viscosity of polystyrene is zero. It should be noted that our limits of error are large (±10%) and that a more sensitive technique might be able to detect the small residual contribution of the polymer to $\eta_s(\omega)$ at 5 GHz. However, it appears that Brillouin scattering will not be useful in determining the high-frequency limiting viscosity in polysytrene. A more flexible chain would be required to shorten the intramolecular relaxation times. However, it is unlikely that relaxation times as short as 10^{-10} s will be observed for conformation changes in most polymers. Brillouin scattering has found great utility in the study of the glass–rubber relaxation in bulk amorphous polymers,[10] but the frequencies are too high to effectively probe the dynamics of intramolecular conformation changes for polymers in solution.

The local (high q) viscosity of polymer solutions has also been probed by several other techniques. Chapoy[11] has used fluorescence depolarization to measure the rotational relaxation time of probe molecules in concentrated polymer solutions. Boss, Stejskal, and Ferry[12] have studied self-diffusion of benzene in polyisobutylene solutions using NMR. Anderson and Liu[13] have measured spin–lattice relaxation times in benzene solutions of poly(methyl methacrylate). All the above studies demonstrated that the rate of the process being studied was determined by a local viscosity which changed only slightly upon addition of large amounts of polymers. Self-diffusion of benzene in PIB[12] correlated very well with the Fujita–Doolittle[14] theory of free volume. The importance of free volume in determining the local viscosity of polymer solutions should be a central concept in future theories of local viscosity. The local translational and orientational motions of the solvent are determined primarily by the available free space.

The concept of local viscosity is important for the understanding of bulk polymerization. While the macroscopic viscosity becomes large, the rate of polymerization will depend on the local mobility of the monomer. The local viscosity has now been studied as a function of conversion in the thermal polymerization of styrene using depolarized Rayleigh spectroscopy.[15] The collective orientational relaxation time increases only 50% after 80% of the reaction is complete. The technique of depolarized Rayleigh spectroscopy should prove to be very useful in the study of the local viscosity of polymer solutions.

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A ^{13}C Nuclear Magnetic Resonance Study of Gel-Forming (1→3)-β-D-Glucans: Molecular-Weight Dependence of Helical Conformation and of the Presence of Junction Zones for Association of Primary Molecules

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ABSTRACT: In order to gain better understanding of conformational behavior, aggregation of the helical segments, and its consequence to the gelation mechanism of gel-forming (1→3)-β-D-glucans, we have undertaken ^{13}C NMR studies of lower molecular-weight glucans of bacterial (1→3)-β-D-glucan (curdlan 13140) obtained from *Alcaligenes faecalis* var. *myxogenes* IFO 13140. It was found that, from the displacements of the ^{13}C chemical shifts of C-1 and C-3 with respect to those of glucans with $\overline{DP}_n < 14$, helix conformation is adopted by the glucans with $\overline{DP}_n \geq 49$. This is consistent with the ^{13}C NMR study of the mixed gel prepared by the mixture of curdlan 13140 and its lower molecular-weight glucans, in which $\overline{DP}_n \leq 20$ gives sharp ^{13}C signals characteristic of the random-coil conformation, suggesting that they are just trapped in the interstices of the gel network. Examination of the variation of the peak intensity vs. $\overline{DP}_n$ exhibits that the helical conformation is associated to form junction zones, presumably composed of the double- or triple-stranded helices. It was also shown that the line width of the glucan with $\overline{DP}_n \geq 49$ is approximately proportional to $\overline{DP}_n$. Thus, the broad linewidths observed in the physically cross-linked glucans and gels are ascribed to the entanglement of the polymers due to the presence of the cross-links. The evaluation of the correlation times of the local motions, on the basis of the approximation by the log χ^2 distribution, showed that there exists distinct difference between the glucans with finite network and the gels with the infinite network.

It is known that (1→3)-β-D-glucans function as structural components in the cell walls of many plants and microorganism and also act as reserve polysaccharides.[3] Thus, (1→3)-β-D-glucans isolated from various sources differ widely in their properties; laminaran is a water-soluble glucan, while pachyman is not; curdlan (from

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Alcaligenes faecalis var. *myxogenes* IFO 13140) is known to form a resilient gel on heating,[4,5] and some fungal branched (1→3)-β-D-glucans such as lentinan[6] (from *Lentinus edodes*), A_3 (ref 7) (from *Pleurotus ostreatus*), and schizophyllan[6] (from *Schizophyllum commune*) form rather soft gels without heating. In addition to the extent of branching and the presence of other glucosidic linkages, molecular weight of polysaccharides may play a dominant role in their properties. In this regard, Ogawa and coworkers[9] showed, on the basis of specific rotation, optical rotatory dispersion and complex formation with Congo Red, that (1→3)-β-D-glucans with $\overline{DP}_n < 25$ (water soluble) adopt a disordered conformation both in neutral and alkaline solution, whereas the glucans with higher $\overline{DP}_n$ (insoluble) adopt an ordered conformation.

In general, polymer gels are composed of the three-dimensional network[10,11] formed by the cross-links, chemically or physically introduced. In particular for polysaccharide gels, primary molecules of finite size are bound together through the formation of crystallites involving bundles of chains, or by multiple-stranded helices.[10] Recently, ^{13}C NMR spectroscopy has been proved to be a very powerful tool to analyze conformation and molecular architecture of both polysaccharides[6-8,12] and chemically cross-linked gels.[13] Moreover, the ^{13}C NMR has been successfully applied to the studies of other bulk materials such as solid rubber and amorphous polymers.[14-20] Accordingly, we showed that the ordered conformation of both the dilute alkaline solution and the resilient gels of the linear (1→3)-β-D-glucan (curdlan 13140) was identified as single helix conformation by ^{13}C NMR spectroscopy.[12] Subsequently, similar single helical conformation was also found in some fungal branched (1→3)-β-D-glucans in an intermediate of NaOH-induced conversion from gel to sol state.[6-8] Thus, it is natural to infer that the junction zone for the gel network in this case should be multiple-stranded helices.[21] In this connection, Takeda et al.[22,23] showed that most of the (1→3)-β-D-glucan molecules in the wet fibrous specimen have a single 7/1 helix and the rest have a triple-stranded 7/1 helical structure. The annealed specimen heated at a temperature above 100 °C with and without the presence of water gave a triple-stranded helix,[22,23] which is in agreement with the results by Marchessault[24] and Sarko.[25] Naturally the portion of the junction zones and their vicinities cannot contribute to the ^{13}C resonance signals,[6-8,12,13] since molecular motion of this region might be too slow to give high-resolution NMR. This is also true if such multiple-helical junction zones might aggregate further to form microfibrils, as can be seen by electron microscope.[5,26,27]

The objective of this work is to clarify the following questions. First, at what chain length does the (1→3)-β-D-glucan adopt helical conformation, and subsequently form junction zones? Second, can the ^{13}C NMR method provide information on the gelation condition of the polysaccharide gel? Third, how is the dynamic feature of polymer chains in the finite and infinite network? We thought that if these questions are resolved we can gain a clue for better understanding for the gelation mechanism of the (1→3)-β-D-glucan, combined with other information such as electron microscopic observation and X-ray diffraction analysis. Thus, we have undertaken to study molecular-weight dependence of the ^{13}C NMR parameters (^{13}C chemical shifts, line width, peak intensity, and relaxation data) by use of curdlan 13140 and its hydrolyzates. Here we show that the physically cross-linked structure appears in a sample of $\overline{DP}_n = 49$, in which the primary molecules adopt helix conformation. Comparison of the peak intensity vs. $\overline{DP}_n$ showed that adopting infinite network, corresponding to the gel formation, might occur at $\overline{DP}_n \sim 300$.

Experimental Section

Lower molecular-weight (1→3)-β-D-glucans were prepared from curdlan 13140 ($\overline{DP}_n = 540$) by hydrolysis with formic acid or sulfuric acid by the method previously reported.[28] Their chemical properties were similar to those of curdlan 13140 ($\overline{DP}_n = 540$), and gel filtration patterns of these preparations on a Sephadex G-150 or Sepharose CL-4B column each gave a symmetical peak, which indicated that they had a nearly normal distribution with regard to the degree of polymerization. The $\overline{DP}_n$ of each glucan was determined by the method of Manners et al.[29] Other (1→3)-β-D-glucans with $\overline{DP}_n = 299$ and 380 were obtained from the culture filtrate of *A. faecais* var. *myxogenes* (IFO 13140) under different fermentation times. All glucans used in the present study were supplied by Takeda Chemical Industries, Ltd. Osaka, Japan.

Those glucans were dissolved or suspended in D_2O by use of 10 mm o.d. sample tubes (80 mg/mL). It was found that only $\overline{DP}_n = 4$ and 8 were soluble in water, but other glucans with higher $\overline{DP}_n$ were insoluble. Heating those aqueous suspensions of acid hydrolyzates at 60 °C for 5 min was found to give a clear solution, but to give precipitates by cooling to ambient temperature, although the original curdlan 13140 gave a resilient gel (low-set gel).[12] The mixed gel composed of small molecular-weight glucan and curdlan 13140 was prepared in a similar manner as in the preparation of the resilient gel after mixing powders thoroughly in a ratio of 12.5 and 87.5%, respectively.

The complex-formation study[9,30] was preformed by use of absorption-maximum shift with Congo Red in the presence of the respective glucans in 0.1 M NaOH. Glucans with $\overline{DP}_n \geq 49$ showed the complex formation, indicating the existence of some ordered conformation.

^{13}C NMR spectra were obtained on a JEOL PFT-100/EC-100 spectrometer operating at 25.03 MHz. The 90° pulse requiring 22 μs was used to accumulate free induction decays with repetition times of 0.6 s. A delay time, 250 μs, between the end of the 90° pulse and the acquisition of the first data point, was introduced. All spectra were recorded using 4K points and a spectral width of 4 kHz. ^{13}C chemical shifts are expressed in parts per million downfield from external tetramethylsilane. Chemical shifts of the narrow components were ±0.1 ppm, while those of wide signals were ±0.5 ppm. The line width was taken as full width at half-height in an expanded spectrum, with an error of ±20–25%. Spin–lattice relaxation times (T_1's) were obtained using the pulse sequence of 180°–t–90°, with an estimated error of ±15%. Nuclear Overhauser enhancements were obtained from the ratio of the intensity of fully decoupled spectra to the intensity of spectra in which the proton noise decoupler was gated off to remove the NOE.[31] The waiting time was taken at least ten times the T_1's.[32,33] The estimated error of the NOE's was ±15%.

Results

Molecular Weight Dependence of ^{13}C NMR Spectra. Figure 1 shows the ^{13}C NMR spectra of (1→3)-β-D-glucans with various $\overline{DP}_n$'s, recorded in a neutral aqueous solution or suspension. Water-soluble glucans with $\overline{DP}_n$'s 4 and 8 gave rise to very sharp ^{13}C signals characteristic of the random-coil conformation.[12] Six intensed ^{13}C resonances are assigned to the C-1, C-3, C-5, C-2, C-4, and C-6 carbons, respectively, from downfield to upfield shift (from left to right). In the glucan with $\overline{DP}_n = 4$, there appear peaks arising from terminal α and β reducing units. Since glucans with $\overline{DP}_n > 14$ are insoluble in water, no ^{13}C resonance was observed among glucans with $\overline{DP}_n = 20$–131, while weak ^{13}C resonances appear in the glucan with $\overline{DP}_n = 14$, presumably due to the presence of water-soluble lower molecular-weight glucans. On the other hand, rather viscous aqueous suspension of glucan with $\overline{DP}_n = 540$,

Table I
Molecular-Weight Dependence of the T_1 (ms),[a] Line Width (Hz),[b] and NOE Values[c]

	$\overline{DP}_n$ = 49			$\overline{DP}_n$ = 131			$\overline{DP}_n$ = 540[d]		
	T_1	line width	NOE	T_1	line width	NOE	T_1	line width	NOE
C-1	87	41	1.8	57	47	1.6	76	161	1.3
C-2	102	23	1.9	69	51	1.4	78		1.5
C-3	105	49	1.8	64	53	1.8	84	167	1.3
C-4	118	22	1.2	93	53	1.5	80		
C-5	129	24	2.0	77	44	1.5	83		1.5
C-6	87	19	2.4	56	25	2.1	54	50	1.6

[a] Estimated error (±15%). [b] Estimated error (±10-15%). [c] Error (±15%). [d] Reference 10.

capable of forming the resilient gel on heating, gives weak but broad ^{13}C resonances.

In alkaline solution, we found that the ^{13}C resonance peaks of those (1→3)-β-D-glucans could be observed.[6-8,12] However, care should be taken in the concentration of NaOH, since conformational transition from helix to random coil is accompanied at higher alkaline concentration (>0.2 M NaOH). Our previous study on ^{13}C NMR showed that conformation of curdlan 13140 in lower alkaline concentration (<0.2 M NaOH) is essentially the same as that of the resilient gel.[12] Thus, we attempted to compare the peak positions, relaxation parameters, and absolute peak intensities of the ^{13}C NMR spectra of the glucans with various $\overline{DP}_n$'s at 0.06 M NaOH, as shown in Figure 2. Also the phenomenon of the alkaline degradation of (1→3)-β-D-glucans[34] is known. At 0.06 M NaOH, however, the alkaline degradation might be neglected, since the peaks asterisked in Figure 2, which are presumably ascribed to the degradation products, showed only minor contribution even in the lower molecular-weight glucans such as $\overline{DP}_n$ = 14 and 20. These peaks were, however, revealed to increase at higher alkaline concentration (0.09 M NaOH). Even at 0.09 M NaOH, no degradation of the higher molecular-weight glucan such as $\overline{DP}_n$ = 104 occurred (spectra not shown).

Interestingly, the line widths and peak positions of C-1 and C-3 are quite different between the glucans with $\overline{DP}_n$ = 14 and 49, the peak position of the latter being displaced downfield compared with that of the former. This is more clearly seen in a plot of the ^{13}C chemical shift vs. $\overline{DP}_n$ (Figure 3). Such a difference of the ^{13}C chemical shifts of C-1 and C-3 was previously explained by the presence of preferred rotamer population around the glucosidic bonds in the helical conformation,[12] in view of he similar difference of the C-1 and C-4 chemical shifts between cyclodextrins and linear (1→4)-α-D-glucans.[35] Although the ^{13}C chemical shifts of the random-coil conformation were varied with the concentration of NaOH (pH), those of the helix form were found to be unchanged up to 0.22 M NaOH concentration, at which the helix form is changed to the random coil.[12] Accordingly, the glucans with $\overline{DP}_n \geq 49$ should adopt the helical conformation. Furthermore, the observation of the reduced peak intensities of the backbone carbons (C-1–C-5), of glucans with $\overline{DP}_n \geq 49$, with respect to C-6 is consistent with taking rather rigid helix conformation with reduced NOE values.

The obvious decrease of the peak intensities of the glucans, especially with higher $\overline{DP}_n$'s (68, 82, and 102), however, cannot be explained by the reduced NOE alone (Figure 2). In Figure 4 is shown the relative peak intensities with respect to those of the $\overline{DP}_n$ = 14, after correction of the individual NOE values. Figure 4 also shows that the peak intensities of the glucans decrease with $\overline{DP}_n$ approximately up to 300. Such an apparent loss of the peak areas is obviously caused by the presence of the

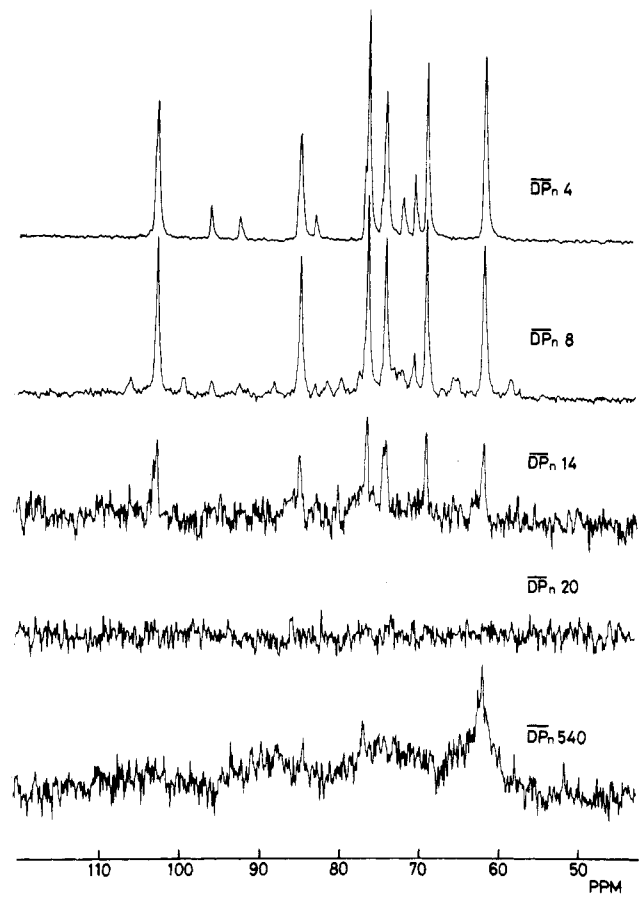

Figure 1. ^{13}C NMR spectra of (1→3)-β-D-glucans with various $\overline{DP}_n$ in neutral aqueous solution or suspension. Six intensed ^{13}C resonances are assigned to the C-1, C-3, C-5, C-2, C-4, and C-6 from downfield to upfield. Accumulation times: 20 000 for $\overline{DP}_n$ = 4, 8, and 14, 51 718 for $\overline{DP}_n$ = 20, and 96 500 for $\overline{DP}_n$ = 540.

physically cross-linked structure (junction zones), since it is expected that molecular motion of the junction zones and their vicinities might be strictly hindered.[36]

In Figure 5, the line widths of C-1, C-3, and C-6 are plotted against the $\overline{DP}_n$'s of the glucans. In contrast to the change of peak intensities in Figure 4, the line width is approximately proportional to the $\overline{DP}_n$, in the helix-forming glucans ($\overline{DP}_n \geq 49$). Table I summarizes the T_1's, line widths, and NOE's for some helix-forming glucans.

^{13}C NMR Spectra of the Resilient Gels Prepared by a Mixture of Curdlan 13140 and Its Acid Hydrolyzate. Along with the same procedure described previously,[12] we prepared a resilient gel consisting of low molecular-weight glucan (12.5%) and curdlan 13140 (87.5%). We noticed appearance of some brittleness in such gels, together with some turbidity. Interestingly, in Figure 6, the coexistence of the broad and sharp signals is clearly seen in the gels involving glucans with $\overline{DP}_n$ = 14 and 20 (bottom and middle traces of Figure 6, respectively). Such sharp ^{13}C

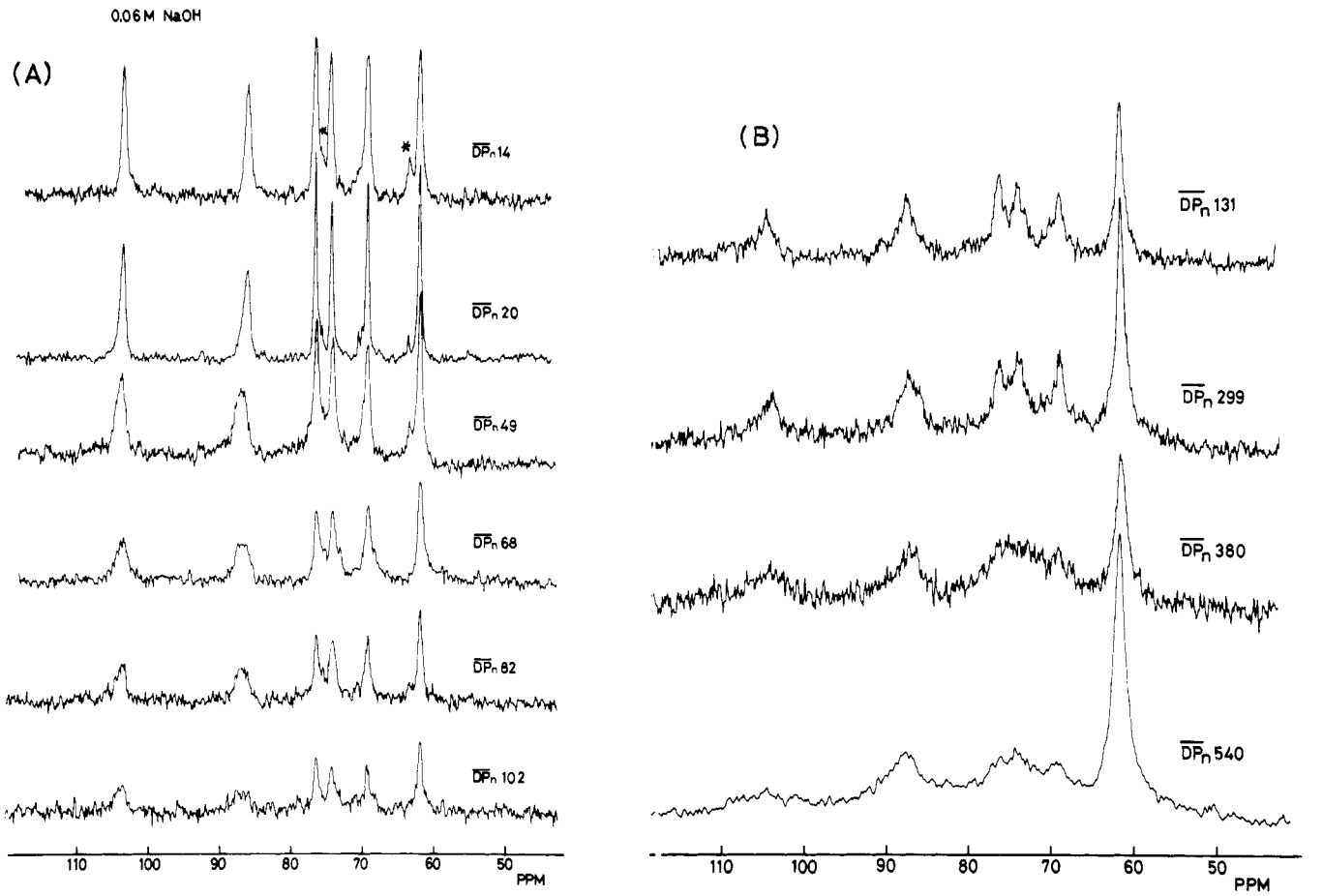

Figure 2. ^{13}C NMR spectra of (1→3)-β-D-glucans with $\overline{DP}_n$ = 14–540 in 0.06 M NaOH solution (for the assignment of peaks see Figure 3). Accumulation times: 20000 for $\overline{DP}_n$ = 14–82, 27198 for $\overline{DP}_n$ = 102, 45900 for $\overline{DP}_n$ = 131, 10941 for $\overline{DP}_n$ = 380, and 77000 for $\overline{DP}_n$ = 540 (note that the last trace was obtained by multiplying the time constant twice as large as the others).

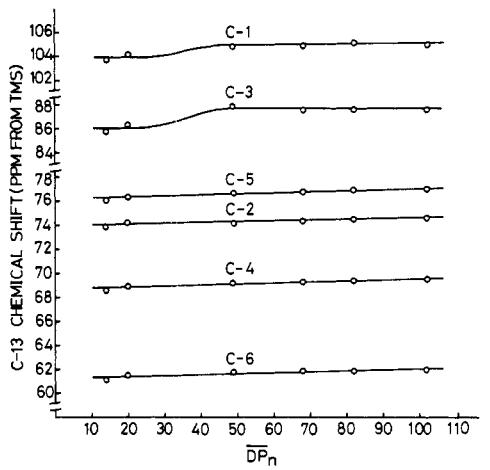

Figure 3. A plot of the ^{13}C chemical shifts vs. $\overline{DP}_n$'s of (1→3)-β-D-glucans with various $\overline{DP}_n$'s (data in 0.06 M NaOH solution).

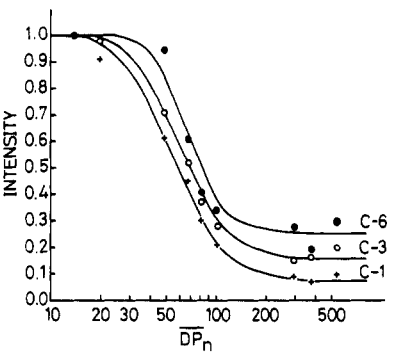

Figure 4. A plot of the peak intensities of C-1, C-3, and C-6, with respect to those of $\overline{DP}_n$ = 14, by taking into account the differences in NOE values vs. $\overline{DP}_n$ of (1→3)-β-D-glucans (0.06 M NaOH solution).

signals, on the other hand, disappear in the gels of the glucans with $\overline{DP}_n \geq 49$. On the basis of the chemical-shift positions, those sharp signals should be unequivocally ascribed to the randomly coiled molecules.[12] This assignment is confirmed by examination of their NOE values, which are almost identical to those in aqueous solution.[12] Furthermore, the ^{13}C chemical shifts of the broad components are exactly the same as those of the resilient gel and also of the glucans with $\overline{DP}_n \geq 49$ in dilute alkaline solution. Accordingly, it can be concluded that the glucan $\overline{DP}_n \geq 49$ participates in the gel network, which is consistent with the finding that the helix form is adopted in the glucans with $\overline{DP}_n \geq 49$. The peak intensities of the broad components were found to be almost identical among three traces in Figure 6, by taking into account the difference of accumulation times. The intensity of the narrow components in the gel involving $\overline{DP}_n$ = 20, however, was reduced by 10% compared with that involving $\overline{DP}_n$ = 14. Possibly, a portion of the glucan with $\overline{DP}_n$ = 20 might be involved in the gel network. In addition, the peak intensity of the sharp components in the gel involving $\overline{DP}_n$ = 14 (Figure 6, bottom) increased about tenfold compared with that in the aqueous suspension (Figure 1) in view of the difference of quantity of $\overline{DP}_n$ = 14 between two samples. A possible explanation of this finding is that isolated oligomers trapped in the interstices of the gel

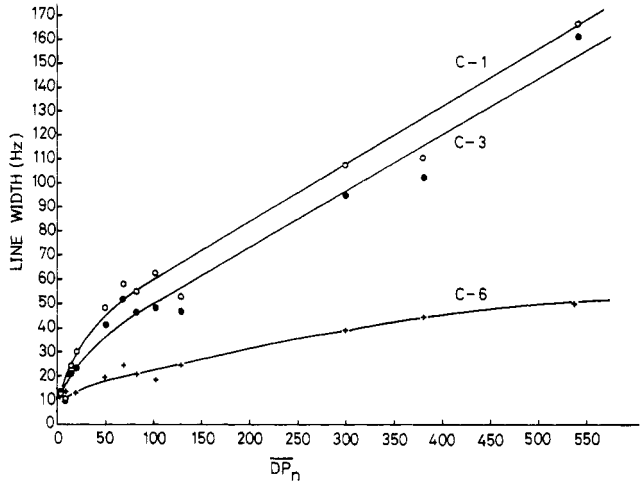

Figure 5. A plot of the line widths vs. $\overline{DP}_n$ of (1→3)-β-D-glucans in 0.06 M NaOH solution.

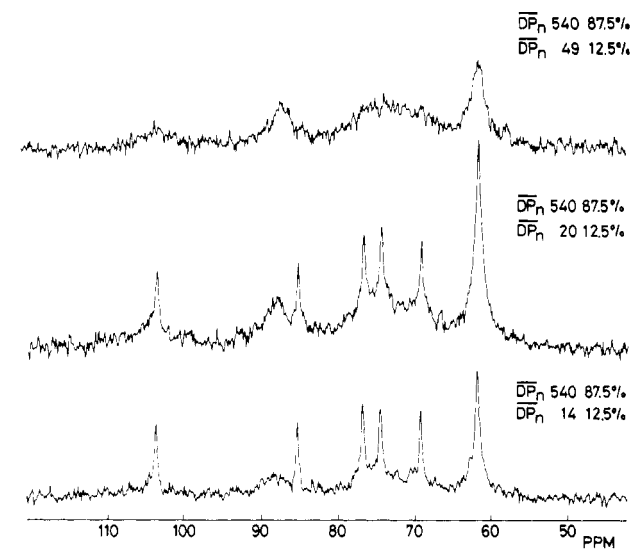

Figure 6. ^{13}C NMR spectra of the resilient gel consisting of curdlan 13140 (87.5%) and lower molecular-weight glucans (12.5%). Top: curdlan and $\overline{DP}_n$ = 49, 92998 accumulations. Middle: curdlan and $\overline{DP}_n$ = 20, 95649 accumulations. Bottom: curdlan and $\overline{DP}_n$ = 14, 46705 accumulations.

network, provided by the swelling of curdlan 13140, seem to prevent molecular association leading to the precipitation. Thus, these same glucans are participated in the gel network, and some are just trapped in the interstices, depending on the distribution of the molecular weight.

The Effect of NaCl to the Formation of Cross-Linked Structure. In addition to the above observation that the line width of the ^{13}C signals was varied considerably with $\overline{DP}_n$, we found that the peak profiles, resembling the cross-linked structure of $\overline{DP}_n$ = 49–131, could be produced by adding 1 M NaCl to the randomly coiled higher alkaline solution (0.38 M NaOH), as shown in Figure 7. Such change could not be observed in the presence of 0.5 M NaCl. This observation is consistent with the result[37] reported by Ogawa et al. They showed that the conformational transition from the random coil to the ordered form was caused by addition of NaCl. However, displacement of the ^{13}C shifts of C-1 and C-3 with or without 1 M NaCl seems to be rather small compared with that shown in Figure 3 (approximately half of the displacement). Moreover, the line widths of C-1–C-5 are considerably smaller (about 40 Hz and one-fourth of the resilient gel). This situation may be realized by the

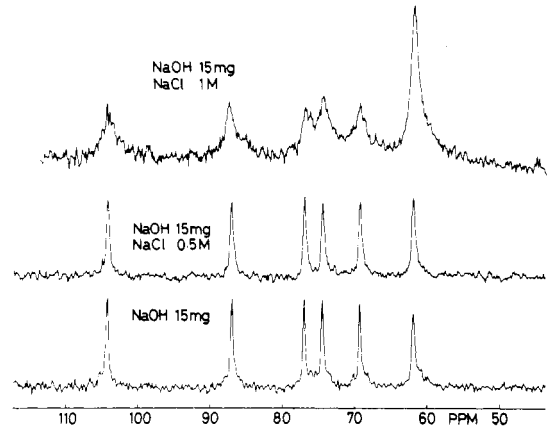

Figure 7. ^{13}C NMR spectra of curdlan 13140 in 0.38 M NaOH and the effect of NaCl to spectral change. Top: 1 M NaCl, 82002 accumulations. Middle: 0.5 N NaCl, 4500 accumulations. Bottom: no salt, 4500 accumulations.

presence of lower degree of cross-linking (junction zones) caused by the effect of NaCl. It should be mentioned here that the rate of the interconversion between the helix and random-coil form may be rather rapid in the NMR time scale, because no separate peaks for the two kinds of conformation were observed in Figure 6.

Discussion

Molecular-Weight Dependence of Helical Conformation and Formation of the Junction Zones. First, it should be emphasized that our ^{13}C NMR observation in gels is solely limited to the rather flexible *single* helical portion where molecular chain is fully exposed to diluent (water in this case) (the correlation times being shorter than 10^{-6} s), in view of the high-resolution condition.[38] Therefore, the ^{13}C resonance peaks of the gel probably arise from free ends or rather flexible middle parts of the single-helical chains tied at the junction zones, in addition to the helical chains trapped in the interstices of the network.[12] Similar interpretation may be applied for acid-hydrolyzed samples with lower $\overline{DP}_n$'s ($49 \leq \overline{DP}_n < 300$) incapable of forming a resilient gel after heating at a temperature above 55 °C, as described below.

In dilute alkaline concentration, it is concluded that, in the (1→3)-β-D-glucans, the shortest chain length to adopt the single helical conformation is about $\overline{DP}_n$ = 49. As judged from the observation of the loss of the peak areas (Figure 4), it follows that the longer the chain length of the primary molecules is the stabler are the multiple-stranded helices. This result is consistent with the theoretical prediction: the triple- and double-stranded helices are energetically more stable than the single helix in (1→3)-β-D-glucan.[25,39-41] Further, the triple-stranded helix was shown to be the major form in the annealed fiber samples of the original glucan as revealed by the X-ray diffraction studies.[22-24] In the dilute alkaline and neutral gel states, however, such conformation of the minimum of energy cannot always be achieved as manifested from the presence of the sizable amounts of the single-helical portion (Figure 2). From the statistical point of view, the extent of forming the multiple-stranded helices may simply depend upon the chain length. Therefore, higher degree of cross-linking may be achieved for the samples with larger $\overline{DP}_n$. This view is supported by the observation that the line widths are varied approximately in proportion to the $\overline{DP}_n$ (Figure 5). This situation is very similar to that of the chemically cross-linked gel of synthetic polymers[13] in which the line width was found to be mainly influenced

Table II
Observed[a] and Calculated T_1 (ms), NOE, and Line Width (Hz) Values and Average Correlation Times[b] of Segmental Motions

	$\overline{DP}_n$ = 49					$\overline{DP}_n$ = 131					$\overline{DP}_n$ = 540				
	p	$\bar{\tau}$, ns	T_1	NOE	line width	p	$\bar{\tau}$, ns	T_1	NOE	line width	p	$\bar{\tau}$, ns	T_1	NOE	line width
obsd			108	1.7	31			72	1.6	50			80	1.4	164
calcd															
I[c]	10	0.4	109	2.1	33	14	2	67	1.8	40	26	30	83	1.3	131
II[d]	8	0.7	98	2.0	20	8	5	73	1.8	53	8	40	90	1.5	123

[a] Average for five carbons (C-1 to C-5). [b] Approximation by the log χ^2 distribution. The parameters p and $\bar{\tau}$ stand for the width parameter and average correlation time, respectively. b = 1000. [c] Not truncated. [d] Slow motions (τ > 1000 ns) are neglected.

by the degree of cross-linking. On the contrary, in spite of the higher $\overline{DP}_n$ (540), the line widths of curdlan 13140 are considerably smaller under the presence of 1 M NaCl in addition to the high concentration of NaOH (0.38 M) (see Figure 7 and also Figure 5). This observation is thus clearly explained in that a very low degree of the physical cross-linking is attained in this case.

The results in the dilute alkaline state seem to be straightforwardly applied to the conformational behavior in the neutral media, as shown partly in the study of the mixed resilient gel (Figure 6) and also the comparative ^{13}C NMR study of the original glucan in the resilient gel and in dilute alkaline solution.[12] However, there appears an apparent distinction between two kinds of preparations, in the neutral medium and in dilute alkaline concentration, for the lower molecular-weight glucans incapable of forming gels. In fact, no ^{13}C NMR signals could be observed in the neutral medium because of precipitation of samples except in the mixed gel (Figure 6). This may be the case in that diluent cannot be effectively held in the interstices, owing to the lack of sufficient physical cross-links to lead to the infinite network. In addition, the multiple-stranded helical portion seems to be more hydrophobic, as suggested by Takeda et al.[22,23] and Marchessault et al.[24] Thus, in the neutral media, it is important to take into account that the hydrophobic portion tends to be further aggregated to form a small crystallite region at higher concentration, as in the present situation, similar to the aggregation of the collagen triple helix in gelatin.[10,42] Therefore, the microfibrils or elementary fibrils observable with an electron microscope[26,27] might be composed of the aggregated multiple-stranded helices. This view may be consistent with the following findings in the resilient gel. First, the resilient gel prepared at higher temperature (high-set gel) exhibits turbidity and the transmittance decreases gradually with increase in temperature from 60 to 100 °C.[43] Second, the gel strength increases with temperature in the region 80–100 °C.[44] Third, gels prepared at 55 °C (low-set gel) tend to show turbidity by standing samples at 4 °C for a long period (say, 1 month), with concomitant occurrence of synerisis. Further, we noticed that the peak intensities of the ^{13}C resonances are considerably decreased for the samples with turbidity and synerisis, compared with those of the freshly prepared low-set gel. Accordingly, these observations are satisfactorily explained by the degree of the physical cross-linking being considerably increased by the aggregation of the multiple-stranded helices as extra cross-links. For this reason, it follows that addition of lower NaOH concentration (<0.2 M) is effective in dispersing the aggregated helices as solution for the acid-hydrolyzed glucans. This is also true for the higher molecular-weight glucans, capable of forming gel on heating, since swelling by water is easily achieved without heating.

Molecular-Weight Dependence of the Segmental Motions. It is likely that the segmental motions of the backbone, in the cases of the physically cross-linked glucans and their gels, may be highly heterogeneous because of a possible distribution of segments in different physical environments. For this reason, it may be more appropriate to employ the model of the log χ^2 distribution of the correlation times[19,45] as an isotropic reorientation, as a first approximation. Intrinsically, such molecular motions of the helix should be anisotropic tumbling motions. Nevertheless, the use of the isotropic tumbling model may be justified by the following reasons, in addition to its simplicity. First, there appears no specific difference of the relaxation parameters in the carbons of C-1 to C-5, in spite of the different orientations of these CH vectors with regard to the helical axis. Second, the isotropic tumbling model[46] was successfully employed to reproduce the relaxation parameters of the helical forms of simple polypeptides.[47,48]

To describe the distribution of the segmental motions, we use the formula proposed by Schaefer,[45] as follows.

$$F^{(p)}(s) = (ps)^{p-1}e^{-ps}/\Gamma(p) \quad (1)$$

with

$$s = \log_b [1 + (b - 1)\tau/\bar{\tau}] \quad (2)$$

where $F^{(p)}(s)$ is the probability density function of the correlation time τ. In eq 1, p is used to describe the width of the distribution of the correlation times. As p becomes larger, the distribution becomes narrower.[19,45] In this approximation, the average correlation time, $\bar{\tau}$, is a parameter of the computation

$$f(w_i) = \int_0^\infty \frac{\bar{\tau}F^{(p)}(s)\{\exp_b s - 1\}\,ds}{\{b - 1\}\{1 + w_i^2\bar{\tau}^2[\{\exp_b s - 1\}/b - 1)]^2\}} \quad (3)$$

instead of

$$f(w_i) = \tau^2/(1 + w_i^2\tau^2) \quad (4)$$

The logarithmic time scale, b, is usually taken as 1000.

The best-fit values of the average correlation times to reproduce the relaxation parameters (average of five carbons) are shown in Table II (calculation I). In agreement with the expectation, the average correlation time of the gel ($\overline{DP}_n$ = 540) is much larger than that of the cross-linked polymers with finite network ($\overline{DP}_n$ = 49 and 131). Unexpectedly, the width parameter, however, must be chosen to become larger with increasing molecular weight of the primary molecules. A similar situation was encountered by Komoroski et al.[18] in that in their temperature-variation study of the relaxation parameters of bulk *cis*-polyisoprene, the width parameter obtained from the log χ^2 distribution resulted in narrow distribution in the increased freedom at higher temperature. As pointed

out by Torchia et al.[49,50] this may be caused by a feature of this approximation that the long tail of the log χ^2 function overemphasizes the contribution of the larger correlation times. This trend is remarkable for the cases with longer average correlation times as in the vicinity of the T_1 minimum. Accordingly, another set of calculations was made for the T_1, NOE, and line widths, by truncating the contribution of the correlation times 500–1500 ns, in the integration of eq 3.[51] By doing this, it is found that the T_1 and NOE values, which are mainly influenced by fast segmental motions, are almost unchanged, although the line width is changed over a factor of 100. In Table II is summarized the results by the use of the truncated log χ^2 distribution at 1000 ns (calculation II). Although the values of the average correlation times are not exceedingly altered by this treatment, the problem of the width parameter is far more improved. In view of the width parameters previously reported for synthetic polymers in solution and solid rubber,[19,45] elastin,[49] and bovine nasal cartilage,[50] the choice of $p = 8$ for the cross-linked helical glucans seems to be reasonable.

Gelation Condition. On the basis of the foregoing results and discussion, it is now reasonable to consider that gelation of the higher molecular-weight linear (1→3)-β-D-glucans arises from the formation of the infinite networks formed by the multiple-stranded helical junction zones.[52] Additionally hydrophobic aggregation of those multiple-stranded helices could be served as the extra cross-links, especially for the high-set gel. For this reason, it seems worthwhile to examine the critical condition, as viewed from ^{13}C NMR parameters, to form the infinite network to lead gelation. Following Flory,[10,11] the critical condition to form infinite network is given by

$$\rho_c = 1/\bar{y}_w \qquad (5)$$

where ρ_c is the density of the cross-linking and $\bar{y}_w$ is the weight-average molecular weight of the primary molecules. If we assume that the distribution of the molecular weight of the respective glucans is identical, $\bar{y}_w \propto \overline{DP}_n$. This assumption may be reasonable for acid-hydrolyzed glucans with $\overline{DP}_n$ = 4–131 in view of their gel filtration patterns on a Sephadex G-150 column, but not strictly for glucans with higher $\overline{DP}_n$'s, 299, 380 and 540, which obtained from the culture filtrate of *A. faecalis* var. *myxogenes* (IFO 13140). Thus the degree of the cross-linking may be $\rho_{eff}\overline{DP}_n$, where ρ_{eff} stands for the effective density of the physical cross-linking.[53] Then, the infinite network may be formed when $\rho_{eff} \geq \rho_c$. Such a condition will be satisfied in the $\overline{DP}_n$ at which the peak intensity becomes constant, ca. 300, as shown in Figure 4. This result is in good agreement with that by Ogawa et al.[9] that the rotation angle of the specific rotation becomes constant at $\overline{DP}_n$ around 200, suggesting the occurrence of gelation. On the contrary, the absorption maximum shift of Congo Red as a result of complex formation with the glucans is shown to occur at $\overline{DP}_n$ ca. 20.[9] Therefore, the shift of the absorption maximum of Congo Red seems to be closely related to helix formation, while the specific rotation is mainly related to the region of the cross-linked structure (multiple-stranded helices). In this connection, Ogawa and Hatano[54] recently showed, on the basis of the circular dichroism measurement for the D-glucan–Congo Red systems, that the single helical part of the D-glucan chain makes the complex with Congo Red.

As pointed out in the introduction, there appears a difference in gelation between the linear and branched (1→3)-β-D-glucans. In the latter, rather soft gels were formed without heating.[6-8] In addition, gel formation of the branched (1→3)-β-D-glucan was observed for the glucan with lower molecular weight[6] (fraction IV, 16200), which corresponds to $\overline{DP}_n$ = 90 incapable of forming gel in the linear glucan. However, our previous studies on the ^{13}C NMR and complex formation with Congo Red in dilute alkaline concentration showed that the physically cross-linked structures are essentially the same for both types of glucans.[6,8] Such an apparent contradiction can be easily compromised by the highly branched structure (tree-like structure) in the branched glucans being more favorable for the formation of physically cross-linked structure (multiple-stranded junctions) without requiring kinked structure in view of the classical theory of gelation.[10,11] Therefore, a much higher degree of the cross-linking may be formed for the branched glucans;[55] in fact, all of the β-(1→3)-linked glucosidic residues are completely suppressed in the gel of the neutral medium.[6-8]

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Carbon-13 Nuclear Magnetic Resonance Study on Sequence Distribution and Anomalous Linkage in Ethylene–Vinyl Alcohol Copolymers

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ABSTRACT: In the ^{13}C-NMR spectra of ethylene–vinyl alcohol copolymers prepared by the hydrolysis of ethylene–vinyl acetate copolymers, six well-resolved methylene carbon lines have been observed, one of which has been assigned to an anomalous 1,4-glycol structure arising from monomer inversion in the radical copolymerization. The six methylene carbon lines can be assigned to five-carbon sequences along a main chain, while the mole fractions of the dyad and triad monomer sequences can explicitly be determined from the intensities of the lines only when the monomer inversion is negligible. Relatively larger amounts of the 1,4-glycol were observed (2 to 6% of the total intensity of the methylene lines) for the samples polymerized at a higher temperature or with higher extents of conversions. From this observation and numerical calculations of "terpolymerization" on the basis of the first-order Markoffian statistics including monomer inversion, the reactivity ratio of the addition of a vinyl acetate monomer in the inverted (head-to-head) mode to the addition in the normal (head-to-tail) mode at an ethylene radical chain end has been determined to be 0.07 at 88 °C and below 0.02 at 60 °C. It has been confirmed that the probability of monomer inversion increases for the copolymerization in comparison with the homopolymerization of vinyl acetate, but, on the other hand, the normal head-to-tail linkage is still predominant even for the addition to an ethylene radical chain end.

I. Introduction

Predominance of head-to-tail linkage in radical polymerization of vinyl monomers has generally been accepted, although three other different modes of linkage are formally possible, viz., head-to-head, tail-to-tail, and tail-to-head, where "inversion" of monomer in polymerization is concerned. (The group =CHR is referred to as the head of a monomer.) This fact has been explained by some qualitative considerations of steric factors and resonance effects[1-3] and by theoretical treatments based on molecular orbital theory.[4] In practice, only small amounts of 1,2-glycol structure have been detected for poly(vinyl alcohol), obtained from the hydrolysis of poly(vinyl acetate): 1.5% at a polymerization temperature of 60 °C and 2.0% at 100 °C.[5,6]

More frequent occurrence of monomer inversion might be expected for ethylene–vinyl monomer copolymerization, because a steric hindrance between substituents is ap-

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