by the direct action of hydriodic acid and phosphorus upon the following: 2-quinolone-4-carboxylic acid; hydantoin-($\Delta^{5,3'}$)-oxindole; hydantoin-($\Delta^{5,3'}$)-5',7'-dibromoxindole and 2,5-diketopiperazine-($\Delta^{3,6,3',3'}$)-di-(oxindole). The ethyl ester of the acid is formed by the action of tin and alcoholic hydrogen chloride on 2-quinolone-4-carboxylic acid.

4. 5-Bromo-isatin, and 5,7-dibromo-isatin will condense with hydantoin and also with diketopiperazine.

NEW HAVEN, CONNECTICUT

[A CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, UNIVERSITY OF WISCONSIN, AND THE OFFICE OF CEREAL CROPS AND DISEASES, BUREAU OF PLANT INDUSTRY]

A METHOD FOR THE DETERMINATION OF URONIC ACIDS[1]

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Introduction

During the course of numerous investigations on the composition of the cell wall of various plants we have had occasion to devote considerable time to the chemistry of the acid polysaccharide constituents. These acidic polysaccharide substances are uronic acids or polymerized anhydride derivatives of such acids. In the past twenty years these substances have attracted considerable attention from numerous investigators, not only because of their almost universal occurrence in the plant world but also because these substances without question play an important role in the carbohydrate metabolism of the cell and also serve as structural components of the cell wall.[2]

An aldobionic acid, glucoso-glucuronic, has been shown by Heidelberger and Goebel to be the fundamental building stone of the polysaccharide derived from Type III pneumococcus and to be an important constituent

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] Tollens, *Ber.*, **41**, 1788 (1908); Ehrlich, *Chem.-Ztg.*, **41**, 197 (1917); *Z. angew. Chem.*, **40**, 1305 (1927); *Biochem. Z.*, **168**, 263 (1926); *ibid.*, **169**, 13 (1926); *ibid.*, **203**, 343 (1928); Nanji, Paton and Ling, *J. Soc. Chem. Ind.*, **44**, 253T (1925); Schmidt and co-workers, *Ber.*, **58**, 1394 (1925); *ibid.*, **59**, 1585 (1926); *ibid.*, **60**, 503 (1927); also *Zeitschrift "Der Papier Fabrikant"*, 26 Jahrgang, Heft 28, 1–7 (1928); Schwalbe, *Ber.*, **58**, 1534 (1925); Marcusson, *Z. angew. Chem.*, **39**, 898 (1926); Schryver and Norris, *Biochem. J.*, **19**, 676 (1925); O'Dwyer, *ibid.*, **20**, 657 (1926); *ibid.*, **22**, 381 (1928); Hägglund and co-workers, *Z. physiol. Chem.*, **177**, 248 (1928); Cretcher and Nelson, *Science*, **67**, 537 (1928); Cretcher and Butler, *ibid.*, **68**, 116 (1928); Candlin and Schryver, *Proc. Roy. Soc., London*, **103B**, 365 (1928); Henderson, *J. Chem. Soc.*, 2117 (1928); Szent-Györgyi, *Biochem. J.*, **22**, 1387 (1928); Rehorst, *Ber.*, **62**, 519 (1929); Ehrlich and Rehorst, *ibid.*, **62**, 628 (1929); Weinmann, *ibid.*, **62**, 1637 (1929); Norris, *Biochem. J.*, **23**, 195 (1929); Butler and Cretcher, THIS JOURNAL, **51**, 1519 (1929); Norman, *Biochem. J.*, **23**, 524 (1929).

in the pneumococcus from Friedländer's bacillus.[3] In our extensive studies on the composition of corn (*Zea Mays*) seedlings we have found that free glucuronic acid is present within the cell, that a polymerized glucuronic acid comprises part of the pectinaceous substances of the cell and cell wall, and that glucuronic acid is also intimately associated with the cellulose of the cell wall.[4]

Methods for the Determination of Uronic Acids.—Recently several methods have been devised for the determination of uronic acids and polyuronic acid substances.[5] These methods are all based on the original determination of Lefèvre and Tollens.[6] Their work showed that when glucuronic acid (anhydride or lactone) is heated with hydrochloric acid sp. gr. 1.06, under conditions similar to those employed for determining pentoses, it is decomposed according to the following equation

$$C_6H_{10}O_7 = C_5H_4O_2 + CO_2 + 3H_2O$$

The yield of furfural is less than the theoretical, whilst that of carbon dioxide is practically quantitative (22.70% of the uronic acid). Since the details of the apparatus and method used by Dore and McKinnis were never published in full and in view of the fact that the method that we have devised is more convenient and more expedient than the method of Nanji, Paton and Ling,[5] we thought it advisable to publish, in detail, the method that we have employed in our researches the past three years.

There are several important factors that Lefèvre and Tollens did not stress sufficiently in the description of their method. To insure complete decarboxylation of uronic acids or polymerized uronic acid substances by heating in 12% hydrochloric acid, it is necessary to conduct the decarboxylation at a bath temperature of 135–140° for at least four hours, preferably five.

The method described below has been used with uniform success on pure crystalline uronic acids, uronic acid lactones and polyuronic acids isolated from citrus pectin and from the cell wall of several plant tissues. The method gives equally satisfactory results on extracts or solutions that contain the afore-mentioned compounds in the presence of organic acids, sugars and polysaccharide substances. We have found that the quantity of carbon dioxide liberated by the action of 12% hydrochloric acid on various plant acids, both hydroxy and dicarboxylic, the sugars and sugar acids and many polysaccharides is very small. In a forthcoming paper we shall publish these results in full along with other data which will

[3] Heidelberger and Goebel, *J. Biol. Chem.*, **70**, 613 (1926); *ibid.*, **74**, 613 (1927).

[4] Forthcoming paper; see also Link, THIS JOURNAL, **51**, 2506 (1929).

[5] Nanji, Paton and Ling, *J. Soc. Chem. Ind.*, **44**, 253T (1925); Dore, THIS JOURNAL, **48**, 232 (1926); McKinnis, *ibid.*, **50**, 1911 (1928).

[6] Lefèvre and Tollens, *Ber.*, **25**, 2569 (1892); *ibid.*, **40**, 4153 (1907).

demonstrate the value of the determination of uronic acid in plant physiological and plant chemical studies.

Description of Apparatus Used.—The apparatus consists essentially of a 500-cc. reaction flask C connected by a ground glass joint to an Allihn 25-cm. reflux condenser D, which in turn is connected to a Truog[7] absorption tower F, through a trap E containing 10% silver nitrate to remove any hydrochloric acid gas that might pass the condenser. Two 20-cm. soda lime towers A, and a bulb water trap B are placed ahead of the reaction flask to remove the carbon dioxide from the air aspirated through the system.

The reaction flask is equipped with a side-arm tube that extends below the neck, to facilitate the removal of the liberated carbon dioxide by the air slowly drawn through the apparatus. The absorbing tower F is connected to a water pump through a safety bottle H of 4-liter capacity. A screw cock, 2, on the rubber tube connection between the tower and safety bottle is used to regulate the rate of aspiration. Heat is applied to the reaction flask by means of an oil-bath heated with an electric hot-plate. All connections are made with heavy walled rubber tubing. The accompanying figure illustrates the details of the apparatus.[8]

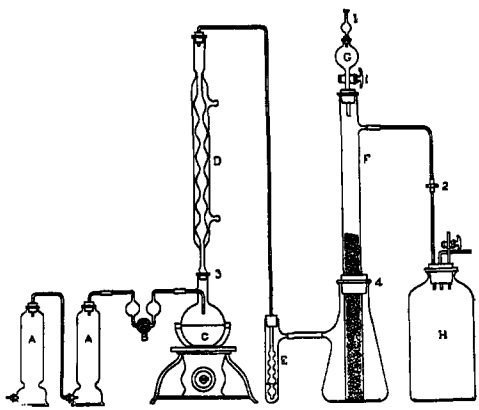

Fig. 1.—Apparatus for the determination of uronic acids.

Analytical Procedure.—The sample (0.2–0.5 g. of uronic acid) or substance to be analyzed is placed in the reaction flask C with 100 cc. of 12% hydrochloric acid (sp. gr. 1.06). A few boiling chips are added to prevent bumping. In dealing with solutions or liquid extracts enough concentrated hydrochloric acid is added to make the concentration equivalent to a 12% solution. The ground glass joint 3 is greased with vacuum stopcock grease to insure an air-tight joint and to prevent the joint from sticking.

The absorbing tower is assembled, filled about two-thirds full with dry glass beads, and connected with the silver nitrate trap E and the safety bottle. The rubber stopper joint 4 between the head tower and the suction flask can be sealed with paraffin to insure an absolutely tight connection. After the dropping funnel G is charged with a measured amount of $N/5$ barium hydroxide solution and put in place at the top of the tower a slow current of carbon dioxide-free air is drawn through the apparatus for fifteen to twenty minutes to remove the air in the system. The dropping funnel G is equipped with a small soda lime tube I. Heat is then applied to the reaction flask. Just as the solution in the flask begins to boil, the barium hydroxide solution is slowly introduced into the tower and washed down with several successive small portions of carbon dioxide-free distilled water. As the solution in the reaction flask C reaches the

[7] Truog, *J. Ind. Eng. Chem.*, **7**, 1045 (1915).

[8] In the method used by Dore the liberated carbon dioxide is collected in potash bulbs. McKinnis used a scrubbing tower (type not described) containing barium hydroxide. As mentioned in the text these authors did not publish the details of the apparatus and procedure used. Nanji, Paton and Ling use several absorption cylinders containing $N/10$ barium hydroxide to collect the liberated carbon dioxide and make use of a gas holder containing air freed from carbon dioxide to sweep out the apparatus.

boiling point and at the moment the barium hydroxide solution is introduced into the tower the air current must be increased sufficiently to prevent the expanding vapors in the reaction flask from passing over into the water trap B and soda lime tower A. The air current is then adjusted so that two to three bubbles per second pass through the tower. The temperature of the oil-bath should be maintained at about 135–140°. After the rate of aspiration and the temperature have been regulated the apparatus requires only intermittent attention. At the end of four to five hours the heating is discontinued and the aspiration is stopped by closing the screw cock 2. The tower is disconnected from the trap E and the stopcock 1 is opened to allow the barium hydroxide solution to flow down into the flask, after which it is again closed. The rubber tube connection is removed from the side arm of the tower, the dropping funnel is taken off and the entire contents of the tower are washed down into the flask with carbon dioxide-free distilled water.

The excess barium hydroxide is titrated with $N/10$ hydrochloric acid, using phenolphthalein as the indicator. Thymolphthalein can also be used with equal success. After the titration is completed, the tower and beads are washed with dilute hydrochloric acid to remove the barium carbonate, washed free from acid and dried in an oven. It is important to remove the condenser from the flask while the flask is still hot to prevent the ground glass joint from sticking. When the samples or solutions to be analyzed for uronic acid contain free carbonates, it is necessary to boil the solution with dilute hydrochloric acid (below 2%) to decompose the carbonate before making the decarboxylation analyses.

Calculation of Results.—One cc. of $N/5$ barium hydroxide solution is equivalent to 0.0044 g. of carbon dioxide. The blank determination on the 12% hydrochloric acid will correct for any carbon dioxide absorbed during the process of charging the apparatus and the titration. It will also detect the presence of minute leaks in the system. The blank determinations should never give titrations larger than 0.20 cc. of $N/10$ hydrochloric acid, which is less than the titration error with the same quantity of barium

SUMMARY OF ANALYTICAL RESULTS

Substance	Carbon dioxide, % Calcd.	Found
Galacturonic acid, $C_6H_{10}O_7$, m. p. 159°, $[\alpha]_D$ +53.40°	22.71	22.44 22.44
Glucuronic acid, $C_6H_{10}O_7$, m. p. 156°, $[\alpha]_D$ +34.0	22.71	22.54 22.57
Glucuronic acid lactone, $C_6H_8O_6$, m. p. 175–176°, $[\alpha]_D$ +19.0°	25.00	24.87 24.89
Barium salt of galacturonic acid, $(C_6H_8O_7)_2Ba$, Ba, 26.85%	16.82	16.62 16.60
Digalacturonic acid, $C_{12}H_{16}O_{12}$, $[\alpha]_D$ +240.80°	24.70	24.45 24.45
Purified lemon pectin	Equiv. to 72.60% uronic acid anhydride[b]	18.15[a] 18.11
Citrus pectin[c]	Equiv. to 64.00% uronic acid anhydride	16.06 15.95

[a] The percentage of carbon dioxide multiplied by 4 gives the percentage of uronic acid anhydride in a sample. The percentage of carbon dioxide multiplied by 5.66 gives the percentage of pectin material.

[b] The uronic acid anhydride values of highly purified pectin preparations vary from 70.00–73.00% (Nanji, Paton and Ling, Ref. 5).

[c] Obtained from the California Fruit Growers Exchange Laboratory, Ontario, California.

hydroxide solution titrated directly. When the blank titration is 0.20 cc. of $N/10$ hydrochloric acid or less, this indicates that only 0.10 cc. of barium solution has been neutralized in the determination by carbon dioxide in the system and admitted in the process of charging the tower.

As an example of the calculation the analytical figures obtained on an authentic specimen of galacturonic acid are given: 0.20-g. samples were decarboxylated at 142° for five hours; 25.00 cc. of $N/5$ barium hydroxide solution was used in the absorption tower; 25.6 cc. of $N/10$ hydrochloric acid equivalent to 12.80 cc. of $N/5$ acid was consumed in the back titration, leaving an equivalent of 10.20 cc. of $N/5$ barium hydroxide neutralized by the liberated carbon dioxide. Multiplied by the factor 0.0044 this is equal to 0.04488 g. of carbon dioxide or 22.44% (theoretical 22.71%).

Addendum January 16, 1930

After the manuscript of the above communication had been sent to the Editor, an article by Ehrlich and Schubert [(*Ber.*, **62**, 1974 (1929))] appeared, in which they discuss (page 2023) the determination of galacturonic acid in pectin substances. The analytical results obtained by our method are in agreement with those of Ehrlich and Schubert. That is, free galacturonic acid and polymerized galacturonic acid anhydride substances can be quantitatively determined by measuring the amount of carbon dioxide liberated by boiling with 12% hydrochloric acid. Ehrlich and Schubert use essentially the same apparatus that van der Haar devised as an improvement of the original of Tollens and Lefèvre Ref. 6. The analytical procedure is modified, however, by conducting the decarboxylation for a period of eight to ten hours instead of three and one-half to four hours. We agree that it is necessary to heat uronic acid substances in 12% hydrochloric acid longer than three and one-half to four hours to ensure complete decarboxylation. However, we have found that the decarboxylation of all the substances tried so far was complete within five hours when the temperature of the oil-bath that contains the reaction flask is maintained at 135–140°. The longer period of eight to ten hours which Ehrlich and Schubert found necessary for complete decarboxylation can possibly be attributed to the fact that the reaction temperature is lower under their conditions, as they do not state the temperature used. It is also possible that the volume of air swept through the apparatus to remove the liberated carbon dioxide is smaller than under our conditions, thus necessitating a longer period for the determination.

Summary

1. An apparatus and method, a modification of the Lefèvre–Tollens method, for the accurate determination of uronic acids by decarboxylation with hydrochloric acid (sp. gr. 1.06) is given.

2. The method is described in detail and the results obtained with authentic specimens of galacturonic acid, glucuronic acid and lactone, the barium salt of galacturonic acid, digalacturonic acid and several pectin preparations are given.

3. In all cases the results obtained approximate the theoretical values.

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