Problems in the Laboratory Diagnosis of Alcaptonuria

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Two patients with urinary findings suggestive of alcaptonuria were observed. One was a two-year-old girl of Turkish descent, presenting with dark-stained diapers, black ear wax, and no other stated problem. The second was a 61-year-old North American Indian woman with long-standing rheumatoid arthritis, bluish sclerae, chronic renal failure, and dark urine. Diagnosis of alcaptonuria was confirmed in the first case by paper chromatographic identification of homogentisic acid in urine and ear wax; also a small amount of a substance with $R_f$ similar to that of homogentisic acid was found in urine of the patient's mother and brother. Whether this implies possibilities for heterozygote detection requires further study. Gentisic acid, a phenolic acid metabolite of acetylsalicylic acid, was responsible for the dark color of the urine in the second patient. This latter finding has not been emphasized in this context, and may cause confusion when patients with arthritis and pigmentation are investigated for possible alcaptonuria.

Additional Keyphrases: homogentisic acid • gentisic acid • inborn errors of metabolism • heterozygotes in alcaptonuria

Alcaptonuria, a defect of tyrosine metabolism, is one of the classical inborn errors of metabolism described by Garrod (1) and is biochemically characterized by the presence of homogentisic acid (HGA) in urine. It has been known as a clinical entity for more than a century (2). The defect, namely absence of the enzyme HGA oxygenase (homogentisate:oxygen oxidoreductase, EC 1.13.1.5), leads to increased urinary excretion of HGA and eventual formation and deposition of dark pigment (a polymer of benzoquinino acid) in sclera, cartilage, and skin, as well as in many other tissues (3). This pigment deposition is the pathological basis of ochronosis (4). The metabolic pathway by which HGA is produced from tyrosine and gentisic acid (GA) from acetylsalicylic acid (aspirin) is shown in simplified form below:

![Diagram showing metabolic pathways of tyrosine and aspirin](#)

Patients are usually asymptomatic until the third decade, when from half to all of them may develop ochronosis (3, 5). Sitaj et al. (6), who studied 102 alcaptonurics from 15 Slovak families, found ochronosis in only 27, the incidence between ages 30 and 80 being only slightly greater than 50%.
We have studied two patients with dark urine suggestive of alcaptonuria. Case No. 1 was a two-year-old girl who presented with dark urine and ear wax. Case No. 2 was a 61-year-old North American Indian woman with long-standing rheumatoid arthritis, blue sclerae, and chronic renal failure. Nonspecific urine screening tests for alcaptonuria were partly positive in both cases. However, chromatography of urine showed HGA, characteristic of alcaptonuria, to be present only in Case No. 1. GA, a metabolite of aspirin, and with a closely similar Rf value, was found in urine from Case No. 2. This latter finding shows that while it is worthwhile to consider the possibility of ochronosis in patients with early-middle-age onset of arthritis, dark urine, and pigmentation of sclerae, it is necessary to distinguish between HGA and GA, as many of these patients are treated with large doses of salicylates.

Case Histories

Case No. 1, Miss S. A., 2 years old, presented with dark stains on her diapers and black ear wax. The data from history and physical examination were entirely within normal limits. Both parents are of Turkish origin, are healthy, and deny consanguinity. A five-year-old brother of the patient is also healthy. Family history added no useful information. Results of urinanalysis on all members of the family are described later.

Case No. 2, Mrs. L. McH., a 61-year-old North American Indian, was admitted for recurrent left-sided chest pain, dyspnea, and fever. She had chronic obstructive lung disease and a 12-year history of arthritis. The arthritis involved both hands with complete luxation of both wrists, many fingers, both hips, the cervical and thoracic spine, and both knees. Movement of the chest suggested subluxation and disorganization of the costochondral joints. She also had bilateral cataracts with severe limitation of vision. Both sclerae were dark blue. Heart disease was common in the patient’s family, both parents dying of this condition. One sister, Mrs. B. H., age 52 years, had had rheumatoid arthritis for 18 years, and in July 1972 had a left total hip replacement. The left femoral head showed degenerative osteoarthritic changes. There was no other significant family history.

Laboratory investigations: Chest roentgenograms showed small effusion at the left base. Roentgenograms of hands, wrists, shoulders, sternoclavicular joints, knees, ankles, and feet showed changes consistent with severe rheumatoid arthritis. Roentgenograms of the cervical spine showed almost complete obliteration and partial ankylosis of the intervertebral discs, subluxation of C2 on C3 and C6 on T1, and partial compression of C4 and C5.

Hemoglobin was 7.6 to 9.9 g/100 ml; leukocytes 16,600 to 7,800/mm³; blood urea nitrogen 32 and 31 mg/100 ml; creatinine 2.4, 2.2, and 1.9 mg/100 ml; creatinine clearance 22.3 ml/min; serum iron 34 μg/100 ml; and iron-binding capacity 300 μg/100 ml, with 11% saturation. Electrolytes and lactate dehydrogenase activity were normal. Urinalysis on admission showed 1 to 5 erythrocytes and 50–100 leukocytes per field, pus, and specific gravity 1.008, and it was noted that the urine darkened on standing.

Before admission the patient was treated with “Entrophen” (enteric coated acetylsalicylic acid) 900 mg three times daily, prednisone 5 mg twice daily, methyprylon 300 mg before retiring, propoxyphene 150 mg every 3 h as required for pain, and Cephalaxin 500 mg four times daily. The final diagnosis was rheumatoid arthritis, chronic obstructive lung disease, bilateral cataracts, urinary tract infection, renal failure, and anemia responsive to iron therapy. The dark urine, pigmentation of sclerae and arthritis suggested the diagnosis of alcaptonuria, and the following investigation was done on the urine.

Materials and Methods

Morning urine specimens were obtained from the patients and relatives (Case No. 1), and the nonspecific screening tests (e.g., FeCl₃) were done according to Henry (7) on fresh specimens. Concentrates of urine were prepared by extracting 10 ml of urine, brought to pH 4.0 with 10-fold diluted HCl, with 20 ml of diethyl ether. The ether phase was transferred to a second tube, evaporated under nitrogen, and the residue dissolved in 0.1 ml of absolute ethanol. Twenty microliters of this solution was equivalent to about 1 ml of the original urine (7). Reference solutions of HGA and GA were prepared by dissolving 5.0 mg in 10 ml of ethanol. Thus, 50 μl of the reference solution was equivalent to about 25 μg of the respective acid. Descending paper chromatography was run in butanol–acetic acid–water (4:1:5 by vol) for 16 h (8). To make the reducing phenolic acids visible, we dried the paper and then sprayed it with 5% ammoniacal silver nitrate (5 g of AgNO₃ plus 10 ml of concentrated NH₄OH, diluted to 100 ml with distilled water). A 0.5-g specimen of ear wax from Case No. 1 was extracted with about 4 ml of ethanol, and this extract similarly chromatographed.

Results

Results of nonspecific tests are summarized in Table 1. Results for the two urine specimens differed in the following respects: ferric chloride test, rapidity of darkening on addition of the ammoniacal solution of AgNO₃, fluorescence in uv light, and intensity of reduction of Benedict’s reagent.

As seen in Figure 1, HGA and GA were separated, the Rf values being 0.8 and 0.9, respectively. Chromatography also confirmed the presence of HGA in both the urine and ear wax of patient S. A. (Case No. 1). However, the urine of Case No. 2 (L. McH) contained GA, a minor metabolite of aspirin. Neither HGA nor GA was detected in control urine extract prepared in the same way.

Figure 2 shows a urinary chromatogram of relatives of patient S. A. (Case No. 1). It may be significant that the urinary extracts of the patient’s brot-
Table 1. Results of Nonspecific Laboratory Tests for HGA in Urine of Patients S. A. and L. McH.

<table>
<thead>
<tr>
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<th>Case No. 1 (S. A.)</th>
<th>Case No. 2 (L. McH.)</th>
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<tr>
<td>Appearance of urine</td>
<td>Darkens from the</td>
<td>Darkens from the</td>
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<td></td>
<td>top within hours</td>
<td>top within hours</td>
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<tr>
<td>Effect of alkalization</td>
<td>Marked darkening</td>
<td>Marked darkening</td>
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<tr>
<td></td>
<td>in 15 min</td>
<td>in 15–30 min</td>
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<tr>
<td>FeCl₃, 3 g/100 ml</td>
<td>No change in color</td>
<td>Stable deep-purple</td>
</tr>
<tr>
<td></td>
<td></td>
<td>color</td>
</tr>
<tr>
<td>Benedict reaction</td>
<td>Muddy green</td>
<td>Green-brown on</td>
</tr>
<tr>
<td></td>
<td>when cool</td>
<td>heating</td>
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<tr>
<td>AgNO₃ + NH₄OH</td>
<td>Black instantly</td>
<td>Black in 10–20 s</td>
</tr>
<tr>
<td>Fluorescence in uv</td>
<td>None</td>
<td>Deep blue</td>
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<td>light</td>
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Fig. 1. Descending paper chromatogram of the ether extract of urine and of ethanolic extract of ear wax. Volumes cited (μl) refer to an ethanol solution of dried extract (see text)
1. normal urine, 40 μl; 2. urine, Case No. 2, 5 μl; 3. GA standard, 40 μl (about 20 μg); 4. ear wax, Case No. 1, 40 μl; 5. HGA standard, 40 μl (about 20 μg); 6. urine, Case No. 1, 5 μl

Fig. 2. Descending paper chromatogram of ether extracts of urine of relatives of propositus (Case No. 1). Volumes cited (μl) refer to ethanol solution of dried extract (see text)
1. father, 40 μl; 2. brother, 40 μl; 3. mother, 40 μl; 4. HGA standard, 40 μl (about 20 μg); 5. ear wax extract of propositus, 40 μl; 6. urinary extract of propositus, 10 μl

er and mother contain a small amount of a reducing substance with \( R_f \) similar to that of HGA.

Discussion

Regarding the nonspecific urine tests, particular attention must be paid to the FeCl₃ reaction. It is stated (7) that urine containing HGA should yield a transitory blue color. However, on no occasion did the urine of Case No. 1 (an authenticated alcaptonuric) display this reaction. The purple color found in the urine in Case No. 2 is typical of salicylate ingestion, but this does not exclude the presence of homogentisic acid, for which the color reaction would be masked.

Another point of confusion is the observation (14) that HGA causes urine to fluoresce. This was not confirmed and we suspect that the cause of fluorescence previously mentioned is GA, the salicylate metabolite, which does produce a deep-blue fluorescence in urine. Of the nonspecific urine tests, that in which ammoniacal silver nitrate is used appears to be the most useful. While the black color developed instantly in the presence of HGA, there was always a 10–20 s delay if only GA was present in the urine.

Detection of heterozygotes in alcaptonuria is an unsolved and interesting challenge. Figure 2 shows the excretion pattern of some relatives of Case No. 1. Of possible interest is the apparent presence of a small amount of a substance with the same \( R_f \) value as HGA in the urine of this patient's brother and mother. This finding was repeatedly made on these subjects, but never for control urine similarly concentrated. The finding of a substance with \( R_f \) similar to that of HGA in the urine of a relative of a patient with alcaptonuria was previously reported only by Neuberger et al. (10). Gas–liquid chromatographic and mass spectrometric studies are obviously required to further substantiate this finding before any conclusions can be made, and these will be the subject of a further report. Previous workers have been unable to detect increased HGA excretion in heterozygotes. However, the complex subject of "normal" HGA excretion (effect of diet, disease, etc.) has not been investigated systematically.

Both HGA and GA are excreted by glomerular filtration and tubular secretion (9–11). This may explain the very high concentration of these compounds in urine, even if the creatinine clearance is
low, as was the case in our patient L. McH. (Case No. 2). Between 1 and 5% of the total urinary metabolites of salicylates is GA (12, 13), and its excretion pattern follows apparent first-order kinetics (11). It is possible that the large amount of urinary GA in our patient resulted from a combination of high dose taken and limited glomerular filtration. Two patients on high doses of salicylates also excreted GA in their urine.

The presence of HGA in ear wax of the patient with alcaptonuria is not surprising in view of the fact that HGA is excreted via the apocrine axillary glands of alcaptonurics (3). Whether the local accumulation of HGA and (or) of the resulting pigment leads to hearing impairment has not been determined. Sitaj (6), who studied a large number of patients with alcaptonuria and ochronosis, reported that 12 of 27 patients with ochronosis had significant hearing loss.

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References

2. Boedeker, C., Ueber das Alcapton: ein neuer Beitrag zur