837. *Submicro-methods for the Analysis of Organic Compounds. Part XXIII.* The Periodate Oxidation of Borohydride-reduced Oligosaccharides*

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Previous work on the application of periodate oxidations on the microgram scale was extended to cover samples previously reduced with borohydride. A total sample of 300—400 μg sufficed for the determination of the oxidation-rate curve based on measurement of the excess of periodate, and for the determination of the formaldehyde produced by the initial selective oxidation of terminal glycitol groups. No separations were needed.

Periodate oxidation of the glycitol end-groups of borohydride-reduced oligosaccharides often provides valuable information about the position of the internal glycosidic linkage. It is also often possible to determine the molecular weight of polysaccharides by a similar procedure. Unreduced oligosaccharides react only slowly with periodate, whereas reduced oligosaccharides generally show a rapid initial reaction in the glycitol residue. At suitable pH values, reduced disaccharides which originally contained a hexose terminal group react rapidly with periodate. The mode of reaction depends on the internal linkage: reduced disaccharides with a 1,2- or 1,5-internal linkage would be expected to consume 3 mol. of periodate in a rapid reaction and evolve 1 mol. of formaldehyde and 2 mol. of formic acid, whereas compounds with 1,3- or 1,4-linkages would consume 3 mol. of periodate and evolve 2 mol. of formaldehyde and 1 mol. of formic acid; compounds with a 1,6-linkage would consume 4 mol. of periodate and evolve 1 mol. of formaldehyde and 3 mol. of formic acid.

These reactions have usually been studied on the macro-scale, and generally involve several distinct operations. After reduction with aqueous sodium borohydride and destruction of the excess with acid, the reduced carbohydrate is isolated by means of ion-exchange resins, or by the preparation of suitable derivatives: the separated material is then regenerated and purified, before being subjected to a normal periodate oxidation; the oxidant consumed and the formic acid and/or formaldehyde evolved are then determined.[1,2]

With the extensive use of thin-layer and paper chromatography, it is common to obtain yields of material of <1 mg., on which such structural studies are required. Hence, the adaptation of these methods to the microgram scale was investigated.

A semimicro-procedure has been described [3] in which, after destruction of the excess of borohydride with acid, the periodate oxidation is carried out *in situ*; but only the formaldehyde was determined, by the use of chromotropic acid [4] or by the phenylhydrazine–hexacyanoferrate(III) [5] method, depending on the pH of the oxidation solution. Owing to the technical difficulties of the microgram scale, it was clear that a submicro-procedure would have to be based on a method not involving separation or preparation of derivatives.

Method for Reduction with Sodium Borohydride.—Because of the minute amounts of materials employed and the relatively high dilutions encountered on the microgram scale, it was not possible to test the completeness of the borohydride reduction of the carbohydrate by any of the usual methods, *e.g.*, failure of a drop of the acidified reduction solution to reduce Fehling's solution, or attainment of constant optical rotation of the

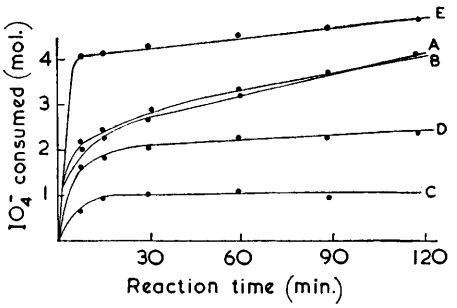
* Part XXII, R. Belcher, G. Dryhurst, and A. M. G. Macdonald, *J.*, 1965, 3964.

[1] M. Abdel-Akher, J. K. Hamilton, and F. Smith, *J. Amer. Chem. Soc.*, 1951, **73**, 4691.
[2] J. H. Dyer, "Methods of Biochemical Analysis," ed. D. Glick, vol. 3, p. 111, Interscience, New York, 1956.
[3] L. Hough, B. M. Woods, and M. B. Perry, *Chem. and Ind.*, 1957, 1100.
[4] J. F. O'Dea and R. A. Gibbons, *Biochem. J.*, 1953, **55**, 580.
[5] L. Hough, D. B. Powell, and B. M. Woods, *J.*, 1956, 4799.

solution. Therefore, a very large excess (at least 20-fold) of borohydride was used, and the reduction was allowed to proceed for at least 14 hr. at room temperature.

In the initial experiments, maltose (30—80 μg.) was reduced with a large excess of borohydride and the excess was decomposed with a 1·0M-acetic acid–acetate buffer of pH 4·5; oxidations were then carried out in a hydrogen carbonate medium (pH *ca.* 8) with various (50—600%) excesses of sodium metaperiodate at 5 and 22°. Oxidation was very slow at 5°; at 22° the results were very indefinite, and plots of the periodate consumption against time showed that the expected rapid oxidation of the terminal glycitol group was overshadowed by extensive over-oxidation of the whole molecule. Periodate oxidation of cellobi-itol (maltitol was not available) without the borohydride treatment showed similar indefinite oxidation patterns at pH 8.

When these tests were repeated with oxidation at pH 5, the terminal glycitol group of cellobi-itol was completely oxidised in 15—20 min. with a 100% excess of 0·025M-periodate and subsequent over-oxidation was slow. Likewise, borohydride-reduced maltose showed the expected oxidation pattern at pH 5, with reasonably rapid selective oxidation of the terminal glycitol group, followed by slow oxidation of the ring and over-oxidation. In order to obtain a pH value of *ca.* 5, it was necessary to decompose the excess of borohydride with 1M-acetic acid; when the acetate buffer solution was used, the pH of the acetate–borate mixture was about 7.



Rates of periodate oxidation of oligosaccharides after reduction with borohydride (100% excess of periodate at pH 5)

A. Maltose. D. Laminaribiose.
B. Lactose. E. β-D-Melibiose dihydrate.
C. Di-*N*-acetylchitobiose.

Determination of Formaldehyde.—With the complicated mixtures arising from the borohydride–periodate procedures, it would be very difficult to obtain reliable figures for the amount of formic or other acid formed in the oxidation of reduced oligosaccharides, without prior separation of the reduced product. Accordingly, attention was confined to the determination of formaldehyde.

The chromotropic acid method described previously [6] had to be modified, because the large amounts of borate produced by decomposition of borohydride on acidification interfered seriously; this is to be expected, since chromotropic acid can be used for the determination of boron.[7] The interference was overcome by the addition of sodium fluoride to form the fluoroborate complex, but the sensitivity of the method was then decreased, and it was necessary to control the amount of fluoride added, to take a larger aliquot than previously [6] for the colour development, and to adjust the reagent concentrations appropriately. The calibration graph for formaldehyde was prepared from erythritol, because the production of formaldehyde was known [6] to be unaffected by pH.

When the recommended method was applied to reduced maltose after the initial rapid periodate oxidation, 2·10 mol. of formaldehyde were found, which agrees well with the expected figure of 2·00.

Application of the Method.—Various disaccharides were analysed by preparing oxidation-rate curves for the products obtained by reduction with borohydride and then

[6] R. Belcher, G. Dryhurst, and A. M. G. Macdonald, *J.*, 1965, 3964.
[7] D. F. Kuemmel and M. G. Mellon, *Analyt. Chem.*, 1957, **29**, 378.

determining the formaldehyde produced at the point of change of direction of the curve, which corresponds to the completion of the rapid oxidation of the terminal glycitol groups. Typical curves are shown in the Figure and results for periodate consumption and formaldehyde production are shown in the Table. Of the compounds studied, only laminaribiose did not react in accordance with theory. However, it has been shown [8] on the macro-scale that the oxidation of laminaribi-itol is anomalous.

The total sample requirement for a six-point rate curve and a formaldehyde determination was 300—400 μg.

Oxidation of borohydride-reduced oligosaccharides

Compound reduced	Mol. periodate consumed		Mol. HCHO produced	
	Found	Calc.	Found	Calc.
Maltose	2·50	3·00	2·10 ± 0·02	2·00
Lactose	2·98	3·00	2·04 ± 0·02	2·00
Di-*N*-acetylchitobiose	1·00	1·00	1·05 ± 0·03	1·00
Laminaribiose	2·07	3·00	1·13 ± 0·04	2·00
β-D-Melibiosedihydrate	4·05	4·00	1·10 ± 0·03	1·00

Experimental

Apparatus and Reagents.—These were the same as described previously [6] with the following exceptions.

Sodium borohydride solution. A freshly prepared aqueous 5% solution of laboratory-reagent grade material was used.

Chromotropic acid solution. Chromotropic acid (1·50 g.) was dissolved in distilled water (100 ml.) and the solution was filtered. A cooled mixture of water (125 ml.) and concentrated sulphuric acid (325 ml.) was added, and the solution was diluted to 500 ml. with concentrated sulphuric acid. It was stored in a dark bottle and prepared afresh every 10 days.

Procedures.—Determination of the periodate consumption of reduced oligosaccharides. A series of 5—6 samples weighing 50—100 μg., was introduced into separate clean dry reaction vessels [6] and dissolved in *ca.* 0·1 ml. of water by careful swirling. Then 6 drops (0·12—0·15 ml.) of freshly prepared 5% sodium borohydride solution were added. The contents of the tubes were thoroughly mixed, the tubes stoppered and the mixture stored for about 14—18 hr. in a cool dark place.

After this time, the excess of borohydride was destroyed by careful dropwise addition of 1M-acetic acid. When all effervescence had ceased, a further 3 drops (0·06—0·07 ml.) of acetic acid were added, so that the pH of the final solution was about 5.

The wall of the vessel was washed down with 0·2—0·4 ml. of water, and the solution was allowed to stand with occasional swirling for several minutes to ensure complete decomposition of the borohydride.

Sufficient sodium periodate solution (usually 60—100 μl. of 0·025M-solution) was then added from an Agla burette so that there was an excess of *ca.* 100% over the expected consumption of the acyclic glycitol end-group. The samples were allowed to react for times usually ranging from 7 min. to 2 hr.

The excess of periodate was then titrated with a suitable standard arsenite solution in hydrogen carbonate medium.[6]

Blanks were carried out in exactly the same way, except that no sample was used.

The results were plotted graphically and the amount of periodate rapidly consumed by the glycitol end-group was obtained by extrapolation of the rate curve obtained.

Determination of the formaldehyde evolved. The borohydride reduction and periodate oxidation procedures were exactly as described above, except that the reaction vessel was a 5·0-ml. volumetric flask; the appropriate time for the periodate oxidation was obtained by reference to the rate-curve mentioned above, at the point when the rapid glycitol end-group oxidation was complete.

When the oxidation was complete, 2·0 ml. of 0·05N-arsenite solution were added to the flask from a pipette and the solution was thoroughly shaken and allowed to stand for a further 15 min. The solution was then diluted to the mark with a saturated aqueous solution of sodium fluoride, and the contents of the flask were thoroughly mixed again. A 2·0-ml. aliquot

[8] M. J. Clancy and W. J. Whelan, *Chem. and Ind.*, 1959, 673.

of this solution was transferred by pipette into a flask of about 25-ml. capacity, followed by 10·0 ml. of chromotropic acid reagent. The resulting solution was thoroughly mixed and the colour developed and measured as described previously.[6]

A calibration graph was prepared with samples of erythritol yielding 5—30 μg. of formaldehyde and taken through the entire procedure, with the exception of the standing period with borohydride. An oxidation time of 15 min. with periodate was used.

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