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N.M.R. STUDIES OF COMPOSITION AND SEQUENCE IN LEGUME-SEED GALACTOMANNANS

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ABSTRACT

After depolymerisation to $\overline{d.p.}_n$ ~20–40, galactomannans from guar, carob (locust bean), lucerne, and clover seeds gave good ^{1}H- and ^{13}C-n.m.r. spectra. The resonances of the anomeric protons and the twelve carbon atoms were fully resolved and easily identified. Measurement of the ratios of corresponding peak-areas gave results for monomeric composition (Gal:Man ratio) in good agreement with those of chemical analysis. Resonances from C-4 of D-mannose residues were split, in evident dependence upon the nearest-neighbour probabilities ("diad frequencies") of D-galactosyl groups along the mannan chains. The relevant peak-area ratios gave values for these probabilities that were roughly consistent with a random arrangement of D-galactosyl groups in all four galactomannans, but they do not exclude the possibility of more complex kinds of arrangement.

INTRODUCTION

Determination of the distribution of (1→6)-linked, α-D-galactopyranosyl side-groups along the (1→4)-linked β-D-mannan chains in legume-seed galactomannans[1,2] has so far been attempted by X-ray diffraction analysis[3], degradation by purified β-D-mannanases[4–6], specific methods of chemical degradation[7,8], methylation analysis after periodate oxidation[9], and theoretical analysis of periodate-oxidation kinetics[10,11].

Agreement between different groups of workers, using different methods, has been poor[3–11]. Part of the difficulty may be due to variation in structure between different samples of galactomannan, isolated from the same species. Since guaran and locust-bean gum can be divided, upon a basis of solubility in water, into fractions of different composition[12], the conditions of extraction must be important. In addition, there may be variation between different strains (varieties) of a given species. Since it is known, for example, that the amylose content of starches from different varieties of maize can vary from 0 to 80%[13,14], this idea must be taken seriously.

Investigation of these points requires the development of simple and rapid methods of structural analysis that are suitable for routine use. The present n.m.r. studies represent an attempt to fill this need. Although the accuracy of the results is not high (±5–10%), it compares favourably with that obtainable by other methods.

Moreover, since only diad frequencies (nearest-neighbour probabilities) are measured, the method will not normally* provide a complete picture of the structure of a galactomannan. The requirements of simplicity and speed are nevertheless met, and the partial characterisation achieved should be particularly useful for studying the fragments of selective enzymic cleavage[1,2,4–6].

EXPERIMENTAL

Materials. — Purified samples of guaran [from *Cyamopsis tetragonoloba* (L.) Taub.] and locust-bean gum (from *Ceratonia siliqua* L.) were supplied by Meyhall Chemical AG, Kreuzlingen, Switzerland. Galactomannans from lucerne (*Medicago sativa* L., Var. "Alfa II") and red clover (*Trifolium pratense* L., Var. "Venla") seeds were prepared as described by Andrews *et al.*[15], but were isolated by freeze-drying, to improve solubility.

Preliminary degradation. — Samples (1 g) of galactomannan were dissolved by grinding in a mortar with syrupy orthophosphoric acid (85% w/w, 100 ml) at 20° for 2 h, followed by mechanical shaking at 4° for 4 weeks. To the resulting solution, in a mortar, ice-cold diethyl ether (500 ml) was added in portions, with vigorous mixing (caution: exothermic reaction; skin- and eye-protection needed). The precipitate was collected by decantation, extracted by repeated grinding with fresh ether, and then dissolved in water (20 ml). Traces of residual phosphoric acid were removed by adding barium carbonate (100 mg), followed by filtration, and the filtrate was centrifuged and freeze-dried. The $\overline{d.p.}_n$ of the degraded samples was determined by the measurement of reducing power[16], with D-mannose as the standard.

N.m.r. spectroscopy. — The samples were dissolved in D_2O at pD 7 (80 mg/ml for ^{13}C; 10 mg/ml for ^{1}H). The deuterium resonance was used as a field-frequency lock, and chemical shifts were expressed relative to internal sodium 3-(trimethylsilyl)-propionate-d_4. All experiments were performed in the Fourier-transform mode, at 25 or 50 MHz for ^{13}C, and at 99.6 MHz for ^{1}H, with JEOL FX 100 (H_0 = 23.5 kG) and Bruker WP 200 (H_0 = 47 kG) spectrometers. All ^{13}C-n.m.r. spectra were acquired by using 8000 data points and a spectral width of 5 kHz. Free-induction decays were accumulated with a 75° pulse and a repetition time of 0.8 s. Spin–lattice relaxation times (T_1) were determined by the inversion-recovery method[17]. Spectra in which the nuclear Overhauser enhancements were removed were also measured, to ensure that relative peak areas represented relative abundances. A probe temperature of 90° was used to diminish viscosity and, thereby, line-width. Peak areas were measured by planimetry. The signal for C-4 of D-mannopyranose in the 25-MHz, ^{13}C-n.m.r. spectra showed a rather small, sequential splitting, and was therefore reconstructed

*Obvious exceptions are unsubstituted and fully substituted mannans, and simple, "block" structures that are tantamount to a mixture of these. In addition, a strictly alternating arrangement of substituted and unsubstituted D-mannose residues would be fully defined by the diad frequencies alone.

in the computer by superposition of three Lorentzian lines of equal width; their positions were obtained from the better-resolved, 50-MHz, ^{13}C-n.m.r. spectrum.

RESULTS AND DISCUSSION

Preliminary degradation of samples. — The key to successful n.m.r. spectroscopy of the galactomannans proved to be an initial, limited depolymerisation, which diminished the viscosity of their solutions and improved their solubility. The choice of cold, concentrated orthophosphoric acid for this purpose was dictated by its excellent solvent power for galactomannans, and its ease of removal by extraction with ether. In addition, this acid was expected to enhance the rate of hydrolysis of the β-D-mannopyranosidic linkages, relative to that of the α-D-galactopyranosidic linkages, by increasing the magnitude of the anomeric effect[18]. Under the conditions described, it was possible to decrease the $\overline{d.p.}_n$ of the samples to 20–40, without any detectable change in monomeric composition.

^{1}H-N.m.r. spectroscopy. — The ^{1}H-n.m.r. spectrum of locust-bean gum and the anomeric region in the spectrum of guaran are shown in Fig. 1. The resonances of the anomeric protons are well separated, and their identification is self-evident from the known[9–11] monomeric compositions of the samples (Gal:Man = 36:64 for guaran and 19:81 for locust-bean gum). The doublet at 5 p.p.m., which is more intense for guaran and must arise from H-1(Gal), has $J_{1,2}$ ~3 Hz, compatible with the expected $^4C_1$ conformation of the α-D-galactopyranose rings. The signal for H-1(Man) at 4.8 p.p.m. has $J_{1,2}$ close to the value of 0.9 Hz observed for monomeric β-D-mannopyranose. Accordingly, all the D-mannopyranose residues in the polymers must be in the expected $^4C_1$ conformation.

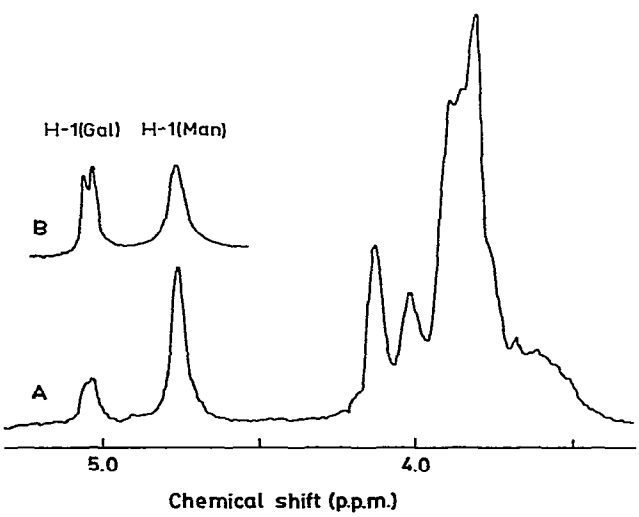

Fig. 1. ^{1}H-N.m.r. spectra (99.6 MHz) of solutions (10 mg/ml) in D_2O (at pD 7 and 90°) of A, carob (locust-bean) gum; and B, guaran.

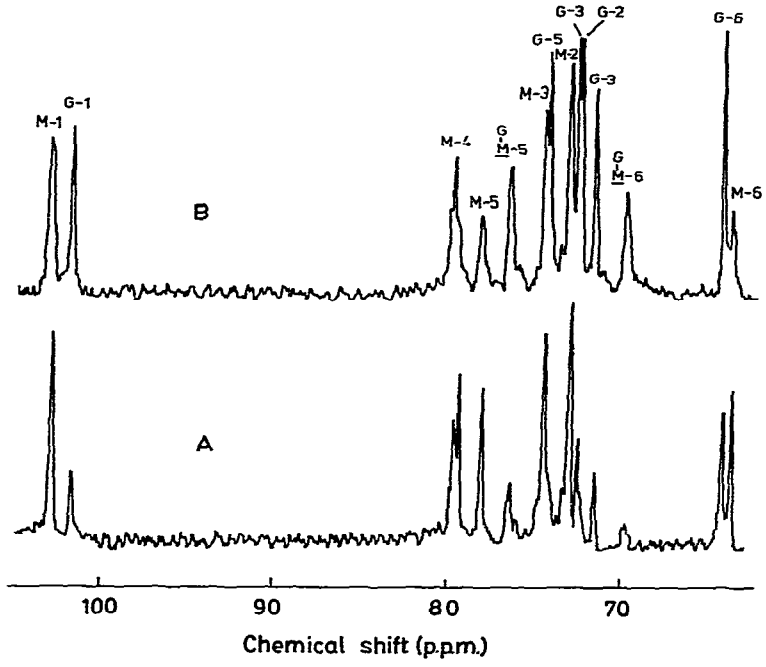

Fig. 2. ^{13}C-N.m.r. spectra (25 MHz) of solutions (80 mg/ml) in D_2O (at pD 7 and 90°) of A, carob (locust-bean) gum; and B, guaran.

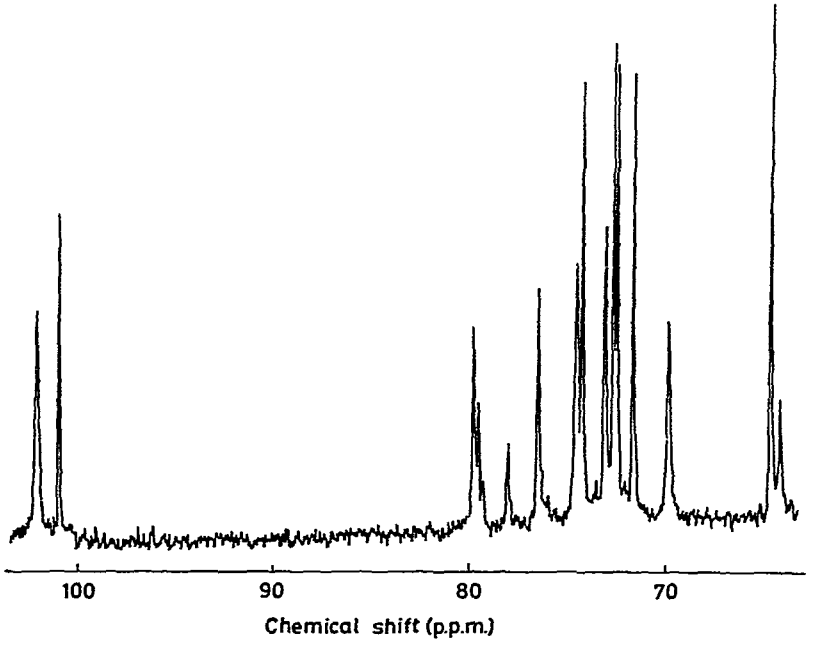

Fig. 3. ^{13}C-N.m.r. spectrum (50 MHz) of a solution (80 mg/ml) in D_2O (at pD 7 and 90°) of the galactomannan from clover seeds.

TABLE I

ASSIGNMENTS OF PEAKS IN ^{13}C-N.M.R. SPECTRA OF LEGUME-SEED GALACTOMANNANS[a]

Type of unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α-D-Galactopyranosyl	101.8	72.3[b]	71.4	72.5[b]	74.1	64.1
β-D-Mannopyranosyl, unbranched at HO-6	103	72.9	74.4	79.3[c] 79.6[d]	78	63.6
β-D-Mannopyranosyl, branched at HO-6	103	72.9	74.4	79.6[c] 79.9[d]	76.4	69.6

[a]Shifts (p.p.m.) downfield from internal sodium 3-(trimethylsilyl)propionate-d_4. [b]These assignments may have to be interchanged. [c]When the preceding D-mannose residue is unbranched. [d]When the preceding D-mannose residue is branched.

^{13}C-N.m.r. spectroscopy. — The 25-MHz, ^{13}C-n.m.r. spectra of locust-bean gum and guaran (Fig. 2) and the 50-MHz, ^{13}C-n.m.r. spectrum of the galactomannan from clover seeds (Fig. 3) illustrate the great promise of ^{13}C-n.m.r. for sequential analysis of polysaccharides. All of the different carbon lines are resolved, and their chemical shifts are recorded in Table I. The resonances associated with the D-galactose and D-mannose residues were distinguished by making use of the different monomeric compositions of the samples, as determined by ^{1}H-n.m.r. and chemical methods. The spectral regions of the anomeric carbons (101.8 and 103 p.p.m.) and the methylene carbons (63.6 and 64.1 p.p.m.) are well documented.

Because the substituted D-mannose residues are branched at O-6, the positions of their C-6 (69.6 p.p.m.) and C-5 (76.4 p.p.m.) resonances are shifted relative to the corresponding resonances of unbranched D-mannose residues, namely, by 6 p.p.m. downfield, and 1.6 p.p.m. upfield, respectively. They are therefore readily identified. Presumably, C-4 of the D-mannose residues resonates at relatively low field because it is involved in a glycosidic linkage. Confidence in the assignments of the resonances for C-2(Man) and C-3(Man) is provided by the fact that these give a spectrum for unbranched D-mannose residues in close agreement with that reported for the 1,4-linked β-D-mannopyranose residues in the mannan from *Rhodotorula glutinis*[19]. Likewise, the assignment given for the carbon resonances of the D-galactose residues accords well with the reported spectrum of monomeric α-D-galactopyranose[20].

The resonances from the D-mannose residues are not so narrow as those from the D-galactose residues. This may reflect a higher reorientational mobility of the latter, but sequence-related heterogeneity of chemical shifts can also be expected to contribute to the width of the carbon resonances from the former. Indeed, the C-4-(Man) resonance showed a clear, sequential splitting. The spectrum taken at 50 MHz (Fig. 3) contained three almost completely resolved lines for C-4. Since all of the D-mannopyranose residues are β-1,4-linked and in the $^4C_1$ conformation, this pattern of lines can only be explained by assuming that the C-4(Man) resonance is sensitive to whether or not the residue linked to O-4 (*i.e.*, the preceding residue in the chain)

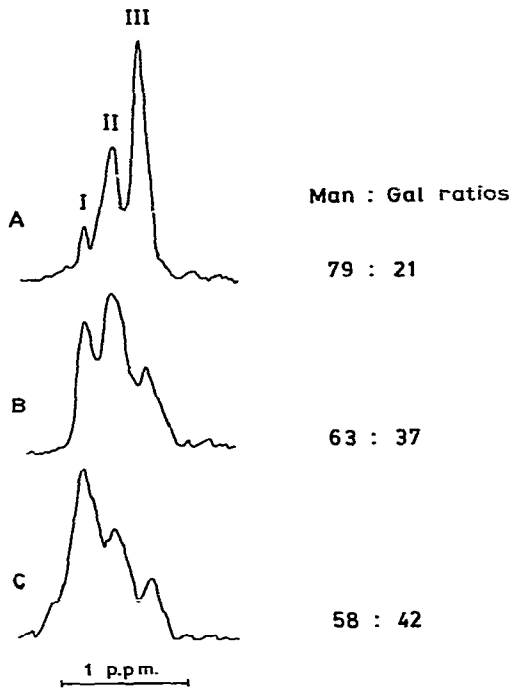

Fig. 4. ^{13}C-N.m.r. spectral region, at 25 MHz, of C-4 of the D-mannose residues in A, carob (locust-bean) gum; B, guaran; and C, the galactomannan from clover seeds. Interpretation (the unit involved is underlined):

(I) Gal Gal (II) Gal and · Gal (III) –Man–Man–
| | |
–Man–Man– –Man–Man– –Man–Man–

is branched; a basis for determining the nearest-neighbour probabilities in the galactomannans is therefore provided.

Fig. 4 shows expansions of the region of C-4(Man) resonances for three different galactomannans. The peak (III) at high field must be due to unsubstituted D-mannose residues that are adjacent to another residue of the same kind, because of its relatively high intensity for locust-bean gum, which contains a relatively small proportion of D-galactose residues. The peak (I) at the lowest field dominates in the spectrum of the sample from clover seeds, which is most highly substituted with D-galactose residues; most probably, therefore, it originates from groups of two contiguous, substituted D-mannose residues. Hence, the intermediate peak (II) must represent a superposition of signals originating from diads in which only one of the two D-mannose residues is substituted (which is also inferred from the relative intensities of the lines). This inference implies that the environment experienced by C-4(Man) is affected to about the same extent by a D-galactosyl group attached to the preceding unit in the chain, as it is by one attached to its own ring. It is then

TABLE II

COMPOSITIONS AND DIAD FREQUENCIES[a] FOR GALACTOMANNANS FROM DIFFERENT SPECIES

Species	*Method*[b]	*Gal:Man ratio*	*Diad frequencies*[a]		
			F_{11}	$F_{12} = F_{21}$	F_{22}
Carob	A	0.30			
	B	0.27	0.08	0.17	0.58
	C	0.24	0.07	0.165	0.60
	(D)		(0.07)	(0.195)	(0.54)
Guar	A	0.59			
	B	0.59	0.30	0.24	0.22
	C	0.56	0.35	0.215	0.22
	(D)		(0.35)	(0.24)	(0.17)
Clover	A	0.85			
	B	0.72	0.56	0.16	0.12
	(D)		(0.52)	(0.20)	(0.08)
Lucerne	A	0.92			
	B	0.79	0.59	0.18	0.05
	(D)		(0.62)	(0.17)	(0.05)

[a]The subscripts 1 and 2 refer to branched and unbranched D-mannose residues, respectively. [b]A, ^{1}H-n.m.r.; B, ^{13}C-n.m.r.; C, periodate oxidation[10,11]; (D), calculated for a random distribution from the Gal:Man ratio measured by Method B.

reasonable to expect that the combined effects of two such groups would give rise to the low-field resonance I, as already inferred.

The diad frequencies for the four galactomannans are given in Table II. For guaran and carob (locust-bean) gum, the results obtained with the same samples by a method based upon periodate oxidation[10,11] are included for comparison. Theoretical values are also included, which show that the results may be conveniently summarised by the statement that they are roughly consistent with a random arrangement of D-galactosyl groups in all four polymers. This finding rules out some of the simpler possibilities that have been considered previously[3-11], such as a strictly alternating arrangement of branched and unbranched D-mannose residues, and structures in which long blocks of contiguous, branched D-mannose residues are considered to alternate with similar blocks of unbranched D-mannose residues. However, more-complex kinds of non-random arrangements, such as those proposed earlier for the same samples of guaran and locust-bean gum[10,11], cannot be inferred or excluded by measurements of diad frequencies alone. Moreover, since only one sample of each galactomannan has been studied, no statement is possible as to whether the present results are typical.

Measurement of monomeric composition (Gal:Man ratio). — The relative areas of the signals for H-1(Gal) and H-1(Man) (Fig. 1) yielded, directly, the mole fractions

of the two monomers presented in Table II. In the ^{13}C-n.m.r. spectra, the relative areas of resonances were not significantly influenced by the nuclear Overhauser enhancements. A maximum value of $T_1 = 0.5$ s was observed for the C-6(Gal) resonance. Under the instrumental conditions chosen, the relative abundance of the two monomers was therefore also obtained directly from the relative peak-areas of corresponding ^{13}C-n.m.r. signals. This approach allowed a number of independent analyses to be carried out, and, by averaging these figures, an improved accuracy was obtained. The averages are given in Table II.

A less direct, but interesting, estimate of the Gal:Man ratio is provided by the sequentially split C-4(Man) resonances. Calculation shows that this ratio is given by the sum of the diad frequencies, $(F_{11} + F_{12})$. As is seen from Table II, the agreement with the other estimates is good, and the internal consistency observed, as well as the observed agreement with the results of the independent, periodate-oxidation method[10,11], provide assurance that the present interpretation of the splitting of the C-4(Man) signal is correct.

Accuracy. — The extent of the preliminary degradation, and the concentrations of the solutions in D_2O, proved to be rather critical. Insufficient degradation and higher concentrations gave spuriously high Gal:Man ratios, probably because of selective aggregation of regions of the chains in which unbranched D-mannose residues preponderated. It is consistent with this that the deviation was greater in the ^1H-n.m.r. results, because the magnetic interaction that is responsible for broadening of the lines is expected to be stronger for protons than for ^{13}C.

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