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Solid state ^{13}C NMR investigation of lipid ligands in V-amylose inclusion complexes

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Abstract

The characteristics of the ligands in inclusion complexes formed from stearic, palmitic, oleic, linoleic, linolenic and docosahexaenoic acids, glycerol monooleate (GMO), glycerol monopalmitate (GMP) and lysophosphatidylcholine (LPC) have been studied by ^{13}C NMR in dry and hydrated forms of the complexes, with ^{13}C labels being used for the carboxyl and C-1(3) glycerol carbons in stearic acid and GMO, respectively. ^{13}C NMR provides definitive proof that V-amylose inclusion complexes have been formed with the mono-carboxylic fatty acids of varying degrees of unsaturation, GMO, GMP and LPC. The chemical shift of the mid-chain methylenes in stearic acid moves about 1.5 ppm upfield upon complexation with the ^{1}H rotating frame relaxation times becoming identical for the lipid and amylose. With the exception of docosahexaenoic acid, the mid-chain methylenes inside the V-helical segments have essentially the same chemical shift for all the other unsaturated fatty acids and lipids investigated. The cross-polarisation dynamics for the carboxyl and glycerol groups in stearic acid and GMO, respectively, have indicated that these bulky polar groups occupy highly mobile conformations in the hydrated complexes which must lie outside the V-helical segments adjacent to the amorphous domains. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: V-amylose; ^{13}C NMR; Lipid ligands

1. Introduction

Amylose present in all non-waxy starches is an essentially linear homopolymer of (1 → 4) linked-α-D-glucopyranose residues. Three distinct polymorphs of amylose exist and are referred to as the A-, B- and V-forms with the V-type only being formed in the presence of a complexing ligand. The A- and B-forms comprise parallel-packed, left-handed double helices (Imberty et al., 1987, 1988a, b). Both these forms contain no internally bound water molecules, but they differ in the packing of the six pairs of helices into bundles with low (A-form) and high (B-form) levels of water in the interstices determined by the lattice symmetry. The A- and B-forms can be considered as extended helices with, unlike the V-form, no hydrogen bonding between consecutive turns of the helices.

In the V-form, a single chain of amylose forms a helix with a relatively large cavity in which various ligands can be situated and the size of the ligand determines the number of glucosyl residues per turn (6, 7 or 8). The single V-helical complexes formed from a range of organic ligands, reviewed by Gidley et al. (1988), have been investigated. The V-forms have relatively large central cavities with a pitch of about 8 Å per turn, whereas the double helical A- and B-forms have a pitch of about 21 Å and there is no internal cavity (Rappenecker et al., 1981; Wu et al., 1978a, b). Some complexation between lipids and amylose evidently occurs during the biosynthesis of non-waxy cereal starches (Morrison, 1988) and during starch thermal processing (e.g. gelatinisation) where added surfactants which complex with amylose are often used to modify and control the starch gel properties.

The only detailed X-ray conformational analysis of the hydrated V-helix, with the original guest molecule in the cavity displaced by water, has been reported by Rappenecker et al. (1981). This clearly defines all bond angles, hydrogen bonding and van der Waals contacts (intra- and interhelical). A key feature of V-amylose is the hydrogen bonding between glucosyl OH-2 and OH-3(2) for all pairs of glucosyl residues in the solid state (Rappenecker et al., 1981) and in solution (St. Jacques et al., 1976), together with that between the helix turns from interactions of

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OH-2 and OH-6(7) which is only possible for the collapsed helices. The chair conformation of a glucopyranose ring is thus comparatively inflexible and the greatest points of helix flexibility are the glycosidic C(4)–O–C(1) bonds, as observed in most α- and β-glucans (Gidley, 1992).

The only structural model of a lipid molecule inside V-amylose is that proposed by Godet et al. (1993) who used the coordinates given by Rappenecker et al. (1981) as their starting point. Acetic acid was then introduced and followed by consecutive methylene groups to model dodecanoic acid inside a V_6 amylose helix. Within the V_6 helix, Godet et al. (1993) contend that only glucosyl H-5 atoms are available for van der Waals contacts with the fatty acid methylenes, implying a hydrophobic internal environment. For the carboxyl group, two low energy solutions were obtained in which the polar group was located near the centre of the cavity, but was prevented from entering by steric and electrostatic repulsions. This would the limit helical segment lengths to two fatty acid molecules situated with terminal methyl groups end-to-end but, as yet, there is no experimental information on the environments of any bulky polar end groups of ligand lipids. For fatty acid complexes, the stoichiometry of amylose is six parts helical to one part amorphous (Karkalas et al., 1986; Raphaelides et al., 1991).

V-amylose inclusion complexes have been investigated by cross polarisation/magic-angle spinning (CP/MAS) ^{13}C NMR by a number of authors (Gidley et al., 1988; Horii et al., 1987; Seneviratne et al., 1991). The results are generally in agreement, with the polysaccharide chemical shifts being fairly insensitive to hydration effects, unlike the A- and B-polymorphs of amylose. The chemical shifts are also not dependent to any significant extent on the number of glucosyl residues per turn in the helix since the chemical shifts of the C-1 and C-4 carbons are primarily determined by the glycosidic bond conformation. The only significant variation observed in the amylose chemical shifts for the different helical forms is the occurrence of the C-1 and C-4 resonances for an eight-fold helix about 1 ppm upfield compared to their six- and seven-fold counterparts (Gidley et al., 1988). The spectra of V-amylose in complexes differ from those of other polysaccharides (α-glucans) in that the C-4 peak at about 82 ppm is better resolved from the combined C-2, -3 and -5 peak. Compared with amorphous or retrograded amylose, the C-3 peak is partially resolved from the C-2 and -5 peak in the complexes. Further, the C-1 peak at about 103 ppm for the complexes is much sharper than the corresponding peak at 101–102 ppm for the amorphous form.

The insoluble V-helical amylose complexes precipitated at neutral pH for non-ionic ligands, and at lower pHs for anionic ligands, such as fatty acids, exist in three polymorphic forms, namely types I, II_a and II_b, each form being characterised by the temperature at which the distinctive endotherm associated with the dissociation of the amylose helix arises (Horii et al., 1987). Their dissociation temperatures are also dependent upon the fatty acid chain length and its degree of unsaturation. The type I forms have lower dissociation temperatures and are considered to be amorphous while the semi-crystalline type II forms give X-ray patterns characteristic of the six-fold single helices in crystallites. The polymorphic form of the helical structure has only relatively minor effects on the chemical shifts of the amylose resonances (Seneviratne et al., 1991).

It has been widely assumed that the small but significant amounts of monoacyl lipids (predominately free fatty acids and lysophospholipids) in cereal starches are present in amylose inclusion complexes since they can be extracted only under extremely rigorous conditions (Morrison, 1988). Indeed, until recently, the evidence for the existence of inclusion complexes has been inconclusive, mainly because they can be formed artifactually in any procedure that allows even limited swelling of the native starch granules. Gidley et al. (1988) concluded from the C-1 and C-4 ^{13}C chemical shifts for single helical amyloses and amorphous material in starch granules that the latter contains a significant fraction of such complexes in local conformations. We have used ^{13}C NMR to provide definitive proof that V-amylose inclusion complexes with the monoacyl lipids present in non-waxy cereal starches do exist in the native starch granules, i.e. they are not artifacts (Morrison et al., 1993a, b). It is somewhat surprising therefore that the chemical shifts and relaxation parameters of the actual ligands in V-amylose inclusion complexes have thus far received so little attention. Bulpin et al. (1982, 1986) studied amylose–sodium myristate interactions in solution, and well resolved signals were observed by solution-state ^{13}C NMR for both the amylose and the lipid at 95°C where the complex should be dissociated. However, at 45°C, where considerable complexation had occurred, the polysaccharide peaks broadened with an overall loss of intensity. Since only the free lipid was being observed by solution-state ^{13}C NMR, no change in the chemical shift for the mid-chain methylenes was reported. A similar effect was noted for ^{23}Na which was interpreted as evidence that the sodium is intimately involved with the helix, although interpretation of the results for quadrupolar nuclei is not always straightforward.

In this investigation, a series of V-amylose inclusion complexes formed from a series of mono-carboxylic fatty acids, namely palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3) and docosahexaenoic (DHA, 22:6) acids, glycerol monooleate (GMO), glycerol monopalmitate (GMP) and lysophosphatidylcholine (LPC) have been characterised by CP/MAS ^{13}C NMR to elucidate the effects of lipid structure, including unsaturation, on their environments in the helical structures. In some instances, normal Bloch decay (single pulse excitation-SPE) spectra have also been obtained to detect the slowly cross-polarising carbons in the complexes and in physical mixtures of amylose and lipids. Further, to probe the positions of bulky end groups in relation to the V-helical structure, ^{13}C labelling

has been used for the carboxyl and C-1(3) glycerol carbons in stearic acid and GMO, respectively. The preparation and thermal properties of the fatty acid inclusion complexes investigated here have been reported elsewhere (Karkalas et al., 1995).

2. Experimental

2.1. Complex preparation

Potato amylose (type III-polydisperse, mean chain length > 600, average degree of branching 6–8), the fatty acids (> 99% purity, unsaturated all *cis*) and monoglycerides were obtained from Sigma. The acyl chain in the LPC used comprises 90% palmitoyl (16:0) with the remaining 10% being mainly stearoyl (18:0). 1-^{13}C-stearic acid and 1(3)-^{13}C-glycerol (99% ^{13}C) were obtained from Prochem Ltd (St Albans, UK). 1(3)-^{13}C-GMO was synthesised from the labelled glycerol by reacting with oleylchloride, and purifying by preparative thin-layer chromatography.

The procedures used for the preparation of the freeze-dried water-washed inclusion complexes from the fatty acids and GMO and the subsequent dissociation, complexing and annealing to give the different polymorphs have been described elsewhere (Karkalas et al., 1995). Amylose was regenerated from its butanol complex by flushing exhaustively with oxygen-free nitrogen, and complexes were then prepared under totally anaerobic conditions to prevent oxidation. In order to avoid the use of dimethylsulphoxide which is also a complexing agent, an alkaline neutralisation route was used to dissolve the amylose and prepare the fatty acid complexes (Karkalas et al., 1995), whilst MG and LPC were dispersed in water to obtain the ligands in a state ready for complexation at neutral pH. After complexation, unsaturated fatty acids are exceptionally resistant to autooxidation (Morrison, 1978; Szejtli et al., 1975), so no oxidative changes are expected to occur during annealing and storage. Complexes used for NMR were both the low-melting type I polymorphs (T_m = 94–100°C) and the higher melting type II polymorphs (T_m = 110–125°C) (Karkalas et al., 1995). For comparative purposes, dry mixtures of amylose and lipids were preprared using a mass ratio of 7:1, close to the stoichiometry of the complexes.

Hydrated complexes containing the ^{13}C-labelled stearic acid were prepared by resuspending freshly precipitated complexes in phosphate buffer at pH intervals from 2.4 to 10, and centrifuging at 20 000*g* to obtain a gel that remained homogeneous in the NMR capsules during MAS. Labelled GMO gels were similarly prepared from neutral aqueous suspension.

2.2. ^{13}C NMR

CP/MAS ^{13}C NMR spectra of the complexes and some of the physical mixtures of amylose and the fatty acids investigated were obtained at 25 MHz using a Bruker MSL 100 instrument. Tetrakis(trimethyl)silane (TKS) with a chemical shift of 3.2 ppm was used as the internal standard. MAS speeds of about 4.5 kHz, a contact time of 1 ms and line broadening factors of either 5, 20 or 35 Hz were used depending upon sensitivity, the smallest factor being used for the measurement of chemical shifts to the nearest 0.1 ppm. ^{1}H rotating frame (^{1}H $T_{1\rho}$) and ^{13}C thermal (^{13}C T_1) relaxation times of the various groups of interest in the dry complexes were determined by standard CP-based pulse sequences.

The mid-chain methylenes in the fatty acids and MGO were clearly visible after about 1000 scans had been accumulated under these conditions for the dry complexes. However, due to the large number of scans required to observe the carboxyl carbons via CP (about 20 000) and the overlap between the amylose and glycerol carbons in the GMO complex, the ^{13}C-labelled stearic acid and GMO were used. Both CP and single pulse excitation (SPE) spectra were obtained for the dry and hydrated complexes prepared using the ^{13}C-labelled compounds. These hydrated complexes could still be spun readily at 4.5 kHz in the CP/MAS probe despite the high water levels which had no adverse effects on the tuning of the probe. For the SPE experiments, a relaxation delay of 20 s was employed which was sufficently long to enable the methylene carbons in the fatty acids to relax following the 90° pulse, but too short for the amylose carbons which had much longer ^{13}C T_1s (see results).

^{31}P NMR spectra of free and complexed LPC were obtained at 40.53 MHz using the Bruker MSL 100 spectrometer.

3. Results and discussion

3.1. Evidence for complexation, 1: saturated acids

The ^{13}C NMR spectra of the type I and II V-helical polymorphs for the amylose–stearic acid complexes are shown in Fig. 1. The polysaccharide chemical shifts are similar to those reported by Gidley et al. (1988), Horii et al. (1987) and Seneviratne et al. (1991) for other V-amylose complexes (although differences of about 1 ppm are evident for the C(1) chemical shifts). In some cases, the resolution between the C(3) and C(2) plus C(5) peaks in the range 71–74 ppm is somewhat better for the type I complex, as found by Seneviratne et al. (1991). However, there is no fundamental reason why the supramolecular packing of V_6 helices should have any significant effect on the amylose chemical shifts. Indeed, the similarity of the amylose and fatty acid chemical shifts for the corresponding stearic and palmitic acid complexes confirm that the spectra of each polymorph are almost identical for all common long-chain fatty acid complexes.

The chemical shift of the mid-chain methylenes occurs at

31.6–31.8 ppm for both the type I and II stearic and palmitic acid complexes (Table 1, no difference was expected between the type I and II polymorphs due to the short-range ordering within the helices being identical). As for the non-waxy cereal starches investigated previously (Morrison et al., 1993a, b), this value lies between those for solid crystals (33.2 ppm for physical mixture of amylose and stearic acid) and solutions (29.7 ppm).of long-chain fatty acids (Table 1). This is a general reflection of the atomic spacings in the crystals being shorter than those in the complexes, as calculated by Godet et al. (1993). The CP-determined ^{1}H $T_{1\rho}$ values of the long chain methylenes are reduced from 20 ms in a physical mixture of amylose and stearic acid (i.e. in the crystalline form) to about 5 ms upon complexation for both the type I and II polymorphs. The fact that both the polysaccharide and the stearic acid protons have the same ^{1}H $T_{1\rho}$ of about 5 ms indicates that they are in sufficiently close contact for spin diffusion between them to occur. Thus, the changes in both chemical shift and ^{1}H $T_{1\rho}$s confirm that complexes have been formed. Given the breadth of the mid-chain methylene peak at 31.6–31.8 ppm, it is doubtful whether small amounts of free acid in the complexes samples could be detected by ^{13}C NMR. However, DSC indicated that there was no endotherm arising from free acid for the palmitic acid complex (the melting point of palmitic acid being some 20°C lower than its amylose complex).

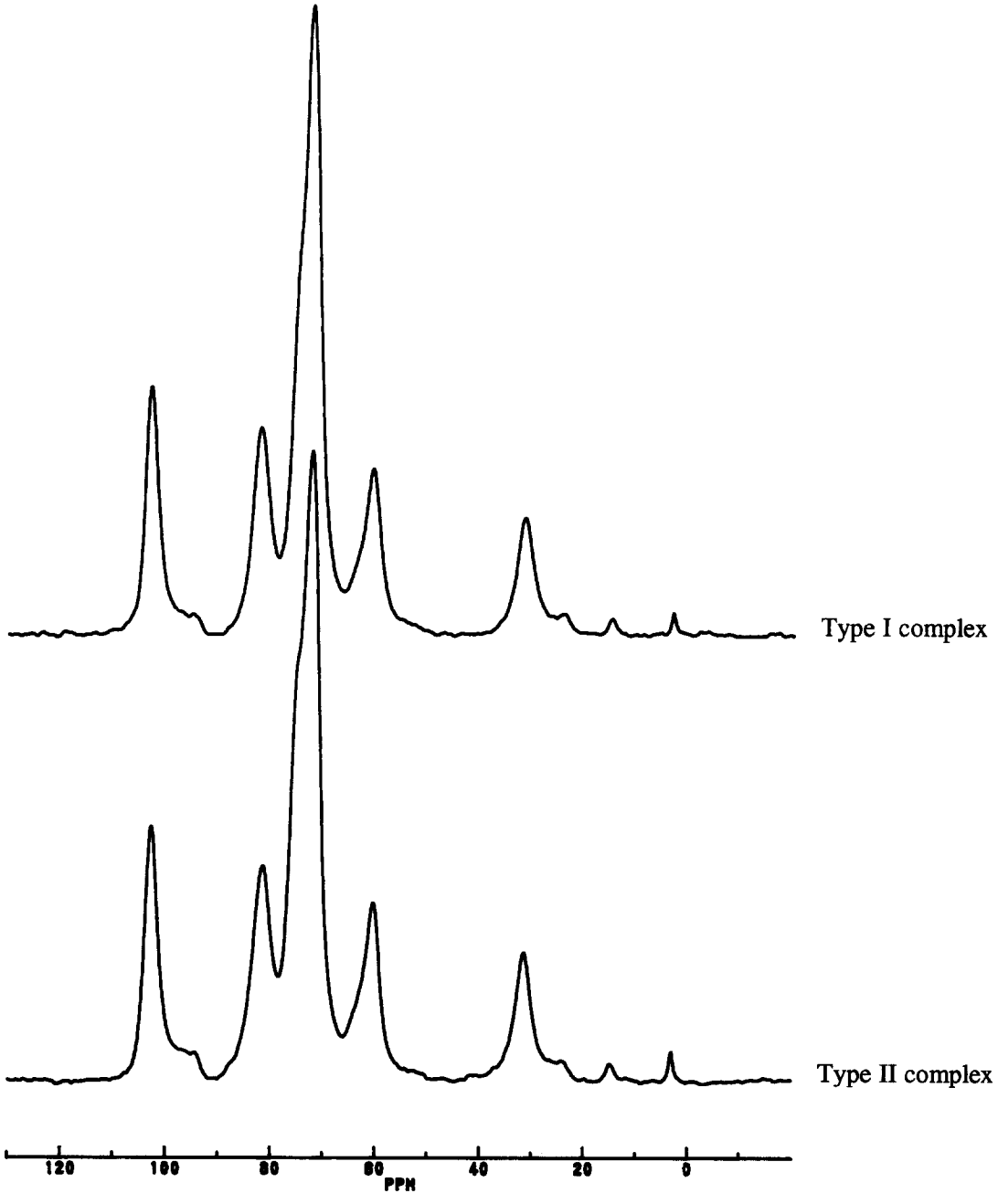

Fig. 1. CP/MAS ^{13}C NMR spectra of type I and II polymorphs of amylose/stearic acid complexes.

Fig. 2 shows the CP/MAS ^{13}C NMR spectra of freeze-dried amylose complexes formed from palmitic acid, GMP and LPC. In all cases, the mid-chain methylene lipid peak has shifted to 31.6 ppm (Table 1), confirming again that complexation has occurred. The chemical shifts of the mid-chain methylenes in crystalline lipids occur in the range 32.5–33.0 ppm (Vanderhart, 1981; Guo et al., 1995) very close to those found here for the crystalline fatty acids (Table 1).

3.2. Evidence for complexation, 2: unsaturated acids

Fig. 3 shows the CP/MAS ^{13}C NMR spectra of the physical mixture and freeze-dried complex for amylose/oleic acid. The same comparison for DHA is shown in Fig. 4, which also includes the SPE spectrum of the physical mixture. If the free lipid is a viscous liquid, as in these particular instances, no peaks should occur in the normal CP spectra obtained with relatively short contact times (~1 ms) due to the weak $^{1}H-^{13}C$ dipolar interactions. Indeed, this has been found to be the case, with the mid-chain methylene peaks only being observed by CP in the inclusion complexes but not in the physical mixtures of the lipids with amylose (Figs. 3 and 4). The lipid peaks are evident in SPE spectra as shown in Fig. 4 for DHA since, provided that the relaxation delay is long enough, this technique is inherently quantitative and does not discriminate between any particular carbon type on the grounds of molecular mobility. Indeed, the chemical shifts obtained for the viscous liquid are extremely similar to those from normal solution state ^{13}C NMR (at 100 MHz with chloroform as solvent) with the peak for the methylene between the double bonds occurring at close to 30 ppm (Table 1).

As anticipated, the chemical shifts in the range 30.4–31.4 ppm for the mid-chain methylenes in the oleic acid, linoleic and linolenic complexes are similar to those described above for the other saturated and mono-unsaturated lipid complexes investigated (Table 1). A larger shift of about 3.5 ppm upfield (from about 30 to 26.5 ppm) occurs upon complexation for the five methylene carbons in the centre of the pentadiene systems in DHA. Although some caution is required since the chemical shift change for DHA is that for forming a complex from a viscous liquid as opposed to a crystalline solid in the cases of stearic and palmitic acids, the corresponding change is about 1.5 ppm downfield (from 29.7 to 31.4 ppm) for the mid-chain methylenes in oleic acid (and also for solutions and melts of saturated acids). The large upfield shift for DHA might reflect stronger contacts with the V-helix where, according to the model proposed by Godet et al. (1993), the strongest van der Waals contacts should be with the glucosyl H-5 atoms. This could well arise from the compact chain folding of DHA within the helix cavity. No significant variations in chemical shift were found upon complexation for the corresponding methylenes in linoleic and linolenic acids.

The solution state ^{13}C NMR spectra of the unsaturated fatty acids comprise a number of alkenic peaks mainly in the range 128–130 ppm (Gunstone, 1991; Gunstone et al., 1994). For DHA, the solid state SPE spectrum of a physical mixture with amylose contains a peak centred at 128.3–128.5 ppm from the six double bonds present (Fig. 4). Upon complexation, there is a loss of resolution for the alkene resonances but there would appear to be no large chemical shift changes (Table 1, $>$ 1 ppm). This might imply that, due to the π-bonding, the glucosyl van der Waals contacts are much weaker than for the methylene

Table 1
Summary of ^{13}C chemical shifts for lipid methylene and alkene carbons in free and complexed forms

Ligand	Complexed	Free	
		Solid[a]	$CHCl_3$ solution/liquid[b]
Mid-chain methylene, $-(CH_2)_n-$			
Stearic and palmitic acids	31.5–31.8	33.2	29.7
GMO	31.6–31.8	33.2–33.6	29.7
Oleic acid	31.0	—	29.7
Linoleic acid	31.4	—	29.7
Linolenic acid	30.4	—	29.7
Methylene adjacent to alkenic groups, $CH_2-CH=CH-(CH_2)_n-CH=CH-CH_2-$			
DHA	26.4 [b]	—	29.5 (30.0)[b] 27.4
Linoleic acid	25.5–27.3	—	27.2
Linolenic acid	26.2–27.2	—	27.2
Alkene, $-CH=$			
Oleic acid	130.1	—	129.7, 130.0
Linoleic acid	129.0	—	127.9–130.2
Linolenic acid	129.0	—	127.2–130.2
DHA	128.4	—	127.0–129.0(128.3, 129.9)[b,c]

Estimated error in the range ± 0.1–0.2 ppm for the chemical shifts; [a] measured in this study; [b] neat liquid lipids, values in parentheses measured in this study by SPE; [c] major peaks.

groups. The similarity in the chemical shifts of the glucosyl C(1) for the DHA and other complexes investigated indicates that the helix is no larger than seven-fold; an eight-fold helix would have given rise to an upfield shift of about 1 ppm to 102 ppm (Gidley et al., 1988).

Horii et al. (1987) reported that the polysaccharide ^{13}C T_1 values for amylose are considerably shorter than for cellulose due to the lower degree of ordering. The values obtained here for the polysaccharide and mid-chain methylene carbons for the stearic, oleic acid complexes are summarised in Table 2. The polysaccharide carbon values follow a similar pattern to those reported by Horii et al. (1987) with C-6 having a much shorter T_1 than the other carbons. The values for the mid-chain methylenes of 0.8–1.0 s compare with that of about 5 s for the amylose/stearic acid physical mixture. This is consistent with the long alkyl chains being less ordered inside the helix than in solid fatty acids, especially since the more unsaturated fatty acids investigated can also fit into the cavity. Although some caution is required over their interpretation, the fairly

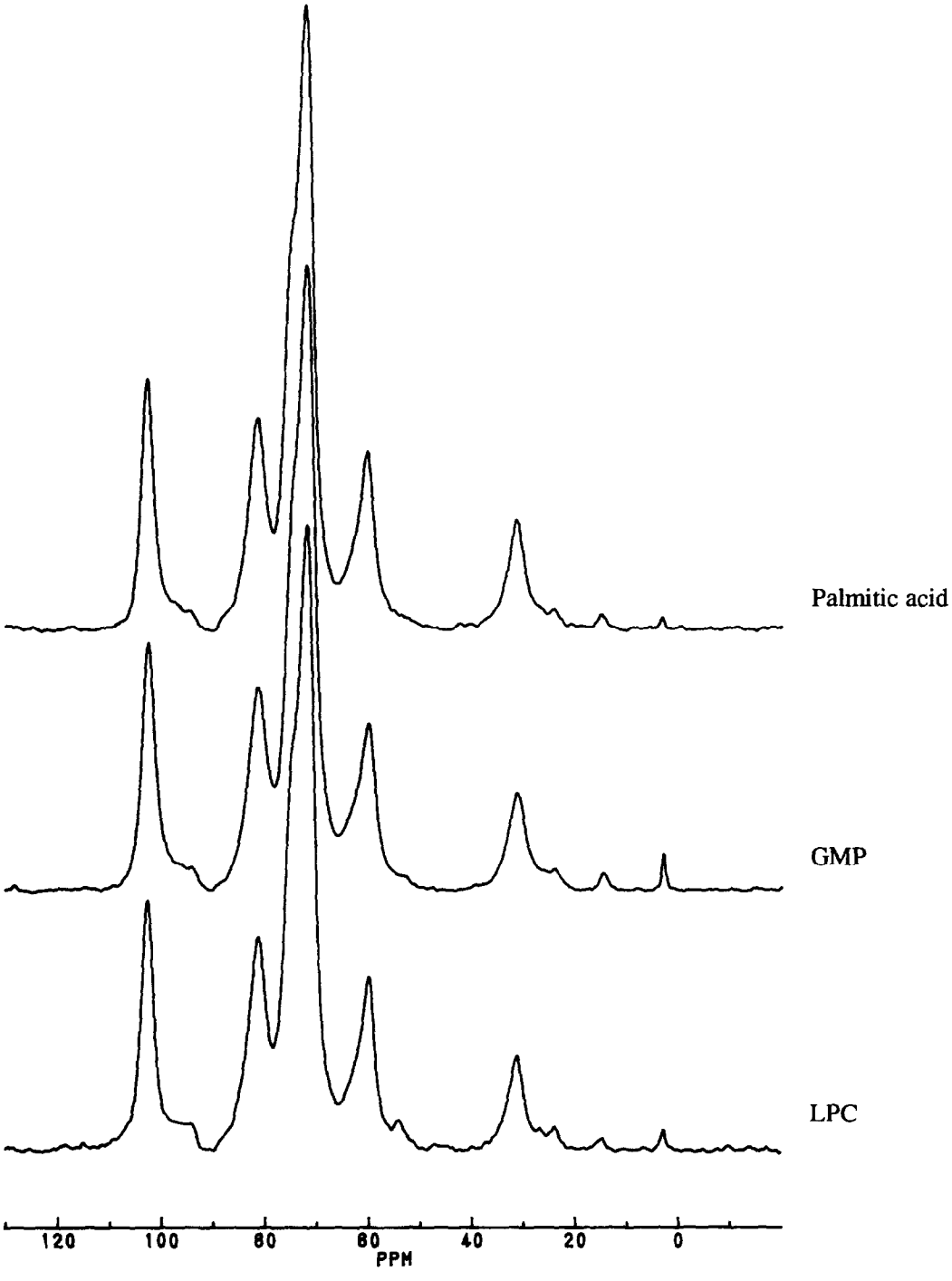

Fig. 2. CP/MAS ^{13}C NMR spectra of freeze-dried amylose complexes formed from palmitic acid, GMP and LPC.

uniform ^{13}C T_1 values for the mid-chain methylenes provides yet further evidence that complexation has occurred.

3.3. Carboxyl environment

It is well known that the chemical shifts of carboxyl carbons in dilute aqueous solution are pH dependent, ranging from about 179 ppm for the fully protonated (acid) form to 184 ppm for the fully ionised (salt) form at high pH (Small, 1986). For example, the pairs of values reported for *n*-decanoic and oleic acids are 181/184 and 179/182 ppm, respectively. A somewhat smaller variation in the range 36–40 ppm is observed (Small, 1986) for the methylene adjacent to the carboxyl group. Fig. 5 shows the effect of pH on the chemical shift of the carboxyl carbon (^{13}C-enriched) in the hydrated gel forms of the amylose/stearic acid complexes with values for the acid and salt forms being about 177 and 182 ppm. The shift of 177 ppm for the salt form is lower than that found in dilute solutions, possibly due to the concentration of the buffer salt used. However, virtually all the carboxylic acid sites would appear to be accessible and the titration curve presented in Fig. 5 for the hydrated gels resembles those for free fatty acids in phospholipid bilayers (Kantor et al., 1978) where the acyl chains are in local non-polar

Table 2
^{13}C Thermal relaxation times for amylose/fatty acid V-helical complexes

Complex	Relaxation time (s)					
	Carbon type					
	Amylose (glucosyl)					Fatty acid
	C-1	C-2,5	C-3	C-4	C-6	Mid-chain $-CH_2$
Stearic acid	47	18	17	33	2.6	0.9
Oleic acid	65	20	25	53	3.3	0.8
DHA	38	17	18	28	3.5	0.6

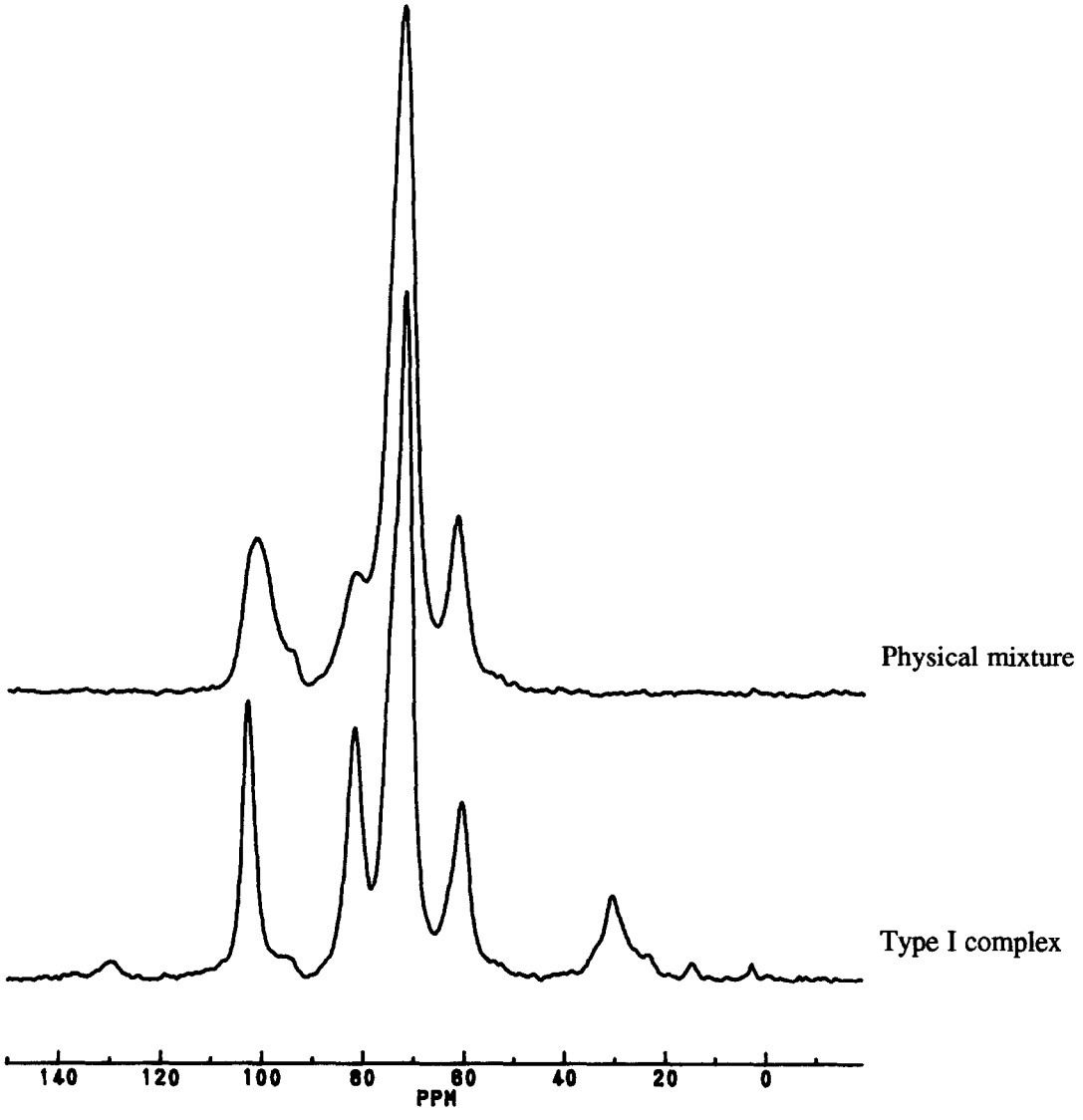

Fig. 3. CP/MAS ^{13}C NMR spectra of amylose/oleic acid samples; physical mixture (mass ratio of 7:1) and type 1 complex.

environments which are ordered often in a "liquid crystalline" form.

Fig. 6 shows (i) the SPE and CP/MAS spectra of the hydrated gel form (pH of 5) of the amylose/stearic acid complex prepared from the ^{13}C-labelled carboxyl precursor and (ii) the CP/MAS spectra of the freeze-dried complexes prepared at 60 and 90°C, also with the ^{13}C-labelled carboxyl precursor. The chemical shifts for the carboxyl groups in the freeze-dried complexes are also included in Fig. 5. The complex prepared at 60°C with a nominal pH of 5 only gives a peak from the acid form close to 177 ppm as for the hydrated gel form. However, the 90°C complex displays a doublet indicating that the salt form is also present and some carboxylate groups may not readily titrate. This situation parallels the behaviour of fatty acids adsorbed on albumin carrier protein where a significant fraction of the total carboxyl sites do not titrate as a result of ion-pair electrostatic interactions (Cistola et al., 1987).

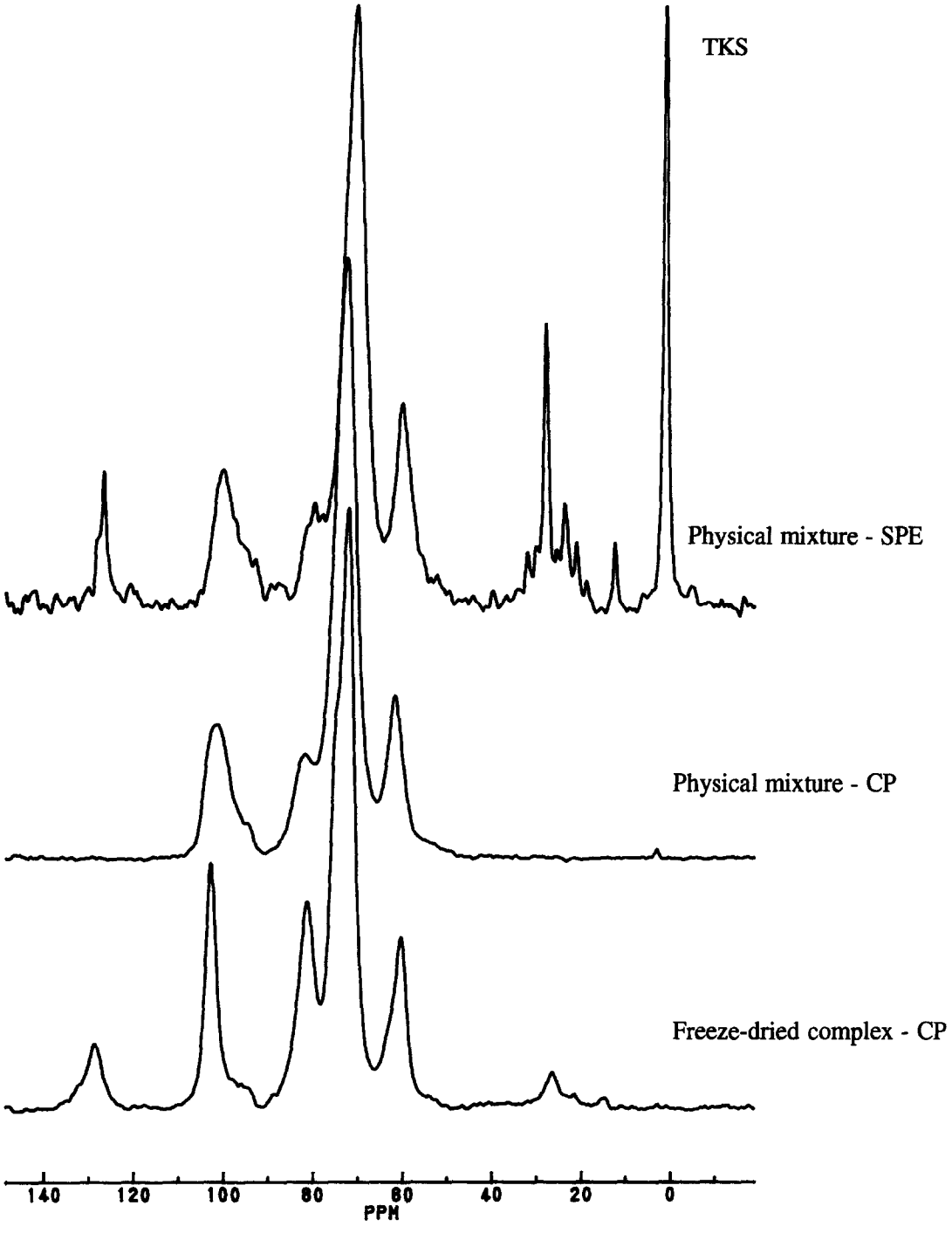

Fig. 4. SPE and CP/MAS ^{13}C NMR spectra of amylose/DHA samples; physical mixture-SPE, physical mixture-CP and freeze-dried complex-CP.

The carboxyl is clearly the dominant peak in the spectra of the freeze-dried amylose/stearic acid complexes (Fig. 6). Remarkably, in the CP spectra of the hydrated complexes obtained with contact times of 1 ms, the carboxyl peaks from the 90% ^{13}C enriched stearic acid are less intense than the polysaccharide peaks (Fig. 6). In contrast, the carboxyl peaks are close to their expected intensity in the SPE spectrum which, since a fairly long recycle time of 20 s was employed, does not discriminate too strongly against carbons on the grounds of mobility. The extremely low CP intensity of the carboxyl carbon clearly arises from an extremely weak dipolar interaction characteristic of an extremely hydrated mobile environment. Thus, it can be concluded that the carboxyl group in the stearic acid complex must lie outside the helix. For the freeze-dried complexes, the CP intensity of the carboxyl group is much greater due to immobilisation which is likely to occur through hydrogen bonding interactions with the extra-helical OH groups of amylose.

3.4. Glycerol environment

Starting with 99% ^{13}C in glycerol C1, a mixture of 49% 1*(3)-^{13}C-GMO and 49% 1(3*)-^{13}C-GMO (esterified at C-1 with C-3 free and vice versa) was obtained via esterification with oleylchloride. Fig. 7 shows the SPE and CP/MAS spectra of the unbuffered hydrated gel form of the labelled amylose/GMO complex, the contact times being 1 and 5 ms for the CP spectra. Compared to the reasonably quantitative SPE spectrum, the intensities of the C-1 and C-3 GMO carbons at 66.0 and 63.5 ppm, respectively (values close to the solution state chemical shifts of 65.1 and 63.5 ppm (Gunstone, 1991; Gunstone et al., 1994), are clearly grossly underestimated by CP. Subtraction of the corresponding spectra for the unlabelled GMO complex left only the C-1 and C-3 peaks, confirming that C-2 was not labelled. This would have given rise to a peak close to 68 ppm (Morrison, 1978; Szejtli et al., 1975).

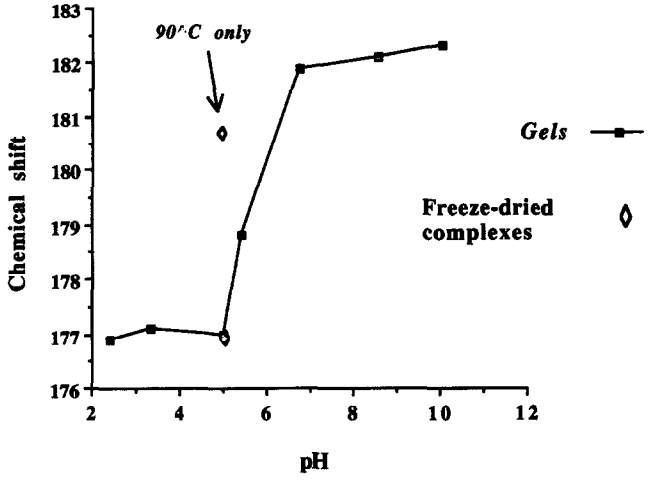

Fig. 5. Effect of pH on chemical shift of carboxyl carbon in the hydrated gel forms of the amylose/stearic acid complexes (– · –). The chemical shifts for the freeze-dried complexes are also included.

It is considered that differences in polarisation rates are mainly responsible for the low intensity of the glycerol peaks in relation to those from the amylose since the measurements on the amylose–stearic acid complex established that the amylose and stearic acid carbons have a common ^{1}H $T_{1\rho}$ (5 ms). Thus, significant differences between the glycerol and amylose carbons would not be expected here since they are in sufficiently close contact for spin diffusion to occur. Since the glycerol carbons are protonated, the discrimination by CP is not quite as severe as for the carboxyl carbon in the stearic acid complex (Figs. 6 and 7) because their polarisation transfer rates are likely to be faster. Therefore, as for the carboxyl group, the CP spectra strongly suggest that the glycerol groups possess considerable mobility through extensive hydration from being positioned outside the helix cavity. Of the two ^{13}C-labelled positions, C-3 (CH_2OH, 63.5 ppm peak) cross polarises the faster indicating that it is less mobile than C-1 (CH_2O-acyl, Fig. 7, compare CP spectra for 1 and 5 ms). This could arise from contacts with either an adjacent amylose helix, analogous to hydrogen bonding between glucosyl residues, or free hydroxyls (C-2 and C-3) on adjacent glycerols. The latter contact is probable bearing in mind that the stoichiometry of MG complexes shows the same amylose:ligand molar ratio as for fatty acid complexes (Karkalas et al., 1986; Raphaelides et al., 1991), and there can only be limited separation between helix segments.

The SPE and CP/MAS spectra of the freeze-dried form of the amylose/GMO complex prepared from the ^{13}C-labelled GMO at C-1 and C-3 are shown in Fig. 8 and, as expected, the C-1 and C-3 peaks dominate, but the resolution is clearly inferior to the hydrated complex due to broadening effects from the additional hydrogen bonding interactions with the extra-helical OH groups.

Since the carboxyl groups of fatty acids lie outside the helix cavity, it was not surprising to find that glycerol esterified to a carboxyl also lay outside. Consequently, it can be concluded that all other polar groups larger than carboxyl will also lie outside the helix cavity. In the case of LPC, this was supported by the fact that no change in the ^{31}P chemical shift was observed upon complexation with amylose: there is also evidence that the phosphorylcholine end of the molecule is accessible to the enzyme phospholipase-B (Acker et al., 1971), which means that this polar end group cannot be in close proximity to the amylose. These observations are consistent with the findings that type I complexes containing fatty acids or monoacyl glycerol ligands, which have compact polar groups, can be annealed to type II polymorphs (Seneviratne et al., 1991; Karkalas et al., 1995). However, complexes with bulkier polar groups (surfactants and LPC) cannot be annealed at higher temperatures to higher-melting more crystalline polymorphs because of steric hindrance to helix packing.

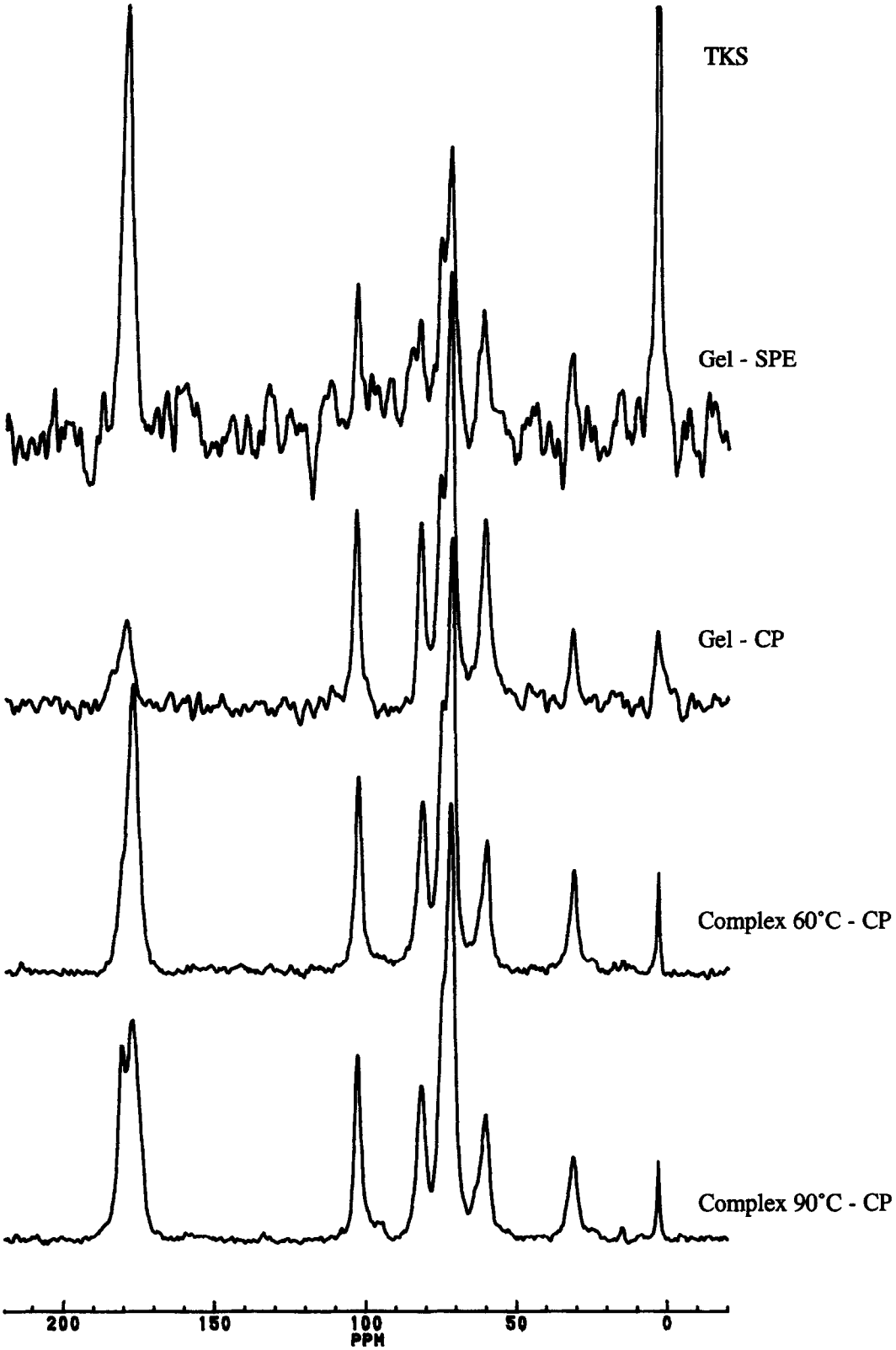

Fig. 6. Comparison of SPE and CP/MAS spectra of the hydrated gel form of the amylose/stearic acid complex prepared from the 1-^{13}C-stearic acid at a pH of 5 and of the CP/MAS spectra of the corresponding freeze-dried complexes prepared at 60 and 90°C. The contact times are 1 and 3 ms for the CP spectra of the freeze-dried complexes and hydrated gel, respectively.

4. Conclusions

The CP/MAS ^{13}C NMR results have proved conclusively that amylose complexes with a range of mono-carboxylic fatty acids with varying degrees of unsaturation. The chemical shift of the mid-chain methylenes in stearic acid moved about 1.5 ppm upfield upon complexation with the ^{1}H rotating frame relaxation times becoming identical for the lipid and amylose. With the exception of DHA, the mid-chain methylenes have the same chemical shift for all the

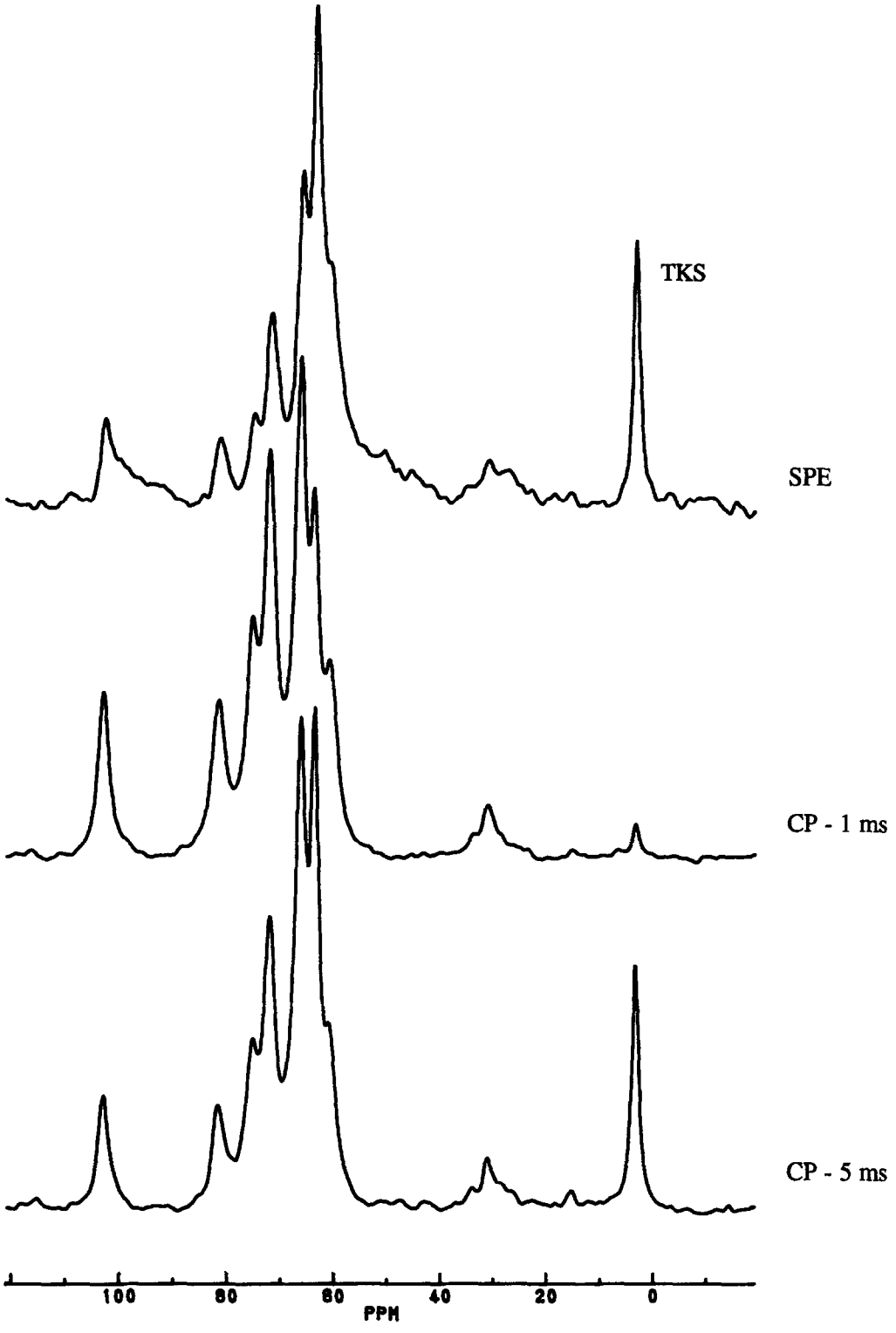

Fig. 7. Comparison of SPE and CP/MAS spectra of the hydrated gel form of the amylose/GMO complex prepared from the ^{13}C-labelled GMO at glycerol C-1(3). The contact times are 1 and 5 ms for the CP spectra.

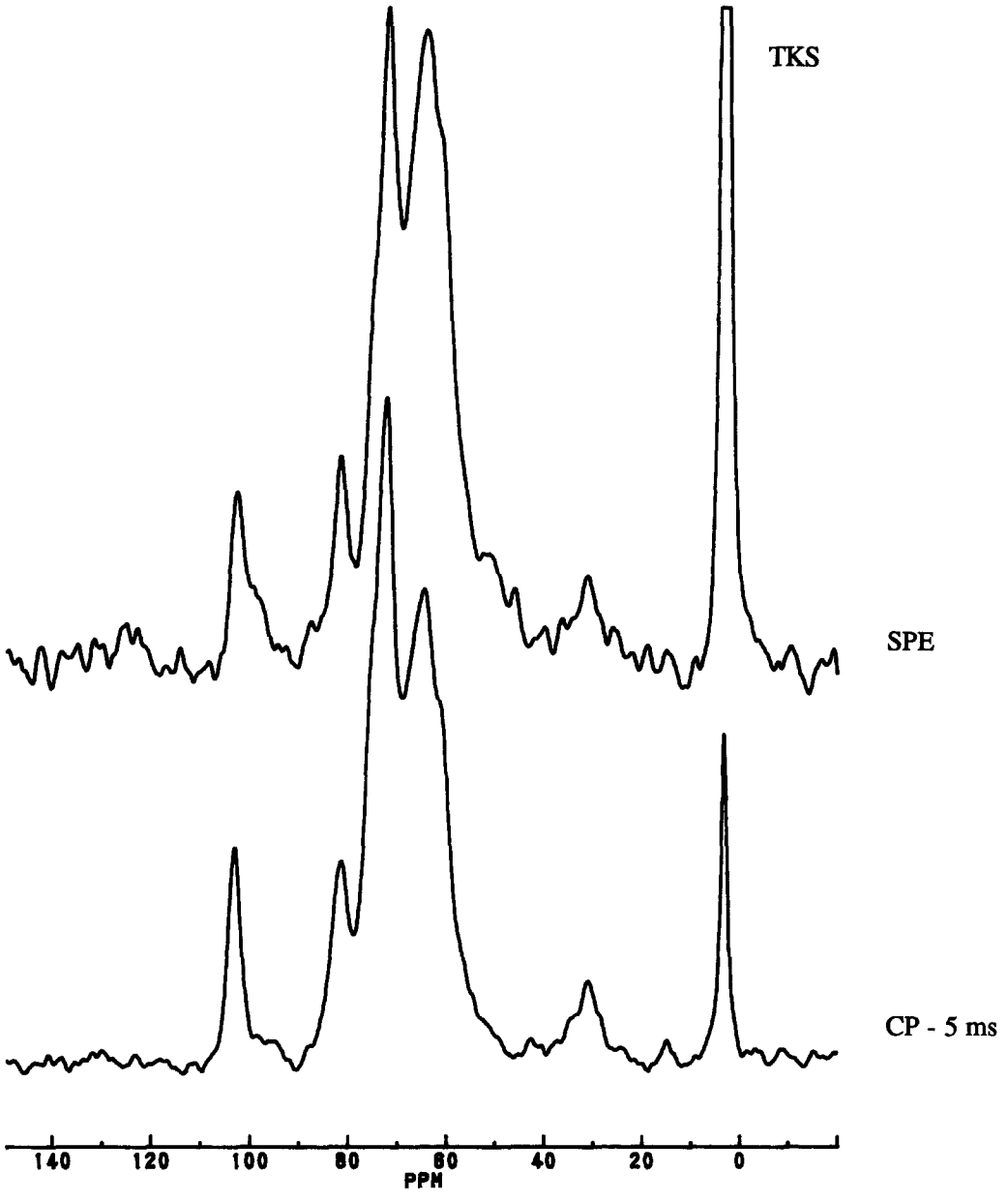

Fig. 8. Comparison of SPE and CP/MAS (1 ms contact time) spectra of the freeze-dried form of the amylose/GMO complex prepared from the ^{13}C-labelled GMO.

other unsaturated fatty acids and lipids investigated. The cross-polarisation dynamics for the carboxyl and glycerol groups in stearic acid and GMO, respectively, have indicated that these groups occupy highly mobile conformations in the hydrated complexes compared to the long chain methylenes situated inside the V-helical cavities.

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