Following Suberization in Potato Wound Periderm by Histochemical and Solid-State ^{13}C Nuclear Magnetic Resonance Methods[1]

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The time course of suberization in wound periderm from potato (*Solanum tuberosum* L.) has been monitored by histochemical and high-resolution solid-state nuclear magnetic resonance (NMR) methods. Light microscopy conducted after selective staining of the lipid and double-bonded constituents shows that suberin is deposited at the outermost intact cell-wall surface during the first 7 d of wound healing; suberization forms a barrier to tissue infiltration at later times. Cross polarization-magic angle spinning ^{13}C NMR spectra demonstrate the deposition of a polyester containing all major suberin functional groups after just 4 d of wound healing. Initially the suberin includes a large proportion of aromatic groups and fairly short aliphatic chains, but the spectral data demonstrate the growing dominance of long-chain species during the period 7 to 14 d after wounding. The results of preliminary ^{13}C-labeling experiments with sodium [2-^{13}C]acetate and DL-[1-^{13}C]phenylalanine provide an excellent prospectus for future NMR-based studies of suberin biosynthesis.

Suberin is an essential plant biopolymer that is synthesized after wounding to protect the tissue from moisture loss and to reduce its susceptibility to bacterial and fungal attack (Kolattukudy, 1984; Goodman et al., 1986). A molecular rationale for suberin's protective function and development has proven elusive, in part because the polymer occurs as an integral unit together with cell-wall carbohydrates and does not dissolve in either aqueous or organic solvents. Nevertheless, we have recently characterized both the structure and dynamic behavior of intact suberin using magic-angle spinning solid-state ^{13}C NMR.

Drawing on related NMR investigations of lignin and cutin (Maciel et al., 1985; Lewis et al., 1987; Zlotnik-Mazori and Stark, 1988), we have successfully estimated the number and type of functional groups present in suberized potato (*So-*

lanum tuberosum L.) cell wall (Garbow et al., 1989; Stark et al., 1989). In addition, NMR spectra and spin-relaxation parameters have provided new information regarding domain formation and attachment sites for the suberin and cell-wall constituents of wound-healing potato tissue (Stark and Garbow, 1992).

A solid-state spectroscopic study of the time course of wound healing follows logically from the work summarized above, since it can be used to characterize key biosynthetic intermediates that involve polyester cross-links or covalent bonds of suberin to the cell-wall matrix. Aided by the development of reliable protocols for removal of unsuberized cell-wall materials (Pacchiano et al., 1993), we have monitored deposition of the aromatic-aliphatic suberin polyester in potato tissue from 0 to 14 d after wound healing. A combination of histochemical methods and CPMAS ^{13}C NMR has been used to follow the suberization process and the incorporation of a ^{13}C label into the biopolyester. Our solid-state investigations also complement reports of soluble aliphatic and aromatic compounds that are possible suberin precursors (Kolattukudy, 1984; Bernards and Lewis, 1992).

MATERIALS AND METHODS

Suberin from Potatoes

Potatoes (*Solanum tuberosum* L. cv Russet Burbank) were peeled, cut into discs, and suberized for time periods of 0 to 14 d as described previously (Kolattukudy and Dean, 1974; Garbow et al., 1989). The wound periderm was separated from unsuberized cell-wall materials using a protocol that included treatments with cellulase (five times), pectinase, and hemicellulase (all enzymes from Sigma); exhaustive Soxhlet extraction was used to separate the waxes (Pacchiano et al., 1993). For a typical growth period, 50 g of peeled potatoes yielded a 500-mg preparation of suberized cell walls.

For biosynthetic labeling experiments, the discs were bathed in (a) distilled water, (b) 5 mM sodium acetate (Fisher Scientific, Springfield, NJ), or (c) 5 mM sodium [2-^{13}C]acetate (MSD Isotopes, Montreal, Canada) in separate experiments. Wound healing was then allowed to proceed for 7 d; for trials

Abbreviations: CPMAS, cross-polarization magic-angle spinning with high-power decoupling; NBS, Nile blue sulfate; PFAS, performic acid-Schiff's reagent.

b and c the humidified air that flowed through the growth chamber was first scrubbed using a potassium hydroxide trap to prevent incorporation of unlabeled carbon dioxide into the suberin polyester. Protocols for suberization, cell-wall removal, and dewaxing have been described elsewhere (Pacchiano et al., 1993).

NMR Spectroscopy

For enzyme-treated and dewaxed potato tissue isolated daily in a 2-week time course of wound healing, dried samples weighing 300 to 400 mg were ground with a mortar and pestle and packed into rotors for NMR studies. Solid-state ^{13}C NMR spectra were collected on a home-built spectrometer operating at a proton Larmor frequency of 127.0 MHz. Samples were spun at the magic angle (54.7°) with respect to the static magnetic field in a double-bearing rotor system (Schaefer et al., 1987) at a rate of 3.0 kHz. CPMAS ^{13}C NMR spectra were obtained at 31.9 MHz following 2-ms matched 50-kHz ^{13}C-^{1}H cross-polarization contacts. High-power proton dipolar decoupling [$B_1(H) = 65$ kHz] was used during data acquisition. Under these conditions, the correct relative intensities are obtained for the peaks observed in each of the spectra (Stejskal et al., 1979; Fukamizo et al., 1986). Additional experimental details are provided in the figure legends.

Histochemistry

In a preparation identical to that described above, potatoes were suberized for periods of 0, 4, 7, 10, 12, and 14 d. Neither unsuberized cell walls nor waxes were removed from the wound periderm. The tissues were first fixed in 10% neutral-buffered formalin for 2 weeks to preserve the chemical constituents and to avoid decay. Dehydration was carried out through a graded ethanol series (70–100%) for periods of 2 to 12 h, and the tissues were then cleared with two 24-h xylene treatments. Infiltration was done stepwise at 60°C with 50 to 100% paraffin:xylene for periods of 2 to 12 h. All potato tissues were then embedded in paraffin, sectioned with a microtome, mounted on slides, and stained progressively with the following reagents: hematoxylin and eosin, for general histological organization; NBS, for lipids; and PFAS, for carbon double bonds (Pearce, 1968; Gahan, 1984; Kiernan, 1990). The tissues were examined with a Nikon S-Ke light microscope at 100X magnification.

RESULTS

Histochemistry

Figures 1 and 2 show representative portions of suberized potato tissue under contrasting growth and staining conditions. Although the cell morphology is not well preserved, these micrographs yield several types of chemical and spatial information about potato wound periderm. Figure 1 illustrates NBS staining of the peripheral regions in 4- and 7-d suberized potato tissue and demonstrates the presence of lipid-like materials. Similar procedures with Sudan dyes have identified suberin in mature root endodermis (Tippett and O'Brien, 1976; Scott and Peterson, 1979) and the hollow-heart tissue of potato tubers (Dean et al., 1977). In analogous

fashion, the PFAS staining in Figure 2 shows the presence of double-bonded carbons in the peripheral regions of tissue sections prepared after 4 and 7 d of suberization. These latter results are consistent with results of EM showing suberin as an electron-dense band (Kolattukudy, 1980) and light microscopy showing autofluorescence produced by its aromatic groups (Pearce and Rutherford, 1981). Neither NBS nor PFAS gives a positive result for unsuberized (0-d) potato samples, but both the cell walls and amyloplasts give the expected positive results in a control experiment with hematoxylin-and-eosin staining (data not shown).

Judged from both NBS and PFAS results, the suberized tissue at both 4 and 7 d is confined to the outermost intact cell-wall surface, in agreement with published reports (Kolattukudy, 1978, 1981). The staining evidence indicates that both saturated (lipid-like) and unsaturated (C=C) bonds are present in solid polymeric form as early as 4 d after wounding. At later times, the development of a covalently bound hydrophobic barrier is suggested by an important negative histochemical result. When wound healing is permitted for 10, 12, or 14 d, the tissues cannot be cleared with xylene, presumably because the aliphatic portions of the suberin polymer are now sufficiently developed to form an effective barrier to solvent penetration. The efficacy of standard clearing procedures is restored if these periderm samples are cut in half to expose unsuberized tissue.

NMR Spectroscopy

Figure 3 shows CPMAS ^{13}C NMR spectra of potato tissue obtained after 0, 4, 7, and 14 d of wound healing and subsequent purification treatments. These spectra, which are representative of NMR data obtained at 12 times during the course of suberization, have features similar to those reported previously (Stark et al., 1989; Kolattukudy et al., 1992). As with the histochemical experiments described above, the NMR spectra allow us to monitor changes in chemical structure at key times during suberization (see below). Quantitative comparisons of the ^{13}C peak intensities for these chemically similar samples are expected to provide an accurate assessment of carbon content, since the 2-ms cross-polarization contact time used to acquire the NMR data minimizes intensity variations due to differing rates of proton-carbon cross-polarization and proton spin relaxation (Stejskal et al., 1979; Fukamizo et al., 1986; Stark and Garbow, 1992).

Immediately after wounding of the tissue, only cell-wall carbohydrates are evident in the CPMAS ^{13}C spectrum. This result is as expected, confirming the negative NBS and PFAS staining tests for lipids and multiple bonds, respectively. The absence of suberin is also corroborated enzymically: 99% of this potato sample is susceptible to a protocol of treatments that degrade cell walls (Pacchiano et al., 1993).

Both aliphatic and aromatic suberin moieties are also absent from the CPMAS ^{13}C NMR spectra of 1- and 2-d potato samples (data not shown), showing that neither biosynthetic intermediates nor the polyester itself have been cross-linked or covalently bound to the cell wall within this time period. Although a variety of hydroxy fatty acids, dicarboxylic acids, and ferulic acid esters may be formed within 1 d of wound healing (Rhodes and Wooltorton, 1978; Cottle and Kolattu-
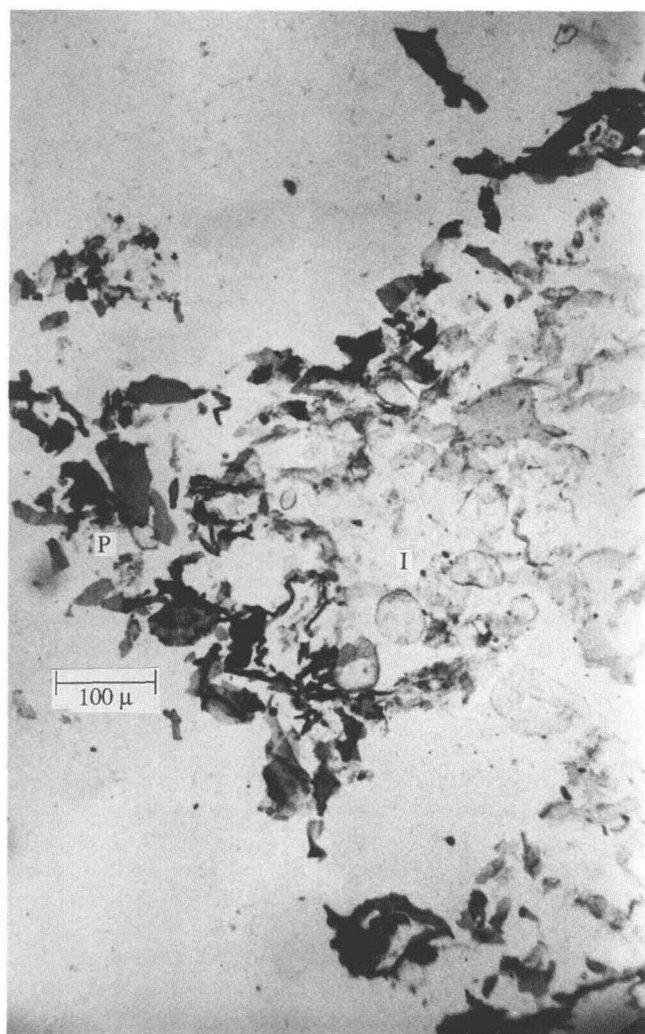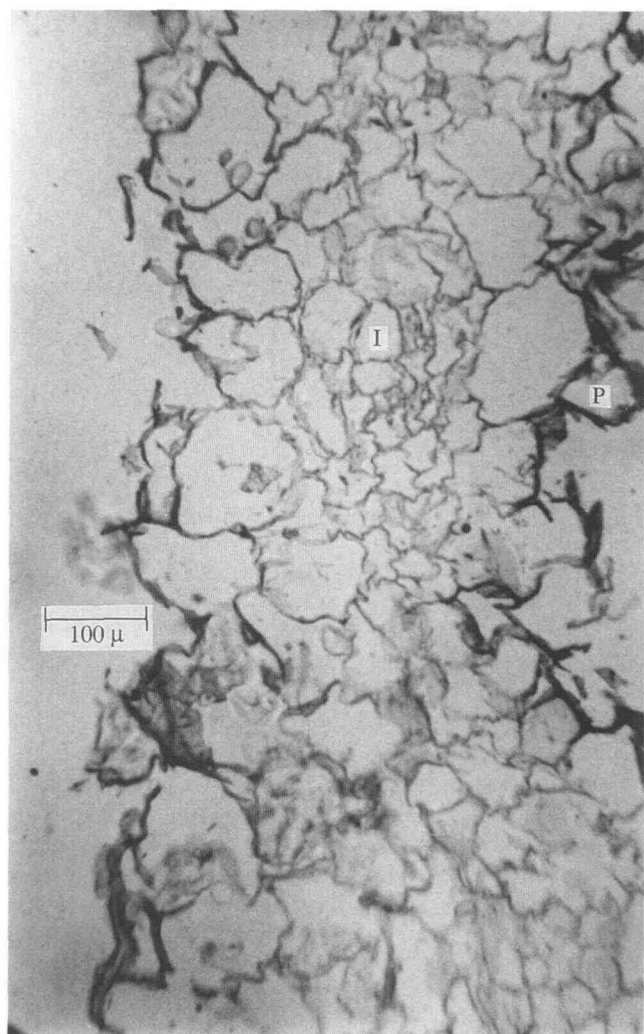
Figure 1. NBS staining of potato tissue suberized for 4 d (left) and 7 d (right). Staining densities are not strictly comparable. I, Internal tissue; P, wound periderm.

kudy, 1982), they are not incorporated into the tissue in sufficient amounts to be detected by solid-state ^{13}C NMR. Our negative spectroscopic evidence for suberization at early times is supported by functional studies of the tissue's resistance to water loss, which indicate that no barrier to diffusion is established within the first 3 to 4 d after wounding (Kolattukudy and Dean, 1974).

After 4 d of incubation, a dramatic buildup of CPMAS ^{13}C NMR signals is observed from both aliphatic and aromatic groups. The appearance of these spectral features reflects the deposition of solid suberin within the potato tissue. The identification of both unsaturated and aliphatic carbon functionalities in the NMR spectrum also confirms our 4-d histochemical evidence for double-bonded carbons and aliphatic lipids. Thus, even before the diffusional barrier at the tissue surface is fully developed (Kolattukudy and Dean, 1974), the chemical structure of the protective polymer includes all major functional groups reported previously for suberized potato tissue (Garbow et al., 1989; Stark et al., 1989): methylenes (20–40 ppm), methoxy carbons (56 ppm), unsaturated

and aromatic groups (105–155 ppm), and carboxyl carbons (172 ppm). The insoluble nature of the potato suberin formed after 4 d suggests that the polyester has become cross-linked and/or covalently bound to the cell wall.

The observation of suberizing cell walls by ^{13}C NMR also complements a recent report of C_{16}-C_{28} alkyl ferulates extracted from wounded potato tubers after 3 to 7 d of growth (Bernards and Lewis, 1992). These soluble long-chain ferulic acid esters, as well as the partial hydrogenolysis products isolated previously from wound-healing potatoes (Kolattukudy and Dean, 1974; Adamovics et al., 1977), have many structural elements in common with our cell-wall-incorporated biosynthetic intermediates. Nevertheless, the spectra in Figure 3 exhibit two features typical of building blocks having shorter alkyl chains. First, the integrated ^{13}C NMR intensity for methylene groups at 20 to 40 ppm is less than two-thirds that of aromatic carbons appearing in the spectrum at 105 to 155 ppm. By contrast, the predicted aliphatic-to-aromatic ratio for long-chain ferulic acid esters is approximately 2.5. Second, the sharp bulk-methylene resonance that dominates
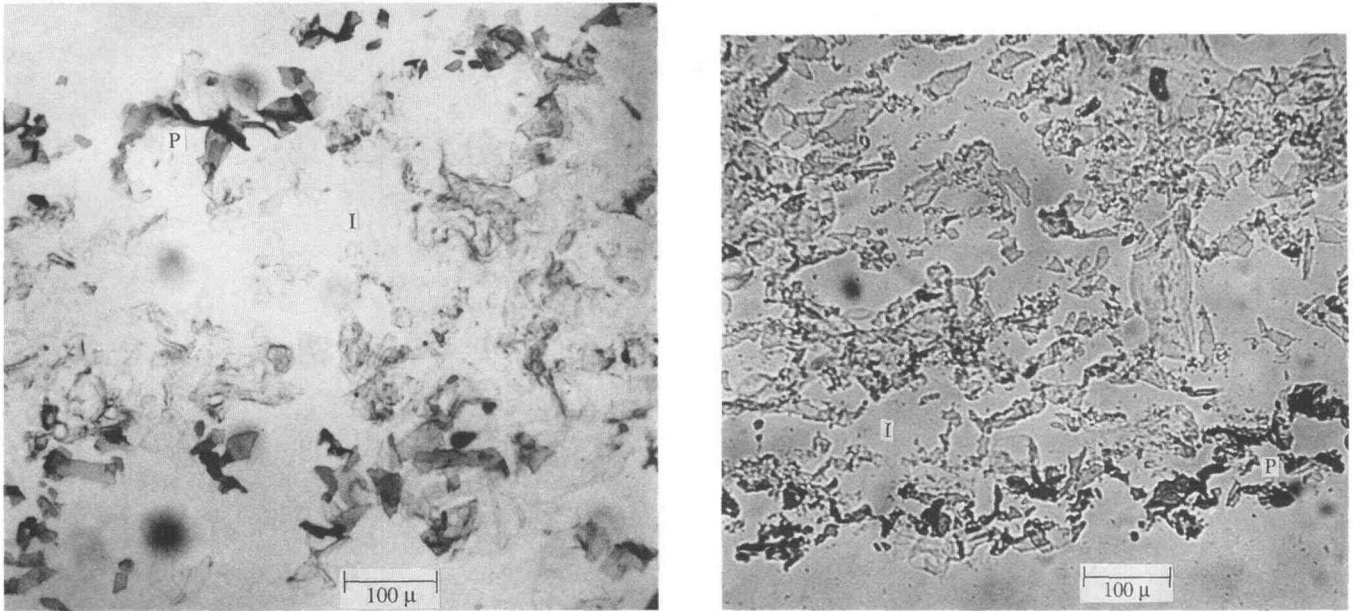
Figure 2. PFAS staining of potato tissue suberized for 4 d (left) and 7 d (right). Staining densities are not strictly comparable. I, Internal tissue; P, wound periderm.

the upfield portion of the spectrum for long-chain cutin polyesters (Zlotnik-Mazori and Stark, 1988) is absent in ^{13}C NMR data for the 4-d suberin. Thus, the methylene carbons of suberin have a heterogeneous distribution of magnetic environments, as might be expected if their neighbors are aliphatic carbons, carboxyl oxygens, or carbohydrate groups. A number of possible structural explanations for the compositional differences between ferulic acid esters and suberin are explored below.

One issue that may be addressed using the ^{13}C NMR spectra obtained at later stages of the wound healing is whether more suberin deposition is occurring as a diffusional barrier becomes established. As a group, the suberin resonances in the 7-d spectrum are largest with respect to the major 72-ppm sugar peak. The data acquired at 8 to 14 d after wounding do not show a consistent pattern of additional suberin formation, so we attribute the apparent maximum in suberin content at 7 d to small variations in the cell-wall-removal treatments (Pacchiano et al., 1993).

A number of subtle modifications to the basic structural elements of the aromatic-aliphatic polyester may be deduced from intensity variations measured across the NMR spectra acquired at longer growth times (see Table I). One trend evident in Figure 3 and Table I is that at longer times, aromatic and unsaturated groups of the suberin (105–155 ppm) are present in greater number compared with its carboxyl carbons (172 ppm). The methylene resonances show a modest increase of intensity and a substantially narrowed chemical shift range, indicating the presence of fewer distinct chain-carbon types and the growing dominance of bulk methylene groups such as those observed in cutin polyesters (Zlotnik-Mazori and Stark, 1988). Almost all of these methylene segments are sufficiently rigid that high-power decoupling is required during acquisition and the ^{13}C NMR signals disap-

pear under delayed-decoupling conditions (data not shown). Thus, as the tissue develops a barrier that can resist water diffusion and prevent clearing with xylene, the principal changes in suberin composition suggest an increase in average chain length and/or light cross-linking of the chains.

Our success in monitoring suberization for wound-healing potatoes by CPMAS ^{13}C NMR encouraged us to combine this approach with biosynthetic ^{13}C-labeling experiments. The uptake of radiolabeled aliphatic and phenolic precursors was reported previously from analysis of suberin depolymerization products (Dean and Kolattukudy, 1977; Cottle and Kolattukudy, 1982). Moreover, a precedent for our investigative approach was provided by experiments in which ^{13}C-labeled ferulic acid was incorporated into the root lignin of wheat seedlings and observed by solid-state ^{13}C NMR (Lewis et al., 1987). Figure 4 illustrates the potential of such investigations for potato suberin, comparing NMR spectra for 7-d wound periderm formed after the cut discs are bathed in water, sodium acetate, and sodium [2-^{13}C]acetate.

The CPMAS ^{13}C NMR spectra are identical for potato tissue suberized after bathing in water or unlabeled acetate (Fig. 4, a and b, respectively), showing that biosynthesis of the protective polyester is insensitive to whether the carbon source is carbon dioxide or acetate. When the spectra of suberin derived from unlabeled and ^{13}C-labeled precursors are compared (Fig. 4, b and c, respectively), it is clear that significant ^{13}C incorporation has occurred at methylene, methoxy, carboxyl, and alkene groups. Among the methylene groups, labeling changes the distribution of peak intensities and may occur preferentially for the mobile population that appears in the NMR spectrum at 30 ppm (Stark and Garbow, 1992). This scrambling of the isotopic label is expected after 7 d, since the acetate may participate in a variety of synthetic and synthesis-breakdown-resynthesis pathways. Moreover,
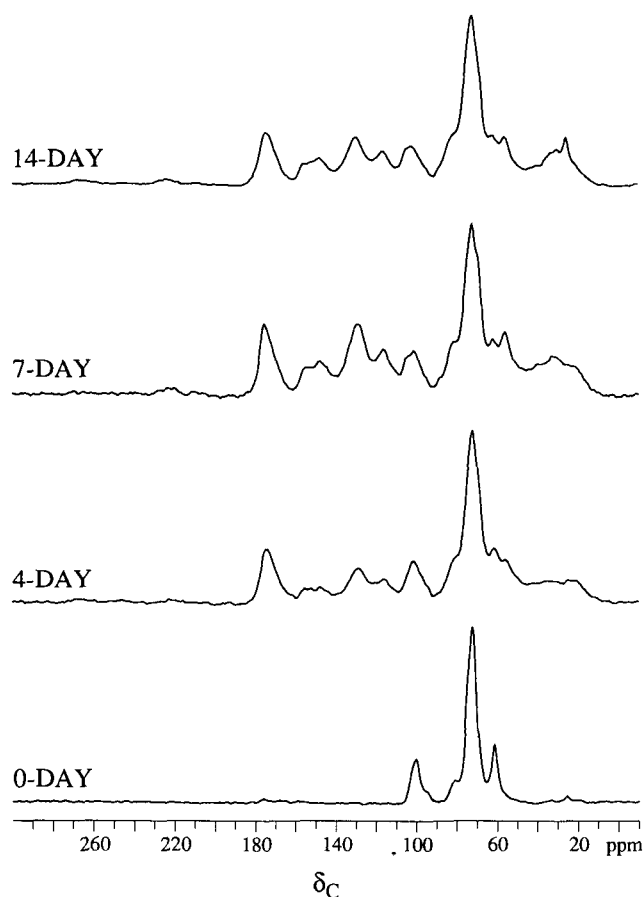
Figure 3. CPMAS ^{13}C NMR spectra (31.9 MHz) of potato tissue after 0, 4, 7, and 14 d of wound healing and a protocol of enzymic and chemical treatments that removes 94 to 99% by weight of the unsuberized cell walls and waxes (Pacchiano et al., 1993). All spectra were collected with 2-ms ^{1}H-^{13}C contacts and 1-s recycle delays to ensure that polarization transfer was complete but intensity losses from proton relaxation were comparable for each resonance (Stejskal et al., 1979; Fukamizo et al., 1986). Chemical shifts are quoted with respect to external Asn (side-chain carbonyl = 175.1 ppm). Vertical scales were adjusted to equalize the height of the 72-ppm peak in each spectrum, focusing attention on suberin behavior with respect to a fixed matrix of cell-wall carbohydrates.

^{14}C label was incorporated into both saturated and unsaturated aliphatic constituents of 7-d periderm when such tissue was incubated briefly with [1-^{14}C]acetate (Dean and Kolattukudy, 1977). We have also incubated potato wound periderm with DL-[1-^{13}C]Phe as a carbon source. Preliminary results show some scrambling and a significant uptake of the label, with approximately 40% of the total ^{13}C NMR signal in the enriched sample arising from the labeled Phe carbon.

DISCUSSION

High-resolution solid-state ^{13}C NMR has been used in conjunction with histochemical methods to monitor the deposition of suberin within potato tissue between 2 and 14 d after wounding. In agreement with prior reports (Kolattukudy, 1980, 1981), we observe a lag period of 2 to 4 d before

Table I. Chemical composition of potato periderm during wound healing

Data are from Bayesian Probability Theory analysis (Bretthorst et al., 1989; Kotyk et al., 1992) of the CPMAS ^{13}C NMR spectra of Figure 3. Reported values are intensities relative to that of the 72-ppm polysaccharide resonance, which is normalized to 1.00 for each spectrum.

Carbon Type[a]	Time after Wounding		
	4 d	7 d	14 d
Methylene (20–40 ppm)	0.21	0.31	0.26
Aromatic/olefinic (105–155 ppm)	0.30	0.57	0.41
Carboxyl (172 ppm)	0.20	0.22	0.18

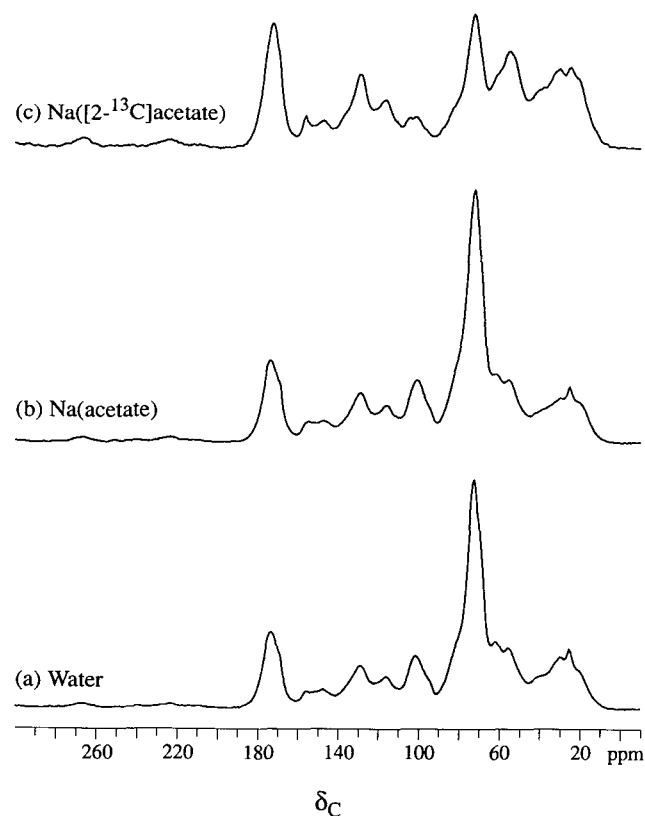[a] Following the assignments of Stark and Garbow (1992).

Figure 4. CPMAS ^{13}C NMR spectra (31.9 MHz) of suberized potato tissue incubated in the presence of (a) water, (b) sodium acetate, and (c) sodium [2-^{13}C]acetate. The spectra were normalized to account for differences in mass and number of acquisitions. Compared with a, integrated intensities for the spectra in b and c are 1.10 and 1.16, respectively.

deposition of the protective polymer. Spectroscopic and histochemical results indicate that the polyester structure contains both aliphatic and aromatic moieties after a time course of 4 d, well before an effective diffusional barrier is established at the wound surface.

Previous workers have detected small amounts (0.002% by weight) of alkyl ferulates in extracts of potato periderm at various stages of wound healing (Bernards and Lewis, 1992). The presence of soluble ferulates in the periderm correlates with suberin formation there, suggesting that these ferulates may play a role in suberin growth. In fact, the insoluble suberin polymer has many compositional and structural features in common with the ferulates. At the same time, it has, on average, shorter alkyl chains and a lower aliphatic-to-aromatic ratio than would be expected for ferulates alone. These compositional differences suggest that other, nonferulate materials also serve as suberin building blocks and/or that structural modification of the ferulates occurs after their initial deposition within the wound-healing matrix.

One possible mechanistic scheme for compositional modification involves anchoring of long ferulate chains into the cuticular waxes, followed by addition of aromatic units to provide cross-links and/or covalent attachments to the cell-wall carbohydrates. Alternatively, the long alkyl chains might be removed and regenerated upon deposition, although such a modification could compromise the waterproofing effectiveness of the initial wound-healing layer. Further investigation of these possibilities is clearly needed.

As building blocks of the intact suberin polyester, alkyl ferulates share the same limitations as suberin depolymerization products. The monomeric lipid products that result from suberin hydrogenolysis or transesterification typically amount to 30 to 70% of the original purified polymeric material (Kolattukudy and Dean, 1974). Thus, the monomers isolated from these reactions, which have long alkyl chains and very few phenolic moieties, need not be strictly representative of the intact suberin polymer.

Between 4 and 7 d, an effective diffusional barrier forms in potato periderm, as measured directly and deduced previously from the release of p-hydroxybenzaldehyde, vanillin, and chlorogenic acid (Cottle and Kolattukudy, 1982). Our histochemical data, which demonstrate resistance to xylene clearing procedures in 10-d potato periderm, provide auxillary evidence for this developing barrier. Examination of our CPMAS ^{13}C NMR results on dewaxed samples reveals several important structural changes occurring in the cell wall as suberization progresses. Both aromatic and alkyl-chain functional groups increase significantly with respect to carboxyl carbons. The diminished chemical-shift dispersion for the bulk-methylene carbons also supports the growing dominance of long-chain species. ^{13}C NMR experiments conducted with delayed and low-power ^1H decoupling demonstrate that essentially all of the alkyl-chain segments of suberin within potato wound periderm are motionally restricted.

Waxes, which were removed from the suberized cell wall prior to NMR analysis, play an important role in the formation of a diffusional barrier (Kolattukudy, 1984), and our previous NMR studies have probed the nature of hydrophobic interactions between the polyester chains and waxes within intact lime cutin (Garbow and Stark, 1990). The

structural changes described above for suberized cell walls may be directly relevant in the formation of a diffusional barrier or in the strengthening of this barrier through covalent anchoring of the aromatic residues to the carbohydrate matrix (Stark and Garbow, 1992). These changes in the polyester matrix may also be required to accommodate and support the wax. Specific details regarding the proximity of various functional groups, covalent bonding and cross-linking patterns, and suberin-wax interactions remain to be elucidated.

With the incorporation of ^{13}C labels into potato suberin, CPMAS ^{13}C NMR has the potential to provide new insights into the chemical structure and biosynthetic pathways involved in wound healing. The central metabolic role of acetyl-CoA may explain why labels that originate with sodium [2-^{13}C]acetate ultimately appear at several chemical sites of the suberin polymer. Nonetheless, ^{13}C incorporation is found to occur preferentially in the mobile population of methylene carbons that resonate at 30 ppm (Stark and Garbow, 1992), consistent with acetate incorporation within a long C_{20} chain (Dean and Kolattukudy, 1977) or a flexible cross-link. Although isotopic scrambling limits the use of acetate precursors for detailed mechanistic studies, even this test case is quite effective in enhancing signals from suberin compared with carbohydrates in the CPMAS ^{13}C NMR spectrum. We are currently exploring the use of alternative precursors and growth conditions to achieve higher levels of ^{13}C enrichment at well-defined suberin sites.

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