Eur. J. Biochem. *130*, 111–115 (1983)

Natural-Abundance ^{13}C-NMR Spectroscopy of Two Glyco-asparagines Derived from the Core of *N*-Glycosidic Carbohydrate Chains

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(Received August 11/October 8, 1982) – EJB 5890

^{13}C-NMR spectroscopic studies were carried out on Manα(1→6)Manβ(1→4)GlcNAcβ(1→4)GlcNAcβ(1→N)-Asn and Manα(1→6)Manβ(1→4)GlcNAcβ(1→4)[Fucα(1→6)]GlcNAcβ(1→N)Asn, which are part of the invariant core of *N*-glycosidically linked carbohydrates of glycoproteins. All resonances of proton-bearing carbon atoms were unambiguously assigned. Where possible, the assignments were confirmed by selective proton-decoupling experiments.

The discovery that glycoconjugates are involved in cell-cell recognition, the immune response, hormone-receptor functions and internalization of various macromolecular materials, has widely stimulated research efforts to characterize these molecules and, especially, their glycan parts. Recent reviews by Sharon and Lis [1] and Montreuil [2] summarize our current understanding of the importance of glycoproteins for the living organism. Although the elucidation of the primary structure of oligosaccharides has become a rather straightforward procedure, at least when sufficient material is available, little is known of the spatial conformation of the glycan chains. Knowledge of the three-dimensional structure is a prerequisite to explain their mode of interaction with ligands. A number of models have been proposed for their spatial conformation, mostly based on model building [3] or theoretical approaches [4], but the experimental evidence for these models is scarce. No crystallographic data exist on major parts of the *N*-glycosidically linked carbohydrates of glycoproteins. One of the largest partial structures crystallized so far is Manα(1→3)Manβ(1→4)GlcNAc [5]. In the few crystallized glycoproteins [6, 7] the glycan part was found to be disordered for the greater part. It is not clear whether this disorder is static, i.e. due to irregular packing of the glycan chains in the crystals, or dynamic, i.e. reflecting mobility of the glycan chains in the crystals.

We decided to initiate an investigation into the dynamic aspects of glycan structures by means of nuclear magnetic resonance (NMR) spectroscopy. NMR relaxation measurements provide an important probe of the dynamics of molecules, since the spin-lattice (T_1) relaxation time, the spin-spin (T_2) relaxation time and the nuclear Overhauser enhancement (NOE) factor are parameters which reflect thermal motions. ^{13}C-NMR of proton-bearing carbon atoms is particularly well suited for the study of dynamics because the relaxation is dominated by the fluctuating dipolar interactions between ^{13}C nuclei and directly bonded protons.

Abbreviations. Fuc, L-fucose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Asn, L-asparagine; NMR, nuclear magnetic resonance; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time; NOE, nuclear Overhauser enhancement; ppm, parts per million.

For the interpretation of relaxation data an assignment of the signals in the ^{13}C spectrum to individual carbon atoms is required. Several reports have been published which contain ^{13}C spectral assignments for partial structures of *N*-glycosidically linked carbohydrates. Dill and Allerhand [8] assigned the resonances of GlcNAcβ(1→N)Asn; Berman et al. [9] published an assignment of Manβ(1→4)GlcNAcβ(1→4)-GlcNAcβ(1→N)Asn; and Nunez et al. [10] interpreted the ^{13}C spectrum of Manα(1→3)Manβ(1→4)GlcNAc. These assignments were based on comparisons with the spectra of monosaccharides and disaccharides.

Here we give a complete assignment of the ^{13}C-NMR spectra of a tetrasaccharide and a pentasaccharide part of the core of *N*-glycosidically linked carbohydrates. Where appropriate, the assignments were corroborated by double-resonance experiments.

MATERIALS AND METHODS

GlcNAcAsn (compound I) was obtained from Vega-Fox Biochemicals (Tucson, AZ, USA). FucGlcNAcAsn (compound II) and Fuc[(Man)$_2$(GlcNAc)$_2$]Asn (compound V) were isolated from the urine of a patient with fucosidosis [11]; (Man)$_2$(GlcNAc)$_2$Asn (compound IV) was obtained from the urine of a patient with Gaucher's disease [12]. The complete structures of these compounds are shown in Fig. 1.

Natural-abundance ^{13}C-NMR spectra were obtained at 50.76 MHz on a Bruker WP-200 spectrometer (Utrecht University) or on a Bruker WM-200 spectrometer (SON-national NMR facility at Nijmegen University). The applied pulse width was 20 µs corresponding to a flip angle of about 75°. The spectral width was 10000 Hz. Sample temperature was 25 °C. Samples (20–25 mg, but compound I 160 mg) were dissolved in 1.4 ml 99.75% ^2H_2O and transferred to a 10-mm NMR tube (Wilmad, USA) after filtering. Chemical shifts, measured digitally, are reported downfield from the ^{13}C resonance of external tetramethylsilane (indirectly to internal acetone in ^2H_2O; δ = 31.40 ppm) with an estimated precision of 0.05 ppm. NOE factors were determined by the method of gated-decoupling; T_1 relaxation times were mea-

sured by the inversion-recovery method. Details will be published elsewhere.

RESULTS AND DISCUSSION

^{13}C-NMR spectra were recorded for the compounds I, II, IV and V. In Fig. 1 the structures of these compounds are listed, together with the numbering system of the constituting residues.

For the assignment of signals in the ^{13}C spectra of I and II use was made of the fully interpreted ^{1}H spectra of these compounds [13]. Therefore, selective proton-decoupling (H1 resonances of GlcNAc and Fuc; the H5 resonance of Fuc) and a series of off-resonance decoupling experiments gave unambiguous assignments for all proton-bearing carbon atoms. The results are listed in Table 1. This assignment resolves the ambiguity in the interpretation of C1 and C5 in the ^{13}C-NMR spectrum of compound I left by Dill and Allerhand [8]. The assignment of the non-proton-bearing carbon atoms is more difficult. The resonance at 173.93 ppm in the spectrum of I could be assigned to the carbonyl carbon of the acetyl group of GlcNAc on the basis of long-range ^{2}J couplings with a methyl group, observed in the proton-coupled spectrum. To discriminate between the Asn side chain carbonyl carbon (Cγ) and the carboxyl carbon (C^{O}) we measured T_1 relaxation times and NOE factors. The results are shown in Table 2. From the observed T_1 values and the NOE factors, the T_1 relaxation times for purely dipolar relaxation (T_1^{DD}) can be calculated ($T_1^{DD} = T_1^{obs} \times 1.98/NOE$; see, e.g. Levy et al. [14]). Because the number of protons close to the Cγ of Asn is twice that of C^{O}, the expected dipolar

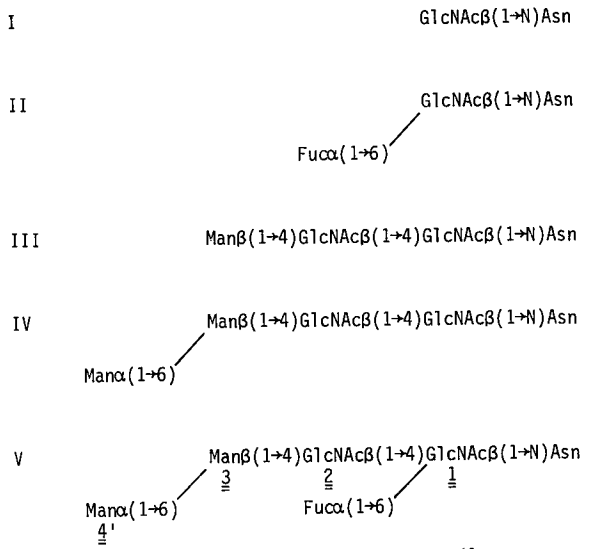

Fig. 1. *Structures of the glyco-asparagines of which the ^{13}C-NMR spectra were completely assigned.* Residue designations used in the text are shown below the monosaccharide residues of compound V

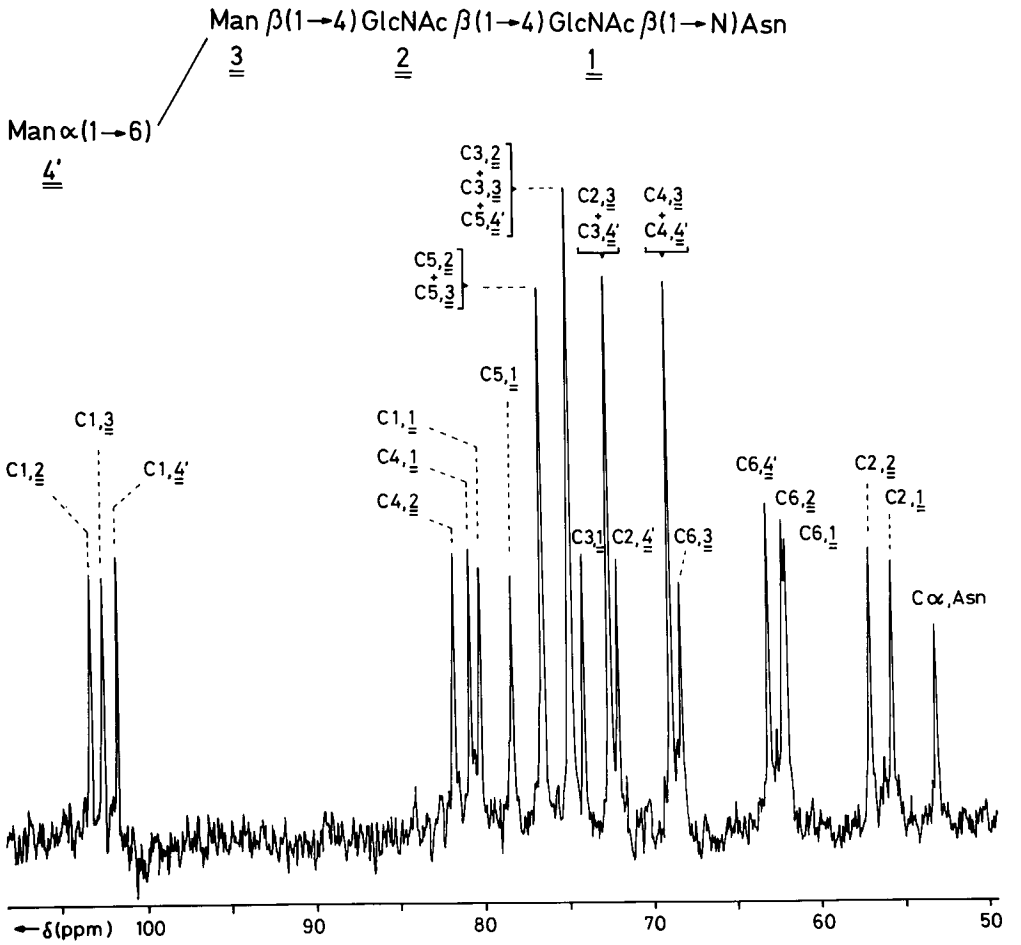

Fig. 2. *Proton-decoupled ^{13}C-NMR spectrum of Manα(1→6)Manβ(1→4)GlcNAcβ(1→4)GlcNAcβ(1→N)Asn (compound IV).* The resonances of the acetyl carbons of the GlcNAc residues, the Cβ, carbonyl and carboxyl atoms of Asn are outside the region shown

Table 1. *^{13}C-NMR chemical shift data of compounds I–V and some reference compounds*
Chemical shifts for I, II, IV and V were measured in neutral ^2H_2O solutions at 25 °C, relative to internal acetone ($\delta \equiv 31.40$ ppm). Chemical shifts for III were measured in H_2O solution at 30 °C, pH 7.8, relative to internal dioxane ($\delta \equiv 67.86$ ppm). For complete structures, see Fig. 1. n.d. = not determined

Residue	Carbon atom	Chemical shift in					
		GlcNAcAsn	FucGlcNAcAsn	Man-(GlcNAc)$_2$Asn	(Man)$_2$-(GlcNAc)$_2$Asn	Fuc[(Man)$_2$-(GlcNAc)$_2$]Asn	
		I	II	III	IV	V	
		ppm					
GlcNAc-1	C1	79.48	79.43	79.50	79.46	79.46	
	C2	55.44	55.36	55.05	54.97	54.90	
	C3	75.60	75.50	73.39	73.38	73.36	
	C4	70.81	70.97	80.16	80.11	79.63	
	C5	78.94	78.10	77.75	77.63	76.54	
	C6	61.84	68.52	61.37	61.30	67.80	
	C = O	173.93	n.d.	n.d.	n.d.	n.d.	
	CH_3	23.40	23.40	n.d.	23.48	23.39	
GlcNAc-2	C1	–	–	102.56	102.68	102.28	
	C2	–	–	56.51	56.30	56.22	
	C3	–	–	74.27	74.12	74.03	
	C4	–	–	80.02	81.03	81.07	
	C5	–	–	75.93	75.80	75.01	
	C6	–	–	61.55	61.48	61.41	
	C = O	–	–	n.d.	n.d.	n.d.	
	CH_3	–	–	n.d.	23.62	23.63	
							Methyl β-mannoside [18]
Man-3	C1	–	–	101.37	101.94	101.88	102.0
	C2	–	–	71.93	71.79	71.69	71.4
	C3	–	–	74.22	74.12	74.03	74.2
	C4	–	–	68.06	68.21	68.13	68.1
	C5	–	–	77.53	75.80	75.71	77.3
	C6	–	–	62.36	67.60	67.52	62.1
							Methyl α-mannoside [18]
Man-4′	C1	–	–	–	101.11	101.01	101.9
	C2	–	–	–	71.30	71.21	71.2
	C3	–	–	–	71.79	71.69	71.8
	C4	–	–	–	68.21	68.13	68.0
	C5	–	–	–	74.12	74.02	73.7
	C6	–	–	–	62.36	62.28	62.1
Fuc	C1	–	100.41	–	–	100.63	
	C2	–	69.53	–	–	69.48	
	C3	–	70.83	–	–	70.83	
	C4	–	73.16	–	–	73.16	
	C5	–	68.11	–	–	68.13	
	C6	–	16.64	–	–	16.68	
Asn	Cα	52.45	52.41	52.58	52.41	52.38	
	Cβ	36.40	36.46	n.d.	36.64	36.27	
	Cγ	174.97[a]	n.d.	n.d.	n.d.	n.d.	
	C^O	175.42[a]	n.d.	n.d.	n.d.	n.d.	

[a] See Table 2.

Table 2. *Assignment of the non-proton-bearing carbon atoms of asparagine for GlcNAcβ(1→N)Asn (compound I)*

Chemical shift	T_1^{obs}	NOE	T_1^{PD}	Assignment
ppm	s		s	
175.42	2.2	0.21	20.7	$-COO^-$ (C^O)
174.97	3.5	0.72	9.6	$-N-C(=O)$ (Cγ)

relaxation is twice as fast for Cγ as for C^O, assuming a reorientational correlation time which is about the same for Cγ and C^O. Thus we could assign these resonances (Table 2). The alternative assignment would imply a reorientational correlation time for Cγ which is about four times that of C^O. This is quite unrealistic, because it would mean a much greater mobility for Cγ than for C^O. Our assignment implies a correction of the assignment made by Dill and Allerhand [8].

For Man(GlcNAc)$_2$Asn (compound III) the ^{13}C spectrum and chemical shift data have been published by Berman et al.

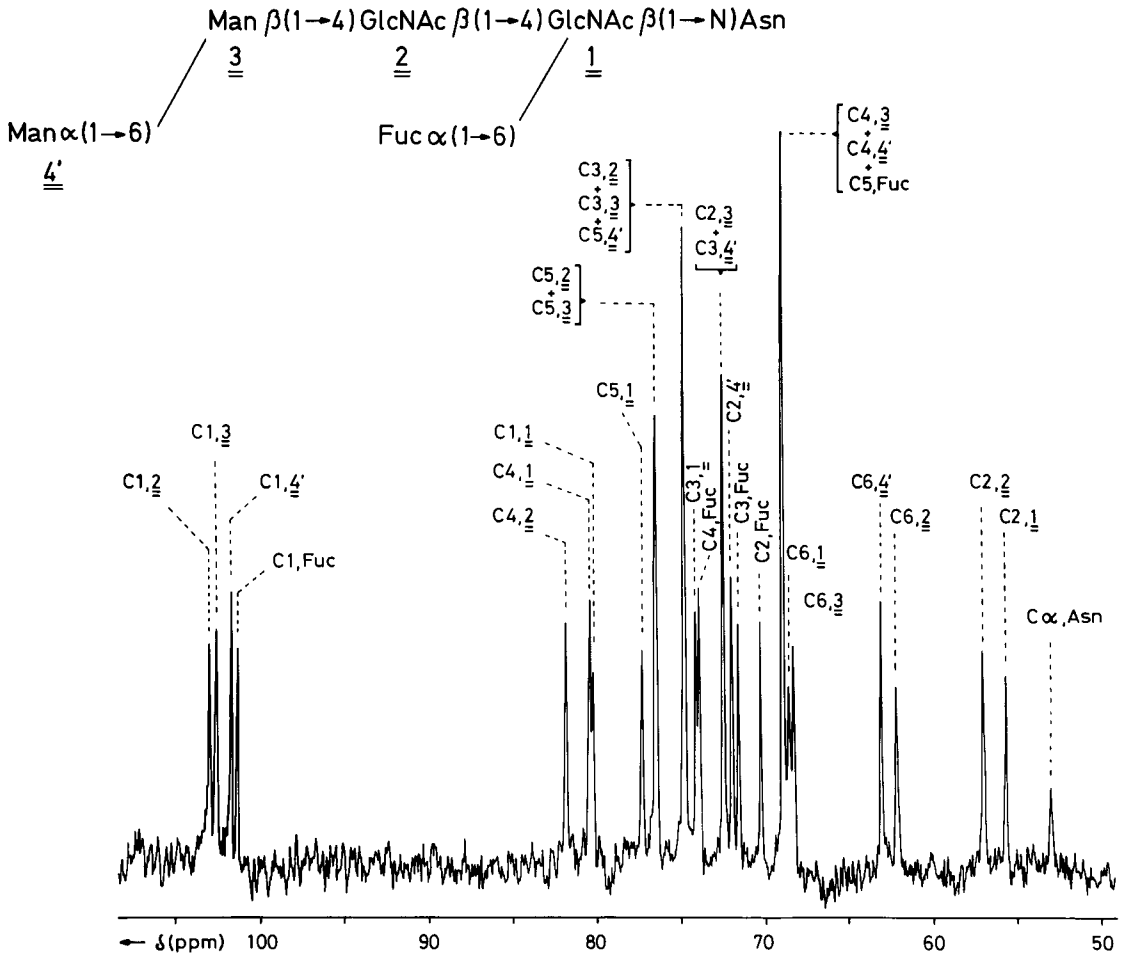

Fig. 3. *Proton-decoupled ^{13}C-NMR spectrum of Manα(1→6)Manβ(1→4)GlcNAcβ(1→4)[Fucα(1→6)]GlcNAcβ(1→N)Asn (compound V).* The resonances of the acetyl carbons of the GlcNAc residues, the methyl group of Fuc, the *Cβ*, carbonyl and carboxyl atoms of Asn are outside the region shown

[9], including an interpretation of the spectrum. We could confirm the major part of their interpretation by a careful comparison of the spectrum of III with the spectra of GlcNAc [8], GlcNAcβ(1→N)Asn (compound I), Manβ-(1→4)Glc [15], GlcNAcβ(1→4)GlcNAc [16], the internal unit of chitotriose GlcNAcβ(1→4)GlcNAcβ(1→4)GlcNAc [17] and methyl β-D-mannopyranoside [18]. However, on this basis we could not discriminate between C5 of GlcNAc-1 and C5 of Man-3. As will be discussed later, this ambiguity and some uncertainties left by Berman et al. [9] could be solved by comparison with the spectra of IV and V.

The broadband proton-decoupled ^{13}C-NMR spectrum of $(Man)_2(GlcNAc)_2Asn$ (compound IV) is shown in Fig. 2. Comparison of this spectrum with that of III reveals the occurrence of six extra signals. One signal has shifted. Comparison of the six extra signals with those of methyl α-D-mannopyranoside [18] gives an assignment for C1, C2, C3, C4 and C5 of Man-4′ of compound IV. The resonance of C6 of methyl α-mannoside [18] is at the same position as C6 of Man-3 in III. In IV, the Man-4′ is linked to C6 of Man-3. Therefore we expect a downfield shift for C6 of Man-3 in IV. This resonance could be identified in a proton-coupled ^{13}C-NMR spectrum by means of its triplet structure at 67.60 ppm at the position of an extra peak. In consequence, the signal at 62.36 ppm is assigned to C6 of Man-4′.

Because the structural-reporter-group region of the ^{1}H spectrum of IV has been interpreted [12], the assignment of C1 and C2 of Man-4′ could be confirmed by selective decoupling experiments, as could the C1 and C2 resonances of Man-3 and the C1 resonances of GlcNAc-1 and GlcNAc-2 in this compound.

Furthermore, from comparison of the ^{13}C spectra of III and IV we can identify C5 of Man-3. Owing to a β-substituent effect this resonance is shifted upfield from 77.53 ppm to 75.80 ppm, while the position of the C5 signal of GlcNAc-1 remains unchanged at 77.7 ppm. This implies a correction of the assignment of the C5 signals of Man-3 and GlcNAc-1 in compound III [9].

The proton-decoupled ^{13}C-NMR spectrum of $Fuc[(Man)_2$-$(GlcNAc)_2]Asn$ (compound V) is shown in Fig. 3. From a comparison of Fig. 2 and 3 the existence of four extra signals in Fig. 3 is obvious. Furthermore, the intensities of two signals are increased and one signal has disappeared. An additional signal in the spectrum of V is at 16.68 ppm (not shown in Fig. 3). Six of the extra signals can easily be assigned by comparison with the Fuc resonances in II. The assignments of C1, C5 and C6 of Fuc were confirmed by selective decoupling of the H1, H5 and H6 resonances, the chemical shifts of which are known [11]. The remaining signal not yet accounted for is that of C6 of GlcNAc-1, which is shifted from 61.30 ppm to 67.80 ppm. Consequently, the ambiguity in assignment of the signals of C6 of GlcNAc-1 and C6 of GlcNAc-2 for compounds III and IV [9] is solved. Furthermore, it is evident from Fig. 2 and 3 that two other resonances have shifted

slightly. These shifts are due to a substituent effect of the Fuc residue on nearby carbon atoms of GlcNAc-1. Similar shift effects are found in the carbon resonances of GlcNAc-1 in I and II. From the extent and direction of these shifts the resonance of C5 of GlcNAc-1 can be unambiguously assigned ($\delta = 77.63$ ppm in IV). This supports the assignment of C5 of GlcNAc-1 at 77.75 ppm in III.

Comparison of the spectra of IV and V also makes it possible to discriminate between the signals of the C4 atoms of GlcNAc-1 and GlcNAc-2: C4 of GlcNAc-2 is at 81.0 ppm in both compounds IV and V, whereas C4 of GlcNAc-1 is at 80.1 ppm in IV and at 79.6 ppm in V. In III, C4 of GlcNAc-2 is at 80.02 ppm. Possibly, elongation of III with an α-linked mannose at C6 of Man-3 causes a conformational change in the glycosidic bond between GlcNAc-2 and Man-3, effecting small changes in the chemical shift positions of both C4 of GlcNAc-2 and C1 of Man-3.

CONCLUDING REMARKS

In the foregoing we presented a complete ^{13}C-NMR spectral assignment of the proton-bearing carbon atoms of a substantial part of *N*-glycosidically linked carbohydrate chains. This assignment will be of aid to the complete interpretation of the ^{13}C-NMR spectra of oligomannoside-type and *N*-acetyllactosamine-type carbohydrates. Because ^{13}C-NMR data under conditions of noise-decoupling are easy to interpret, as compared to ^{1}H-NMR data, such an assignment will allow the study of the dynamics of these carbohydrates at an atomic level. Also detailed information on the mode of action of glycosidases may be obtained. Finally, conformational analysis of carbohydrate chains by means of ^{13}C-NMR spectroscopy may furnish insight into their spatial structure, and this will be of value for studies into the interaction of glycan chains with, for example, lectins.

Thanks are due to Dr H. van Halbeek for helpful discussions in preparing this manuscript. This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO), by the Netherlands Foundation for Cancer Research (KWF, grant UUKC-OC 79-13), and by the *Centre National de la Recherche Scientifique (Laboratoire Associé no. 217: Relations structure-fonction des constituants membranaires)*.

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