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Biochemistry of Suberization

ω-HYDROXYACID OXIDATION IN ENZYME PREPARATIONS FROM SUBERIZING POTATO TUBER DISKS[1]

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ABSTRACT

A cell-free extract obtained from suberizing potato (*Solanum tuberosum* L.) tuber disks catalyzed the conversion of 16-hydroxy[G-^{3}H]hexadecanoic acid to the corresponding dicarboxylic acid with NADP or NAD as the cofactor, with a slight preference for the former. This ω-hydroxyacid dehydrogenase activity, located largely in the 100,000*g* supernatant fraction, has a pH optimum of 9.5. It showed an apparent *Km* of 50 μM for 16-hydroxyhexadecanoic acid. The dehydrogenase activity was inhibited by thiol reagents, such as *p*-chloromercuribenzoate, N-ethylmaleimide, and iodoacetamide, and this dehydrogenase is shown to be different from alcohol dehydrogenase. That 16-oxohexadecanoic acid was an intermediate in the conversion of 16-hydroxyhexadecanoic acid to the corresponding dicarboxylic acid was suggested by the observation that the cell-free extract also catalyzed the conversion of 16-oxohexadecanoic acid to the dicarboxylic acid, with NADP as the preferred cofactor. The time course of development of the ω-hydroxyacid dehydrogenase activity in the suberizing potato disks correlated with the rate of deposition of suberin. Experiments with actinomycin D and cycloheximide suggested that the transcriptional processes, which are directly related to suberin biosynthesis and ω-hydroxyacid dehydrogenase biosynthesis, occurred between 72 and 96 hours after wounding. These results strongly suggest that a wound-induced ω-hydroxyacid dehydrogenase is involved in suberin biosynthesis in potato disks.

ω-Hydroxyacids and dicarboxylic acids constitute two of the major aliphatic components of suberin (12). Little is known about the biosynthesis of these components of suberin. It has been suggested that the ω-hydroxylating enzyme system and the enzyme(s) which catalyzes the oxidation of ω-hydroxyacids to the corresponding dicarboxylic acids are the key enzymes involved in suberin biosynthesis (11, 12, 14). As yet, ω-hydroxylation of fatty acids has not been demonstrated in cell-free preparations from higher plants. Attempts to demonstrate ω-hydroxylation in cell-free preparations from suberizing potato slices have not been successful, as yet. The second enzyme, which should be involved in the suberization process, namely ω-hydroxyacid dehydrogenase, has not been demonstrated in suberizing tissues.

Here, we describe the preparation and some properties of a cell-free extract from suberizing potato tissue disks which oxidizes ω-hydroxyacids to the corresponding dicarboxylic acids. We have demonstrated that such a cell-free extract also oxidizes ω-oxoacid to the corresponding dicarboxylic acid. We have also shown that the changes in ω-hydroxyacid dehydrogenase activity correlate with the time course of suberin formation. Evidence is presented which suggests that the transcriptional and translational processes directly related to suberization and ω-hydroxyacid dehydrogenase biosynthesis occur about 72 to 96 hr after preparing the potato disks. A preliminary report of this work has been presented (1).

MATERIALS AND METHODS

Potato tubers (*Solanum tuberosum* L., Russet Burbank) were grown at the Othello Experimental Farm of Washington State University. The tubers (8-16 oz) were washed free of soil and stored at 7 C.

Substrates and Reagents. Dithioerythritol, NAD, NADP, N-ethylmaleimide, cycloheximide, and actinomycin D were purchased from Sigma Chemical Co. Omnifluor was purchased from New England Nuclear Corp., *p*-chloromercuribenzoate from Nutritional Biochemicals, iodoacetamide from Aldrich Chemical Co., and PVP from GAF of New York. Hexadecane-1,16-dioic acid was purchased from K. & K. Lab., and 16-hydroxyhexadecanoic acid from Aldrich Chemical Co.

16-Hydroxy[G-^{3}H]hexadecanoic acid (277.4 Ci/mol) was prepared in the following manner. About 100 mg of methyl 16-acetoxyhexadecanoate (a generous gift from A. P. Tulloch) was exposed to 6 Ci $^3H_2$ for 15 days according to the Wilzbach's method at New England Nuclear Corp. Rigorous purification of the acetoxymethyl ester by repeated TLC followed by hydrolysis and purification of the hydroxyacid by TLC with ethyl ether-hexane-formic acid (60:40:2, v/v), gave chemically and radiochemically pure 16-hydroxy[G-^{3}H]hexadecanoic acid.

16-Oxo[G-^{3}H]hexadecanoic acid was synthesized from 16-hydroxy[G-^{3}H]hexadecanoic acid by CrO_3-pyridine complex treatment. CrO_3-2Pyr complex was prepared as previously described (22). One mg of 16-hydroxy[G-^{3}H]hexadecanoic acid in a small volume of dry CH_2Cl_2 was mixed with 2 ml of the above complex and stirred for 15 min. The solvent was removed under a stream of N_2, the residue was washed with small volumes of ethyl ether seven to eight times, and the ether washings were combined. The remaining residue was washed with H_2O and the combined water washings were extracted with the combined ether washings. The ether phase was washed with H_2O until the ether layer was free from yellow color, then washed with 5% HCl and saturated NaCl solution, and dried over anhydrous sodium sulfate. The ether was evaporated and the ω-oxoacid was purified by TLC with ethyl ether-hexane-formic acid (60:40:2, v/v) as the developing solvent.

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Substrates were dispersed in H_2O as described below. The substrate (2–3 mg) was dissolved in 10 ml of ethyl ether containing 2 mg of Tween 20. After the ether was evaporated off by N_2, the required volume of H_2O or buffer was added to the substrate and the mixture was sonicated three times for 5 sec with the needle probe of Biosonik III at full power.

Chromatography and Radioactivity Measurement. TLC was done on 0.5-mm or 1-mm (20 × 20 cm) layers of Silica Gel G activated overnight. Lipids were visualized under UV after spraying the chromatogram with a 0.1% ethanolic solution of 2′,7′-dichlorofluorescein. The thin layer plates were scanned with a Berthold thin layer scanner. Radio GLC was performed with a Perkin-Elmer 811 gas chromatograph equipped with a flame ionization detector and an effluent splitter; the effluent was continuously monitored with a Barber-Colman radioactivity monitor.

For radioactivity measurement, the liquid samples and the TLC fractions were taken in scintillation vials; 15 ml of a scintillation fluid consisting of 30% ethanol in toluene containing 0.4% (w/v) Omnifluor was added to each vial and then assayed for tritium in a Packard Tri-Carb liquid scintillation spectrometer, model 3003. Internal standard of [^{3}H]toluene was used to determine counting efficiency, which was usually 14% for ^{3}H. All counting was done with a standard deviation less than 3%.

Determination of Protein. Protein was estimated by the method of Lowry *et al.* (20). Protein in the aliquots of the cell-free extract was precipitated by adding 10% trichloroacetic acid, and the precipitate collected by centrifugation was triturated with 80% acetone-H_2O and centrifuged. The precipitate was dissolved in 0.1 N NaOH and assayed for protein with BSA as standard.

Preparation of Suberizing Potato Disks. Potato tubers were immersed in 2% (w/v) hypochlorite (30% [v/v] Clorox) solution for 5 min and thoroughly washed with H_2O. They were broken into two halves by hand, and disks, 10 mm in diameter and approximately 2 mm in thickness, were cut from the broken surface of the potato tuber by punching tissue cylinders with a cork borer and subsequent sectioning with a slicer. The disks were rinsed with sterile H_2O, blotted with tissue papers, and incubated on a rubberized mesh in Petri dishes under a slow current of humid air (0.6 1/hr) at about 22 C for the required time. All operations from breaking the potato tubers to placing slices in Petri dishes were performed under sterile conditions in a glove box. All of the utensils were autoclaved and precautions were taken to prevent bacterial contamination. Tissue slices were examined for bacterial contamination by incubating them in a Petri dish on agar containing 27% of nutrient broth for 2 to 3 days.

Preparation of the Cell-free Extract. Potato disks suberized for 6 days were ground in an Omni-Mixer for 1 min at 80% of the full speed with insoluble PVP (100 mg/disk) and 0.2 M K-phosphate (1 ml/disk) (pH 6.5) containing 1 mM dithioerythritol and 1 mM magnesium chloride. The resulting mixture was centrifuged at 27,000*g* for 20 min. To the supernatant, powdered ammonium sulfate was added slowly to obtain 80% saturation. After stirring for an additional 45 min, the precipitate, which was collected by centrifugation, was dissolved in minimum amounts of 0.1 M glycine-NaOH buffer (pH 9.5) containing 1 mM dithioerythritol and 1 mM magnesium chloride. Aliquots were assayed for ω-hydroxyacid oxidation as described in the following section.

Assay for ω-Hydroxyacid Dehydrogenase Activity. The assay for ω-hydroxyacid dehydrogenase activity of the cell-free extract was done as described below unless otherwise mentioned under "Results and Discussion." Aliquots of the cell-free extract containing 0.25 mg protein were incubated for 1 hr at 30 C with 0.1 mM 16-hydroxy[G-^{3}H]hexadecanoic acid, 0.5 mM NAD, 0.5 mM NADP, 0.1 M glycine-NaOH buffer (pH 9.5), 1 mM dithioerythritol, and 1 mM magnesium chloride in a total volume of 1 ml. The reactions were terminated by adding 1 ml dilute HCl, and the lipids were extracted three times by vigorously shaking the incubation mixture with chloroform (5 ml each time) in a 60-ml separatory funnel. The extracts were collected in a test tube, the solvent was evaporated off under N_2, the inner walls of the test tube were washed down with approximately 2 ml of diethyl ether, and the ether was evaporated off. The lipids were dissolved in 0.1 ml tetrahydrofuran; 10 μl of the solution was assayed for radioactivity to determine the total recovery of ^{3}H. In each assay, the recovery was always better than 85%. Another aliquot (25 μl) of the solution was applied to 0.5-mm thick layers of Silica Gel G with unlabeled 16-hydroxyhexadecanoic acid and hexadecane-1,16-dioic acid as internal standards. The chromatograms were developed with ethyl ether-hexane-formic acid (60:40:2, v/v) as the solvent in unlined tanks. The lipids were located under UV after spraying the plates with 0.1% ethanolic dichlorofluorescein, the silica gel from the area of hexadecane-1,16-dioic acid was scraped, and the radioactivity was measured in a scintillation spectrometer.

Experiments with Actinomycin D. Thirty-five disks suberized for appropriate lengths of time were incubated aerobically with 10 ml of actinomycin D solution (5 μg/ml) for 4 hr at 30 C, washed thoroughly with sterilized H_2O, blotted with filter paper, and put back into the suberizing jar. Similar treatments with water were performed for control. After suberization for a total of 5 days, 10 disks were taken out for making acetone powder and subsequent measurement of ω-hydroxyacid dehydrogenase activity, as described in the legend of Figure 3. Another 10 disks were taken out after 2 additional days of suberization (*i.e.*, at the 7th day) for quantitation of suberin formed.

Preparation of Acetone Powder. Suberized potato disks (15–20) were homogenized with 20 to 25 ml of cold acetone (−20 C) for 45 sec in an Omni-Mixer at 80% of the full speed, and were quickly filtered and homogenized again with 20 to 25 ml of cold acetone for another 30 sec. The residue was filtered, air-dried, and stored in a small vial at −20 C until used.

Quantitation of the Aliphatic Components of Suberin. Fifteen potato disks suberized for appropriate periods of time were freeze-dried, powdered with mortar and pestle, transferred into a centrifuge tube, and washed twice successively with a 1:1 mixture of $CHCl_3$-CH_3OH, CH_3OH, and tetrahydrofuran. To the suspension of the powder in tetrahydrofuran was added 100 μg of hexadecane-1,16-dioic acid as an internal standard, and the resulting mixture was refluxed with $LiAlH_4$ for 24 hr. The reaction mixture was decomposed with H_2O, acidified, and extracted with chloroform. The diol in the hydrogenolysate was isolated by TLC and analyzed by gas chromatography as described before (14).

Enzymic Oxidation of 16-Oxohexadecanoic Acid. Protein (100 μg) was incubated at 30 C for 20 min with 1 mM NADP and 0.1 mM 16-oxo[G-^{3}H]hexadecanoic acid (2.86 × 10^6 cpm) in a total volume of 1 ml of 0.1 M glycine-NaOH buffer (pH 9.5) containing 1 mM dithioerythritol and 1 mM magnesium chloride. Since 16-oxohexadecanoic acid and its oxidation product, hexadecane-1,16-dioic acid, are not resolved well on TLC, the incubation was terminated by adding sodium borohydride, which specifically reduced the substrate 16-oxohexadecanoic acid to 16-hydroxyhexadecanoic acid, allowing a clean separation of the product, hexadecane-1,16-dioic acid, from the substrate. The $NaBH_4$-treated incubation mixture was acidified and the lipids were extracted with chloroform and then subjected to TLC with unlabeled 16-hydroxyhexadecanoic acid and hexadecane-1,16-dioic acid as internal standards. The silica gel from the region occupied by hexadecane-1,16-dioic acid was scraped and radioactivity was measured.

RESULTS AND DISCUSSION

Since dicarboxylic acids constitute a major component of the aliphatic monomers of the suberin deposited in the wound periderm of potato tubers (14), suberizing potato slices would appear to be an ideal system in which to study the enzymic synthesis of dicarboxylic acids. To determine whether suberizing potato disks contain an enzyme catalyzing the conversion of ω-hydroxyacids to dicarboxylic acids, a cell-free extract prepared from potato disks suberized for 6 days was incubated with 16-hydroxy[G-^{3}H]hexadecanoic acid, NAD, and NADP, and the lipid products were subjected to TLC. The radio TLC of the lipids showed only two peaks, one with an R_F identical to that of the substrate, and the faster moving component had an R_F identical to that of hexadecane-1,16-dioic acid. The latter was not present in the incubation mixture containing boiled cell-free extract.

For further identification of the product, the silica gel from the area corresponding to hexadecane-1,16-dioic acid was scraped and the product was eluted with ethyl ether, treated with 14% BF_3-CH_3OH, and subjected to radio GLC. All of the radioactivity was contained in a compound which had a retention time identical to that of authentic dimethyl hexadecane-1,16-dioate; thus, the enzymic reaction product was identified as hexadecane-1,16-dioic acid. Therefore, we concluded that 16-hydroxyhexadecanoic acid was converted exclusively into hexadecane-1,16-dioic acid.

Subcellular Localization. In order to determine the subcellular localization of the ω-hydroxyacid dehydrogenase, 1,000*g* particles, mitochondrial, microsomal, and 100,000*g* soluble fractions prepared by differential centrifugations were assayed for the dehydrogenase activity (Table I). The major portion of the dehydrogenase activity was present in the soluble fraction. For further experiments, the 100,000*g* supernatant fraction, or for convenience the 27,000*g* supernatant, was used as the enzyme source. In *Vicia faba* epidermis, the ω-hydroxyacid dehydrogenase activity was also found mainly in the soluble fraction (13). In rat liver preparations, ω-hydroxyacid dehydrogenase was found both in the soluble and in the microsomal fractions, although the former contained more activity than the latter (3).

Time Course and Effect of Protein Concentration. The rate of conversion of 16-hydroxyhexadecanoic acid to the dicarboxylic acid was linear with respect to protein concentration up to at least 0.15 mg/ml. The rate was linear up to at least 90 min of incubation time. In subsequent experiments, protein concentrations and incubation periods were chosen so that the rates were linear.

Cofactor Requirements. The effect of addition of various cofactors on the rate of conversion of 16-hydroxyhexadecanoic acid into the dicarboxylic acid is shown in Table II. NAD gave appreciable rates, whereas NADP gave a higher rate than that obtained with NAD. The maximal rate of conversion was observed when both NAD and NADP were included in the reaction mixture. Alcohol dehydrogenases of potato which catalyze oxidation of aliphatic alcohols, terpene alcohols, and aromatic alcohols have specificity for NAD, NADP, and NADP, respectively (5, 6). NADP is the preferred cofactor for the conversion of ω-hydroxyfatty acid to dicarboxylic acid, catalyzed by the enzyme preparation from epidermis of *V. faba* (13). In contrast to this plant enzyme, NAD is the preferred cofactor for the oxidation of ω-hydroxyfatty acids by animal microsomes and microbial systems (2, 17, 18, 21), as well as the benzyl alcohol dehydrogenase of *Pseudomonas* (23). It is important to note that these reactions are involved in catabolic pathways, while the present enzyme is probably involved in the biosynthesis of suberin. Addition of CoA did not affect the conversion of 16-hydroxyhexadecanoic acid to the dicarboxylic acid, suggesting that an acyl-CoA is not the product of this reaction. It is possible that, *in vivo*, the diacid product is transferred to the suberin polymer either directly from an acyl-enzyme intermediate or via the CoA ester. Such a reaction may not be detected in crude extracts because of the thioesterases which are most probably present in such preparations. Since the addition of both NAD and NADP in equimolar amounts gave the highest rate, both cofactors were used to assay ω-hydroxyacid dehydrogenase activity.

Effect of pH. Measurements of the rate of conversion of 16-hydroxyhexadecanoic acid into the dicarboxylic acid at different pH values showed a sharp pH optimum at 9.5 (Fig. 1). As pH increased from 7 to 9, there was a gradual increase in the ω-hydroxyacid dehydrogenase activity. This rate almost doubled when pH increased from 9 to 9.5, and at pH 10 the rate dropped

Table I. Subcellular Localization of ω-Hydroxyacid Dehydrogenase Activity

Each reaction mixture contained 0.5 mg NAD, 0.5 mg NADP, 1.92 × 10^6 cpm of 16-hydroxy[G-^{3}H]-hexadecanoic acid (100 nmole), 125 µg protein, 0.2 M pH 9.5 glycine-NaOH buffer, and 1 mM DTE in a total volume of 0.5 ml. The reaction was carried out at 30 C for 2 hr. For preparation of pellets, 2% soluble PVP was used and 15% insoluble PVP was used for the preparation of 100,000*g* supernatant.

	Dicarboxylic Acid Formed
	(nmoles/hr/mg protein)
1,000*g* pellet	4.8
15,000*g* pellet	6.8
100,000*g* pellet	3.2
100,000*g* supernatant	31.6

Table II. Cofactor Requirement for the Conversion of 16-Hydroxyhexadecanoic Acid into Hexadecane-1,16-Dioic Acid Catalyzed by the Cell-Free Preparation from Suberizing Potato Disks

The reaction mixture contained 1.64 × 10^{-4} M 16-hydroxy-[G-^{3}H]hexadecanoic acid (5 × 10^6 cpm), 0.32 mg protein, 1 mM DTE, and 0.1 M pH 9.5 glycine-NaOH buffer in a total volume of 1 ml. The incubation was done for 1 hr at 30 C.

Cofactor	Dicarboxylic Acid Formed
	(nmoles/hr/mg protein)
Boiled	7.5
No Cofactor	13.8
NAD (1 mM)	30.7
NADP (1 mM)	36.2
NAD (0.5 mM) + NADP (0.5 mM)	38.3
NAD (0.5 mM) + NADP (0.5 mM) + 1 mM CoASH	36.9

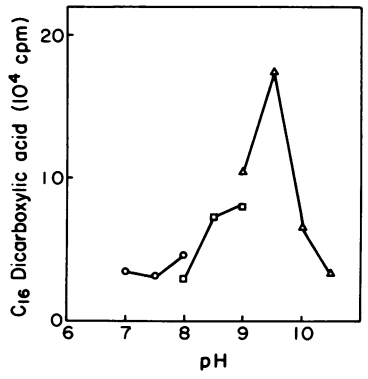

FIG. 1. Effect of pH on the rate of conversion of 16-hydroxyhexadecanoic acid to hexadecane-1,16-dioic acid by the cell-free extract from potato disks suberized for 6 days. Each reaction mixture contained 0.25 mM NAD, 0.22 mM NADP, 1 × 10^6 cpm of 16-hydroxy[G-^{3}H]hexadecanoic acid, 0.5 mg protein, 0.1 M buffer, 1 mM dithioerythritol, and 1 mM $MgCl_2$ in a total volume of 3 ml. The reaction was carried out at 30 C for 90 min. ○: phosphate buffer; □: tris-HCl buffer; △: glycine-NaOH buffer.

by a factor of 4. This result compares fairly well with that obtained with ω-hydroxyacid dehydrogenase from hog liver, which has a pH optimum of 10 (21). The conversion of ω-hydroxyfatty acid to dicarboxylic acid catalyzed by enzyme preparation from the epidermis of *V. faba* occurred maximally at pH 8 (13).

Effect of Substrate Concentration. As the concentration of 16-hydroxyhexadecanoic acid increased, the rate of oxidation increased linearly at low substrate concentrations, and further increase showed a typical substrate saturation pattern (Fig. 2). Double reciprocal plots were linear and apparent K*m* was 50 μM. The ω-hydroxyfatty acid dehydrogenase from the epidermis of *V. faba* showed a K*m* value of 12.5 μM for 16-hydroxyhexadecanoic acid (13). Because the substrate was dispersed in a detergent and an aldehyde intermediate is probably involved, the K*m* value should be taken with usual precautions.

Effect of Inhibitors. Conversion of 16-hydroxyhexadecanoic acid into the dicarboxylic acid catalyzed by the cell-free extract was completely inhibited by 0.1 mM *p*-chloromercuribenzoate, whereas 1 mM N-ethylmaleimide and 1 mM iodoacetamide inhibited the enzymic oxidation by about 80%. These results suggest that a thiol group(s) is essential for the activity of the enzyme, as previously observed wth ω-hydroxyacid dehydrogenase from epidermis of *V. faba* (13) and other fatty alcohol and aldehyde dehydrogenases (17, 18). A 60% stimulation in the activity of ω-hydroxyacid dehydrogenase by 1 mM dithioerythritol supports the conclusion that the thiol group(s) is essential for the activity of the enzyme.

16-Oxohexadecanoic Acid as an Aldehyde Intermediate. In an attempt to demonstrate that 16-oxohexadecanoic acid is the aldehyde intermediate involved in the enzymic oxidation of 16-hydroxyhexadecanoic acid to hexadecane-1,16-dioic acid, the cell-free extract prepared from potato disks suberized for 6 days was incubated with 16-oxo[G-^{3}H]hexadecanoic acid and NADP as described under "Materials and Methods." After treatment with $NaBH_4$, the reaction mixture was acidified and extracted with $CHCl_3$, the lipid products were subjected to TLC, and the radioactivity in the area corresponding to hexadecane-1,16-dioic acid was determined. The product of the reaction was identified as hexadecane-1,16-dioic acid by radio GLC, as described elsewhere in this section. NADP was found to be the preferred cofactor for the conversion of the ω-oxoacid to the dicarboxylic acid, and NAD was virtually ineffective, whereas the conversion of 16-hydroxyacid to the dicarboxylic acid occurred maximally in the presence of both NAD and NADP as shown earlier. The conversion of the ω-oxoacid to the dicarboxylic acid catalyzed by the cell-free extract is suggestive evidence for the hypothesis that the conversion of the ω-hydroxyacid to the dicarboxylic acid involves the ω-oxoacid as an intermediate. ω-Oxoacid also appears to be involved as an intermediate in the conversion of the ω-hydroxyacid to the dicarboxylic acid catalyzed by the cell-free extract of *V. faba* epidermis (13). The conversion of the ω-hydroxyacid to the dicarboxylic acid appears to be a two-step process, oxidation of the ω-hydroxyacid to the ω-oxoacid followed by oxidation of the ω-oxoacid to the dicarboxylic acid. It is possible that in the cell-free extract, either a single enzyme catalyzes both steps or there are separate enzymes catalyzing the two steps. Our recent preliminary experiments indicate that ω-hydroxyacid dehydrogenase and ω-oxoacid dehydrogenase are two distinct enzymes. The rate of conversion of the ω-oxoacid to the dicarboxylic acid was about 10 times higher than that of the ω-hydroxyacid to the dicarboxylic acid, suggesting that the conversion of the ω-hydroxyacid to the ω-oxoacid is the rate-limiting step in the synthesis of the dicarboxylic acid from the ω-hydroxyacid.

Time Course of Formation of the Aliphatic Components of Suberin and Induction of ω-Hydroxyacid Dehydrogenase Activity. It has been demonstrated that formation of aliphatic components of suberin is induced as a result of wounding the potato tuber, and its time course has been determined (14). About 3 days after preparation of the slices, the formation of aliphatic components of suberin started and became quite rapid between the 5th and 6th day, and leveled off after the 7th day (14). These results obtained with potato tissue cylinders (1 × 2 cm) might not be applicable to the thin disks used for the present study, as metabolic changes brought about by wounding are known to depend on the thickness of the tissue (15). We determined the time course of formation of aliphatic components of suberin in potato disks as described under "Materials and Methods," and found results similar to those previously obtained for potato tissue cylinders (Fig. 3). Since formation of aliphatic components of suberin is wound-induced, it was expected that the enzymes involved in suberization (*e.g.* ω-hydroxylase and ω-hydroxyacid dehydrogenase) might be also induced after wounding. Potato disks were suberized for different lengths of time and ω-hydroxyacid dehydrogenase activity was measured in disks as described under "Materials and Methods." For measurement of the dehydrogenase activity, only NADP was used as cofactor.

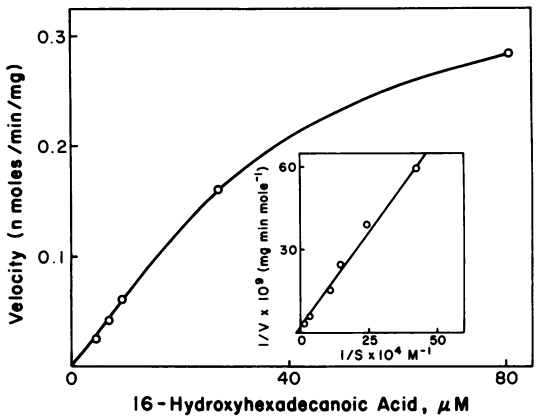

FIG. 2. Effect of the concentration of 16-hydroxyhexadecanoic acid on the rate of its oxidation to the dicarboxylic acid by the cell-free preparation from potato disks suberized for 6 days. Each reaction mixture contained, in a total volume of 4 ml, 0.5 mg protein, 1 mM NADP, 1 mM NAD, 1 mM dithioerythritol, 1 mM $MgCl_2$, and 0.1 M glycine-NaOH buffer (pH 9.5). Incubation was done for 1 hr at 30 C.

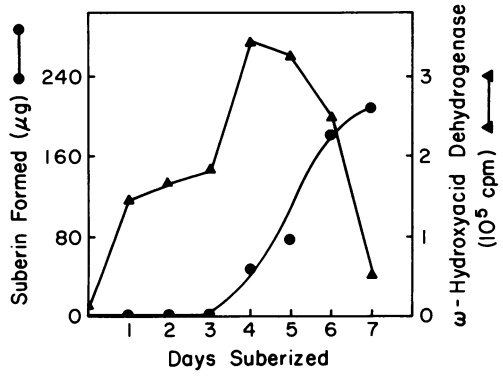

FIG. 3. Time course of formation of the aliphatic components of suberin (●——●) and changes in the ω-hydroxyacid dehydrogenase activity (▲——▲). Aliphatic components of suberin were measured as described under "Materials and Methods" and the values are expressed in μg of octadecene-1,18-diol obtained/15 disks. The ω-hydroxyacid dehydrogenase was assayed as follows. Twenty-five mg of acetone powder prepared from 15 suberized disks was suspended in 1 ml of 0.1 M glycine-NaOH buffer (pH 9.5) for 1 hr and centrifuged at 27,000*g* for 20 min. The supernatant was used for dehydrogenase activity measurement. The incubation mixture contained 0.1 mg protein, 1 mM NADP, 0.2 mM 16-hydroxy[G-^{3}H]hexadecanoic acid (1.96 × 10^6 cpm), and 0.1 M glycine-NaOH buffer (pH 9.5) in a total volume of 0.5 ml. The incubation was done at 30 C for 1 hr, and the product was measured.

The use of NAD was avoided to eliminate the possible contribution of alcohol dehydrogenase to the ω-hydroxyacid dehydrogenase activity. The ω-hydroxyacid dehydrogenase activity, hardly detectable in fresh potato disks, slowly increased until the 3rd day, peaked on the 4th or 5th day, and decreased after the 5th day, and this activity was barely detectable after the 7th day (Fig. 3). This ω-hydroxyacid dehydrogenase activity profile is compatible with that of the time course of formation of aliphatic components of suberin.

Recent [1-^{14}C]acetate incorporation studies with suberized potato disks have shown that the amount of radioactivity incorporated into the aliphatic monomers of suberin slowly increased until the 3rd day, peaked on the 5th day, and slowly decreased after the 5th day (8). The changes in the ω-hydroxyacid dehydrogenase activity observed in the present study correlate well with the time course of [1-^{14}C]acetate incorporation into aliphatic monomers of suberin and the deposition of suberin. These results suggest that the ω-hydroxyacid dehydrogenase activity being studied is induced by wounding and is involved in suberin biosynthesis.

Wound-induced respiration in potato tubers has been extensively studied (10, 16, 19, 24). It has been observed that it increases rapidly after wounding, peaks between 24 and 48 hr, and then slowly decreases, whereas the formation of the aliphatic components of suberin starts only after 72 hr of wounding. These observations show that the formation of aliphatic components of suberin is preceded by wound-induced respiration even though the latter might be a prerequisite for the former.

Effects of Cycloheximide and Actinomycin D. In an attempt to determine whether the increase in the activity of ω-hydroxyacid dehydrogenase and suberization require protein synthesis, the effects of cycloheximide and actinomycin D on these two processes were examined. The potato disks, suberized for different lengths of time, were incubated with cycloheximide solution (5 μg/ml) for 6 hr, placed on filter paper moistened with cycloheximide solution, and suberized for the total of 7 days. Their suberin content was measured as described under "Materials and Methods." The disks suberized for 1, 2, and 3 days before cycloheximide treatment contained very little suberin (< 15 μg/15 disks), whereas disks suberized for 4 and 5 days before cycloheximide treatment had 115 and 141 μg of suberin/15 disks respectively. These results suggest that either the biosynthesis of the enzyme(s) or the biosynthesis of protein(s) which activates the preformed inactive enzyme(s) is involved in the formation of the aliphatic components of suberin, and that this protein synthesis takes place before the 4th day after wounding.

To determine the approximate time when the gene transcription, involved in the formation of the aliphatic components of suberin, takes place, experiments with actinomycin D, which inhibits nucleic acid synthesis (4, 9), were performed as described under "Materials and Methods." The findings that the maximum inhibition of the formation of aliphatic components of suberin occurred when the disks were treated with actinomycin D 3 days after cutting and that little inhibition occurred when similar treatment was done 4 days after cutting (Fig. 4) suggest that the transcriptional processes directly related to the biosynthesis of aliphatic components of suberin take place between the 3rd and the 4th day after wounding under the conditions used in the present experiments. Inhibition of the formation of aliphatic components of suberin in disks treated after 1 and 2 days of suberization suggests that the DNA synthesized during this period might be directly or indirectly involved in suberin synthesis. There is also a possibility that actinomycin D applied after 1 and 2 days of aging persisted in the tissue, and thus, affected the transcriptional processes directly involved in suberization. To determine whether the appearance of ω-hydroxyacid dehydrogenase activity was affected by actinomycin D treatment, disks suberized for 1, 2, 3, and 4 days were incubated with actinomycin D and water (control) for 4 hr, and the dehydrogenase activity was measured on the 5th day as described under "Materials and Methods." Maximum inhibition in the level of the dehydrogenase activity in the disks treated with actinomycin D after 3 days (Fig. 4) suggests that the transcriptional process involved in the synthesis of this enzyme occurred 72 hr after wounding. The simultaneous inhibition of the suberin formation and ω-hydroxyacid dehydrogenase activity by actinomycin D and water strongly suggests that the ω-hydroxyacid dehydrogenase examined in the present study is involved in suberin biosynthesis.

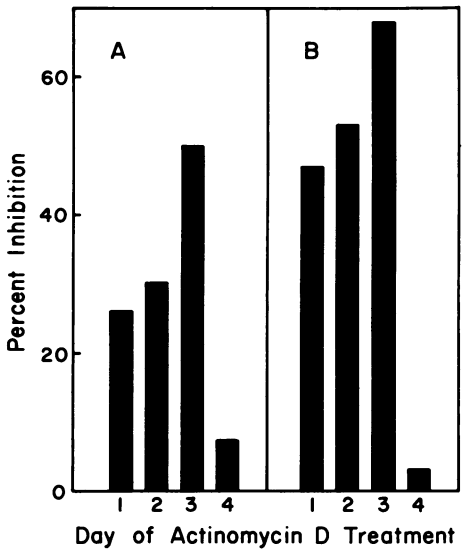

FIG. 4. Effect of actinomycin D on the formation of aliphatic components of suberin (A) and the development of ω-hydroxyacid dehydrogenase activity (B). Ten disks suberized for 1 to 5 days were treated with actinomycin D as described under "Materials and Methods," and were suberized for a total of 7 days, and the amount of aliphatic components of suberin, expressed in μg of octadecene-1,18-diol obtained/disk, was measured. Ten disks suberized for 1, 2, 3, and 4 days were treated with actinomycin D, and suberized for a total of 5 days, at which time the dehydrogenase activity was measured as described in the legend of Figure 3. Since treatment of disks with water after 1, 2, 3, 4, and 5 days of aging resulted in substantial (≈ 50%) inhibition of suberization and development of ω-hydroxyacid dehydrogenase activity, the per cent inhibition due to actinomycin D was calculated as a percentage of water-treated control. In the untreated control, the amount of suberin and dehydrogenase activity were 80 μg/disk and 0.64 nmol/min·mg protein, respectively.

Evidence that ω-Hydroxyacid Dehydrogenase is Different from Alcohol Dehydrogenase. Since alcohol dehydrogenase of potato does catalyze the oxidation of alcohols which are significantly larger than ethanol, even though only at very slow rates (5), it might also catalyze the oxidation of ω-hydroxyacid. It was important to ascertain whether alcohol dehydrogenase and ω-hydroxyacid dehydrogenase are two separate enzymes. The following experimental evidence demonstrated that ω-hydroxyacid dehydrogenase is different from alcohol dehydrogenase. (*a*) Alcohol dehydrogenase has an absolute requirement for NAD as cofactor, whereas ω-hydroxyacid dehydrogenase preferred NADP as a cofactor. (*b*) Changes in alcohol dehydrogenase activity during the suberization process do not match with the changes in ω-hydroxyacid dehydrogenase in the sense that (*i*) in fresh disks, ω-hydroxyacid dehydrogenase was hardly detectable, whereas alcohol dehydrogenase activity was quite high, and (*ii*) the alcohol dehydrogenase activity peaked on the 3rd day, whereas ω-hydroxyacid dehydrogenase activity peaked on the 4th day of suberization. (*c*) The changes in the ω-hydroxyacid dehydrogenase activity of the tissue correlated with the time course of the suberin formation, suggesting that this enzyme is

specifically involved in suberin biosynthesis. (*d*) Preliminary experimental results indicate that these two enzymes are separable by certain protein fractionation techniques (unpublished results).

CONCLUSIONS

The results presented in this paper show that the cell-free extract obtained from 6-day suberized potato disks contain ω-hydroxyacid dehydrogenase and ω-oxoacid dehydrogenase activities which can catalyze the conversion of an ω-hydroxyfatty acid to the corresponding dicarboxylic acid. The development of ω-hydroxyacid dehydrogenase activity is correlated with the time course of synthesis of aliphatic components of suberin. The final proof for induction of the synthesis of this enzyme must await isolation and purification of this enzyme, possibly followed by a direct measurement of the level of this enzyme by immunological techniques. Even though it appears that wounding induces the synthesis of this enzyme, the nature of the inducing agent is unknown. Preliminary experimental results indicate that washing of the slices inhibits the suberization process (B. B. Dean and P. E. Kolattukudy, unpublished results), and therefore it is possible that a chemical(s) produced at the wound triggers a series of events which results in suberization. Even though the dramatic metabolic changes which occur during the first 2 days after wounding have been studied extensively (10, 16, 19, 24), the relationship between such changes and the suberization process remains obscure. It is possible that the wound-induced processes can be viewed as a developmental process in which the respiratory changes and the subsequent cell divisions are followed by the final event of suberization, which is a differentiation process resulting in the formation of a protective cover on the wound. With the recent demonstration that suberization is a process involved in the wound-healing of not only storage tissues but also of aerial parts of plants such as fruits and leaves (7), it has become increasingly clear that this wound-induced process is of general significance.

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