

Paramagnetic Ion Spin-Spin Coupling as Direct Evidence for Cooperative Ion Binding to Higher Plant Cell Walls

Peter L. Irwin, Michael D. Sevilla¹, James J. Shieh, and Carla L. Stoudt

Agricultural Research Service, Eastern Regional Research Center, U.S. Department of
Agriculture, Philadelphia, PA 19118

Electron spin resonance {ESR} spin-spin coupling experiments were performed to estimate Mn^{2+} and Cu^{2+} near neighbor distances, thereby determining if carboxylate-divalent cation complexes potentiate ion association at adjacent sites on cell wall polyuronides. Distances were estimated to be 12 and 14Å for Cu^{2+} and Mn^{2+} , respectively. At the maximal bound ion concentration, the lattice constant { κ } was ca. 2.5 indicating that approximately 6 paramagnetic ion near neighbor spin-spin interactions occur per dipole in the nearly-filled lattice. Competitive ion exchange with Ca^{2+} was found to reduce the Mn^{2+} spin-spin line broadening at similar total bound [Mn^{2+}]; this could only be observed if Ca^{2+} competes with Mn^{2+} at adjacent sites. These data offer strong support for the sequential-cooperative ion binding mechanism.

Polyuronides are a major component of the primary cell wall and middle lamella of higher plant cortical tissues (1, 2). These polymers are efficient cation exchangers (3, 4) and, thus, can affect ion activity, transmembrane potential and electrolyte flux (5). In addition, the divalent salts of pectic substances

¹Current address: Department of Chemistry, Oakland University, Rochester, MI 48063.

appear to play some structural role in adjacent cell wall-to-wall adhesion (6-9) as well as exert steric control over the activity of certain hydrolytic enzymes (7, 8).

Solution studies on the binding behavior of polygalacturonate and polyguluronate (10-16) indicate that a molecular size dependent cooperative ion binding takes place (12, 13). Some authors (11, 15) have proposed that these acidic polymers bind divalent cations in electronegative cavities between chains like "eggs in an egg box". Little information has been available with regard to the ion binding mechanism or the polyuronide-cation aggregate structure in solid matrices (3-5, 17).

In this paper we will present direct physical evidence (18, 19) which supports the concept that carboxylate group-divalent cation complexes potentiate ion association at adjacent binding sites (the sequential binding mechanism, 18). We will also present evidence that the cation-polyuronide aggregate structure is similar to the model proposed for polyuronides in solution (11), that polymer blocks exist in three dimensional lattices low in methyl ester content and that subtle conformational changes can be observed with this ESR technique (19). This method could prove useful in the elucidation of the structural mechanisms of certain developmental changes in plant tissues (e.g., ripening, abscission, extensive growth, etc.; 6-9, 19).

Materials and Methods

Intact cortical tissues of Malus pumila {cv. Golden Delicious} fruit were used throughout these experiments. Tissue fixation and determination of free {e.g., nonmethylated} uronic acid concentrations were described previously (18).

For the ESR experiments, intact tissues {1x1x4mm} were equilibrated at pH 5 in water for 15 min. and decanted; this procedure was repeated thrice. About 10-40 mg dry weight of cell wall complex was used for each treatment combination. Samples were equilibrated {22±2°} in 10 ml of various concentrations of Mn^{2+} (18, 19), Mn^{2+} with Ca^{2+} (18) or Cu^{2+}

(19). After ca. 24 hrs., the solutions were decanted and tissue specimens washed thrice in {pH 7} water, allowing 15 min. equilibration in each wash. After an additional 1 hr. soak, this washing procedure was repeated. Samples were dehydrated with ethanol and critical point dried as described previously (18, 20). Some samples were rehydrated in a saturated water vapor chamber (19) at 160 torr and 22°C. All samples were immediately loaded and sealed in 3x120 mm quartz ESR tubes. After each experiment, the samples were removed, washed in methanol, vacuum dried at 35°C, weighed, and dry-ashed for atomic absorption spectrophotometric analysis of Mn^{2+} , Ca^{2+} and/or Cu^{2+} using standard procedures (18, 19).

Mn^{2+} and Cu^{2+} ESR spectral parameters, Mn^{2+} linewidths $\{\Delta H_{[dI(H)/dH]_{max}}\}$ and dimer-only nearest neighbor distance parameter $\{d\}$ calculations were as previously described (18, 19). All experiments in this report were run at near liquid N_2 temperatures $\{-176$ to $-180^\circ C\}$ to avoid certain relaxation time phenomena which can be problematic (19). Cu^{2+} linewidths were calculated directly from the first derivative g_{\perp} component (19) as the difference in field strength $\{G\}$ between the maximum and minimum amplitude. This empirical measure of linewidth was found to be directly proportional to the true linewidth as determined by an anisotropic simulation routine (21); this simulation showed that the true linewidth $\{\Delta H_{[dI(H)/dH]_{max}}$, Figure 3} was equal to the empirical measure, described above, divided by 1.17 (19).

Results and Discussion

The results in Figure 1 demonstrate that cell wall bound Mn^{2+} is associated with an acid titratable site; similar results have been obtained with Ca^{2+} . Polyuronides are the most likely ion binding species since they are the predominant anionic component (2, 20) in these tissues.

From the standpoint of the ESR experiment, ionically bound paramagnetic ions can be treated as fixed points which interact

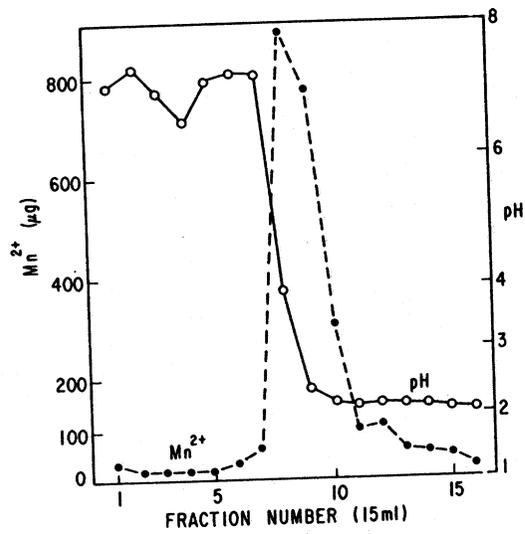


Figure 1. Titration of Mn^{2+} off intact cell wall matrices under mildly acidic conditions. Reproduced with permission from Ref. 18. Copyright 1984, Biochimica et Biophysica Acta.

only through their couplings with each other (22). Thus, any ith spin in the midst of other paramagnetic transition ions bound within a solid matrix, such as these cell wall matrices, has a total spin interaction profile equal to the sum of the spin-spin and exchange interactions (22). In this summation, the series converges to a value which depends only on those dipoles in the vicinity of each ith spin and, thus, is the same for all the dipoles of the same type in the lattice. Of these terms, spin-spin interactions cause line broadening (23) and are proportional to the inverse cube of the distance between interacting spins ($1/r_{ij}^3$; 22-26) as described by the square root of the second moment ($\langle \Delta v^2 \rangle^{1/2}$; 23, 26, 27) of a Gaussian line:

$$\langle \Delta v^2 \rangle^{1/2} = \{3/5 \cdot g^4 \beta^4 h^{-2} \cdot S[S+1] \cdot \sum_i 1/r_{ij}^6\}^{1/2}; \text{ (1; units in Hz)}$$

whereupon g , β and h have their usual values and S represents the total electron spin of the ion ($S=5/2$ for Mn^{2+} and $1/2$ for Cu^{2+}). In nonmagnetic insulators (28), linewidths and -shapes agree with basic Van Vleck theory (23). However, as paramagnetic ions approach one another ($r < 5-10 \text{ \AA}$; 25) the linewidths are affected by exchange coupling (29). While $\langle \Delta v^2 \rangle$'s are independent of the exchange term ($2J_{ij} \vec{S}_i \cdot \vec{S}_j = 2J_{ij} S_i S_j \cos \theta$, where J_{ij} is the "exchange integral" and θ is the angle formed between the ith-jth spins and the magnetic field; 22, 30) the lineshapes no longer remain Gaussian, become mixed (27) and line narrowing results (23, 29, 31, 32). Figures 2 and 3 illustrate that, relative to the spin-spin interactions, we have little exchange since the linewidths of the bound dipoles only broaden as the concentration increases.

We (18) have simplified the $\langle \Delta v^2 \rangle$ relationship by making use of the fact that, for a Gaussian line, the linewidth is $2\langle \Delta v^2 \rangle^{1/2}$. In this relationship

$$\Delta \Delta H_{[dI(H)/dH]_{\max}} = 28690 \cdot GA^3 \cdot \{S[S+1]\}^{1/2} \cdot \kappa/d^3; \text{ (2; units in G)}$$

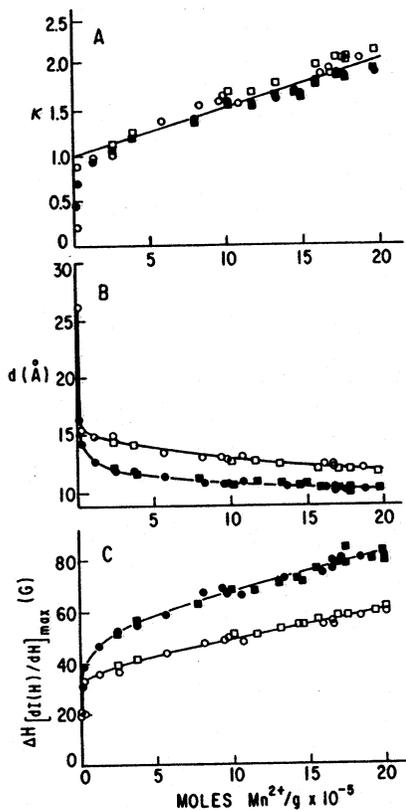


Figure 2. Cell wall bound Mn^{2+} concentration dependency of linewidth $\{\Delta H [dI(H)/dH]_{max}\}$, dilute limit nearest neighbor distance parameters $\{d \text{ assuming } \kappa=1\}$ and lattice constants $\{\kappa\}$. Open symbols represent hydrated samples, closed symbols represent dry samples. Squares and circles represent the results of two independent experiments. The d values used to calculate κ were 13.72 and 16.3 Å for dry and hydrated tissues, respectively. Reproduced with permission from Ref. 19. Copyright 1985, Biochimica et Biophysica Acta.

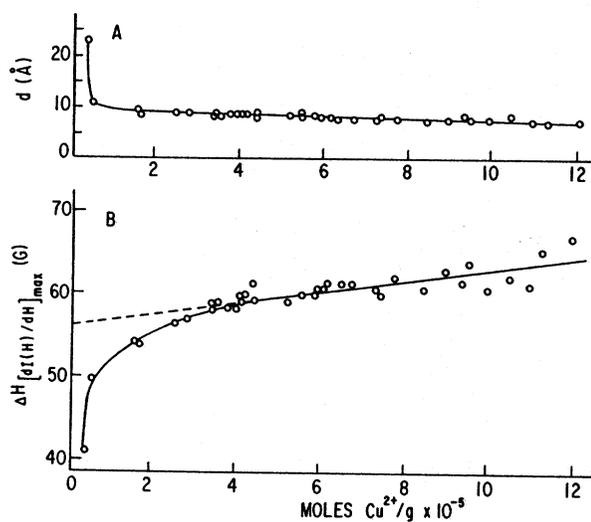


Figure 3. Cu^{2+} linewidth $\{\Delta H_{[dI(H)/dH]_{max}}\}$ and dilute limit nearest neighbor distance parameters' dependency $\{d \text{ assuming } \kappa = 1\}$ on cell wall bound Cu^{2+} at -180°C . All samples were dehydrated. The dilute limit $\Delta H_{[dI(H)/dH]_{max}}$ intercept $\{\text{dotted line}\}$ is ca. 56 G. Reproduced with permission from Ref. 19. Copyright 1985, Biochimica et Biophysica Acta.

$\Delta\Delta H_{[dI(H)/dH]_{\max}}$ is the incremental change in linewidth relative to a dilute glassy matrix containing Mn^{2+} or Cu^{2+} ; d is the nearest neighbor distance parameter. The lattice constant $\{\kappa\}$ depends upon the arrangement of the ions in the lattice and is unity for a 2-spin system, 1.42 for a linear array of spins and about 2.85 for a filled cubic lattice (18). We can closely approximate the dimer-only d values $\{\text{when } \kappa=1\}$ from the extrapolated zero concentration $-\Delta H_{[dI(H)/dH]_{\max}}$ intercept for Mn^{2+} {Figure 2} and Cu^{2+} {Figure 3}; this calculation provides the most reliable results when utilizing Van Vleck theory (23). Using this approach, the dimer-only d values were calculated {equation 2} to be ca. 12 and 14Å {dehydrated samples} for Cu^{2+} and Mn^{2+} , respectively. This small difference in d could be due to the fact that Cu^{2+} , unlike other transition ions such as Mn^{2+} , loses a portion of its shell of hydration upon binding (33, 34) which causes these ions to have an almost covalent character probably resulting from a more compact lattice structure (19). These distances (12x the intrachain carboxylate spacing; 15, 35) are observed at very low bound ion concentrations and argue for a special form of cooperative ion binding which we have referred to as sequential (18). This mechanism is also suggested by the relatively small change in d , after the initial rapid increase, as the lattice fills with Mn^{2+} or Cu^{2+} . The relatively small increase in d as the binding sites gradually fill is likely due to a change in the number of near neighbors ($\sim \kappa^2$; 19, 26). Figure 2A shows calculated κ values assuming a constant d . If the sequential ion binding model is true, κ should approach zero only at low concentrations {e.g., ca. 10^{-5} moles/g} where a significant portion of the i th dipoles experience no interactions with neighboring spins. Figure 2A clearly demonstrates this feature. From the κ - $[Mn^{2+}$ or $Cu^{2+}]_{\text{bound}}$ relationship we calculate that the κ_{\max} value is ca. 2.5. We can utilize κ_{\max}^2 to closely approximate the number of nearest neighbors even for samples having an extended array because of the decreased weighting of distant interactions. The

values for κ_{\max}^2 indicate that the average number of near spin-spin interactions per *i*th spin is ca. 6 and is evidence for an egg box-like divalent cation-polyuronide aggregate structure (10, 11, 16, 36, 37) of the nearly-filled lattice. The large κ_{\max}^2 also argues for the existence of pockets or regions of nonmethylated polymer existing contiguously in the cell wall/middle lamella matrix (19) since κ_{\max}^2 is larger than expected for a highly methylated pectic polysaccharide matrix (20). Upon equilibrium hydration {Figure 2B and C, open symbols} the dimer-only distance parameter increases about 2Å {10G decrease in $\Delta H[dI(H)/dH]_{\max}$ as $[Mn^{2+}]_{\text{bound}}$ approaches 0}. This change is not likely to be due to the hydration of the transition ions alone because bound divalent cations, such as Mn^{2+} , have a rather significant aquoshell even in the dehydrated state (19). This observation, in conjunction with other data (19), indicate that the higher order structure of cell wall polyuronides changes as a function of temperature, degree of hydration and polymer size.

Lastly, if the sequential ion binding hypothesis is true, competitive exchange with a nonparamagnetic ion should reverse the broadening effect. We find that the Mn^{2+} spin-spin line broadening effect is largely lost when cell wall material is exchanged with both Mn^{2+} and Ca^{2+} {Figures 4 and 5} while keeping the total bound Mn^{2+} constant. For example, both first derivative spectra in Figure 4 are obtained from wall material having equivalent levels of bound Mn^{2+} {ca. 4×10^{-5} moles/g}; however, spectrum B is qualitatively similar to those obtained from dilute Mn^{2+} glycerol/water solutions at $-176^\circ C$. The small hyperfine lines seen in the least broadened spectrum {B} are due to the effect of the crystal field (38) on the Zeeman levels. Spectra with qualitatively identical crystal field splittings were obtained from dilute Mn^{2+} glasses at $-176^\circ C$ and are evidence that the crystal field contribution to lineshape is effectively the same in both cases. We have also found that the Mn^{2+} line broadening, which results from Mn^{2+} near neighbor

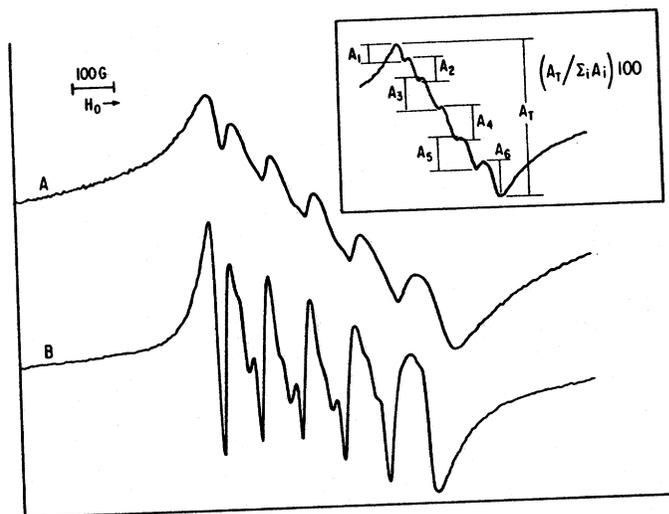


Figure 4. First derivative ESR spectra of cell wall absorbed Mn^{2+} as a function of bound Ca^{2+} . The sample represented in spectrum A has ca. 4×10^{-5} moles Mn^{2+}/g dry weight $\{X_{Mn^{2+}} = 1\}$; the sample represented in B has an equivalent level of paramagnetic ion but $X_{Ca^{2+}}=0.7$. Inset spectrum: Illustration of the empirical line broadening factor, $LBF = \{A_T / \sum_i A_i\} 100$. Reproduced with permission from Ref. 18. Copyright 1984, Biochimica et Biophysica Acta.

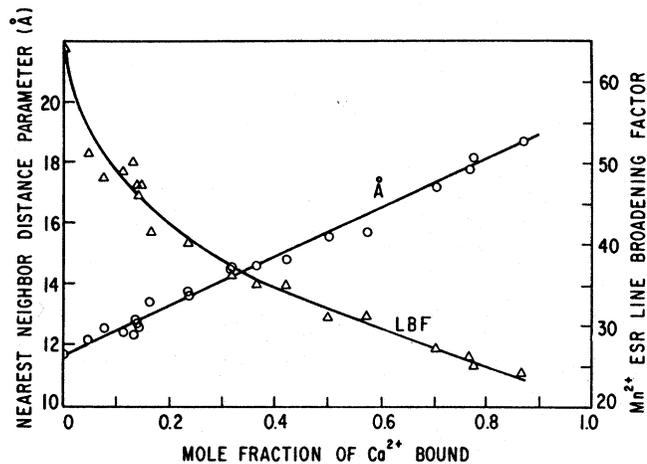


Figure 5. Changes in linewidth as measured by the line broadening factor {LBF, triangles} and calculated paramagnetic ion nearest neighbor distance parameters {d, circles} as a function of $X_{Ca^{2+}}$. The fraction of sites filled by Mn^{2+} are approximately the same ($22.4 \pm 1.5\%$) for each sample though the LBF values vary between 64 and 30; thus, the change in line broadening can only be associated with a competitive binding effect. Reproduced with permission from Ref. 18. Copyright 1984, *Biochimica et Biophysica Acta*.

6. Hall, M. In "Quality in Stored and Processed Vegetables and Fruits"; Academic: London, 1981; pp. 53-64.
7. Demarty, M.; Morvan, C.; Theiller, M. Plant Cell Environ. 1984, 7, 441-8.
8. Ferguson, I. Plant Cell Environ. 1984, 7, 477-89.
9. Tefper, M.; Taylor, J. Can J. Bot. 1981, 59, 1522-5.
10. Gidley, M.; Morris, E.; Murray, E.; Powell, D.; Rees, D. J. Chem. Soc. Chem. Commun. 1979, 990-2.
11. Grant, G.; Morris, E.; Rees, D.; Smith, P.; Thom, D. FEBS Lett. 1973, 32, 195-8.
12. Kohn, R.; Larsen, B. Acta Chem. Scand. 1972, 26, 2455-68.
13. Kohn, R. Pure Appl. Chem. 1975, 42, 37197.
14. Morris, E., Rees, D.; Thom, D., Boyd, J. Carbohydr. Res. 1978, 66, 145-54.
15. Rees, D.; Morris, E.; Thom, D.; Madden, J. In "The Polysaccharides"; Academic: New York, 1982; pp. 195290.
16. Thom, D.; Grant, G.; Morris, E.; Rees, D. Carbohydr. Res. 1983, 100, 2942.
17. Van Cutsem, P.; Gillet, C. Plant Soil 1981, 62, 36775.
18. Irwin, P.; Sevilla, M.; Shieh, J. Biochim Biophys. Acta 1984, 805, 186-90.
19. Irwin, P.; Sevilla, M.; Stoudt, C. Biochim. Biophys. Acta 1985, 842, 7b-83.
20. Irwin, P.; Pfeffer, P.; Gerasimowicz, W.; Pressey, R.; Sams, C. Phytochem 1984, 23, 2239-42.
21. Lefebvre, R., Maruani, J. J. Chem. Phys. 1965, 42, 1480-96.
22. Pryce, M.; Stevens, K. Proc. Phys. Soc. London 1950, A63, 3651.
23. Shinar, J.; Jaccarino, V. Phys. Lett. 1938, 91A, 132-4.
24. Damoder, R.; More, K.; Eaton, G.; Eaton, S. Inorg. Chem. 1984, 23, 1320-6.
25. Eaton, S.; More, K.; Sawant, B.; Eaton, G. J. Am. Chem. Soc. 1983, 105, 6560-6.

spin-spin interactions, is reduced greatly as the mole fraction of $[Ca^{2+}]_{bound}$ $\{X_{Ca^{2+}}$, Figure 5} increases at the same total bound $[Mn^{2+}]$. The linear relationship observed between d and $X_{Ca^{2+}}$ is expected if a lattice consists of a uniform sequential array of bound ions (39). These data also indicate that the binding of Ca^{2+} to cell wall polyuronides is similar to Mn^{2+} .

Conclusions

From these studies (18, 19) we conclude that certain divalent cations bind to the cell wall polyuronides in a spatially sequential fashion. This form of association can be thought of as a special case of cooperative whereby the binding of some initial cation lowers the potential energy for binding only at nearby sites {e.g., 1-2x the intrachain carboxylate distance}. This type of ion binding is implied by the proposed egg box structure (11) and is supported by these data (18, 19). Our results also indicate that a large proportion of pectic substances exist contiguously in three dimensional lattices low in methyl ester content and that the relative positions of the point dipoles increases ca. 2-3Å upon hydration, probably under the influence of alterations in the conformation of the polyanionic ligand. Other data (19) indicate that hydration-induced changes {Figure 2} in the apparent relative position of bound Mn^{2+} is at least partially controlled by the polymer's structure.

Literature Cited

1. Albersheim, P.; Muhlethaler, K.; Frey-Wyssling, A. J Biophys. Biochem Cytol. 1960, 8, 501-6.
2. Knee, M.; Bartley, I. In "Recent Advances in the Biochemistry of Fruits and Vegetables"; Academic: London, 1981; pp. 133-48.
3. Dainty, J.; Hope, A. Aust J. Biol. Sci. 1959, 12, 395-411.
4. Gillet, C.; LeFebvre, J. J. Exp. Bot. 1978, 29, 1155-9.
5. Van Cutsem, P.; Gillet, C. J. Exp. Bot. 1982, 33, 847-53.

26. Van Vleck, J. Phys Rev. 1948, 74, 1168-83.
27. Judeikis, H. J. Appl Phys. 1964, 35, 2615-7.
28. Gulley, J.; Hone, D.; Scalapino, D., Silbernagel, B.
Phys. Rev. B 1970, 1, 1020-30.
29. Gorter, C.; Van Vleck, J. Phys. Rev. 1947, 72, 1128-9.
30. Anderson, P.; Weiss, P. Rev. Mod Phys. 1953, 25, 269-76.
31. Shia, L.; Kokoszka, G. J. Chem. Phys. 1974, 60, 1101-5.
32. Smith, T.; Pilbrow, J. Coord. Chem. Rev. 1974, 13, 173-8.
33. Deiana, S.; Erre, L.; Micera, G.; Piu, P.; Gessa, C.
Inorg. Chim. Acta 1980, 46, 249-53.
34. Deiana, S.; Micera, G.; Muggiolu, G.; Gessa, C.; Pusino, A.
Colloids Surf. 1983, 6, 17-25.
35. Morvan, C.; Demarty, M.; Thellier, M. Plant Physiol.
1979, 63 1117-22.
36. Gidley, M.; Morris, E.; Murray, E.; Powell, D.; Rees, D.
Int. J. Biol. Macromol. 1980, 2, 332-4. 37. Morris, E.;
Powell, D.; Gidley, M.; Rees, D.
J. Mol. Biol. 1982, 155, 507-16.
38. McMillan, J. In "Electron Paramagnetism"; Reinhold: New
York, 1968; pp. 199-204.
39. Abragam, A.; Bleaney, B. In "Electron Paramagnetic
Resonance of Transition ions"; Clarendon: Oxford, 1970; p.
521.

RECEIVED December 20, 1985

Reprinted from ACS SYMPOSIUM SERIES No. 310
Chemistry and Function of Pectins
Marshall L. Fishman and Joseph J. Jen, Editors