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QUANTIFICATION OF THE STRUCTURAL FEATURES OF STARCH POLYSACCHARIDES BY N.M.R. SPECTROSCOPY

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ABSTRACT

N.m.r. spectra (^1H and ^{13}C) of starch-derived polysaccharides contain several resolvable resonances due to minor structural features, which can be used for quantitative analysis. Thus, the degree of branching of amylopectins and native and degraded starches can be non-destructively determined and the reducing residues of degraded starches can be quantified. ^{13}C-N.m.r. spectroscopy can also distinguish between glucose and the reducing residues of higher glucans.

INTRODUCTION

Starch polysaccharides (and glycogen) are (1→4)-α-D-glucans with branch points involving α-(1→6) linkages[1]. The extent of this branching, which occurs largely in the amylopectin component, varies with the botanical origin and may be important in determining the type of granular crystallinity[2]. The degrees of branching or unit chain-lengths of amylopectins and glycogens from various sources have been determined using combined enzymic and chemical methods[2,3]

N.m.r. spectroscopy can be used for the quantification of such minor structural features provided that the appropriate signals are resolved. Thus, for amylopectins and glycogens, integration of the signals from either branch points or non-reducing terminal units (one for each branch point[1]) could be used to obtain the degree of branching. ^1H-N.m.r. spectroscopy can distinguish between anomeric protons involved in α-(1→4) and α-(1→6) linkages and has been used to estimate the degree of branching of glycogens and amylopectin beta-limit dextrins[4,5] but not of intact amylopectins and starches. Published ^{13}C-n.m.r. spectra[5–7] of intact and degraded amylopectins and glycogens show minor peaks which have been assigned[7] to terminal non-reducing units, but quantification has not been attempted. We now describe experimental conditions for determining the degrees of branching of starches and amylopectins using ^1H- and ^{13}C-n.m.r. spectroscopy.

An additional structural feature which is produced during the degradation of starch (by enzymes or acid) is the terminal reducing unit, usually determined by colorimetric assay[8,9]. We now show that ^1H- and ^{13}C-n.m.r. spectroscopy can give

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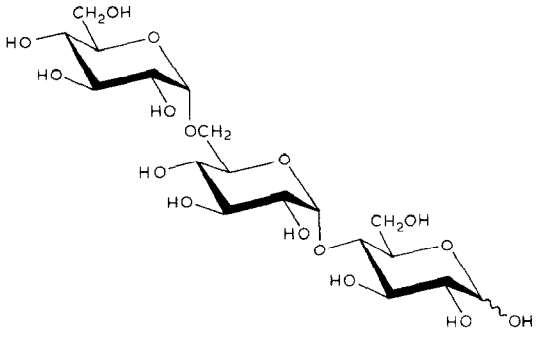
the same information simultaneously with quantification of the degree of branching.

RESULTS

^{1}H-N.m.r. spectroscopy. — Panose (**1**) is a model compound for the minor structural features of starch, as it contains terminal reducing and non-reducing glucose residues and α-(1→6) and α-(1→4) linkages. The low-field ^{1}H-n.m.r. spectrum of **1** comprises (Fig. 1a) four doublets assigned[4] to H-1 of the α and β forms of the reducing unit (5.27 and 4.68 p.p.m., respectively), the central unit (5.40 p.p.m.), and the (1→6)-α-linked terminal unit (5.00 p.p.m.). In the spectrum of maltoheptaose (Fig. 1b), all the anomeric protons except those from reducing units contribute to the doublet at 5.4 p.p.m. and hence the number of terminal reducing residues can be obtained by integration. Only one of the H-1 doublets due to the reducing unit need be integrated since the anomeric equilibrium ($\alpha\beta$-ratio 43:57) for (1→4)-α-D-glucans is independent of chain length. Fig. 1c shows a typical partial spectrum of a native starch (waxy maize). Although the multiplet structures are lost on going from the low-molecular-weight model compounds (Figs. 1a and 1b) to starch, the resonances due to the main chain and branch points are clearly resolved. Partial degradation of starches produces terminal reducing units without destroying all the branch points; signals from both these features are therefore observed in the ^{1}H-n.m.r. spectrum (Fig. 1d).



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In order to quantify these structural features, the repetition time of spectral acquisition should be greater than 5 times the longest T_1 of the relevant signals. The T_1 values for a number of samples are shown in Table I. Inspection of the data in Table I shows that, at 90°, repetition times of >5 s are necessary for intact starches and amylopectins, whereas degraded starches require longer repetition times (at least 10 s). Lowering the sample temperature would reduce the T_1 values and hence the necessary repetition times for quantitative analysis. However, there are advantages in operating at high temperatures, as peaks are sharper and there-

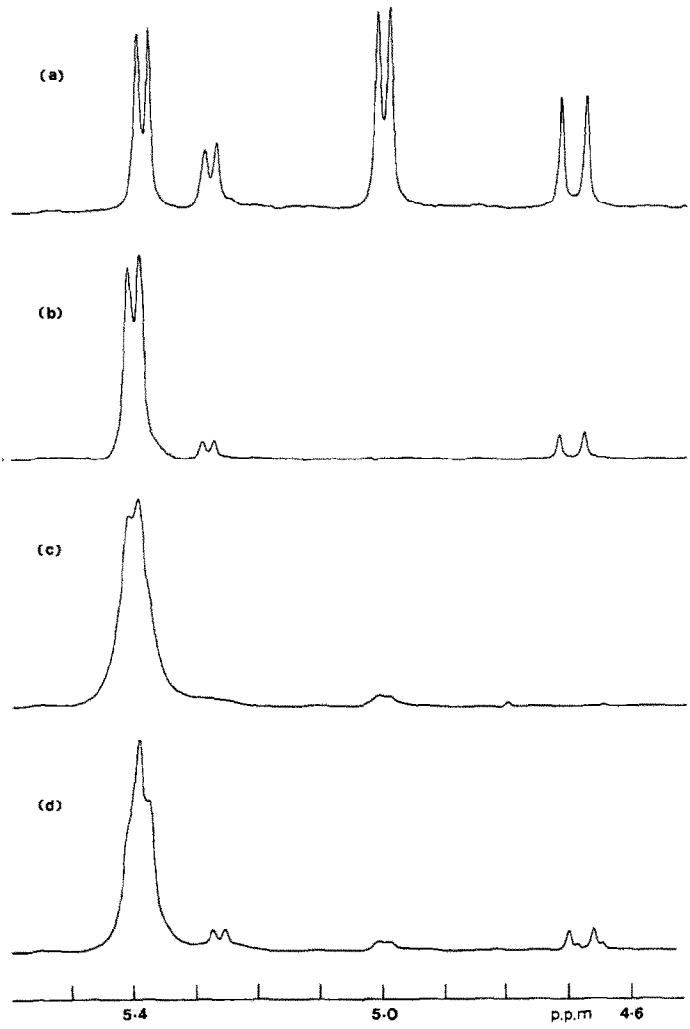

Fig. 1. Partial ^{1}H-n.m.r. spectra (D_2O, 90°) of (*a*) panose, (*b*) maltoheptaose, (*c*) waxy-maize starch, and (*d*) degraded starch MD 02 (ex Alwitt). Peaks at 5.40, 5.00, 5.27, and 4.68 p.p.m. are assigned to H-1 of (1→4)- and (1→6)-α-linked units, and the α and β forms of reducing units, respectively.

fore better separated, and the residual solvent (HOD) signal is shifted to higher field and is less likely to interfere with the signals of interest.

Quantitative results obtained using ^{1}H-n.m.r. spectroscopy are shown in Table II and compared, where possible, with values determined by other methods. The main source of error in the n.m.r. method is in the integration, but a comparison of different methods coupled with the use of repeat samples suggests that errors in determinations of the proportions of branch points or reducing residues are $<\pm 5\%$, *i.e.*, comparable in accuracy to those obtainable using enzymic/chemical procedures[2].

TABLE I

T_1 VALUES (s) FOR H-1 SIGNALS OF STARCH POLYSACCHARIDES AND MODEL COMPOUNDS AS DETERMINED USING THE INVERSION-RECOVERY METHOD AT 90° AND 2% CONCENTRATION

	Main chain	*Branch point*	*Reducing end-group*	
	(5.40 p.p.m.)	*(5.00 p.p.m.)*	*(5.27 p.p.m.)*	*(4.68 p.p.m.)*
Waxy-maize starch	0.70	0 82	—	—
Degraded starch SA6 (ex Avebe)	0.80	0.52	2.0	2.2
Maltoheptaose	0.75	—	2.2	2.1
Panose	1.5	2.0	3.6	2.2

TABLE II

QUANTIFICATION OF MINOR STRUCTURAL FEATURES OF STARCH POLYSACCHARIDES BY ^{1}H-N M.R SPECTROSCOPY[a]

	Ratio of α-(1→4) to α-(1→6) linkages	*Ratio of mid-chain residues to reducing termini*
Waxy-maize starch	20 (20)[2]	—
Potato amylopectin	23 (22–23)[2]	—
Tapioca amylopectin	17.5 (18–19)[2]	—
Mussel glycogen	10.0 (10)[3]	—
Degraded starch SA6	23	21
Degraded starch MD 01	20	25
Degraded starch MD 02	26	8.0

[a]Errors are estimated to be $<\pm 5\%$. Previously determined values are shown in parentheses.

^{13}C-N.m.r. spectroscopy. — Characteristic signals attributable to the minor structural features of wheat amylopectins have been described[7]. The spectrum of waxy-maize starch (Fig. 2a) is very similar to that of wheat amylopectin[7]. The most useful minor resonance for purposes of quantification is that at 70.1 p.p.m. due to C-4 of non-reducing terminal units[7]. Another possible diagnostic signal is that (67–68 p.p.m.) for C-6 involved in α-(1→6) linkages[5,7,10]. A broad resonance at this position has been observed for glycogens[7] and Fig. 2a shows a similarly broad signal for waxy-maize starch. The observed breadth is presumably[7] due to the restricted motion of the trisubstituted branching residue within the polymer. A second broad signal at 71 p.p.m. in Fig. 2a is probably[7] due to C-5 of branching residues.

The ^{13}C-n.m.r. spectrum (Fig. 2b) of panose contains the expected resonance (66.7 p.p.m.) for C-6 involved in a glycosidic linkage. The spectrum was assigned by comparison with the observed chemical shifts for maltotriose[11], our assignments agreeing with previous assignments for the central[12] and terminal[6] units. Several signals due to the terminal reducing residue are well separated in Fig. 2b, in particular all peaks between 74 and 97 p.p.m. Minor resonances in some of these positions are also clearly resolved in spectra of degraded starches (*e.g.*, Fig. 2c). As the

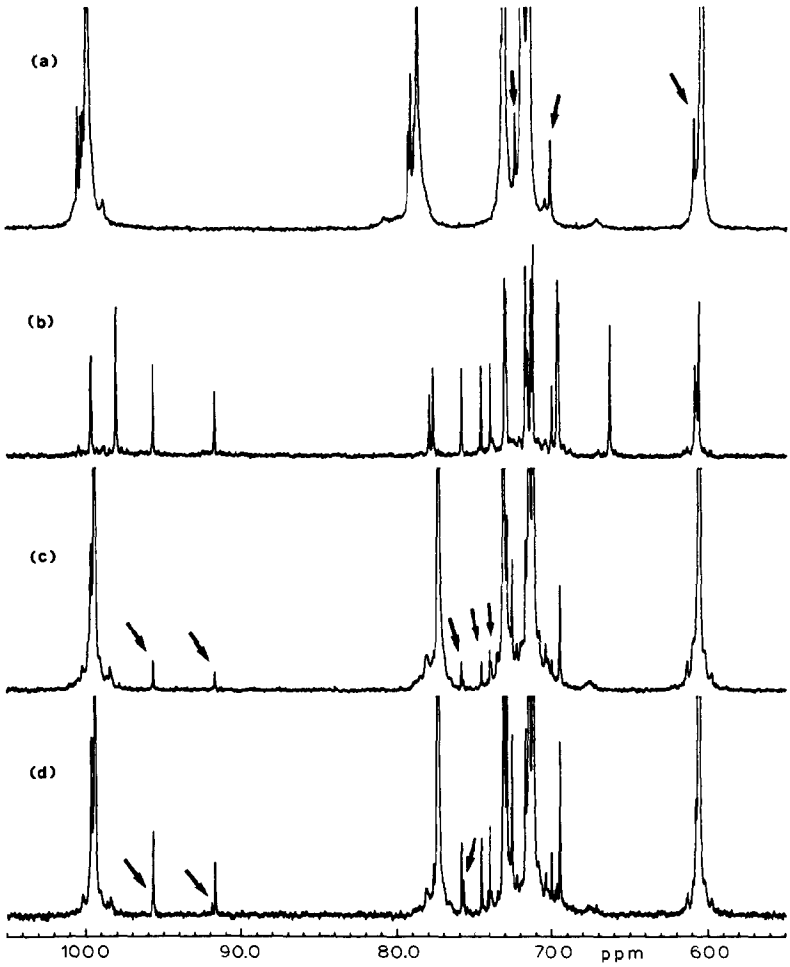

Fig. 2. ^{13}C-N.m.r. spectra (90°) of (*a*) waxy-maize starch in Me_2SO-d_6, (*b*) panose in D_2O, (*c*) degraded starch MD 01 (ex Alwitt) in D_2O, and (*d*) degraded starch MD 02 (ex Alwitt) in D_2O. Arrowed resonances in (*a*), (*c*), and (*d*) arise from non-reducing terminal groups, reducing terminal groups, and glucose, respectively.

characteristic non-reducing-terminal resonance at 70 p.p.m. is also clearly resolved, simultaneous quantification of both non-reducing and reducing terminal units is possible.

Extensively degraded starches contain significant amounts of glucose as well as higher gluco-oligosaccharides. Some of the ^{13}C signals for glucose show significant differences in chemical shift compared to the corresponding signals from the maltose reducing-unit[11], whereas the chemical shifts of reducing-unit resonances from maltose and maltotriose[11,13] are virtually identical to those of higher oligomers[13]. Fig. 2d shows the ^{13}C spectrum of an extensively degraded starch with several resolved glucose resonances. Thus, there are three pieces of quantitative

TABLE III

T_1 VALUES OF MAIN-CHAIN AND DIAGNOSTIC MINOR ^{13}C RESONANCES OF STARCH POLYSACCHARIDES AT 90°

	Main chain			*Non-reducing terminus*	*Reducing terminus*		*Glucose*		
	C-1	*C-2,3,4,5*	*C-6*	*C-4*	*C-1α*	*C-1β*	*C-3β/5β*	*C-1α*	*C-1β*
Waxy-maize starch (10% in Me_2SO-d_6)	0.19	0.1–0.3	0.12	0.25	—	—	—	—	—
Degraded starch MD 01 (10% in D_2O)	0.25	0.15–0.3	0.16	0.5	0.6	1.0	—	—	—
Degraded starch MD 02 (10% in D_2O)	0.26	0.2–0.35	0.19	0.45	0.6	0.95	2.7	3.1	2.4

TABLE IV

QUANTITATIVE ^{13}C-N.M.R ANALYSIS OF BRANCHED (1→4)-α-D-GLUCANS (ERRORS ±5%)[a]

	Ratio of mid-chain residues to non-reducing termini	*Ratio of mid-chain to reducing residues*	*Ratio of glucose to other reducing residues*
Waxy-maize starch	20.5 (20)	—	—
Degraded starch MD 01	11.8	24 (25)	0.1
Degraded starch MD 02	6.3	7.5 (8.0)	0.25
Mussel glycogen	10.5 (10.0)	—	—

[a]Figures in parentheses refer to values determined by ^{1}H-n.m.r. spectroscopy (Table II).

information available from such spectra, namely the proportions of reducing and non-reducing terminal units and the amount of glucose. It is interesting to note that, even in significantly degraded starches (Fig. 2d), the resonance (67–68 p.p.m.) of C-6 in the branching residue is very broad.

For quantitative analysis, T_1 values need to be determined and representative data are presented in Table III. Another possible source of error in quantitative ^{13}C-n.m.r. spectroscopy is differential n.O.e. This problem can be overcome, and quantitative spectral data obtained, by using the gated-decoupling pulse sequence[14]. Results obtained using this method and employing repetition times greater than 5 times the relevant T_1 values are shown in Table IV.

DISCUSSION

The results presented above show that (*a*) the degree of branching of intact starches and amylopectins can be obtained from ^{1}H- and ^{13}C-n.m.r. data, and (*b*) for degraded starches, the number of terminal reducing units can be determined using either ^{1}H- or ^{13}C-n.m.r. spectroscopy, the degree of branching can be derived from ^{1}H-n.m.r. data, and ^{13}C-n.m.r. data can be used to quantify both free glucose and non-reducing terminal units.

Where both ^{1}H- and ^{13}C-n.m.r. spectroscopy can be used to provide the same information, the former technique is preferred due to its higher sensitivity. For example, in order to quantify the branching in amylopectin, the ^{1}H-n.m.r. method typically requires 30-min acquisition time for a 2% sample, whereas a 10% sample requires an acquisition time of 8–24 h for quantitative ^{13}C-n.m.r. analysis.

^{1}H-N.m.r. spectroscopy appears to be the method of choice for determining the degree of branching of starch-derived polysaccharides, as it gives the desired information directly. Alternative methods[3,9] are considerably more complex and time-consuming, as they involve debranching with isoamylase followed by separate assays for reducing power and total carbohydrate content. Although the n.m.r. method gives no direct information on the distribution of branch points, a combination of partial enzymic degradation (using, for example, beta-amylase, phos-

phorylase, and/or debranching enzymes) with ^{1}H(and ^{13}C)-n.m.r. analysis should prove useful in studies of the fine structure of amylopectins and degraded starches.

The sample requirement (minimum 1 mg) for ^{1}H-n.m.r. spectroscopy is sufficiently low that analysis of chromatography fractions is possible. Thus, gel-permeation chromatography fractions could be used to monitor the variation in degrees of branching with molecular size for both native and degraded starches.

A further application of this technique may be in the determination of degrees of branching in amyloses. Previously[9], such determinations have relied on the separate colorimetric assay of small precentages of both reducing and non-reducing terminal units, whereas ^{1}H-n.m.r. spectroscopy could determine the extent of branching directly.

A major characteristic of degraded starches (maltodextrins) is the d.e. (dextrose equivalent) which is defined as the percentage of reducing residues in the polysaccharide. Quantitative analysis of reducing residues in degraded starches is achieved conventionally using separate assays for total carbohydrate and reducing power[8,9]. ^{1}H-N.m.r. spectroscopy provides an alternative, direct method of analysis with the added advantage that degrees of branching can be determined simultaneously.

EXPERIMENTAL

^{13}C-N.m.r. spectra were obtained at 90° using either a Bruker CXP 300 or WP 200 instrument, operating at 75.46 and 50.32 MHz, respectively, and ^{1}H-n.m.r. spectra were recorded at 200.13 MHz using a Bruker WP200 spectrometer at 90°. ^{13}C-N.m.r. spectra of intact amylopectins and starches were obtained using Me_2SO-d_6 as solvent. For all other experiments, D_2O was used and samples were exchanged with D_2O prior to analysis in order to reduce interference from the residual solvent resonance. This solvent-suppression method is preferred for quantitative analysis, as other techniques (*e.g.*, selective irradiation) may cause intensity distortions in the signals of interest. ^{1}H and ^{13}C chemical shifts are referenced to external Me_4Si (0 p.p.m.) and 1,4-dioxane (67.4 p.p.m.), respectively. Spin–lattice relaxation times (T_1) were determined using the inversion–recovery pulse sequence (180°–τ–90°–acquire). Comparative integration of signals was achieved by either excision and weighing, using the spectrometer integration routine, or by calculating the product of peak height and width at half height.

Tapioca amylopectin was prepared by Dr. P. Bulpin of this laboratory, using the method of Banks and Greenwood[15]. All other materials were commercial samples.

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