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Electron Spin Resonance Studies of Starch–Water–Probe Interactions

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SUMMARY

Interactions between starch, water and stable nitroxide radicals were studied by electron spin resonance. The motional properties of TEMPO, 4-(2-bromoacetamido)TEMPO (BrAcTEMPO), 5-DOXYL-stearic acid and 16-DOXYL-stearic acid probes as well as a label covalently attached to amylopectin were investigated in concentrated (10–50%) starch–water systems as a function of temperature, concentration of polymer and storage period. Compared with the free probes in solution, TEMPO and BrAcTEMPO showed slower tumbling rates in starch–water dispersions or gels, suggesting a higher microviscosity in the probe's environment. The spectra, however, remained motionally narrowed. In contrast, the three line spectra of the fatty acid probes in solution became highly anisotropic in the presence of starch. The results indicated that these probes were highly immobilized at room temperature by the starch granules or by the polysaccharide gel matrix. These interactions are weakened at elevated temperatures where the spectra revealed the presence of both motionally narrowed and motionally slowed spin populations. The nitroxide label on the amylopectin exhibited a much slower mobility than the corresponding free probe as well as being found to be more motionally sensitive to temperature changes; such motional behavior was interpreted as reflecting contributions from rotation of the label around the chain backbone as well as local segmental motion of the polymer chain itself. Starch gels doped with free probes or the spin labelled amylopectin displayed no change in the motion of the nitroxide group upon storage, i.e. the tumbling rates did not follow the time-dependent conformational changes associated with the retrogradation phenomenon.

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INTRODUCTION

Electron spin resonance (ESR) techniques have been extensively used to characterize the structure and molecular dynamics of biological as well as synthetic macromolecular systems (Berliner, 1976; Knowles *et al.*, 1976; Miller, 1979; Boyer & Keinath, 1980; Woodward & Bovey, 1980; Devaux & Seigneuret, 1985). Most of the ESR work falls into two categories: 'spin-probe' experiments in which the paramagnetic molecule is present as a free or guest molecule and 'spin-label' studies where the paramagnetic species is incorporated covalently into the polymer chain. Experimental evidence has shown that the molecular motions of the probe are closely related to those of the polymeric matrix, which in turn are dictated by the structure/organization of the polymer as well as its response to the environment (solvent–temperature). Two techniques have been mainly used to assess the motion of the spin probe/label in ESR studies. The first employs the calculation of the correlation frequency of the tumbling probe while the second is based on the changes in the extrema separation or lineshapes of the spectra of stable nitroxide radicals with temperature. For the latter, the outermost peak-to-peak separation narrows with increasing motion of the probe due to averaging of the anisotropic hyperfine interaction and the anisotropy of the *g*-tensor. In most cases of polymeric materials this change occurs rather rapidly at a temperature that is found to correlate well with the glass transition temperature (Kumler *et al.*, 1977; Tormala & Weber, 1978). In view of such dependence of spectral parameters on the state of the polymeric material in terms of its chain segmental motion and its interactions with the probe, as well as the recently reported calorimetric evidence for second-order transitions in heated starch/water systems (Maurice *et al.*, 1985; Biliaderis *et al.*, 1986), it seemed of interest to explore the phase transitions of starch using ESR. In this respect, ESR offers the advantage of probing these phenomena in concentrated samples (which are 'transparent' to microwave radiation) and of providing motional information for processes occurring over a timescale of 10^{-11}–10^{-7} s.

Two recent studies have employed ESR methodologies to investigate starch–water interactions using PDTEMPONE (*N*-oxo-2,2,6,6-tetramethylpiperidine-D_{16}-4-ketone), TEMPO (2,2,6,6-tetramethyl-piperidine-1-oxyl) and 16-DOXYL-stearic acid as spin probes (Pearce *et al.*, 1985; Windle, 1985). Although the line shapes of PDTEMPONE and TEMPO were not affected, their rotational correlation times (τ_c) in the presence of starch showed slower motion. On the other hand, the spectrum lineshape of starch/water/16-DOXYL stearic acid was

markedly different from the three line spectrum of the probe alone in solution, thus indicating a much stronger interaction between starch and spin probe. In the light of these findings, it was decided to further examine starch–water–probe interactions using several stable nitroxide probes as well as a spin-labelled amylopectin preparation over a range of starch concentrations, temperatures and storage periods.

EXPERIMENTAL

The waxy maize and wheat starch (21% amylose) samples used in this study were obtained commercially. The spin probes, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), 4-(2-bromoacetamido)TEMPO (BrAcTEMPO), 16-DOXYL-stearic acid (16-DSA) and 5-DOXYL-stearic acid (5-DSA) were products of Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). The chemical structures of these probes are shown in Fig. 1. Spin labelling of amylopectin was carried out

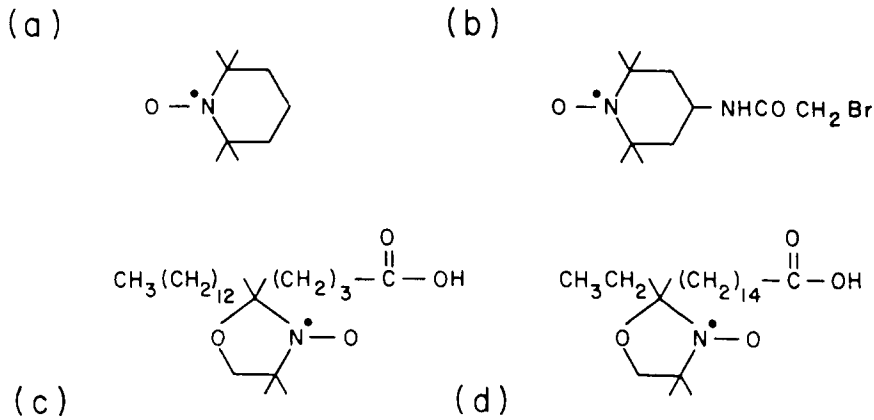
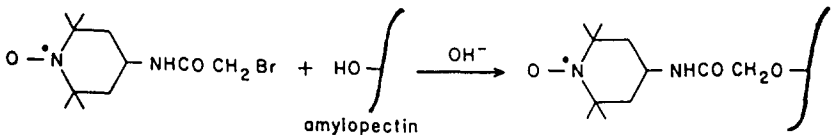


Fig. 1. Chemical structures of the spin probes used in this study: (a) TEMPO; (b) 4-(2-bromoacetamido)TEMPO; (c) 5-DOXYL-stearic acid; (d) 16-DOXYL-stearic acid.

using the method described by Aplin & Hall (1979). Solutions of well-dispersed waxy maize starch (15 mg ml^{-1} of 4% NaOH) were reacted overnight at 20°C with BrAcTEMPO (0·25 mg(mg of polysaccharide)$^{-1}$) dissolved in acetone (50 mg ml^{-1}). This reaction yields stable acetamido ether linkages to free hydroxyl groups, presumably distributed at random sites on the polysaccharide chains:



The reaction mixture was then neutralized with a dilute HCl solution and exhaustively dialyzed against phosphate buffer (50 mM, pH 7·0) and distilled water, to remove unbound label, using a Spectrapor membrane (molecular weight cutoff 6000–8000 daltons), until no detectable residual ESR signal was observed in the dialyzate. Subsequent freeze drying resulted in a spin-labelled amylopectin (slAMY) preparation which could be readily solubilized in hot water. Double integration of the ESR signals from BrAcTEMPO solutions of known concentrations provided standards for determining the spin concentrations of the unknown labelled material. These measurements indicated that one nitroxide group was present per ~200 anhydroglucose residues, assuming uniform substitution on the molecule. This low level of modification ensures negligible spin–spin, dipolar broadening between radicals of close proximity. This condition was further verified in parallel experiments where the amount of the spin label was varied in the derivatization procedure; at the above level of label no contributions to line-widths were found from spin exchange or dipole–dipole interactions between spins.

Starch samples for ESR analysis were prepared by suspending starch granules in the probe or slAMY solutions and allowing a period of 4 h at 20°C before measurements. The starch–water–probe (label) dispersions were then drawn into a 50 μl disposable glass capillary (0·8 mm i.d., Accupette) and placed inside a quartz tube (3 mm o.d.). All spectra were recorded with a Varian E-109 spectrometer (X-band), equipped with a nitrogen gas flow variable temperature controller. Typical operating conditions were: scan range, 250 G; modulation amplitude, 0·8 G; microwave frequency, 9·31 GHz; microwave power, 12·5 mW; time constant, 0·25 s; and scan time, 8 min. For all heating–cooling experiments the temperature was varied in 5°C increments between 10 and 95°C and the samples were allowed to come to temperature equilibrium (usually 5–6 min) before recording the spectra. Typical ESR spectra for the probes or slAMY/starch mixtures are presented in Fig. 2. For rotational correlation time (τ_c) measurements, the following equation was used for spectra that were motionally narrowed (i.e. three lines (Knowles *et al.*, 1976)):

$$\tau_c \text{ (in seconds)} = 6{\cdot}5 \times 10^{-10} \times W_0 \times [(h_0/h_{-1})^{1/2} - 1] \qquad (1)$$

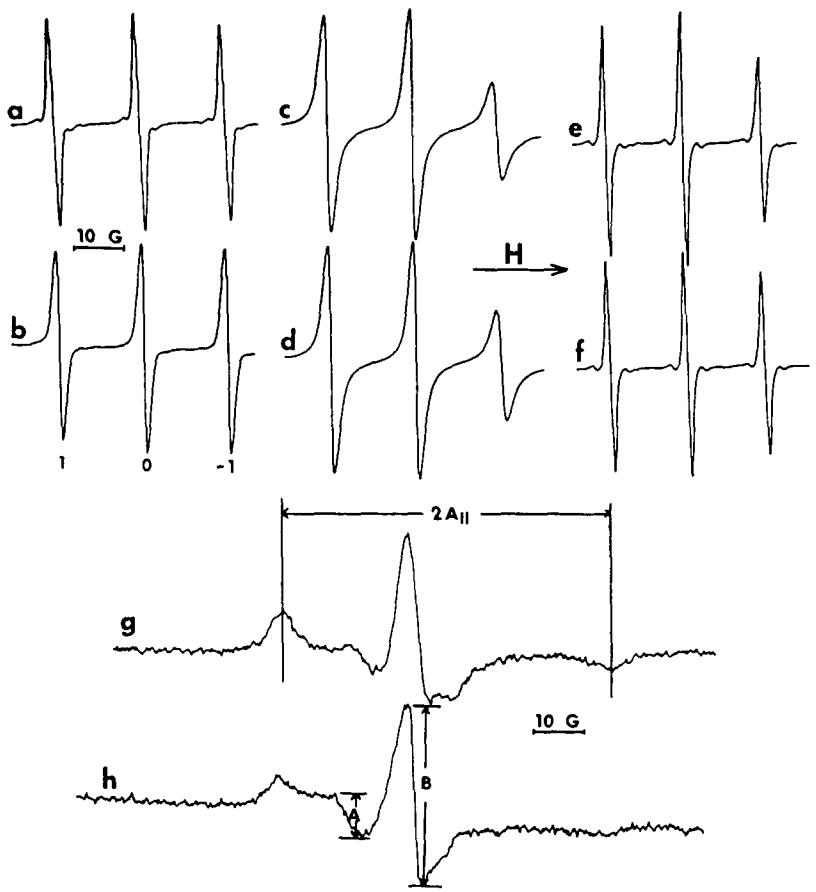


Fig. 2. Typical ESR spectra at 20°C: (a) 30% wheat starch in aqueous TEMPO (25 μM); (b) 30% wheat starch in aqueous BrAcTEMPO (250 μM); (c) 0·75% (w/v) slAMY solution; (d) 30% wheat starch in aqueous (0·75% w/v) slAMY; (e) aqueous 5-DSA (150 μM); (f) aqueous 16-DSA (150 μM); (g) 10% wheat starch in aqueous 5-DSA (150 μM); (h) 10% wheat starch in aqueous 16-DSA (150 μM).

where h_0 and h_{-1} are the peak-to-peak heights of the mid-field and high-field lines (shown in Fig. 2(b)) and W_0 is the line width of the central peak (0). For a more accurate estimation of the latter, the central peak was rescanned using a scan range of 20 G. Certain samples of the starch gels were stored at 25°C and scanned over a period of 7 days to explore any changes in the motional behavior of the radicals as a result of the time-dependent recrystallization phenomena in the polysaccharide gel matrix. For fatty acid probe–starch mixtures, where the spectra were indicative of very slow tumbling rates, the parameters used to describe the spectral

response to temperature changes were the extrema separation $2A_{||}$ (5-DSA, Fig. 2(g)) and the ratio of A/B peaks (16-DSA, Fig. 2(h)). These parameters have been frequently used in systems exhibiting motionally slowed spectra (Morrisett *et al.*, 1975; Novosad *et al.*, 1976; Vaughan *et al.*, 1980; Simatos *et al.*, 1981).

Differential scanning calorimetry (DSC) of aqueous starch and starch–fatty acid probe mixtures was carried out using a DuPont 9900 thermal analyzer equipped with a 910 cell base and a pressure DSC cell. Operation and calibration of this equipment were essentially as described by Biliaderis *et al.* (1985). Data analysis for transition temperatures and enthalpy changes (J g^{-1} of material) was performed using the DuPont software programs. The starch samples used for calorimetry were defatted by hot extraction with 85% methanol (48 h).

RESULTS AND DISCUSSION

Motional behavior of TEMPO, BrAcTEMPO and slAMY: temperature, concentration and time effects

Representative ESR spectra at 20°C of starch–water (30% w/w) suspensions for TEMPO (25 μM) and BrAcTEMPO (250 μM) are shown in Fig. 2(a) and 2(b), respectively. A sharp three line spectrum in both cases was obtained, similar to those of the probes alone. This behavior is generally interpreted in terms of isotropic motion. However, the calculated correlation times for starch–water systems were found to be larger than the values for the probes in water: 0·048 ns vs. 0·012 ns for TEMPO and 0·060 ns vs. 0·028 ns for BrAcTEMPO. Measurements of line-widths (not shown) over the entire temperature range revealed that line broadening from intermolecular interactions (dipolar exchange broadening) is not the cause for changes in the τ_c at the spin-probe or slAMY concentrations employed herein. Hence, motional modulations are mostly responsible for lineshape changes which are manifested primarily in the peak height ratios. These results also confirm the findings of Windle (1985) and Pearce *et al.* (1985). The observed slower motion in the presence of starch suggests that the probe molecules experience an environment of higher microviscosity. Pearce *et al.* (1985) have recently reported that TEMPO does not irreversibly interact with the granular material and thus it can be removed by washing. After centrifugation of the starch–water–probe mixtures, the τ_c of TEMPO and BrAcTEMPO in the supernatants were found to be identical to those of the aqueous solutions of the probes. Therefore, considering that the probe molecules

partition between the interior amorphous granular regions of the starch and the aqueous phase surrounding the granules, the slower mobility can be explained in terms of differences in the properties of sorbed and bulk water and/or weak interactions between the probe and the granular structure. Calorimetric and NMR measurements as well as dielectric relaxation spectra of water associated with macromolecules (Harvey & Hoekstra, 1972; Kuntz & Kauzmann, 1974; Yang & Rupley, 1979) do distinguish between bound water, which has a relaxation time near 10^{-9} s, and the bulk-like water that gives a relaxation time of about 2×10^{-11} s. As such, the ESR spectra of probes, which do not interact with the starch, must represent an average tumbling behavior of spin-probe molecules distributed between the sorbed and the more mobile bulk water. Thus, the recorded average motional freedom of the probe would be expected to become more restricted in the presence of starch. In this respect, it is of interest to note that similar changes in the motional behavior of TEMPONE were observed in hydrated powders of lysozyme containing water at levels above the amount required for completion of the monolayer hydration shell (Rupley *et al.*, 1980). Hilton *et al.* (1977) have reported that the properties of water sorbed on macromolecules can best be described by those of a viscous liquid.

In contrast to the symmetrical three line spectra of the free probes, slAMY exhibited a marked decrease in the lineheight of the high-field line (Fig. 2(c)). As a result, the calculated τ_c for the attached label was 0·5 ns (20°C), about 15 times larger than for the free BrAcTEMPO. This implies that the motional freedom of the nitroxide group is substantially reduced upon covalent attachment of the label to the polysaccharide chain backbone. Two processes can contribute to the tumbling rate of a label attached to a polymer in solution. The first is the rotation about single bonds in its linking unit with the macromolecule, and the second is the motion due to the polymer itself which consists of both segmental and rotational motions of the entire molecule. However, for large macromolecules, end-over-end motion does not occur within the ESR timescale (i.e. $\tau_c > 10^{-7}$ s; Knowles *et al.* (1976)) and consequently the contribution from the overall tumbling of the molecule is negligible. The ESR spectrum of slAMY in solution must therefore represent motional contributions of the spin label itself with respect to the polymer backbone as well as local segmental motion of the polymer chains. The latter is also substantiated by the studies of Florine *et al.* (1984) on the molecular motion of spin-labelled dextrans in dilute aqueous solutions. These authors, using a label rigidly bound to dextran (i.e. minimizing free rotation of the nitroxide about bonds joining it to the macromolecule), interpreted the τ_c of 0·5–1·2 ns values as reflecting averaged contribu-

tions from a relatively rapid motion of chain ends (it becomes more pronounced with shorter chains) and a slower short-range segmental reorientation that spans the entire length of the polymer chain. Unlike the free probes, the rotational mode of slAMY was essentially unaffected by the presence of starch granules. The 30% starch in aqueous (0·75% w/v) slAMY gave spectra and a τ_c value similar to the labelled material in solution (Fig. 2(c) vs. Fig. 2(d)). This is not surprising if one considers that the spin label no longer has access to the internal granular phase due to the molecular size of the branched polysaccharide molecule. Thus, the attached nitroxide groups always find themselves in the bulk, largely mobile, aqueous phase surrounding the granules.

Figure 3 shows the temperature dependence (heating and cooling modes) of the correlation times for the motion of the probes and slAMY in aqueous solution alone or in the presence of 30% wheat starch. Correlation times were calculated using eqn (1) and assuming isotropic motion. As the temperature increases from 20 to 95°C, the τ_c of free TEMPO and BrAcTEMPO tends to decrease slightly, reaching a limiting value at high temperatures. However, greater responses of τ_c to temperature changes were observed for the slAMY solution. This implies that the proportion of motion contributed by segmental reorientation of the polymer backbone increases more rapidly with

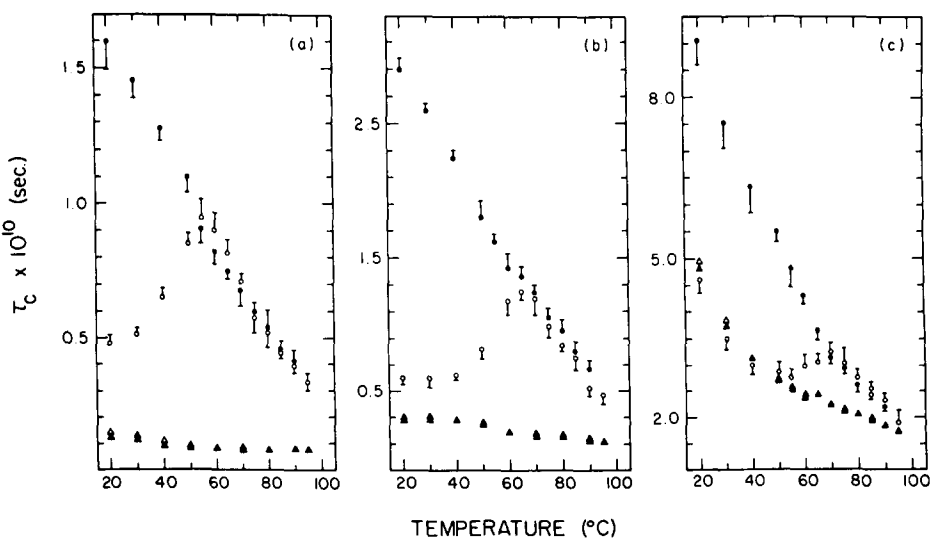


Fig. 3. Temperature dependence of τ_c: (a) TEMPO (25 μM), (b) BrAcTEMPO (250 μM) and (c) slAMY (0·75 w/v) solutions alone (△, heating; ▲, cooling) or with 30% wheat starch (○, heating; ●, cooling).

temperature. For aqueous starch suspensions with the probes or the slAMY, there was an increase in τ_c at temperatures between 30 and 65°C or 50 and 70°C, respectively, indicating an increasing microviscosity in the environment of the nitroxide radical. Proton NMR studies have in fact shown that water mobility does decrease in this temperature region, presumably by increased water adsorption on the granular material (Collison & McDonald, 1960). At temperatures above the gelatinization of starch, τ_c drops gradually and thus implies a progressive increase in water mobility which can be attributed to the increased motion of the polymer chains within the gel matrix. The above spectral behavior is consistent with the narrowing in water line-width obtained from NMR measurements on suspensions of granular starches (Jaska, 1971). Upon cooling the starch gels, τ_c increases monotonically (Fig. 3). The dependence of τ_c on starch concentration for aqueous starch suspensions (20°C) or gels at 90 and 20°C is summarized in Fig. 4. The spectral lineshape remained motionally narrowed over the starch concentration and temperature ranges examined. There was only a slight increase in τ_c with increasing concentration of starch granules for measurements at 20°C. However, the most pronounced effect on the τ_c was demonstrated in the cooled (20°C) gels for all nitroxide radicals. It is well known that for molecules undergoing isotropic rotational motion

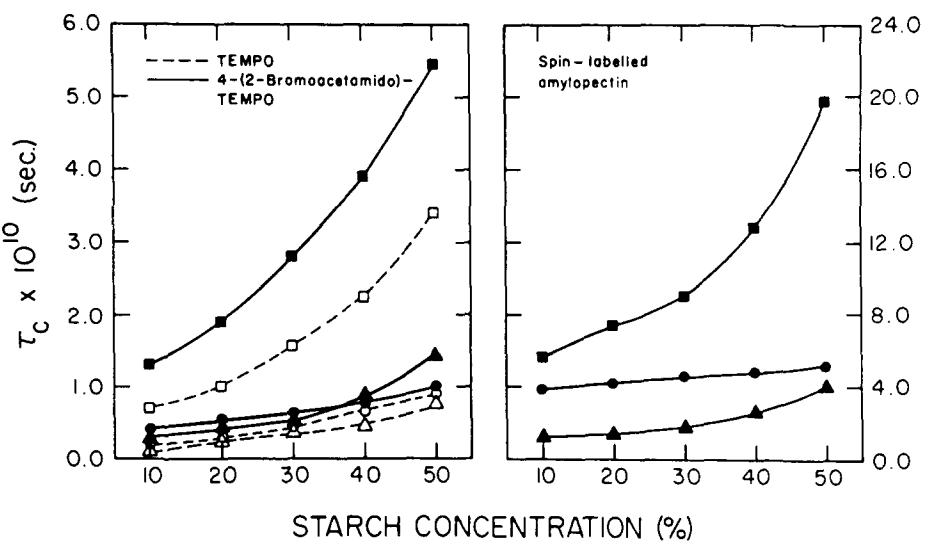


Fig. 4. Starch concentration dependence of τ_c for TEMPO (25 μM), BrAcTEMPO (250 μM) and slAMY (0·75 w/v) — wheat starch granule suspensions at 20°C (○, ●); gels at 90°C (△, ▲); gels at 20°C (□, ■).

the resistance to rotation follows the Stokes–Einstein relationship, according to which τ_c increases with viscosity (Freed, 1976). One would therefore expect the τ_c to mirror viscosity changes in the probe's medium. It is also noteworthy that Aplin & Hall (1977) reported that in solutions of various polysaccharide hydrocolloids, despite their considerable effect on the macroscopic viscosity of the system, the motional behavior of small nitroxide molecules was essentially unaffected. However, these workers employed much lower polysaccharide concentrations (1–3%) than the range adopted in this study. The data shown in Fig. 4 (gels at 20°C) indicate that τ increases with polymer concentration but generally remains within a factor of 3–5 between 10 and 50% starch. Over the same concentration range, however, the bulk viscosity, although not measured, must rise several orders of magnitude. Hence, changes in τ_c appear to mainly reflect local or microviscosity changes rather than increases in the bulk viscosity.

In view of the well-known time-dependent changes in the structure of starch gels, as a result of the aggregation–recrystallization phenomena, we attempted to follow the changes in ESR spectral parameters during storage (25°C) of spin probed/labelled wheat starch gels. The effects of ageing on the τ_c of gels doped with slAMY are shown in Fig. 5. Aged gels of varying starch contents exhibited very little change in τ_c. Similar

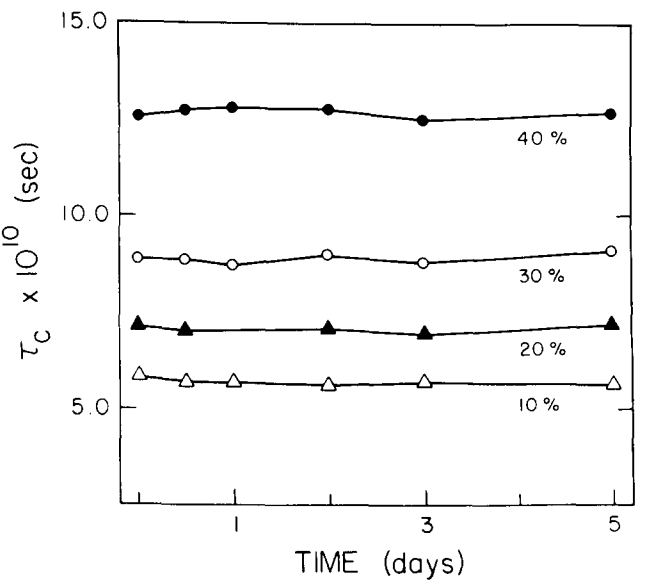


Fig. 5. Time dependence of τ_c in wheat starch gels of varying starch content prepared in slAMY (0·75% w/v) solution.

trends were also seen for gels probed with TEMPO and BrAcTEMPO. However, calorimetric studies for these samples (data not shown) demonstrated the appearance of recrystallized material even within the first day of ageing. Thus, conformational changes associated with the retrogradation process do not seem to impose motional restrictions to the nitroxide label. Instead, the radical appears to experience a stable microenvironment. In the case of slAMY, steric factors due to the bulkiness of the nitroxide group could be responsible for such behavior; polymer regions containing modified anhydroglucose units cannot be included in the ordered chain domains (crystallites) and thus retain a relatively constant tumbling rate.

Interactions between starch and fatty acid probes

Of particular interest to the present ESR studies, within the series of spin probes examined, has been the abrupt change in the motional behavior of the fatty acid probes caused by the starch. Thus, although both 5-DSA and 16-DSA in aqueous solution (150 μM) exhibited a three line spectrum (Fig. 2(e) and (f)), addition of starch imparted a marked decrease in their motion (Fig. 2(g) and (h)). These spectra are characteristic of a highly immobilized probe (i.e. powder-like spectrum). Similar behavior was also shown with defatted starch samples. Thus, in contrast to TEMPO and BrAcTEMPO, the fatty acid probes seem to adsorb strongly on the granules. Indeed, when the starch fraction was separated by centrifugation, no ESR signal was detected in the supernatant. Even after multiple washings, the spectra of the starch granules showed very little desorption of both 5-DSA and 16-DSA. These observations are in agreement with the findings of Pearce *et al.* (1985) on the interactions of 16-DSA with wheat starch. When TiO_2 or Avicel (microcrystalline cellulose) were used as adsorbents (at concentrations of 10–30%, w/w) the obtained spectra (data not shown) were still motionally narrowed, indicating a freely tumbling probe. These results suggested that, under the conditions employed in this study, no detectable pertubations occur in the solubility of the probes when present in a solid–liquid system. Furthermore, they do demonstrate the specificity of the 5-DSA and 16-DSA in interacting with the starch granules.

In view of the well-known interaction between fatty acids and amylose, the ESR studies were extended using a waxy maize starch sample ($< 1\%$ amylose). The spectral response of 5-DSA to changes in temperature (heating–cooling) for 30% wheat and waxy maize starches is shown in Figs 6 and 7, respectively. The dominant feature observed in the spectra at low temperatures is the broadened anisotropic line

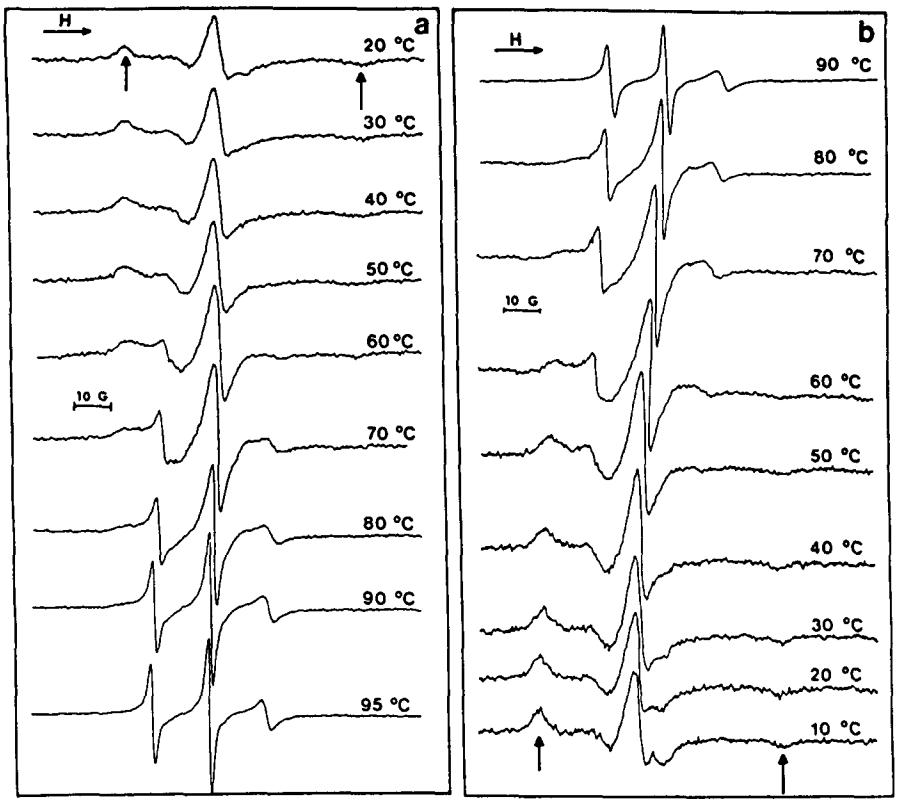


Fig. 6. ESR spectra of 30% (w/w) wheat starch in aqueous 5-DSA (150 μM). Relative modulation amplitudes from top to bottom: (a, heating) 12·5, 12·5, 12·5, 12·5, 12·5, 12·5, 8·0, 5·0, 4·0; (b, cooling) 5·0, 8·0, 12·5, 16·0, 20·0, 20·0, 20·0, 20·0, 20·0.

pattern, i.e. strong interaction between the probe molecules and the starch granules results in very little motional averaging. As the samples are heated, however, line narrowing and gradual disappearance of the powder-like spectrum occur. In fact, there is a downward shift of the high-field line along with a concomitant increase in the signal intensity, suggesting a more rapid motion of the probe. At temperatures around 50–70°C, where increased chain motion due to second-order and melting transitions of the starch granule is anticipated (Biliaderis *et al.*, 1986), the spectral lineshape suggests the coexistence of two components of different mobilities. With further increase in temperature the nitroxide motion becomes more rapid and only the motionally narrowed spectra are observed. Although the nitroxide hyperfine splittings of the fast components for the 5-DSA and 16-DSA doped starch gels were

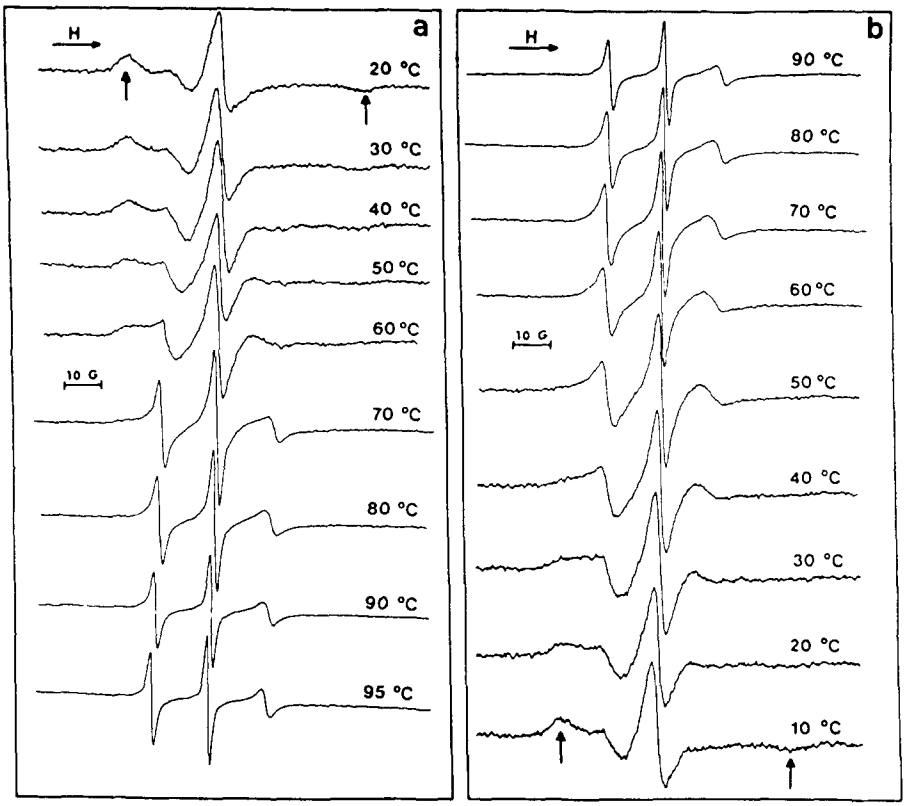


Fig. 7. ESR spectra of 30% (w/w) waxy maize starch in aqueous 5-DSA (150 μM). Relative modulation amplitudes from top to bottom: (a, heating) 12·5, 12·5, 12·5, 10·0, 10·0, 5·0, 3·2, 2·0, 2·0; (b, cooling) 2·0, 3·2, 5·0, 6·3, 10·0, 12·5, 16·0, 16·0, 20·0.

very close to those of the probes in aqueous solution, the calculated τ_c for gels at 90°C (Table 1) indicate that motion is still considerably restricted when compared with the corresponding motional properties of TEMPO and BrAcTEMPO (Fig. 3). Upon cooling (Figs. 6(b) and 7(b)), the broad (slow) components (indicated by arrows in Figs 6 and 7) again start to develop at 70°C for the wheat and at 40°C for the waxy maize starch gel. Finally, gels at 10°C gave almost identical spectra to those of the starch suspensions before heating.

Figure 8 presents a summary of the evolution of spectral parameters as a function of temperature for 5-DSA ($2A_{\parallel}$, extrema separation) and 16-DSA (A/B ratio) probes incorporated into aqueous wheat and waxy maize starch systems. The separation of the hyperfine extrema for both starches (Fig. 8(a)) changes rapidly from a value near the rigid limit

TABLE 1
Correlation Times (τ_c, ns) for 5-DSA and 16-DSA Probes in Aqueous Solutions (150 μM) and in Starch Gels at 90°C

Fatty acid probe	*Aqueous solution*	*Starch gel*		
		Wheat (10%)	*Wheat (30%)*	*Waxy maize (30%)*
5-DSA	0·035	0·921	1·801	1·437
16-DSA	0·018	0·470	0·782	0·730

(64–68 G) to the isotropic value (31–34 G) upon heating at temperatures where melting of starch crystallites occurs. Evidence for the latter is obtained by the DSC thermal profiles of the granular starches (Fig. 9, curves a_1 and b_1). Similar trends were also apparent for the motional behavior of 16-DSA, as assessed by the lineheight ratio, A/B (Fig. 8(b)), i.e. more abrupt changes in the slopes are seen at 60–70°C. In two recent calorimetric studies (Maurice *et al.*, 1985; Biliaderis *et al.*, 1986) it was suggested that second-order transition phenomena (glass transition) take place upon heating starch–water systems at the leading edge of the melting endotherm. In this respect, the ESR technique, using fatty acid probes, can detect the T_g-related onset in molecular motion of the starch molecules, as has been frequently shown in the field of synthetic polymers (Kumler *et al.*, 1977; Tormala & Weber, 1978). Measurements of $2A_{\|}$ and A/B parameters during the cooling cycle for the starch gels (Fig. 8) illustrate the progressive appearance of the motionally slow component.

In an effort to further elucidate the nature of fatty acid spin-probe–starch interactions, calorimetric studies were also carried out using defatted starch samples to eliminate any competing effects from lipids present *in situ* within the granules. DSC measurements of wheat starch–probe mixtures (Fig. 9, curves a_3 and a_4) showed enthalpy changes (3·6 and 3·8 J g^{-1}) over the temperature region where melting of amylose–lipid complexes usually takes place (115–135°C). It appears, therefore, that both 5-DSA and 16-DSA can induce formation of V-amylose crystallites despite the presence of the bulky nitroxide group on the aliphatic chain. A small fraction of the probes also remained free, as evidenced by the transition at 70–80°C (indicated by the arrows (Fig. 9)). On the other hand, the thermal curves of waxy maize starch showed no evidence for such complex formation, as one would in fact anticipate in the absence of amylose (Fig. 9, curves b_3 and b_4). Instead, the DSC curves exhibited only the melting endotherms for the unbound probes.

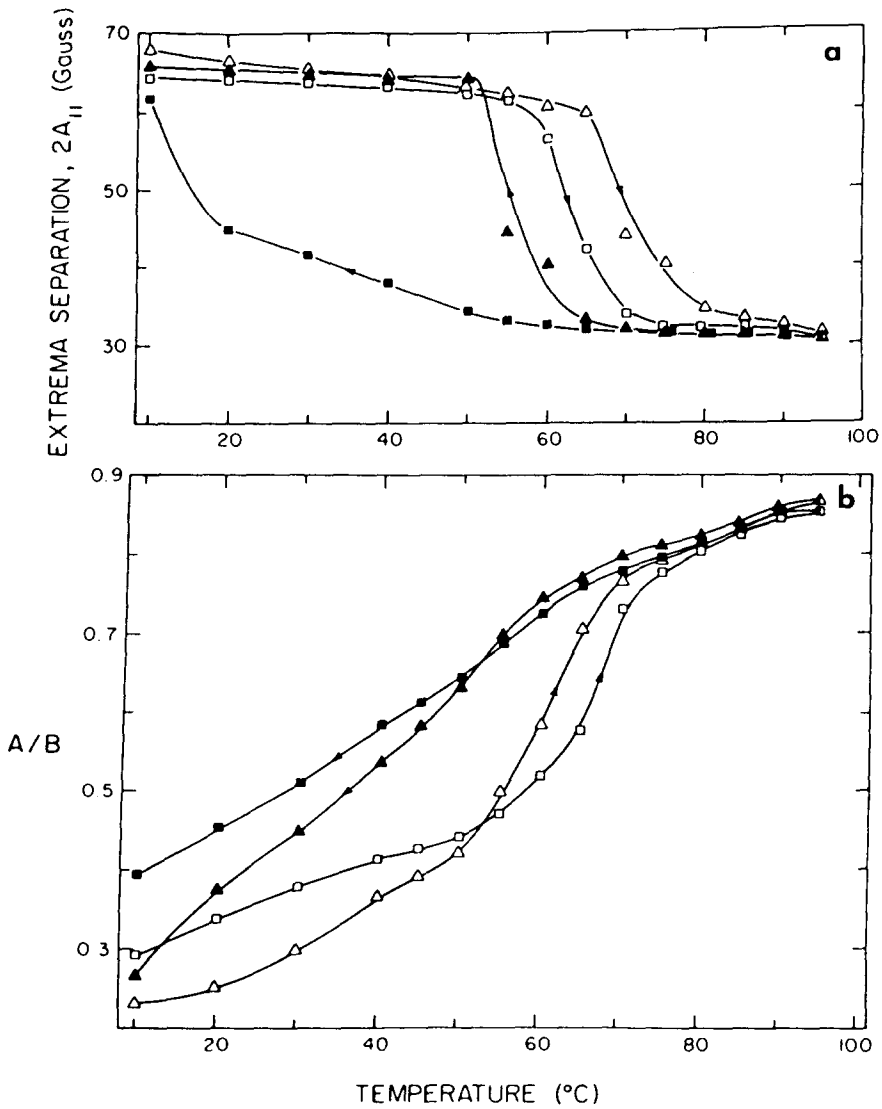


Fig. 8. Temperature dependence of the outermost peak-to-peak separation ($2A_{||}$) and the A/B spectral parameters for 30% waxy maize (□, heating; ■, cooling) and wheat (△, heating; ▲, cooling) starches in aqueous 150 μM 5-DSA (a) or 16-DSA (b).

Inclusion of these probes into the structure of V-amylose is expected to substantially reduce their molecular motion (the complex disorganizes only above 110°C). However, since similar temperature dependence of the ESR spectra (Figs 6 to 8) was obtained for both wheat and waxy maize starches, it is reasonable to suggest that the recorded spectral

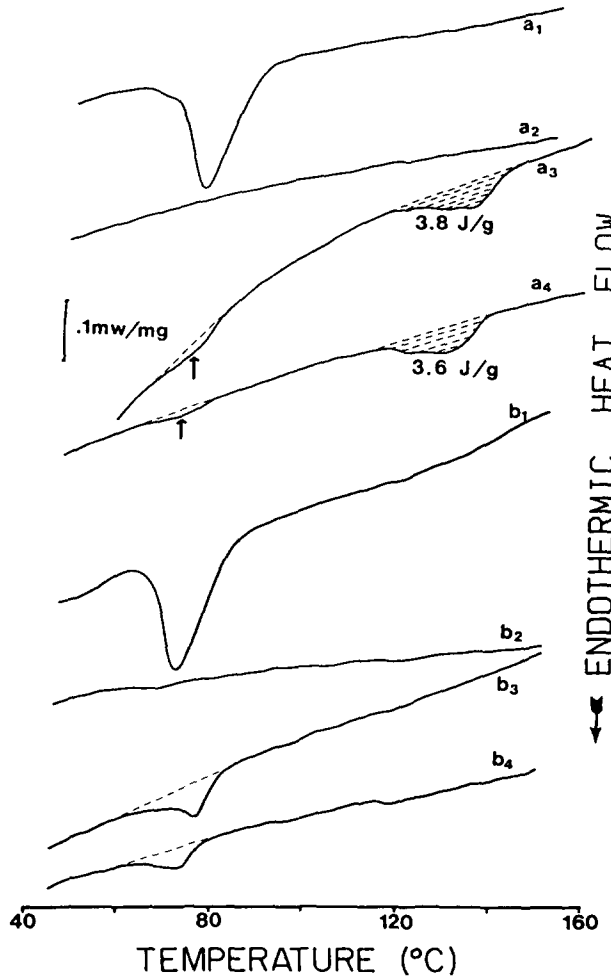


Fig. 9. DSC thermal curves of 30% starch (w/w)–water/probe (200 μg) mixtures: a_1, wheat starch (5·20 mg); a_2, re-scan of a_1; a_3, re-scan of wheat starch (4·44 mg)/5-DSA; a_4, re-scan of wheat starch (5·08 mg)/16-DSA; b_1, waxy maize starch (4·94 mg); b_2, re-scan of b_1; b_3, re-scan of waxy maize starch (4·72 mg)/5-DSA; b_4, re-scan of waxy maize starch (4·94 mg)/16-DSA.

changes mainly represent the motional consequences of weak interactions with the starch probe molecules. The latter is further supported by the fact that the fatty acid probes exhibited similar motional responses to temperature changes (20–95°C) when present in aqueous corn starch maltodextrin (30% w/w) media of *DE* 6–25 (data not shown). Because of the relatively short chain length of these materials, formation of semi-crystalline aggregates of helical chains (like in V-amylose) is hindered.

However, it is known that helicogenic agents do induce complex formation even with short chain maltooligosaccharides (Jane *et al.*, 1985) which can undergo order–disorder transitions at much lower temperatures than the V-amylose complex. Thus, on the basis of the ESR data presented herein, it is plausible to suggest that the thermally reversible spectral behavior of 16-DSA and 5-DSA in starch gels may reflect association–dissociation phenomena between these probes and short chain segments of the polysaccharide molecules (possibly including the amylopectin component).

Overall, the ESR approach in 'viewing' the temperature-dependent motional properties of fatty acid spin probes in starch based media is schematically summarized in Fig. 10. At low temperatures, the probes have a very slow tumbling rate, far below the 10^{-7} s limit where motional averaging starts to occur with conventional ESR, i.e. the spectrum is powder-like. At elevated temperatures, however, two forms can be distinguished within the timescale of the ESR experiment, and the resulting composite spectrum is attributable to superposition of motionally narrowed and motionally slowed spin populations. The existence of two

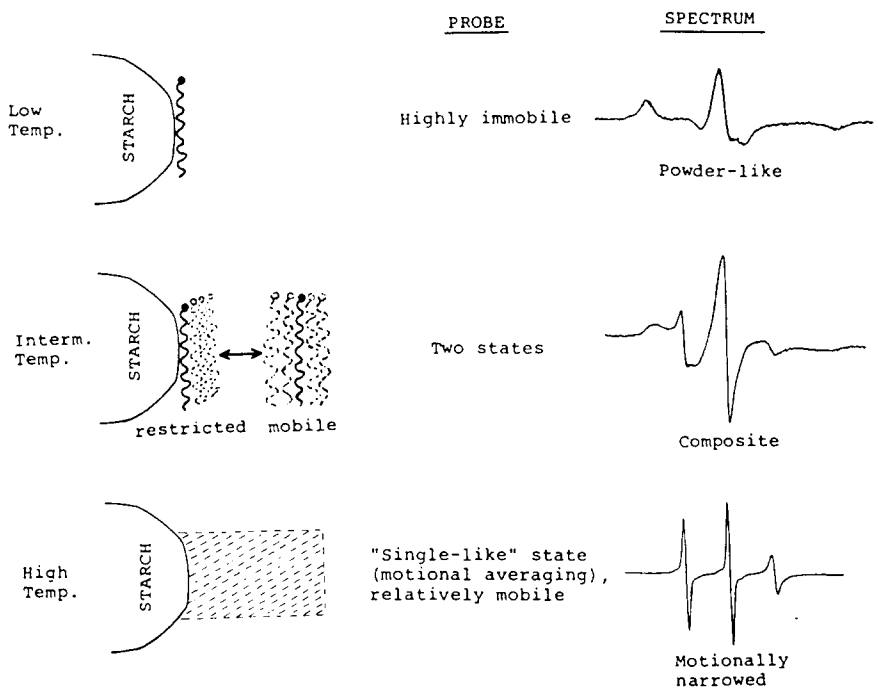


Fig. 10. Schematic representation of temperature-modulated interactions between starch and fatty acid spin probes as 'viewed' by ESR.

distinct mobile states has been reported for various polymer systems doped with nitroxide probes. In general, the bimodal distribution of nitroxides has been explained on the basis of microstructure heterogeneity present in polymeric materials or as reflecting dynamic equilibria such as adsorption–desorption phenomena at solid–liquid interfaces and exchange equilibria at the boundaries of biological macromolecular complexes (Fox *et al.*, 1974; Veksli *et al.*, 1976; Griffith & Jost, 1979; Liang *et al.*, 1980; Martini, 1984; Devaux & Seigneuret, 1985). Although the nature of forces governing the interaction between the probes and the starch is not clearly understood at present, it appears that changes in the tumbling behavior of the probes are related to the temperature-dependent segmental mobility of the polymer chains which must dictate the stability of the probe–polysaccharide complexes. Finally, with further increase in temperature the nitroxide motion of the overall spin population is relatively fast to allow for distinction between two states. Under these conditions, extensive motional averaging occurs and thus a single-like state seems to exist (region designated by the shaded area in Fig. 10).

CONCLUSIONS

The motional behavior, as revealed by ESR, of free nitroxide spin probes and a label attached covalently to amylopectin (low level of substitution) in aqueous starch systems was influenced by the presence of the polysaccharide. At room temperature, the spectra of starch samples containing TEMPO or BrAcTEMPO were motionally slower, as compared with those of the free probes in solution, suggesting that the nitroxides experience different microenvironments, i.e. they partition between the sorbed and the more mobile bulk water surrounding the granules. Nevertheless, the spectral lines remained motionally narrowed (three lines) over the entire range of starch concentrations (10–50%) and temperature (10–95°C) studied. Covalent attachment of BrAcTEMPO to the amylopectin caused considerable diminution in its molecular tumbling rate. The correlation times of spin-probed or -labelled samples increased with starch content as well as upon gelatinization of the granules, while temperature had the opposite effect, i.e. with increasing temperature the nitroxide motion became more rapid. On the other hand, the local dynamics of the nitroxide radical were not sensitive to the aggregation–recrystallization phenomena occurring in ageing starch gels, as the τ_c values remained relatively constant during storage.

The spectra of both fatty acid probes (5-DSA and 16-DSA) in starch suspensions or gels at room temperature were of a broadened anisotropic line pattern, thus indicating a strong adsorption/binding of the probes by the starch. With increasing temperature, however, the spectra revealed the presence of two components characterized by different mobilities: a narrow (fast) component which predominates at high temperature and a broad (slow) component which apparently originates from the interaction between the probe and the starch molecules. From the ESR data presented herein, it is suggested that the tumbling behavior of these probes is modulated by the temperature-dependent segmental motion of the polymer chains at both granular and gel state. From a technological viewpoint, the implications of such strong affinity between starch and fatty acid probes become obvious when one considers that lipids do alter the functional characteristics (e.g. solubility, stickiness, staling rate, etc.) of this polysaccharide in starch-based food systems by complexing with amylose. Thus, preferential adsorption of fatty-acid-like molecules at the surface of the starch granules would facilitate these interactions during thermal processing.

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