438. *Determination of the Degree of Polymerisation of Reducing Oligosaccharides.*

By Stanley Peat, W. J. Whelan, and J. G. Roberts.

A microchemical method has been developed for the determination of the degree of polymerisation of reducing oligosaccharides. The technique involves reduction with sodium borohydride and the use of the anthrone reagent.

In our studies of polysaccharide structure by the method of linkage analysis [1] and of the products of enzymic breakdown of polysaccharides [2,3,4] we have frequently isolated hitherto unknown oligosaccharides, a first essential in the examination of which is the measurement of molecular weight. The yield in which these substances are obtained often means that, at most, only a few milligrams are available and attention was therefore directed to microchemical methods. Such a method has been developed and is applicable to certain classes of reducing oligosaccharides. Non-reducing oligosaccharides present special problems which are not considered here.

All known reducing oligosaccharides are so constituted that they contain only one free reducing group per molecule and a stoicheiometric determination of this group provides a measure of molecular weight. If the monosaccharide components of an oligosaccharide are known, then the molecular weight may be conveniently expressed as the degree of polymerisation (DP), *i.e.*, the number of monosaccharide units per molecule. A review of existing methods of assay of reducing-end groups revealed many unsatisfactory features. A widely used method is based on the oxidation, by alkaline hypoiodite, of the potential aldehydic group to carboxyl.[5] This reagent gives satisfactory results with glucose and maltose but overconsumption of iodine becomes progressively more marked as the degree of polymerisation of the oligosaccharides increases.[6] Satisfactory results for maltodextrins were obtained with the Somogyi copper reagent [6,7] but it was clear that this method could not be generally applicable unless reference compounds of similar structure to the unknown were available. The same is probably true of alkaline ferricyanide oxidation which has been applied to maltodextrins.[8] K. H. Meyer's 3 : 5-dinitrosalicylate reagent [9] was at first claimed to have the properties sought, but later work [10] revealed complicating features. After preliminary trials of other possible methods, the following procedure was adopted, in which advantage is taken of the fact that sodium borohydride quantitatively converts the reducing group of a sugar into the corresponding alcohol group. The reducing power of the acid hydrolysate of the oligosaccharide is compared with that of the acid hydrolysate of the borohydride-reduced oligosaccharide. An oligosaccharide of which the degree of polymerisation is n will yield, when hydrolysed by acid, n reducing monosaccharide molecules before, and $(n - 1)$ molecules after, treatment with sodium borohydride; measurement of the reducing powers of the two acid hydrolysates therefore enables n to be calculated. It is to be noted that hydrolysis of the borohydride-reduced oligosaccharide also liberates one mol. of a non-reducing sugar alcohol.

In early experiments copper reagents [7,11] were employed to measure reducing power but the interference caused by the borate ion (from the borohydride) could not be eliminated We therefore examined the colorimetric anthrone–sulphuric acid reagent [12] which was found not to be affected by borate and has the additional advantage that the hydrolysis of the oligosaccharide and the colour development, which is the basis of the estimation,

[1] Peat, Whelan, and Edwards, *J.*, 1955, 355.
[2] Whelan and Roberts, *Nature*, 1952, **170**, 748.
[3] *Idem*, *J.*, 1953, 1298.
[4] Whelan and Bines, *Biochem. J.*, 1955, **61**, i.
[5] " Polarimetry, Saccharimetry and the Sugars," Nat. Bur. Stand., Circular C440, Washington, 1942, p. 208.
[6] Whelan, Bailey, and Roberts, *J.*, 1953, 1293.
[7] Somogyi, *J. Biol. Chem.*, 1945, **160**, 61.
[8] Nussenbaum and Hassid, *Analyt. Chem.*, 1952, **24**, 501.
[9] Meyer, Noelting, and Bernfeld, *Helv. Chim. Acta*, 1948, **31**, 103.
[10] Meyer, Wyk, and Feng, *ibid.*, 1954, **37**, 1619.
[11] Shaffer and Hartman, *J. Biol. Chem.*, 1921, **45**, 377.
[12] Dreywood, *Ind. Eng. Chem. Analyt.*, 1946, **18**, 499.

are accomplished simultaneously by the hot acid reagent. The method is not suitable for pentose oligosaccharides, since the colours developed by pentoses with the anthrone reagent are too transient. For hexose oligosaccharides however, anthrone is to be preferred to copper reagents, inasmuch as the former gives the same intensity of colour with different hexoses, *e.g.*, glucose and fructose, mannose and galactose.[13] Consequently oligosaccharides constituted of two different monosaccharides may be assayed by this method whereas copper reagents could not be employed because of the widely varying copper-reducing powers of different hexoses.[14]

Some results obtained with polymers of glucose and glucose–fructose are shown in the Table, in which oligosaccharides with degrees of polymerisation ranging from 2 to 5 have been studied. An obvious limitation to the examination of larger molecules is the fact that as the degree of polymerisation increases the difference between the reducing powers of the hydrolysate of the reduced and the unreduced oligosaccharide decreases. The reproducibility of the analysis is such that 7 probably represents the upper limit for the degree of polymerisation.

Quadruplicate estimations of anthrone-reducing powers were made in the present investigations but duplicate estimations would probably be sufficient for the smaller molecules. For example, the average degree of polymerisation found for maltose was 2·0, permutation of eight individual readings showing a variation in estimation of degree of polymerisation from 1·9 to 2·1. For the pentasaccharide (Found : average DP 5·0) the variation was from 4·3 to 5·7. Unexpectedly, maltulose required several days for complete reduction in contrast to the oligosaccharides (including turanose) listed in the Table which

Degree of polymerisation of some reducing oligosaccharides.

Oligosaccharide	Symbol	Reducing power quotient (Q) *	Degree of polymerisation $[Q/(Q - 1)]$
Cellobiose	○$^{\beta}_{4}$●	2·02	2·0
O-β-D-Fructofuranosyl-(2→6)-D-glucose	□$^{\beta}_{6}$●	2·02	2·0
Gentiobiose	○$^{\beta}_{6}$●	2·00	2·0
Laminaribiose	○$^{\beta}_{3}$●	1·94	2·1
Maltose	○$^{\alpha}_{4}$●	1·98	2·0
*iso*Maltose	○$^{\alpha}_{6}$●	2·00	2·0
Turanose	○$^{\alpha}_{3}$■	1·88	2·1
Maltotriose	○$^{\alpha}_{4}$○$^{\alpha}_{4}$●	1·49	3·0
Panose	○$^{\alpha}_{6}$○$^{\alpha}_{4}$●	1·49	3·0
Maltotetraose	○$^{\alpha}_{4}$○$^{\alpha}_{4}$○$^{\alpha}_{4}$●	1·33	4·0
Pentasaccharide	○$^{\alpha}_{4}$○$^{\alpha}_{6}$○$^{\alpha}_{4}$○$^{\alpha}_{4}$●	1·25	5·0

○ = D-glucopyranose; ● = free reducing glucose unit; □, ■ = corresponding symbols for D-fructose.

* Quotient of reducing powers developed by the unreduced and reduced oligosaccharides.

were treated with borohydride for one hour only. No evidence appeared in any of these experiments to suggest that borohydride causes the reductive scission of glycosidic linkages, as claimed by Hough, Jones, and Richards.[15] This claim has been investigated and has not been confirmed.[16]

Experimental

Sugars.—Glucose, maltose, and cellobiose were commercial specimens. Turanose was prepared from melezitose,[17] gentiobiose from glucose,[18] the pentasaccharide by α-amylolysis of waxy-maize amylopectin, and *O*-β-D-fructofuranosyl-(2→6)-D-glucose by the action of yeast invertase on a mixture of methyl β-fructofuranoside and glucose.[19] Panose was the gift of

[13] Yemm and Willis, *Biochem. J.*, 1954, **57**, 508.
[14] Ref. 5, p. 190.
[15] Hough, Jones, and Richards, *Chem. and Ind.*, 1953, 1064.
[16] Whelan and Morgan, *Chem. and Ind.*, 1955, 1449.
[17] Hudson and Pacsu, *J. Amer. Chem. Soc.*, 1930, **52**, 2519.
[18] Peat, Whelan, and Hinson, *Nature*, 1952, **170**, 1056.
[19] Whelan and Jones, *Biochem. J.*, 1953, **54**, xxxiv.

Dr. S. C. Pan. Laminaribiose, *iso*maltose, and malto-triose and -tetraose were prepared by partly hydrolysing laminarin, dextran, and amylose, respectively, and fractionating the mixtures on charcoal–Celite.[6]

Anthrone and Sodium Borohydride Reagents.—Anthrone (1 g.) was dissolved in " AnalaR " sulphuric acid (*d* 1·840) to give 500 ml. of solution which was stored at 0° and renewed after 2 days.

The sodium borohydride solution was 2·5% (w/v) and was freshly prepared. In order that the unreduced sample of sugar solution should contain the same concentration of borate ion as that subjected to reduction there was added sodium borohydride (2·5%, w/v) inactivated by dissolution in N-sulphuric acid.

Standardisation of the Anthrone Reagent.—An approx. 0·1% solution of glucose is required. The concentration need not be known accurately since units of concentration are not involved in the ratio of colour intensities given by the reduced and the unreduced specimen of the test sugar.

To five E.E.L. colorimeter tubes (10 ml. capacity, Evans Electroselenium Ltd.) were added water (3 ml.) or glucose solution (0·5, 1·0, 1·5, and 2·0 ml.), the glucose solutions being diluted to 3 ml. with water. Inactivated borohydride solution (0·2 ml.) and 5N-sulphuric acid (0·8 ml.) were then added, the contents mixed, and the tubes dipped in cold water. Anthrone reagent (6 ml.) was added, slowly from a burette, the mixture being stirred with a glass rod; the addition occupied 0·5 min. Each tube was covered with a glass bulb, transferred to a boiling-water bath for 7·5 min., and cooled for 3 min. in water, and the optical density at 680 mμ (Ilford no. 608 filter) was measured in the E.E.L. colorimeter against water. Each standardisation was performed in duplicate and the curve relating mean optical density to glucose concentration plotted. The upper region of the curve always showed a slight departure from linearity. Under these conditions glucose and fructose give the same colour yield, but sorbitol gives no colour at an equivalent concentration.

Determination of Degree of Polymerisation.—Four samples of the test sugar (0·15—0·20 mg. in 3 ml. of solution) were treated with inactive sodium borohydride and freshly standardised anthrone reagent, as described for glucose. Four similar samples were each treated with active borohydride (0·2 ml.) for 1 hr. at room temperature. 5N-Sulphuric acid (0·8 ml.) was added, followed by anthrone reagent as before. The mean optical density of each batch of sugar was then converted into glucose equivalents by using the calibration curve, and the values so obtained were used to give the degree of polymerisation as described in the Discussion.

We thank the Brewing Industry Research Foundation for a maintenance grant (to J. G. R.).

UNIVERSITY COLLEGE OF NORTH WALES, BANGOR. [*Received, January 16th,* 1956.]