A MICROMETHOD FOR THE DETERMINATION OF URONIC ACIDS

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(Received for publication, October 9, 1933)

The original macromethod of Tollens and Lefevre (1) for the determination of uronic acids has undergone various modifications in the hands of different investigators (2–8). All of the modifications are based on the principle that the simple uronic acids and the complex polyuronide substances yield carbon dioxide quantitatively when distilled with 12 per cent hydrochloric acid. In some the carbon dioxide is determined gravimetrically, in others by titrimetric methods. The size of the sample used varies between the limits of 0.2 to 1.0 gm. and the period of heating from 4 to 8 hours.

Recently Buston (9) described a micromethod embodying titrimetric technique for the determination of uronic acid anhydride groups in pectic substances. In conjunction with the studies on the carbonyl sugar acids in progress in this laboratory, one of us (K. P. L.) and a collaborator1 were engaged with the development of a satisfactory micromethod for their determination prior to the time of the appearance of Buston's method. The details had not been completely worked out, consequently it appeared advisable to evaluate the merits and defects of Buston's method in conjunction with our work.

Buston's method is open to criticism. The apparatus is very

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by grants from the University Research Fund.

1 The assistance of Dr. Eugene Schoeffel in the preliminary work is acknowledged. We are also indebted to Mr. Sam Morell for various helpful suggestions contributed to the titrimetric modification.

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fragile and difficult to clean. We were also impressed by the fact that the method appeared to be supported by rather meager analytical data. The data were confined entirely to such complex substances as calcium pectate \((C_{25}H_{46}O_{36}Ca_2)\), sodium pectate \((C_{35}H_{46}O_{52}Na_4)\), and pectin, whose exact composition might still be considered an open question. With substances of such complexity the substitution of one or more residues of a pentose sugar by hexose residues or the loss of a methoxyl or acetyl residue, would alter but slightly the elementary composition. Thus the galacturonic acid anhydride figures obtained might approximate the values expected on the basis of the empirical formulæ presented, even though the complex molecules are actually not identical with these formulæ. Furthermore, within the limits of the analytical figures reported, considerable quantities of impurities might be present.

In the method described herewith the desirable features of Buston's procedure are employed along with certain parts of the excellent apparatus described by Clark (10) for the microdetermination of methoxyl groups. With authentic preparations of \(d\)-glucuronic and \(d\)-galacturonic acid, various methoxyl derivatives of \(d\)-galacturonic acid, and highly purified polyuronide substances, the optimum temperature and the duration of the heating period necessary to effect complete decarboxylation were ascertained. It was found that when the decarboxylation is performed at a bath temperature of 133–136\(^\circ\), results in agreement with the theoretical values were obtained, provided the hydrolysis and decarboxylation period was conducted for a period of not less than 120 minutes. With authentic polygalacturonic acid anhydride preparations, \((C_6H_8O_6)_n\), the duration of the reaction had to be extended to \(2\frac{1}{2}\) hours. Highly purified specimens of alginic acid, \((C_6H_8O_6)_n\), required \(3\frac{1}{2}\) hours.

It is well known that the accurate measurement of small quanti-
ties of carbon dioxide by either gravimetric or titrimetric method presents formidable difficulties. The numerous factors involved have been thoroughly presented in the various treatises dealing with organic microanalysis (11, 14, 15), hence they need not be restated here.

We have explored both the gravimetric and titrimetric methods using the same conditions for the decarboxylation. Although we have been able to obtain consistent results with the titrimetric method presented below, we prefer the gravimetric method since it appears to be more accurate. It is also less tedious and more rapid. It should be emphasized that the titrimetric procedure is preferable when the temperature and atmospheric conditions are such as to make it difficult to obtain accurate weighings of the absorption tubes by the gravimetric method.

Since the gravimetric technique employed for the estimation of the carbon dioxide is identical with that developed by the late Professor Fritz Pregl for the determination of carbon in organic substances, the details need not be given. It should, however, be pointed out that accurate and reliable results cannot be realized unless the conditions originally prescribed by Pregl (11) and later by others (14–16) for the manipulation of the absorption tubes and the microbalance be followed rigidly.

EXPERIMENTAL

Description of Apparatus—The apparatus consists of a reaction flask, A, with a capacity of 25 cc., which is connected to an air condenser, B, through a standard ground joint No. 6. The air condenser is 25 cm. long and terminates in a trap, C, containing silver sulfate dissolved in concentrated sulfuric acid, to retain any hydrogen chloride gas that might pass the condenser from the flask A. The joint in trap C is also standard No. 6. From trap C a side arm leads to the absorption tubes, or bottles D and E, through a 2-way stop-cock, S. The absorption system in the titrimetric method consists of small Jena gas wash bottles of 20 cc. total capacity, equipped with interchangeable ground glass stoppers carrying the inlet and outlet tubes. The inlet tube terminates in a Jena sintered glass disk (porosity No. 0) which breaks the gas stream into a spray of fine bubbles to insure efficient absorption. The bottles are connected to a filter pump through a
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needle valve, $F$, which enables the rate of aspiration to be controlled accurately. A safety bottle, $G$ (2.5 liter capacity equipped with a stop-cock), is shunted in to serve as a reservoir to equalize the flow of gas through the system. The aspirated air is freed from carbon dioxide by a train consisting of a small tower, $H$, filled with ascarite or soda-lime and a gas bottle, $I$, of the type described above, filled with saturated barium hydroxide solution. A small mercury trap, $J$, prevents the liberated CO$_2$ from backing up into the purification train. The reaction flask $A$ is placed in an oil bath which is heated with a microburner. The air condenser is protected from the heat of the bath with a piece of sheet asbestos, $KK'$, which is placed over the oil bath and around the condenser. The reaction flask should be immersed in the oil bath to such a depth that the levels of the reaction mixture in flask $A$ and the outside oil are the same. The thermometer should not touch the sides of the bath. In order to promote smooth boiling a small boiling rod is placed in the flask. It consists of a piece of glass tubing, approximately 2 mm. internal diameter and 5 cm. long, sealed at one end and also about 5 mm. from the other end. The open end is fire-polished. The rod is introduced open end down. All the rubber connections should consist of properly treated and aged thick walled tubing of the type used in the Pregl carbon and hydrogen microdeterminations.

Analytical Procedure for Titrimetric Method—A sample of 9 to 11 mg. is weighed out accurately in a boat made from about one-third of a cigarette paper. The paper boat is folded carefully and introduced into the reaction flask.$^4$ 7 cc. of 12 per cent HCl saturated with sodium chloride are then added, the boiling rod introduced, and the flask connected to the apparatus. The trap $C$ is previously filled with a few drops of concentrated sulfuric acid containing silver sulfate. The 2-way stop-cock is set to establish a direct connection to the needle valve. The air in the apparatus is then removed by drawing a small but steady stream of CO$_2$-free air through the system. This requires about 10 minutes. Meanwhile 20 cc. of 0.02 $\text{n}$ Ba(OH)$_2$ are added to each of the gas bottles $D$ and $E$ which are then connected to the apparatus with

$^4$ Blank determinations on the cigarette paper used showed that no appreciable quantities of CO$_2$ are produced by the action of the hydrochloric acid.
Fig. 1. Apparatus for the microdetermination of uronic acids and polyuronide substances
thick walled tubing as shown in Fig. 1. The heating is now started without altering the initial rate of aspiration. (A small burner is convenient for this purpose.) When the temperature of the oil bath reaches 100° the stop-cock S is turned so that the air stream passes through the gas bottles. As the temperature approaches 120° care must be taken to maintain sufficient aspiration to prevent excessive back pressure on mercury trap J. The contents of the flask begin to boil at approximately 120° and equilibrium is soon reached. The burner is adjusted so that the temperature of the oil bath is maintained between the limits of 135–137°. The aspiration is adjusted so that a small, steady stream of bubbles passes through the gas bottles. At the end of 2 hours or a longer period, depending on the substance, the heating is discontinued and the stop-cock S is turned to its former position. The gas bottles D and E are disconnected, the ground glass heads replaced by rubber stoppers, and the barium carbonate allowed to settle.

The titration of the barium hydroxide solution from the gas wash bottles should always be preceded by the following method of standardizing the relative strength of the acid and the alkali solutions. Approximately 20 cc. of the standard barium hydroxide are withdrawn from the burette into a small flask (25 cc.). By means of a standardized 5 cc. Ostwald pipette, two aliquots are then rapidly pipetted into 125 cc. Erlenmeyer flasks containing 20 cc. of carbon dioxide-free water and 3 drops of phenolphthalein. The aliquots are then titrated with 0.01 N sulfuric acid. Not more than two aliquots should be pipetted at one time. The aliquots should agree within 0.04 cc. of 0.01 N acid. These titrations give the acid-alkali ratio which must be determined each time a sample is analyzed, since it may vary slightly from time to time. In exactly the same manner two 5 cc. aliquots from each of the gas bottles D and E are then titrated.

The barium hydroxide solution is accurately standardized once each week with analytically pure potassium acid phthalate.

The calculations are made as follows:

Let A equal cc. 0.01 N acid equivalent to 5 cc. alkali

Let B = 0.01 " 5 " aliquots of bottle D
Let C = 0.01 " 5 " " " E

Each gas bottle contains 20 cc. of standard alkali. 1 cc. of 0.01 N H₂SO₄ is equivalent to 0.00022 gm. of CO₂; therefore,

\[
\frac{4 ((A-B) + (A-C))(0.00022)(\text{acid factor})(100)}{\text{Weight of sample}} = \text{per cent CO}_2
\]
The following representative calculation is based on figures obtained in a determination of the uronic acid content of methyl-d-galacturonide dihydrate, C₇H₁₂O₇.₂H₂O.

**Acid-Alkali Ratio**—5 cc. of 0.02 N Ba(OH)₂ (factor = 1.005) required 9.45 cc. of 0.01 N sulfuric acid. Therefore the 0.01 N H₂SO₄ has a factor of 1.050.

**Titration of Aliquots from Bottles D and E**

Two 5 cc. aliquots from bottle D = 7.38

\[ \frac{7.38}{7.38 \text{ cc.}, \text{ average}} \]

Two 5 cc. aliquots from bottle E = 9.34

\[ \frac{9.34}{9.345 \text{ cc.}, \text{ average}} \]

**Calculation**

\[ \frac{4 \times (9.45 - 7.38) + (9.45 - 9.34)) \times (0.0022 \times 1.05 \times 100)}{0.01119 \text{ gm.}} = 18.00 \% \text{ CO}_2 \]

The theoretical for the methylgalacturonide dihydrate is 18.03 per cent.

Achromatic indicators described by Smith (17) may be used in place of phenolphthalein as suggested in Buston’s article (9). Such indicators were tried but we feel that they offer only slight, if any, advantages over phenolphthalein.

**Analytical Procedure for Gravimetric Modification**—In the gravimetric method the standard Pregl soda-lime absorption tubes ((11) p. 45) are used to collect the carbon dioxide liberated. A Schwartz U-tube equipped with ground glass stoppers, containing concentrated sulfuric acid and a few glass beads is connected directly to the side arm of the decarboxylation apparatus in place of the 2-way stop-cock S. The other outlet of the U-tube is connected to a second Schwartz U-tube containing dehydrite (18) or porous anhydrous calcium chloride (grain size) as prescribed by Pregl ((11) p. 54). The first U-tube contains just enough concentrated sulfuric acid to cover the beads in the bottom of the tube. The flow of the gas bubbles through this tube should be about 18 to 24 per minute.

The 2-way stop-cock S is next, being joined to the side arm of the second U-tube containing the dehydrite or the calcium chloride. The Pregl soda-lime tubes follow; they are connected to one of the limbs of the stop-cock S. The Pregl tubes are connected with the
regular thick walled rubber tubing (8 mm. outer diameter, 2 mm. bore) treated as prescribed for the carbon and hydrogen microdetermination ((11) pp. 54–57). A Pregl calcium chloride tube with two connecting tubes bent at right angles ((11) p. 54) is placed between the second soda-lime absorption tube and one limb of the Y-tube that leads to needle valve F. The other limb of the Y-tube is connected to the remaining limb of stop-cock S. This tube prevents the second Pregl soda-lime tube from absorbing water from the aspiration system. The other aspects of the procedure used in the gravimetric method are comparable to the details given above for the titrimetric method. It should be reemphasized that the soda-lime tubes should be manipulated as prescribed for the Pregl carbon and hydrogen microdetermination ((11) pp. 43–53, 80–87).

Sources of Errors—The micromethod presented is recommended only for pure uranic acids or their derivatives. Other carbohydrate substances like the sugars, starch, cell wall polysaccharides, and certain organic acids yield small quantities of carbon dioxide when boiled with 12 per cent hydrochloric acid. While the quantity of carbon dioxide liberated from these substances frequently does not interfere with the accuracy of the macromethods (7), the same is not necessarily true in the microdetermination. Consequently the method is not recommended for uranic acid determinations on plant extracts or crude polysaccharide preparations containing small quantities of uranic acids. The most important sources of error in the uranic acid microdetermination are the rate of aspiration and the length of the heating period. Low results are invariably obtained when the rate of aspiration is too rapid and when the heating period is too brief. A very slow aspiration accompanied with the existence of an occasional back pressure in the course of the determination can likewise produce low results.

The titrimetric modification is obviously subject to the various sources of error that accompany the handling of dilute standard solutions. Consequently the standard precautions recommended for titrimetric work must be rigidly observed. With the gravimetric modification, the commonest source of error is invariably due either to the improper handling of the absorption tubes or to adverse temperature and atmospheric conditions that influence the constancy of the absorption tubes and the microbalance. The
TABLE I  
Summary of Analytical Results

<table>
<thead>
<tr>
<th>Substance</th>
<th>Size of sample</th>
<th>Length of heating</th>
<th>Calculated</th>
<th>Found by gravimetric method</th>
<th>Found by titrimetric method</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>d</em>-Galacturonic acid monohydrate, C₄H₁₀O₇·H₂O; m.p. 159; [α]₀ = +53.4°</td>
<td>10 mg.</td>
<td>2 hrs.</td>
<td>20.75</td>
<td>20.88 20.86 20.85</td>
<td>20.90 20.99 20.40</td>
</tr>
<tr>
<td><em>d</em>-Glucuronic acid, C₄H₁₀O₇; m.p. 163; [α]₀ = +34.0°</td>
<td>10 mg.</td>
<td>2 hrs.</td>
<td>22.68</td>
<td>22.10 21.88 21.98</td>
<td>22.34 22.11 22.26</td>
</tr>
<tr>
<td>Methyl-<em>d</em>-galacturonide dihydrate,* C₃H₁₂O₇·2H₂O; m.p. 112-114°; [α]₀ = +127.6°</td>
<td>10 mg.</td>
<td>1½ hrs.</td>
<td>18.03</td>
<td>18.34 18.01 18.26</td>
<td>18.00 17.91 18.30</td>
</tr>
<tr>
<td>Methyl-<em>d</em>-galacturonide methyl ester monohydrate,* C₄H₁₀O₇·H₂O; m.p. 140-141°; [α]₀ = +124.1°</td>
<td>10 mg.</td>
<td>2½ hrs.</td>
<td>18.33</td>
<td>17.80 18.65 18.12</td>
<td>18.45 18.32 18.40</td>
</tr>
<tr>
<td>Methylglycoside methyl ester polygalacturonide,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₄H₁₀O₇·COOH₃(C₅H₇O₃·COOCH₃)ₙ·CH₃OH·(OCH₃)COOCH₃; [α]₀ = +198°</td>
<td>10 mg.</td>
<td>2 hrs.</td>
<td>22.21</td>
<td>22.30 22.99 22.99</td>
<td>22.59 22.23 22.24</td>
</tr>
<tr>
<td>Alginic acid, (C₅H₇O₃)ₙ;† [α]₀ = −136.0°</td>
<td>10 mg.</td>
<td>3 hrs.</td>
<td>25.00</td>
<td>24.50 24.05 24.45</td>
<td>22.81 23.93 23.40</td>
</tr>
<tr>
<td>Polygalacturonide, (C₅H₇O₃)ₙ;† [α]₀ = +259.0°</td>
<td>10 mg.</td>
<td>2½ hrs.</td>
<td>25.00</td>
<td>25.31 24.39 25.21</td>
<td>24.28 24.17 24.51</td>
</tr>
</tbody>
</table>

* Prepared from a polygalacturonide isolated from citrus pectin after the method of Morell and Link (19).
† Results calculated on ash- and methoxyl-free basis.
sources of these errors have been fully described in the handbooks dealing with the carbon and hydrogen microdetermination (11, 14–16). The various substances used in the absorption and gas washing trains must obviously be changed at frequent intervals in order that fresh effective reagents are always at hand. The first Pregl soda-lime tube can be used for seven to eight determinations—the second tube can be safely used for twelve to fifteen determinations.

SUMMARY

1. A micromethod with either a volumetric or gravimetric modification is given for the accurate determination of uronic acids and uronic acid derivatives by decarboxylation with hydrochloric acid (sp. gr. 1.06).

2. The results obtained with authentic specimens of d-galacturonic acid, d-glucuronic acid, methyl-d-galacturonide dihydrate, methyl ester methyl-d-galacturonide monohydrate, a methylglycoside methyl ester polygalacturonide, C₆H₈O₆COOCH₃ (C₅H₄O₄COOCH₃)ₙ, C₅H₁₀O₃ (OCH₃) COOCH₃, a pure polygalacturonide, (C₆H₈O₆)ₙ, and alginic acid, (C₆H₇O₆)ₙ, are presented (Table I).

3. In all cases, the results obtained by either the volumetric or the gravimetric modification approximate the theoretical values.

4. The micromethod presented is capable of giving results of the same order of accuracy as the best macromodifications.

BIBLIOGRAPHY

A MICROMETHOD FOR THE
DETERMINATION OF URONIC ACIDS
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