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[CONTRIBUTION FROM DIVISION OF APPLIED BIOLOGY, NATIONAL RESEARCH LABORATORIES]

Oxidation of Carbohydrates with Periodate in the Warburg Respirometer[1,2]

BY A. S. PERLIN

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A modified periodate oxidation of carbohydrates is described, the reaction being carried out in dilute bicarbonate solution and an atmosphere of carbon dioxide in the Warburg respirometer. Formic acid produced in the oxidations is thereby conveniently estimated on the micro scale as an increase in pressure due to the carbon dioxide displaced from solution. The results obtained with a wide variety of simple carbohydrates and polysaccharides are in good agreement with theory or with the results obtained by established methods. The modified reaction may be especially useful in conjunction with new isolation and fractionation techniques of carbohydrate chemistry when often only small quantities of material are available.

Carbohydrates which contain at least three adjacent hydroxyl groups or an aldehyde adjacent to a hydroxyl group yield formic acid when oxidized with periodate.[3-5] A knowledge of the quantity of acid produced, therefore, is often of assistance in elucidating structure and affords an index of the number of end groups present in many polysaccharides, including starch and glycogen.[6] Several methods, recently reviewed by Morrison, *et al.*,[7] have been devised for direct titration of formic acid, but they generally require macro to semi-micro quantities of the carbohydrate.

The manometric technique used for measuring acid production in biochemical studies[8] suggested that the formic acid released during periodate oxidation of carbohydrates might also be estimated in the Warburg respirometer.[8] Thus, if the oxidation were performed in bicarbonate buffer the quantity of acid produced should be indicated by the observed increase in pressure due to carbon dioxide displaced from solution. Because minute amounts of gas exchange are measurable with the respirometer, the reaction would permit oxidations on the micro scale and would provide a continuous record of acid production.

Periodate oxidations are usually carried out at a hydrogen-ion concentration below *p*H 5, because in alkaline media over-oxidation and other side-effects have been observed.[9-12] Since no appreciable concentration of bicarbonate exists below *p*H 5[13] more alkaline conditions were necessary in our experiments. However, it was found possible to maintain the *p*H slightly above 5 by use of dilute bicarbonate (<0.02 *M*) and an atmosphere of pure carbon dioxide. This small departure from the more usual hydrogen-ion concentration had no apparent effect on the oxidation of a wide range of carbohydrates. Figure 1, illustrating rates of formic acid production (carbon dioxide evolved) at *p*H 5.7 and 16.7° in the Warburg respirometer, shows that all of the oxidations proceeded smoothly to completion with evolution of close to the theoretical quantities of carbon dioxide. The results were typical of all aldoses, hexitols, uronic acids, glycosides and non-reducing oligosaccharides examined. The scale of the oxidations is indicated by the fact that samples of from 0.2 mg. (glucose) to 2 mg. (raffinose) were sufficient. The oxidation of glucose, representative of the aldoses, incidentally constitutes a novel manometric estimation of these reducing sugars. In agreement with results obtained by the titration method,[14-17] a reducing disaccharide having a 1:6 linkage (*e.g.*, melibiose) yielded the theoretical quantity of acid but those

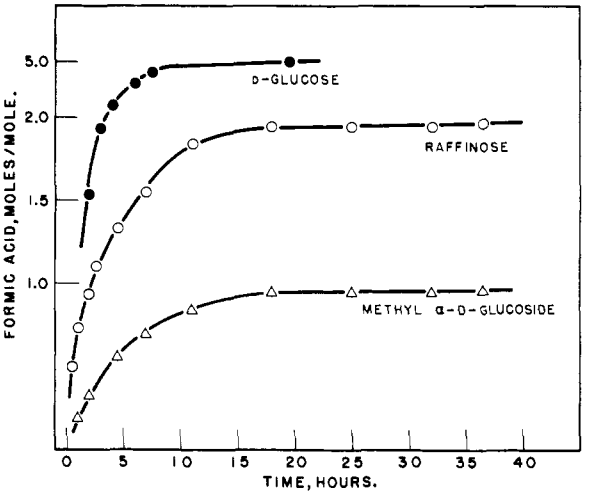

Fig. 1.—Rates of formic acid production in the oxidation of some simple carbohydrates at *p*H 5.7.

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having 1:4 linkages (*e.g.*, maltose and lactose) were over-oxidized.

The quantity of acid produced during oxidation of some polysaccharides (3–4 mg. samples) at *p*H 5.7 in the respirometer was in close agreement with that found by titration of the samples oxidized at *p*H 4.5 (Fig. 2). However, the reaction rates in

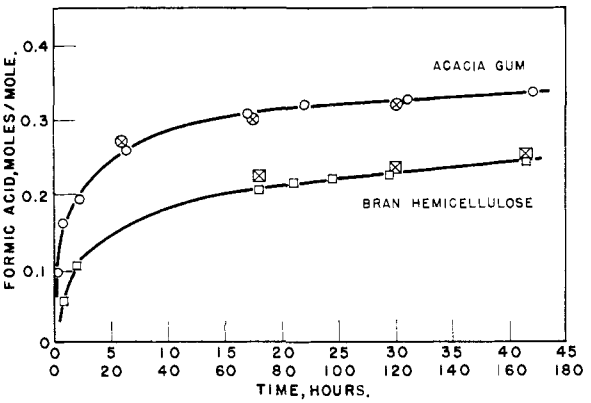

Fig. 2.—Rates of formic acid production in the oxidation of polysaccharides at *p*H 5.7. Crossed symbols represent acid values by titration (Warburg oxidations, upper time scale; titration method, lower time scale).

the respirometer were approximately four times as rapid which may be related to the difference in hydrogen-ion concentration. When the manometric procedure was used to estimate the average chain length of glycogens, extensive over-oxidation was observed; curve I (Fig. 3) is typical of several glycogens examined. However, the extent of over-oxidation was reduced considerably but without greatly altering the rate of the main reaction by a small decrease in the concentration of bicarbonate and a consequent slight lowering of the *p*H. Thus at *p*H 5.4 (curves II and III, Fig. 3) the evolution of carbon dioxide almost ceased in 25 to 30 hours reaction time when the yield corresponded to chain lengths of 10 (II) and 12 (III), in good agreement with values obtained by titration (Fig. 3, and ref.

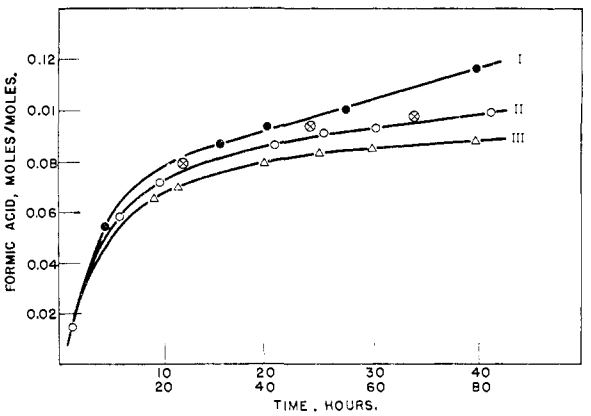

Fig. 3.—Rates of formic acid production in the oxidation of glycogens: I, glycogen (Eastman Kodak Co.) at *p*H 5.7; II, same sample at *p*H 5.4; crossed circles are acid titration values for this glycogen (lower time scale); III, human liver glycogen.

18). The oxidation of glycogens will be described in greater detail in a forthcoming publication.

It may be noted that since excess periodate is not destroyed in estimating formic acid by this method, the same sample can be used at the end of the oxidation for measurement of the periodate consumed.

Oxidations were not carried out below *p*H 5.4 since insufficient bicarbonate was present. However, by employing vessels of larger capacity (*e.g.*, 25 ml.) than the conventional Warburg vessels (15 to 16 ml.) and greater volumes of bicarbonate of suitable concentration it may be possible to extend significantly the lower *p*H limit. On the other hand, the technique also affords a convenient measure of formic acid in oxidations at hydrogen-ion concentrations up to *p*H 7.5 to 8 which is more suitable for some simple carbohydrates[12,15,19] where titration cannot be used.

The results indicate that the formic acid produced in the oxidation of many carbohydrates can be estimated satisfactorily in the Warburg respirometer, but conditions might have to be altered somewhat depending on the substrate. It is anticipated that this technique may be especially useful in conjunction with new methods for isolation and fractionation of carbohydrates, such as paper chromatography, when often only small quantities of material are available.

Experimental

Apparatus.—A conventional constant volume type of Warburg respirometer was used. The apparatus and methods for its use are described in detail by Umbreit, *et al.*[13] The respirometer was calibrated by periodate oxidation of a standard solution of erythritol under a given set of conditions (see below) and subsequent oxidations of the materials described were carried out in the same manner. The bath temperature was 16.7 ± 0.05°. During the oxidation period the vessels were protected from light,[20] which was conveniently achieved by dissolving a small quantity of black dye in the bath fluid.

Occasionally during prolonged oxidation periods the manometer fluid passed out of the graduated range due to a pronounced change in barometric pressure. One method for reading the manometer in such circumstances is given in ref. 13, p. 41. In our experiments, the manometric fluid was Brodie solution,[13] having density 1.033, but use of a fluid of somewhat greater density would largely eliminate the adverse effects of external pressure changes.

Oxidations with Periodate in the Warburg Respirometer.—A typical oxidation at *p*H 5.7 was carried out as follows: 1.9 ml. of 0.02 *M* sodium bicarbonate and 1.0 ml. of 0.1 *M* sodium metaperiodate were pipetted into the vessel chamber; 0.1 ml. of the bicarbonate solution and 0.5 ml. of solution containing 1.0 mg. (0.00515 millimole) of methyl α-D-glucoside were added to the side arm. A second vessel, which served as the reagent blank, contained water (0.5 ml.) in place of the glycoside solution. A stream of carbon dioxide was flushed through the apparatus for one hour, and equilibration was then carried out for at least one hour during which a check for possible leaks was made. After mixing the contents of the chamber and side-arm, changes in pressure were noted at intervals as desired. At 20 hours, the difference in pressure between the sample and blank manometers was virtually constant at 69 mm. which, according to the calibration, corresponded to 0.0049 millimole of formic acid or 0.95 mole per mole of glycoside (Fig. 1).

In the oxidation of glycogen at *p*H 5.4 (Fig. 3), 3.0 ml. of 0.008 *M* sodium bicarbonate, 0.5 ml. of 0.23 *M* sodium metaperiodate and 0.5 ml. of glycogen solution were used. An estimate of the periodate consumed in this oxidation illustrates the method employed. The contents of the

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vessel were transferred to a 125 ml. flask with 15 ml. of water and acidified by addition of 0.1 ml. of 0.5 *N* sulfuric acid. After five minutes, 1.5 g. of sodium bicarbonate, 5 ml. of 0.03 *M* sodium arsenite and 1.0 ml. of 10% potassium iodide were added in succession.[10] Twenty minutes later excess arsenite was titrated with 0.01 *N* iodine. A glycogen sample of 10.8 mg. required 12.9 ml. of iodine, which corresponded to a periodate consumption of 0.97 mole per mole.

The *p*H was measured by transferring the contents of a vessel after equilibration, or at the end of a run, to a *p*H-meter having glass electrodes. A fine stream of carbon dioxide gas was passed into the solution during the measurement making it difficult to obtain an accurate reading. The *p*H values quoted are therefore approximate.

Estimation by Titration of the Formic Acid Produced in Periodate Oxidation (Figs. 2 and 3).—In a typical experiment 100 mg. of glycogen, dissolved in 25 ml. of water, was treated with 0.2 *M* sodium metaperiodate (25 ml.), in the dark at 16.6°. At suitable intervals aliquots (10 ml.) of the solution and of a reagent blank were withdrawn and, 20 minutes after the addition of 0.5 ml. of ethylene glycol and 2.0 ml. of 10% potassium iodide, were titrated with 0.02 *N* sodium thiosulfate.[18]

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Chromatographic Adsorption. III. Investigation of the Isomer Distribution during Fischer Methyl D-Galactoside Formation

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The changing distribution of isomers during the formation of methyl D-galactosides by the Fischer method at room and reflux temperatures using 0.5 and 4% hydrogen chloride was studied by means of chromatography and polarimetry. It was found that β-isomers are formed first and change to α-isomers, principally α-D-galactopyranoside, the change being accelerated at higher temperatures or hydrogen chloride concentrations. The change of furanosides to pyranosides which takes place simultaneously is, contrary to the commonly accepted view, a less important reaction, at least in this case. Conditions under which maximum yields of the various isomers may be expected are included.

Introduction

In the past, study of the rearrangements taking place during glycoside formation by the Fischer method has been seriously handicapped by the lack of easy reliable methods by which the various isomers can be quantitatively determined. An early investigation of this reaction by Levene, Raymond and Dillon[3] indicated that furanosides were formed first and changed slowly to pyranosides as the reaction continued. Until very recently no good method for the quantitative determination of α- and β-isomers has been known and therefore changes from α- to β-forms or *vice versa* could not be followed. In 1946 Binkley and Wolfrom[4] reported the chromatographic separation of the anomeric forms of the penta-O-acetyl-D-glucopyranoses. More recently Hough, Jones and Wadman[5] reported the chromatographic separation of methyl α- and β-L-rhamnopyranosides. Very recently Augestad, Berner and Weigner[6] have reported the chromatographic separation of methyl fructosides and methyl galactosides using a powdered cellulose column. It has been found that Florex XXX, a fuller's earth type of adsorbent, can be used for separation of sugar mixtures[7] and that with the proper pretreatment[8] essentially 100% recovery of the individual sugars can be expected. It was thought that if methyl galactosides could be separated by this adsorbent, a quantitative method for their determination might be realized. In addition, it was hoped that data might be obtained for a comparison of the relative capacities of Florex and cellulose columns for the separation of methyl galactosides.

In the present investigation, methyl galactoside mixtures formed under various conditions of hydrogen chloride concentration and temperature were analyzed by passage in methanol through a Florex XXX column by a procedure previously[8] described. The β-isomers, with negative rotations in methanol, were eluted from the column first, followed by the α-isomers, with positive rotations. Although separation of furanosides from pyranosides was not possible with this adsorbent, a complete separation of α- and β-isomers was obtained. The distribution of furanoside and pyranoside in each fraction can be calculated from the specific rotations of the two components, the weight of the fraction, and the ml.-degree area under the elution curve provided only that no more than traces of other substances are present. This is believed to be the case, as the most important by-products of the reaction, D-galactose dimethyl acetal and the methyl galactoseptanosides would be expected in very small amounts only. D-Galactose dimethyl acetal, for

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