The Identification of 2-Dimethylamino-6-hydroxypurine and Its Ribonucleoside in Urine of Normal and Leukemic Subjects*

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SUMMARY

Urine from normal and leukemic subjects on controlled low-purine diets was fractionated by ion-exchange column chromatography, and the various effluent peaks exhibiting absorbance at 260 nm were subjected to two-dimensional filter paper chromatography for further resolution. It appeared from gross examination of the chromatograms that there were a greater number and concentration of blue fluorescent substances in much of the leukemic urine. Two of these compounds have been identified as 2-dimethylamino-6-hydroxypurine and its ribonucleoside.

A number of methylated purines have been found in human urine by Weissmann, Bromberg, and Gutman (33). These include 1-methylhypoxanthine, 7-methylguanine, 8-hydroxy-7-methylguanine, 6-hydroxymethylaminopurine. Their presence has been confirmed by Park et al. (24), who also reported increased excretion of 1-methylhypoxanthine, 7-methylguanine, and 8-hydroxy-7-methylguanine in a number of leukemic patients. In a continuation of a study of urinary pyrimidines and purines in normal and leukemic urines by a combination of ion-exchange and filter paper chromatography (1), a greater number and concentration of blue fluorescent substances were observed in much of the leukemic urine. Two of these compounds have been identified as 2-dimethylamino-6-hydroxypurine and its ribonucleoside. Elion et al. (13) have synthesized 2-dimethylamino-6-hydroxypurine, and its biological occurrence as a minor base in RNA has been reported by Smith and Dunn (29).

MATERIALS AND METHODS

Normal subjects and leukemic patients of various types were maintained on a controlled low-purine diet for 5 days prior to the collection of a 24-hour urine specimen. Aliquots of the urine were subjected to ion-exchange chromatography by modification of a procedure described earlier (1). The urine was added to a column of Dowex 2-chloride (×8, 200–400 mesh) anion exchange resin, and gradient elution was accomplished by the gradual delivery of a solution of 0.1 M acetic acid and 0.025 M ammonium chloride into a mixing chamber containing a constant volume of solution, which initially consisted of 0.22 M ammonium hydroxide and 0.025 M ammonium chloride. A gradual fall in pH from 10.4 to about 7.5 was achieved, and the eluate from the column was collected in 2.5-ml. fractions. In most cases a volume of urine equivalent to 6 mg. of creatinine was applied to a column 24 × 0.75 cm., and the volume in the mixing chamber was 250 ml.

Some urine was also fractionated on a larger scale, with 75 per cent of a 24-hour volume, by the following procedure: One-fourth of a total 24-hour collection of urine was reduced in vacuo to approximately 50 ml. To this was added an equal volume of solution #1 (0.6 gm. ammonium formate and 15.0 ml. of concentrated ammonium hydroxide per liter), the pH of the mixture was adjusted to 10.4 with additional ammonium hydroxide, and after filtration the material was added to a column (46 × 2.5 cm.) of Dowex 2-formate resin (×8, 200–400 mesh).
The ammonium formate solution \#1 was employed as the influent until 40 fractions of 25 ml. each of effluent had been collected. Creatine and N-methyl-2-pyridone-5-carboxamide were thus removed. At this stage solution \#2 (0.6 gm. ammonium formate and 3.8 ml. of formic acid per liter) was started into a mixing chamber containing 2500 ml. of solution \#1, thus initiating gradient elution. Pyrimidines consisting primarily of 5-ribosyluracil and uracil were eluted first, followed by the various purines, with uric acid being eluted last. The combined effluents containing the purines (except for uric acid) from three such fractionations on Dowex 2-formate were lyophilized, and ammonium formate was removed by sublimation at 55°-60°C. The residue was taken up in 50 ml. of 0.01 N HCl, added to a column (46 X 2.5 cm.) of Dowex 50-H+ (X12, 200-400 mesh) which was in equilibrium with 0.01 N HCl.

The ultraviolet absorbancy at 260 m\(\mu\) of the individual fractions collected from the columns was recorded. Portions of fractions comprising various ultraviolet-absorbing peaks were subjected to two-dimensional paper chromatography with isopropyl alcohol, water, and HCl (35), and with n-butyl alcohol, water, and ammonia (20). The chromatograms were examined over a short-wave ultraviolet lamp (253.7 m\(\mu\)), and two spots frequently observed were characterized by bright blue fluorescence and did not appear to correspond to purines previously reported in urine (24, 33, 34).

To obtain these substances in larger quantity with sufficient purity for identification purposes, fractions from the large-scale procedure containing relatively high concentrations of the compounds in question were streaked along the base line of filter paper and subjected to one-dimensional chromatography. The bands corresponding to the compounds to be isolated were cut from several papers and were concentrated by a technic previously described (9). The substances thus purified were used for determination of \(R_f\) values and electrophoretic mobility and were rechromatographed and eluted for determination of ultraviolet absorption spectra with a Beckman DK-2 recording spectrophotometer.

### RESULTS

Of the known compounds initially available for direct comparison, 6-hydroxy-2-methylaminopurine (N2-methylguanine) seemed most closely related to one of the substances isolated from urine, as judged by absorption spectral characteristics and chromatographic properties. However, no difficulty was encountered in distinguishing standard 6-hydroxy-2-methylaminopurine from the urinary compound. A sample of 2-dimethylamino-6-hydroxypurine (N2-dimethylguanine) was then obtained for direct comparison, and the data obtained left little doubt that the isolated substance was 2-dimethylamino-6-hydroxypurine.

In Table 1 \(R_f\) values for the urinary compound are given in solvents which provided some separation of the N2-methylated guanines by the chromatographic methods described earlier (14). A number of other chromatographic solvents were tested, and, although the isolated compound had

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>RF VALUES (X100) IN VARIOUS SOLVENT SYSTEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Hydroxy-2-methylaminopurine</td>
<td>FORM t-Bu 68</td>
</tr>
<tr>
<td>2-Dimethylamino-6-hydroxypurine</td>
<td>FORM t-Bu 50</td>
</tr>
<tr>
<td>Compound isolated from urine</td>
<td>HAc n-Bu 37</td>
</tr>
</tbody>
</table>

The composition of the various solvents is as follows: FORM t-Bu = tert-butyl alcohol, methyl-ethylketone, water, formic acid (40:30:15:15), HAc n-Bu = n-butyl alcohol, water, glacial acetic acid (50:25:25), HCl i-Pr = isopropyl alcohol, water, concentrated HCl (65:18.4:16.6), FORM EtAc = ethyl acetate, formic acid, water (70:20:10), EtAc form = upper phase from a mixture of ethyl acetate, water, formic acid (60:35:5).
the same Rf values as 2-dimethylamino-6-hydroxypurine, separation from the 2-monomethyl-substituted guanine on the same chromatogram was not obvious. The color of the urinary compound on chromatograms observed over an ultraviolet lamp was variable, depending on what chromatographic solvents were used and how soon the chromatogram was examined after removal from the solvent. It varied from a dark absorbing spot in ammoniacal and neutral solvents to an intense bright blue fluorescent substance in the isopropyl alcohol: H2O: HCl solvent. With the latter solvent the color of the urinary compound on chromatograms observed over an ultraviolet lamp was variable, depending on what Chromatographic solvents were used and how soon the chromatogram was examined after removal from the solvent. It varied from a dark absorbing spot in ammoniacal and neutral solvents to an intense bright blue fluorescent substance in the isopropyl alcohol: H2O: HCl solvent. With the latter solvent TABLE 2

ELECTROPHORETIC MOBILITIES OF URINARY COMPOUND AND TWO METHYLATED GUANINES

<table>
<thead>
<tr>
<th>Compound</th>
<th>0.02 M Citrate pH 3.0</th>
<th>0.01 M Borate pH 9.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound isolated from urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Dimethylamino-6-hydroxypurine</td>
<td>4.5 cathodal</td>
<td>0</td>
</tr>
<tr>
<td>6-Hydroxy-2-methylaminopurine</td>
<td>4.5 cathodal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.8 cathodal</td>
<td>0</td>
</tr>
</tbody>
</table>

* 4 hours, 300 volts, Whatman #1 filter paper.

6-hydroxy-2-methylaminopurine exhibited considerably less fluorescence, and the color was darker. In side-by-side chromatographic comparisons of the isolated compound, synthetic 2-dimethylamino-6-hydroxypurine, and 6-hydroxy-2-methylaminopurine in a variety of solvents, the isolated compound routinely exhibited the same gradations in color as the authentic 2-dimethyl-substituted guanine.

In Table 2 the electrophoretic mobilities of the compounds are given, and in Table 3 ultraviolet absorption spectra are presented. Elion, Lange, and Hitchings (13) and Smith and Dunn (29) have reported previously spectral characteristics for 2-dimethylamino-6-hydroxypurine.

After fractionation of the purines in a number of the leukemic urines with the small column of Dowex 2 resin, subsequent chromatography revealed the presence of 2-dimethylamino-6-hydroxypurine in the effluent adenine peak, and occasionally it also extended into the adjacent hypoxanthine peak. Comparable aliquots of the effluent peaks obtained from normal and leukemic urines were used for paper chromatography, and only once was the dimethyl-guanine derivative observed in the normals (seven cases), whereas it was easily detected in two-thirds of the leukemic specimens (30 cases of a variety of types including acute and chronic granulocytic, lymphocytic, and myelocytic). The compound was routinely detected chromatographically in the effluents from the Dowex 50 columns used in the large-scale procedure with both normal and leukemic urine; but it was not confined to a single peak, and a quantitative estimation of its level has not as yet been undertaken.

With a visual inspection of the chromatograms, a rough classification of the concentration could be made on the basis of spot size and intensity, and it appeared that the acute leukemias and chronic lymphocytic leukemias were associated with an increased excretion of this compound in comparison with what was found in normals and chronic granulocytic leukemias.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 1</th>
<th>pH 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λmax</td>
<td>λmin</td>
</tr>
<tr>
<td>2-Dimethylamino-6-hydroxypurine</td>
<td>256, 285</td>
<td>233</td>
</tr>
<tr>
<td>Compound isolated from urine</td>
<td>255, 285</td>
<td>234</td>
</tr>
</tbody>
</table>

* Inflection.

The ribosyl derivative of 2-dimethylamino-6-hydroxypurine was isolated from the urine of a normal subject. It was noted on paper chromatograms prepared from an effluent fraction of the purines obtained from chromatography on Dowex 2-formate resin, and a sufficient quantity was isolated by paper chromatographic technics to permit identification. Its Rp value in sec-butyl alcohol saturated with water was 0.35, but it remained at the origin when borate was added (8). The markedly lower Rp value in the presence of borate was consistent with a ribonucleoside structure. The chromatographic and electrophoretic properties (migration toward the anode in 0.01 M borate buffer, pH 9.2) of the urinary compound agreed with those reported by Smith and Dunn for 2-di-

TABLE 3

ULTRAVIOLET ABSORPTION CHARACTERISTICS OF AUTHENTIC 2-DIMETHYLMINO-6-HYDROXYPURINE AND THE COMPOUND ISOLATED FROM URINE

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 1</th>
<th>pH 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

* Inflection.
methylated purines observed by Weissmann et al. (33)—namely, 7-methylguanine, 1-methylguanine, 6-dimethylamino-6-hydroxypurine, as well as some of the urinary methylation of yeast.

be the only methylated base in the serine sRNA. 2-dimethylamino-6-hydroxypurine was found to be the only methylated base in the serine sRNA obtained by hydrolysis was identified as 2-dimethylamino-6-hydroxypurine on the basis of its absorption spectra and also by direct chromatographic comparison with an authentic standard, with the solvents listed in Table 1. A careful identification of the nucleoside has been made in urine from only one subject, but a blue fluorescent spot in the proper chromatographic position for the compound has been observed in urine from a number of patients.

DISCUSSION

The list of methylated purines found as minor constituents of ribonucleic acids has continued to expand since the initial report of Littlefield and Dunn (19). It now includes 1-methylguanine (2, 29), 2-dimethylamino-6-hydroxypurine (29), 6-hydroxy-2-methylaminopurine (2, 29), 7-methylguanine (12), 2-methyladenine (18, 19), 6-methylaminopurine (2, 18, 19), 6-dimethylaminopurine (18, 19), and 1-methyladenine (11, 12). These methylated purines occur in the sRNA (10, 5) involved in the transfer of amino acids, but they are not incorporated directly into the RNA (30). The methyl groups originate from methionine (6, 22, 23), and the methylation is carried out at the level of the pre-formed polynucleotide structure by a soluble enzyme system, RNA methylase (15). Further purification of the system has demonstrated that the transmethylation is performed by S-adenosylmethionine and that specific enzymes may be involved for the particular base methylated (16, 17). The function of these minor bases in the sRNA has not yet been determined, and evidence by Starr (31) indicates that the methylation is not essential for the amino acid acceptor role of the sRNA. The question as to a possible coding function of the methylated bases was raised by Cantoni et al. (7) in a recent investigation in which 2-dimethylamino-6-hydroxypurine was found to be the only methylated base in the serine sRNA of yeast.

It seems probable that 2-dimethylamino-6-hydroxypurine, as well as some of the urinary methylated purines observed by Weissmann et al. (33)—namely, 7-methylguanine, 1-methylguanine, 6-hydroxy-2-methylaminopurine, 1-methylhypoxanthine, and 8-hydroxy-7-methylguanine—represent metabolic end-products of the sRNA. All but the last two are known components of sRNA, and it is not unlikely that 1-methylhypoxanthine may be formed biologically from 1-methyladenine (or its corresponding nucleoside or nucleotide). If 7-methylguanine is a precursor of 8-hydroxy-7-methylguanine, an oxidase other than xanthine oxidase would presumably be required, since it has been demonstrated that 7-methylguanine is refractory to xanthine oxidase (34, 36).

Other mechanisms may be mentioned which might contribute to the urinary level of the methylated purines, but available information is not adequate to assess them critically. First, it is conceivable that in some abnormal states an excessive methylation of nucleic acid might occur, with a resultant increase in the level of the methylated purines excreted. The excessive methylation might be accomplished by donors other than S-adenosylmethionine, since Magee and Farber (21) have reported methylation of liver RNA and DNA after intraperitoneal administration of a C14-labeled carcinogen, dimethylnitrosamine. Second, methylation of purines other than in nucleic acid linkage may occur. The early high specific activity of urinary 7-methylguanine and 8-hydroxy-7-methylguanine observed after administration of labeled glucose to some individuals with gout, polycythemia vera, and myeloid metaplasia (37, 38) could be consistent with some non-nucleic acid purine methylations. Axelrod and Daly (3) obtained extracts of rabbit tissues which could methylate adenine to 3-methyladenine, but the latter compound could not be detected in DNA or RNA, and it or the corresponding hypoxanthine derivative has not been identified in urine. Remy reported an S-adenosylmethionine transmethylase of E. coli that could N-methylate some unnatural synthetic 2-amino-substituted purines, but it exhibited no appreciable activity toward guanine (26, 27). However, in a footnote to a later report concerning a mammalian system for S-methylation of 6-thio-substituted purines, he stated that E. coli extracts containing an active 2-amino-purine transmethylase system synthesized 6-hydroxy-2-methylaminopurine in the presence of S-adenosylmethionine and guanosine-8-C14; thus, the nucleoside or nucleotide derivative may be the natural methyl acceptor, rather than guanine (28). Third, an enhanced level of urinary methylated purines may reflect a lowered metabolism of these compounds, such as demethylation or cleavage of the ring structure. Townsend and Robins (32) have recently demonstrated that methylation at position 7
renders guanosine and xanthosine very susceptible to ring opening between C-8 and N-9 under mild conditions of temperature and pH. Thus, if 7-methylnucleosines monophosphate from the sRNA were biologically degraded to 7-methylguanosine, it is possible that a portion of it might undergo cleavage of the imidazole ring yielding a 5-formylaminopyrimidine derivative. Demethylation of 6-methylaminopurine has been demonstrated with a rat liver homogenate (100,000 X g supernatant fraction), which yielded hypoxanthine and uric acid as two of the products (27), but biological demethylation of the naturally occurring methylated guanines has not been shown, and they are either refractory to xanthine oxidase or acted on at an exceedingly slow rate (34, 36).

The increased urinary excretion of some of the methylated purines in many of the leukemic patients is of considerable interest, but the significance of that observation awaits further investigation of the biochemical mechanisms involved.

ACKNOWLEDGMENTS

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