A MICRODETERMINATION OF HYDROXYL AND ACETATE CONTENT OF SUGARS AND GLYCOSIDES

by

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A THESIS

submitted to the

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

May 1943

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ACKNOWLEDGEMENT

The author wishes to express appreciation to Dr. B. E. Christensen for his generous assistance and encouragement during the course of this work.

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INTRODUCTION

ACETATE DETERMINATION. In connection with the studies of a fructosan in Yucca mohavensis, it was necessary to devise micro-methods for the determination of both the acetate and hydroxyl groups of sugars and sugar derivatives.

Numerous reagents and methods have been published for the microdetermination of both O- and N-acyl groups based on alkaline and acid hydrolysis. An alkalimetric method of R. Kuhn and H. Roth (19) employs 1N NaOH for the hydrolyzing agent. The method of A. J. Bailey and R. J. Robinson (2) uses a 0.04N NaOH and refluxes the sample for 12 to 35 hours. Barium hydroxide has also been used (18). The acid hydrolyzing agents generally used are either sulfuric acid or p-toluene sulfonic acid. (27). The selection of the hydrolyzing agent depends on the solubility of the acetate, the possibility of side reactions and other factors. After hydrolysis the volatile acidic reaction products are subjected to steam distillation and the distillate is collected in several portions. Each portion is then titrated separately with standard alkali. From the total amount of alkali consumed the percent acyl is determined. A. Elek

and R. A. Harte (5) using p-toluene-sulfonic acid as hydrolysis agent improved the titration procedure by titrating the acetic acid liberated iodimetrically with standard 0.01N sodium thiosulfate.

These micro-methods require a special apparatus. The procedure is rather long and very close attention is required.

Using the above procedures on the acyl derivative of the fructosan, mentioned previously, satisfactory results were not obtained in this laboratory.

The fact that the acetyl esters of sugars are easily hydrolyzed with dilute alkali at room temperature has been known for sometime. In 1902 (17) and 1904 (1), a study of the rate of the hydrolysis of acetylated monoses and bioses was made. The hydrolysis was carried out in an alcohol-water solution with 0.04N NaOH solution. It was generally concluded for most of the sugars used that K (velocity coefficient) was not constant, but decreased as the change proceeded, indicating that the acetyl groups were not separated at the same rate. Armstrong and Arup (1) in order to obtain further evidence as to the manner in which the acetyl groups were removed extended the study to methyl glucosides of α and β glucose and α and β galactose. These workers

found that these glucosides were hydrolyzed at about the same rate and that K remained fairly constant for about three-fourths of the hydrolysis. Near the end the change proceeded far less rapidly.

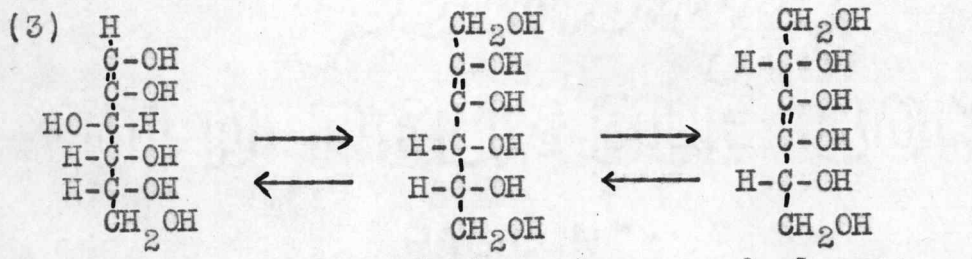
The action of alkali on sugars has been studied quite extensively. Lobry de Bruyn and Alberda van Ekenstein (2) have postulated a series of equilibrium reactions for d-glucose and d-galactose in an alkaline solution.

(1) d-galactose $\rightleftharpoons$ d-tagatose $\rightleftharpoons$ d-talose $\rightleftharpoons$
l-sorbose $\rightleftharpoons$ d-α-galtose $\rightleftharpoons$ d-β-galtose

(2) d-glucose $\rightleftharpoons$ d-fructose $\rightleftharpoons$ d-mannose $\rightleftharpoons$
d-pseudo fructose $\rightleftharpoons$ d-α-glutose $\rightleftharpoons$ d-β-glutose

Nef (22) and his co-workers postulated for these hexoses the existence of a series of enediols in an alkaline solution.

(3)



d-glucose (1,2 enediol) d-glucose (2,3 enediol) d-glucose (3,4 enediol)

Wm. Lloyd Evans (6-12) and his collaborators with the above equilibrium reactions in mind have studied the influence of temperature and the concentration of

alkali on the possibility of the shifting of this equilibrium for both monosaccharides and disaccharides. This was done by the characterization of volatile acids and the residues which result from the splitting of the enediols.

For the purpose of this paper, the conclusion can be drawn from the above work that oxidation of reducing sugars takes place much less readily with alkaline solutions of normality less than one-tenth at a temperature of 25° or less. Nef (22) stated that the enediols do not exist in solutions of an alkalinity less than N/30 and that they begin to decompose in systems of alkalinity above N/20. It thus becomes obvious that the alkaline hydrolysis micro-method of Kuhn and Roth would not give satisfactory results for reducing sugars.

Since the fructosan studied in this laboratory was non-reducing it was possible to obtain very satisfactory results by dissolving the sample in 95 percent alcohol and then hydrolyzing with an aqueous 0.1N NaOH solution at room temperature for 4 to 15 hours and back titrating the excess alkali with standard sulfuric acid. This method has been used successfully on a macro scale for fructose pentaacetate hydrolyzing

at zero degrees with 0.1N NaOH for 3-1/2 hours (16). It has also apparently been used on triacetyl-α-phenyl d-xyloside (21).

In view of the above, a micro-method for the determination of acetates of sugars and sugar derivatives was investigated.

HYDROXYL DETERMINATION. Many methods have been devised for the quantitative estimation of hydroxyl groups. The preparation of an acyl derivative (usually acetyl) and the estimation of the acid formed on hydrolysis of it requires a quantity of material for the preparation of the derivative. Oftentimes the derivative is difficult to prepare and its purification laborious. The method of T. Zerewitinoff (32) based upon the observation of L. Tschugaeff (29) involves the action of a Grignard reagent, methyl magnesium iodide, with an active hydrogen in the organic compound and a measurement of the methane liberated. The method was reduced to micro quantities by B. Flaschenträger (13) and was improved by A. Soltys (28). The method requires a special apparatus and is inconvenient as well as time consuming. Great care must be taken to remove traces of water.

A semi-micro method using acetyl chloride as the esterifying agent has been developed in this laboratory (4).

Many modifications and further applications of the original method of A. Verley and F. Bölsing (30) using a mixture of pyridine and acetic anhydride as the esterification agent have been published. V. L. Peterson and Edward S. West (26) showed that the reagent could also be used for the quantitative acetylation of sugars and sugar derivatives as well as various other compounds. The procedure was rather flexible. A weighed quantity of the sample was heated with a known amount of the pyridine-acetic anhydride mixture (2 volumes of pyridine to 1 of acetic anhydride), in a small tube attached with a condenser at 37° - 80° C. for 24 to 48 hours. The mixture was then poured into ice water and the amount of acid liberated was titrated with standard base. A blank containing no sample was run at the same time.

Edward S. West and co-workers (31) have modified the procedure slightly to be applicable for lipids and hydroxylated fatty acids. M. Freed and H. M. Wynne (14) simplified and shortened the procedure by merely boiling the pyridine-acetic anhydride solution of the sample in an open test tube for 1 minute. When the solution had cooled, it was poured into water. The test tube was rinsed with water and alcohol and the amount of acid liberated was titrated with a standard base.

Recently a micro-method has been developed in this laboratory by J. W. Peterson, K. Hedberg, and B. E. Christensen (25). The esterification is carried out with pyridine and acetic anhydride in a hermetically sealed tube. The method was found to give satisfactory results with a number of alcohols, phenols and poly-hydroxy compounds.

The micro-method above is here being extended to include sugars and glucosides.

EXPERIMENTAL

ACETATE DETERMINATION OF SUGARS AND GLYCOSIDES. The sugar and glycoside acetates used in this investigation were prepared from pyridine-acetic anhydride mixtures using essentially the procedure of Haworth and Percival (15).

To 3.0 g. of sugar (or glycoside) was added 35 ml. of pyridine. The solution was agitated intermittantly until all the sugar had dissolved. Then 30 ml. of acetic anhydride was slowly added with stirring and the solution allowed to stand for approximately 24 hours at room temperature. This mixture was then poured with vigorous stirring onto 500 g. of ice. The white solid, which usually formed immediately, was filtered, washed with cold water, and dried. Further purification by recrystallization from alcohol could be accomplished when necessary.

Using the above procedure with galactose an oil was formed when the reaction mixture was poured upon ice. This oil was dissolved in hot alcohol and crystallized upon cooling.

Several of the β-glucoside tetracetates used were prepared by the method of Bembry and Powell (3).

In order to establish the optimum conditions for the quantitative hydrolysis of these esters a number of initial experiments were executed.

Rate of Hydrolysis. Studies with sucrose octa-acetate indicated that hydrolysis of this compound was complete in 3-1/2 hours (see Figure 1).

Similar tests with glucose, maltose and lactose indicated complete hydrolysis in 3-1/2 hours in the case of the disaccharides while glucose gave results higher than theoretical. On continued hydrolysis maltose (21 hours), lactose (19 hours) as well as galactose (14 hours) gave high and erratic results.

Since this excess was best accounted for on the basis of enolization, splitting and oxidation of the breakdown products of the sugar, test runs were made using a layer of a hydrocarbon to shield the acetates from the air.

The results of these experiments are given in Table I.

Concentration of the Alkali. Due to the critical nature of the concentration factor (6-12), several experiments were conducted to establish optimum condition. According to Nef (23), enolization and breakdown of reducing sugars does not occur in solutions of less than N/30 base. The results of a series of experiments in which this factor was tested are tabulated in Table II.

TABLE I

Acetate	Temperature (°C)	Time (Hours)	Concentration of Base	Percent Hydrolysis
Glucose	20°	6	0.045	98.6
(With a	20°	26	0.045	102.
layer of	20°	72	0.045	116.
a hydro-				
carbon)				

TABLE II

Acetate	Temperature (°C)	Time (Hours)	Concentration of Base	Percent Hydrolysis
Glucose	20°	10	0.09	105.
	20°	34	0.09	114.
	20°	60	0.09	126.
	20°	6	0.045	102.
	20°	24	0.045	106.
	20°	60	0.045	113.
	0°	4	0.045	101.
	0°	26	0.045	103.
	20°	4	0.015	101.
	20°	7	0.015	103.6
	20°	19	0.015	103.8
	0°	12	0.015	96.6
	0°	24	0.015	100.6
	0°	41	0.015	100.5
	0°	68	0.015	100.6
Maltose	20°	11	0.015	98.8
	20°	24	0.015	103.
	0°	45	0.015	98.2
	0°	69	0.015	98.8
Lactose	20°	8	0.015	98.5
	20°	18	0.015	99.6
Sucrose	20°	11	0.015	99.3
	0°	45	0.015	95.3
	0°	68	0.015	97.3

Procedure of Analysis. On the basis of these studies the following procedure was adopted.

A 5 to 10 mg. sample of the acetate is weighed out in a small glass boat and is placed in a 25 cc. Erlenmeyer flask. The sample is then dissolved in 95 percent alcohol, 2 to 5 cc. depending upon the solubility of the acetate, warming if necessary to obtain solution. Four ml. of 0.045 N. sodium hydroxide (carbonate free) is added from a micro-burette. The flasks are stoppered and shaken mechanically or intermittently by hand for 3-1/2 to 10 hours at room temperature. The excess alkali is neutralized with dilute sulfuric acid (0.05N) using phenolphthalein indicator. The percent acetate is calculated as follows:

$$\% (CH_3COO) = \frac{(\text{ml. of base} - (\text{ml. of acid} \times T.)) \times N_b \times 5900}{\text{mg. of sample}}$$

T = ratio of acid to base
N_b = normality of base

Using this procedure the data in Table III were obtained.

TABLE III

Name of Acetate	Number of Determination	$\%CH_3C(=O)-O$ Theory	$\%CH_3C(=O)-O$ Found (Average)	Average Deviation Parts per 1000
Glucose	7	75.6	77.1	5.9
Galactose	3	75.6	76.4	2.2
Sucrose	14	69.6	69.7	4.6
Lactose	7	69.6	69.7	3.7
Maltose	7	69.6	69.7	2.2
Raffinose	6	67.2	67.0	2.0
Inulin	5		61.7	6.8
Glycogen	7		61.4	10.0
A Fructosan	5		61.8	3.2
Salicin	7	59.5	59.5	3.1
Amygdalin	6	55.0	55.6	7.5
Digitalin	5		46.6	2.6
Phloridzin	4	56.6	55.0	3.6
Arbutin	4	61.0	60.8	2.5
Aesculin	3	53.4	54.6	6.8
Eugenol-β-d-glucoside	7	47.8	48.5	6.2
Phenyl-β-d-glucoside	4	55.8	55.2	6.8
Guaiacol-β-d-glucoside	6	52.1	52.0	7.7
p-Cresol-β-d-glucoside	3	53.8	54.4	2.4

HYDROXYL DETERMINATION OF SUGARS AND GLYCOSIDES.

The procedure developed in this laboratory (25) for the microdetermination of hydroxyl groups was followed. Since some sugars were slow to dissolve in the pyridine-acetic anhydride mixture the period of acetylation was extended to 48 hours. The method as reported is as follows:

Reagents. C.P. acetic anhydride, redistilled and acetate-free, kept in a well-stoppered screw cap bottle; C.P. pyridine, redistilled and water free; and 0.04N sodium hydroxide, carbonate-free.

Apparatus. The reaction vessel consists of a melting point tube, 3 mm. in diameter and 6 cm. in length, made from a soft glass test tube.

Two medicine droppers, for the delivery of acetic anhydride and pyridine, respectively, are made by drawing one end of a 6 mm. soft glass tubing to a fine capillary and equipping the other end with a rubber policeman.

Glass plungers, 1 mm. x 0.5 cm., are made from soft glass rod.

A microcentrifuge.

Analytical Procedure. Introduce 2 to 4 mg. of the compound into a weighed reaction tube employing the technique described by Niederl for filling Rast tubes (24). Then weigh the tube. By means of a dropper introduce

20-30 mg. (4-6 drops) of pure acetic anhydride. Centrifuge and reweigh. Add 10 to 12 drops of pyridine. This amount is about double that reported but is advantageous for obtaining solution of sugars more rapidly. Insert a small glass rod in the tube, seal, then shake well to insure complete mixing and set aside for 48 hours. At the same time, run a blank to determine the volume of standard base required to neutralize the acid derived from 1 mg. of acetic anhydride.

Place the reaction tube in a 50 ml. Erlenemeyer flask, add 5 ml. of water, and then break the tube by means of a stout stirring rod. Titrate released acid with 0.04N sodium hydroxide. The percent hydroxyl can then be calculated by means of the formula:

$$\% \text{ (OH)} = \frac{\text{(m.e. of anhydride used--m.e. of acid found) X 1700}}{\text{Mg. of sample}}$$

where m.e. of acid found = ml. x normality, m.e. of anhydride used = mg. of anhydride X ratio X normality, and ratio = ml. of base required to neutralize acid derived from 1 mg. of anhydride.

Using this procedure the data in Table IV were obtained.

TABLE IV

Name of Glycoside	Number of Determinations	%Hydroxyl (Theory)	%Hydroxyl (Found)	Average Deviation; pt/1000
Sugars				
Glucose ($1H_2O$)	2	42.9	42.6	3.5
Galactose	2	47.2	45.9	8.7
Fructose	5	47.2	40.2	16.
Mannose	2	47.2	47.4	15.
Xylose	2	45.3	43.7	4.6
Rhamnose (hydrate)	2	37.3	36.6	9.6
Sucrose	2	39.8	39.7	2.5
Maltose ($1H_2O$)	2	37.8	37.5	9.3
Lactose ($1H_2O$)	2	37.8	37.5	12.
Raffinose Hydrate	1	31.5	31.6	0
Glucosides				
Salicin	2	29.7	29.2	5.
Phloridzin ($2H_2O$)	2	25.2	25.5	20.
Digitalin	2		20.1	0
Amygdalin ($3H_2O$)	2	23.3	23.3	4.3
Arbutin ($1H_2O$)	2	29.3	28.5	17.
Aesculin ($2H_2O$)	2	22.6	22.4	2.2

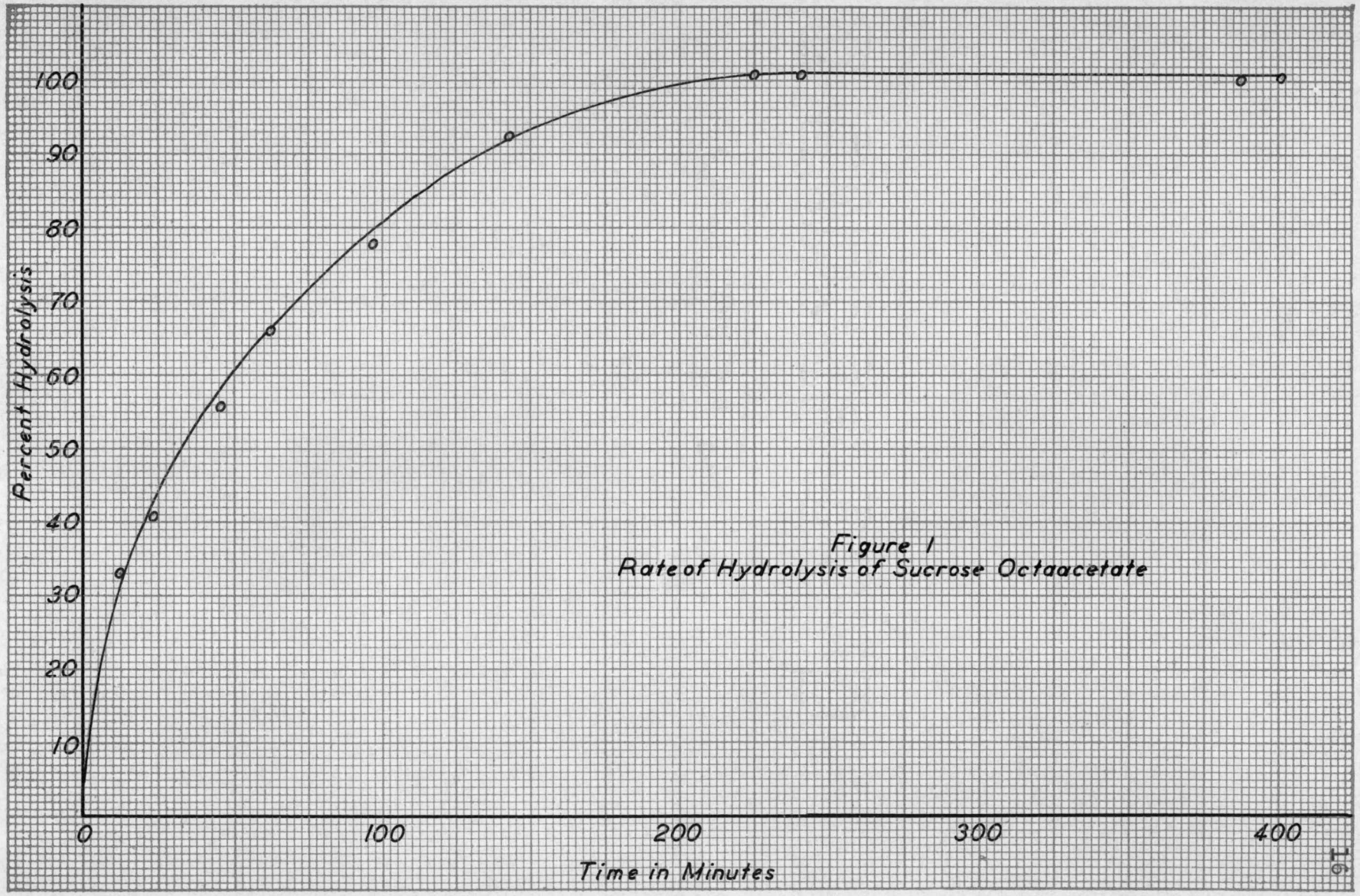


Figure 1
Rate of Hydrolysis of Sucrose Octaacetate

DISCUSSION

As indicated by the results in Table III, the determination of the acetate with 0.045N NaOH at room temperature is complete in approximately 4 hours. These conditions appear to be satisfactory in all tests except those applied to the reducing monosaccharides.

In the case of these reducing sugars, the minimum concentrations necessary for enolization and splitting as reported by Nef were confirmed. Lower concentrations of alkali were not satisfactory for analytical purposes.

Since hydrolysis of glucose pentaacetate even with 0.015N NaOH at 20°C gave high results, oxidation is evidently taking place. At 0° C. more normal results are obtained due to slow rate of hydrolysis and probably absence of oxidation.

From the data in Table I it appears probable that increased acidity results from a self oxidation as well as a mechanism of atmospheric oxidation. However, this question is still unsettled.

According to the information in Table III, this procedure gives fairly consistent results with an average deviation of approximately 1/2 percent, which is quite satisfactory for a microdetermination of this type.

The procedure developed in this laboratory (25) for the microdetermination of hydroxyl groups was applied to sugars and glycosides. Only minor modifications were made. The amount of pyridine was increased to hasten solution of the sugars and the period of acetylation was extended to48 hours. The results tabulated in Table IV are in good agreement with the precision obtained using ordinary alcohols.

Since this method is relatively easily carried out, it gives considerable promise as a tool for analysis of sugars and glycosides.

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