MICROESTIMATION OF URONIC ACIDS*

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(Received for publication, March 6, 1940)

It has long been known that glucuronic acid when boiled in HCl in the presence of naphthoresorcinol forms a purple ethersoluble pigment. The reaction, first described by Tollens (1), is given also by galacturonic acid, and has been widely used as a qualitative test for the presence of these substances. The limitations on its specificity are summarized in van der Haar’s (2) monograph on the sugars.

Recently Salt (3) attempted to apply the reaction to the estimation of glucuronic acid in urine, with only partial success. His efforts were directed to the preparation of urine fractions which would yield clean violet colors, unmixed with the pinkish tones which are so troublesome in tests on whole urine. His procedure consisted in the separation of glucuronides from other urinary constituents by fractional precipitation with lead salts; the formation of the colored derivative with naphthoresorcinol, however, was performed in the classical manner, which, as will be seen, would be unlikely to yield reproducible results.

The need for a simple and reasonably accurate method of determining uronic acids in urine led us to examine the Tollens reaction itself, to see whether it might not be adapted to quantitative use by modifying the conditions under which it is carried out. It soon became evident that the conditions ordinarily employed are far from optimal for color development. However, when various factors had been adjusted so as to produce maximum amounts of pigment, the sensitivity was markedly increased and the color intensity was found to be a linear function of the concentration of uronic acid.

* The work reported in this communication was carried out under a grant from the W. K. Kellogg Foundation.
The following factors required modification from the original form of the reaction: (1) Time of heating with HCl. Maximum color development required several hours, instead of the 1 minute boiling period of the classical test. (2) Concentration of HCl. The optimum acid concentration was 3 parts of concentrated HCl to 7 of aqueous solution, instead of equal parts of each. (3) Concentration of naphthoresorcinol. The intensity of color developed by a given quantity of uranic acid was found to vary with the amount of naphthoresorcinol used. However, the smaller the excess of this reagent, the cleaner were the colors obtained. The amount of naphthoresorcinol added to each test was accordingly reduced to one-tenth of the amount recommended by Tollens. (4) Dissolution of the pigment. The extractability of the pigment from the reaction mixture by ether was enhanced by reducing the concentration of acid prior to extraction. Other immiscible solvents (benzene, ethyl acetate, petroleum ether, chloroform) either failed to extract the pigment or yielded unsatisfactory colors.

Color intensity was measured in the Pulfrich stufenphotometer. Since the pigment has a well defined absorption maximum at 578 nm, Filter S-57 is well suited for its measurement. Under the conditions finally adopted as standard, samples could be used containing from 10 to 40 μ of glucuronic acid, or from 7 to 30 μ of galacturonic acid, in a volume of 3.5 ml. or less.

Details of the Estimation

Reagents Used—
Naphthoresorcinol (c.p., Eimer and Amend), 1 per cent solution in 95 per cent ethyl alcohol. Store in a brown bottle in the refrigerator.
Ethyl ether, peroxide-free.
Sodium sulfate, anhydrous.
Hydrochloric acid, concentrated, c.p.

Procedure for Glucuronic Acid—Place a sample containing from 10 to 40 μ of free glucuronic acid in 3.5 ml. of water in a centrifuge tube of 10 or 15 ml. capacity. Add exactly 0.1 ml. of naphthoresorcinol solution, followed by 1.5 ml. of concentrated HCl. Mix by rotating the tube. Place in a boiling water bath or steam bath and heat, uncovered, for 4.5 hours; mix by rotating
the tubes every half hour for the first 2 hours. Cool and centrifuge at 2000 r.p.m. for 10 minutes. Remove the bulk of the supernatant with a capillary pipette, avoiding disturbance of the precipitate, and dilute the remaining drop or so of fluid with about 10 times its volume of water. Extract the aqueous suspension with successive small portions of ether (about 1 ml. at a time), using a capillary pipette to mix the materials thoroughly, and transferring the violet-colored extracts to a test-tube. The total volume of pooled extracts should not exceed 6 ml. Dry the extract by adding to it a small amount of anhydrous sodium sulfate. Filter through fine filter paper (Whatman No. 44 is satisfactory) into a 10 ml. volumetric flask, using the capillary pipette to transfer the solution to the filter and washing the filter paper dropwise with as much fresh ether as the capacity of the flask will allow. With careful washing, the same funnel and paper can be used for a series of extracts. Make up to volume. Read the solutions as soon as possible in the stufenphotometer, using Filter S-57 and 20 mm. cells, against an ether blank. If there is any delay between the preparation of the solutions and their reading, protect them from light and keep in the refrigerator. With good ether, they may be kept overnight, though this is to be avoided whenever possible.

Procedure for Galacturonic Acid—For galacturonic acid, the working range is from 7 to 30 \( \gamma \) per sample, and 2 hours on the steam bath suffice for maximum color development.

Calculation of Results—The color intensities developed by known amounts of glucuron\(^1\) and galacturonic acid monohydrate\(^1\) are shown graphically in Fig. 1. The density is that of a 20 mm. layer of solution. Equations for the conversion of density \((D)\) to mg. were derived empirically from these data, and are represented by the straight lines shown in Fig. 1. The equations are the following.

\[
\frac{(D - 0.08)}{16.7} = \text{mg. glucuronic acid (as } C_6H_{10}O_7) \\
\frac{(D - 0.08)}{24.5} = \text{mg. galacturonic acid (as } C_6H_{12}O_7) 
\]

The constant 0.08 is in effect a correction for the amount of light absorption produced by the reagents alone. (Blank deter-

\(^1\) Authentic specimens of these substances were kindly furnished by Dr. Walther Goebel of the Rockefeller Institute.
minations yield pale pinkish, straw-colored extracts.) Its possible variation from one batch of naphthoresorcinol to another must be controlled by determining a blank for each new lot of reagent, as the commercial c.p. grade is brown.

The range of concentration can be extended in preliminary estimations by reading very strong colors in a 10 mm. cell and doubling the value of the density thus obtained. Such a reading should be followed by a new determination with a smaller sample, calculated to fall within the specified working range, where the conversion formula is known to be valid.

A number of substances, including pentoses, interfere with the qualitative test by developing pink colors which obscure the definitive purple pigment. Such substances will of course interfere with the quantitative estimation as well, since any light absorption of extraneous origin which falls within the range of the filter

![Graph](https://via.placeholder.com/150)

**Fig. 1.** The relation between the size of the sample of uronic acid and the intensity of color developed.
will register as glucuronic acid. For example, a pure specimen of $d$-arabinose formed a dark blue precipitate with naphthoresorcinol under the conditions described here. This precipitate yielded a rose-colored ethereal extract, with an absorption maximum in the range of Filter S-47. However, it also showed absorption in the range of Filter S-57, of the same order of intensity as had been obtained with the glucuronic acid pigment. It is therefore essential that preparations of uronic acid for estimation be free of contaminants which produce overlapping absorption.

Salt (3) has described a method of preparing suitable fractions from urine.

In an unknown preparation it is of course necessary to determine whether one is dealing with glucuronic or galacturonic acid, in order to calculate the results. This can readily be done by making use of the rate of color development as a criterion. The rates of color development of the two acids are shown in Fig. 2, from which it will be seen that they are clearly distinguishable.

The applicability of the method to a series of preparations from
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urine was tested by recovery experiments, in which known amounts of glucuronic acid were added to samples of previously analyzed material. These experiments are summarized in Table I. The largest discrepancy between found and calculated values amounted to 3\(\gamma\) in 39\(\gamma\). Duplicates as a rule agreed within 1 or 2\(\gamma\).

Observations on the Reaction

One feature of the reaction whereby the pigment is formed warrants discussion. Not only does the amount of pigment vary with the amount of naphthoresorcinol, but the color developed by a constant amount of glucuronic acid was found to be linear with the logarithm of the naphthoresorcinol concentration (see Fig. 3). From this we infer that naphthoresorcinol, besides

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>In original sample Found</th>
<th>In sample + 0.018 mg. known glucuronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac23</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.008</td>
<td>0.026</td>
</tr>
<tr>
<td>Sm5</td>
<td>0.018</td>
<td>0.036</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.011</td>
<td>0.029</td>
</tr>
<tr>
<td>Sm6</td>
<td>0.021</td>
<td>0.039</td>
</tr>
</tbody>
</table>

reacting with uronic acid, undergoes some other process which gradually reduces the amount available for pigment formation. Visible evidence of such a side reaction is to be found in the formation of a grayish precipitate in blank determinations containing only naphthoresorcinol, water, and HCl. The development of the precipitate in the blank roughly parallels, in time, the development of the deep blue uronic acid precipitate in the test. The rates of the two processes are sufficiently similar to result in effective competition between them. Hence the initial relative concentrations of naphthoresorcinol and uronic acid are significant and must be controlled carefully; that is, the size of the sample must fall within a relatively narrow range, and the amount of naphthoresorcinol used must be measured accurately.
if color development is to be proportional to the amount of uronic acid present.

Another aspect of the above is to be seen in the difference in behavior between glucuronic and galacturonic acids. Galacturonic acid reacts about twice as rapidly as glucuronic acid, reaching maximum color development in 2 as against 4.5 hours. Moreover, the intensity of color developed per mg. is considerably greater for galacturonic acid, as can be seen from the conversion formulae. In the case of galacturonic acid, the corrected density must be divided by 24.5 to obtain the weight in mg., where-

![Fig. 3. The relation between the intensity of color developed by a constant amount (0.031 mg.) of glucuronic acid and the amount of naphthoresorcinol used. D refers to density as defined for Fig. 1.](image)

as for glucuronic acid it is divided by only 16.7. The difference in the amount of pigment formed by the two compounds may well be related to the difference in rate of color development. In the presence of galacturonic acid, pigment formation by naphthoresorcinol takes place rapidly, before much of the reagent becomes unavailable by the side reaction. In other words, relatively more naphthoresorcinol is available for pigment formation during the reaction period as a whole in the case of galacturonic acid. It follows from these considerations, and is borne out experimentally, that uronic acid in combination must be hydrolyzed prior to the addition of naphthoresorcinol.
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SUMMARY

Free glucuronic or galacturonic acid can be estimated photometrically by a procedure based on the Tollens reaction.

The method is described for samples containing from 10 to 40 $\gamma$ of glucuronic acid, or from 7 to 30 $\gamma$ of galacturonic acid.

These acids can be distinguished from one another by their different rates of pigment formation with naphthoresorcinol.

BIBLIOGRAPHY

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J. Biol. Chem. 1940, 134:143-150.

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