow rate was 100 ml per minute. The material having the same elution time as authentic p-hydroxybenzaldehyde was collected in a small vial which was connected to the column outlet with polyethylene tubing. The tubing and vial were washed with 2 containing 100 PC radium source was used (3). The temperature of the column was 215° and the gas flow rate was 100 ml per minute. The material having the same elution time as authentic p-hydroxybenzaldehyde was collected in a small vial which was connected to the column outlet with polyethylene tubing. The tubing and vial were washed with 2 x NH₄OH to dissolve the product, and the ultraviolet spectrum of the solution was determined.

Another sample of the isolated amine was oxidized with periodate and the proportions of p-hydroxybenzaldehyde and methylamine were compared. p-Hydroxybenzaldehyde was determined spectrophotometrically. Methylamine was steam distilled from an aliquot of the reaction mixture and determined by reaction with fluorodinitrobenzene (4). Results with the latter method varied as much as 20% when standard solutions of methylamine were analyzed.

**Assay of Synephrine**

Synephrine in urine was measured by utilizing the previously described assay procedure for the metanephrines (1), except that the spectrophotometric readings were taken at both 330 μp (absorption maximum for p-hydroxybenzaldehyde) and 350 μp (absorption maximum for vanillin). The amounts of p-hydroxybenzaldehyde (equivalent to synephrines) and vanillin (equivalent to metanephrines) in the same solution were determined by solving a simultaneous equation which included the absorbancy measured at the two wave lengths. 2 The 24-hour urine sample used for this isolation of synephrine contained 600 μg of the metanephrines and 1000 μg of synephrines.

**RESULTS**

The following data conclusively identify the unknown amine as synephrine.

**Paper Chromatography and Color Reactions**—Results of twodimensional paper chromatography of the amine isolated from urine are shown in Fig. 1. It can be seen that the unknown amine, when mixed with authentic synephrine and chromatographed, gave only one spot after spraying the paper with diazotized p-nitroaniline. On the other hand, a mixed chromatogram of the unknown compound and norsynephrine (α-(amino-methyl)-p-hydroxybenzyl alcohol) clearly showed two spots, thus proving that the unknown amine was not norsynephrine. On all chromatograms, the unknown compound and synephrine gave similar colors with spray reagents, pink with diazotized p-nitroaniline and yellow with diazotized sulfanilic acid. Spraying with 1% NaIO₄ in 5% Na$_2$CO₃ resulted in the oxidation of both synephrine and the unknown compound to yield an aldehyde which absorbed ultraviolet light from a Woods lamp (360 μp). The aldehyde produced in this way on paper also gave the pink and yellow colors with diazotized p-nitroaniline and sulfanilic acid, respectively.

**Identification of p-Hydroxybenzaldehyde and Methylamine**—Identification of p-hydroxybenzaldehyde was established by gas chromatography and spectrophotometric analysis of the aldehyde recovered from the column. Table I shows that gas chromatography serves as an excellent procedure for separating and identifying aromatic aldehydes. The aldehyde formed through the periodate oxidation of synephrine and the unknown amine had exactly the same relative retention time as authentic p-

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**Table I**

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Relative retention time*</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-Methoxybenzaldehyde</td>
<td>0.35</td>
</tr>
<tr>
<td>o-Methoxybenzaldehyde</td>
<td>0.39</td>
</tr>
<tr>
<td>p-Methoxybenzaldehyde</td>
<td>0.53</td>
</tr>
<tr>
<td>o-Vanillin</td>
<td>0.72</td>
</tr>
<tr>
<td>Vanillin</td>
<td>1.90</td>
</tr>
<tr>
<td>Isovanillin</td>
<td>2.05</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>3.95</td>
</tr>
<tr>
<td>Aldehyde from periodate oxidation of synephrine</td>
<td>3.95</td>
</tr>
<tr>
<td>Aldehyde from periodate oxidation of unknown</td>
<td>3.95</td>
</tr>
</tbody>
</table>

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*Retention times are relative to methyl stearate set at 1.00 on an ethylene glycol adipate polyester column.

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**Fig. 2. Absorption spectra of \( p \)-hydroxybenzaldehyde (solid line) and the aldehyde from the unknown amine (formed by periodate oxidation) recovered from the gas chromatography step (dotted line).** Effluent gas was collected only during the time interval when \( p \)-hydroxybenzaldehyde was known to come off.

**Fig. 3. Fluorescence spectra of aqueous solutions at pH 1 of synephrine (solid line) and the unknown amine (dotted line).** Both compounds had the same excitation peak of 275 \( \mu \text{m} \). The unknown amine sample was obtained from the electrophoretic step of the isolation procedure.

hydroxybenzaldehyde. Furthermore, spectrophotometric analysis of the unknown aldehyde recovered from the column revealed the same absorption peak as \( p \) hydroxybenzaldehyde (Fig. 2). The spectrum differed at the lower wave lengths from the spectrum of untreated \( p \)-hydroxybenzaldehyde because of contaminants formed through decomposition of the polyester column under the conditions used.

As expected, synephrine and the unknown amine gave essentially equivalent amounts of methylamine and \( p \)-hydroxybenzaldehyde after oxidation with periodate. The ratio of volatile amine to aldehyde was 1.1 for synephrine and 1.2 for the unknown amine. The dinitrophenyl derivative of the apparent methylamine was further characterized by noting the ratio of absorbancy at 350 to 390 \( \mu \text{m} \). The derivatives of authentic methylamine and of the unknown gave ratios of 2.1 which is characteristic of a primary amine (4). Another aliquot of the volatile amine when chromatographed on paper (n-butanol-concentrated acetic acid-water, 50:12:50; butanol layer) as the hydrochloride gave only one ninhydrin positive spot with an \( R_F \) of 0.48. One spot, with the same \( R_F \), was obtained on mixing the unknown with authentic methylamine hydrochloride. A mixture of the unknown and ethylamine hydrochlorides gave two spots, \( R_F \) 0.48 and \( R_F \) 0.60. Methylamine and ethylamine hydrochlorides when chromatographed alone had \( R_F \) values of 0.51 and 0.63, respectively. Similar quantities of ammonium chloride chromatographed under the same conditions, alone or with the amine, could not be detected.

**Spectrophotofluorometry**—The fluorescence characteristics of the unknown compound and of synephrine were found to be similar. In each case the excitation peak of aqueous solutions at pH 1 was at 275 \( \mu \text{m} \) and the fluorescence peak at 320 \( \mu \text{m} \) (uncorrected) (Fig. 3). Similar fluorescence characteristics were observed for tyrosine. According to Teale and Weber (5), tyrosine has a corrected excitation maximum at 275 \( \mu \text{m} \) and a fluorescence maximum at 303 \( \mu \text{m} \). The isolated urinary amine, when treated with \( \alpha \)-nitoso-\( \beta \)-naphthol, gave a yellow color and the characteristic fluorescence of \( \alpha \)-substituted phenols (6). The color yield from the reaction of the urinary amine with this reagent was identical to that obtained with an equivalent amount of authentic synephrine, the equivalence being based on the spectrophotometric assay of the \( p \)-hydroxybenzaldehyde formed from each after oxidation with periodate.

**DISCUSSION**

The primary amine, norsynephrine, was first identified in biological material by Erspamer (7) who isolated it from octopus salivary glands and called it octopamine. Subsequent studies in this laboratory (1) and by Kakimoto and Armstrong (8) indicated its presence in mammalian urine. A significant elevation of the concentration of octopamine was also noted in tissues after the administration of monoamine oxidase inhibitors (8). The \( N \)-methyl derivative, synephrine, however, was not found in these investigations.

In preliminary attempts to measure the urinary excretion of these products, six urines from normal subjects have been examined carefully for the presence of synephrine and norsynephrine. In only one instance was more than a trace of norsynephrine detectable by two-dimensional paper chromatography. In this instance, the patient excreted a total of 3.3 mg of synephrine, of which only 16% was the unmethylated product. The synephrines and metanephrines have been determined in the same urine samples with the use of the assay method for the metanephrines together with the simultaneous equation given in the footnote. In 12 normal subjects, the range of excretion of the metanephrines was 200 to 450 \( \mu \text{g} \) per day (average 400 \( \mu \text{g} \)) compared to 100 to 1900 \( \mu \text{g} \) per day (average 650 \( \mu \text{g} \) per day) for the synephrines. Administration of monoamine oxidase inhibitors to patients has been shown to increase the excretion of both the metanephrines and synephrines.

The structural similarity between synephrine and epinephrine has suggested a possible parallel between their biosynthetic pathways. It is likely that the major route of synephrine synthesis is initiated by \( \beta \)-hydroxylation of tyramine to form norsynephrine. In this laboratory, the \( \beta \)-hydroxylation of tyramine to norsynephrine has recently been accomplished in an adrenal-slice system identical to that necessary for the formation of norepinephrine by \( \beta \)-hydroxylation of Dopamine (9). This finding, and the observation that tyramine \( \beta \)-oxidase activity was found in the same areas of the brain that are rich in Dopamine \( \beta \)-oxidase (10), suggest that the \( \beta \)-hydroxylation of tyramine and Dopamine is catalyzed by the same enzyme. The \( N \)-methylolation of norsynephrine to synephrine may very well be

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catalyzed by the same enzyme which converts norepinephrine to epinephrine.

The finding of increased excretion of synephrine during treatment with monoamine oxidase inhibitors is strong evidence that a major route of metabolism for this amine involves monoamine oxidase. p-Hydroxymandelic acid, the acid metabolite which would result from oxidation of synephrine, has been identified in human urine (11). From other studies it is known that the weak pressor and inotropic activity of synephrine is potentiated by monoamine oxidase inhibition (12), but the exact pharmacological significance of synephrine and of its accumulation during monoamine oxidase inhibition remains to be elucidated. Although the synephrines may prove interesting only from a metabolic standpoint, the possible physiological implications of the close chemical relationship of the synephrines to epinephrine and norepinephrine merit consideration.

It should be pointed out that the ortho and meta hydroxy analogues of tyramine are also present in urine (13) and are not of dietary origin. The β-oxidation of these amines would yield the corresponding ortho and meta analogues of norsynephrine. In the case of the meta compound, N-methylation would yield neosynephrine, a well known and potent vasoconstrictor. These possibilities are also being investigated.

SUMMARY

Synephrine was conclusively identified in human urine by means of separation on ion exchange columns, two-dimensional paper chromatography, paper electrophoresis, color reactions, spectrophotometric and fluorometric characteristics, and by the identification and determination of the products formed by periodate degradation of the amine.

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REFERENCES

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