

An Electron Spin Resonance Study of Native and Gelatinized Starch Systems¹

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ABSTRACT

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Wheat, corn, and waxy corn starches were slurried at three water/starch ratios (0.5:1, 1:1, and 2:1), with a hydrophobic probe, TEMPO-laurate, and a hydrophilic probe, TEMPO. Samples containing TEMPO at the two higher water ratios gave electron spin resonance (ESR) signals that were symmetrical and that had high tumbling rates. At the low water/starch ratio, the signals became distorted and tumbling rate decreased. For the 1:1 and 2:1 samples, the ESR signal was an average of that for the probe inside and outside the starch granules. A probe-binding study revealed that TEMPO-laurate and 16-DOXYL stearic acid were readily washed from

starch with ether but were bound to the starch in the presence of water. ESR spectra of the TEMPO-laurate or 16-DOXYL stearic acid plus starches had distorted line shapes and tumbling rates clustered in the range of 10^8 /sec. This differed greatly from the results obtained using TEMPO, and verified probe binding. The molecular motion of TEMPO-laurate and 16-DOXYL stearic acid was not dependent on the amount of water in the starch-probe system. Addition of amylose or amylopectin to TEMPO-laurate in water resulted in distorted spectra with lower values for tumbling rates, suggesting that the probe was bound to both polymers.

Spin labeling has become an attractive technique to extend the use of electron spin resonance (ESR) spectroscopy to include biological, diamagnetic samples. Spin labeling involves placing a stable nitroxide radical into a diamagnetic system and monitoring changes in its ESR spectrum (Berliner 1976).

The line shape of an ESR signal is determined by the radical's immediate molecular environment. Because the spectrum of a spin label rotating freely in solution is much different from one experiencing restricted molecular motion, the resulting spectra can be used to investigate the local environment of the biological system of interest (Likhtenstein 1976). Griffith et al (1965) developed a mathematical formula to calculate the rotational correlation time, τ , of a free radical, based on the heights and widths of the spectral lines. By monitoring the reciprocal ($1/\tau$), a value that reflects the tumbling rate of the radical, information about its immediate environment can be obtained (Berliner 1979).

Nishiyama et al (1981) used rotational correlation times to show that lipid was immobilized in a gluten matrix by strong interaction with protein. Spin labeling also has been used to examine the conformation of amylose in aqueous solution (Ebert 1984), and to study interactions of starch, water, and fatty acid (Pearce et al 1985). Windle (1985) used a ketone-substituted probe to define three distinct starch/water conditions occurring during potato starch gelatinization.

The objective of this study was to use probes of differing hydrophobic-hydrophilic properties to study starch/probe interactions. These could be quantitated by monitoring changes in the tumbling rate during starch gelatinization and retrogradation.

MATERIALS AND METHODS

Materials

Starch. A commercially isolated unmodified wheat starch was obtained from Midwest Solvents Co. (Atchison, KS). Corn starch was obtained from the CPC Co. (Argo, IL), and waxy corn starch was provided by Clodualdo Maningat (Kansas State University). Potato amylose and amylopectin (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO).

Spin labels. The following nitroxide spin labels were obtained from Aldrich Chemical Co. (Milwaukee, WI): 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), and the derivatives of 5-ketostearic acid, 12-ketostearic acid, and 16-ketostearic acid (5-, 12-, and 16-DOXYL stearic acid, respectively). A 12-carbon fatty

acid derivative of TEMPO, TEMPO-laurate, as well as TEMPO-benzoate, were obtained from Molecular Probes (Junction City, OR).

Solvents and other reagents. Solvents and other chemicals were all reagent grade.

Preparation of Spin-Labeled Samples

TEMPO samples. TEMPO was diluted with water to a concentration of $2.5 \times 10^{-3} M$ and the solution added to wheat, corn, and waxy corn starches. Samples were equilibrated at room temperature by gentle stirring for 24 hr before ESR spectra were taken. Starch solutions were gelatinized by heating samples in a water bath to 70°C for 20 min.

TEMPO-laurate. TEMPO-laurate was dissolved in ether ($2 \times 10^{-5} M$), and the ether was allowed to evaporate before distilled water was added. Separation of starch from solution was accomplished by centrifugation in a benchtop centrifuge for 30 min at 300 *rcf*. ESR spectra were obtained for both the supernatant and starch pellet.

Binding study. Samples were prepared using TEMPO-laurate and 16-DOXYL stearic acid as described above. The subsequent washing protocol is outlined in Figure 1.

Amylose/amylopectin. Potato amylopectin (0.1 g) was dissolved in 3 ml of water containing $2 \times 10^{-5} M$ TEMPO-laurate. The solution was sonicated for 30 min at full power in a Sonac S-2 sonicator (Chicago, IL) to solubilize the probe and amylopectin.

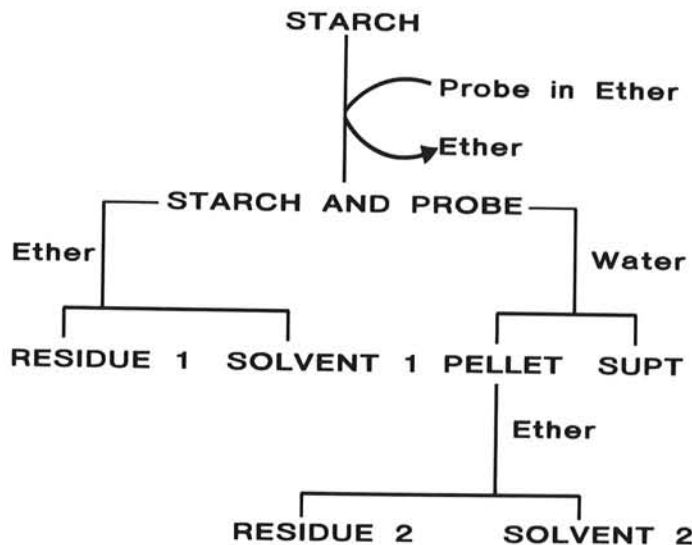


Fig. 1. Flowchart for probe binding study.

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Potato amylose (0.1 g) was dissolved in 3 ml of 1N KOH and sonicated for 30 min. TEMPO-laurate ($2 \times 10^{-5} M$) was added as an ether solution, and the ether was allowed to evaporate. The sample plus probe was sonicated for an additional 10 min to solubilize both polymer and probe. The sample then was neutralized with 1N HCl. ESR spectra were taken immediately after neutralization.

ESR. ESR measurements were taken at room temperature on an IBM/Bruker ESR ER 200D-SRC spectrometer. Center field for all spectra was 3,475 gauss. No attempt was made to exclude oxygen. Rotational correlation times were calculated from Griffith's formula (Snipes and Keith 1970):

$$\tau = 6.5 \times 10^{-5} W_0[(h_0/h + 1)^{1/2} + (h_0/h - 1)^{1/2} - 2]$$

where W_0 is the width of the center field line in gauss, and h_0 , $h + 1$, and $h - 1$ are the heights of the center field, low field, and high field lines, respectively. Most results are expressed as tumbling rates or the reciprocal of τ . Most instrumental settings were identical for all ESR spectra that were taken. Variations in phase and instrument gain were made for each spectrum to optimize signal amplitude and line symmetry.

Mixtures of water, glycerol, and TEMPO ranging in viscosity from 1.5 to 2.7 centipoise (Lange 1967) were prepared by carefully stirring TEMPO/water solutions with appropriate amounts of glycerol.

Final TEMPO concentration was $2.5 \times 10^{-3} M$.

RESULTS AND DISCUSSION

Water/Glycerol Viscosity

The Stokes equation suggests that if a spin label behaved ideally as a rigid sphere rotating in a medium of viscosity η , τ would equal $4\tau_\eta \cdot a^3/3kT$, where a equals the radius of the sphere, T equals the absolute temperature, and k equals the Boltzmann constant (Poole 1983). To verify this for the TEMPO spin label used in subsequent studies, tumbling rates (the reciprocal

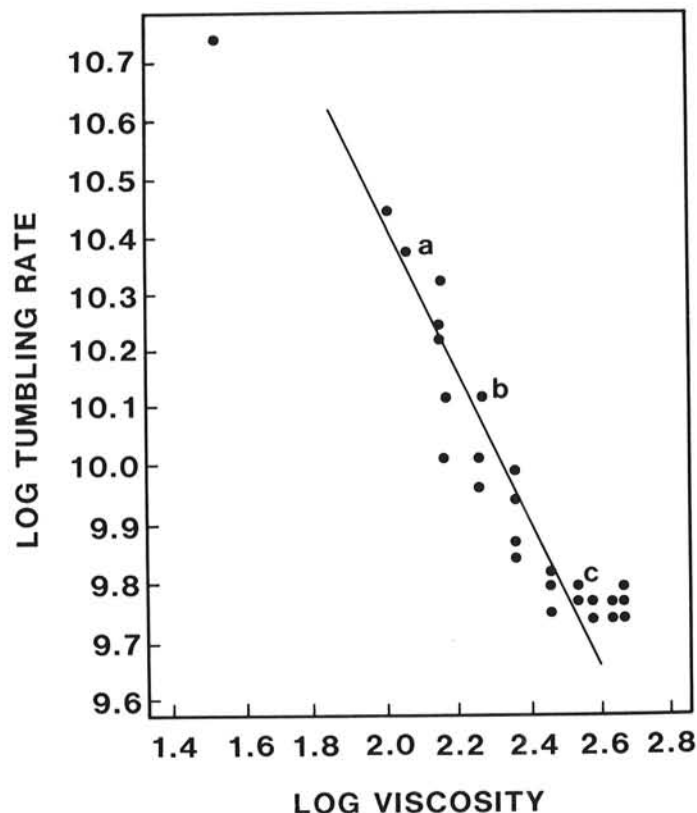


Fig. 2. Tumbling rate of TEMPO-glycerol/water mixtures as a function of viscosity. Axes show log of tumbling rate (sec) and log of viscosity (cP). A, 25% glycerol/75% water; B, 50% glycerol/50% water; and C, 75% glycerol/25% water.

rotational correlation times) for a series of water, glycerol, and TEMPO mixtures were determined by ESR. Figure 2 shows a log-log plot of τ^{-1} as a function of viscosity. A linear relationship was found ($R = 0.879$), in agreement with Stoke's equation. If we limit the working range of log viscosity between 1.6 and 2.5 the agreement is even better ($R = 0.954$). This is in agreement with Cooke (1974) and Snipes and Keith (1972), who determined the same relationship using phospho and keto derivatives of TEMPO. It also confirms the ability of this probe (and τ^{-1}) to be used as a measure of freedom of molecular motion.

Starch Studies with TEMPO

Verification of the Stokes equation suggests that spin labeling may be an attractive technique to examine the interactions of starch and water or starch, water, and fatty acid. To test this, each starch was hydrated to three different water/starch ratios (0.5:1, 1:1, and 2:1). In each case, TEMPO was dissolved in the water to achieve a final concentration of $2.5 \times 10^{-3} M$. The water contents were chosen to create a range of water-to-starch environments; the 1:1 and 2:1 samples produced slurries. The 0.5:1 water level samples were moist powders, indicating that all the water was involved in hydrating the starch granules.

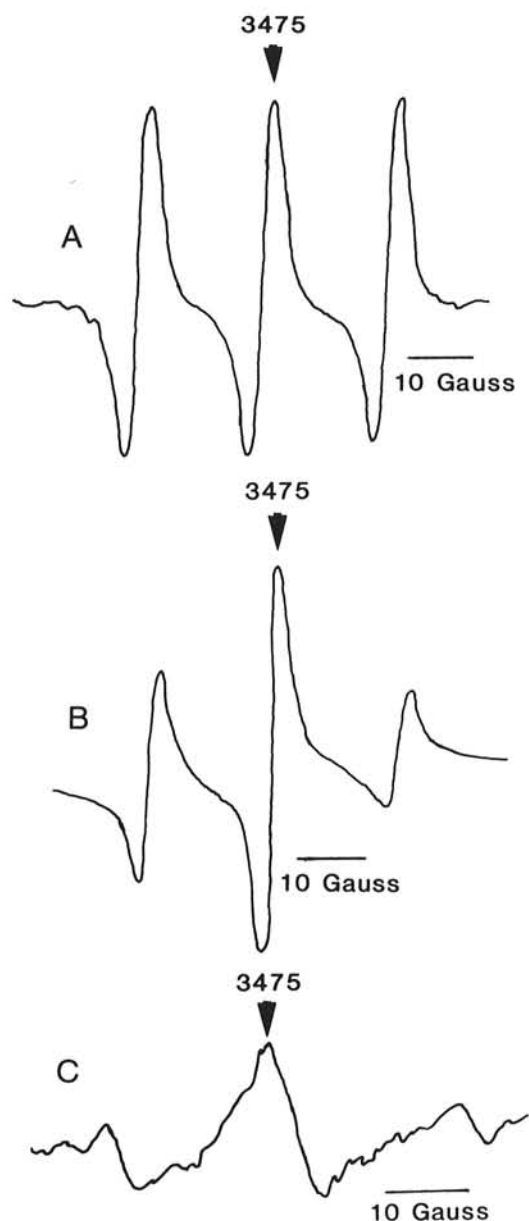


Fig. 3. Electron spin resonance spectra of TEMPO: A, dissolved in water; B, 0.5:1 water/wheat starch; and C, 0.5:1 water/wheat starch after gelatinization.

Spectra from the 1:1 and 2:1 samples displayed the symmetrical triplet signal characteristic of a TEMPO probe rotating freely in solution (Fig. 3A). They did not differ significantly in appearance from the spectrum of TEMPO in water, but their hyperfine lines had broadened slightly. The spectrum from the 0.5:1 sample, however, was distorted (Fig. 3B). Its high-field and low-field lines were greatly reduced in size, and all three lines were broadened. The spectra were essentially the same for wheat, corn, and waxy corn starch. These observations are consistent with a signal originating from a probe with restricted rotational motion (Berliner 1976).

Examination of tumbling rates (τ^{-1}) verified these observations. The τ^{-1} values for all three starches at the three water/starch ratios are given in Table I. For each 0.5:1 sample, τ^{-1} was between 2.67×10^8 and $4.44 \times 10^8 \text{ sec}^{-1}$. At the 1:1 ratio, τ^{-1} had increased to 4.66×10^9 to $6.38 \times 10^9 \text{ sec}^{-1}$. At the highest water level, τ^{-1} had increased to between 2.18×10^{10} and $3.82 \times 10^{10} \text{ sec}^{-1}$. For comparative purposes, the tumbling rate for TEMPO in pure water is approximately $2.00 \times 10^{11} \text{ sec}^{-1}$.

At the higher dilutions (1:1 and 2:1), the water/starch system is discontinuous. The granules contain water and are also surrounded by free water. When a spin label is added to this system, it is distributed throughout both systems and, therefore, experiences two different molecular environments. In the 0.5:1 water/starch samples, there is essentially no excess water outside the granules. In this case the ESR signal observed is that of the probe inside the starch granule. For the discontinuous 1:1 and 2:1 water/starch samples, rotational correlation times are presumably the average of signals produced by the radical in free water and inside the starch granule.

Gelatinization

As starch is gelatinized, the starch granules swell, hydrogen bonds between molecules are disrupted, and liberated hydroxyl groups become hydrated (Collision 1968). The net result of those changes, coupled with the loss of soluble material to solution, causes the aqueous suspension to increase in viscosity.

Samples of water, starch, and TEMPO at the described water/starch ratios were heated to 70°C for 20 min. ESR spectra were obtained for the samples 2 hr after they had cooled and again after 72 hr.

Spectra taken 2 hr after the samples cooled (Fig. 3C) were essentially identical in appearance to those taken before heating. Signal amplitude increased greatly for all three starches at all three water levels, but line shapes remained unchanged. Tumbling rates did not change appreciably from their corresponding unheated values (Table II). Samples of 0.5:1 had τ^{-1} values of 10^8 sec^{-1} , whereas 1:1 samples were in the 10^9 sec^{-1} range. Samples at the 2:1 level were approximately 10^{10} sec^{-1} .

The increase in solution viscosity caused by starch gelatinization is easily observed visually. However, the average environment of the probe apparently does not change upon heating. Thus, τ^{-1} cannot be used to follow starch gelatinization.

Retrogradation

To determine the effects of retrogradation on τ^{-1} , the above

samples were stored for three days after gelatinization before ESR spectra were taken. Tumbling rates for wheat, corn, and waxy corn starches at the three water/starch ratios did not change as a result of three days of storage. These data appear to be inconsistent with that of Windle (1985), who found that τ decreased by 30% upon storage, indicating a corresponding increase in gel viscosity.

Binding Study

Amylose is known to complex with hydrophobic straight-chain molecules such as fatty acids (Krog 1981). A hydrophobic spin label, TEMPO esterified to lauric acid (TEMPO-laurate), was used to attempt to measure this interaction.

To identify the occurrence of a probe/starch complex, a binding study was performed (Fig. 1). If the probe were bound or complexed with amylose or amylopectin, it should not be extractable, even with a suitable solvent. A bound probe will remain in the granule to give an ESR signal, albeit one that may be reduced in magnitude and/or symmetry. An unbound probe, by the same logic, should be extracted in the solvent.

TEMPO-laurate applied as an ether solution was readily extracted from wheat starch and corn starch by ether. No signal was found in the starch residue. That evidence suggested that the probe had not bound to the starch. A second experiment was conducted to determine if binding was caused by the addition of water. Samples were prepared as above, then slurried with water to achieve a ratio of 1:2:0.002 for water, starch, and probe. For both starches, the TEMPO-laurate signal was found in both the supernatant and the pellet. The dried pellet was then extracted with ether and filtered. ESR spectra taken on both the resulting solution and the residue showed that the radical was not extracted with the ether solution, but remained with the starch. Thus, the probe bound to the starch, but only after adding water.

This finding defines a critical difference between TEMPO and TEMPO-laurate in the water, starch, and probe system. Whereas TEMPO remained in solution before heating and gave a signal that was an average of two water environments, TEMPO-laurate clearly did not. It bound or interacted with the starch granule, and its correlation time was not affected by the amount of water ultimately in the system. The results of the washing experiment suggested that the binding occurs because of the presence of water. Water enhances hydrophobic interactions between molecules (e.g., starch and TEMPO-laurate).

The same binding study was repeated using 16-DOXYL stearic acid. Results for both wheat starch and corn starch were identical to those obtained using TEMPO-laurate. The probe was readily extracted from dry starch with ether, but bound to the starch in the presence of water.

Waxy corn starch displayed anomalous behavior in the binding study. The behavior of waxy corn starch is addressed in an accompanying publication (Nolan et al 1986).

Studies with Fatty Acid Spin Labels

Verification of TEMPO-laurate's binding to starch involved a repetition of the initial starch, water, and probe experiment using TEMPO-laurate and 16-DOXYL stearic acid instead of TEMPO.

TABLE I
Tumbling Rates of TEMPO in Water/Starch Samples Before Heating

Starch	Water/Starch Ratio	Tumbling Rates ^a (sec ⁻¹)
Wheat	0.5:1	2.67×10^8
	1:1	6.38×10^9
	2:1	2.87×10^{10}
Regular corn	0.5:1	3.92×10^8
	1:1	4.66×10^9
	2:1	3.82×10^{10}
Waxy corn	0.5:1	4.44×10^8
	1:1	5.40×10^9
	2:1	2.18×10^{10}

^aStandard deviation for 0.5:1 samples was 5.0×10^7 , for 1:1 samples 2.1×10^8 , and for 2:1 samples 2.5×10^9 .

TABLE II
Tumbling Rates of TEMPO in Water/Starch Samples After Heating

Starch	Water/Starch Ratio	Tumbling Rates ^a (sec ⁻¹)
Wheat	0.5:1	2.00×10^8
	1:1	2.99×10^9
	2:1	2.82×10^{10}
Regular corn	0.5:1	3.25×10^8
	1:1	2.60×10^9
	2:1	1.43×10^{10}
Waxy corn	0.5:1	3.44×10^8
	1:1	5.85×10^9
	2:1	3.71×10^{10}

^aStandard deviations for 0.5:1 samples was 6.7×10^7 , for 1:1 samples 3.9×10^8 , and for 2:1 samples 2.48×10^9 .

The fatty acid probes were added to each sample to achieve a ratio of 1:2:0.002 for water, starch, and probe. Samples were equilibrated for 24 hr before ESR spectra were taken.

For wheat, corn, and waxy corn starch, spectra of the 0.5:1 samples appeared similar to equivalent starch/TEMPO samples. Signals from the 1:1 and 2:1 water/starch samples were so distorted that it was difficult to identify the triplet (Fig. 4A). Baselines were curved and signal symmetry was lost (Fig. 4B and C). Tumbling rates for all starch samples at all water/starch ratios were between $2.78 \times 10^8 \text{ sec}^{-1}$ and $5.51 \times 10^8 \text{ sec}^{-1}$ (Table III). When considered in conjunction with τ^{-1} values that are identical over a

TABLE III
Tumbling Rates of TEMPO-Laurate in Water/Starch Samples Before Heating

Starch	Water/Starch Ratio	Tumbling Rates ^a (sec ⁻¹)
Wheat	0.5:1	3.50×10^8
	1:1	2.65×10^8
	2:1	3.72×10^8
Regular corn	0.5:1	2.78×10^8
	1:1	2.81×10^8
	2:1	2.51×10^8
Waxy corn	0.5:1	3.26×10^8
	1:1	3.50×10^8
	2:1	5.51×10^8

^a Standard deviation for 0.5:1 samples was 3.98×10^7 , for 1:1 samples 1.98×10^7 , and for 2:1 samples 3.43×10^7 .

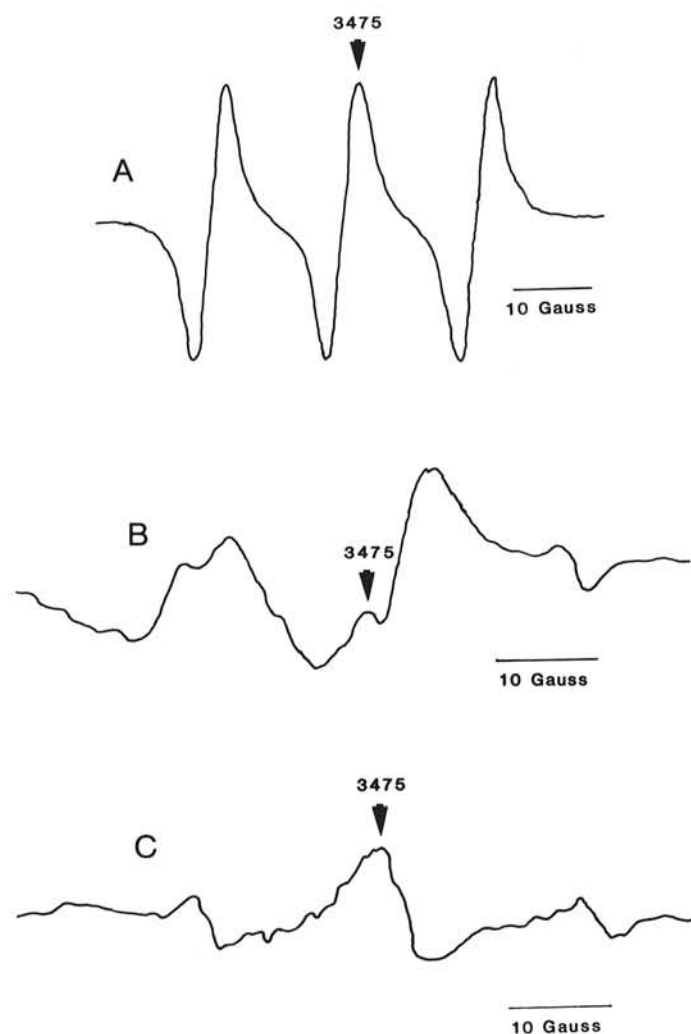


Fig. 4. Electron spin resonance spectra of TEMPO-laurate: **A**, dissolved in ether; **B**, 1:1 water/corn starch, and **C**, 1:1 water/waxy corn starch.

wide range of free water (50–200% water), the distorted ESR spectra of TEMPO-laurate and 16-DOXYL stearic acid support the concept of starch/probe binding.

Gelatinization

For both wheat and regular corn starch plus TEMPO-laurate at the 1:1 and 2:1 ratios, spectral features became so distorted after gelatinization that calculation of τ^{-1} became unreliable. Baseline distortions became more pronounced and identification of individual triplets was difficult. Signals from the 0.5:1 samples remained unaffected. The basis for this behavior is not known.

Tumbling rates that could be calculated from the above spectra were all clustered in the narrow range between $2.10 \times 10^8 \text{ sec}^{-1}$ and $7.06 \times 10^8 \text{ sec}^{-1}$. The extensive distortions present after heating to gelatinization temperatures suggest that gelatinization increases the interaction of probe with starch or somehow changes the environment of the bound TEMPO-laurate and 16-DOXYL stearic acid to further restrict its motion. This decreased mobility may reflect more complexation of the probe (i.e., more of it bound) by starch or a perfection of those complexes formed before gelatinization.

Amylose/Amylopectin Binding Study

The data reported to this point present impressive, yet incomplete, evidence that fatty acid spin labels bind to starch. As a final test, potato amylose and amylopectin were individually dissolved with $2.5 \times 10^{-5} M$ TEMPO-laurate and examined by ESR. Ratios of starch, water, and probe ratios were kept constant at 0.1:2:0.002. Dissolution of molecules was ensured by sonication.

The ESR spectrum of $2 \times 10^{-5} M$ TEMPO-laurate dissolved in water is shown in Figure 5A. It is a symmetrical triplet signal exhibiting rapid isotropic motion ($\tau^{-1} = 4.87 \times 10^{10} \text{ sec}^{-1}$). When amylopectin was present, the spectrum changed (Fig. 5B). All three lines broadened, and the high-field and low-field lines diminished greatly in size. The tumbling rate decreased to $5.03 \times 10^9 \text{ sec}^{-1}$.

Similar changes in ESR spectra resulted from the addition of amylose. An ESR signal of TEMPO-laurate in a neutral solution

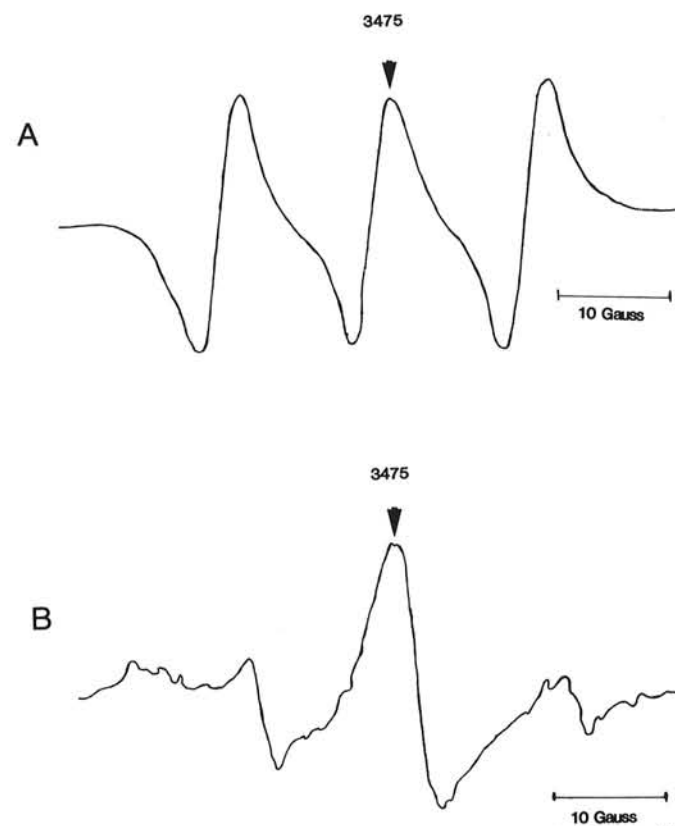


Fig. 5. Electron spin resonance spectra of TEMPO-laurate: **A**, sonicated in water; and **B**, with 0.1 g amylopectin added.

has a τ^{-1} of $3.21 \times 10^{10} \text{ sec}^{-1}$ and was an unbound symmetrical signal. When amylose was added, the spectrum became distorted, and τ^{-1} decreased to $6.18 \times 10^9 \text{ sec}^{-1}$. Both changes are indicative of starch/probe binding. Moreover, the data offer evidence that TEMPO-laurate binds effectively to both amylose and amylopectin.

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LITERATURE CITED

- BERLINER, L. J., ed. 1976. Pages 1-4 in: Spin Labeling: Theory and Applications. Academic Press: New York.
- BERLINER, L. J., ed. 1979. Pages 126-129 and 116-124 in: Spin Labeling II: Theory and Applications. Academic Press: New York.
- COLLISION, R. 1968. Swelling and gelation of starch. Pages 168-173 in: Starch and Its Derivatives. J. A. Radley, ed. Chapman and Hall: London.
- COOKE, R. 1974. Spin label probes of the intracellular aqueous environment. Colloq. Int. CNRS 246:283.
- EBERT, B. 1984. Helical conformation of amylose in aqueous solution. II. Electron spin resonance, fluorescence depolarization, and sedimentation measurements. Biopolymers 23:2543.
- GRIFFITH, O. H., CORNELL, E. W., and McCONNELL, H. M. 1965. Nitrogen hyperfine tensor and g tensor of nitroxide radicals. J. Chem. Phys. 43:2909.
- KROG, N. 1981. Theoretical aspects of surfactants in relation to their use in breadmaking. Cereal Chem. 58:158.
- LANGE, N. A. 1967. Page 1681 in: Handbook of Chemistry. McGraw-Hill: New York.
- LIKHTENSTEIN, G. I. 1976. Pages 1-17 and 22-36 in: Spin Labeling Methods in Molecular Biology. Academic Press: New York.
- NISHIYAMA, J., KUNINORI, T., MATSUMOTO, H., and HYONO, A. 1981. ESR studies on lipid-protein interaction with gluten. Agric. Biol. Chem. 45:1953.
- NOLAN, N. L., HOSENEY, R. C., and FAUBION, J. M. 1986. The effect of fatty acid spin labels upon waxy corn starch. Cereal Chem. 63(4):291.
- PEARCE, L. E., DAVIS, E. A., GORDON, J., and MILLER, W. G. 1985. Application of electron spin resonance techniques to model starch systems. Food Microstruct. 4:83.
- POOLE, C. P. 1983. Electron Spin Resonance: A Comprehensive Treatise on Experimental Techniques. John Wiley & Sons: New York.
- SNIPES, W., and KEITH, A. 1970. Spin labels extend applications of ESR. Res. Dev. Feb.:22-26.
- SNIPES, W., and KEITH, A. 1972. Viscosity of cellular protoplasm. Science 183:666.
- WINDLE, J. J. 1985. An ESR Spin probe study of potato starch gelatinization. Staerk 37:121.

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