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Biochemistry of Suberization

INCORPORATION OF [1-^{14}C]OLEIC ACID AND [1-^{14}C]ACETATE INTO THE ALIPHATIC COMPONENTS OF SUBERIN IN POTATO TUBER DISKS (*SOLANUM TUBEROSUM*)[1]

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ABSTRACT

Biosynthesis of the aliphatic components of suberin was studied in suberizing potato (*Solanum tuberosum*) slices with [1-^{14}C]oleic acid and [1-^{14}C]acetate as precursors. In 4-day aged tissue, [1-^{14}C]oleic acid was incorporated into an insoluble residue, which, upon hydrogenolysis ($LiAlH_4$), released the label into chloroform-soluble products. Radio thin layer and gas chromatographic analyses of these products showed that ^{14}C was contained exclusively in octadecenol and octadecene-1,18-diol. OsO_4 treatment and periodate cleavage of the resulting tetraol showed that the labeled diol was octadec-9-ene-1,18-diol, the product expected from the two major components of suberin, namely 18-hydroxyoleic acid and the corresponding dicarboxylic acid. Aged potato slices also incorporated [1-^{14}C]acetate into an insoluble material. Hydrogenolysis followed by radio chromatographic analyses of the products showed that ^{14}C was contained in alkanols and alkane-α,ω-diols. In the former fraction, a substantial proportion of the label was contained in aliphatic chains longer than C_{20}, which are known to be common constituents of suberin. In the labeled diol fraction, the major component was octadec-9-ene-1,18-diol, with smaller quantities of saturated C_{16}, C_{18}, C_{20}, C_{22}, and C_{24}-α,ω-diols. Soluble lipids derived from [1-^{14}C]acetate in the aged tissue also contained labeled very long acids from C_{20} to C_{28}, as well as C_{22} and C_{24} alcohols, but no labeled ω-hydroxy acids or dicarboxylic acids were detected. Label was also found in *n*-alkanes isolated from the soluble lipids, and the distribution of label among them was consistent with the composition of *n*-alkanes found in the wound periderm of this tissue; C_{21} and C_{23} were the major components with lesser amounts of C_{19} and C_{25}. The amount of ^{14}C incorporated into these bifunctional monomers in 0-, 2-, 4-, 6-, and 8-day aged tissue were 0, 1.5, 2.5, 0.8, and 0.3% of the applied [1-^{14}C]oleic acid, respectively. Incorporation of [1-^{14}C]acetate into the insoluble residue was low up to the 3rd day of aging, rapid during the next 4 days of aging, and subsequently the rate decreased. These changes in the rates of incorporation of exogenous oleic acid and acetate reflected the development of diffusion resistance of the tissue surface to water vapor. As the tissue aged, increasing amounts of the [1-^{14}C]acetate were incorporated into longer aliphatic chains of the residue and the soluble lipids, but no changes in the distribution of radioactivity among the α-ω-diols were obvious. The above results demonstrated that aging potato slices constitute a convenient system with which to study the biochemistry of suberization.

Cutin and suberin are polymeric substances which constitute the structural component of the outer layer of plants. Cutin, which is associated with aerial parts of plants, is composed mainly of aliphatic components, and its major constituents are dihydroxy C_{16} fatty acids, 18-hydroxy-9,10-epoxy C_{18} fatty acids, and trihydroxy C_{18} fatty acids. Also found are smaller amounts of ω-hydroxy C_{16} and C_{18} fatty acids, as well as smaller quantities of phenolic materials (4, 7, 10, 11). Suberin, which is mainly associated with the below ground portions of plants and periderms, is composed of aliphatic components (25–50%) and phenolic materials (50–75%). The major aliphatic components of suberin are ω-hydroxyfatty acids and dicarboxylic acids; among them C_{16} and C_{18} predominate, but longer chains (C_{20}–C_{28}) are also found. Fatty acids and alcohols with chain lengths longer than C_{20} are also significant constituents of suberin (1–5, 8, 10, 12).

Biosynthesis of cutin has been studied and pathways for the biosynthesis of cutin monomers are fairly well established (4, 7). However, virtually nothing is known about the biosynthesis of suberin. It was found that [1-^{14}C]oleic acid applied to the surface of rapidly growing potato tubers was incorporated into the major C_{18} aliphatic components of the suberin in the skin (4). Because it is difficult to obtain uniform tissue of suitable size for biosynthetic studies throughout the year, this technique did not seem promising. Recently, it was found that the composition of the aliphatic monomers deposited at the wound periderm of potato tuber slices is identical to that of the natural skin (suberin) of potatoes (6). The time course of suberin deposition during the wound-healing process has been determined in potato tuber slices; after a 2- to 3-day lag period following wounding, suberin is synthesized rapidly for 3 or 4 days (6). As uniform tissue slices can be readily prepared, and because the induction of suberin biosynthesis can be monitored in this system by a nondestructive and quick technique (6), we thought that this tissue would serve as a good model system for investigating the biochemistry of the suberization process.

In this paper, we report that [1-^{14}C]acetate and [1-^{14}C]oleic acid are incorporated into the major aliphatic components of suberin in potato tuber disks. The changes in the rates of incorporation of these exogenous precursors follow a time course consistent with the time course of deposition of suberin, as measured by the development of resistance of the tissue surface to diffusion of water vapor as well as by gas chromatography.

MATERIALS AND METHODS

Substrates and Reagents. Sodium[1-^{14}C]acetate (58.1 Ci/mol) and [1-^{14}C]oleic acid (57 Ci/mol) were purchased from Amersham/Searle. The labeled oleic acid (50 μCi) was dispersed in 2.5 ml of H_2O containing about 1 mg of Tween 20 with the needleprobe of a Biosonik III sonicator at full power (2 × 10

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sec). $LiAlH_4$ was purchased from Pierce Chemical Co., and BF_3 in methanol was prepared by bubbling BF_3 gas into absolute methanol until 14% by weight of BF_3 was dissolved.

Tissue Preparation. Potato tubers, *Solanum tuberosum* var. White Rose, were purchased from a local grocery store and used immediately. The tubers were immersed in a 1.5% hypochlorite (25% Clorox) solution for 5 min, the solution was poured off, and the tubers were allowed to dry. The tubers were then broken in cross-section by hand to expose the internal tissue, and from the vascular region, tissue cylinders were removed with a sharp cork borer (1 cm diameter). Uniform disks, 2 mm in thickness, were cut with a tissue slicer; they were placed in sterile distilled deionized H_2O as they were cut, and were rinsed twice with fresh sterile distilled deionized H_2O after each 100 disks were prepared (10–25 min). Disks which were not used immediately after cutting were placed on rubberized mesh screens in wide mouth gallon jars at 20 C in the dark, and they were aerated with humidified air at 0.6 l/hr. All of the above operations were performed under sterile conditions. Periodic checks for bacterial contamination were made by placing five disks from aged samples on complete nutrient agar at 25 C for 3 days.

Incorporation of Substrates. In 50-ml Erlenmeyer flasks containing 0.5 ml (50 μCi acetate or 10 μCi oleic acid) of substrate solution, 15 disks were placed and disks were individually bathed so that the entire surface was coated with the substrate. The disks were then spread on the bottom of the flask, a KOH trap for CO_2 was placed on top of the flask, and the tissue was incubated in a shaking water bath at 30 C for the desired amount of time. Water was added dropwise periodically if necessary to keep the tissue moist. At the end of the incubation period, the disks were rinsed (4 × 25 ml) with water and frozen until all of the samples were collected.

Depolymerization of Suberin and Analysis of Products. The disks were homogenized in a 2:1 mixture of chloroform and methanol with a 15-ml TenBroeck homogenizer. The homogenate was left for 1 hr at room temperature and centrifuged at 24,000*g* for 30 min. The residue was resuspended in methanol and centrifuged at 24,000*g* for 30 min. The procedure was repeated twice using a 1:1 mixture of chloroform and methanol: once with methanol, and twice with tetrahydrofuran. All of the supernatants were pooled for extraction of the soluble lipids, and the final residue was transferred into a 50-ml round bottomed flask with dry tetrahydrofuran (30 ml). Excess $LiAlH_4$ was added to the flasks, and the contents were refluxed for 24 hr. The reaction mixture was carefully added to water (50 ml) with vigorous stirring, and the mixture was acidified with concentrated HCl (5–10 ml). The lipid material was extracted with chloroform (3 × 150 ml) and the chloroform extracts were pooled and evaporated to dryness with a rotary evaporator. These hydrogenolysis products were then subjected to TLC with ethyl ether-hexane-methanol (40:10:5, v/v) as the developing solvent. The alcohol and diol components isolated by TLC were acetylated and the products were subjected to TLC on silica gel with hexane-ethyl ether (7:3, v/v) as the developing solvent. The acetate derivatives thus isolated were subjected to TLC on silica gel plates impregnated with 5% $AgNO_3$ using hexane-ethyl ether as the developing solvent (9:1 for alcohol acetates and 4:1 for diol acetates).

Analysis of Soluble Lipids. For the extraction of the soluble lipids, the combined supernatants obtained from the above tissue extraction procedure were acidified and extracted with chloroform (3 × 150 ml). The pooled chloroform extract was evaporated to dryness with a rotary evaporator. In order to examine the labeling of the phospholipids, a portion (5 × 10^5 cpm) of the lipids was subjected to TLC under N_2 with chloroform-methanol-glacial acetic acid-water (170:25:25:4, v/v/v/v) as the developing solvent in one direction, followed by chloroform-methanol-7 N ammonium hydroxide (65:30:4, v/v/v) in the second direction. Neutral lipids were analyzed by TLC with hexane-ethyl ether-formic acid (40:10:1, v/v/v) as the developing solvent. To determine the chain length distribution of the fatty acids, a portion (5 × 10^5 cpm) of the soluble lipids was refluxed with 14% BF_3 in methanol for 3 hr, and after addition of water, the products were extracted with chloroform (3 × 100 ml). The solvent was removed using a rotary evaporator and the methyl esters were isolated by TLC with hexane-ethyl ether-formic acid (65:35:2, v/v/v) as the developing solvent. The fatty acid methyl esters were also separated according to the degree of unsaturation by TLC on silica gel impregnated with 5% $AgNO_3$ using hexane-ethyl ether (9:1, v/v) as the developing solvent. Hydrocarbons from potato tuber skin and the wound periderm were isolated by subjecting the soluble lipids, obtained by Soxhlet extraction of crude suberin samples prepared as described previously (6), to TLC in unlined tanks with hexane as the developing solvent. Labeled hydrocarbons were similarly isolated from the soluble lipids derived from [1-^{14}C]acetate.

Preparation of Derivatives. Acetates of alcohols and diols were prepared by treating them with a 2:1 mixture of acetic anhydride and pyridine at room temperature overnight. The reaction mixture was then added to water, acidified with concentrated HCl, and extracted with chloroform (3 × 100 ml). The solvent was removed with a rotary evaporator and the acetates were purified by TLC using hexane-ethyl ether (35:15, v/v) as the developing solvent. Determination of the location of the double bond in the diol diacetate fraction obtained from suberin was done by treating the sample (1.0 × 10^5 cpm) with ~1% OsO_4 in dioxane for 2 hr at room temperature. The reaction was stopped by the addition of 1 ml of a saturated aqueous solution of sodium sulfite followed by 4 ml of methanol. The mixture was centrifuged, and the pellet was resuspended in methanol and recentrifuged. The pooled supernatant was extracted twice with ethyl ether, and the combined ether extracts were evaporated to dryness and the products were subjected to TLC with hexane-ethyl ether (70:30, v/v) as the developing solvent. The fraction corresponding to the tetraol diacetate, recovered from the silica gel, was treated with 50 mg of powdered KIO_4 in 5 ml of pyridine under N_2 with vigorous stirring for 4 hr. About 0.5 ml of a 0.5 mM aqueous solution of $KMnO_4$ containing 2 mM Na_2CO_3 was added and stirred for 2 additional hr. The reaction mixture was decolorized with sodium bisulfite, diluted with 2 volumes of water, and acidified. The lipids were extracted with chloroform, the solvent was removed with a rotary evaporator, and the products were refluxed with an excess of $LiAlH_4$ in tetrahydrofuran. The reduction products, isolated as described above, were subjected to TLC and the diol fraction was isolated, acetylated as above, and subjected to radio gas chromatographic analysis.

Chromatography. TLC was done on 0.5-mm or 1.0-mm Silica Gel G plates (20 × 20 cm) activated overnight at 110 C. Components on the thin layer chromatograms were located by viewing the plates under UV light after spraying them with 2′, 7′-dichlorofluorescein or by charring with 5% potassium dichromate in 50% H_2SO_4. Thin layer chromatographic fractions were eluted from the silica gel with a 2:1 mixture of chloroform and methanol followed by absolute methanol.

Radio GLC of the alcohol acetates, diol diacetates, alkanes, and the fatty acid methyl esters was done with 5% OV-1 on 80- to 100-mesh Gas-chrom Q in a coiled stainless steel column (243.8 × 0.6 cm). The fatty acid methyl esters were also chromatographed on 10% diethylene glycol succinate on 80- to 100-mesh Gas-chrom Q in a coiled stainless steel column (243.8 × 0.6 cm). All radio gas chromatography was done with a Perkin-Elmer 801 gas chromatograph attached to a Barber-Colman radioactivity monitor. Combined gas chromatography and mass spectrometry of alkanes was done with an Aerograph 550 gas chromatograph attached to a Perkin-Elmer Hitachi RMU 6 D

mass spectrometer with a Biemann separator interphase. Mass spectra were recorded at the apex of the gas chromatographic peaks with 70 ev ionizing voltage.

Determination of Radioactivity. A Berthold thin layer scanner was used to monitor radioactivity on the thin layer chromatograms. Radioactivity in the thin layer chromatographic fractions was determined by assaying aliquots of the samples for ^{14}C after they were eluted from the silica gel. A Packard liquid scintillation spectrometer was used with a counting efficiency of about 70% as determined by [^{14}C]toluene standards. All counting was done with a standard deviation of less than 3%. Autoradiography of the thin layer chromatograms was performed with Kodak medical no-screen x-ray film.

Diffusion Resistance and Respiration Measurements. The resistance of the tissue surface to diffusion of water vapor was determined every time that samples were removed for incorporation of labeled substrate. Weight loss was measured over a 30-min period and resistance of the tissue to water loss was calculated as reported earlier (6). Production of CO_2 by a sample of 40 tissue cylinders (1 × 2 cm, 68.5 g), contained in a wide mouth quart jar, was continuously monitored with a model 215A Beckman infrared analyzer.

RESULTS AND DISCUSSION

The time course of suberin deposition during wound-healing has been studied using cylinders of potato tuber tissue (6); however, thin slices of tissue are more convenient for biosynthetic studies. As tissue thickness is known to affect metabolic changes in potato tissue drastically (9), the time course of suberization was determined in tissue disks. It was found that by the 4th day of aging, suberin deposition began as measured by diffusion resistance or gas chromatography (Fig. 1). Suberization was rapid for the next 3 days and then leveled off as previously observed in tissue cylinders. The amount of suberin/unit surface area in the disks was only about 25% of that in the tissue cylinders. Consistent with these values, the diffusion resistance of the surface of disks to water vapor was also only 25% of that of the tissue cylinders. However, on a weight basis, the amount of suberin present in the disks was about 10 times that present in the cylinders as might be expected from the high ratio of the surface area to weight of the disk.

Incorporation of [1-^{14}C]Oleic Acid into Suberin. Since the major aliphatic components of the suberin in the wound periderm of potato slices are ω-hydroxyoleic acid and the corresponding dicarboxylic acid, oleic acid would seem to be the likely precursor of these suberin monomers. Therefore, [1-^{14}C]oleic acid was applied to potato disks which were aged for 4 days, as suberin deposition is known to be quite rapid at this period. After a 6-hr incubation with the labeled substrate, the soluble lipids were removed by thorough extraction with solvents as described under "Materials and Methods," and the insoluble residue was found to be labeled. Exhaustive hydrogenolysis of this residue showed that about 5% of the total radioactivity applied to the tissue slices was contained in the lipid products obtained from the hydrogenolysis step. TLC of the hydrogenolysis products revealed that >50% of the total radioactivity was present in the diol fraction. Since hydrogenolysis converts both ω-hydroxyfatty acids and dicarboxylic acids into indistinguishable diols, the labeled diol fraction probably represents both of these classes of monomers. Radio gas chromatography of the diol fraction (as diacetates) showed that the bulk of the ^{14}C was contained in one component which had a retention time identical to that of octadecene-1,18-diol diacetate. Diols longer than C_{18} could not be detected even upon injection of larger amounts of radioactivity. These results are consistent with the chemical composition of potato suberin in that the longer chain ($>C_{18}$) aliphatic components of suberin are saturated, whereas the major C_{18} component is monounsaturated.

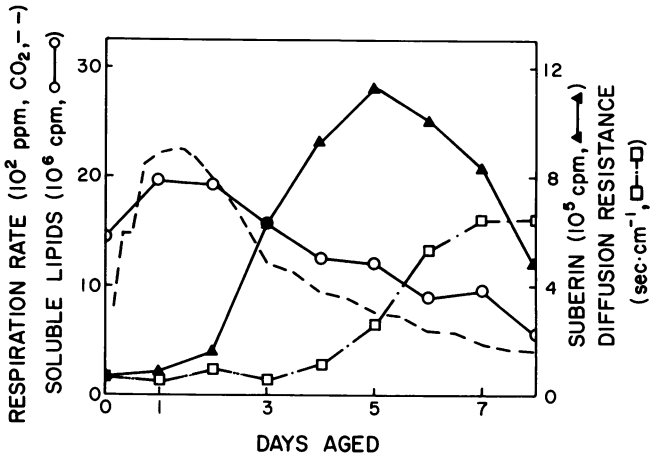

FIG. 1. Incorporation of [1-^{14}C]acetate into soluble lipids (O——O) and suberin (▲——▲) in aging potato disks; CO_2 production (- - -) and diffusion resistance (□- · -□) are also indicated.

If the exogenous [1-^{14}C]oleic acid is directly converted into ω-hydroxyoleic acid and/or the corresponding dicarboxylic acid of suberin, the labeled diol diacetate derived from them should be Δ^9 monounsaturated. In order to determine whether this was the case, the labeled diol diacetate was treated with OsO_4 to convert the olefinic functions to *vic*-diols. Recovery of the aliphatic components after treatment with OsO_4 and subsequent TLC showed that all of the radioactive diol diacetate had been converted to a much more polar material. This polar material was treated with KIO_4, followed by $LiAlH_4$ reduction of the resulting hydroxyacids. TLC of the products revealed only one labeled component, and it had an R_F identical to that of C_9 diol. This component was acetylated and subjected to radio gas chromatography, which showed the presence of only one radioactive component, and it had a retention time identical to that of C_9 diol diacetate. These results show that the original labeled C_{18} diol was octadec-9-ene-1,18-diol, which is the expected reduction product of the major aliphatic components of suberin of the wound periderm. Therefore, it is quite clear that exogenous oleic acid is directly converted into the major aliphatic monomers of suberin in aging potato disks.

Relationship between the Duration of Aging and the Incorporation of [1-^{14}C]Oleic Acid into Suberin. If the incorporation of exogenous [1-^{14}C]oleic acid into the insoluble material in the aged potato slices represents biosynthesis of suberin, the rate of incorporation should reflect the rate of deposition of suberin. In order to test this possibility, labeled oleic acid was incubated with disks which had been aged for 0, 2, 4, 6, and 8 days, and the hydrogenolysis products derived from the insoluble material were examined as described above. No radioactivity could be detected in the diol fraction obtained from fresh tissue. Octadecene-1,18-diol obtained from each of the aged tissue samples was labeled; the amounts of ^{14}C recovered in the diol were 1.4, 2.5, 0.8, and 0.3% in 2-, 4-, 6-, and 8-day aged tissues, respectively. Thus, the maximum rate of incorporation of exogenous [1-^{14}C]oleic acid into the major aliphatic components of suberin occurred after about 4 days of aging, and this result is consistent with the time course of development of diffusion resistance of the tissue surface.

Incorporation of [1-^{14}C]Acetate into Suberin. Very long chain ($>C_{20}$) aliphatic components are characteristic constituents of suberin. However, since these monomers are saturated, exogenous [1-^{14}C]oleic acid would not be expected to be converted into such components. Therefore, [1-^{14}C]acetate was used as a substrate to follow the biosynthesis of these very long chain ($>C_{20}$) components. After incubation of 6-day aged disks with

this labeled material for 6 hr, the tissue was homogenized and the soluble lipids and other soluble metabolites were removed by thorough extraction with solvents as described under "Materials and Methods." The resulting insoluble material was found to be labeled, and hydrogenolysis of the residue released radioactivity into chloroform soluble products. These products were subjected to TLC which revealed only two major radioactive components, and the R_F values of these two fractions indicated that they were alkanols and alkane-α,ω-diols. TLC of the alcohol acetates on silica gel impregnated with 5% $AgNO_3$ showed that virtually all (90–95%) of the alcohols present in this fraction were saturated. Radio gas chromatography of the alkanol fraction (analyzed as acetates) showed a homologous series of labeled fatty alcohols from C_{16} to C_{28} with the very long chains ($>C_{20}$) predominating (Fig. 2A). The distribution of the radioactivity in this fraction was consistent with the quantities of fatty alcohols and acids that are constituents of wound suberin (6), so that all of the components appeared to have similar specific radioactivities.

The diol fraction could conceivably contain some phenolic materials as covalently attached ferulic acid is known to be present in the suberin deposited in the wound periderm (unpublished results). The hydrogenolysis product of ferulic acid is not well resolved from alkane-α,ω-diols by the TLC used for the isolation of the diol fraction. Therefore, the diol fraction was extracted with 5% NaOH to remove phenolic components. Only about 2% of the label was found in the phenolic fraction and, therefore, this fraction was not further analyzed. The aliphatic diol fraction was acetylated and subjected to TLC which showed only one radioactive component with an R_F identical to that of octadecene-1,18-diol diacetate. This component, when analyzed by radio gas chromatography, showed one major peak with a retention time identical to that of octadecene-1,18-diol diacetate and four smaller peaks with retention times corresponding to those of C_{16}, C_{20}, C_{22}, and C_{24} diol diacetates (Fig. 2B). Thin layer chromatography of the diol diacetate fraction with $AgNO_3$-impregnated silica gel showed saturated and monounsaturated components, with R_F values corresponding to hexadecanediol diacetate and octadecenediol diacetate, respectively. Radio gas chromatography of the monounsaturated fraction showed only one radio gas chromatographic peak with a retention time identical to that of octadecene-1,18-diol diacetate, whereas the saturated fraction showed C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, and C_{26} diol diacetates. In order to determine the location of the double bond in the labeled unsaturated diol, the total diol diacetate fraction was treated with OsO_4. TLC revealed two labeled components: one had an R_F corresponding to hexadecanediol diacetate, indicating that it was saturated; and the other was a much more polar material, presumably a tetraol diacetate. The polar material was subjected to periodate degradation as described above for the unsaturated diol derived from [1-^{14}C]oleic acid. Radio gas chromatographic analysis showed that C_9 diol was the only final labeled product (Fig. 2C), proving that the original C_{18} diol was octadec-9-ene-1,18-diol. Radio gas chromatography of the saturated diol diacetates obtained after the osmilation step gave results similar to those obtained using the $AgNO_3$ TLC. These results demonstrate that [1-^{14}C]acetate was incorporated into all of the major aliphatic components of suberin in aging potato slices and that the extent of incorporation into each component was consistent with the composition of the suberin.

Incorporation of [1-^{14}C]Acetate into Soluble Lipids in Suberizing Potato Disks. In tissues which rapidly synthesize cutin, soluble monomers are barely detectable (7), suggesting that a very efficient transfer of monomers to the polymer occurs in such tissues. In order to determine whether such is the case in suberin-synthesizing tissues, the soluble lipids derived from [1-^{14}C]acetate in the suberizing potato disks were analyzed. A small amount (<2%) of the radioactivity in the soluble fraction was found to be in C_{22} and C_{24} alcohols (after transesterification with BF_3 in methanol). TLC of the hydrogenolysate of the soluble lipids showed some labeled material with an R_F corresponding to alkane-α,ω-diol. However, acetylation and repeated TLC and subsequent radio gas chromatography showed that this material was not alkane-α,ω-diol and, therefore, no soluble ω-hydroxy-acids or dicarboxylic acids existed in this tissue. Our analysis of the hydrogenolysate of the soluble lipids from suberizing potato tissue by combined gas chromatography and MS also failed to detect any alkane diols. Since the presence of very long chain

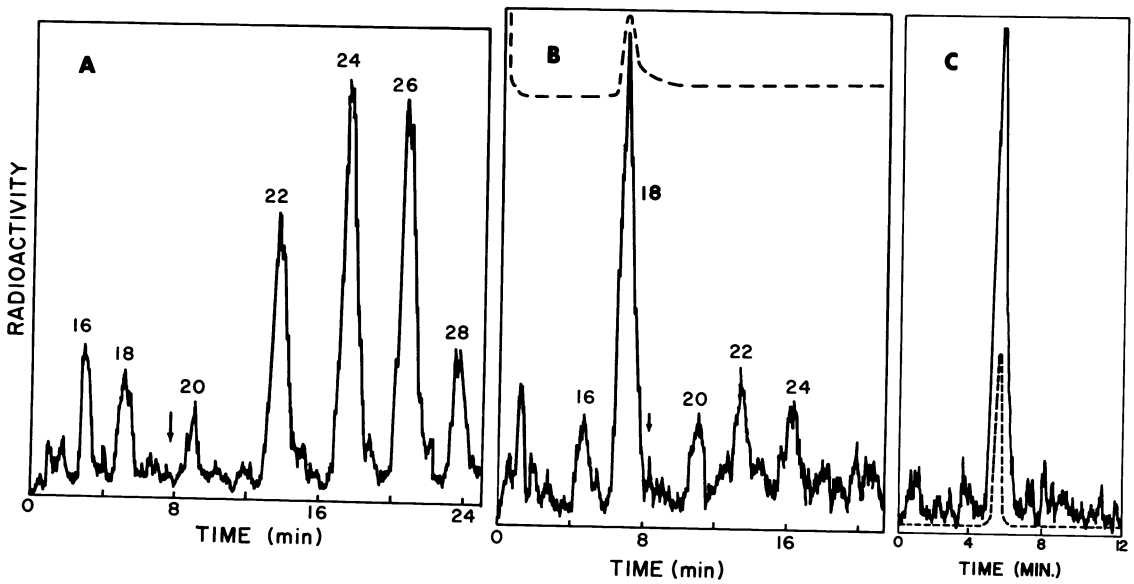

FIG. 2. Radio gas chromatogram of acetates prepared from the alcohols (A) and diols (B) isolated from the hydrogenolysate of suberin from 6-day aged potato disks which had been incubated with [1-^{14}C]acetate for 6 hr. The column temperature was held at 250 C and 270 C for A and B, respectively, for 8 min and subsequently programmed to 310 C at 12.5 C/min. The inlet pressure of carrier gas Ar was 50 p.s.i. The number on each peak represents the carbon chain length, which was determined by comparison of the retention times with those of authentic standards. C: radio gas chromatogram of the diacetate prepared by the periodate degradation of the polar fraction obtained by OsO_4 treatment of the diol fraction shown in A; column temperature 200 C, Ar at 20 p.s.i. The dashed lines in B and C represent the flame ionization detector response of the coinjected octadec-9-ene-1,18-diol diacetate and nonane-1,9-diol diacetate, respectively.

($>C_{20}$) aliphatic monomers is a characteristic of suberin, such aliphatic chains might also occur in the soluble lipids of suberizing tissue. Therefore, methyl esters prepared from the soluble lipids derived from [1-^{14}C]acetate in 6-day aged tissue were analyzed by radio gas chromatography, and the major labeled fatty acids were found to be C_{20} to C_{28} (32%), C_{16} (34%), and C_{18} (34%). $AgNO_3$ TLC and radio gas chromatography showed that the very long chain (C_{20}–C_{28}) fatty acids were saturated, whereas the C_{16} fraction contained both saturated and monounsaturated acids, and the C_{18} components were found to be saturated, monounsaturated, and diunsaturated.

Analysis of the soluble lipids from the normal potato tuber skin and the wound periderm showed the presence of *n*-alkanes which were identified by combined gas chromatography and MS. The normal skin had C_{25} as its major hydrocarbon and smaller amounts of C_{23} and C_{27} (Fig. 3a), whereas the major hydrocarbons from the wound periderm were found to be C_{23} and C_{21} (Fig. 3b). TLC of the soluble lipids derived from [1-^{14}C]acetate in 5-day aged tissue revealed the presence of labeled alkanes. As the duration of aging increased, the amount of label incorporated into hydrocarbons changed in a manner similar to that observed for the aliphatic suberin components described below. Radio gas chromatography of the alkane fraction showed labeled C_{19}, C_{21}, C_{23}, and C_{25} alkanes (Fig. 3c), and the distribution of radioactivity in this fraction was consistent with the composition of the alkane fraction isolated from the wound periderm described above. These observations are consistent with previous findings that hydrocarbons of the surface lipids of plants contain longer carbon chains than the internal hydrocarbons (7).

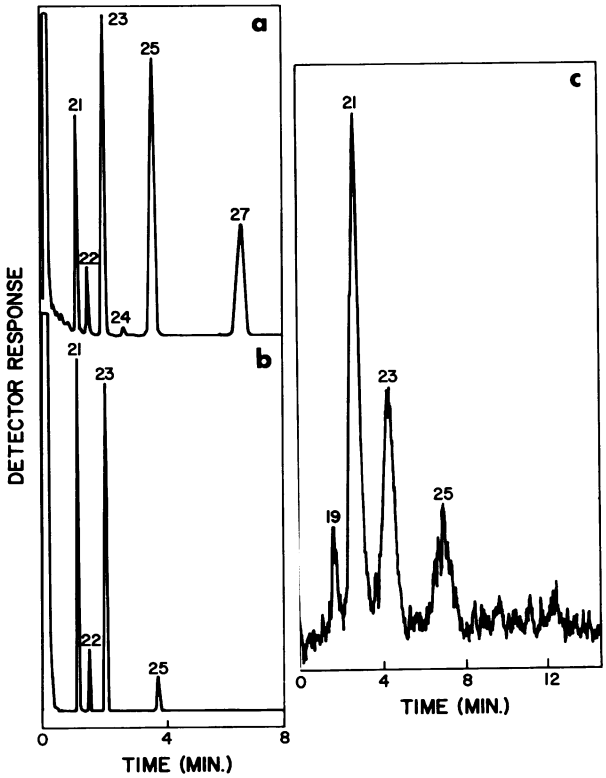

FIG. 3. Gas chromatogram of *n*-alkanes isolated from (A) potato tuber skin and (B) wound suberin. A coiled glass (183 × 0.31 cm o.d.) column packed with 5% OV-1 on 80- to 100-mesh Gas-chrom Q was held at 240 C with the carrier gas He at 27 p.s.i. C: radio gas chromatogram of *n*-alkanes isolated from 5-day aged potato disks which had been incubated with [1-^{14}C]acetate for 6 hr. The OV-1 column described in the text was held at 272 C with the carrier gas Ar at 20 p.s.i. The number on each peak represents the chain length, which was determined from the mass spectrum and by comparison of the retention times with those of authentic standards.

Relationship between Duration of Aging and Incorporation of [1-^{14}C]Acetate into Suberin and Soluble Lipids. In order to determine whether the rate of acetate incorporation into the suberin monomers accurately reflects the rate of deposition of suberin, the effect of the duration of aging on incorporation of acetate into the suberin monomers was determined. A time course of incorporation was done to determine the linear range of incorporation so that rates could be compared. Incorporation of the label into soluble lipids was linear up to 4 hr of incubation, whereas the rate of incorporation into suberin was linear up to 6 hr. As our major objective was to measure incorporation into suberin, a 6-hr incubation period was used for subsequent experiments.

Incorporation of the label from [1-^{14}C]acetate into the insoluble residue was very low up to the 3rd day of aging. Labeling of the residue was rapid during the next 4 days of aging, and subsequently the rate decreased (Fig. 1). These changes in the rate of labeling were consistent with the time course of deposition of suberin as measured by diffusion resistance (Fig. 1).

In order to determine the time course of synthesis of the individual aliphatic components of suberin, the hydrogenolysates of the labeled insoluble material derived from [1-^{14}C]acetate were analyzed by TLC and radio gas chromatography. In the fresh tissue, no radioactivity could be found in the alkanols obtained from the insoluble material. The rate of incorporation of labeled acetate into the alkanols in the aged tissue reflected the rate of incorporation into the total insoluble material. The distribution of ^{14}C among the various fatty alcohols obtained from the insoluble material (Table I) showed that increasing periods of aging resulted in the incorporation of increasing amounts of label into the longer chains. For example, only about 30% of the label contained in this fraction was in chains longer than C_{20} in the 1- or 2-day aged tissue, whereas in 3- and 5-day aged tissue, these very long chains contained 67 and 85% of the ^{14}C, respectively. Labeled diols were not detected in the insoluble residue until the tissue was aged for 3 days. The rate of incorporation of label into the diol fraction was very low in 3-day aged tissue, increased up to 5 days of aging, and then decreased. The distribution of the radioactivity among the diols did not change during aging. The rates of conversion of exogenous precursors into suberin and the distribution of radioactivity in the aliphatic monomers of suberin observed in these experiments are consistent with the time course of deposition and the

Table I. Distribution of Radioactivity in the Fatty Alcohols Isolated from the Hydrogenolysate of the Insoluble Residue Obtained from Suberizing Potato Disks Incubated with [1-^{14}C]Acetate

Chain length distribution was determined by radio-gas chromatography of the alcohol acetates. Percentages were obtained by triangulation of the radio gas chromatographic peaks. The chromatographic conditions were the same as those described in Figure 2.

Days Aged	Radioactivity						
	C_{16}	C_{18} s+u[1]	C_{20}	C_{22}	C_{24}	C_{26}	C_{28}
	% total						
0	ND[2]	ND	ND	ND	ND	ND	ND
1	20	32	18	14	17	ND	ND
2	27	30	14	12	15	3	ND
3	13	12	11	26	24	17	ND
4	11	9	8	27	26	17	4
5	6	6	4	30	39	36	10
6	10	9	6	18	24	22	11
7	14	12	6	19	21	15	12
8	26	14	12	18	11	9	12

[1]S, saturated; u, unsaturated. [2]ND, not detected.

known composition of wound suberin.

The rate of incorporation of [1-^{14}C]acetate into soluble lipids was very rapid in 1- and 2-day aged tissue, and it decreased slowly as the tissue aged for longer periods of time (Fig. 1). Because the 6-hr incubation time was beyond the linear range, the observed incorporation of ^{14}C into soluble lipids probably does not reveal true quantitative differences in the rate of synthesis of soluble lipids. The distribution of radioactivity in the fatty acids of the total soluble lipids showed that longer aging periods resulted in increased incorporation of label into longer chain fatty acids (Table II). Preliminary fractionation of the soluble lipids prior to analysis of the fatty acids revealed that the very long chains were present mainly in the neutral lipids. The incorporation of labeled acetate into fatty acids longer than C_{18}, previously observed in 23-hr aged potato slices (13), probably reflects the initiation of the suberization process.

Analysis of the fatty acids in the soluble lipids derived from [1-^{14}C]acetate showed that incorporation into saturated fatty acids increased up to 3 days of aging, after which no change was observed (Table III). The monounsaturated fraction showed only labeled $C_{18:1}$ during the first 2 days of aging, after which time increasing incorporation into $C_{16:1}$ was found. Although the appearance of labeled $C_{16:1}$ in the soluble lipids coincided with the onset of suberization, the relationship, if any, between $C_{16:1}$ and suberization is not obvious. $C_{18:2}$ was the only labeled diunsaturated fatty acid detected in this analysis, and after 2 days of aging, the proportions of ^{14}C found in this acid declined.

In order to determine whether any detectable change in the incorporation of [1-^{14}C]acetate into a specific soluble lipid coincided with the onset of suberization, soluble lipids were fractionated by TLC and the distribution of radioactivity among the various lipids was examined by autoradiography. Neutral lipids and phospholipids were further subfractionated by TLC using developing solvents described under "Materials and Methods," and radioactivity in the individual components was recorded on x-ray film. No changes in labeling, which would suggest a precursor role for suberin, were detected in any fraction. There was, however, an increase in incorporation into phospholipids as the tissue aged for 1 or 2 days.

CONCLUSIONS

The results presented in this paper show that [1-^{14}C]oleic acid and [1-^{14}C]acetate are incorporated into the major aliphatic components of suberin, and that the changes in the rates of incorporation are consistent with the time course of deposition of suberin as measured by diffusion resistance. These findings demonstrate that suberization in the wound periderm involves an induction period of 2 to 3 days after wounding, followed by a period of rapid synthesis of suberin (about 3 days), after which the rate of synthesis of the aliphatic monomers decreased. Since suberin at the wound is synthesized by cells which do not synthesize suberin in the intact tuber, it is probable that some factor, released or produced at the wound, directly or indirectly triggers suberization. Consistent with this hypothesis is the recent finding that thorough washing of cut tissue with water results in the inhibition of suberin formation (unpublished results), thus raising the possibility of isolating some suberization-inducing factor(s). Isolation and characterization of such factors would be crucial to our understanding of the sequence of biochemical processes which ultimately leads to suberin synthesis. Our recent observation, that in aging potato slices, abscisic acid promotes the synthesis of the major aliphatic components of suberin (unpublished results), raises the possibility that suberization may be under hormonal control.

Table II. Distribution of Radioactivity in Fatty Acid Methyl Esters Obtained from the Total Soluble Lipids Derived from [1-^{14}C]Acetate in Suberizing Potato Disks

Fatty acid methyl esters were analyzed by radio gas chromatography as described under Materials and Methods. Column temperature was 220 C for 8 min after which the temperature was programmed to 310 C at 10 C/min; inlet pressure of carrier gas was 42 psi.

Days Aged	Radioactivity						
	$C_{16\ s+u}$[1]	$C_{18\ s+u}$	C_{20}	C_{22}	C_{24}	C_{26}	C_{28}
	% total						
0	35	50	6	9	ND[2]	ND	ND
1	29	40	12	11	8	ND	ND
2	35	47	9	5	5	ND	ND
3	37	35	10	11	7	ND	ND
4	36	31	10	14	12	ND	ND
5	33	37	6	9	9	7	ND
6	34	34	6	8	10	4	4
7	41	28	7	11	9	3	3
8	39	27	7	10	6	4	8

[1] s, saturated; u, unsaturated. [2] ND, not detected.

Table III. Distribution of Radioactivity in Saturated and Unsaturated Fatty Acids Obtained from the Total Soluble Lipids Derived from [1-^{14}C]Acetate in Suberizing Potato Disks

Fatty acid methyl esters were separated into saturated, monounsaturated and diunsaturated fractions by $AgNO_3$-thin-layer chromatography, and each fraction was subjected to radio gas chromatography as described under Materials and Methods.

Days Aged	Radioactivity			
	Saturated[1]	Monounsaturated		Diunsaturated
		16:1	18:1	18:2
	% total			
0	47	ND[2]	29	24
1	60	ND	15	25
2	73	ND	11	16
3	86	6	17	1
4	82	7	8	3
5	72	6	14	8
6	78	7	8	7
7	75	9	12	4
8	83	6	8	3

[1] The saturated fraction contained C_{18} to C_{28} as shown in Table II.

[2] ND, not detected.

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