A METHOD FOR THE QUANTITATIVE DETERMINATION OF MENTHOL GLYCURONIC ACID IN URINE.

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The study of glycuronic acid is important because it is concerned with an important detoxication mechanism of the body, and because of its possible relationship to carbohydrate metabolism. Our present knowledge of its synthesis in the body is meager and there is a distinct disagreement among the findings of the various investigators who have worked on the problem (1, 2). Much of this conflict in the experimental findings is undoubtedly due to the lack of a reliable quantitative method for glycuronic acid, or rather conjugated glycuronic acids, which can be applied to urine.

One of the oldest and most frequently used methods for determining conjugated glycuronic acids is based on optical rotation measurements. There are several serious disadvantages and objections to this method of analysis. In the first place, urine is an unsatisfactory solvent medium for polariscopic measurements, since its high concentration of salts and its variable hydrogen ion concentration introduce factors which can neither be controlled nor corrected satisfactorily. In the second place, the method fails completely unless the other optically active substances are removed. Glucose and other sugars may be removed by fermentation, but there is evidence that glycuronic acid is also slightly fermentable (3, 4). Finally, very few conjugated glycuronic acids have been prepared in a pure state and their optical rotation as well as the rotation of their salts studied with sufficient accuracy to be of value for quantitative analysis.

Another quantitative method proposed is based on the conversion of glycuronic acid to furfural when distilled with 12
per cent hydrochloric acid. This method, as outlined by Tollens (5), is purely empirical since the amount of furfural obtained is only one-third of the amount required by theory. The method further assumes that all conjugated glycuronic acids yield a definite amount of furfural, whereas Mann, Kruger, and Tollens (6) found that the amount varied with the individual acids.

As early as 1887 Thierfelder (7) found that a mol of glycuronic acid possessed the same reducing power on Fehling solution as a mol of glucose (1). No advantage of this property was taken until recently when Biberfeld (8) determined free glycuronic acid in urine by Bang’s method and found that 1 mol of glycuronic acid was equivalent to 1 mol of glucose. The method, as employed by Biberfeld, is not directly applicable to conjugated glycuronic acids nor can it be used in the presence of other reducing substances, especially sugars. As already pointed out, sugars are especially objectionable since no common satisfactory method exists for their removal from urine without also destroying or removing some of the glycuronic acid.

Other means of determining glycuronic acid have been proposed from time to time. Thus, Neuberg and Neimann (9) suggested a determination of phenol glycuronic acid by simultaneous hydrolysis and oxidation of the compound with hydrochloric acid and bromine under pressure and determining the resulting saccharic acid as the silver salt. As the method does not give very accurate results and requires the use of sealed tubes, it has claimed little attention. Another method, devised by Vitali (10) for urochloralic acid, which requires a laborious reduction of trichloroethyl alcohol to alcohol (which is then determined), has met a similar fate.

Since none of the methods were satisfactory and since it was planned to investigate the relationship of glycuronic acid to glucose metabolism as studied by means of phlorhizin and pancreatic diabetes, efforts were made to develop a method which could be applied to urines containing sugar.

Attention was first directed to the furfural distillation method since it was hoped that by incorporating the modification of Pervier and Gortner (11) of steam distilling the furfural out of the reaction mixture, the great source of error, namely the destruction of furfural by prolonged contact with strong
acids, could be eliminated. It was found, however, that the conversion of glycuronic acid to furfural was so slow that a 0.2 gm. sample of menthol glycuronic acid yielded only a little more than one-half of the theoretical amount of furfural after 8 hours of continuous distillation and after 1,200 cc. of distillate had been collected.

The possibility of utilizing the reducing action of glycuronic acid and applying a quantitative sugar method for its determination was next investigated. This study lead to the development of a satisfactory method which is presented in detail.

Since it seemed almost impossible to find a general method which could be applied to all conjugated glycuronic acids, it seemed best to develop a method which was specific for one, but which, with slight modifications, could be extended to the determination of other conjugated glycuronic acids. Menthol glycuronic acid was chosen, for the acid can readily be isolated from urine and prepared pure so as to be suitable for a standard. It, furthermore, has the advantage of being easily soluble in ether and of being readily hydrolyzed by dilute acids. Menthol, itself, is a substance which is readily obtainable, and which is well suited for feeding experiments since animals tolerate it well and in considerable amounts.

The quantitative method developed for menthol glycuronic acid consists in the extraction of the compound from urine by means of ether, hydrolysis of the conjugated acid with a dilute mineral acid, and finally a quantitative determination of the liberated glycuronic acid by a sugar method. Since sugar and other common reducing substances are insoluble in ether the method is decidedly specific. By a preliminary ether extraction of the urine made alkaline with sodium carbonate, the specificity can be further enhanced since a number of substances such as aromatic hydroxy compounds, which might react with the sugar reagents, are thus removed, while the sodium salt of menthol glycuronic acid, which is insoluble in ether, stays behind. Then, on acidification the free acid is extracted. So far this preliminary extraction has not been found necessary. The hydrolysis of menthol glycuronic acid is brought about by boiling it with $\mathbf{N}$ hydrochloric acid under a reflux condenser.
Menthol Glycuronic Acid in Urine

The hydrolysate after neutralization and dilution to a definite volume is analyzed either by the Benedict or the Folin-Wu sugar method. Before Benedict's method could be applied to the dilute solutions used, the method had to be modified and rigorously standardized before trustworthy analytical results were obtainable. It was found that the oxidizing value of Benedict's solution is markedly influenced by the concentration of sodium carbonate, or, more exactly, by the hydroxyl ion concentration of the solution. To control this factor it was necessary, first, to add a constant weighed amount of anhydrous sodium carbonate, and secondly, to determine the oxidizing value of Benedict's solution for a series of dilutions of varying concentrations such as would be obtained by titrating with solutions of different strengths of glycuronic acid. From the data obtained Chart 1 was constructed from which the oxidizing value, i.e. the glycuronic acid equivalent per cc. of Benedict's solution, can be read directly. Although Benedict's solution when carefully prepared according to directions and with pure chemicals has a constant and definite strength, it seemed desirable to be able to standardize the solution with glucose. It was found that while 1 mol of glycuronic acid has approximately the same reducing power as 1 mol of glucose, a slight difference exists since the concentration of sodium carbonate influences the oxidation of glycuronic acid slightly more than it does the oxidation of glucose. A 0.125 per cent solution of glucose is recommended for the standardization of Benedict's solution. Curiously, while 1 mol of glycuronic acid is equivalent to 1 mol of glucose in its power to reduce Benedict's solution, 1.5 mols of glycuronic acid are necessary for a similar action on the reagent of Folin and Wu.

By using the Benedict as a macro, and the Folin-Wu as a micro method, it is possible to get good results over a wide range of concentrations of conjugated glycuronic acids in urine. As the size of the sample can also be varied, the method is applicable to the whole range of concentrations from the highest possible to a few milligrams per 100 cc. The method, furthermore, possesses an accuracy equal to that of the quantitative sugar methods with the added advantage that it is more specific, which is of great importance when analyzing urine containing more than
one reducing substance. While the method has been applied only to menthol glycuronic acid, there is no reason why it cannot be extended to the analysis of other glycuronic acids since a great many of them are soluble in ether and are hydrolyzed by dilute acids. Preliminary work on thymol glycuronic acid seems to indicate that the time of hydrolysis may have to be lengthened, but fortunately glycuronic acid is not destroyed by prolonged boiling with dilute acid. The analysis of phenol glycuronic acid and others of physiological importance will be studied in detail and reported later.

EXPERIMENTAL.

Preparation of Menthol Glycuronic Acid.—This compound is best isolated from urine by Bang's method (12) which consists in the precipitation of the ammonium salt of menthol glycuronic acid by half saturating the urine with ammonium sulfate. The complete procedure for obtaining the compound was as follows: 2 gm. of menthol were mixed with hot water and allowed to stand until it was liquefied when it was emulsified by shaking and administered to the rabbit through a stomach tube. The urine excreted during the 24 hours following the feeding was collected, made alkaline with ammonium hydroxide, heated to boiling, half saturated with solid ammonium sulfate, and filtered hot. On cooling the ammonium salt separated out almost completely, less than 0.4 gm. per 100 cc. remaining in solution. The product obtained was generally colorless and free from gummy impurities, and a second precipitation with ammonium sulfate rendered it pure white. The free acid was prepared by dissolving the ammonium salt in the minimum amount of hot water and adding a calculated amount of N hydrochloric acid to liberate completely the free acid. On cooling the acid crystallized out and one additional crystallization from hot water was sufficient to render the compound analytically pure. Unfortunately, the acid contains a variable amount of water. Fromm and Clemens (13) state that it has 1.5 molecules of water of crystallization, but repeated analyses showed a content somewhat midway between that calculated for 1.5 and 2 molecules. Over fused calcium chloride or sulfuric acid it lost water rapidly until its water content corresponded to 1 molecule of water of
crystallization. After this the rate of loss was slow, indicating that the vapor tension of the monohydrate is slightly higher than that of the drying agent. Because of this variable water content, the samples used for analysis were dried to constant weight at 100° under vacuum and all calculations based on anhydrous menthol glycuronic acid.

TABLE I.

<table>
<thead>
<tr>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>per cent</td>
<td>per cent</td>
<td>gm. per 100 cc.</td>
<td>cc.</td>
<td>mg.</td>
</tr>
<tr>
<td>0.1834</td>
<td>0.107</td>
<td>9.26</td>
<td>22.4</td>
<td>2.42</td>
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<td>9.94</td>
<td>20.0</td>
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<td>0.2292</td>
<td>0.134</td>
<td>10.99</td>
<td>17.3</td>
<td>2.31</td>
</tr>
<tr>
<td>0.2750</td>
<td>0.161</td>
<td>12.55</td>
<td>13.9</td>
<td>2.23</td>
</tr>
</tbody>
</table>

CHART 1.

**Standardization of Benedict's Solution.**—A weighed sample (180 to 275 mg.) of anhydrous menthol glycuronic acid was refluxed with 10 cc. of N hydrochloric acid for 15 minutes to complete hydrolysis. On cooling the solution was neutralized with N sodium hydroxide and diluted in a volumetric flask to such a volume that the glycuronic acid content was 0.10 to
0.015 per cent. With such dilute solutions, Benedict's reagent had to be carefully standardized for varying concentrations of sodium carbonate. As the concentration depends both on the amount initially present (the amount in the reagent itself and the amount added); and on the final dilution, i.e. the volume of the reagent plus the volume added by titration; it was necessary, first to add a constant weighed amount of anhydrous sodium carbonate, and secondly, to determine the oxidizing power (the glycuronic acid equivalent) of Benedict's solution for the range of dilutions produced by adding from 10 to 25 cc. in the titration. The results recorded in Table I were obtained by using 10 cc. of Benedict's solution and 2 gm. of anhydrous sodium carbonate. The titrations were carried out in a 100 cc. Erlenmeyer flask instead of an open porcelain dish to minimize both the loss of water by evaporation and the reoxidation of the reduced copper.

Three different samples of Benedict's solution were found identical in strength. Besides having been standardized with menthol glycuronic acid, they were also standardized with a 0.125 per cent solution of glucose. In each titration 2 gm. of anhydrous sodium carbonate were added.

Found:
10 cc. of Benedict's solution required 16.6 cc. of a 0.125 per cent glucose solution.
10 cc. of Benedict's solution required 16.6 cc. of a 0.139 per cent glycuronic acid solution.
1 cc. of Benedict's solution = 2.08 mg. glucose or 2.30 mg. glycuronic acid.

Ratio: Glycuronic acid: glucose = 1.105.

Calculated:
Glycuronic acid: glucose = 1.077 (assuming 1 mol of glycuronic acid is equivalent to 1 mol of glucose).

Standardization of the Folin-Wu Reagent.—A sample of anhydrous menthol glycuronic acid was hydrolyzed as described above, neutralized, and diluted to such a volume that the solution contained approximately 250 mg. of glycuronic acid per 100 cc. The reducing value of this solution was determined in the precise manner as that of an unknown sugar solution.

45.86 mg. of menthol glycuronic acid were dissolved in 100 cc. of water.
1 cc. contained 0.459 mg. menthol glycuronic acid, or 0.267 mg. glycuronic acid.
674 Menthol Glycuronic Acid in Urine

Found:

The reducing value of 1 cc. = 0.177 mg. glucose, therefore,

and

the reducing value of 1.51 mg. glycuronic acid = 1.0 mg. glucose.

Extraction of Menthol Glycuronic Acid.—Menthol glycuronic acid was quantitatively removed from urine with ether, using a continuous extractor of the type designed by Clausen (14). The apparatus was, however, modified so as to be adapted to the analysis of 10 cc. samples. The lower or constricted portion of the extraction tube was 180 mm. long and had an internal diameter of 14 mm. The lower end of the inner tube was a small perforated bulb such as is commonly employed in aeration with the exception that the perforations came out obliquely upwards instead of laterally. A well insulated air bath supplied with a 75 watt nitrogen-filled lamp produced a well controlled and steady flow of ether through the extractor. With this apparatus complete extraction was effected in 2½ hours. It is advisable, however, when using a new extractor to redetermine the time necessary for complete extraction, and for very important analyses to run the second sample a half an hour longer to determine whether the extraction of the first sample was complete. Since it is common practice in extractions to saturate the solution with ammonium sulfate, the procedure was tried, but it hindered rather than aided the extraction.

Procedure of Analysis.—A 10 cc. sample of filtered urine (5 cc. if the concentration of menthol glycuronic acid was known to be high) was transferred to the extraction tube and acidified with 1 cc. of 20 per cent sulfuric acid. 50 cc. of ether were placed in the boiling flask, and the extraction was carried out for 2½ hours. The ether extract was then quantitatively removed to a 100 cc. Erlenmeyer flask, the ether boiled off, and the residue hydrolyzed by refluxing it for 15 minutes with 10 cc. of N hydrochloric acid. On cooling the solution was made neutral to litmus with N sodium hydroxide. If the approximate concentration of menthol glycuronic acid was not known, the solution was diluted to a small volume (25 cc. or even less) and the approximate concentration of glycuronic acid determined, using an aliquot. If the concentration was found to be 0.1 per cent or higher
the Benedict method was employed, while for lower concentra-
tions the Folin-Wu method was used. For the Benedict method
the solution was diluted in a volumetric flask to such a volume
that the concentration of glycuronic acid was 0.10 to 0.15 per
cent. It was filtered to remove undissolved menthol which has a
tendency to adhere to glass and thus cause imperfect drainage of
the burette. 10 cc. of Benedict's reagent and 2 gm. of anhydrous
sodium carbonate (weighed to within 0.05 gm.) were placed in
a 100 cc. Erlenmeyer flask and heated to boiling. After the
sodium carbonate had dissolved, the glycuronic acid solution
was added from a burette at such a rate that constant boiling
of the reagent could be maintained. Near the completion of
the titration the solution was added drop by drop, allowing at
least 10 seconds to elapse between each addition. The com-
plete disappearance of the blue color (green, if the solution was
yellow) was taken as the end-point. With a little practice the
end-point of duplicate samples could easily be checked to 0.1 cc.

Calculation (Benedict's method).

\[
10 \times \frac{\text{Glycuronic acid equivalent per cc. of Benedict's solution}}{\text{Volume after dilution}} \times \frac{\text{Volume after dilution}}{\text{Titration in cc.}} \times \frac{\text{Total volume of urine}}{\text{Volume of urine taken}} = \text{Gm. glycuronic acid in total volume of urine}
\]

The Folin-Wu method was used without modification. The
glycuronic acid solution was diluted so that its concentration
was between 0.01 and 0.015 per cent. The reducing power in
terms of glucose of this solution was determined, and the result
multiplied by the factor, 1.51, gave the glycuronic acid content.

A Typical Analysis.—This analysis was made on a 24 hour
specimen of urine collected after a rabbit had been fed 3.5 gm.
of menthol.

<table>
<thead>
<tr>
<th>Volume of urine</th>
<th>122 cc.</th>
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<tbody>
<tr>
<td>&quot; samples extracted</td>
<td>5.0 &quot;</td>
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</table>

Analysis by the Benedict Method.

<table>
<thead>
<tr>
<th>Volume of hydrolyzed extract</th>
<th>50 cc.</th>
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<tbody>
<tr>
<td>Titration, I</td>
<td>19.2 cc.</td>
</tr>
<tr>
<td>II</td>
<td>19.4 &quot;</td>
</tr>
</tbody>
</table>

* Obtained from Chart 1.
Menthol Glycuronic Acid in Urine

Found. I. 1.49 gm. glycuronic acid or 2.56 gm. menthol glycuronic acid.
II. 1.48 gm. glycuronic acid or 2.54 gm. menthol glycuronic acid.

Analysis by the Folin-Wu Method.
Volume of hydrolyzed extract ............................................ 250 cc.
Strength of glucose standard ........................................... 0.15 mg. per cc.
Reading of standard. I. 20 mm. Reading of sample. I. 18.0 mm.
II. 20 " II. 18.3 "

Found. I. 1.54 gm. glycuronic acid or 2.62 gm. menthol glycuronic acid.
II. 1.51 gm. glycuronic acid or 2.58 gm. menthol glycuronic acid.

By Direct Isolation of the Ammonium Salt.
Ammonium menthol glycuronate isolated from 105 cc. of urine ............................................. 1.75 gm.
Ammonium menthol glycuronate calculated for 122 cc. of urine ............................................. 2.04 "
Ammonium menthol glycuronate remaining in solution . . 0.45 "
Total .......................................................... 2.49 "
Calculated as menthol glycuronic acid ......................... 2.36 "

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<tbody>
<tr>
<td></td>
<td>cc.</td>
<td>mg.</td>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>10</td>
<td>114.5</td>
<td>114.0</td>
<td>Benedict.</td>
</tr>
<tr>
<td>2†</td>
<td>10</td>
<td>91.7</td>
<td>91.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>91.7</td>
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</tr>
<tr>
<td>4</td>
<td>10</td>
<td>36.7</td>
<td>34.6</td>
<td>Folin-Wu.</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>45.9</td>
<td>47.0</td>
<td>&quot;</td>
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<td>6</td>
<td>10</td>
<td>45.9</td>
<td>46.1</td>
<td>&quot;</td>
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</table>

* Sample 1 also contained 1 gm. of glucose.
† The extract of Sample 2 was hydrolyzed 2 hours.

SUMMARY.

The quantitative methods for free and conjugated glycuronic acids are discussed and their defects pointed out.

A new quantitative method is proposed for menthol glycuronic acid, which depends on the extraction of the compound from urine with ether, with subsequent hydrolysis and the determination of the liberated glycuronic acid by means of either the
Benedict or the Folin-Wu sugar method. Modifications of the Benedict method which are necessary to make the method applicable to dilute reducing solutions are described.

BIBLIOGRAPHY.


Addendum.—After this paper had been sent to press, an article by Csonka (Csonka, F. A., *J. Biol. Chem.*, 1924, lx, 545) appeared in which a determination for benzoyl glycuronic acid is outlined. The method is similar to the one presented above in that the benzoyl glycuronic acid is hydrolyzed by means of dilute acid and the liberated glycuronic acid determined by Benedict’s quantitative sugar method. It differs, however, in the isolation of the conjugated acid from urine. The compound is precipitated with basic lead acetate, and the lead subsequently removed by means of hydrogen sulfide.

In the development of the quantitative method for menthol glycuronic acid, its removal from urine by means of basic lead acetate was considered but abandoned in favor of the extraction method; because the latter procedure is less cumbersome, is more applicable to small volumes of urine and small amounts of glycuronic acid, and is more specific since basic lead acetate will also precipitate other reducing substances, especially sugars. Since it is very likely that benzoyl glycuronic acid is soluble in ether, it ought to be readily and accurately determined with the method outlined for menthol glycuronic acid.
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