Molecular Organization in Starches: A ^{13}C CP/MAS NMR Study

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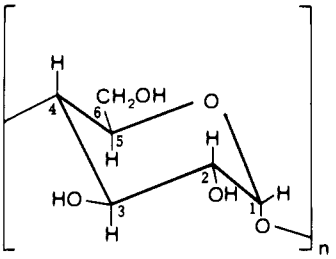
Abstract: Cross polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectra have been obtained for starches from various botanical origins and analyzed by comparison with model materials. All features of starch ^{13}C CP/MAS spectra are accounted for by a combination of amorphous (single chain) and ordered (double helix) components. The proportion of each of these structural types present in native starches can be estimated from ^{13}C CP/MAS spectra. This provides the first approach to the quantitative analysis of starch molecular structures; the values obtained can be compared with estimates of longer range ordering from X-ray powder diffraction. C-1 and C-4 carbons (i.e., those sites involved in glycosidic linkage) show large chemical shift displacements between amorphous and crystalline materials indicating substantial conformational differences. Chemical shift displacements between the two common crystalline types (A and B) of starches are much smaller but model crystalline materials show polymorphic variations in C-1 multiplicity which can be ascribed to known differences in the symmetry of double helix packing. The chemical shift ranges observed for spectral features of amorphous materials suggest that part of the amorphous component of starches has a well-defined conformation.

Starch is the major energy reserve of all higher plants and is an important constituent of the human diet. The two main components of starch, amylopectin and amylose, are both high molecular weight, polydisperse (1→4)-α-D-glucans (I). Amylopectins are highly branched through (1→6)-α-linkages (at, on average, one residue in 18–25), and amyloses are linear or lightly branched.[1] Starches from most botanical sources contain 15–30% amylose although varieties which can produce essentially 100% amylopectin ("waxy") and up to 75% amylose ("amylose extender") are known.



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All starches are biosynthesized as granules containing densely packed polysaccharide and relatively little water. Granules take on a variety of three-dimensional shapes and vary in size from less than 1 μm to more than 100 μm depending on botanical source.[2] A common feature of starch granules, though, is the presence of regions of long-range molecular order ("crystallinity") as shown by X-ray powder diffraction. Two distinct types of X-ray diffraction pattern are commonly observed (A and B), characteristic of cereal grain starches and tuber, fruit, and stem starches, respectively.[2] From X-ray diffraction studies of amylose fibers, Wu and Sarko[3,4] demonstrated that both A- and B-type structures contain ordered arrays of double helices. Individual double helices in these two structures have very similar, if not identical, conformations but there are substantial differences in packing with the A-type adopting a nearly close-packed arrangement[4] and the B-type structure being more open[3] and containing correspondingly more water (Figure 1). There is good evidence[2] that the amylopectin component is responsible for the observed long-range order through regular packing of double helices formed from adjacent branches (but not involving branch points). This proposal is consistent with the currently accepted "cluster" model for the molecular structure of amylopectin[2,5,6] (Figure 2).

To date, the only available non-invasive probe of starch granule organization has been X-ray diffraction, a technique which reports on the regularly repeating nature of double helical molecular structures but which does not detect irregularly packed structures. High-resolution solid-state NMR, by contrast, is expected to be sensitive to structural organization at the molecular level and should therefore complement information obtained from X-ray diffraction.

Although resonances due to starch have been identified in ^{13}C cross polarization and magic angle spinning (CP/MAS) NMR spectra of flours and seeds,[7,8] no detailed spectral analysis has previously been attempted. We now report the results of a detailed ^{13}C CP/MAS NMR study of starches and model compounds which provides novel information on starch polymorphism and allows the first approach to the quantitative analysis of molecular structures within starches.

Experimental Section

All starch samples were obtained commercially. To provide amorphous starch-based materials, potato and maize starch were each dissolved in deionized water (1% w/v) by heating in a sealed tube at 160 °C for 20 min. Aliquots of the solutions were (i) lyophilized and (ii) added to 4 volumes of ethanol and left for 16 h at 20 °C: the resulting precipitate was collected, thoroughly washed with ethanol and then diethyl ether, and finally air dried. Linear amorphous material was prepared from amylose (synthesized with potato phosphorylase[9]) by lyophilization of a 0.5% aqueous solution.

To act as models for starch ordered structures, highly crystalline materials were prepared from both branched and linear (1→4)-α-D-glucans. Low molecular weight branched material was prepared by acid degradation of waxy maize starch[10] (to give a Nägeli-type amylodextrin[11]), fractioned by selective precipitation from water/pyridine/methanol mixtures[11], and crystallized from hot 30% aqueous solution by

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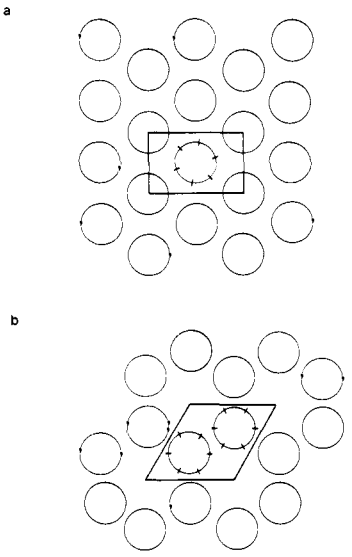

Figure 1. Representation of helix packing and unit cells in (a) A-type and (b) B-type crystalline (1→4)-α-D-glucan (adapted from ref 3 and 4). Each circle represents a view down the center of a double helix. Positions of C-1 sites are shown by spokes for helices within unit cells.

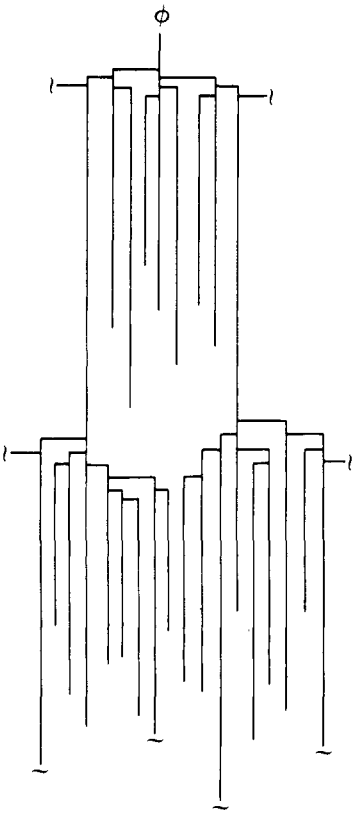

Figure 2. Schematic diagram showing structural features present in amylopectins. Each molecule has a single reducing terminal residue (ϕ) and extends via clusters of branch points.[2,5,6]

incubation at 15 °C.[12] Low molecular weight linear (1→4)-α-D-glucan (average degree of polymerization 11.2 by ^{1}H NMR[13]) was prepared by the action of the debranching enzyme isoamylase (EC 3.2.1.68) from *Cytophaga sp.*[14] (a gift from Dr. I. Fleming, Glaxo) on mussel glycogen (Sigma) and fractionated by dialysis. Crystallization of the unfractionated material from hot 30% aqueous solution at 15 °C resulted in essentially pure B-type material as judged by X-ray diffraction (Figure8 a) which showed no significant intensity due to the characteristic "halo"

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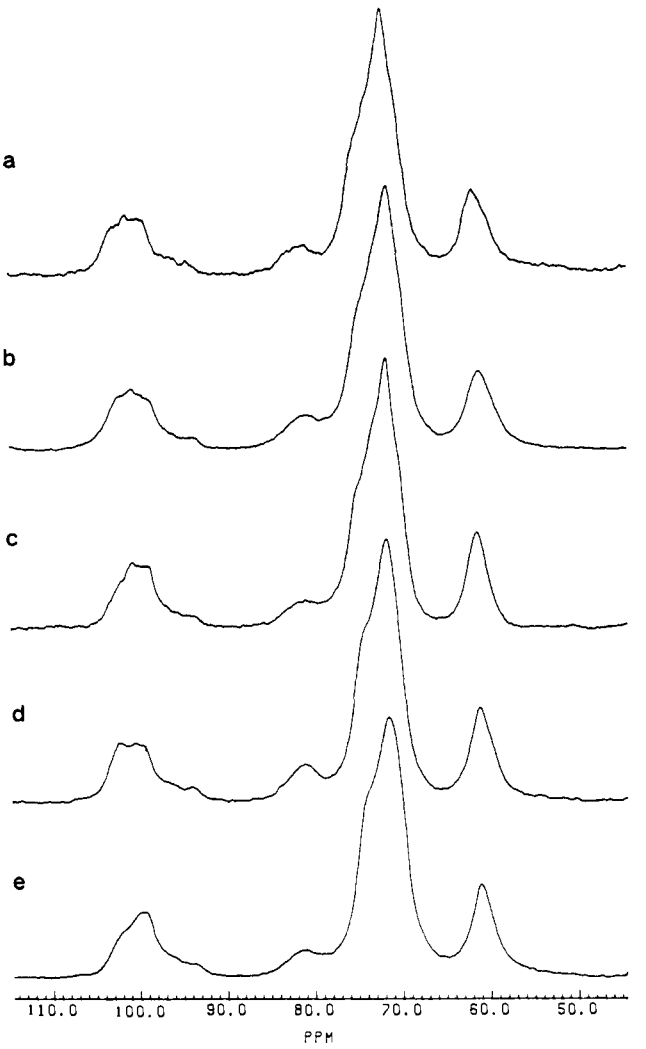

Figure 3. ^{13}C CP/MAS NMR spectra of (a) rice, (b) maize, (c) waxy maize, (d) amylomaize, and (e) potato starches.

of amorphous starch-based materials (Figures 7b and 8b) in the region 10–25 2θ°. Essentially pure A-type material (X-ray diffraction pattern: Figure 7a) was prepared from a dialyzate fraction of average degree of polymerization 8.0 (^{1}H NMR[13]) by incubation of a hot 50% aqueous solution at 30 °C. Full details of the preparation and crystallization of debranched mussel glycogen will be published elsewhere.[15] These crystalline materials are considered to be good models for the ordered structures in starches as they are formed from chains of similar length to the branches of amylopectins (average length 18–25) which are responsible for the long-range ordering in starch granules[2] (through regular packing of double helices formed from adjacent branches).

X-ray diffraction patterns were obtained with a Phillips powder diffractometer (PCW 1050/1390) mounted on a PW 1730/10 sealed-tube X-ray generator operating at the Cu Kα wavelength (1.542 Å).

Solid-state CP/MAS carbon-13 spectra were obtained at 75.46 MHz on a Bruker CXP-300 spectrometer with a standard Bruker "Andrews"-type probe head. Spinning rates of 3–3.5 kHz and spin locking and decoupling fields of ~50 kHz were used. Spectra are referenced to external Me_4Si via the residual "rotor" peak (from perdeuterated PMMA) at 44 ppm. A contact time of 1 ms was used for all the standard spectra with a recycle time of 2 s. At least 1000 scans were averaged for each spectrum. Other parameters were the following: spectral width 30 kHz; acquisition time 30 ms; time domain points 2K; transformation size 8K; line broadening 20 Hz.

Results

The results of a ^{13}C CP/MAS NMR survey of a variety of starches are shown in Figure 3. Substantial similarities are observed in all spectra with resolved resonances in the ranges 60–64 and 94–105 ppm. By comparison with observed chemical shifts

(15) Gidley, M. J.; Bulpin, P. V., in preparation.

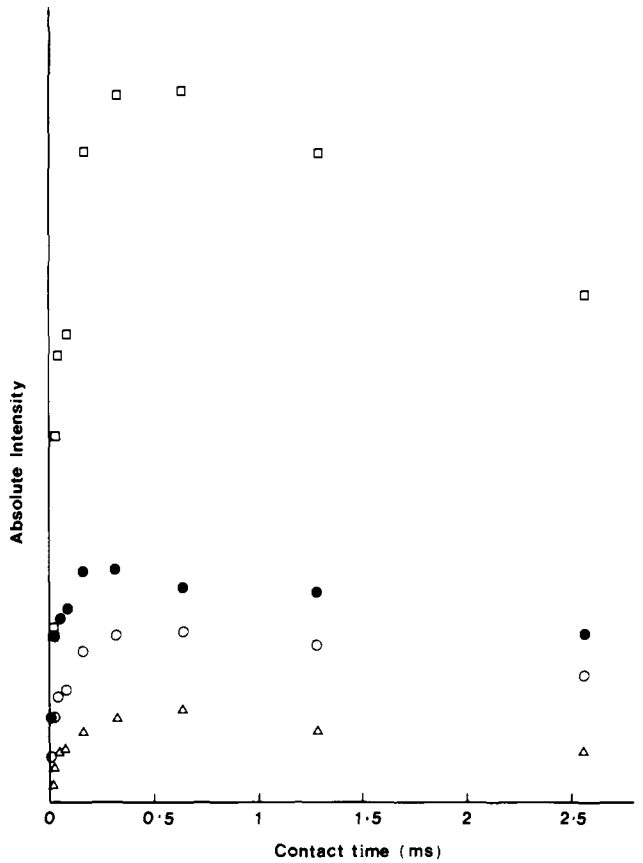

Figure 4. Variation of intensity as a function of Hartman–Hahn contact time for waxy maize starch at (△) 82, (○) 101, (●) 62.5, and (□) 73 ppm, respectively.

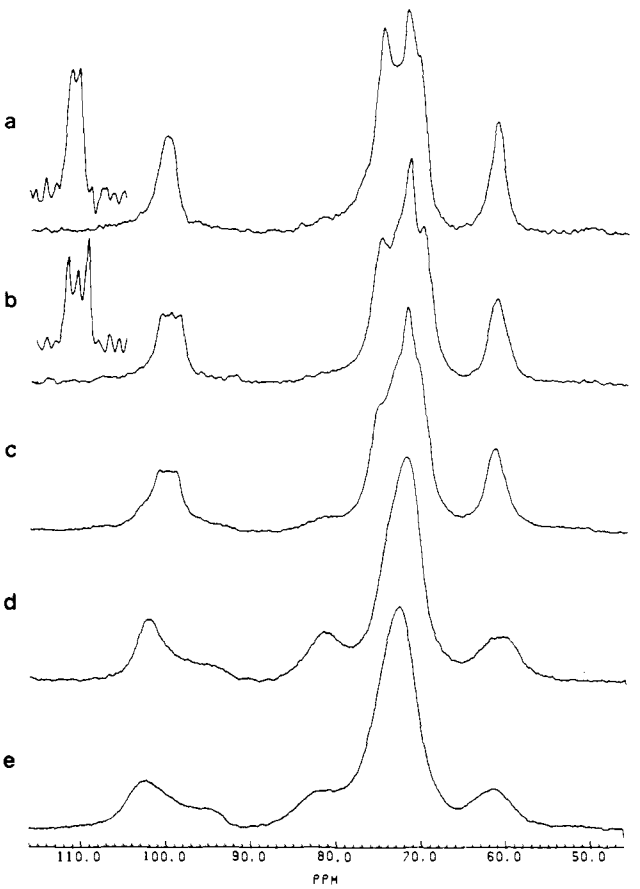

Figure 5. ^{13}C CP/MAS NMR spectra of (a) B-type crystalline debranched glycogen, (b) A-type crystalline debranched glycogen, (c) A-type crystalline acid-degraded waxy maize starch, (d) gelatinized and ethanol-precipitated potato starch, and (e) lyophilized amylose. Insets in parts a and b are resolution enhancements of the 95–105 ppm region.

in solution,[16] these peaks may be assigned to C-1 and C-6 sites, respectively.[8] A further low-intensity resonance at 80–83 ppm is also partially resolved in all spectra. The major signal intensity in all spectra (Figure 3) is in the range 68–77 ppm and is presumably due to C-2, -3, -4, and -5 sites. The observed spectral similarity for maize (ca. 25% amylose), waxy maize (ca. 0% amylose), and amylomaize (ca. 70% amylose) demonstrates that the amylose/amylopectin ratio is not the dominant factor in determining the relative intensity of spectral features. Although resonances due to amylopectin branching residues are observed in solution-state ^{13}C spectra of starches[13] (notably at 61, 70, and 73 ppm), such signals are not resolved in ^{13}C CP/MAS spectra due to the width of resonances and the low concentration of branch points (maximum of 5% for the starches studied).

Further insights into the origins of starch CP/MAS NMR spectral features could be gained if reliable signal integration could be carried out. To allow a quantitative comparison of peak intensities within a ^{13}C CP/MAS spectrum, carbon sites with the slowest cross polarization rates (T_{CH}^{-1}) should be allowed sufficient time to be magnetized fully via spin-locked protons, and ideally this should have occurred before significant proton relaxation (time constant: $T_{1\rho}(H)$) takes place,[17] i.e. $T_{1\rho}(H) \gg T_{CH}$. This condition can be checked and approximate values of $T_{1\rho}(H)$ and T_{CH} obtained, by measuring individual signal intensities as a function of the cross polarization contact time[18,19] (t_{cp}). The main result of such an experiment on both waxy maize and potato starches is that all spectral features (except C-6 resonances) have the same relative intensities within the spectrum for all contact times in the range 10 μs to 10 ms, i.e., T_{CH} and $T_{1\rho}(H)$ values are each similar for all sites except C-6 (H-6). Resonances at 60–63 ppm (C-6) are more efficiently cross-polarized than other resonances, i.e., T_{CH} is shorter, presumably because C-6 is a methylene carbon whereas all other sites are methine.[18] Figure 4 illustrates these results by showing signal intensities at 101, 82, 73, and 62.5 ppm for waxy maize starch as a function of contact time. Closely similar results are obtained for potato starch.

Approximate values of T_{CH} and $T_{1\rho}(H)$ can be obtained by analysis of the initial exponential rise in intensity at short contact times and the exponential decrease in intensity at long contact times, respectively.[18,19] When this approach is used, $T_{1\rho}(H)$ values for these four signals are all found to be in the range 4–6 ms, i.e., similar to previously determined values for other polymers.[18] T_{CH} values are 30–40 μs for the C-6 (methylene) resonances and 70–90 μs for methine carbon resonances (101, 82, and 73 ppm) in line with the expectation[18] that, other factors being equal, cross polarization should be twice as efficient in CH_2 as in CH groups.

As all T_{CH} values are substantially shorter than $T_{1\rho}(H)$ values, integration of spectral signals is possible provided that the contact time is sufficient to allow complete cross polarization.[17,18] In obtaining the spectra shown in Figure 3, a contact time of 1 ms was employed: at this is more than ten times T_{CH} values, a comparison of peak intensities in these spectra can be made. Thus, assigning the intensity of the C-1 resonance (94–105 ppm) as 1.0, signals in the chemical shift ranges 57–66, 66–79, and 79–85 have intensities of 1.0, 3.4–3.6, and 0.6–0.4, respectively, for the starches studied (Figure 3). Signal intensity in the range 79–85 ppm therefore does not correspond to all sites of a particular glucan carbon. As further aids to spectral assignment, models for the crystalline and amorphous regions present within starch granules[2] were prepared and analyzed by ^{13}C CP/MAS NMR.

Crystalline (1→4)-α-D-glucan oligomers (derived from debranched glycogen) of both A- and B-type have similar ^{13}C

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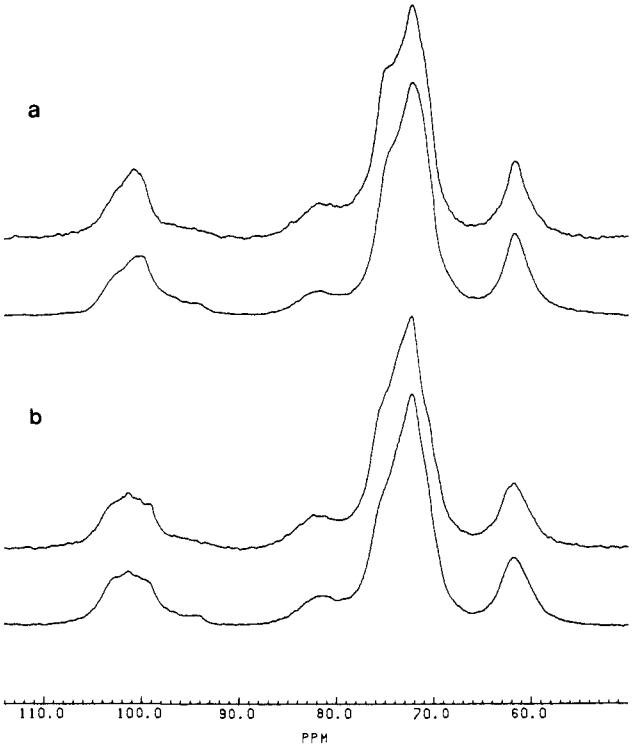

Figure 6. (a) Comparison of the ^{13}C CP/MAS spectrum of potato starch (upper trace; as Figure 3e) with (lower trace) a composite of 50% of the spectral intensity of B-type crystalline debranched glycogen (Figure 5a) and 50% of the spectral intensity of ethanol-precipitated potato starch (Figure 5d). (b) Comparison of the ^{13}C CP/MAS spectrum of maize starch (upper trace; as Figure 3b) with (lower trace) a composite of 42% of the spectral intensity of A-type crystalline debranched glycogen (Figure 5b) and 58% of the spectral intensity of ethanol-precipitated potato starch (Figure 5d).

CP/MAS NMR spectra (Figure 5a,b) the most notable exception being the C-1 region where the A-type shows 3 peaks at 100.4, 99.2, and 98.2 ppm (Figure 5b) and the B-type has 2 peaks[30] at 100.0 and 99.2 ppm (Figure 5a). Crystalline material (A-type) was also obtained from low molecular weight branched (1→4)-α-D-glucan produced by acid degradation of waxy maize starch and containing an average of 28 glucose residues and 1.4 branch points per molecule (^1H NMR[13]). The ^{13}C CP/MAS spectrum of this material (Figure 5c) shows similar features to that of linear crystalline material of the same polymorphic form (Figure 5b) with the exception of extra signal intensity at 81–83, 102–105, and possibly 94–98 ppm.

Amorphous samples (by X-ray diffraction) prepared from both maize and potato starches by thorough gelatinization followed by either precipitation with ethanol or lyophilization all give essentially identical ^{13}C CP/MAS spectra (Figure 5d) and show substantial differences to spectra of crystalline mateirals (Figure 5a–c).

Particularly striking differences are observed in the C-1 region, where peak intensity is shifted to low field compared to crystalline material (Figure 5a–c), and at 81–83 ppm, where a peak of similar intensity to C-1 and C-6 is observed for amorphous starch but where there is almost no signal intensity for crystalline materials. By comparison with chemical shifts observed in solution,[16] this latter resonance is assigned to C-4 sites in the amorphous sample. Linear amorphous material, prepared by lyophilization of a dilute aqueous solution, gives a similar ^{13}C CP/MAS spectrum (Figure 5e) to that of amorphous starch. Signal intensity at 81–83, 94–98 and 102–105 ppm for crystallized branched material (Figure 5c) can now be assigned to "amorphous" sites within the sample. The presence of some amorphous character is not surprising in view of the obvious disruptive influence of branch points on the packing of ordered structures.

A comparison of Figures 3 and 5 shows that all the spectral features of native starches can be accounted for by a combination

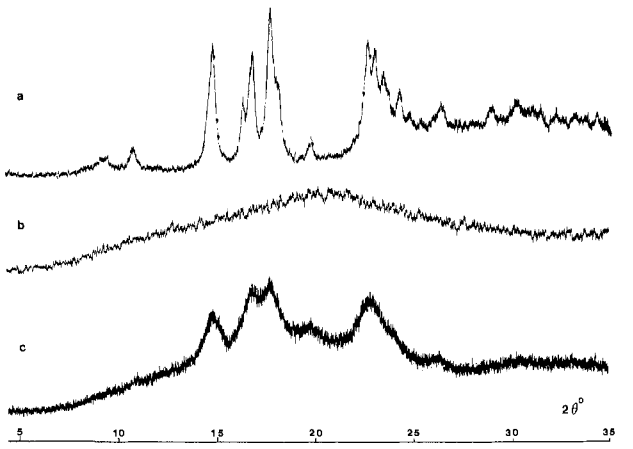

Figure 7. X-ray powder diffraction patterns obtained from (a) A-type crystalline debranched glycogen (^{13}C CP/MAS spectrum shown in Figure 5b), (b) gelatinized and ethanol-precipitated maize starch (^{13}C CP/MAS spectrum essentially identical with Figure 5d), and (c) maize starch (Figure 3b).

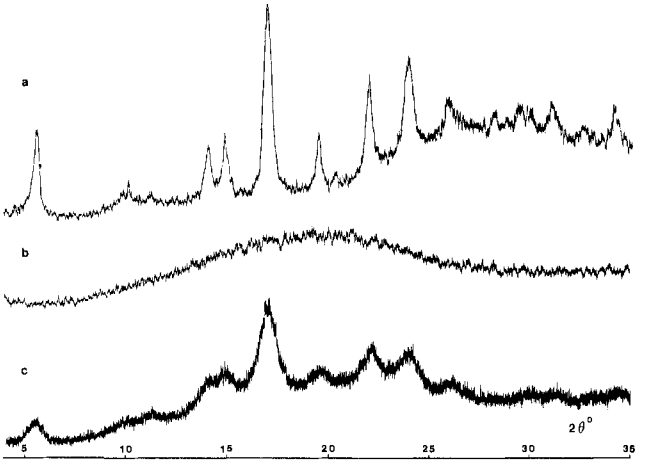

Figure 8. X-ray powder diffraction patterns obtained from (a) B-type crystalline debranched glycogen (^{13}C CP/MAS spectrum shown in Figure 5a), (b) gelatinized and ethanol-precipitated potato starch (Figure 5d), and (c) potato starch (Figure 3e).

of the observed spectral features of crystalline and amorphous model materials. Therefore, taking a spectroscopic combination of data for crystalline (Figure 5, parts a and b) and amorphous (Figure 5d) materials, the possibility of synthesizing starch ^{13}C CP/MAS spectra can be investigated. Figure 6 shows how this approach can successfully generate essentially identical spectra to those obtained from natural materials for both maize (A-type) and potato (B-type) starches. For maize starch the optimum mixing ratio of A-type ordered to amorphous material is 42:58 and for potato starch (using B-type ordered material) the equivalent ratio is 50:50. Employing the same approach for rice, waxy maize, and amylomaize starches, optimum ordered amorphous mixing ratios are 49:51, 53:47, and 38:62, respectively. The accuracy of matching observed spectra to mixtures of the two types of model spectra is estimated to be approximately ±2%, i.e., 50:50 implies 48:52 to 52:48.

Similarly, model crystalline and amorphous materials account for all the observed features of X-ray powder diffraction patterns for both A-types (Figure 7) and B-type (Figure 8) starches, although peaks due to the native materials (Figures 7c and 8c) are significantly broader than those due to model crystalline materials (Figures 7a and 8a) showing that there is greater long-range ordering of double helices in the latter. Estimates of the proportion of regularly repeating structures in starches have been obtained[2,20] by treating powder diffraction patterns as a combination of dif-

(20) Nara, S. *Staerke* **1978**, *30*, 183–186.

fraction peaks (arising from regularly repeating structures) superimposed on a featureless background halo (due to all other material). When this approach is used the estimated percentage of regular repeating structures in starches has been found to be approximately 25% for potato[20,21] and amylomaize[21] and 38–39% for maize, waxy maize, and rice starches.[20]

Discussion

A major finding of this work is that ^{13}C CP/MAS NMR spectra of starches can be completely accounted for by a combination of features due to two types of polysaccharide structures viz. double helix ("crystalline") and single chain ("amorphous"). This analysis provides the first quantitative molecular description of structures present within starch granules. Furthermore, the estimated proportions of each type of structure can be compared to previous quantitative analyses of X-ray diffraction patterns. Not surprisingly, such a comparison shows that the percentage of double helices (by NMR) is never less than the percentage crystallinity (by X-ray diffraction). However, for some starches comparative percentages are very similar (e.g., maize; 42% double helix and 39% crystallinity[20]) whereas in others (e.g., potato) estimates of double helix content (50%) are far greater than those of crystallinity (approximately 25%[20,21]). Planned investigations involving the analysis of starch samples by both techniques should throw further light on such comparisons of levels of structural organization. Similarly, this approach should provide valuable quantitative insights into the changes in starch molecular structures caused by enzymatic breakdown (i.e., analogous to the mobilization of energy reserves in plants) and processing conditions relevant to food production (e.g., heat/moisture treatment).

A related polysaccharide which has previously been studied in some detail by ^{13}C CP/MAS NMR is cellulose (1→4)-β-D-glucan)[22-28] for which a similar combination of "crystalline" and "amorphous" features has been shown to account for the observed spectra of natural materials.[22-25]

In ^{13}C CP/MAS spectra of amorphous starch samples (Figure 5, parts d and e), resolved resonances cover greater chemical shift ranges than model crystalline materials (Figure 5, part a–c) as might be expected. However, for the C-1 resonance, a substantial portion of the signal intensity is localized over a narrow (100–104 ppm) chemical shift range suggesting that at least part of the amorphous component in starches has a well-defined conformation.

The large differences observed between ^{13}C CP/MAS NMR spectra of single-chain (Figure 5, parts d and e) and double-helical (Figure 5, parts a–c) materials suggest substantial conformational differences between the two types of structures. C-1 and C-4 resonances show particularly significant differences with chemical shifts being displaced by up to 5 ppm for C-1 and by at least 6 ppm for C-4 (Figure 5). These are the two carbon sites involved in glycosidic bonds and would therefore be expected to be the most sensitive to polysaccharide conformation as this is defined by the geometry of glycosidic linkages.[29] Further small differences between single-chain and double-helical materials are apparent in the 70–77 ppm region of the spectrum (Figure 5), but these are not as pronounced as those for C-1 and C-4 resonances.

As described earlier, the chemical shift range of C-1 sites in model crystalline materials (Figure 5, parts a–c) depends on the polymorphic form. Moreover, a difference in multiplicity in the C-1 region for the two polymorphic forms is apparent (Figure 5, parts a and b), i.e., 3 peaks for A-type and 2 for B-type. X-ray diffraction studies of amylose fibers[3,4] have shown that polysaccharide conformations in the two polymorphic forms are very similar if not identical; it therefore seems unlikely that the observed multiplicities could be due to conformational differences within double helices. However, consideration of the packing of helices in the two structural types (Figure 1) does provide a plausible explanation. As the double helices have regular sixfold repeats and the two strands are conformationally indistinguishable,[3,4] there are only six possible environments relative to neighboring helices for C-1 sites in either polymorphic form (Figure 1). The effects on chemical shifts of surrounding helices will presumably be restricted to nearest neighbors and therefore considerations of symmetry with respect to adjacent helices could be reflected in resonance multiplicities. In the A-type structure (Figure 1a) there is a twofold symmetry, and hence 3 different environments, of C-1 sites with respect to adjacent double helices in line with the observation (Figure 5b) of threefold multiplicity.[31] Similarly, in the B-type structure (Figure 1b), the threefold symmetry of adjacent helices is consistent with the observed twofold multiplicity (Figure 5a). In principle, other resonances in spectra of the two crystalline materials could show similar multiplicities. However, although polymorphic differences are apparent in the C2–C5 region of the spectrum (Figure 5, part a and b), the only other resolved resonance is that due to C-6 which, as it is exocyclic, is less conformationally restricted and indeed has essentially the same chemical shift range for all samples studied.

Conclusions

^{13}C CP/MAS spectra of starches can be analyzed in terms of two types of polysaccharide structure, i.e., single chain ("amorphous") and double helix ("crystalline"). Quantitative comparisons of degrees of molecular ordering (double-helix content) with longer range ordering (crystallinity) can be obtained from ^{13}C CP/MAS and X-ray diffraction data, respectively. Chemical shift displacements (particularly for C-1 and C-4 sites) demonstrate substantial conformational differences between amorphous and crystalline starch components. The similarities in chemical shifts observed for materials of A- and B-type crystallinity are consistent with X-ray fiber diffraction data[3,4] which suggest very similar double-helix conformations for the two polymorphs. Furthermore, differences in the multiplicity of the C-1 signal for the two forms can be explained in terms of known,[3,4] helix packing arrangements. A well-defined conformation for part of the amorphous component of starches is suggested by the chemical shift ranges observed in spectra of model materials. It is anticipated that ^{13}C CP/MAS NMR will become an important tool in studies of starch structures and their manipulation.

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Registry No. Starch, 9005-25-8; amylose, 9005-82-7.

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(30) Although a doublet is not unequivocally proven in Figure 5a as the depth of the central minimum is of the same order as the noise, we consistently observe a doublet having the same two peak chemical shifts for repeated spectral acquisitions of three separate preparations of model B-type material.
(31) Spectral simulation showed that the C-1 signal in Figure 5b can be accurately reproduced by three Lorentzian lines of essentially equal (34:35:31) area but differing intensities and line widths. The central peak was found to have the greatest line width and the highest field peak the narrowest. Such relative line width variations presumably explain the observed unequal intensities in the resolution-enhanced spectrum (inset in Figure 5b).