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STRUCTURAL STUDY OF AMYLOSE POLYMORPHS BY CROSS-POLARIZATION–MAGIC-ANGLE SPINNING, ^{13}C-N.M.R. SPECTROSCOPY

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ABSTRACT

C.p.–m.a.s. ^{13}C-n.m.r. spectra and ^{13}C spin–lattice relaxation times (T_1) at room temperature have been measured for recrystallized samples of different polymorphs of amylose. Although the spectra of A- and B-amyloses in the dry state are very broad and almost structureless, the respective resonance lines of the samples soaked in H_2O narrow markedly, and fine splittings can be observed in the C-1 lines, namely, a triplet for the A-type crystal form and a doublet for the B-type crystal form. These multiplicities had already been reported for native A- and B-starches, but the resolution is much higher for the recrystallized samples. In contrast, all resonance lines are composed of single signals for the hydrated and anhydrous V-amyloses, without any significant effect of hydration. It is also found that ^{13}C T_1 values of the crystalline components of amyloses are much shorter than those of celluloses. This suggests that the enhanced torsional motion about the α-D-(1→4)-glycosyl linkages is allowable for amyloses, even in the crystalline region, because of the flexible, helical structure.

INTRODUCTION

Amylose, which constitutes one of the main components of starches, together with the branched amylopectin, is a polymer of D-glycosyl residues joined by (1→4)-α-D-glycosyl linkages. In crystals, this molecule adopts *n*-fold helical structures with relatively large *n* (*n* = 4–8), while cellulose, having (1→4)-β-D-glycosyl linkages, forms an almost linear, twofold helical structure. Three distinct polymorphs have been recognized, and are referred to as A-, B-, and V-amyloses. Recent X-ray analyses[1–4] have revealed that the amylose molecules possess a 6_1 right-handed, double helix in both A- and B-amyloses, whereas they form a 6_1 left-handed, single helix in V-amyloses. These two types of helices differ greatly in pitch per turn for one single helix; 20.82–21.06 Å for the A- and B-forms, and 7.92–8.04 Å for the V-forms, respectively. As a result, V-amylose has a relatively

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large cavity in the center of the helix, allowing formation of complexes with small organic molecules and with iodine, but there is no such space in A- and B-amyloses, where the helices are rather extended.

The A- and B-amyloses are thought to have similar molecular conformations, but to differ with respect to the packing of the helical chains. Furthermore, water molecules are situated in different positions in the different crystal forms. These features of amylose with respect to chain conformation, helical structure, and helix packing have been revealed by a large number of X-ray analyses. However, the detailed morphological structure, including the noncrystalline structure, is not yet clear (as in the case of cellulose), and also, little is known about the molecular mobility in the solid state. Some interesting results[5–10] for native starches have already been reported on using the cross-polarization–magic-angle spinning (c.p.–m.a.s.), ^{13}C-n.m.r. technique, but there has been no c.p.–m.a.s. ^{13}C-n.m.r. study of amylose samples crystallized under well defined conditions We have now applied the c.p.–m.a.s. ^{13}C-n.m.r. technique, found to be very powerful in characterizing the crystalline and noncrystalline regions of cellulose[11–16], to the structural analysis of recrystallized samples of different polymorphs of amylose.

EXPERIMENTAL

Commercial amyloses with $\overline{M}$ 2,900, 16,000, and 1,000,000, provided by the Hayashibara Institute of Biological Chemistry and Wako Chemicals, Ltd., were used for preparation of A-, B-, and V-amyloses without further purification. Amyloses with $\overline{M}$ 2,580 and 7.3×10^5 were kindly supplied by Professor S. Hizukuri of Kagoshima University for preparation of highly crystalline A-amylose and of amorphous amylose.

According to Hizukuri *et al.*[17], A-amylose crystallizes from a relatively concentrated aqueous solution at higher temperatures, while B-amylose forms from a rather dilute solution at lower temperatures. We prepared highly crystalline A-amylose by crystallizing amylose of $\overline{M}$ 2,580 from a 45% (w/w) aqueous solution at 30°. Highly crystalline B-amylose was successfully prepared from a 10% (w/w) aqueous solution of amylose with $\overline{M}$ 2,900 at 23°.

The A- and B-amyloses can also be prepared by solid-state deacetylation of amylose triacetate[2] Because this method is convenient for samples with higher molecular weight, we prepared high-molecular-weight A-type and B-type amyloses as follows First, amylose triacetate was prepared in formamide[18] from commercial amylose ($\overline{M}$ 10^6) and then converted into the KOH-amylose complex by the solid-state deacetylation with 3:1 (v/v) ethanol–water and 0.2M potassium hydroxide. After the alkali–amylose had been exposed to 80% relative humidity for 1 h at 85°, A- and B-amylose samples were prepared by soaking in de-ionized water for 1 h at room temperature and at 90°, respectively.

V-Amylose can be precipitated from a solution of starch or amylose by such an alcohol as 1-butanol When the molecular weight is not high enough, however,

B-amylose is formed under the same conditions. We prepared V-amylose by the method by Buleon *et al.*[19], using the low-molecular-weight amylose ($\overline{M}$ 16,000); 0.1g per dL of M NaOH solution was dialyzed at room temperature against 3:2 (v/v) water–ethanol. A flocculent, crystalline precipitate produced after one day was centrifuged off, and washed repeatedly with the same mixed solvent as used for dialysis. The undried sample thus obtained is referred to as V_h-amylose. Anhydrous V-amylose, (herein after referred to as V_a-amylose), was also obtained by drying the V_h-amylose sample to constant weight under diminished pressure at room temperature. X-Ray diffraction patterns for the respective samples sealed in capillary tubes were obtained by using nickel-filtered CuKα radiation with Rigaku–Denki Rotaflex RU-100, to confirm the existence of the corresponding crystal structures before n.m.r.-spectral measurements.

C.p.–m.a.s. ^{13}C-n m.r. measurements were carried out at room temperature with a JEOL JNM-FX200 spectrometer operated under a static magnetic field[9,15] of 4.7 T; ^{1}H and ^{13}C r.f. field strengths, $\gamma B_1/2\pi$, were 69 kHz for the c.p. process, while the ^{1}H dipolar decoupling field was lessened to 54 kHz. Each sample was packed into a m.a.s. rotor with an O-ring seal[9,15] together with sufficient de-ionized water or the solvent used for crystallization, unless otherwise stated. The m.a.s. rate was 3.2–3.5 kHz, and the contact time was 2.0 ms throughout this work. ^{13}C-Chemical shifts relative to tetramethylsilane (Me_4Si) were determined by using the peak at 33.6 p.p.m. of a small chip of crystalline linear polyethylene inserted as the internal standard.

RESULTS AND DISCUSSION

1. C.p.–m.a.s. ^{13}C-n.m.r. spectra of amylose polymorphs. — A-Amylose. Fig. 1 shows the 50-MHz, c.p.–m.a.s. ^{13}C-n.m.r. spectra of highly crystalline A-amylose in the dry and hydrated states. The respective resonance lines of the dry sample are very broad, and almost structureless, as is frequently observed for amorphous polymers. Similar broad spectra were also obtained for starches, as well as lyophilized amylose in the dry state[7–9]. This sort of broadness is mainly caused by the distribution of the isotropic chemical shifts, due to nonequivalent magnetic environments which are possibly produced by the differences in molecular conformation and packing. Irrespective of the low resolution, however, the contributions from the crystalline and noncrystalline components, as in the cases of starches[8], can be recognized. A relatively narrow peak at ~101.5 p.p.m. belongs to the crystalline component of the C-1 line, while the downfield shoulder and the upfield tailing of this line are both assigned to the noncrystalline part. In addition, the broad resonance at ~82 p.p.m. is due to the noncrystalline component of the C-4 site.

When this sample is soaked in de-ionized water, the respective lines narrow markedly, and the fine splittings can be observed, as shown in Fig. 1b. Similar marked effects of hydration were observed for starches[7,9], but the resolution of

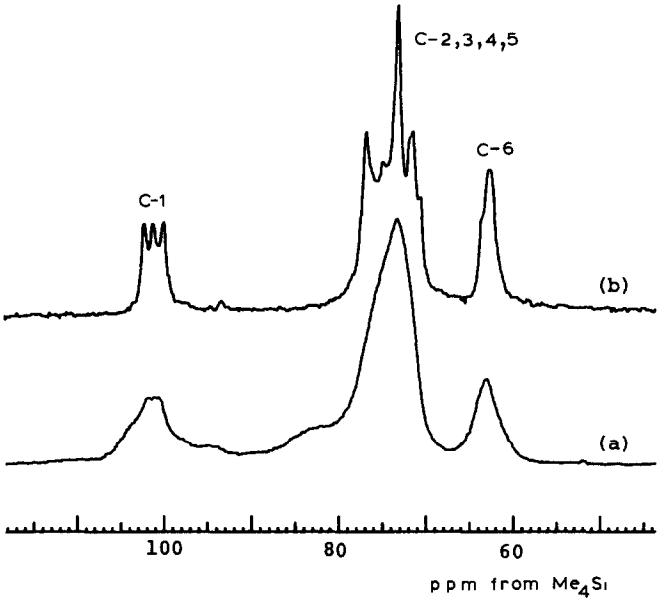

Fig 1 50-MHz, c.p –m a s ^{13}C-n m r. spectra of highly crystalline A-amylose; (a) dried, (b) hydrated

each line is much higher for this recrystallized sample. In particular, the C-1 line clearly splits into a triplet with almost identical intensity, although this triplet was not so easily observed for corn starch[7–9]. It is therefore concluded that a triplet appears in the C-1 line for a recrystallized A-type sample and corn starch (frequently classified as an A-starch) which both have the crystal structure referred to as that of A-amylose.

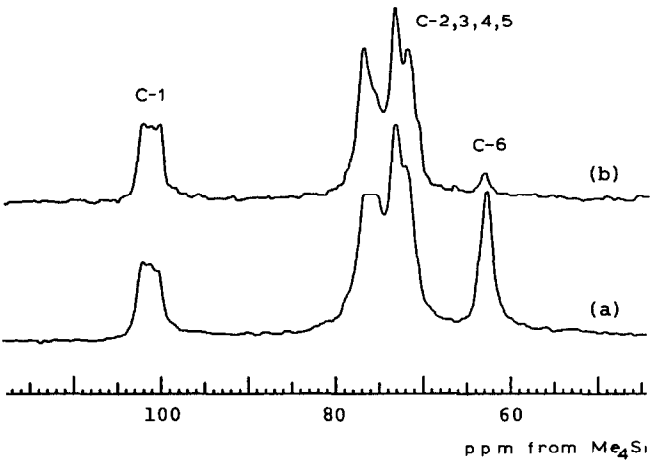

Fig 2 50-MHz, c p –m a s ^{13}C-n m r spectra of high-molecular-weight A-type amylose in the hydrated state, (a) whole spectrum, (b) crystalline component.

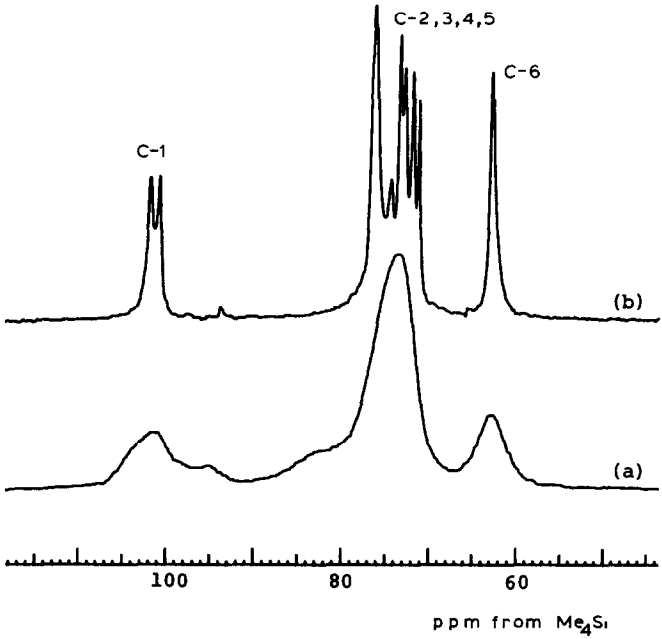

Fig 3. 50-MHz, c p.–m.a s ^{13}C-n.m r spectra of highly crystalline B-amylose, (a) dried, (b) hydrated

In the chemical-shift range of 70–80 p.p.m., several sharp lines also appear, but their assignments are not easy at present, because these resonances are probably also composed of lines with multiplicities. In contrast, the C-6 resonance at 62.7 p.p.m. is relatively simple; only a small additional shoulder appears at 63.5 p.p.m.

On the other hand, hydration also induces the disappearance of the non-crystalline resonances which are recognized in C-1 and C-4 sites of the dried sample as already described. Similarly, almost the same contributions from the non-crystalline region have also been found to disappear in the spectra of starches[7–9]. This sort of disappearance suggests the increase in the fraction of the ordered region, which is composed of double helices of amylose or amylopectin. Thus, it should probably be assumed that some D-glucosyl residues of the double helices may be unwound and disordered from their ends upon drying. However, such disordering of the helices are thought to be reversible, because almost the same c.p.–m.a.s. ^{13}C-spectra as shown in Fig. 1b were obtained for both never-dried and rehydrated samples.

Fig. 2a shows the c.p.–m.a.s. ^{13}C spectrum of the high-molecular-weight, A-type amylose in the hydrated state. The triplet in the C-1 line is not clear in this spectrum, as is frequently seen for corn starch[7–9]. However, the triplet is more clearly recognized in the spectrum (Fig. 2b) of the crystalline component, which was selectively recorded as a component having a longer ^{13}C spin–lattice relaxation time (T_1) by using Torchia's pulse sequence[20]. It has therefore been confirmed that

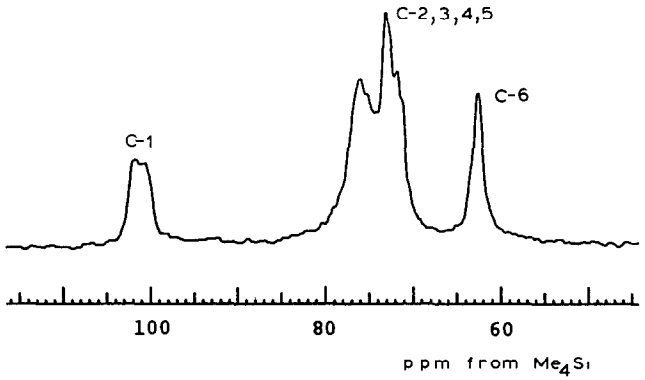

Fig. 4 50-MHz, c p –m a s ^{13}C-n.m r spectra of high-molecular-weight B-type amylose in the hydrated state.

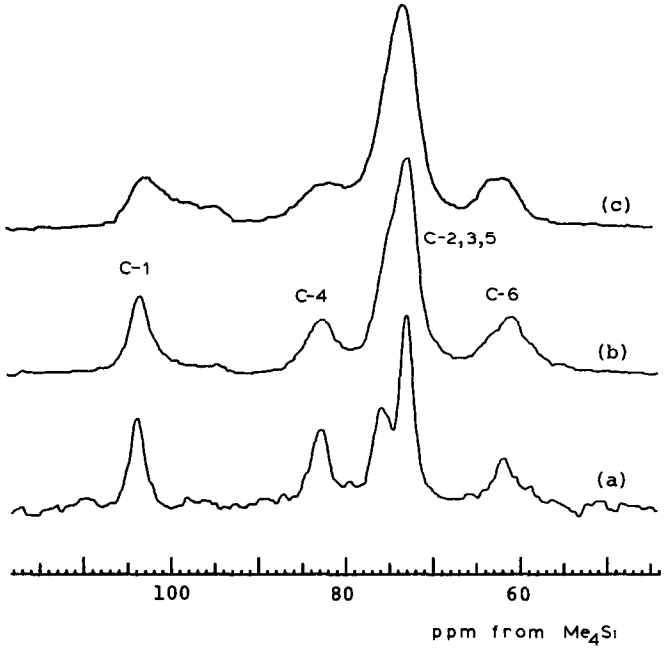

Fig. 5 50-MHz, c p –m a.s ^{13}C-n.m r. spectra of (a) V_h-amylose, (b) V_a-amylose, and (c) amorphous amylose

this crystal form is A-amylose, in good agreement with the previous X-ray crystal analysis[2]. Somewhat less resolution of the triplet, compared to that of the highly crystalline sample (Fig. 1b), may be due to less ordering in the crystalline region. In addition, smaller intensity of the C-6 line is due to the considerably shorter T_1 of the line, compared to the values of other lines (when Torchia's pulse-sequence is used, the intensities of the respective lines depend on their T_1 values).

B-Amylose. Fig. 3 shows the c.p.–m.a.s. ^{13}C spectra of the highly crystalline

B-amylose in the dried and hydrated states. The spectrum of the dried sample is also very broad, and almost indistinguishable from that of the dried A-amylose (see Fig. 1a). However, striking changes in the spectrum also occur in this sample upon addition of water; the C-1 line splits into a doublet with almost equivalent intensity, and several sharp lines appear in the range of 70–80 p.p.m. A similar doublet in the C-1 site was also observed for potato starch[7–9], although the resolution was much lower for that sample. Because potato starch also has the B-type crystal form, it has been confirmed that the C-1 doublet is well associated with the B-type crystal structure of amylose. As the discussion of the effects of hydration is almost identical to that for A-amylose, it is not repeated herein.

In Fig. 4 is shown the c.p.–m.a.s. ^{13}C spectrum of the high-molecular-weight, B-type amylose. The C-1 line appears to split into a doublet, irrespective of quite low resolution, indicating that this sample has the B-type crystal form, in accord with the results of X-ray analysis[2]. Because no contribution from the noncrystalline region can be observed in the C-1 and C-4 lines, all of the D-glucosyl residues may be wound up to form the double helices without any large disorder, as in the case of the high-molecular-weight A-type amylose.

V-Amylose. Fig. 5a shows the c.p.–m.a.s. ^{13}C-n.m.r. spectrum of V_h-amylose. Although each line is fairly broad, compared to the cases of A- and B-amyloses, the spectrum clearly indicates that this sample has a crystal form differing from those of A- and B-amyloses. Especially, the C-4 line is well separated from the lines of the C-2, C-3, and C-5 atoms in the upfield region.

In Fig. 5b is shown the c.p.–m.a.s. ^{13}C spectrum of anhydrous V-amylose (V_a-amylose), which was obtained by drying the V_h-amylose sample to constant weight under diminished pressure at room temperature. The chemical shift of each line stays almost constant upon drying, although the resolution is somewhat decreased. The absence of any change in chemical shift suggests that there is no large change in helical structure and packing for the anhydrous amylose; this appears to be in good accord with the X-ray diffraction results[1,3].

For comparison, the spectrum of amorphous amylose is shown in Fig. 5c; it

TABLE I

^{13}C CHEMICAL SHIFTS OF DIFFERENT CRYSTALLINE POLYMORPHS OF AMYLOSE

Crystal form	*Chemical shift (p p m.)*			
	C-1	*C-4*	*C-2,3,5*	*C-6*
A-Amylose	102 30, 101 32	76.90, 76 32[a], 75 05, 73 20		63.67[a]
	100.05	71 94[a], 71.45, 70.77[a]		62 74
B-Amylose	101 71, 100 74	76.12, 74 27, 73 20		62.69
		72 72, 71 74, 71.06		
V_h-Amylose	103.85	83 03	76.03, 73 11	62 21
V_a-Amylose	103 76	83 13	73 79	61 92

[a]Shoulder

is almost identical with the spectra of lyophilized amylose and gelatinized and ethanol-precipitated potato starch[8]. These spectra of amorphous materials seem to be somewhat similar to the spectrum of V_a-amylose, mainly because, in both cases, the C-4 resonance lines appear at positions well separated from the C-2, C-3, and C-5 resonance lines. However, the shape of the C-1 line of the amorphous sample greatly differs from that of V_a-amylose. The former line looks like a composite of a relatively sharp resonance and upfield tailing, while the latter is composed of a rather sharp, single line. Moreover, the chemical shifts of the downfield C-1 line, as well as the C-4 line, are ~1 p.p.m. smaller than the corresponding values for V_a-amylose. These differences must stem from the significant differences in molecular conformations and packing between these two samples.

2. Chemical shifts and molecular conformation. — The ^{13}C chemical shifts of the respective carbon atoms of amylose are summarized for different polymorphs in Table I. Because not enough data for oligosaccharides having α-(1→4)-glycosidic linkages are available, it is not possible to ascertain relationships between ^{13}C chemical shifts and torsion angles associated with the α-(1→4)-glycosidic linkages, similar to that for cellulose and oligosaccharides having β-(1→4)-glycosidic linkages[12,14,16]. However, the chemical shifts of the C-1 and C-4 atoms of V_h- and V_a-amylose are much higher than those of those in A- and B-amyloses, compared to the differences in chemical shifts within the V_h- and V_a-amyloses, or within the A- and B-amyloses. This indicates that the C-1 and C-4 chemical shifts are dependent on the helical structure, and thus, on the torsion angles ϕ and ψ about the α-(1→4)-glycosidic linkages.

In contrast to such a large difference in chemical shift, fine splittings within 1–2 p.p.m , such as multiplicities in the C-1 lines of A- and B-amyloses were not unambiguously explained in terms of a specified, structural factor. In particular, the cause of the fine splittings of the C-1 and C-4 lines of cellulose is still a subject of continual dispute[16,21,22]. However, Marchessault and his coworkers[7,10] recently proposed that the multiplicities of the C-1 lines of A- and B-amyloses are associated with the number of asymmetric units, on the basis of results for the crystalline methyl α- and β-D-xylosides[23]. According to the $P2_1$ space group assigned for A-amylose[4], where the 2_1 axis is perpendicular to the six-residues-per-turn strands of the double helix, maltotriose (or half a turn) must be regarded as being an asymmetric unit. This is well correlated to the triplet of the C-1 line for recrystallized, A-type amylose and native sarch. For B-amylose, the space group $P3_121$ specifies an asymmetric unit of maltose (or one-third of a turn)[4]. This is reflected in the doublet signal of the C-1 line for recrystallized, B-type amylose and native starch.

The explanation in terms of the asymmetric unit also seems valid for V-amyloses. In V_h-amylose, all D-glycosyl residues are identical, because the 6-hydroxyl groups of one helix turn are in equivalent positions, and thus the formation of symmetric, intramolecular hydrogen-bonds is allowable[4]. In contrast, molecular sixfold symmetry in the helix is not present for V_a-amylose, and a 2_1

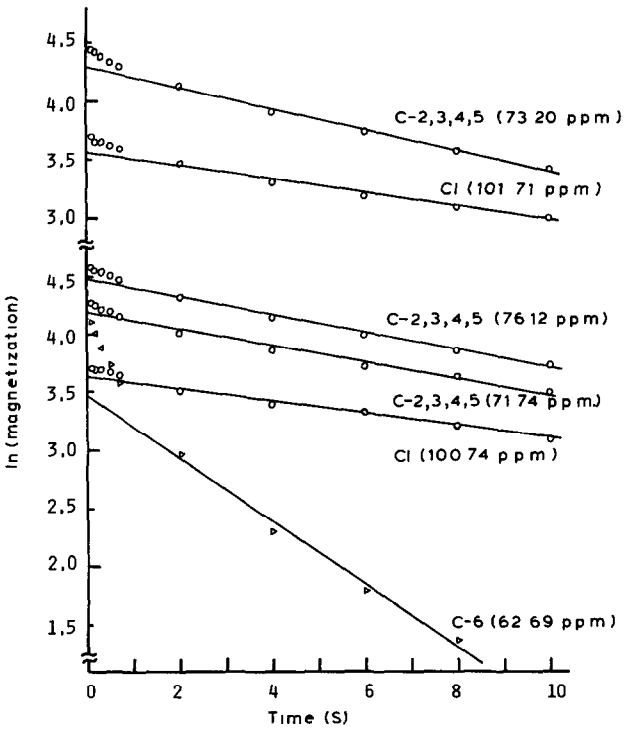

Fig. 6 Semilogarithmic plots of ^{13}C magnetization *vs.* time in ^{13}C spin–lattice relaxation for highly crystalline B-amylose in the hydrated state.

screw axis exists along the helix axis because of the difference in O-6 positions. Nevertheless, the helix backbone still resembles a sixfold helix[4]. Therefore, the asymmetric unit of a single D-glycosyl residue is present at least for ring-carbon atoms of both V_h- and V_a-amyloses, which corresponds well to the singlet signals for the respective resonances.

On the other hand, we have correlated the C-6 chemical shifts to the torsion angles χ about the exocyclic C-5–C-6 bonds for D-glucoses and disaccharides having D-glucose residues[12,14,16,24]. According to this result, the chemical shift of 62.69 p.p.m. of the C-6 atom in B-amylose corresponds to the *gauche-trans* (*gt*) orientation of the CH_2OH group, which is in good agreement with the results of X-ray analysis[2]. In A-amylose, however, the orientation of the CH_2OH group has not been unambiguously determined by X-ray analysis; it may be either all *gt* or a mixture of *gt* and *tg*. Our n.m.r. results suggest that the CH_2OH group of A-amylose also adopts the *gt* orientation, because the C-6 chemical shifts are almost the same for both crystals, irrespective of the existence of the small downfield shoulder. In comparison with the C-6 chemical shifts of A- and B-amyloses, the corresponding values of V_h- and V_a-amyloses are as low as 62.2–61.9 p.p.m. Because such values are very close to the upper limit for the *gg* orientation[12,14,16,24], the CH_2OH groups are assumed to possess the *gg* orientation for both V-amyloses.

TABLE II

^{13}C SPIN–LATTICE RELAXATION TIMES (T_1) OF THE RESPECTIVE CARBON ATOMS OF DIFFERENT POLYMORPHS OF AMYLOSE

Sample	T_1 *(s)*			
	C-1	*C-4*	*C-2,3,5*	*C-6*
A-Amylose, dried	21.7	13 5		5 8
soaked in H_2O	13.5 (102.30 p.p.m.)	16.1 (76.90 p.p.m)		4 0
	15.6 (101.32 p.p.m)	12.8 (73.20 p.p.m.)		
	16.1 (100.05 p.p.m.)	12 2 (71.45 p.p m.)		
B-Amylose, dried	19.6	13 5		5 3
soaked in H_2O	17.2 (101 71 p.p.m.)	12 8 (76.12 p.p.m)		3 7
	19.2 (100 74 p.p.m.)	11 4 (73.20 p.p.m.)		
		13 9 (71 74 p.p m.)		
V_h-Amylose	11 1	11.2	8.6	0 9
			8.4	
V_a-Amylose	7.1	5.8	5.9	0 7

This assumption is in good accord with the conclusion of X-ray analysis for V_h-amylose[3], but in conflict with that for V_a-amylose[1]. In the latter case, the X-ray analysis revealed that the CH_2OH groups adopt three different rotational positions close to *gt, gg,* and *tg* on successive residues. This conflict may be due to the broadness of the C-6 lines and, more importantly, the possibility of transition among the orientations must be considered, because the molecular mobility of the CH_2OH groups is quite high, as will be described.

3. Spin–lattice relaxation times and molecular mobility. — Fig. 6 shows semilogarithmic decay curves of the respective peak intensities in ^{13}C spin–lattice relaxation which were obtained by using the pulse sequence including the c.p. technique[20] for the highly crystalline B-amylose soaked in H_2O. It is clear that each decay curve is well described as a single exponential, although a minor component (<15%) also appears in the initial part. (Such a rapid-decay component is >50% for the C-6 line, but the cause is not clear at present.) Of these two, the component with longer T_1 values can be assigned to the crystalline component, because the longer T_1 value relates to less mobility under this experimental conditions. In addition, the resolution of each line of the longer component is much higher, compared to that of the shorter T_1 component. On the other hand, the shorter T_1 component may be the contribution from the disordered region. Two similar components have also been observed for the dry A-amylose, as well as for B-amylose and V-amylose in both states.

The T_1 values of the crystalline components of amylose polymorphs are summarized in Table II. It was found that pyranose-ring carbon atoms of A- and B-amyloses have T_1 values of the order of 11–22 s and almost no hydration effect on these T_1 values appears. Because the C-6 atoms possess two protons chemically bonded, T_1 values experimentally observed should be multiplied by a factor of 2 for

comparison with the results for ring-carbon atoms. As a result, the T_1 values of C-6 are not significantly short compared to those of the ring-carbon atoms, suggesting no great difference in mobilities of the ring-carbon atoms and the C-6 atoms.

Similar results have been obtained for crystalline cellulose I of cotton, but the orders of the T_1 values of this material are 160–210 s for ring-carbon atoms and 140–210 s for C-6 atoms, that is, ~10 times longer than the corresponding values for A- and B-amyloses. Such a large difference in the T_1 value indicates that the molecular mobilities differ greatly from each other; amylose molecules undergo significantly enhanced molecular motion, whereas the motion is much hindered in cellulose. Furthermore, this sort of difference in molecular mobility is likely to stem from the structural differences between the two molecules; amylose chains adopt a six-fold helical structure, whereas cellulose chains are almost linearly extended as a result of the twofold helix. In the cases of A- and B-amyloses, the relatively flexible six-fold helix may allow enhanced torsional motion about the α-D-(1→4)-glycosyl linkages.

It should be pointed out that the respective lines of the multiplicities of the C-1 resonances for A- and B-amyloses exhibit different T_1 values. Although the difference is not very large, it suggests that each D-glycosyl residue in the asymmetric units undergoes somewhat different molecular motion. Similarly, smaller differences also appear in the ^1H spin–lattice relaxation times $T_{1\rho H}$ in the rotating frame for A-amylose. As a result of the difference, each line of the triplet becomes nonequivalent in the spectra obtained by using longer contact-times.

As described in the Introduction, V-amyloses form six-fold single helices whose pitch per turn is ~2.6 times as long as that for A- and B-amyloses. Therefore, the flexibility of V-amylose chains is predicted to be higher than that of the considerably extended helix for A- and B-amyloses. Rather short T_1 values of V-amyloses suggest that this prediction is plausible. Further decreases of T_1 values observed in V_a-amylose may be due to the unstable helical structure which is probably induced by the removal of water and 1-butanol. In contrast to the cases of A- and B-amyloses, the C-6 atoms of both types of V-amylose have much shorter T_1 values than those of the ring-carbon atoms, suggesting high molecular mobility, possibly about the exocyclic C–C bonds. Such high mobility may be associated with possible transitions among the *gg*, *gt*, and *tg* orientations

Finally, it should be noted that significant decreases of T_1 values are observed for A- and B-starches, although the values are almost the same as those of A- and B-amyloses in the dried state[9]. Previously[9], this phenomenon was explained in terms of the enhancement of flexibility of helical chains by water. However, this explanation is not suitable for the cases of A- and B-amyloses, because here is almost no effect of hydration for them. The true origin of the hydration effect on starches is not clear at present, but it may be associated with the existence of the branches at one end of the helix.

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