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Note

A ^{2}H-n.m.r. spectral method for determination of the degree of polymerization of reducing oligosaccharides

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A wide variety of chemical, colligative, and hydrodynamic properties has been used to determine the degree of polymerization (d.p.) and number-average molecular weights of oligosaccharides[1]. Of the chemical methods available, most relay on the characteristic reactivity of the terminal carbonyl group with oxidizing reagents. However, these analyses are often complicated by the additional reactivity of other functional groups in the oligosaccharide or by the primary structure of the oligosaccharide[2–11]. For these reasons, the estimation of the d.p. by these methods is best applied to structurally characterized, short polysaccharides having little or no branching.

The impetus for the present study was the desire to develop, for the estimation of the d.p. of polysaccharides, a chemical–spectroscopic method which is convenient to use and in which complicating side-reactions are minimized. It has been found that both of these criteria can be satisfied by reduction of the polysaccharide with NaB^2H_4, followed by hydrolysis, and estimation of the incorporated deuterium by using ^{2}H-n.m.r. spectroscopy. Furthermore, the short relaxation-time of the ^{2}H nucleus facilitates the acquisition of spectra in reasonably short periods of time, giving the method an advantageous sensitivity compared to that of other existing n.m.r. methods.

EXPERIMENTAL

Materials. — NaB^2H_4 came from MSD Isotopes (St. Louis, MO); 2,4-pentanedione, from Eastman (Rochester, NY); Dowex-50 (H^+) ion-exchange resin (200 mesh), deuterium-depleted water, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose, from Sigma Chemical Co. (St. Louis, MO); and amylopectin, from Nutritional Biochemicals Corp. (Cleveland, OH). All other reagents were of analytical grade.

Amylopectin hydrolysis. — Partial hydrolysis of amylopectin with acid was

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carried out on a 0.5-g sample according to the procedure of Whelan[7]. As hydrolysis proceeded, 25-mL samples (60 mg) were withdrawn, cooled, and made neutral with 2 mL of NaOH. The mixture (5 mL) was diluted two-fold, and set aside to await determination of d.p. by periodate oxidation[12].

Determination of d.p. by the ^{2}H-n.m.r.method. — Samples prepared from fractions of the amylopectin hydrolyzate and from D-gluco-oligosaccharides were treated similarly. Typically, 50 mg of solid NaB^2H_4 was added to 40–50 mg of an oligosaccharide in 20 mL of water, the solution incubated for 30 min at 70°, and then treated with an excess of Dowex-50 resin, the resin removed by filtration, and the filtrate made 0.2M in HCl, incubated for 12 h at 70°, and evaporated. The boric acid was removed by repeatedly dissolving the residue in methanol and evaporating under diminished pressure. The residue was dissolved either in distilled or deuterium-depleted water, and the relative concentration of D-(1,1-$^2H_2$)glucitol arising from terminal D-glucose residues was determined by digital integration of the ^{2}H resonance in the ^{2}H-n.m.r. spectrum. Total glucose was estimated in the same manner, following a second reduction with NaB^2H_4. The d.p. of the sample was calculated from the ratios of peak intensities before and after the final reduction with NaB^2H_4.

Determination of d.p. by sodium periodate oxidation. — The d.p. values of the hydrolyzed starch fractions were determined by the rate of periodate oxidation, as described by Whelan[7]. Attempts to measure the concentration of formaldehyde by using chromotropic acid[7] or AHMT[13] proved unreliable, due to the absence of color-complex formation (in the chromotropic acid assay) or the low rate of colored complex formation. The most convenient and reliable method was found to be the Nash reagent assay[14].

N.m.r.-spectral conditions. — ^{2}H-N.m.r. spectra were recorded without field lock, at 30 MHz (4.7 T) with a JEOL-FX200 instrument. Spectra were collected in 2048 data point blocks, using a 90° observation pulse and a sweepwidth of 5 kHz (total acquisition time, 204 ms).

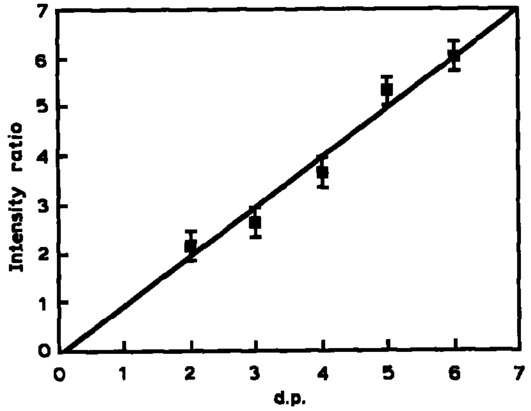

Fig. 1. The ratio of ^{2}H signal intensities of NaB^2H_4-reduced D-gluco-oligosaccharides, before and following complete hydrolysis with acid *vs.* the actual d.p. of these standard samples. [Errors in the calculated values were estimated from the reproducibility of integrated peak intensities.]

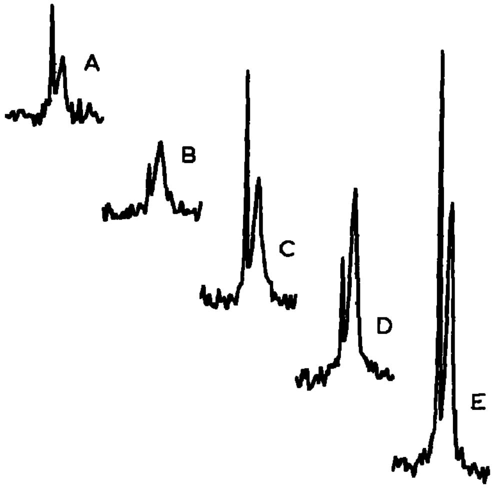

Fig. 2. ^{2}H-N.m.r. spectra of D-glucose–D-[1,1-$^2H_2$]glucitol mixtures prepared from partially hydrolyzed and NaB$^2H_4$-reduced starch fractions. [Spectra of samples prepared from starch are an average of 10,000 scans (total acquisition time ~30 min). The total hydrolysis time prior to reduction was A, 30 min; B, 1 h; C, 2 h; D, 3 h; and E, 4 h.]

RESULTS

Fig. 1 shows the actual d.p. of standard D-gluco-oligosaccharides plotted against the d.p. calculated by using the ^{2}H-n.m.r. method. D.p. values were calculated by taking the ratio of ^{2}H signal intensities of the NaB$^2H_4$-reduced sample before and following complete hydrolysis with acid. A linear least-squares fit to the data yielded a line of unit slope (1.05 ±0.08), with a standard deviation in the calculated d.p. values of 0.36.

Fig. 2 shows ^{2}H-n.m.r. spectra of D-glucose–D-[1,1-$^2H_2$]glucitol mixtures prepared from partially hydrolyzed and NaB$^2H_4$-reduced starch fractions. The narrower signal lying farthest downfield in each of the spectra arises from the small amount of deuterium in the solvent. The variation in intensities of the solvent peak results from the limited number of data points defining the signal. In contrast, the signal arising from the alditol is adequately defined, owing to its greater linewidth. The intensity increase in the C_2H_2OH resonance with hydrolysis time reflects the greater number of terminal residues. The expected first-order behavior of the acid-catalyzed hydrolysis of the starch sample[15,16] is illustrated in Fig. 3, where the log of the d.p. is plotted as a function of hydrolysis time. The error in each data point was estimated from the signal-to-noise ratios of the ^{2}H-n.m.r. signals. Hence, the error is largest for short hydrolysis times, where the signal-to-noise ratio is relatively low. For comparison, Fig. 3 shows similar data obtained by using the periodate oxidation method[7], in which the d.p. of the unknown is calculated by comparing the rate of formation of formaldehyde resulting from oxidation to the

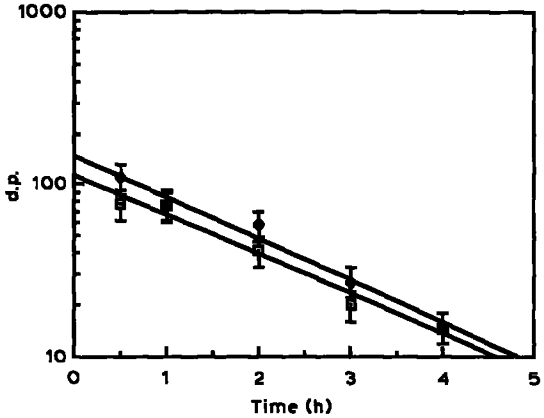

Fig. 3. Calculated d.p. as a function of starch hydrolysis time. [The d.p. was determined by the periodate oxidation method (◆) and the ^{2}H-n.m.r. method (□). Lines in the Figure represent linear, least-squares fits to the ^{2}H-n.m.r. data and the periodate oxidation data.]

oxidation rate of a known standard tetrasaccharide. The absolute error involved in the determination of the concentration of formaldehyde results in considerable error in the rates determined by a linear least-squares fit to the data, with the relative error larger for lower absolute oxidation rates. These errors are reflected in the large errors for hydrolysis times of <2 h.

With the exception of the data points of 2 h, the d.p. values calculated by using the ^{2}H-n.m.r. method agree, within experimental error, with those calculated by using the periodate oxidation method. Extrapolation to zero time, using a linear least-squares fit of the ^{2}H-n.m.r. data, yielded 112.7 ±9.0 for the d.p. of the unhydrolyzed starch, whereas a d.p. of 146 ±14.7 is found by using the periodate oxidation data.

DISCUSSION

From the data presented in Fig. 3, it appears that the relative errors in d.p. measurements using the ^{2}H-n.m.r. method are only slightly smaller than those found by using the periodate oxidation method. However, although the accuracy of the ^{2}H-n.m.r. method can be improved by obtaining spectra having greater signal-to-noise ratios than those shown in Fig. 2, namely, by averaging a greater number of spectral acquisitions, the accuracy of the periodate oxidation method is limited by the absolute error in the determination of formaldehyde concentration and the total number of data points. Because the relaxation time of ^{2}H nuclei in these samples is quite short (<10 ms), the signal-to-noise ratios obtained per unit of spectral-acquisition time are governed by the spectral resolution desired. In practice, resolution of the sample from the solvent signal required a minimum of ~100 ms per scan (about half the time used in acquiring the spectra of Fig. 2).

Of the methods at present available for determining the d.p. of oligosaccharides, oxidative methods involving oxidation by periodate, hypoiodate, ferric ion, and cupric ion are nonstoichiometric, the total amount and the rate of oxidation varying as a function of the primary structure of the oligosaccharide[1–8]. As a result, these methods can only be used if a nominal structure of the unknown has been determined and an appropriate standard can be found. For undetermined structures, the two remaining stoichiometric methods[10,11], which are specific for either reducing aldoses or nonreducing terminal residues, appear to be the methods of choice. Although the ^{2}H-n.m.r.-spectral method is probably slightly less sensitive than the other methods in this group, it has the advantage of being faster and involving fewer experimental manipulations. In addition, the very short relaxation time of the ^{2}H nucleus makes the method more sensitive than either ^{1}H- or ^{13}C-n.m.r. spectroscopy if one considers the signal-to-noise ratios obtained per total time spent in acquiring spectra.

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