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Biosynthesis of Cutin

ENZYMATIC CONVERSION OF ω-HYDROXY FATTY ACIDS TO DICARBOXYLIC ACIDS BY CELL-FREE EXTRACTS OF *VICIA FABA* EPIDERMIS[1]

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ABSTRACT

Long chain dicarboxylic acids are constituents of the protective biopolymers cutin and suberin of plants. Cell-free extracts from the excised epidermis of *Vicia faba* leaves catalyzed conversion of 16-hydroxy[G-^{3}H]hexadecanoic acid to the corresponding dicarboxylic acid with nicotinamide-adenine dinucleotide phosphate as the preferred cofactor. This enzymatic activity, located largely in the 100,000*g* supernatant fraction, had a pH optimum near 8. This dehydrogenase showed an apparent Km of 1.25×10^{-5} M and 3.6×10^{-4} M for 16-hydroxyhexadecanoic acid and NADP, respectively. Modification of the substrate, either by esterification of the carboxyl group or by introduction of another hydroxyl group at C-10, resulted in a substantial (two-thirds) decrease in the rate of reaction, and hexadecanol was not a good substrate. The enzyme was inhibited by thiol reagents such as N-ethylmaleimide and *p*-chloromercuribenzoate. The aldehyde intermediate was trapped by the inclusion of dinitrophenyl hydrazine in the reaction mixture, and the 16-oxo compound was regenerated and identified. Furthermore, synthetic 16-oxo-[G-^{3}H] hexadecanoic acid was readily converted to the dicarboxylic acid by the cell-free preparation. These results demonstrate that epidermis of *Vicia faba* contains an ω-hydroxyacid dehydrogenase and an ω-oxoacid dehydrogenase.

Long chain dicarboxylic acids are produced by animals (3, 4, 25, 26, 29, 30), microbes (10, 16, 17), and higher plants (5, 8, 9, 11, 12, 26, 27, 31). In animals, such acids appear to be intermediates in a catabolic pathway, namely ω-oxidation, which has no known specific functions, although under certain abnormal conditions, such as ketosis, an increased production of dicarboxylic acids has been observed (23, 24). In plants, on the other hand, dicarboxylic acids are constituents of the protective polymers, cutin and suberin. Free, very long chain (C_{28} and C_{30}) dicarboxylic acids occur also in the surface lipids of plants, particularly on spores (1, 20). Since fossil spores are preserved in peat and various sediments, in lignite, coal, and possibly even pre-Cambrian shales, dicarboxylic acids have also attracted the attention of geochemists.

The most likely precursor of dicarboxylic acids would be the corresponding ω-hydroxyacids. Cutin and suberin of higher plants contain both dicarboxylic acids and ω-hydroxyacids. However, oxidation of ω-hydroxyacids to the corresponding dicarboxylic acids does not appear to have been demonstrated in higher plants. Since the epidermal layer of cells seems to be the site of cutin biosynthesis (14), we examined this tissue for the presence of enzymes that can catalyze the formation of dicarboxylic acids. In this paper, we describe the enzymatic conversion of 16-hydroxypalmitic acid to the corresponding dicarboxylic acid by cell-free extracts prepared from excised epidermis of young *Vicia faba* leaves.

MATERIALS AND METHODS

Plants. Broad bean (*Vicia faba*) plants were grown from seed purchased from the Burpee Co., in a soil-sand-peat moss (1:1:1) mixture under wide spectrum Gro-lux (very high output) lights (about 1200 ft-c) supplemented with incandescent lights with 16-hr days. The most rapidly expanding leaves (about 4 × 2.5 cm) were used for enzyme preparations.

Substrates and Reagents. 16-Hydroxy[G-^{3}H]hexadecanoic acid and 10,16-dihydroxy[G-^{3}H]hexadecanoic acid were prepared by tritium gas exposure according to Wilzbach's method, followed by rigorous purification as previously described (11). The unlabeled 16-hydroxyhexadecanoic acid was a generous gift from Dr. A. P. Tulloch and the 10,16-dihydroxyhexadecanoic acid was isolated from *V. faba* cutin as described previously (15). For specificity studies, methyl esters of substrate acids were prepared with 14% BF_3 in methanol as described below. [1-^{14}C]Hexadecanol was prepared from [1-^{14}C]hexadecanoic acid (Amersham-Searle Corp.) by $LiAlH_4$ reduction followed by thin layer chromatographic purification on Silica Gel G with hexane-ethyl ether-formic acid (65:35:2, system A) as the developing solvent. 16-Oxo[G-^{3}H]hexadecanoic acid was synthesized by V. P. Agrawal of this laboratory by oxidation of 16-hydroxy[G-^{3}H]hexadecanoic acid with CrO_3 in pyridine. The oxo-acid was purified by TLC on Silica Gel G with ethyl ether-hexane-formic acid ((25:25:1, v/v) as the developing solvent. The substrates were dispersed in H_2O with the aid of Tween-20 and sonication as described before (15). Authentic standards of hexadecane-1,16-dioic acid and 7-hydroxyhexadecane-1,16-dioic acid were prepared by oxidation of 16-hydroxyhexadecanoic acid and 10,16-dihydroxyhexadecanoic acid, respectively, with CrO_3 in 95% acetic acid

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at room temperature; the 7-oxo-dioic acid was reduced to the corresponding hydroxydioic acid by $NaBH_4$ treatment. The diacids (as dimethyl esters) were purified by TLC as described above (system A). Cofactors and inhibitors were purchased from Sigma Chemical Co.

Enzyme Preparation. The epidermal layer of cells was excised from young, rapidly expanding *V. faba* leaves and these tissue slices were placed in ice-cold 0.1 M sodium phosphate buffer (pH 8) containing 0.3 M sucrose, 5×10^{-4} M dithioerythritol, 10^{-3} M $MgCl_2$, and about 200 mg of insoluble PVP. The epidermal strips, usually collected from 20 to 30 leaf pairs, were ground in this medium with a TenBroeck homogenizer. The homogenate was centrifuged at 27,000*g* for 20 min and the supernatant was used as the enzyme source. In the subcellular localization experiments, the homogenate was first centrifuged at 3000*g* for 5 min and the resulting supernatant was centrifuged at 15,000*g* for 20 min to obtain the mitochondrial fraction. Further centrifugation of this 15,000*g* supernatant at 105,000*g* for 90 min provided the microsomal fraction and the soluble supernatant.

Assay for ω-Hydroxyacid Dehydrogenase. Usually the reaction mixtures, which contained 100 to 200 μg of protein, 10^{-3} M NADP, 0.3 M sucrose, 5×10^{-4} M DTE, 10^{-3} M $MgCl_2$, and 16-hydroxy[G-^{3}H]hexadecanoic acid in a total volume of 1.5 ml, were incubated at 30 C for 2 to 4 hr. At the end of the incubation period, the reaction was stopped by the addition of a few drops of concentrated HCl and the reaction mixture was mixed with about 100 ml of a 2:1 mixture of chloroform and methanol to give a single phase. After this mixture was shaken with 30 ml of 1 N HCl, the chloroform layer was withdrawn. The aqueous phase was extracted twice with 50-ml portions of chloroform and the pooled chloroform extract was evaporated to dryness under reduced pressure. The lipids thus recovered were refluxed with 14% BF_3 in methanol for 16 hr. The reaction products, recovered in the usual manner, were subjected to TLC (system A) after the addition of authentic unlabeled dimethyl hexadecane-1,16-dioate. The dimethylester region was located under UV light after spraying the chromatogram with an ethanolic solution of 2,7-dichlorofluorescein. The ^{3}H contained in the silica gel scraped from this region was assayed directly with a liquid scintillation spectrometer.

Detection of the 16-Oxohexadecanoic Acid Intermediate. The reaction mixture containing 5×10^{-5} M 16-hydroxy[G-^{3}H]-hexadecanoic acid, 10^{-3} M NADP, 150 μg of protein, and the other components indicated in the standard assay, in a total volume of 1.5 ml, was saturated with dinitrophenyl hydrazine. After incubation at 30 C for 5.5 hr, the products were extracted and methylated as described in the previous section. The products were subjected to TLC on Silica Gel G (system A). The radioactive material contained in the silica gel recovered from the diester region was eluted with a 2:1 mixture of chloroform and methanol. This material was refluxed with 100 mg of pyruvic acid in a 5:1 mixture of dioxane and H_2O for 40 min. The products were extracted with chloroform and evaporated to dryness under reduced pressure. In order to specifically reduce any regenerated aldehyde, this material was treated with $NaBH_4$ in methanol for 15 min at room temperature. The products isolated by solvent extraction were again treated with 14% BF_3 in methanol and the methylated products were subjected to TLC on Silica Gel G (system A). The labeled material from the methyl 16-hydroxyhexadecanoate region was recovered and acetylated with a 2:1 mixture of acetic anhydride and pyridine at room temperature overnight. The acetylated product was subjected to TLC with hexane-ethyl ether (3:2) as the developing solvent. The labeled material with an R_F identical to that of methyl 16-acetoxyhexadecanoate was recovered and subjected to radio GLC as described under Figure 2b. When the regenerated aldehyde fraction was subjected to TLC, no label appeared in the region of methyl 16-hydroxypalmitate; however, a labeled material with an R_F slightly less than that of dimethyl hexadecane-1,16-dioate was noted. This material was recovered and treated with $NaBH_4$ in methanol at room temperature for 15 min. The products were isolated and subjected to TLC with methyl 16-hydroxyhexadecanoate as the standard.

Chromatography. Thin layer chromatography was performed in lined tanks with 0.5-mm layers of Silica Gel G activated at 110 C overnight. Radio GLC was performed with a Perkin-Elmer gas chromatograph attached to a Barber-Colman radioactivity monitor. Gas chromatographic conditions used are described under the appropriate figures.

Determination of Radioactivity. Radioactivity in soluble lipid samples and thin layer chromatographic fractions was determined in a counting solution consisting of 0.4% (w/v) Omnifluor dissolved in 30% ethanol in toluene with a Packard liquid scintillation spectrometer. Radioactivity on thin layer plates was also monitored with a Berthold thin layer scanner. All assays were conducted with a standard deviation less than 3% and counting efficiencies were determined with internal standards (^{3}H, 14% and ^{14}C, 72%).

Protein Determination. Protein was estimated by the method of Lowry *et al.* (21), after precipitation with 10% trichloroacetic acid. The precipitate was washed with 80% acetone and dissolved in 0.1 N NaOH before the assay.

RESULTS AND DISCUSSION

C_{16} and C_{18} dicarboxylic acids constitute a common component of cutin of all plants thus far examined (14). Because the epidermal layer of cells seems to be the site of synthesis of cutin, a cell-free extract prepared from the excised epidermis of *V. faba* leaves was incubated with the suspected precursor, 16-hydroxy[G-^{3}H]palmitic acid, NAD, and NADP. The methyl esters of the chloroform-soluble products were subjected to TLC (Fig. 1a). The only major labeled product found had an R_F identical to that of authentic dimethyl hexadecane-1,16-dioate. Gas chromatographic examination of this product showed that all the ^{3}H in the product was contained in a compound which had a retention time identical to that of authentic dimethyl hexadecane-1,16-dioate (Fig. 1b). Thus, 16-hydroxyhexadecanoic acid was converted exclusively into hexadecane-1,16-dioic acid.

Cofactor Requirements. The effect of addition of various cofactors on the rate of dicarboxylic acid formation from 16-hydroxyhexadecanoic acid in epidermal extracts is shown in Table I. NADP was the preferred cofactor and NAD gave very low yields of the dicarboxylic acid. 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, 7, 16, 17, 22), as well as the benzyl alcohol dehydrogenase of *Pseudomonas* (28). It is noteworthy that these reactions are involved in catabolic pathways while the present enzyme is presumably involved in the biosynthesis of cutin. Addition of CoA did not affect the conversion of the ω-hydroxyacid to the dicarboxylic acid, suggesting that an acyl-CoA is not the product of this reaction. However, it is possible that, *in vivo*, the diacid product is transferred to the cutin 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 that are most probably present in such preparations. Addition of ATP and CoA did not increase the rate of dicarboxylic acid formation, suggesting that activation of the carboxyl group is not required for the

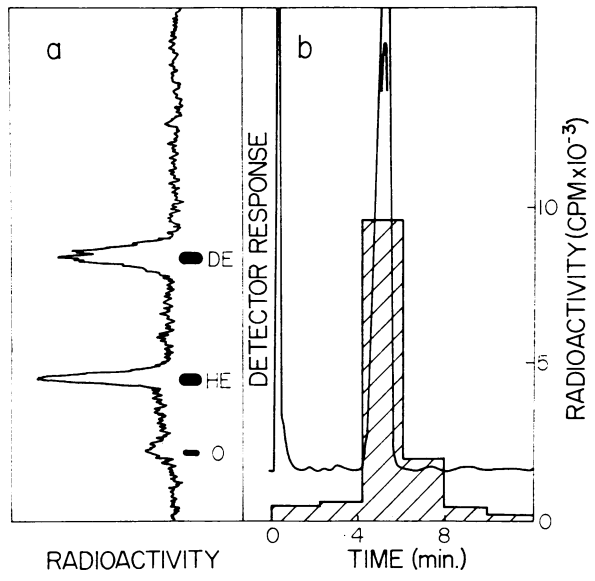

FIG. 1. (a) Radio thin-layer chromatogram of methylated lipid products obtained by incubation of 16-hydroxy[G-^{3}H]hexadecanoic acid with an extract of *V. faba* epidermis and NADP for 4 hr at 30 C. O: origin; HE: authentic methyl 16-hydroxyhexadecanoate; DE: authentic dimethyl hexadecane-1,16-dioate. TLC was performed on 0.5-mm Silica Gel G with hexane-ethyl ether-formic acid (65:35:2 v/v) as the developing solvent. The chromatogram was monitored with a Berthold thin-layer scanner. (b) Radio gas-liquid chromatogram of the dimethyl hexadecane-1,16-dioate fraction isolated from the thin-layer chromatogram shown in Figure 1a. The bar graph represents the radioactivity which was assayed in the material trapped by passing the effluent through pasteur pipets packed with glass wool. GLC was performed with a coiled stainless steel column (152.4 × .64 cm) packed with 5% OV-1 on 80–100 mesh Gas Chrome Q, held at 210 C with an argon flow rate of 120 cm^3/min.

Table I. *Effect of Cofactors on Conversion of 16-Hydroxyhexadecanoic Acid to Hexadecane-1,16-dioic Acid*

Reaction mixtures contained, where indicated, 1 mg of NAD, 1 mg of NADP, 10 mg of ATP, 1 mg of CoA, 5.3 × 10^6 cpm 16-hydroxy[G-^{3}H]hexadecanoic acid, and 300 μg of protein in a total volume of 3 ml of 0.1 M phosphate buffer, pH 7.9, containing 0.3 M sucrose, 5 × 10^{-4} M dithioerythritol, and 10^{-3} M $MgCl_2$. Incubation was at 30 C for 90 min.

Additions	Product
	cpm × 10^{-5}
None	2.1
NAD	2.4
NADP	8.5
NAD + NADP	5.8
NADP + CoA + ATP	7.1
NADP + CoA	8.9
NAD + NADP − boiled enzyme	1.6

oxidation at the ω-carbon. Usually, the inclusion of these two cofactors resulted in a decrease in dicarboxylic acid formation, presumably due to oxidative degradation of the substrate or the dicarboxylic acid. On the other hand, hydroxylation at the C-10 position of ω-hydroxyhexadecanoic acid required activation of the carboxyl group (32).

Aldehyde Intermediate. There is little doubt that a 16-oxo intermediate is involved in the conversion of 16-hydroxyhexadecanoic acid into the dicarboxylic acid. However, the 16-oxo compound did not accumulate in the reaction mixtures, although small amounts of ^{3}H (<10% of the ^{3}H found in the dicarboxylic acid) could be detected in a material tentatively identified as the 16-oxo compound. Therefore, the 16-oxo intermediate is either enzyme bound or it is rapidly oxidized to the dicarboxylic acid. In order to test these possibilities, dinitrophenyl hydrazine was included in a reaction mixture which contained the enzyme, the labeled ω-hydroxy acid, and NADP. Thin layer chromatographic analysis of the chloroform-soluble products, as methyl esters, revealed the presence of labeled dimethylester of dicarboxylic acid and, possibly, a slightly more polar product which was incompletely resolved from dimethyl hexadecanedioate. This presumed mixture was isolated and then treated with pyruvic acid in order to regenerate the aldehyde from the suspected dinitrophenyl hydrazone. The products isolated by solvent extraction were treated at room temperature with $NaBH_4$ in order to convert any aldehyde generated into the alcohol, and the chloroform-soluble products were isolated and subjected to TLC (Fig. 2a). A radioactive product with an R_F identical to that of the methyl ester of 16-hydroxyhexadecanoic acid was found, and this product contained 15% of the ^{3}H contained in the original mixture of products; the remaining portion of the radioactivity was in the dimethyl hexadecane-1,16-dioate fraction. The methyl 16-hydroxyhexadecanoate fraction generated from the dinitrophenyl hydrazone was acetylated, and the only labeled product showed an R_F identical to that of authentic

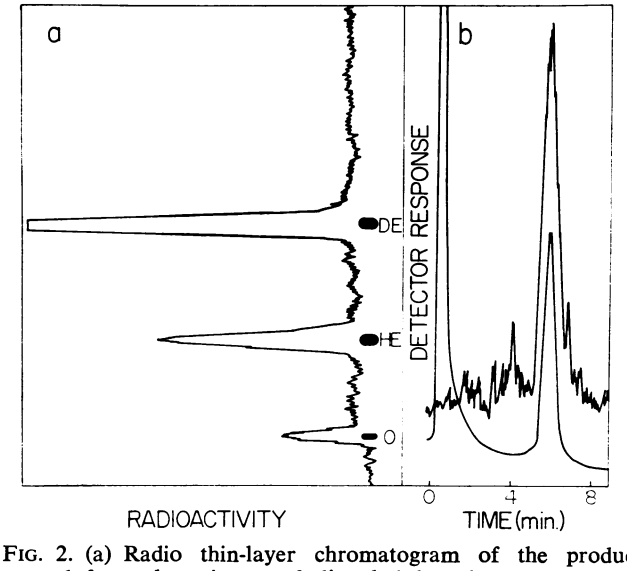

FIG. 2. (a) Radio thin-layer chromatogram of the products generated from the mixture of dimethyl hexadecane-1,16-dioate and the hydrazone of methyl 16-oxohexadecanoate by sequential treatment of this mixture with pyruvic acid and $NaBH_4$. The dioate-hydrazone mixture was derived from a reaction mixture containing *V. faba* extract (150 μg of protein), 10^{-3} M NADP, 5 × 10^{-5} M 16-hydroxy[G-^{3}H]hexadecanoic acid, saturating concentration of dinitrophenyl hydrazine, and the other usual components of the standard dehydrogenase assay described under "Material and Methods." Incubation was for 5.5 hr at 30 C. O: origin; HE: authentic methyl 16-hydroxyhexadecanoate; DE: authentic dimethyl hexadecane-1,16-dioate. (b) Radio gas-liquid chromatogram of acetylated HE fraction of Figure 2a. The smooth bottom tracing represents the flame ionization detector response obtained from the co-injected authentic methyl 16-acetoxyhexadecanoate. The top tracing shows the radioactivity as recorded by a Barber-Colman radioactivity monitor attached to the gas chromatograph. GLC was performed with a coiled stainless steel column (152.4 × .64 cm) packed with 5% OV-1 on 80–100 mesh Gas Chrome Q, held at 198 C with an argon flow rate of 120 cm^3/min.

methyl 16-acetoxyhexadecanoate when subjected to thin layer chromatography. Radio GLC showed that all of the radioactivity coincided with the coinjected authentic methyl 16-acetoxyhexadecanoate (Fig. 2b). These experimental results demonstrate the formation of the aldehyde intermediate during the enzymatic conversion of 16-hydroxyhexadecanoic acid to the corresponding dicarboxylic acid. Furthermore, these preparations converted synthetic 16-oxohexadecanoic acid to the dicarboxylic acid at a rate nearly 10 times higher than the rate of formation of the dicarboxylic acid from the 16-hydroxy compound. An aldehyde intermediate could not be shown in the rat liver microsomal preparations which catalyzed conversions of ω-hydroxy acid to the corresponding dicarboxylic acid but no trapping experiments were reported (2). It is possible that in the membrane preparations, such as those obtained from the rat liver, the ω-hydroxyacid dehydrogenase and the aldehyde dehydrogenase are tightly coupled, while in the soluble preparations the two activities are not very tightly coupled.

Subcellular Localization. In order to determine the subcellular localization of the ω-hydroxyacid dehydrogenase, mitochondrial, microsomal, and the 100,000*g* soluble fractions prepared by differential centrifugations were assayed. The major portion (about 80%) of the dehydrogenase was present in the soluble fraction, while both the mitochondrial and microsomal fractions contained about 10% each of the total activity. Therefore, for further experiments, the 100,000*g* supernatant fraction, or for convenience the 27,000*g* supernatant, was used as the enzyme source. 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).

Effect of pH. Very little dehydrogenase activity could be observed below pH 6. As the pH increased from 6 to 8, there was a dramatic increase in the ω-hydroxyacid dehydrogenase activity in phosphate buffer (Fig. 3). At pH 8, borate buffer gave only about half of the rate observed with phosphate buffer of the same pH. Therefore, we routinely used phosphate buffer at pH 8.

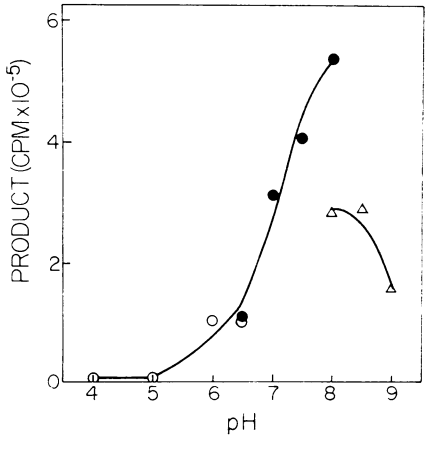

FIG. 3. Effect of pH on the rate of conversion of 16-hydroxyhexadecanoate to hexadecane-1,16-dioic acid by *V. faba* epidermal extracts. A 27,000*g* supernatant fraction of *V. faba* epidermal extract was 80% saturated with ammonium sulphate and the precipitated protein was dissolved in 0.05 M phosphate buffer. To each reaction mixture was added 0.2 ml of this enzyme solution. The reaction was carried out at 30 C for 2 hr in a total volume of 3.4 ml of 0.1 M appropriate buffer, containing 1 mg NADP and 16-hydroxy[G-^{3}H]hexadecanoic acid (2.6×10^6 cpm). ○: citrate-phosphate buffer; ●: phosphate buffer; Δ: borate buffer.

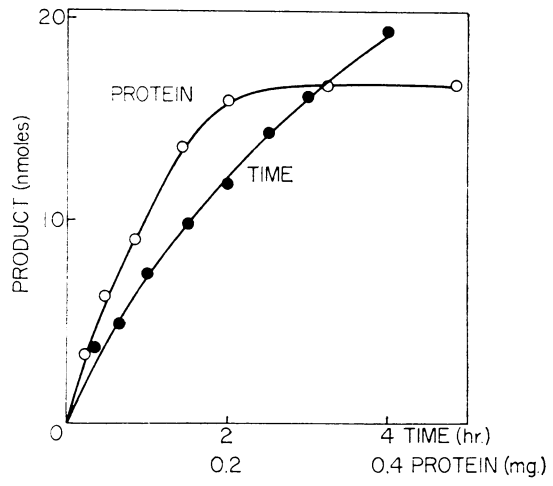

FIG. 4. Effect of time and protein concentration on the rate of conversion of 16-hydroxyhexadecanoic acid to hexadecane-1,16-dioic acid by *V. faba* epidermal extracts. Each reaction mixture contained 10^{-3} M NADP, and 5×10^{-5} M 16-hydroxy[G-^{3}H]hexadecanoic acid in a total volume of 1.5 ml 0.1 M sodium phosphate buffer, pH 8, containing 0.3 M sucrose, 5×10^{-4} M dithioerythritol, and 10^{-3} M $MgCl_2$. Each reaction mixture in the time-course experiment contained 200 μg of protein, and in the protein dependence experiment, 3 hr assays were done; in both cases incubation temperature was 30 C.

Effect of Time and Protein Concentration. Rate of conversion of ω-hydroxyhexadecanoic acid to the dicarboxylic acid increased linearly with an increase in protein concentration up to about 200 μg under the standard assay conditions (Fig. 4). The rate of reaction was linear up to about 4 hr of incubation time (Fig. 4). With μM concentration of the substrate, there was a lag period of about 1 to 1.5 hr after which the rate was linear for up to at least 4 hr. Because at higher concentrations of substrate such a lag period was not observed, we did not further examine this lag period. All further experiments were done within the linear range of protein concentrations and time of incubation.

Effect of Concentration of ω-Hydroxyhexadecanoic Acid and NADP on Rate of Dicarboxylic Acid Formation. As the concentration of ω-hydroxyhexadecanoic acid increased, the rate of dicarboxylic acid formation increased in a linear fashion, and further increases resulted in a typical substrate saturation pattern (Fig. 5). The double reciprocal plot gave a straight line from which apparent *Km* and V_{max} of 1.25×10^{-5} M and 17.4 μmoles/l-mg-hr, respectively, were calculated. Because the substrate was not in a true solution and an aldehyde intermediate is involved, these values should be taken with the usual precautions. It is obvious, however, that at fairly low concentrations of the substrate, moderately good rates of dicarboxylic acid formation were observed. From the data obtained with rat liver microsomes (2), it seems that this enzyme also showed substrate saturation at about the same concentration as that observed with the plant enzyme. The enzyme preparation also showed a typical substrate saturation pattern with increasing concentrations of NADP (Fig. 6). The double reciprocal plot gave a straight line from which an apparent *Km* value of 3.6×10^{-4} M was calculated for NADP.

Substrate Specificity. Even though fatty alcohol dehydrogenase from higher plants has not been studied, rapid oxidation of exogenous labeled fatty alcohol has been observed in plant tissues (13). In order to test whether the oxidation of the ω-hydroxyacid observed in our preparation is due to such an enzyme, the oxidation of hexadecanol was examined (Table

II). This alcohol was converted to hexadecanoic acid at only one-third of the rate of oxidation of ω-hydroxyhexadecanoic acid. Esterification of the carboxyl group of the ω-hydroxyhexadecanoic acid resulted in a substantial (64%) decrease in the rate of oxidation of the primary alcohol function at the ω-carbon. Presence of another hydroxyl group in the middle of the chain also decreased the rate of oxidation of the primary alcohol at the ω-carbon. For example, 10,16-dihydroxyhexadecanoic acid was oxidized at only 36% of the rate observed with 16-hydroxyhexadecanoic acid. The methyl ester of the dihydroxy acid was oxidized more slowly than the free dihydroxy acid. From these limited studies of substrate specificity, it seems that the dehydrogenase of the epidermal extract is, indeed, specific for ω-hydroxyacids. It is likely that this enzyme is involved in the biosynthesis of the dicarboxylic acids found in the cuticular polymer, cutin.

Inhibitors. Conversion of 16-hydroxyhexadecanoic acid to dicarboxylic acid, catalyzed by the epidermal enzyme preparation, was severely inhibited by thiol reagents such as N-ethylmaleimide and *p*-chloromercuribenzoate (Table II). These

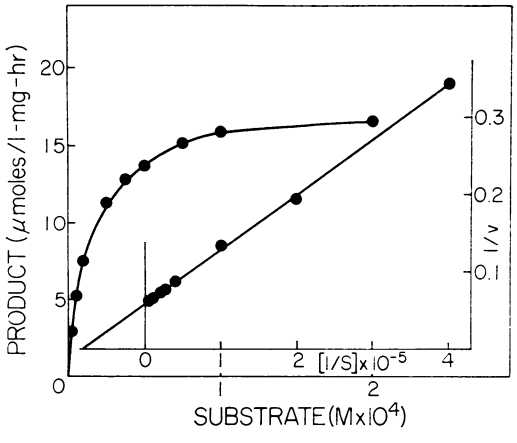

FIG. 5. Effect of the concentration of 16-hydroxyhexadecanoic acid on the rate of its oxidation to the dicarboxylic acid by the *V. faba* cell-free preparation. Each reaction mixture contained, in a total volume of 1.5 ml, 140 μg of protein, 10^{-3} M NADP, and the other components indicated under Figure 4. Incubation was for 2 hr at 30 C.

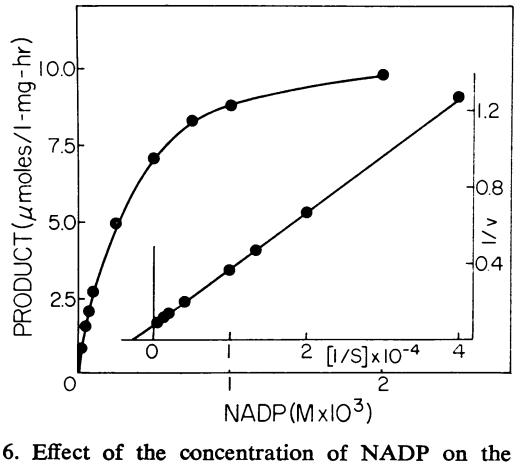

FIG. 6. Effect of the concentration of NADP on the rate of oxidation of 16-hydroxyhexadecanoic acid to the dicarboxylic acid by the *V. faba* cell-free preparation. Each reaction mixture contained, in a total volume of 1.5 ml, 180 μg of protein, 2.5×10^{-5} M 16-hydroxy[G-^{3}H]hexadecanoic acid, and the other components indicated under Figure 4. Incubation was for 2 hr at 30 C.

Table II. *Substrate Specificity and Inhibitors of ω-Hydroxyacid Dehydrogenase from V. faba Epidermis*

In experiment 1 the reaction mixtures contained 150 μg of protein, 5×10^{-5} M 16-hydroxy[G-^{3}H]hexadecanoic acid, and 10^{-3} M NADP in a total volume of 1.5 ml of 0.1 M sodium phosphate buffer, pH 8, containing 10^{-3} M $MgCl_2$ and 0.3 M sucrose. Incubation was at 30 c for 3 hr. The rate of formation of hexadecane-1,16-dioic acid without any inhibitor was 11.1 μmoles/1-mg-hr. In experiment 2 similar experimental conditions were used except that 5×10^{-4} M dithioerythritol was included, and the concentration of each substrate was 10^{-4} M. In this experiment, the oxidation rate for 16-hydroxyhexadecanoic acid was 9.3 μmoles/1-mg-hr.

Substrate or Inhibitor	Rate
	% of control
Experiment 1	
No inhibitor	100
N-Ethylmaleimide (10^{-3} M)	37
p-Chloromercuribenzoate (5×10^{-4} M)	16
Experiment 2	
16-Hydroxy[G-^{3}H]hexadecanoic acid	100
Methyl 16-hydroxy[G-^{3}H]hexadecanoate	36
10,16-Dihydroxy[G-^{3}H]hexadecanoic acid	36
Methyl 10,16-dihydroxy[G-^{3}H]hexadecanoate	24
[1-^{14}C]Hexadecanol	33

results are similar to those observed with other fatty alcohol and aldehyde dehydrogenase preparations (18, 19).

Conversion of ω-hydroxyhexadecanoic acid to the corresponding dicarboxylic acid did not require activation of the carboxyl group. In contrast, the other steps which are involved in the synthesis of cutin monomers from palmitic acid, namely, hydroxylation at C-10, as well as the synthesis of the polymer from monomers, require activation of the carboxyl group (6, 14, 32). It seems that the small amount of free ω-hydroxyacid released from the enzyme system, which is involved in the hydroxylations and the acyl transfers, is incorporated into cutin after oxidation to the dicarboxylic acid by the soluble dehydrogenase reported in this paper. This type of a dehydrogenase must also participate in the biosynthesis of suberin, the protective polymer of the underground parts of plants, because dicarboxylic acids constitute one of the major components of this polymer (12, 27). Recently, an ω-hydroxyacid dehydrogenase and an ω-oxoacid dehydrogenase were found to be induced in suberizing potato slices (Agrawal and Kolattukudy, unpublished). Thus, these ω-hydroxyacid dehydrogenases probably play a key role in the biosynthesis of the protective polymers of plants.

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