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STRUCTURAL ANALYSIS OF COMB-LIKE, AMYLOSE DERIVATIVES BY ^{13}C-N.M.R. SPECTROSCOPY

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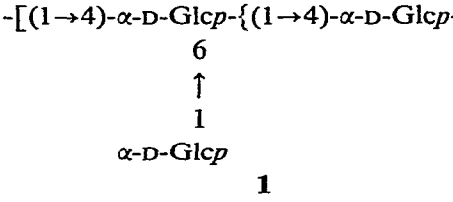
ABSTRACT

^{13}C-N.m.r. spectra have been recorded for previously reported, comb-like derivatives of amylose produced by orthoester and Helferich condensations of D-glucose to amylose. As known from monomeric studies, the Helferich condensation conditions (the presence of mercury salts) favor α-D-glucosylation, and orthoester condensation conditions favor β-D-glycosylation. It was anticipated that, for these polymer condensations, the Helferich and orthoester condensations would also favor α- or β-D-glycosylation, respectively. The ^{13}C-n.m.r. spectra of representative products of the Helferich and orthoester condensations confirmed the presence of 4,6-di-*O*-substituted α-D-glucopyranosyl residues, and also the degree of polymer linearity derived from independent, analytical data. However, these spectra indicate extensive, if not exclusive, β-D-glycosylation for both the Helferich and the orthoester conditions. These results were obtained by using the product from an enzymically synthesized, strictly linear amylose in the Helferich condensation reaction.

INTRODUCTION

Several series of comb-like D-glucans have been produced by chemical means[1–4]. Linear D-glucans, namely, amylose and cellulose, were chemically modified by the introduction of trityl and dicarbanilate groups. The modified D-glucans were then linked to monosaccharides *via* the Helferich[5], the Bredereck[6], and the orthoester[7] condensations. The protective groups could then be removed, yielding a branched analog of the starting D-glucan. For example, when amylose, essentially a linear

D-glucan containing α-D-(1→4)-linkages, was subjected to these conditions, single (1→6)-linked, D-glucopyranosyl groups were incorporated into the polymer. Due to the chemical nature of the side-group additions, a random distribution of branch-point positions can be expected along the amylose backbone chain. However, an average frequency of branching can be established that allows the representation of an average, repeating sub-unit (**1**), where *n* is the average number of α-D-glucopyranosyl residues between 4,6-di-*O*-substituted α-D-glucopyranosyl residues.



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For most glycoside syntheses, the *trans*-1,2 configuration of the product is favored when participating groups are present on C-2 (*e.g.*, in the Koenigs–Knorr reaction, the Bredereck reaction, and the orthoester method), and β-D-glycosylation is favored for tetra-*O*-acetyl-α-D-glucopyranosyl bromide or 1,2-orthoacetate-3,4,6-tri-*O*-acetyl-α-D-glucopyranose. The modification of the Koenigs–Knorr reaction by Helferich *et al.*[5,8] is of interest because, when employed with monomeric sugars, the less easily obtained α-D-glycosylic addition occurs, this being ascribed to the presence of mercury salts[9,10]. Therefore, these reaction conditions, when employed with linear D-glucans, could be expected to produce similar products differing only in the stereochemistry of the anomeric carbon atoms of the side-group D-glucopyranosyl groups.

Condensations of D-glucose with amylose have been performed under Helferich[1,2], Bredereck[1,2], and orthoester[3] conditions. Minor modifications of the reaction conditions (*e.g.*, molar ratios of reagents) resulted in products of differing degrees of linearity. It is fairly easy to establish the degree of branching of the products *via* the dicarbanilate (nitrogen) content of the resulting, protected intermediate[1]. Similarly, the type of branching [*e.g.*, (1→2)- or (1→3)-linkages] can be established by permethylation–fragmentation analysis of the branched D-glucan. However, the nature of the newly formed anomeric linkages is difficult to establish. Most techniques that distinguish between anomers (*e.g.*, specific rotation) yield only a single value that is a composite representing all anomeric linkages present. As the starting amylose was exclusively α-D-linked, all branched D-glucans resulting will contain large proportions of α-D-linked D-glucosyl residues. Therefore, for confidence in the assignment of the α or the β anomeric configuration to the newly formed branching-linkages, it is necessary clearly to distinguish properties associated with these new linkages from properties associated with the α-D-(1→4)-linkages of the D-glucosyl residues of the original, amylose backbone.

We have previously published the ^{13}C-n.m.r. spectra of a series of dextran-like D-glucans of bacterial origin[11,12]. A wide range of degrees of branching was observed for these D-glucans, as well as a wide variety of resonances arising from

α-D-(1→2)-, α-D-(1→3)-, α-D-(1→4)-, and α-D-(1→6)-linkages. These dextran spectra established the following points. High-temperature, spectral-acquisition conditions give improved resonance resolution. Various diagnostic, linkage-type resonances indicate the type of branching present, and the intensity of these diagnostic resonances provides a good estimate of the degree of branching of the D-glucan. In general, the spectrum of a polymer may be considered to be a composite of the individual spectra of the D-glucosyl residues present in the average, repeating sub-unit of that polymer, structure **1** being an example.

A convenient approach for ^{13}C-n.m.r.-spectral analysis of a D-glucan is to consider four regions independently; (*a*) the 60–70-p.p.m. region, which contains free and linked C-6 resonances, (*b*) the 70–75-p.p.m. region, which contains most of the free C-2, C-3, C-4, and C-5 resonances, (*c*) the 75–85-p.p.m. region, which contains the linked C-2, C-3, and C-4 resonances, and (*d*) the 95–107-p.p.m. region, which contains the C-1 resonances. Importantly for this report, most ^{13}C-n.m.r. resonances for each type of D-glucopyranosyl residue are dependent on the anomeric configuration of that residue. As could be expected, the C-1 resonances are the most highly dependent on the anomeric stereochemistry. It had previously been observed that C-1 resonances of glycopyranosides are dependent on the anomeric linkage-type, to the extent that, for recordings at ambient temperature, the α-D-linkages are in the 95–101-p.p.m. region, and the β-D-linkages, in the 103–105-p.p.m. region[13]. We have confirmed this effect for 90° spectra of α-D-glucopyranosyl residues, as all anomeric resonances that have thus far been recorded for dextrans lie[11,12] between 96.3 and 101.7 p.p.m. Also, the anomeric resonances of 3- and 6-*O*-substituted β-D-glucopyranosyl residues (ambient temperature, aqueous solution) have been found[14,15] to be, respectively, at 103.8 and 104.2 p.p.m. Therefore, ^{13}C-n.m.r. spectroscopy can identify the presence of α or β anomeric linkages in a D-glucan. The extent to which the absence of either α- or β-linkages can be established depends on the signal-to-noise ratio of the specific spectrum under consideration. For Fourier-transform conditions, this signal-to-noise ratio is a function of the number of acquisitions and of the sample size. In general, ^{13}C-n.m.r. contributions of glucose residues can be detected when these specific residues form at least five percent of the total number of residues in the average, repeating sub-unit.

RESULTS AND DISCUSSION

The ^{13}C-n.m.r. spectra of modified amylose dissolved in deuterium oxide, at 90°, were recorded (see Fig. 1 and Table I). These spectra were quite different from the spectra previously recorded for dextran-like D-glucans[11,12]. The ^{13}C-n.m.r. spectrum (ambient temperature, aqueous solution) of amylose previously recorded[14] contains the expected six resonances. Glycogen is similar to amylose in containing a large proportion of 4-*O*-substituted α-D-glucopyranosyl residues, but it has a much smaller proportion of 4,6-di-*O*-substituted α-D-glucopyranosyl residues. For increased resolution, and direct comparison with previous spectra[12], the ^{13}C-n.m.r. spectrum

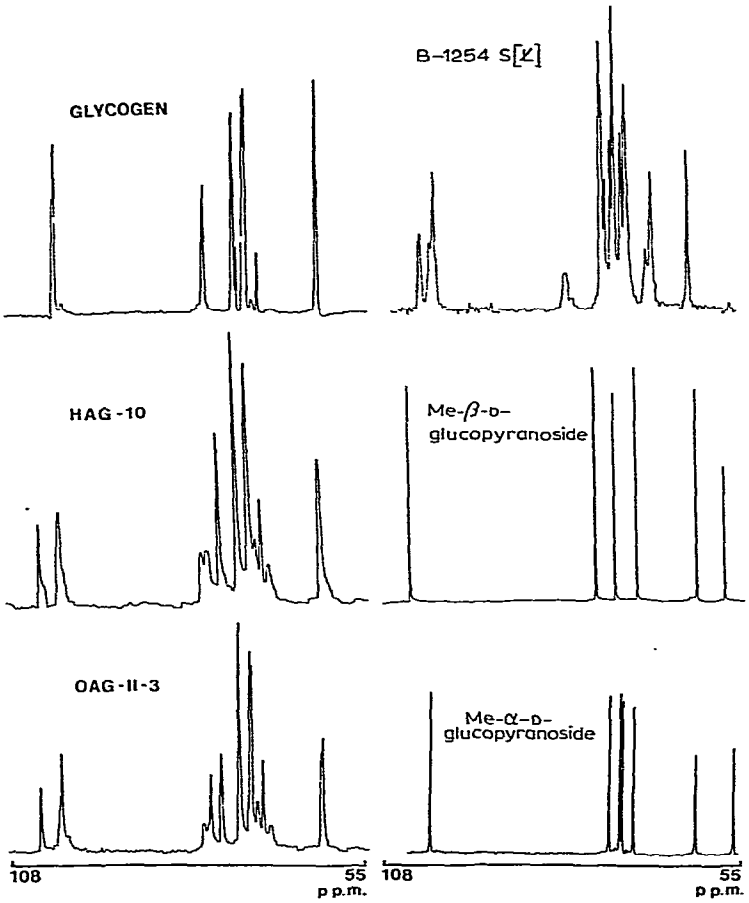

Fig. 1. ^{13}C-N.m.r. spectra at 90° for rabbit-liver glycogen; the comb-like, amylose products of the Helferich condensation (HAG-10), and the orthoester condensation (OAG-II-3); dextran B-1254 fraction S[L]; and the anomers of methyl D-glucopyranoside.

of rabbit-liver glycogen was recorded at 90°, and six major resonances (at 62.51, 73.41, 75.02, 79.37, and 101.43 p.p.m.) were observed (see Fig. 1 and Table I). These resonances, then, constitute the major chemical-shifts associated with the 4-*O*-substituted α-D-glucopyranosyl residue, and are in agreement with the amylose data[14] reported (ambient temperature). Previously, we recorded the ^{13}C-n.m.r. spectra of dextrans containing 4,6-di-*O*-substituted α-D-glucopyranosyl residues[11,12], and identified resonances at 68.5, 80.4, and 101.6 p.p.m. as being diagnostic of this residue. Comparative spectral data for dextran B-1254 fraction S[L] are given in Fig. 1 and Table I (see ref. 12 for a discussion of the structure and nomenclature of this dextran fraction). We have also recorded the ^{13}C-n.m.r. spectrum (90°) of methyl α-D-glucopyranoside, and have found that the 70–75-p.p.m. region contains resonances quite similar to those for linear dextran, which is composed of 6-*O*-substituted α-D-glucopyranosyl residues. In addition, highly branched dextrans contain large per-

CHEMICAL SHIFTS[a] FOR ^{13}C-N.M.R. SPECTRA OF D-GLUCANS AND D-GLUCOPYRANOSIDES AT 90°

Rabbit-liver glycogen	*Glucan OAG-II-3*	*Glucan HAG-10*	*Dextran B-1254 fraction S[L]*	*Methyl α-D-glucopyranoside*	*Methyl β-D-glucopyranoside*
Acquisitions (in thousands)					
47	*5*	*4*	*6*	*1*	*1*
	104.39	104.36			104.85
101.43	101.20	101.43	101.58	101.76	
100.39 s[b]			100.07 s		
			99.42		
	80.27	80.25	80.40		
79.37	79.10	79.66			
	77.91	77.62			77.59
					77.50
			75.07	75.06	
75.02	74.90	74.96	74.87 s		74.73
74.52 s			74.37 s		
73.41	73.26 s		73.50 s	73.41	
73.18	73.03	73.14	73.12	73.19	
72.36 s	72.29 s	72.32 s	71.95	71.73	
71.44 s	71.40 s	71.42 s	71.43		71.51
	70.42 s	70.41 s	71.22 s		
			68.47 s		
			67.80		
	62.59	62.68	62.51	62.70	62.63
62.51	62.40				
				56.80	58.54

[a]Chemical shifts in p.p.m. relative to tetramethylsilane. [b]The symbol s refers to a shoulder or weak resonance.

centages of a wide variety of variously *O*-substituted, α-D-glucopyranosyl residues, and yet their 70–75-p.p.m. regions (*e.g.*, for dextran B-1254 fraction S[*L*]) are not greatly different from that for linear dextran. From this observation, it is inferred that the corresponding methyl β-D-glucopyranoside and variously *O*-substituted β-D-glucopyranosyl residues will have similar ^{13}C-n.m.r.-spectral patterns. The ^{13}C-n.m.r. spectrum (90°) of methyl β-D-glucopyranoside has been recorded (see Fig. 1 and Table I), and it is quite different from that of the corresponding α anomer.

Comparative ^{13}C-n.m.r. spectra are, therefore, available for the four D-glucopyranosyl residues that could be present in the comb-like, modified amyloses, these being the 4-*O*-substituted α-D-glucopyranosyl residue, the 4,6-di-*O*-substituted α-D-glucopyranosyl residue, the α-D-glucopyranosyl terminal group, and the β-D-glucopyranosyl terminal group. Such data allow a detailed analysis of the spectra of the comb-like, modified amylose glucans to be made. Two modified amyloses have been studied, namely, glucan OAG-II-3, which is the product of an orthoester condensation[3], and glucan HAG-10, the product of a Helferich conden-

sation (employing an enzymically synthesized, linear amylose). A comparison of the chemical-shift data from the spectra of these two modified amyloses (see Table I) shows relatively few differences, but the spectra do differ in terms of relative resonance-intensity (see Fig. 1). In general, the resonances associated with glycogen (or linear amylose) are more intense in the spectrum of glucan OAG-II-3. The presence of strong resonances at ~77.6 and 104.4 p.p.m. in both of these spectra is of great importance. The resonance at 104.4 p.p.m. lies well inside the region associated with β-D-anomeric linkages, and is in accord with the expected structure for glucan OAG-II-3, the product of an orthoester condensation. The resonance at ~77.6 p.p.m. is also in accord with a β-D-linked, terminal D-glucopyranosyl group, as the spectrum of methyl β-D-glucopyranoside contains a well defined doublet at 77.50 and 77.59 p.p.m., resonances that have not been observed in the spectrum of any exclusively α-D-linked D-glucan that has been studied. The dextrans containing 4,6-di-*O*-substituted α-D-glucopyranosyl residues exhibit an anomeric resonance at 100.0 p.p.m. that has been associated with the anomeric carbon atom of the terminal D-glucopyranosyl group. This 100.0-p.p.m. resonance is not detectable in the spectrum of either glucan OAG-II-3 or glucan HAG-10. It is, therefore, concluded that both glucans, OAG-II-3 and HAG-10, contain an amylose backbone chain that is periodically branched through 4,6-di-*O*-substituted α-D-glucopyranosyl residues having β-D-(1→6)-linkages to terminal D-glucopyranosyl groups; this indicates that the orthoester condensation conditions had, as expected, resulted in β-D-glucosylation, but that the Helferich condensation conditions failed to contribute any detectable α-D-glucosylation. Such a result could suggest that Helferich condensations may, in general, fail to yield α-glycosylation when condensation with a polymer is involved; however, a second effect must be considered for this addition. Excoffier *et al.*[16] examined condensations that afford oligosaccharides under Helferich conditions, and concluded that condensations to primary alcohols cause only 1,2-*trans*-glycosylation. Their observations are in accord with our conclusion that the Helferich condensation resulted in the β-D-glucosylation of amylose. Therefore, it is possible that, when similar Helferich condensations are performed on the secondary hydroxyl groups of D-glucans, the expected α-D-glycosylation will occur.

The ^{13}C-n.m.r. spectra of glucans HAG-10 and OAG-II-3 are not identical, but all differences result from changes of relative resonance–intensities. For example, in the anomeric region, the ratio of the resonance intensities at 104.4 and 101.4 p.p.m. is greater for glucan HAG-10. As the 104.4-p.p.m. resonance is associated with the β-D-anomeric branching, this ratio implies that glucan HAG-10 is more branched than glucan OAG-II-3. For the 75–85-p.p.m. region, the 80.4-p.p.m. resonance is associated with the 4,6-di-*O*-substituted α-D-glucopyranosyl residue (dextran B-1254 fraction S[L] and the 79.3-p.p.m. resonance, with the 4-*O*-substituted α-D-glucopyranosyl residue (glycogen). A general correlation for the degree of branching of polymers has been obtained by comparing ^{13}C-n.m.r. resonance-heights[11,12]. For a direct comparison of two resonances in the same spectrum, care must be taken that both nuclei have approximately the same spin–lattice times (T_1) and nuclear

Overhauser effect (n.O.e.). Glucan HAG-10 has a relative-intensity ratio of the 79.3 and 80.4-p.p.m. resonances of 1.0, which, for equal T_1 and n.O.e., would imply that $n = 1$, comparing favorably with the nitrogen data of $n = 0.86$ (when, by definition, $n = 1/z - 1$). The 79.3:80.4-p.p.m. resonance-intensity ratio calculated for glucan OAG-II-3 is 2.7:1, implying that $n = 2.7$ for this polymer, as compared to the previously reported[3] value of $n = 2$.

Although the T_1 and n.O.e. for different nuclei in the same polymer can be quite different, the ratios of these peaks can provide a general index for comparing the degrees of linearity of different polymers. For amylose derivatives of increasing linearity, an estimation of the degree of branching based on the 79.3:80.4-p.p.m. intensity ratio will require progressively higher signal-to-noise ratios, as the relative intensity of the 80.4-p.p.m. resonance weakens for linear D-glucans. However, the 77.6:79.3-p.p.m. resonance-intensity ratio is a convenient alternative for estimation of the degree of branching. For example, the 80.4-p.p.m. resonance is associated with the 4,6-di-*O*-substituted α-D-glucopyranosyl residue, and the 77.6-p.p.m. resonance, with the β-D-linked (terminal) D-glucopyranosyl group. For each branch-point residue, there is a terminal residue, giving a one-to-one correspondence. For HAG-10, the 77.6:80.4-p.p.m. relative-intensity ratio is 3.1:1, and for OAG-II-3, it is 3.4:1. The 77.6-p.p.m. resonance is the more intense, as it represents an unresolved doublet (as observed in the spectrum of methyl β-D-glucopyranoside), and the side group probably has greater mobility than the backbone chain. As the 79.3:80.4-p.p.m. ratio for HAG-10 is approximately unity when $n = 0.9$, it may be assumed that, for a polymer having $n = 1$, the intensity ratio of 80.4:79.3 p.p.m. will approximately equal 0.9:1. On multiplying the intensity ratios of (80.4:79.3 p.p.m.) by (77.6:80.4 p.p.m.), the value for (77.6:79.3 p.p.m.) is obtained, and this is equal to ~2.6:1. Therefore, an approximate value for n can be obtained for a comb-branched amylose by taking the resonance-intensity ratio 77.6:79.3 p.p.m. and multiplying it by 2.8. As the 77.6-p.p.m. resonance is (*a*) diagnostic for the terminal β-D-glucopyranosyl group, (*b*) a relatively intense resonance, and (*c*) situated in a region where resonances for the α-D-linked D-glucopyranosyl residues are not found, the 77.6:79.3-p.p.m. resonance-intensity ratio should be measurable for relatively linear polymers, thus providing an additional check on the anomeric resonance-intensity ratio.

Anomeric peak-height ratios were not chosen for linearity comparisons. For glucan HAG-10 (see Fig. 1), the resonances at 104.4 and 101.4 p.p.m. were of almost equal height, but the peak at 101.4 p.p.m. was almost twice as broad as that at 104.4 p.p.m., indicating that the peak areas, but not the peak heights, are in agreement with the expected distribution of anomeric resonance-intensity.

Finally, the comparison spectrum of rabbit-liver glycogen deserves some comment. The spectrum of the glycogen showed broader resonances than those for dextran, recorded under similar conditions. It is possible that this resonance broadening for glycogen is due to the dendritic structure normally assigned to this polymer. The glycogen spectrum depicted in Fig. 1 does not show this line broadening, as a high signal-to-noise data set was acquired, and these data were processed by convolution-

difference, resolution enhancement (c.d.r.e.)[17] to achieve a high-resolution spectrum. The spectrum of rabbit-liver glycogen contains the six resonances expected for a glycan composed of a large percentage of 4-*O*-substituted α-D-glucopyranosyl residues, and the minor resonances associated with branching. Previous experience with the predominantly α-D-(1→6)-linked dextrans had shown that the linked C-6 resonance is located at 67.8 p.p.m. For branched dextrans, an additional resonance at 62.7 p.p.m., representing the free C-6, was present. These linked and free C-6 resonances have proved to be relatively insensitive to structural changes of dextrans, with only ~62.7 and ~67.8-p.p.m. resonances present in the 60–70-p.p.m. spectral region, the ratio of the peaks being dependent on the degree of dextran branching. The absence of any 62.7-p.p.m. resonance for glucan HAG-10 and glucan OAG-II-3 is not so surprising, as other ^{13}C-n.m.r.-spectral regions clearly indicate branching by β-D-(1→6)-linkages, and all the spectral implications of this type of branching are still not certain. However, glycogen is considered to contain only α-D-linkages, a concept supported by the absence of anomeric-linkage resonances in the β-D-linkage spectral-region (downfield from 102 p.p.m.). A minor anomeric resonance, and additional minor resonances in the 70–75-p.p.m. region, indicate that branching of glycogen contributes observable resonances. At present, an explanation for the absence of a linked C-6, minor resonance from the 65–70-p.p.m. spectral region of glycogen is not evident.

Most glycogens have an average, unit-chain length of 10–14 residues between branch points[18]. A portion of the rabbit-liver glycogen employed for recording of the ^{13}C-n.m.r. spectrum was subjected to permethylation–fragmentation analysis[19,20]. The peracetylated aldononitrile (PAAN) derivatives[21] of the products in the hydrolyzate yielded a chromatogram having only three peaks, corresponding to the 2,3,4,6-tetra-*O*-methyl (6.4%), the 2,3,6-tri-*O*-methyl (88.3%), and the 2,3-di-*O*-methyl (5.3%) PAAN glucose derivatives (percentages expressed as percent area of the uncorrected, hydrogen-flame-detector chromatogram). These data indicate one branching residue for every 13.8 4-*O*-substituted α-D-glucopyranosyl residues. Therefore, the percentage of branching residues for glycogen is near to the lower limits of ^{13}C-n.m.r. detection. However, the absence of any resonance at ~62.7 p.p.m. in this high signal-to-noise spectrum is an unexpected result. These ^{13}C-n.m.r. data for rabbit-liver glycogen are in accord with similar data acquired for oyster glycogen, the only differences being minor changes in the relative resonance-intensity.

EXPERIMENTAL

The preparation and the physical properties of the orthoester condensation product[3], OAG-II-3, have previously been reported ($1/z$ = 2.20 nitrogen content; $1/z$ = 3.0 methylation analysis). The Helferich condensation product, HAG-10, was prepared as previously described[1] ($1/z$ = 1.86 nitrogen content), but employing an enzymically synthesized, strictly linear amylose as the backbone. Methyl β-D-glucopyranoside was obtained from Pfanstiehl Inc., Waukegan, Ill.

The ^{13}C-n.m.r. conditions and sample preparation methods have been de-

scribed[11,12]. In general, a Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 system was employed in the Fourier-transform mode. Samples (~200 mg) were dissolved in deuterium oxide (5 ml).

The permethylation analysis methods for glycogen have been described[19,20], and the glycogen sample was obtained from Sigma Chemical Co., St. Louis, Mo. The production and characterization of dextran B-1254 fractions S[L] has been described[22].

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