Technical methods

Simple colour reaction for alkaptonuria

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A simple colour reaction on aqueous extracts of urine from 10 alkaptonuric subjects has been developed for the detection of alkaptonuria. Such extracts invariably give a pinkish brown colour with trace amounts of copper in alkaline medium but aqueous extracts of urine from normal and other pathological cases failed to give this colour.

Alkaptonuria is an inborn error associated with the excretion of homogentisic acid in the urine. The most commonly used test for its detection are the darkening of urine on exposure to air, reduction of Benedict's solution, colour reactions with FeCl₃. However, none of these tests are specific for alkaptonuria. While investigating some cases of alkaptonuria, a simple but specific colour reaction was developed for detecting such cases.

METHODS

Freshly voided urine samples (10-15 ml) from alkaptonuric subjects were concentrated on a boiling water bath. The concentrates were extracted with 3 ml of n-butanol. The butanol extracts were then partitioned with 5 ml of distilled water and the aqueous extracts separated for use in the subsequent work. This process removed most of the urinary pigments.

To 2 drops of the aqueous extract in a test tube, 2 drops of CuSO₄ (0.01%), were added followed by 5 ml of distilled water and 2 drops 0.1N NaOH. The tube was agitated immediately after the addition of alkali and left aside for 20 minutes when a pinkish brown colour developed.

These aqueous extracts were usually subjected to paper chromatography in a butanol:acetic acid:water (4:1:5) system to confirm the presence of homogentisic acid.

RESULTS AND DISCUSSIONS

Fresh urine samples of alkaptonuric subjects give a reddish brown colour on addition of alkali. In our experimental conditions, the addition of alkali has been shown to have no effect on the colour formation. However, under the same set of experimental conditions, addition of trace amounts of copper was shown to increase the colour intensity five-fold as indicated in Table I, and prompted us to study the effect of other oxidants on colour production. Such studies showed that mild oxidants like H₂O₂, FeCl₃ have very little effect on the colour intensity (Table I). Moreover, the colour chromogen obtained with trace amounts of copper is distinctly different from the reddish brown colour obtained by alkali with fresh urine from alkaptonuric subjects, suggesting that the coloured substance produced in the presence of copper possibly has a different absorption maximum.

The evidence suggests that homogentisic acid excreted in the urine of alkaptonuric subjects is responsible for this colour formation with copper. This was further confirmed by a study of the aqueous extract from an alkaptonuric child (3 months old). This gave the same colour reaction and on paper chromatography showed a single ammonical silver nitrate-positive spot corresponding to homogentisic acid.

However, the mechanism of this colour formation is not clear. It can be said that it is not due to the biuret reaction as the amount of copper used is far below the optimum copper required for biuret reaction. Since homogentisic acid is structurally related to quinone, solutions of hydroquinone have been subjected to the colour reaction. Hydroquinone gives a similar colour but it fades after a short time. It is inferred that the stability of the colour with aqueous extract of alkaptonuric urine is possibly due to the CH₂ COOH side chain of homogentisic acid present in such extract. The effect of copper is perhaps due to its mild oxidizing action. Other possibilities such as the formation of copper chelates are not ruled out.

Aqueous extracts of urine of normal and some other pathological cases (jaundice, amino aciduria, proteinuria, glycosuria) failed to give this colour reaction. Because of its simplicity and specificity, this colour reaction can be safely used for screening alkaptonurics.

TABLE I

EFFECT OF SOME OXIDANTS ON THE COLOUR INTENSITY

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Optical Density</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>0.092</td>
</tr>
<tr>
<td>B</td>
<td>0.018</td>
</tr>
<tr>
<td>C</td>
<td>0.023</td>
</tr>
<tr>
<td>D</td>
<td>0.027</td>
</tr>
</tbody>
</table>

1A contains 2 drops of aqueous extract, 2 drops of CuSO₄, 5 ml distilled water, and 2 drops of NaOH. Intensity of colour measured in Lumetron photoelectric colorimeter, at 530 mµ with water blank. Colour intensity measured after 20 minutes.

B Same as in A except copper sulphate was not added.

C Same as in A except 2 drops of 0.01% FeCl₃ replacing copper sulphate.

D Same as in A except 2 drops of dilute H₂O₂ (1 in 20 dilution) replacing copper sulphate.

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