AN ELECTRON SPIN RESONANCE STUDY OF NATIVE STARCH SYSTEMS

by

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Introduction

Electron Spin Resonance Spectroscopy is the most powerful tool available for the detection of free radicals. It utilizes the unique paramagnetic properties associated with an unpaired electron to identify free radicals in both aqueous and non-aqueous systems. An inherent disadvantage of the technique is its ineffectiveness in diamagnetic samples. ESR cannot identify or isolate a compound which has only paired electrons. To extend the use of ESR to include biological, diamagnetic systems, spin labelling has become an attractive technique. Spin labelling involves placing a stable nitroxide radical into a diamagnetic system and monitoring changes in its ESR spectrum.

The lineshape 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. Griffith et al (1965) developed a mathematical formula used to calculate the rotational correlation time of a free radical, based on the heights and widths of the spectral lines. Thus, by monitoring the tumbling rate of the radical, information about its immediate environment can be perceived.

The objectives of this study were to use spin labelling to study native and gelatinized starch systems. Specifically, the goal was to identify interaction occurring between probes and starch, and to use this information to monitor the molecular

changes which occur during starch gelatinization and retrogradation. Application of spin labelling to study dough systems was also initiated.

REVIEW OF LITERATURE

Electron Spin Resonance Spectroscopy

Electron Spin Resonance Spectroscopy (ESR) is the most powerful tool available for identifying free radicals (Poole, 1983). Most radicals are generated during homolytic chemical reactions, which separate the two electrons which would normally form a chemical bond. The products of such a reaction contain an unpaired electron and are called free radicals. Therefore, whether naturally-occuring or synthetic, radicals all contain an odd number of electrons. They are common intermediates in photosynthesis, combustions, explosions and halogenations (Pryor, 1966).

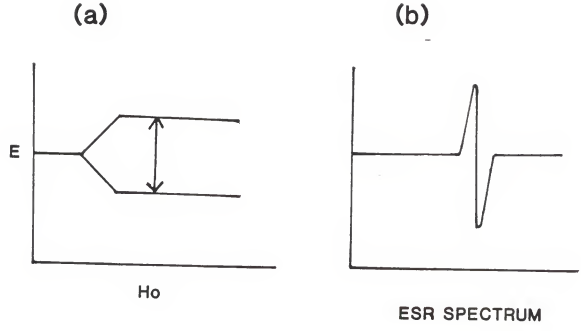
The presence of an unpaired electron makes compounds extremely reactive, and some radicals are consequently short-lived. If their half-lives are less than minutes, detection by ESR is difficult. However, for more stable radicals, their unique magnetic properties are used by ESR to facilitate their identification. Most substances are diamagnetic if they are placed between the poles of a powerful magnet. All of the paired electrons in the molecule align themselves to oppose the external magnetic field. As a result, they will exert a force to move out of the magnetic field. Substances with unpaired electrons are paramagnetic, and will respond quite differently to the presence of strong magnetic fields. The paramagnetic contribution of the odd electron opposes the diamagnetic contribution of all the paired electrons. In this case, the substance will be drawn into the magnetic field and be observed by the ESR (Poole, 1983).

ESR is based on the premise that an electron possesses both a spin and a magnetic moment. When a strong external magnetic field is applied, the electron's magnetic moment will orient either parallel or antiparallel to the field. These two orientations correspond to two different energy levels in which the electron can exist. By supplying electromagnetic energy in the microwave region, the ESR spectrometer causes promotion of electrons from the lower energy level to the higher energy level (Poole, 1983). The population of electrons now exists such that their spins are in a resonance condition between the two energy levels.

The spectrometer makes use of the phenomena by supplying a constant level of microwave power to the sample while increasing the strength of the magnetic field it experiences. At a characteristic (or critical) field strength, spin resonance is induced and, as a consequence, microwave energy is absorbed. An ESR spectrum is therefore a plot of microwave energy absorbed versus increasing field strength. The transition that results in absorption is the single energy level for an electron being split into two at a specific magnetic field strength (Fig. 1a). Transitions between the two levels produces a line on the ESR spectrum. The symmetrical band generally presented is, in fact, the first derivative of the ESR absorption line (Fig. 1b).

If the odd electron in a radical is located on an atom that has a nucleus with a magnetic moment (ie. with an excess proton), this magnetic moment interacts with the electron and splits its energy level further (Fig. 1c). From interaction with this

Figure 1. ESR Spectra.
(a) Transition for a single electron in a magnetic field.
(b) First derivative ESR spectrum for this transition.
(b) Transition for an electron interacting with a proton in a magnetic field.
(d) First derivative ESR spectrum for the transition allowed by (c).


(a)
(b)
E
Ho
ESR SPECTRUM
(c)
(d)
E
Ho
ESR SPECTRUM

excess proton, each electron energy level is split into two. In the figure, transitions denoted by arrows are those having the same nuclear spins. Only transitions with the same spins are "allowed". This phenomena is referred to as hyperfine splitting and, because of it, most ESR spectra consist of more than one line (Pryor, 1966). For the example discussed here, the spectrum displays two lines (Fig. 1d). In general, when n equivalent protons interact with an electron, n + 1 lines appear in the spectrum (Likhtenshtein, 1976).

Spin Resonance Studies of Grain-Native Radicals

Studies utilizing ESR have identified the presence of radicals in a number of intact grains including rice, tomato, cabbage, carrot, beet, barley and, most importantly, wheat (Windle and Evans, 1970). The radicals were stable both at room temperature and in the presence of oxygen. When the wheat was milled to bran and flour, the radical signal was found almost exclusively in the bran fraction. It was stable indefinitely in the presence of air. Storage apparently does not affect this radical since an intact, twenty-five year old wheat kernel produced the same radical signal as a two month old kernel. The free radical content of a new seed decreased as moisture content increased, but was restored to original intensity upon drying. Illumination by ultraviolet light enhanced the radical signals (Windle and Evans, 1972).

Induced Radicals

Dronzek and Bushuk (1973) found positive evidence for the formation of free radicals in dough during mixing by using a methylacrylate copolymerization reaction. This finding was

verified by the fact that 1,1-diphenyl-2-picryl-hydrazyl (DPPH) is decolorized during mixing, indicating that free radicals were being scavenged. The rate of decolorization of DPPH was decreased by sequential removal of free lipids and bound lipids from the flour (Nishiyama et al., 1978). These combined results led to the conclusion that free radicals produced during lipid oxidation are formed during mixing of dough. These authors assume that these radicals are responsible, to at least some extent, for changes in the rheological properties of the dough. No ESR evidence has been found to support this model.

Free radicals were produced in flour, glutenin and gliaden by fine grinding at -196° C in the absence of oxygen. ESR signals were present only if samples were maintained at this temperature, and disappeared rapidly when samples were stored at 25° C (Redman, 1966). Free radicals have also been produced by subjecting flour to electric discharge and gamma-irradiation (Washik and Bushuk, 1973). These radicals were inert to water vapor but were scavenged (destroyed) quickly by atmospheric oxygen. Bread baked with flour irradiated at low dosage levels had a moderate increase in loaf volume. Increased levels of radiation treatment resulted in a gradual decrease in loaf volume (Lee, 1973). Loaf volumes tended to decrease as the elapsed time between irradiation and baking increased. This finding, in conjunction with the general concomitant reduction in the ESR signal, indicated that the free radicals generated in the flour by irradiation did not cause a permanent strengthening of the gluten so as to produce increases in loaf volume. The radicals

disappeared via second order kinetics, which suggested that they interacted with species that could destroy them. The most probable candidates for these species are the hydroxyl groups of carbohydrates, specifically starch, along with water and other hydrogen donating groups. All of the above could scavenge the free radicals and cause the observed decrease in loaf volume over time.

Spin Labelling

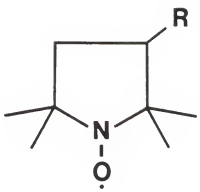
ESR studies of grain and its products have limited, as few samples are paramagnetic under condition conducive to dough preparation and baking. Without external perturbation, stable free radicals were not present and ESR analysis was impossible (see above). A method of extending ESR use to diamagnetic systems is spin labelling. This involves placing a known radical into a biological diamagnetic sample and using ESR to monitor the radical's activity (Berliner, 1976).

Spin labels are most commonly nitroxide free radicals (Fig. 2a, 2b &2c). To be usable, the radical should be stable in both aqueous and nonaqueous states indefinitely.Other properties essential to a spin label are that its ESR spectrum be sensitive to the nature of the system of interest and be simply interpreted. Finally, the label must not peturb the host system to cause changes on its own (Likhtenshtein, 1976).

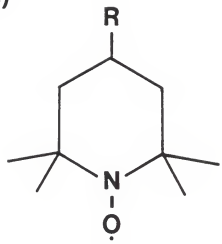
Through proper selection of a nitroxide, the various components of a biological system can often be spin labelled quite specifically, either covalently or by non-covalent interaction (Berliner, 1976). Because there are no interfering signals from the unlabelled environment, the diamagnetic nature

Figure 2. (a) & (b) Five and six-membered cyclic nitroxides.
(c) Cyclic nitroxide as a stearic acid derivative.

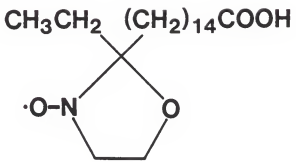
(a)



(b)



(c)



of the system becomes an asset rather than a liability. Information derived from an ESR spectrum of spin labelled biological macromolecules show changes in the local environment not seen for the molecule as a whole. This differs favorably from physiochemical measurement (absorbance, viscosity, circular dichroism) that show changes averaged over the entire molecule (Snipes and Keith, 1970).

The general electronic structure of stable nitroxide free radicals is such that the unpaired electron interacts with the nucleus of the nitrogen atom (Berliner, 1979). This hyperfine coupling interaction splits the absorption into three lines of equal intensity and equal spacing. The width of the three absorption lines depends on various environmental factors and can vary for each of the three hyperfine components. Since all three are known to be of equal intensity, a broad line has less amplitude on a derivative absorption curve (Likhtenshtein, 1976). This can be used to show that the motion of the free radical is somehow restricted by its environment.

Most nitroxide spin labels fall into one of two general classes. The first class includes five or six-membered cyclic nitroxides with various substituent functional groups to allow the probe to interact with (ie. to label) a system of interest (Fig. 2a and 2b). The functional group may be one which facilitates hydrogen bonding, hydrophobic interactions, or covalent bonding of the macromolecule, as desired. A disadvantage of this type of probe is that motion between the nitroxide moiety and the macromolecule is allowed by the linkage. This infers that the motion of the nitroxide group may differ

from that of the macromolecule as a whole (Berliner, 1979). This is a problem when the motion or mobility of the probe in its local environment is under study.

The second class of spin labels compensates somewhat for this problem (Fig. 2c). It positions the nitroxide more closely to the rest of the molecule, allowing for attachment of the probe's carboxylic acid group to ketone sites on the host. Further, the mobility of the nitroxide now will be more indicative of that of the molecule as a whole (Snipes and Keith, 1970).

Analysis of a spectrum from a spin-labelled sample involves consideration of line broadening. Line broadening occurs due to three separate phenomena. First, the presence of oxygen, which is paramagnetic, can cause local variations in the net magnetic field surrounding the nitroxide. Because different nitroxide molecules experience different net magnetic fields, spectral lines are broadened. Second, line shape is altered by too high a concentration of spin label. Electron exchange can occur, and with an increase in nitroxide concentration, the three distinct lines will all broaden into a single unresolved line. Finally, line widths are most dramatically altered by restricted molecular motion. The spectrum given by a spin probe either adsorbed onto a molecule or in a highly viscous medium will have an entirely different line shape from that of the probe in a solvent (Berliner, 1976). This is, in fact, the phenomena often used in the study of local molecular environments by ESR.

Perhaps the most useful information obtained from a spin

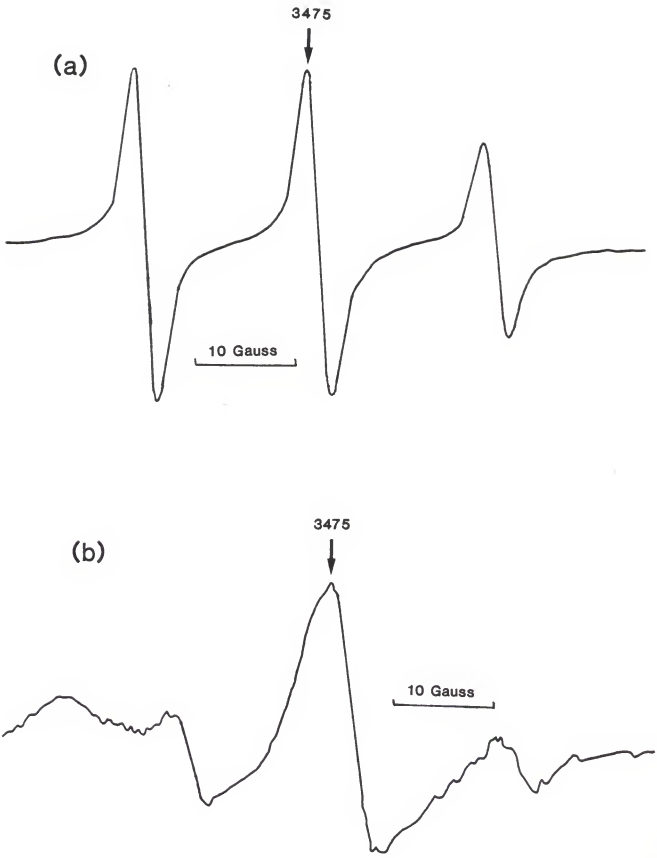
label spectrum is the rotational correlational time, tau (τ), indicative of the tumbling rate of the molecule. Griffith et al. (1965) developed equations to calculate τ which relate the line widths of the nitroxide spectrum to the effects of restricted molecular motion. According to Griffith,

$$\tau = 6.5 \times 10^{-10} \text{ Wo } [(Ho/H-1)^{1/2} + (Ho/H1)^{1/2} - 2]$$

where Wo is the line width of the center field line (in Gauss), and Ho,H_1 and H_{-1} are heights of the center field, low field, and high field lines, respectively. A nitroxide radical, tumbling freely in solution, would have a rotational correlation time between 10^{-8} and 10^{-10} seconds and would exhibit isotropic motion (Snipes and Keith, 1970). If motion is anisotropic, it is nonrandom with respect to orientation and will be inadequately described by τ . Within the useful range of correlation times (10^{-8} to 10^{-10} seconds), the anisotropic dipolar contributions are averaged out by their random distribution and rapid motion (Poole, 1983). For motion more restricted than this, the line broadening equations cannot be applied, and a more empirical method must be used to quantitatively estimate molecular mobility (Likhtenshtein, 1976).

A spectrum showing isotropic motion results in nitrogen hyperfine lines that are approximately equal in line width and symmetrical in appearance (Fig. 3a). If molecular motion is restricted by an increase in viscosity, contributions from the anisotropic parameters are entered into the ESR spectrum. The result is a loss of symmetry among the lines, with the high field line having a greater width than the other two lines (Fig. 3b). This spectral distortion can be quantitated by the decrease in

Figure 3. (a) ESR Spectrum of a Radical with Rapid Isotropic Motion.
(b) ESR Spectrum of the same Radical in a More Viscous Environment.


3475
(a)
10 Gauss
3475
(b)
10 Gauss

the rotational correlation time. By monitoring changes in τ, the molecular environment of the free radical can be monitored (Berliner, 1976).

Spin Labelling Applications

Snipes and Keith (1973) calculated rotational correlation times for glycerol/water/spin label mixtures and established a linear logarithmic relationship between τ and viscosity. Using a ketone spin label, they concluded that the intracellular viscosity was greatly increased by the presence of internal membranes. Sachs and Latorre (1974) used an alcohol derivative of the same probe and found that the τ of the spin label inside muscle fibers was about five times greater than in water. Much of this increase was attributed to the probe binding to other cell constituents. Cooke (1974) reconciled both viewpoints by showing that the τ of an intracellular probe increases as the hydrophobicity of the probe increases. Note that all these studies were successful in showing that molecular motion was more restricted inside cells than in dilute salt solutions.

Nishiyama, Kuniyori, and Matsumoto (1981) used rotational correlation times to show that lipid is immobilized in a gluten matrix by strong interaction with protein. Spin labelling has also been successfully used to examine the conformation of amylose in aqueous solution (Ebert, 1984). Windle (1985) used a ketone substituted probe to define three distinct starch/water conditions occurring during potato starch gelatinization.

Starch

The starch granule is composed of two molecular types,

amylose and amylopectin. Amylose constitutes roughly twenty-five percent of the granule by weight (Whistler, 1984). It is essentially linear and consists of several hundred D-glucose units linked by α-1,4 glucosidic bonds. The bulk of the granule is amylopectin, a highly branched molecule composed of many short (25 to 30 glucose units) α-1,4 linked chains that are attached through α-1,6 branch points. In both molecular weight and volume, amylopectin is much larger than amylose (Potter and Hassid, 1948). Stable genetic varieties of some cereals (corn, barley, sorghum) exist in which the starch is nearly ninety-nine percent amylopectin and only trace amounts of amylose. These are the so-called "waxy" starches (Osman, 1967).

The arrangement of the molecules within the starch granule directly determines its properties. Kassenbeck (1979) distinguishes three types of organization: the radial arrangement of amylose molecules, amylose in amorphous arrangement, and an arrangement of amylopectin clusters such that crystallites are in tangential lamellae. Growth rings are seen in all starches except barley, and display the growth of the granule in an outward direction from the hilum. The thickness of the growth ring corresponds to the length of an amylopectin molecule (Yamaguchi et al., 1979). Wheat and maize starches observed through Transmission Electron Microscopy show growth rings and a pronounced radical periodicity of 60-70 angstroms. This period corresponds to the average spacing of amylopectin clusters and supports the idea that amylopectin molecules have a generally radial orientation. Amorphous areas in the granule occur when molecules are less dense and not closely associated.

Starch granules are birefringent and show a characteristic "maltese cross" pattern when viewed with polarizing optics (Whistler and Turner, 1955). This implies that there is a high degree of regular repeating molecular orientation within the granules. Cereal starches show weaker birefringence than do tuber starches; high amylose cereal starch generally has a weak birefringence. Banks & Greenwood (1975) showed X-ray diffraction patterns supporting amylopectin, rather than amylose, as the principle crystalline component of starch. Robin et al. (1974) support this conclusion.

Starch granules have a limited capacity for absorbing cold water and swelling reversibly, which indicates a limited degree of elasticity of the intermicellar network (Leach, 1965). The amount of sorbed water is dependent on the temperature, pH, and specific botanical species of starch. Intact starch granules under normal atmospheric conditions contain between 10% and 17% moisture. Schierbaum et al. (1962) postulated that the first 14% of the water is strongly bound, water between 14% and 30% is absorbed in the capillaries of the granule, and the remainder is unbound. During reversible swelling, water penetrates into the amorphous regions of the granules and forms hydrogen bonds with the free hydroxyl groups on the starch molecule.

Gelatinization

When starch is heated in an aqueous medium (at least 30% water), or treated with specific chemical reagents, a sequence of changes collectively termed gelatinization occurs (D'Appolonia, 1971). The appearance of the granules is unaffected until a

critical temperature is reached. At this temperature, the granules swell enormously and lose their birefringence. Both swelling and loss of birefringence are irreversible, and begin at the botanical center, or hilum, of the granule (Leach, 1965). The loss of birefringence is widely accepted as evidence of starch gelatinization (Collision, 1968). Gelatinization begins where the bonding is weakest, in the accessible amorphous intermicellar areas of the granules (Sterling, 1968). For a specific granule, the gelatinization transition is sharp, taking place over a 1-2 degree temperature range. However, starch granules in a sample do not lose birefringence at the same temperature, but instead reveal a gelatinization temperature range of 8-10 degrees Celsius. This is consistent with the variation in internal structure expected in a population of biological macromolecules. Specifically, it suggests that varying degrees of association exist in the amorphous and crystalline regions of individual granules (Collision, 1968).

As starch is heated in aqueous medium above its gelatinization temperature, hydrogen bonds continue to be disrupted, the liberated hydroxyl groups are hydrated, and the granules continue to swell (Collision, 1968). Two stages of starch gelatinization have been revealed by X-ray diffraction (Katz, 1928). The first stage occurs at approximately 60° C and reveals a loss of crystalline structure corresponding to the loss of birefringence. The second stage is typified by a change to a diffraction amorphous pattern, accompanied by continual swelling and uptake of water. Granules lose soluble carbohydrates to solution and become distorted in shape around 70° C, but they

keep their identity on heating to above 90° C (Leach, 1965).

The most noticeable gross change in the starch suspension during continual heating is the increase in consistency or apparent viscosity, which results from granule swelling and the binding of solubilized molecules. These changes are coupled with a reduction in free water due to its uptake by the granule (Whistler, 1984). The temperature at which this increase in viscosity occurs depends on the concentration of the suspension. Gelatinization and the attandant changes in the starch molecule is an important phenomena in the production of all starch-containing foods. Because of this, several instruments have been designed to measure and monitor gelatinization. The most prevalent, the amylograph and viscometer, make use of the above-mentioned change in solution viscosity as the measuring technique (Pyler, 1964).

Retrogradation

As a starch paste cools, the starch molecules become less soluble and begin to aggregate. Given time, they will crystallize (delRosario & Pontireros, 1983). For linear polymers such as amylose, straight chains will orient themselves in a parallel fashion to facilitate maximum hydrogen bonding between hydroxyl groups on adjacent chains. As this bonding occurs, hydration of the chains by water is replaced by direct hydrogen bonding between the chains. Thus, the molecules bound together to form aggregates that are insoluble in water. This reformation of crystalline order by the starch gel is known as retrogradation (Harris et al., 1965). It is typified by an increase in rigidity

and opacity of the gel and a decrease in the ability of the starch granules to complex with iodine or to be acted upon by amylases (Ott and Hester, 1965).

The highly branched structure of amylopectin molecules reduces their ability to align and crystallize, and thereby limits gel formation. Only under special conditions such as reduced water levels, freezing, or prolonged aging will amylopectin dispersions show retrogradation effects (Osman and Cummisford, 1959). The hydroxyl groups tend to attract and hold water molecules through the same associative forces. The retrogradation of amylopectin is more complex in food systems (ie bread) and is not well understood.

Linear amylose is considered primarily responsible for retrogradation and will form firm gels alone at concentrations as low as 1.5% (Osman, 1967). In some gels, amylose is thought to link together intact starch granules or fragments, providing additional structure in the network. It also is the chief material that forms the gel network which binds and entraps unabsorbed water (Ott and Hester, 1965).

Starches containing both amylose and amylopectin retrograde readily at relatively low concentrations. Retrogradation rate is affected by the type and size of the starch granules, their age and previous treatment, paste concentration, temperature, pH, and non-starch component in the system (Schoch and Elder, 1955). Hollo and coworkers (1960) concluded that retrogradation takes place in three stages. The first consists of chain stretching due to the breaking of intermolecular bonds which maintain the helical configurations. This is followed by the loss of bound

water and subsequent reorientation of the molecules. Retrogradation is completed by hydrogen bond formation between adjacent molecules forming a crystalline structure.

Doughs

In breadmaking, the dough mixing process has two basic objectives: the thorough and uniform dispersion of all ingredients to form a homogeneous mixture, and the physical development of flour storage proteins into a continuous structure possessing the optimum degree of plasticity, elasticity, & viscous flow (Pyler, 1979). The chemical and rheological changes that occur during dough mixing are numerous and complex. Dough formation involves hydrating and disaggregating the protein matrix to produce gluten, and the subsequent spreading of gluten over the surface of the free starch to form a continuous matrix that will permit gas retention (Farrand, 1943).

After mixing, dough is a complex, viscoelastic system. At the molecular level, it is composed of high molecular weight polymers (primarily proteins) linked by hydrogen bonds, hydrophobic interactions, ionic linkages, van der Waals forces, and covalent disulfide bonds (Hlynka, 1964). Wehiri and Pomeranz (1969) suggest that covalent and ionic bonds primarily increase the cohesiveness of doughs, while dipole, hydrogen, and hydrophobic bonds contribute to elasticity and plasticity. The formation of complexes between lipid and protein is of particular importance in the development of a proper gluten extensibility and gas retention (Grosskreutz, 1961). Through reduced sulfhydryl-disulfide bond interchange reactions, the

dough becomes sufficiently mobile to endure the stress of mixing. Both disulfide bonds and free sulfhydryl groups contribute to the development of the three-dimensional gluten network in mixing. Reduction of only 7% of the disulfide bonds in a dough produces dramatic changes in its physical properties (Tsen and Hlynka, 1963).

The consistency changes which occur during mixing are easily observed visually and by touch. Quantitatively, the mixograph and farinograph both monitor dough development and stability and can identify the optimum mixing time (Pyler, 1979). No method has been developed to monitor the viscosity changes which occur during dough mixing.

Materials and Methods

Materials

Starch

A commercially isolated unbleached wheat starch was obtained from Midwest Solvent. Co. of Atchison, Kansas. Regular corn starch was isolated and purchased from the CPC Company, while waxy corn starch was a gift from Clodualdo Maningnat. Amylose and amylopectin (from potato, 99% pure) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Hard red winter wheat bread flour obtained from Ross Mills (Wichita, KS) was used in mixing doughs.

Spin Labels

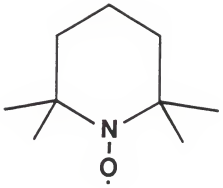
Aldrich Chemical Company (Milwaukee, WI) supplied the following nitroxide spin labels; 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), and the derivatives of 5-ketostearic acid, 12-ketostearic acid , and 16-ketostearic acid (5, 12, and 16-DOXYL stearic acid). A twelve carbon fatty acid derivative of TEMPO, TEMPO-laurate, and TEMPO-benzoate, were obtained from Molecular Probes (Junction City, OR). Figure 4 presents the structures of these molecules.

Solvents

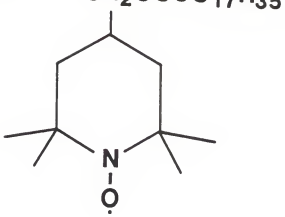
Solvents for the spin labels were distilled water and Reagent grade ethyl ether, obtained from Aldrich Chemical Co. (Milwaukee, WI). Glycerol, potassium hydroxide, and hydrochloric acid were all reagent grade and purchased from Fisher Scientific Co. (New Jersey). All were diluted to 1M concentration before

Figure 4. Structures of Nitroxide Spin Labels.
(a) TEMPO
(b) TEMPO-Laurate
(c) TEMPO-Benzoate
(d) 5-DOXYL Stearic Acid
(e) 12-DOXYL Stearic Acid
(f) 16-DOXYL Stearic Acid

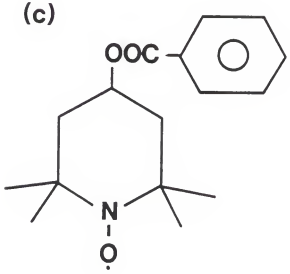
(a)



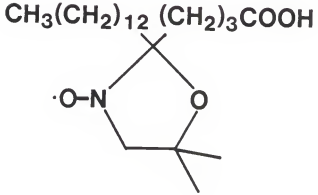
(b)


$CH_2OCOC_{17}H_{35}$

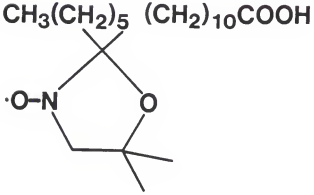
(c)


OOC

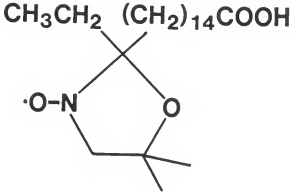
(d)


$CH_3(CH_2)_{12}$ $(CH_2)_3COOH$

(e)


$CH_3(CH_2)_5$ $(CH_2)_{10}COOH$

(f)


CH_3CH_2 $(CH_2)_{14}COOH$

using.

Preparation of Spin-Labelled Samples

TEMPO Samples

TEMPO was diluted with water to a concentration of 2.5 x 10^{-3} M and the solution added to wheat, regular corn, and waxy corn starches. Samples were equilibrated by gentle stirring for 24 hours at room temperature before ESR spectra were taken. Starch solutions were gelatinized by heating samples to 70^{o} for twenty minutes in a water bath and then allowing them to cool.

TEMPO-Laurate

The procedure described above was used for TEMPO- laurate samples, with the exception that the probe was diluted to 2 x 10^{-5} M in ether, which was allowed to evaporate off before distilled water was added in the desired amount. Where separation of starch from solution was required, it was accomplished by centrifugation in a benchtop centrifuge for thirty minutes at 300 RCF. ESR spectra were taken for both the supernatant and starch pellet, after which the supernatant was discarded. The pellet was then slurried with water to a 2/1 water/starch ratio, stirred, and re-centrifuged.

TEMPO Doughs

Doughs were mixed in a National Manufacturing Co. 10 gram mixograph for periods of 2 minutes, 4 minutes, and 6 minutes. Doughs contained 63% water (flour basis) and contained TEMPO at a final concentration of 2.5 x 10^{-3}M.

DOXYL Stearic Acid

Gluten was isolated from regular hard wheat flour by washing. Doughs were kneaded in a stream of water until no starch remained in the wash water. The resulting gluten was freeze-dried and ground with a mortar and pestle before using. 16-DOXYL stearic acid was dissolved in ether and added to each gluten sample to achieve a final concentration of 5 nmol probe/ 0.2 g gluten. Ether was allowed to evaporate off before ESR spectra were taken.

Microscopy

Wheat, regular corn, and waxy corn starches were suspended in a water/starch/spin label mixture of 1/2/.002 with 16-DOXYL stearic acid, TEMPO-benzoate, and TEMPO-laurate, as described above. Samples were obtained from the pellet after centrifugation as well as its accompanying supernatant. Bright field and polarized images were examined on the same fields with a Zeiss Universal Microscope equipped with Olympus objectives and camera system. Where appropriate, pictures were taken using Kodak Tri-X Pan film.

Amylose/Amylopectin

Potato amylopectin (0.1 g) was dissolved in 3 ml water containing 2 x 10^{-5} M TEMPO-laurate. The solution was sonicated for thirty minutes at full power in a Sonac S-2 sonicator to affect complete solubilization of the label and amylopectin.

Potato amylose (0.1g) was dissolved in 3 ml 1N KOH and sonicated for thirty minutes. TEMPO-laurate (2 x 10^{-5} M) in ether was added, and the ether was allowed to evaporate off. Sample plus probe were sonicated for an additional ten minutes to ensure complete solubilization of both starch and probe. 1N HCl

was then added to neutralize the sample to pH 7. ESR spectra were taken immmediately after neutralization.

Electron Spin Resonance

ESR measurements were performed on an IBM Bruker ESR ER 200D-SRC Spectrometer at room temperature. Center field for all spectra was 3475 Gauss. Rotational correlation times were calculated from Griffith's formula,

$$\tau = 6.5 \times 10^{-5} \text{ Wo } [(ho/hl)^{1/2} + (ho/h\text{-}l)^{1/2} - 2]$$

where Wo is the width of the center field line in Gauss, and ho, hl, and h_{-1} are the heights of the center field, low field, and high field lines, respectively. Most instrumental settings were identical for all ESR spectra that were taken. Variations in phase adjustment and the gain setting were made for each spectra to optimize signal amplitude and line symmetry.

Results and Discussion

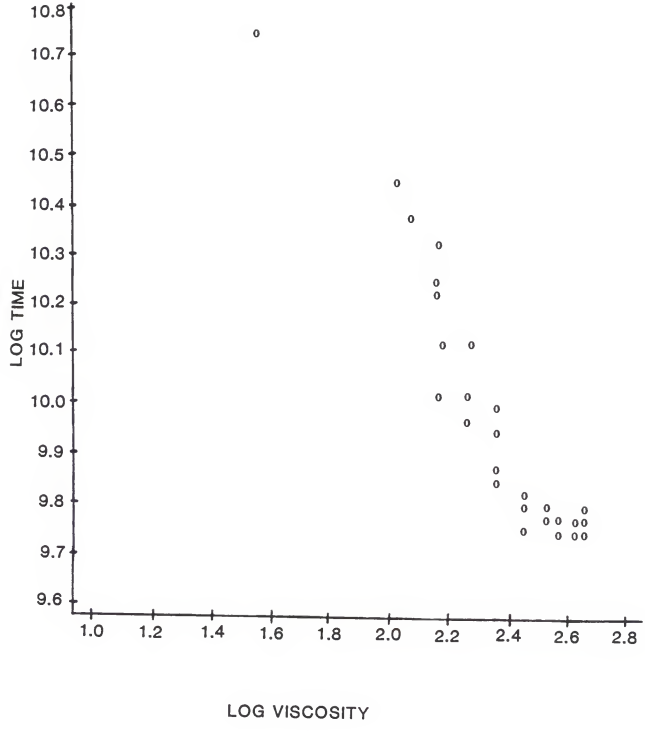
Water/Glycerol Viscosity Study

Snipes and Keith (1972) successfully used spin labelling to identify restricted molecular motion in intracellular membranes. The foundation of their work, along with that of Cooke (1974), was the assumption that the rotational correlation time, τ, does indeed increase linearly with viscosity. If the spin label behaves ideally as a rigid sphere rotating in a medium of viscosity η, τ would obey the Stokes equation: $\tau = 4\pi\eta a^3/3hT$, where a = the radius of the sphere, T = the absolute temperature, and k = the Boltzmann constant (Berliner, 1976). A change in the structure (viscosity) of the water surrounding the spin label should, therefore, cause a linear change in the value of τ.

To verify this for the TEMPO spin label used in subsequent studies, the rotational correlation times for water/glycerol/TEMPO mixtures were determined by the ESR. Figure 5 shows a log-log plot of τ as a function of the derived viscosity values for each solution (Lange, 1967). A linear relationship is displayed ($P < .001$), in agreement with the Stokes equation. This verifies the work of Cooke (1974) and Snipes and Keith (1972), who determined the same relationship using phospho and keto derivatives of TEMPO, as well as demonstrating the ability of this probe (and τ) to be used as a measure of freedom of molecular motion (see below).

The fact that TEMPO obeys Stokes equation is remarkable. The approximation of the spin label as spherical is less than

Figure 5. Rotational Correlation Time of TEMPO/Glycerol/ Water Mixtures as a function of Viscosity.


LOG TIME
10.8
10.7
10.6
10.5
10.4
10.3
10.2
10.1
10.0
9.9
9.8
9.7
9.6
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
LOG VISCOSITY

perfectly accurate. Also, most solutions have some long-range order which results in anisotropic motion. Each of these phenomena could, individually, lead to a nonlinear relationship between viscosity and correlation time. Agreement with the Stokes equation permits τ to be used as an appropriate "viscosity" for the rotation and diffusion of TEMPO molecules.

Starch Studies with TEMPO

Verification of the Stokes equation suggests that spin labelling may be an attractive technique to examine starch/water interactions and starch/water/fatty acid interactions. This work conducts a study assessing the bonding of several labels using both hydrophilic and hydrophobic probes combined with wheat, corn, and waxy corn starches.

Wheat starch, regular corn starch, and waxy corn starch were each mixed with water to three different ratios (0.5:1, 1:1, 2:1), and allowed to completely hydrate. In each case, TEMPO was dissolved in the water to achieve a final concentration of 2.5 x 10^{-3} M. Thus, the water/starch ratio changed accordingly. The water contents were chosen to create a range of potential water/starch/spin label environments. The 1:1 and 2:1 samples thus produced slurries. The 0.5:1 water level samples were moist powders, indicating that all the water was involved in hydrating the starch molecules. ESR spectra were obtained for each.

At each water/starch ratio, spectra appeared the same for all three types of starch. The 1:1 and 2:1 samples displayed a symmetrical triplet signal characteristic of a TEMPO probe rotating freely in solution (Fig. 6b & 6c). They did not differ

significantly in appearance from the spectrum of TEMPO in water (Fig. 6d), but their hyperfine lines had broadened slightly. The spectrum from the 0.5:1 ratio, however, did show distortions (Fig. 6a). Its high field and low field lines were greatly reduced in size, and all three lines were broadened. These observations are consistent with the signal originating from a probe where rotational motion is restricted (Berliner, 1976).

An examination of rotational correlation times verifies this. Table I lists τ values for all three types of starch at all three water/starch ratios. For each starch at a 0.5:1 dilution, τ was between 2.35 x 108 and 4.34 x 108 sec-1. At a 1:1 dilution, τ had increased to 4.66 x 109 to 6.38 x 10^9 sec-1. At the highest water level (least viscous) 2:1 dilution, tau increased to between 2.18 x 1010 and 3.82 x 1010 sec -1. For comparative purposes, the rotational correlation time for 2.5 x 10-3 M TEMPO in pure water is approximately 2.00 x 1011 sec-1. This value is known to vary between 1011 and 1012 sec-1 due to probe-probe exchange reactions, and is not always reproducible. A minor error in measuring peak heights can lead to a large change in τ for TEMPO in water.

At the higher 1:1 and 2:1 dilutions, the water/starch system is discontinuous by nature. The granules contain molecules of water inside them, yet are surrounded in solution by free water. When a spin label is added to this system, it is distributed throughout both systems and experiences two different molecular environments. In the 0.5:1 water/starch samples, there is little excess water outside the granules to form an aqueous system. The ESR signal observed is that of the

Figure 6. ESR Spectra of Water/Starch Samples with TEMPO at Room Temperature.
(a) 0.5:1 (b) 1:1 (c) 2:1

(d) 2.5 x 10-3 M TEMPO in Water.

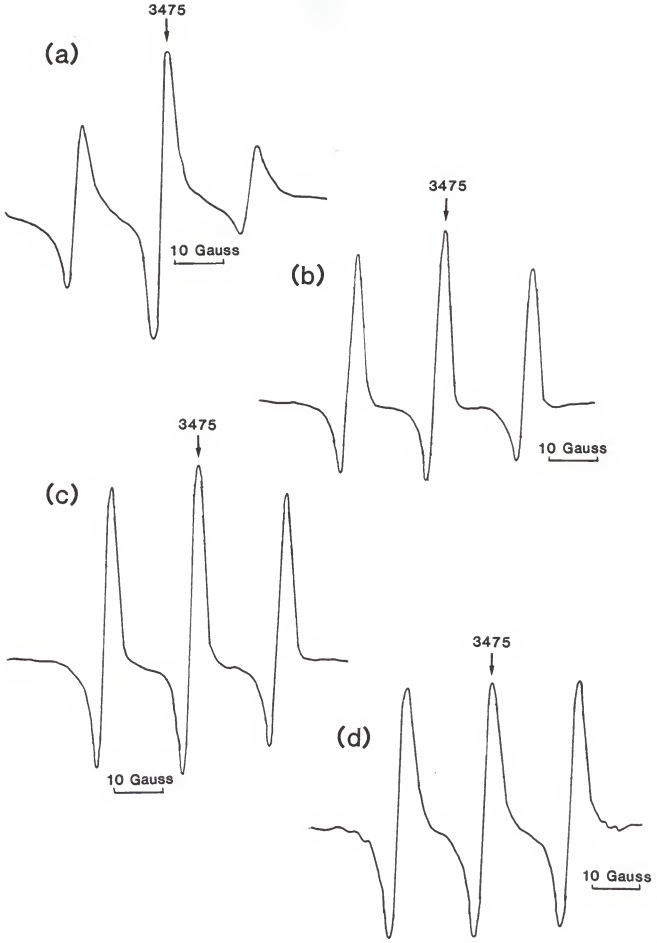

3475
(a)
10 Gauss
3475
(b)
10 Gauss
3475
(c)
10 Gauss
3475
(d)
10 Gauss

Table I. Rotational Correlation Times for Water/Starch Samples with TEMPO before heating.

Starch	Ratio Water/Starch	Correlation Timea (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}

a Standard 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}.

probe in the water inside the starch granule. For the less viscous 1:1 and 2:1 water/starch samples, there is clearly excess water present to suspend the starch granules in solution. For these samples, examination of both spectral appearance and the rotational correlation times reveals that the probe's molecular motion is in all probability an **average** of that given by the radical in pure water and that inside the starch granule.

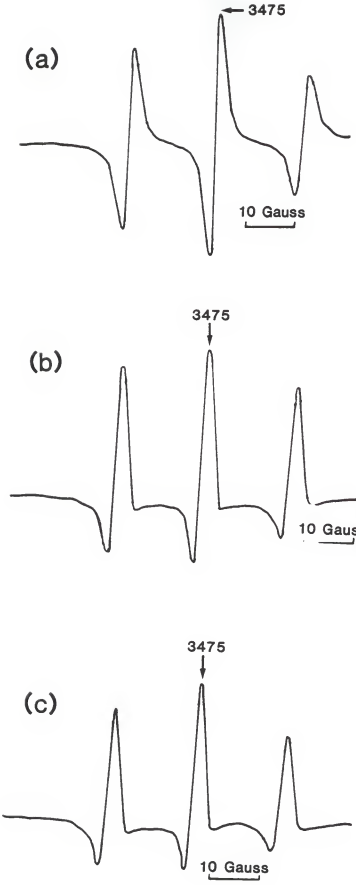
Gelatinization

Gelatinization of native starch can be achieved by heating a sample that contains over thirty percent water (D'Appolonia, 1971). As starch is heated above its minimum gelatinization temperature, individual starch granules swell enormously, hydrogen bonds are disrupted, and released hydroxyl groups become hydrated (Collision, 1968). The net result of these changes plus loss of soluble material to solution is that the aqueous suspension increases in viscosity.

Water/starch/TEMPO samples from the previous experiment were heated to 70o C for twenty minutes and allowed to cool. ESR were run on all samples two hours after thay had cooled, and again three days later. Changes in viscosity and the interaction of starch and water caused by by gelatinization were expected to result in changes in τ .

Spectra taken immediately after the samples cooled were virtually identical in appearance to those run before heating (Fig. 7). Signal amplitude increased greatly for all three starches at all three water levels, but line shapes remained the same as before. Rotational correlation times did not change

Figure 7. ESR Spectra of Water/Starch Samples with TEMPO After Heating.
(a) 0.5:1 (b) 1:1 (c) 2:1


(a)
3475
10 Gauss
(b)
3475
10 Gauss
(c)
3475
10 Gauss

significantly (Table II). Samples diluted to 0.5:1 had τ values of 108 sec^{-1}, while 1:1 samples were in the 109 sec^{-1} range, and 2:1 samples were approximately 1010 sec^{-1}.

The consistencies observed in spectral appearance and correlation times before and after gelatinization have several implications. First, they reveal that the motion of TEMPO in the water inside the starch granule is restricted. Strong absorption of water by starch results in a system in which the viscosity is increased and the probe finds itself in a more restrictive environment. Second, they reveal that before heating, in the ungelatinized state, the ESR signal is an average of that given by the radical in pure water and that inside the starch granule (see page 33). Finally, the molecular changes which occur during starch gelatinization changes this. The previous distribution of water is altered, resulting in the production of a continuous system. After gelatinization, the rotational correlation time can be used to measure the overall viscosity.

Retrogradation

The above samples were cooled after gelatinization and stored for three days before ESR spectra were obtained, to determine the effects of retrogradation on τ. In a similar study, Windle (1985) found that the correlation time decreased by 30% upon storage, indicating a corresponding decrease in gel viscosity. The data reported here do not support this conclusion. Rotation correlation times for wheat, regular corn, and waxy corn starches at all three water/starch ratios did not change three days after heating (Table III). They remained essentially the same as they were before and after heating. Some

Table II. Rotational Correlation Times for Water/Starch Samples with TEMPO after heating.

Starch	Ratio Water/Starch	Correlation Timea (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	1.43×1010
Waxy Corn	0.5:1	3.44×10^{8}
	1:1	5.85×10^{9}
	2:1	3.71×10^{10}

[a] Standard 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}.

Table III. Rotational Correlation Times for Water/Starch Samples with TEMPO. Three Days after Gelatinization.

Starch	Ratio Water/Starch	Correlation Timea (sec^{-1})
Wheat	0.5:1	2.50×10^{8}
	1:1	3.76×10^{9}
	2:1	3.54×10^{10}
Regular Corn	0.5:1	2.46×10^{8}
	1:1	3.17×10^{9}
	2:1	4.46×1010
Waxy Corn	0.5:1	2.83×10^{8}
	1:1	4.69×10^{9}
	2:1	1.75×10^{10}

[a] Standard Deviation for 0.5:1 Samples was 1.4×10^{7}, for 1:1 samples 4.6×10^{8}, and for 2:1 samples 6.3×10^{9}.

actually increased. After retrogradation, the probe can be used to measure the environment, which is continuous and remains the same. Because of this, the correlation time is primarily responsive to the total amount of water present at gelatinization and then afterwards, in the initial gel state. The ESR signal in native starch is a composite of label inside and outside the starch granule, while that from the gelatinized state is more uniform and representative of the whole. The changes in the distribution of the water caused by gelatinization and, particularly, retrogradation, may not cause enough change in the probe's environment to result in changes in τ .

Starch Studies with TEMPO Laurate

Amylose is known to complex with hydrophobic straight chain molecules. To utilize this, the experiment detailed above was repeated using a more hydrophobic spin label, TEMPO esterified to lauric acid, TEMPO-laurate. A probe with a different side chain was desirable, as interaction of spin labels has been shown to be through the probe side chain rather than through the nitroxide moiety (Miller, 1979). This is particularly true in starch where straight chain fatty acids are well known to interact (complex) with amylose in its helical conformation. TEMPO laurate would permit an examination of water/starch/fatty acid interactions and possibly demonstrate interaction.

TEMPO laurate was dissolved in ether and placed on each starch sample in liquid form. The ether was evaporated off in air, leaving the spin label uniformly dispersed on the starch. Water was added to the desired 0.5:1, 1:1, and 2:1 levels, and

solutions were equilibrated for twenty-four hours. The final TEMPO- laurate concentration was 2 x 10-5 M with respect to water.

ESR spectra taken before heating for both wheat starch and regular corn starch showed line broadening in different manifestations. For both starches, the spectra of the driest samples appeared identical to those in which regular TEMPO was used as label at similar water contents (Fig. 8a, 8b, & 8c). The signal was essentially a triplet, which was both broadened and distorted, the result of restricted molecular motion.

The signals from the 1:1 and 2:1 samples were significantly different from those of the 0.5:1 samples. For both starches, 1:1 signals were so distorted that it was difficult to identify the triplet (Fig. 9a, 9b, 9c). Baselines were curved and signal symmetry was lost. Spectra from the 2:1 samples were similar to this regardless of the starch (Fig. 10a, 10b, 10c).

Rotational correlation times for all starch samples at all water/starch ratios were between 1.78 x 108 sec-1 and 7.67 x 108 sec-1 (Table IV). This is a relatively narrow range and represents a clear difference from the wider range observed when regular TEMPO was used as a spin probe.

TEMPO laurate in each sample produces an ESR spectrum which differs dramatically from that of the probe alone, dissolved in ether (Fig. 11). When considered in conjunction with τ values that are identical over a wide range of free water (50% water to 200% water), binding of the probe or its strong interaction with the granule is clearly suggested. Alternatively, the results may be a result of TEMPO- laurate's insolubility in water.

Figure 8. ESR Spectra of Water/Starch/TEMPO-Laurate in a 0.5/1/ 2 x 10^{-5} M ratio.
(a) Wheat (b) Regular Corn (c) Waxy Corn

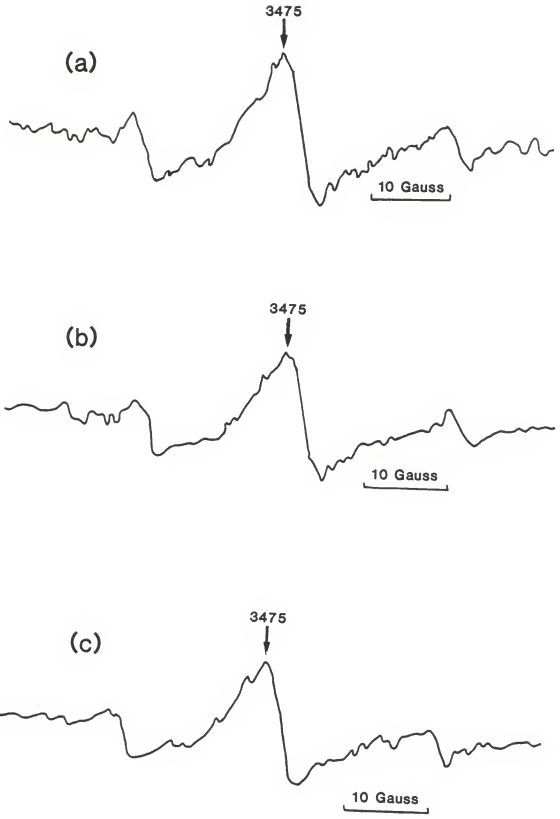

3475
(a)
10 Gauss
3475
(b)
10 Gauss
3475
(c)
10 Gauss

Figure 9. ESR Spectra of Water/Starch/TEMPO-Laurate in a 1/1/ 2 x 10^{-5} M ratio.
(a) Wheat (b) Regular Corn (c) Waxy Corn

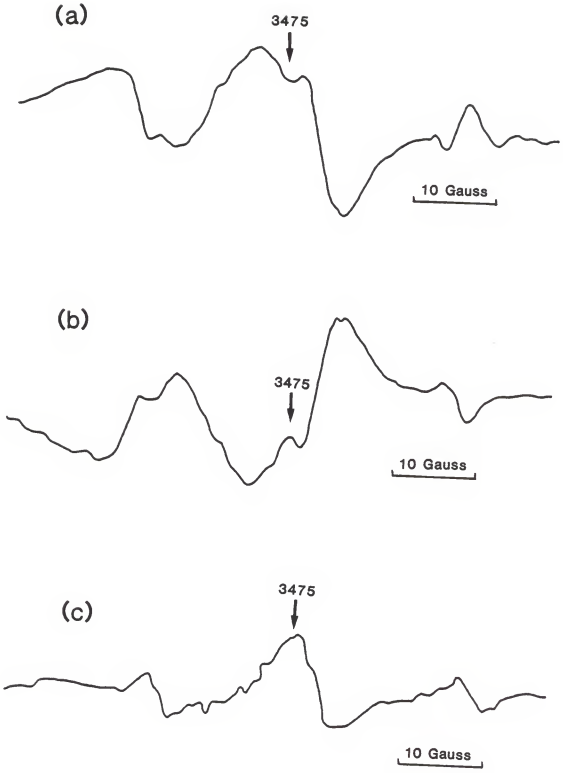

(a)
3475
10 Gauss
(b)
3475
10 Gauss
(c)
3475
10 Gauss

Figure 10. ESR Spectra of Water/Starch/TEMPO-Laurate in a 2/1/ 2 x 10-5 M ratio.
(a) Wheat (b) Regular Corn (c) Waxy Corn

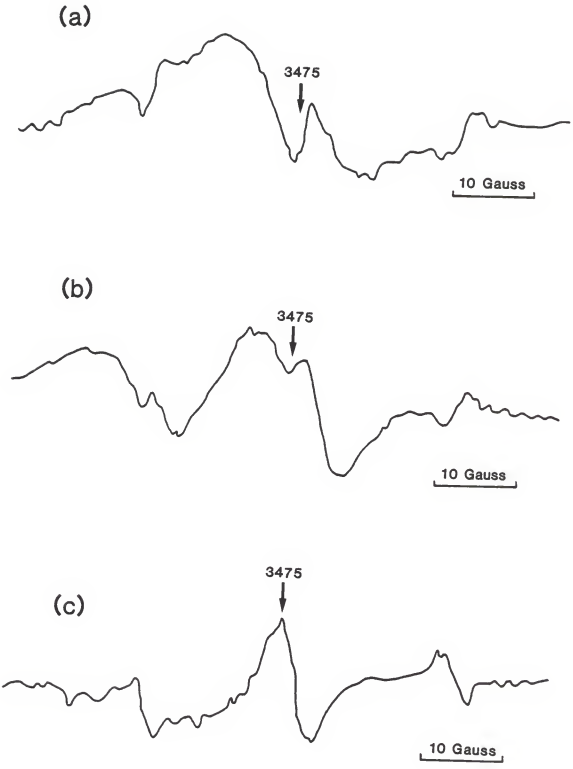
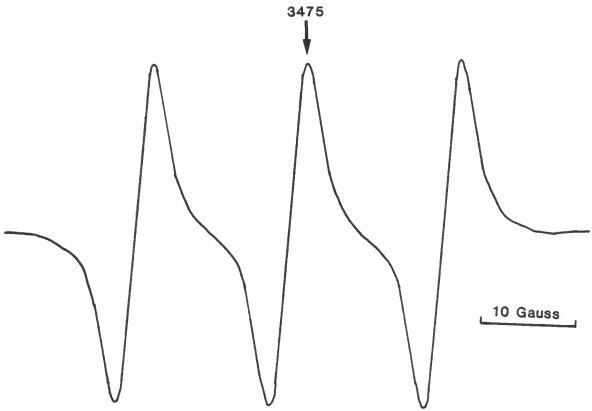

(a)
3475
10 Gauss
(b)
3475
10 Gauss
3475
(c)
10 Gauss

Table IV. Rotational Correlation Times for Water/Starch Samples with TEMPO-Laurate before heating.

Starch	Ratio Water/Starch	Correlation Timea (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 x 107, for 1:1 samples 1.98 x 107, and for 2:1 samples 3.43 x 107.

Figure 11. ESR Spectrum of TEMPO-Laurate in Ether.

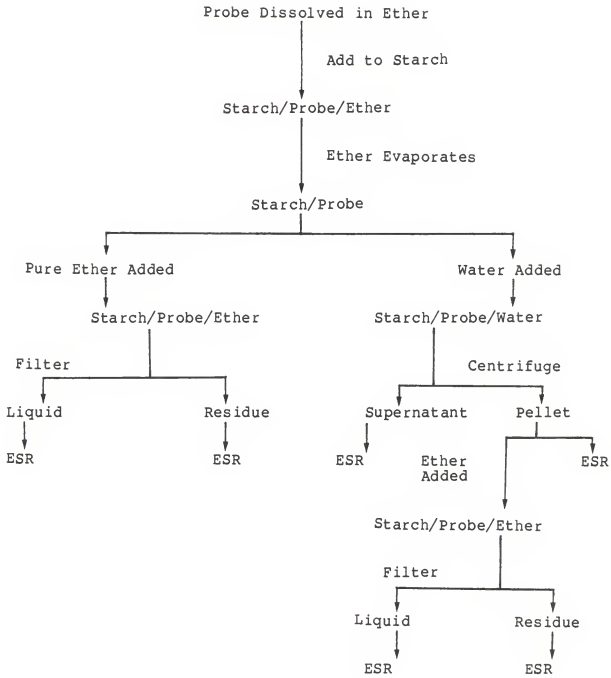

3475
10 Gauss

TEMPO-Laurate Binding Study

To test this latter possibility, a binding study was done (Figure 12). If a probe is, in fact, bound or complexed with amylose or amylopectin, it should not be extractable with a a suitable solvent. Probe that is bound will remain in the granule to give an ESR signal, albeit one that may be reduced in magnitude and/or symmetry. Unbound probe, by the same logic, should be lost in (and identified in) wash solution. TEMPO-laurate was dissolved in ether and added to wheat starch. The ether was allowed to evaporate, leaving starch and probe. The sample was extracted with ether, and both the resulting solution and residue were examined by ESR. The TEMPO-laurate signal was easily extracted from the starch by ether. No signal was found in the starch residue.

While that evidence suggested that the probe had not bound to the starch, a second experiment was conducted to determine if binding was caused by water. TEMPO-laurate was added to the starch in ether solution, and the ether allowed to evaporate. The starch/probe sample was then slurried with water for one hour at a water/starch/probe ratio of 1/2/.002. The sample was then centrifuged, and both the supernatant and pellet were examined by ESR. 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 were run on both the resulting solution and the residue. The radical signal was not extracted with the ether solution, but remained with the starch. Binding of the probe to starch occurred only after "wetting"

Figure 12. Flow Scheme for Starch/Probe Binding Study.


Probe Dissolved in Ether
Add to Starch
Starch/Probe/Ether
Ether Evaporates
Starch/Probe
Pure Ether Added
Water Added
Starch/Probe/Ether
Starch/Probe/Water
Filter
Centrifuge
Liquid
Residue
Supernatant
Pellet
ESR
ESR
ESR
Ether Added
ESR
Starch/Probe/Ether
Filter
Liquid
Residue
ESR
ESR

with water. This finding defines a critical difference between TEMPO and TEMPO-laurate in the water/starch/probe experiments. While TEMPO remained "in solution" before heating, and is an average of two types of water environments, TEMPO-laurate clearly is not. It binds to or interacts with the starch granule,and its correlation time is not affected by the overall amount of water ultimately in the system. The results of the washing experiment suggest that the binding occurs through interaction with water. Its presence enhances hydrophobic interactions among other molecules.

Gelatinization

Following gelatinization, few changes in ESR spectra occurred from the above samples. For both wheat starch and regular corn starch at the 1:1 and 2:1 ratios, spectral lines became so distorted that calculation of τ became unreliable (Fig. 14a, 14b, 15a, 15b). Baseline distortions became more pronounced and identification of individual triplets was difficult. This was additional evidence that TEMPO-laurate was interacting with the starch in a manner that TEMPO did not. The spectral distortions observed implied binding of the hydrophobic probe. Interestingly, signals from the 0.5:1 samples remained unaffected (Fig. 13).

Rotational correlation times that could be calculated from these spectra were all, once again, clustered between 2.10×10^8 sec $^{-1}$ and 7.06×10^8 sec $^{-1}$, a narrow range. In this case, with so many signals too distorted to permit calculation of τ, visual examination of spectra is the most effective comparative technique. The extensive distortions present after heating to

Figure 13. ESR Spectra of 0.5/1/ 2 x 10-5 M Water/Starch/ TEMPO-Laurate Samples after Gelatinization. (a) Wheat (b) Regular Corn (c) Waxy Corn

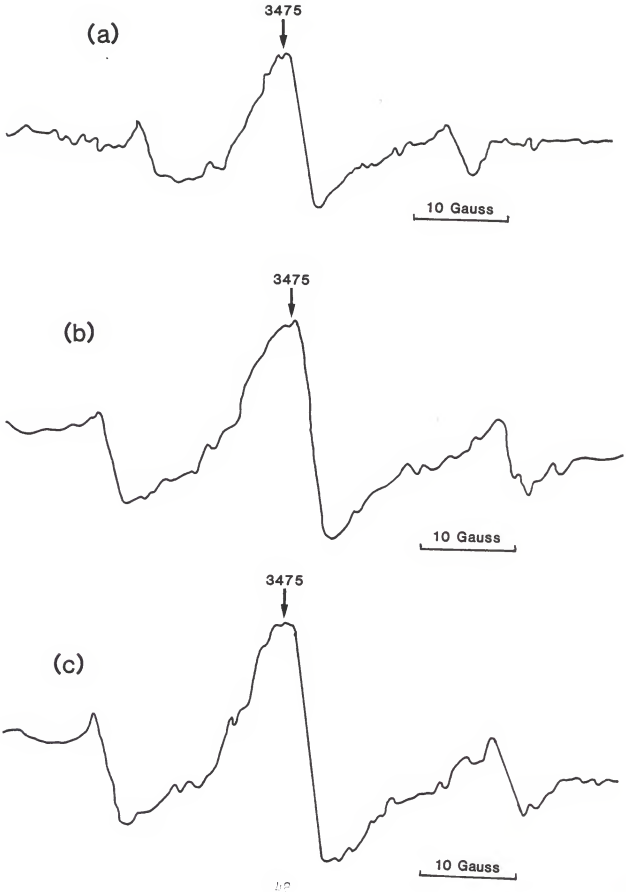

(a)
3475
10 Gauss
(b)
3475
10 Gauss
(c)
3475
10 Gauss

Figure 14. ESR Spectra of 1/1/ 2 x 10-5 M Water/Starch/ TEMPO-Laurate Samples after Gelatinization. (a) Wheat (b) Regular Corn (c) Waxy Corn

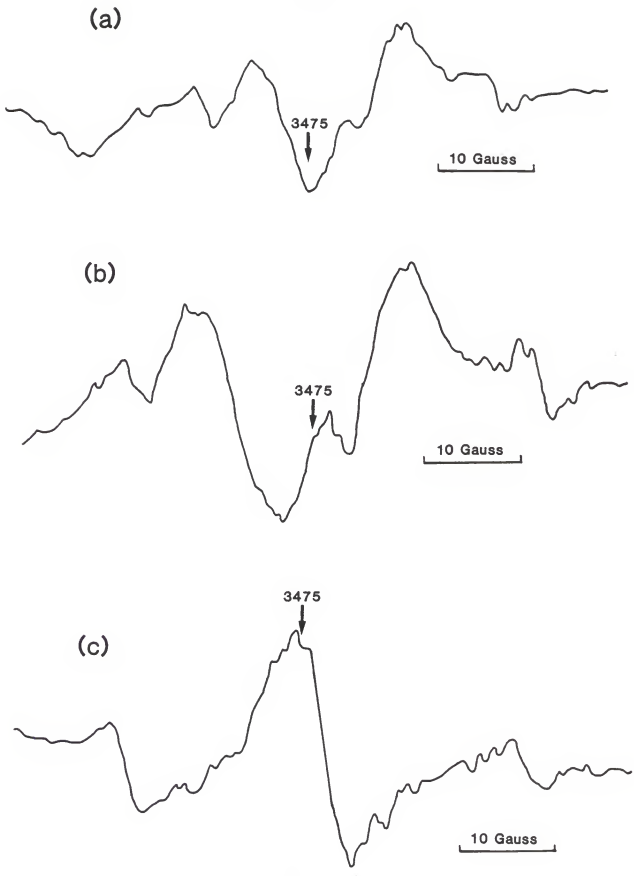
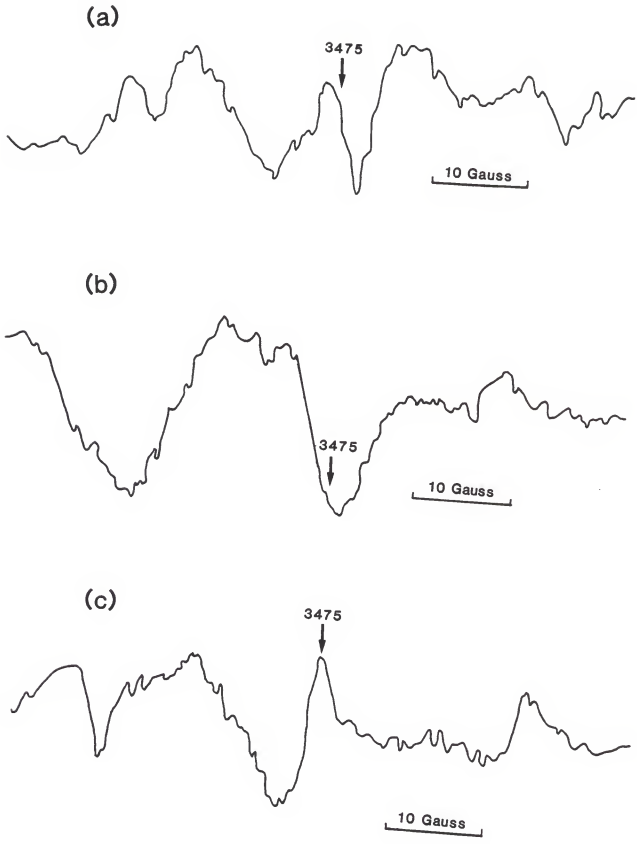

(a)
3475
10 Gauss
(b)
3475
10 Gauss
3475
(c)
10 Gauss

Figure 15. ESR Spectra of 2/1/ 2 x 10-5 M Water/Starch/ TEMPO-Laurate after Gelatinization.
(a) Wheat (b) Regular Corn (c) Waxy Corn


(a)
3475
10 Gauss
(b)
3475
10 Gauss
(c)
3475
10 Gauss

gelatinization temperatures suggest that gelatinization increases the interaction of probe with starch or somehow changes the environment of the bound TEMPO-laurate so as to further restrict its motion. It isn't unreasonable to suggest that this decreased mobility may reflect more complexation (ie more of it bound) of the probe by amylose or a perfection of the complexes formed prior to gelatinization.

16-DOXYL Stearic Acid and Starch

The objective in using 16- DOXYL stearic acid was to again examine possible starch-probe interactions. Specific questions involved differences in binding or interaction strength between this probe and the TEMPO derivatives. The stearic acid moeity should, at least in theory, facilitate starch-probe interaction. This was observed previously with TEMPO-laurate and starch in the presence of water (see page 47). The binding study outlined in Figure 12 was repeated using wheat starch, regular corn starch, and 16-DOXYL stearic acid.

Wheat starch and regular corn starch were both treated with 16-DOXYL stearic acid that was dissolved in ether. The ether was allowed to evaporate. The sample was dried and extracted with ether. The 16-DOXYL stearic acid signal was easily washed off the starch and found in the ether solution. No signal was observed in the starch residue.

A second washing procedure was used to determine if the probe would bind to starch as a result of treatment with water. Wheat and regular corn starch were treated with 16-DOXYL stearic acid to achieve a starch/water/spin label ratio of 1/2/.002.

Samples were slurried with the aqueous probe for one hour and then centrifuged. Both the supernatant and pellet fractions were examined with the ESR. Both starches gave similar results: a weak ESR signal in both the supernatant and pellet. This suggested that part of the label was bound to the starch. After three washings, only the pellet retained an ESR signal. The dried starch was then extracted with ether. The ESR radical signal was found only in the starch residue. We therefore concluded that 16-DOXYL stearic acid bound to starch only after "wetting" with water.

Waxy Corn Starch

Throughout the binding studies with both TEMPO-laurate and 16-DOXYL stearic acid, waxy corn starch gave unusual behavior. Waxy corn starch was treated with the probes to achieve the same starch/water/spin label ratio of 1/2/.002 that was used for wheat starch and regular corn starch. Waxy corn starch, upon standing with water, gave a gel that could not be separated by centrifugation.

In addition , the ESR spectra with waxy corn starch and TEMPO-laurate in water (Fig.13c, 14c, 15c) were not nearly as distorted as those of wheat starch and regular corn starch. Clearly, waxy corn starch interacts quite differently with the fatty acid labels than do wheat starch and regular corn starch.

Microscopy

To gain some insight regarding the interaction of waxy corn starch and 16-DOXYL stearic acid, the labelled starch pellet and supernatant were examined by bright field and polarizing microscopy. For each starch, samples were prepared as detailed

in the washing experiment.

Waxy and regular corn starch controls (ie without spin label) both behaved and appeared normal (Fig. 16a and 16c). Both sedimented essentially completely upon centrifugation, giving supernatant devoid of granules. Pellets contained granules that were both intact and retained their birefringence (Fig. 16b & 16d). Few, if any, granule fragments were observed in any preparation from control starch pellets. Thus, the process of centrifugation and re-suspension is not producing (inducing) granular damage. When regular corn starch was incubated with TEMPO-laurate, it behaved similarly to the control. Its microscopic appearance was identical to equivalent preparations from starch lacking the label.

When either 16-DOXYL stearic acid or TEMPO-laurate were added to waxy corn starch, granular appearance and structure was altered radically. The cloudy supernatant referred to above contained a great deal of granular remnants, fragments, and starch granules in various stages of disintegration (Fig. 17a). Examination of this material using polarizing optics showed that little, if any, of it retained sufficient native secondary structure to be birefringent (Fig. 17b). Examination of the last material to sediment, ie the top of the starch pellet, revealed similar, yet less severe, changes in the starch (Fig. 17c & 17d). Here, granular remnants were conspicuous, as were granules in the process of disintegrating. Here, however, ten to twenty percent of the granules as well as some of the larger fragments retained birefringence (Fig. 17d). Starch from the bottom third

Figure 16. Photographs of Control Starch Pellets (No Label).
(a) & (b) Regular Corn Starch.
(c) & (d) Waxy Corn Starch.

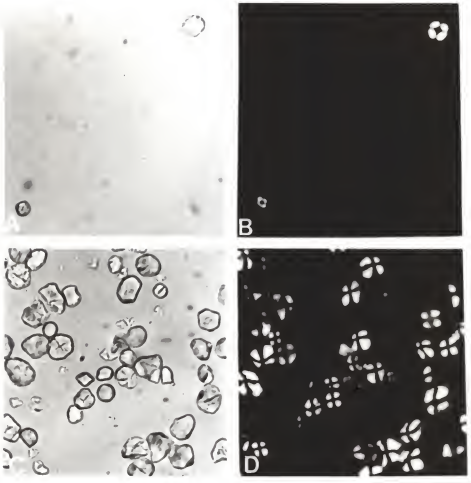
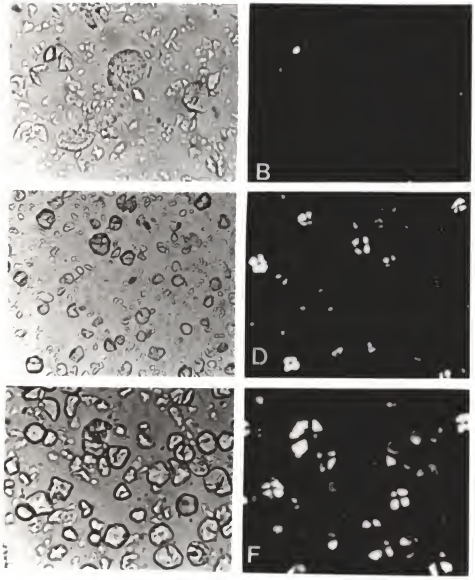

A
B
C
D

Figure 17. Photographs of Waxy Corn Starch treated with TEMPO-Laurate.
(a) & (b) Supernatant.
(c) & (d) Top of pellet.
(e) & (f) Bottom third of pellet.


B
D
F

of the pellet, the first to sediment, was also disrupted (Fig. 17e & 17f). When compared to the material above it in the pellet and supernatant, disruption was less severe, but still clearly present. It is important to note that the changes in granular structure were of the same type as that observed above, only less severe. Nonbirefringent granule remnants were present, along with large pieces of broken granules. Many of the larger remnants retained birefringence, as did the 40-50% of the granules that appeared to be unaffected by the presence of TEMPO-laurate.

The pattern of granular disruption was quite unexpected, particularly since the granules were not exposed to significant amounts of heat at any time during the experiment. In addition, the presence of fatty acid alone has not been reported to cause similar changes in granular structure. Still, changes observed (radial disintegration, loss of birefringence) are nearly identical to those observed as waxy corn starch gelatinizes. Those granules most affected sedimented poorly or not at all, remaining in the supernatant, as would be expected for damaged starch. Those least affected or not visually affected pelleted readily (see Fig. 17e & 17f).

The presence of this fatty acid spin label apparently destabilizes waxy corn starch to such a degree that gelatinization occurs at room temperature. This phenomemon is not without precedent, as DMSO will cause granule disintegration at room temperature. The TEMPO-laurate affect is apparently more specific, as it occurs only with waxy starch.

It remained possible that although spin label-starch

interaction took place, the granule disintegration or disruption occurred due to the stress encountered during centrifugation. To test this, waxy corn starch plus TEMPO-laurate were allowed to stand without centrifugation. Samples from the re-suspended starch (Fig. 18a & 18b) clearly show that granule disintegration was taking place without prior centrifugation.

Wheat starch, which pelleted cleanly in studies designed to take ESR spectra (see above), did not change in its microscopic appearance due to interaction with TEMPO-laurate (Fig. 19a & 19b). Granules were not swollen or fragmented, and they all retained their birefringence. This does not rule out the possibility that some sort of destabilizing interaction may have, in fact, taken place here. For this starch, the changes may not be manifested at room temperature.

The inclusion of TEMPO with a different adduct, benzoate (TEMPO-benzoate), also resulted in distruption of waxy starch granules. In this instance, the granule disintegration and loss of birefringence was much less severe than that encountered with TEMPO-laurate (Fig. 20a & 20b). The same types of changes were, however, still evident. Figure 20c, for example, contains granular remnants, granules in the process of coming apart radially (arrow), and intact granules. It appears that the probe-starch interaction that results in destabilization is not due only to the fatty acid substituent on 16-DOXYL stearic acid and TEMPO-laurate, but is mediated by both spin label and adduct. The severity, or perhaps more accurately, the extent of the destabilization does appear to be specific to the adduct

Figure 18. Photograph of Waxy Corn Starch with TEMPO-Laurate with no Centrifugation. Sample from starch kept in Suspension.

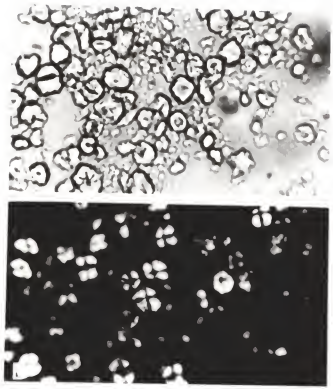


Figure 19. Photograph of Wheat Starch with TEMPO-Laurate.

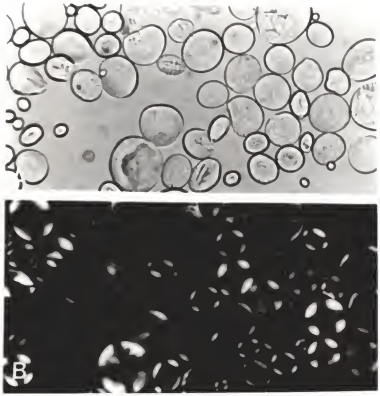
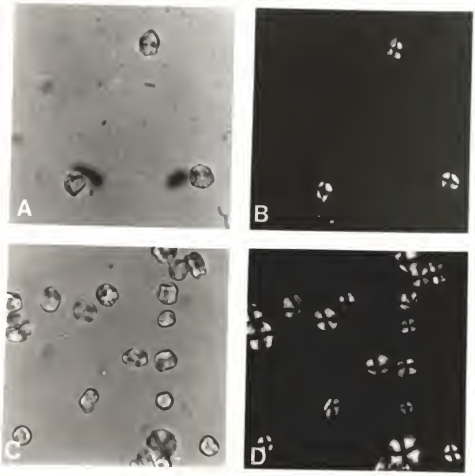

B

Figure 20. Photographs of Waxy Corn Starch and TEMPO-Benzoate.
(a) & (b) Supernatant.
(c) & (d) Pellet.


A
B
C
D

esterified to the spin label itself.

The implications of these results are significant. Waxy starch is almost exclusively amylopectin, and under normal conditions, does not gelatinize without exposure to higher temperatures (Osman, 1959). Only in the presence of such destabilizing compounds as DMSO have wheat starch and regular corn starch been reported to lose birefringence without heating (D'Appolonia, 1959). Clearly, both 16-DOXYL stearic acid and TEMPO-laurate exert the same type of effect on waxy starch as DMSO. Both probes are long chain fatty acid derivatives which interact in some way with the starch to destabilize its helices. This interaction with the host is a clear violation of a spin label's requirement to remain unreactive in a system. Because the radical's signal is preserved, ESR analysis is still possible. Yet the probes clearly generate changes in their environments that are not well understood and are difficult to monitor. More study of this phenomena is warranted before the use of 16-DOXYL stearic acid and TEMPO laurate in starch systems will be clearly understood.

Amylose/Amylopectin Binding Study

The experiments completed so far presented impressive, yet inconclusive evidence that fatty acid spin labels bind to starch. As a final test, amylose and amylopectin from potato were individually dissolved into solution with 2 x 10-5 M TEMPO laurate and examined with the ESR. Amylopectin was dissolved in water, while amylose was dissolved in 1M KOH and neutralized to pH 7 with 1M HCl. Starch/water/probe ratio was kept constant at 0.1/2/.002, while dissolution of starch was ensured by sonication.

The ESR spectrum of 2 x 10 -5 M TEMPO-laurate dissolved in water in shown in Fig. 21a. It clearly is a symmetrical triplet signal exhibiting rapid isotropic motion (τ = 4.87 x 1010 sec-1). When amylopectin was added, however, the spectrum changed (Fig. 21b). All three lines exhibited broadening, and the high field and low field lines diminished greatly in size. Tau decreased to 5.03 x 109 sec-1.

Similar changes result for amylose, as shown in Fig. 22. An ESR signal of TEMPO-laurate in a neutral solution has a τ of 3.21 x 1010 sec -1 and an unbound symmetrical signal. When a small amount of amylose is added, the spectrum becomes distorted, and τ decreases to 6.18 x 109 sec-1.

Similar visual changes were observed previously during the starch gelatinization study with TEMPO. In those cases, the changes did not represent probe binding, but a change in the system's overall viscosity. In the present study, viscosity is clearly not the cause. Amylopectin and amylose were added to the solutions in such trace amounts (0.1g/3 ml water) that their effects upon viscosity are negligible. As shown by the starch/water/TEMPO-laurate data, TEMPO-laurate is insensitive to the total amount of water in the system. It binds to both components of starch and consequently endures a restriction in molecular motion. Its binding also catalyzes gelatinization of starch in the absence of heat by an unexplained mechanism.

DOXYL stearic acid Probes with Gluten

The 5, 12, and 16-DOXYL stearic acid probes used by Nishiyama et al (1981) to study lipid-protein interactions in

Figure 21. ESR Spectra of TEMPO-Laurate.
(a) Dissolved in Water.
(b) Dissolved in Water with Amylopectin.

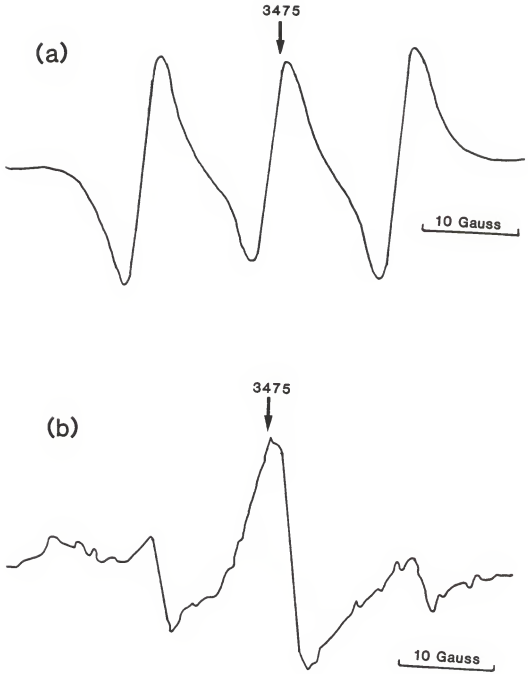
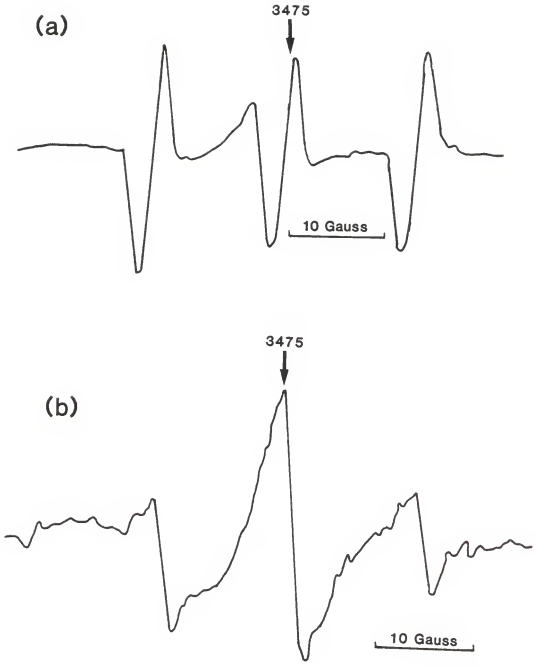

3475
(a)
10 Gauss
3475
(b)
10 Gauss

Figure 22. ESR Spectra of TEMPO-Laurate.
(a) Dissolved in a neutralized solution.
(b) Dissolved in a neutralized solution with Amylose.


(a)
3475
10 Gauss
(b)
3475
10 Gauss

gluten were used to study gluten binding. Nishiyama's results suggested that these spin labels are selectively, if not specifically, incorporated into the lipid environment of the gluten. By monitoring changes in the correlation time of each spin label in gluten, the authors could characterize the environment at the depth of 5, 12, and 16 carbon atoms of the stearic acid chain, respectively. Their conclusions provided information on the physical state of the lipid in gluten, including the presence of a lipid-protein complex in hydrated gluten. This complex is believed to be maintained by hydrogen bonding, hydrophobic interactions, and van der Waals forces.

A logical first step in gaining confidence with DOXYL stearic acid was to reproduce this work. Samples of regular gluten and defatted gluten were incubated individually with 16-DOXYL stearic acid dissolved in ether. After removing the ether by evaporation, spectra were obtained on the ESR. Correlation times for the label in gluten from both regular and defatted flour fell between 1.7×10^{-9} seconds and 2.6×10^{-9} seconds, which compared favorably with the previously reported values of 0 to 2×10^{-9} seconds.

Dough Mixing Times

The consistency changes that occur during the mixing of dough are easily observed visually and by touch. Viscosity changes accompany gluten development, as water is redistributed during hydration of various flour components (Farrand, 1943). This redistribution of water was examined by introduction TEMPO into flour/water doughs and monitoring the probe's ESR spectrum

over a range of dough mixing times.

A mixograph curve (Fig. 23) revealed that the optimum mixing time was four minutes at an absorption of 63%. Samples were mixed for two minutes (undermixed), four minutes (optimum mix), and eight minutes (overmixed). ESR spectra were taken for each dough. Table V shows that, regardless of the mixing time, the rotational correlation time did not change. For each sample, τ was between 2.16 x 108 sec-1 and 2.47 x 108 sec-1.

At first glance, this seems quite puzzling. An observer can easily discriminate viscosity and textural changes occurring over the range of mixing times used, yet τ values seemed to be insensitive to these macroscopic changes. One reasonable explanation is that TEMPO is not the an appropriate spin label for the flour-water dough. As demonstrated by the starch experiment (see page35), TEMPO does not bind to the sample appreciably. It remains "in solution" and the resulting correlation time varies only according to the water content of the system. The same phenomenon appears to be active in doughs. The aqueous environment remains the same over the range of mixing times. Since TEMPO itself is in solution, its aqueous environment is constant. Consequently, it does not and, for this system, cannot distinguish between free and bound water. The visual and tactile changes seen with mixing times are changes in the distribution of the water, to which TEMPO is not sensitive. τ simply does not vary in solution.

To successfully accomplish a study examining changes due to mixing time, a probe must be chosen which will bind to or

Figure 23. MixographCurve for Flour/Water Dough(63% Absorption) containing 10-5 M TEMPO.

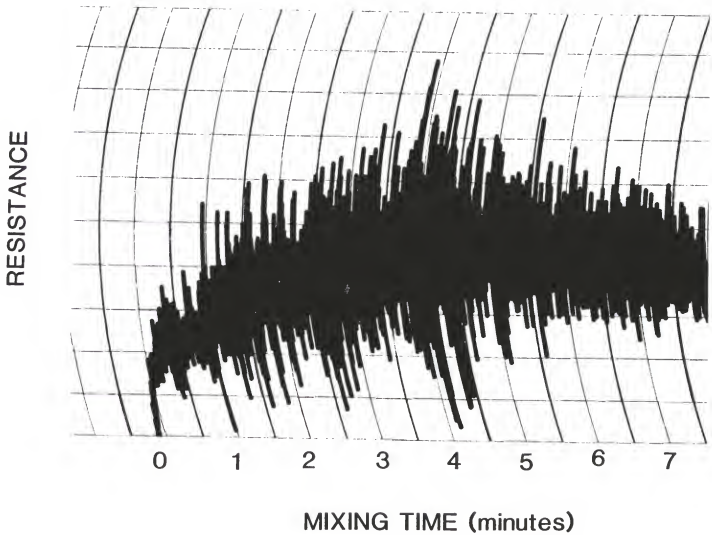

RESISTANCE
0
1
2
3
4
5
6
7
MIXING TIME (minutes)

Table V. Rotational Correlation Times for Undermixed, Optimum Mix, and Overmixed Flour/Water Doughs with TEMPO.

Mixing Time		Correlation Time (sec-1)a
Undermixed	1	2.34×10^8
	2	2.38 x 108
	3	2.24×10^8
Optimum	1	2.16 x 108
	2	2.34×10^8
	3	2.23 x 108
Overmixed	1	2.29×10^8
	2	2.47 x 108
	3	2.38×10^8

[a] Standard deviation for undermixed doughs was 7.2 x 106, for optimum mix 9.07 x 106, and for overmixed 9.0 x 106.

interact with a specific flour component or class of components. Then, as the environment of the dough, as viewed by the attached label, changes, these changes will be reflected by τ . More work needs to be done in this area.

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SUMMARY

Wheat, corn, and waxy corn starches were slurried at three water/starch ratios (0.5:1/ 1:1, 2:1), with a hydrophobic probe, TEMPO-laurate, and a hydrophilic probe, TEMPO. The two higher water systems were discontinuous by nature, as water existed both inside and outside the starch granules. When placed in pure water, TEMPO's molecular motion (1011 sec-1) is not restricted and it gave symmetrical ESR signals with high correlation times. Samples containing TEMPO at high water ratios gave ESR signals that were symmetrical and that had high correlation times. For 1:1 samples, tau was in the 109 sec^{-1} range, while for 2:1 samples it increased to 1010 sec-1. As the amount of available water was decreased, the signals became distorted and correlation times were decreased. This was attributed to TEMPO's distribution in the water both inside and outside of the starch granules. For the intermediate 1:1 and 2:1 samples, the ESR signal is an average of that for the probe inside and outside the starch granules. After gelatinization, the samples became more homogeneous, and the probe's signal could be used to measure the system's overall viscosity.

ESR spectra taken with TEMPO-laurate and the three starches at the same three dilutions gave distorted lineshapes and correlation times clustered in the same range (108 sec-1). This differed greatly from the results obtained using TEMPO, and suggested probe binding. TEMPO-laurate's molecular motion was clearly not dependent on the amount of water in the system.

To verify that this phenomena was not a result of TEMPO-

laurate's insolubility in water, a binding study was conducted. TEMPO-laurate and 16-DOXYL stearic acid were readily washed from starch with ether, but were shown to become bound to the starch when mixed with water.

Addition of amylose and amylopectin to TEMPO-laurate in water resulted in distorted spectra with slower values for tau. This suggests that the probe is bound to both polymers.

Microscopy studies of waxy corn starch with TEMPO laurate, 16-DOXYL stearic acid, and TEMPO benzoate showed that all three probes caused gelatinization of the starch at room temperature. Broken granules and loss of birefringence was most prevalent in waxy starch that had not sedimented well during centrifugation. Regular corn and wheat starch were less affected by the probes.

AN ELECTRON SPIN RESONANCE STUDY OF NATIVE STARCH SYSTEMS

by

NANCY L. NOLAN

B.A. Rhode Island College 1983

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the
requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

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ABSTRACT

Wheat, corn, and waxy corn starches were slurried at three water/starch ratios (0.5:1, 1:1, 2:1), with a hydrophobic probe, TEMPO-laurate, and a hydrophilic probe, TEMPO. The two higher water systems were discontinuous by nature, as water existed both inside and outside the starch granules. When placed in pure water, TEMPO's molecular motion (10^{11} sec^{-1}) is not restricted and it gave symmetrical ESR signals with high correlation times. Samples containing TEMPO at high water ratios gave ESR signals that were symmetrical and that had high correlation times. As the amount of available water was decreased, the signals became distorted and correlation times decreased. This was attributed to TEMPO's distribution in the water both inside and outside of the starch granules. For the intermediate 1:1 and 2:1 samples, the ESR signal is an average of that for the probe inside and outside the starch granules. After gelatinization, the samples became more homogeneous, and the probe's signal can be used to measure the system's overall viscosity.

ESR spectra taken with TEMPO-laurate and the three starches at the same three dilutions gave distorted lineshapes and correlation times clustered in the same range (10^{8} sec^{-1}). This differed greatly from the results obtained using TEMPO, and suggested probe binding. TEMPO-laurate's molecular motion was clearly not dependent on the amount of water in the system.

To verify that this phenomena was not a result of TEMPO-laurate's insolubility in water, a binding study was conducted. TEMPO-laurate and 16-DOXYL stearic acid were readily washed from

starch with ether, but were shown to become bound to the starch when mixed with water.

Addition of amylose and amylopectin to TEMPO-laurate in water resulted in distorted spectra with slower values for tau. This suggests that the probe is bound to both polymers.

Microscopy studies of waxy corn starch in solution with TEMPO-laurate, 16-DOXYL stearic acid, and TEMPO-benzoate showed that all three probes caused gelatinization of the starch at room temperature. Regular corn and wheat starch were less affected by the probes.