Biosynthesis of Hydroxyfatty Acid Polymers. Enzymatic Synthesis of Cutin from Monomer Acids by Cell-Free Preparations from the Epidermis of *Vicia faba* Leaves†

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ABSTRACT: A particulate preparation from epidermal extracts of young *Vicia faba* leaves catalyzed the incorporation of palmitic acid, 16-hydroxypalmitic acid, and 10,16-dihydroxypalmitic acid into an insoluble material with adenosine triphosphate and coenzyme A as the required cofactors. Sequential treatment of the insoluble residue with hydrolytic enzymes, and chemical depolymerization studies, demonstrated that these acids were esterified to cutin, the hydroxyfatty acid polymer of plant cuticle. The apparent K_m values for palmitic acid, 16-hydroxypalmitic acid, and 10,16-dihydroxypalmitic acid were 2.0×10^{-5}, 6.7×10^{-5}, and 1.1×10^{-4} M, respectively. Fatty acids from C_{10} to C_{18}, and C_{18}-hydroxyfatty acids could function as substrates, but the C_{16} family of acids was preferred, consistent with the chain length of acyl moieties found in *V. faba* cutin. Methyl esters and fatty alcohols were not significantly incorporated into cutin, but acetates of hydroxy acids were incorporated with 60–70% efficiency of the parent acid, suggesting that the carboxyl end of the incoming monomer was transferred to free hydroxyls of the polymer. With free acids as substrates the system had two pH optima, near 7.0 and near 8.5, while with palmitoyl-coenzyme A a single optimal pH (near 8.5) was observed, indicating that activation of the carboxyl group and transacylation occur optimally at pH 7.0 and pH 8.5, respectively. Sulfhydryl inhibitors did not decrease the incorporation of palmitoyl-CoA into cutin, but diisopropyl fluorophosphate (10^{-4} M) inhibited incorporation by 50%. Mild sonication of the particulate preparation provided an enzyme system that required exogenous purified cutin as a primer. Transacylase activity was proportional to the amount of *V. faba* cutin added up to a level of 3 mg/ml. This primer-dependent system required the same cofactors and utilized the same substrates as the particulate preparation. A variety of chemically distinct cutins from several plant species could function as primers, but *V. faba* cutin was strongly preferred. Cellulose, glycerol, hexadecanol, cholesterol, and polyethylene glycol were ineffective as acyl acceptors. Methylation of the carboxyl groups of *V. faba* cutin had no effect on its efficiency as a primer, indicating that free carboxyl groups of cutin were not involved in the esterification process. Acetylation of *V. faba* cutin decreased primer efficiency by 70%, confirming the requirement for cutin hydroxyl groups as acyl acceptors. Cutin prepared from young *V. faba* leaves was a more efficient primer than that prepared from mature, fully expanded leaves, suggesting that the enzyme prefers the less developed polymer. Treatment of *V. faba* cutin with cutinase doubled the efficiency of the primer, but extended treatment decreased the priming efficiency. Chemical treatments of cutin which increased the number of hydroxyl groups or opened the polymer matrix also increased priming efficiency. Enzyme preparations capable of esterifying acyl moieties to cutin were also obtained from *V. faba* flowers and the epidermis of *Senecio odoris* leaves. This is the first report on an enzyme which catalyzes the formation of a hydroxyfatty acid biopolymer, cutin.

Polymeric materials provide the protective coverings of all living organisms. In animals, this function is performed by protein or chitin, while in plants a similar function is performed by cutin, a meshwork of polymerized, cross-esterified hydroxyfatty acids (Mazliak, 1968; Martin and Juniper, 1970; Kolattukudy and Walton, 1972a). Varying proportions of a C_{18} and a C_{16} family of monomer acids are found in cutin. Oleic acid, 18-hydroxyoleic acid, 18-hydroxy-9,10-epoxystearic acid, and 9,10,18-trihydroxystearic acid, along with their Δ^{12} analogs, constitute the bulk of the C_{18} family of cutin acids, while palmitic acid, 16-hydroxypalmitic acid, and 10,16-dihydroxypalmitic acid and/or its positional isomers comprise the C_{16} family (Kolattukudy and Walton, 1972a; Walton and Kolattukudy, 1972a; Kolattukudy *et al.*, 1973). The C_{16} family of acids is found in the cutin of most plants and these acids are the major components of the cutin of *Vicia faba* (Kolattukudy and Walton, 1972a,b; Walton and Kolattukudy, 1972a).

Previous studies with *V. faba* leaf slices (Kolattukudy, 1970a; Kolattukudy and Walton, 1972b) strongly suggested that palmitic acid is hydroxylated in turn at C-16 and C-9 or C-10 and then incorporated into cutin. In such studies, virtually all of the hydroxylated C_{16} fatty acids were found in the insoluble cutin, with barely detectable amounts in the soluble lipids, suggesting that free monomers are not formed (Kolattukudy, 1970a; Kolattukudy and Walton, 1972b). Additionally, activation of the carboxyl group was required for hydroxylation of 16-hydroxyplamitic acid to 10,16-dihydroxypalmitic acid in cell-free preparations from *V. faba* leaf epidermis (Walton and Kolattukudy, 1972b), and the major part of the 10,16-dihydroxypalmitic acid formed from 16-hydroxypalmitic acid was found in an insoluble polymer (P. E. Kolattukudy, unpublished). Therefore, it was suggested that activated fatty acids were sequentially hydroxylated and, subsequently, the hydroxyacyl moieties were transferred to the growing cutin polymer (Kolattukudy and Walton, 1972a). However, the biological polymerization process has not been heretofore studied, except for an earlier report of preliminary findings (Croteau and Kolattukudy, 1973).

In this paper we describe a particulate enzyme preparation which catalyzes incorporation of monomer acids into cutin with

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ATP and CoA as cofactors. A primer-dependent enzyme system that catalyzes the same reaction, but which requires exogenous, purified cutin as a primer, is also described.

Experimental Section

Plants. Broad bean (*Vicia faba*) plants were grown from seed purchased from the Burpee Co., in a soil–sand–peatmoss (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. Unless otherwise specified, the most rapidly expanding young leaves (about 4 × 2.5 cm) from plants no higher than 20 cm were used for enzyme preparations.

Substrates and Reagents. 1-^{14}C-labeled fatty acids from C_{10} to C_{18}, purchased from Amersham-Searle, were diluted with unlabeled authentic acids to a specific activity of 5 Ci/mol. [1-^{14}C]Palmitoyl-CoA, purchased from New England Nuclear Corp., was diluted with unlabeled palmitoyl-CoA (Sigma Chemical Co.) to a specific activity of 1 Ci/mol. [G-^{3}H]-16-Hydroxypalmitic acid and [G-^{3}H]-10,16-dihydroxypalmitic acid were prepared by tritium exposure according to Wilzbach's method, and rigorously purified as previously described (Walton and Kolattukudy, 1972b; Croteau and Kolattukudy, 1973). [G-^{3}H]-10,16-Dihydroxypalmitic acid was used at a specific activity of 282 Ci/mol for initial experiments (definition of reaction, cofactor requirements, localization) and was then diluted with unlabeled material to 25.7 Ci/mol for all further experiments. [G-^{3}H]-16-Hydroxypalmitic acid was used at a specific activity of 12.5 Ci/mol. [10-^{14}C]Palmitoleic acid was purchased from Schwarz/Mann, Orangeburg, N. Y. Epoxidation with *m*-chloroperbenzoic acid in CH_2Cl_2 (Walton and Kolattukudy, 1972a) afforded [10-^{14}C]-9,10-epoxypalmitic acid, which on $LiBH_4$ reduction yielded a mixture of 9- and 10-hydroxyplamitic acid. The mixture was diluted with unlabeled acid to a specific activity of 4.87 Ci/mol. [10-^{14}C]-9,10-Dihydroxypalmitic acid (4.87 Ci/mol) was prepared by treatment of [10-^{14}C]-9,10-epoxypalmitic acid with glacial acetic acid followed by hydrolysis with alcoholic KOH. [18-^{3}H]-18-Hydroxystearic acid (19.3 Ci/mol) was obtained by hydrogenation of [18-^{3}H]-18-hydroxyoleic acid, which was previously synthesized (Croteau and Kolattukudy, 1974). [18-^{3}H]-9,10,18-Trihydroxyoctadecanoic acid (32.2 Ci/mol) was derived from previously synthesized [18-^{3}H]-18-hydroxy-9,10-epoxyoctadecanoic acid (Croteau and Kolattukudy, 1974) by acetoxylation followed by hydrolysis. A mixture of [18-^{3}H]-9,18- and -10,18-dihydroxyoctadecanoic acids (19.3 Ci/mol) was prepared by $NaBH_4$ reduction of [18-^{3}H]-18-hydroxy-9,10-epoxyoctadecanoic acid. Other derivatives used as substrates in this study were prepared as described previously (Kolattukudy and Walton, 1972b). Acids were reduced to the corresponding alcohols with $LiAlH_4$ in tetrahydrofuran and they were methylated with 14% BF_3 in methanol. Hydroxy acids were acetylated with acetic anhydride–pyridine (2:1). Mild oxidation of [G-^{3}H]-16-hydroxypalmitic acid with CrO_3 in 95% acetic acid yielded [G-^{3}H]hexadecane-1,16-dioic acid (specific activity 6.76 Ci/mol). All substrates were rigorously purified by thin-layer chromatography as described below, and were dispersed in water by sonication with the aid of Tween-20 as described earlier (Kolattukudy, 1970a).

Cofactors, inhibitors, cellulase, pectinase, and Pronase were purchased from Sigma Chemical Co. The sources of other reagents and chemicals are cited elsewhere (Walton and Kolattukudy, 1972a).

Preparation of the Particulate Enzyme. The epidermis excised from young, rapidly expanding *V. faba* leaves was ground in a Ten-Broeck homogenizer in cold 0.05 M sodium phosphate buffer (pH 7.0) containing 0.25 M sucrose, 2.5×10^{-3} M dithioerythritol, and 10^{-3} M $MgCl_2$. The homogenate was centrifuged at 3000*g* for 20 min and the resulting pellet was rehomogenized in the same buffer and centrifuged again. This second pellet, suspended in the same medium as above, was used as the source of the particulate enzyme.

Preparation of the Primer-Dependent Enzyme. Sonication of the particulate preparation described above at 4° (6 × 5 sec with the needle probe of a Brownwill Biosonic III at full power) followed by centrifugation at 3000*g* yielded a supernatant fraction. This supernatant was used as the source of the primer-dependent enzyme, or was centrifuged again at 105,000*g* for 90 min.

Assay for Acyl-CoA–Cutin Transacylase. In the assay of the particulate enzyme, centrifuge tubes containing the enzyme preparation and appropriate amounts of cofactors and substrate were incubated (sealed under N_2) at 30° in a vigorously shaking water bath for periods up to 4 hr. An identical procedure was followed in assaying the primer-dependent enzyme, with the exception that a cutin primer was also added to the incubation mixture. The cutin primer was suspended in buffer by thorough homogenization or sonic dispersion. Further details of the experiments are supplied elsewhere.

At the end of the incubation period, the insoluble material was recovered from each sample by centrifugation at 27,000*g* for 20 min. The insoluble residues were then transferred with methanol into 10 × 50 mm cellulose extraction thimbles. After washing the residue thoroughly with methanol it was extracted (Soxhlet) with $CHCl_3$ overnight to remove any remaining soluble lipids. Each extracted residue was then homogenized in 5 ml of H_2O and the suspension was transferred to a counting vial. After the addition of 15 ml of Aquasol (New England Nuclear Corp.) followed by shaking to form a gel, the ^{3}H or ^{14}C was assayed directly in a liquid scintillation spectrometer. Identical procedures were used in experiments with both the particulate enzyme and the primer-dependent enzyme systems.

The overall counting efficiency of this Aquasol gel-suspension technique was approximately 7% for ^{3}H and 70% for ^{14}C, as determined by parallel experiments in which the hydrogenolysis ($LiAlH_4$) products of the thoroughly extracted residue were assayed by liquid scintillation spectrometry. As a further check on the assay procedure, the combustion products of the radioactive residue, recovered from the counting gel as described below, were assayed for radioactivity, and the results confirmed the counting efficiency of the gel-suspension technique.

In order to examine the possibility of formation of soluble products, the 27,000*g* supernatant described above was acidified and thoroughly extracted with chloroform. The analysis of soluble lipids is described elsewhere in the Experimental Section.

Recovery of the Radioactive Residue. In order to recover the residue for further analysis, the counting gel was liquified in hot acetone and the insoluble material was removed by filtration and thoroughly washed.

Enzymatic and Chemical Depolymerization of Biosynthetically Labeled Cutin. In the enzymatic depolymerization study, the thoroughly washed and dried residue recovered from the counting gel was sequentially treated with pectinase, cellulase, Pronase, and cutinase as described previously (Croteau and Kolattukudy, 1973), and after each treatment, the insoluble residue remaining was recovered and the radioactivity was measured by the Aquasol gel-suspension technique described above. The soluble lipids released by cutinase treatment were recovered by extraction with chloroform and they were ana-

lyzed by thin-layer chromatography.

For chemical depolymerization studies, the insoluble residues recovered from several large-scale experiments were pooled, and were thoroughly mixed by grinding in a mortar and pestle (specific activity 53,500 dpm/mg). Portions of the residue (16 mg each) were subjected to the following procedures: (a) $LiAlH_4$ reduction in tetrahydrofuran as previously described (Walton and KolattuKudy, 1972a), (b) reduction with excess $NaBH_4$ in tetrahydrofuran under reflux for 24 hr, (c) reduction with excess $LiBH_4$ in tetrahydrofuran under reflux for 24 hr, (d) hydrolysis with 10% KOH in 95% ethanol under reflux in a N_2 atmosphere for 6 hr, (e) methanolysis with 14% BF_3 in methanol under reflux for 12 hr. The reaction mixtures were processed by standard techniques (Walton and Kolattukudy, 1972a), and the soluble lipids were recovered by extraction with $CHCl_3$. The residue remaining after each treatment was recovered by filtration and any remaining traces of soluble lipids were removed by thorough solvent extraction. The final residue was dried and weighed, and the radioactivity remaining in it was determined by the Aquasol gel-suspension technique described above.

Preparation of Cutin Primers. Cutin prepared from fresh tissue by the combination of enzymatic and chemical techniques described previously (Walton and Kolattukudy, 1972a) was finely powdered in a Wiley mill and kept frozen until used. The physiological age of the tissue employed is noted in the text.

Modification of Cutin Primers. Carboxyl groups of *V. faba* cutin were methylated by treatment of powdered cutin with an excess of 14% BF_3 in methanol overnight at room temperature. The hydroxyl groups were acetylated by treating the cutin powder with excess acetic anhydride–pyridine (2:1) overnight at room temperature. In order to cleave vicinal diol functions and generate two primary alcohol functions, cutin powder prepared from golden delicious apples was treated with a 20-fold excess of KIO_4 in pyridine under N_2 with vigorous stirring at room temperature overnight. The cutin, recovered by centrifugation, was thoroughly washed with H_2O and then treated with a tenfold excess of $NaBH_4$ in H_2O for 3 hr at room temperature (such treatment reduces epoxide functions also). Double bonds of apple cutin were converted to vicinal diols by treating the cutin powder with an excess of a 0.1% solution of OsO_4 in pyridine–ethyl ether (1:6) at room temperature for 5 hr with vigorous stirring. Aqueous methanolic $Na_2S_2O_3$ was then added to decompose the osmic esters, and the modified cutin was recovered by centrifugation. For cutinase treatment, 5-mg portions of *V. faba* cutin were incubated with a cutinase preparation (Purdy and Kolattukudy, 1973) in 2 ml of 0.2 M glycine–NaOH buffer (pH 10) for periods of time up to 30 min. The reaction was stopped by adding acetone and the partially hydrolyzed cutin was recovered by centrifugation.

After each treatment, the cutin powder was repeatedly washed by suspension in an appropriate solvent followed by centrifugation. After final washing with H_2O, the cutin was dried and kept frozen until used. Cutin used in control experiments was subjected to identical treatments except for the absence of the modifying reagent.

Determination of Hydroxyl Value. The hydroxyl value of cutin (a measure of free hydroxyl functions per unit weight) was determined by acetylation with [1-^{14}C]acetic anhydride (specific activity 17.4 μCi/mmol, Amersham-Searle Corp.) as follows. A 3-mg portion of cutin was treated with 0.1 ml of labeled acetic anhydride and 0.1 ml of pyridine in a sealed tube at room temperature for 48 hr. Excess reagent was decomposed with 50% aqueous methanol and the residue was transferred into a soxhlet thimble and it was thoroughly extracted with $CHCl_3$. The ^{14}C incorporated into cutin was determined by the Aquasol gel-suspension technique described above.

Chromatography. Thin-layer chromatography was performed with activated 0.5- or 1-mm layers of silica gel G. Hydrogenolysis products were analyzed with ethyl ether–hexane–methanol (8:2:1, v/v) as the solvent, while hydrolysis products and methanolysis products were fractionated with ethyl ether–hexane–methanol–formic acid (40:10:1:2, v/v). Most substrates were purified using the above system, or with hexane–ethyl ether–formic acid (65:35:2, v/v) as the solvent system. Except as otherwise noted, the details of other thin-layer chromatographic, gas chromatographic (gc), and gc–mass spectrometric techniques were essentially the same as those used previously (Walton and Kolattukudy, 1972a; Kolattukudy and Walton, 1972b).

Determination of Radioactivity. Radioactivity in soluble lipid samples and thin-layer chromatograms was determined by liquid scintillation spectrometry as described before (Kolattukudy, 1966). Thin-layer plates were also monitored with a Berthold thin-layer scanner. Procedures for radiogas chromatographic analysis have been described previously (Kolattukudy and Walton, 1972b).

Protein Determination. Protein was routinely estimated by the method of Lowry *et al.* (1951), after precipitation with 10% trichloroacetic acid, washing with 80% acetone, and solubilization in 0.1 N NaOH. With the particulate preparation, the Lowry method gave only 43.5% of the protein value obtained by Kjeldahl determination, and, therefore, all of the results are expressed as Kjeldahl protein.

Results and Discussion

Esterification of 10,16-Dihydroxypalmitic Acid to Cutin by a Particulate Enzyme Preparation. Previously it was found that in crude homogenates of *V. faba* epidermis most of the 10,16-dihydroxypalmitic acid formed from 16-hydroxypalmitic acid was located in the insoluble polymer, indicating that an active polymerizing enzyme was present in the homogenate (Kolattukudy, unpublished). Incubation of a 3000*g* pellet suspension, prepared from excised epidermis of young *V. faba* leaves, with [G-^{3}H]-10,16-dihydroxypalmitic acid, ATP, and CoA gave rise to a radioactive insoluble residue. Exhaustive solvent extraction removed no further ^{3}H from the residue. Treatment of the residue with $LiAlH_4$ in tetrahydrofuran, followed by thin-layer chromatography of the resulting $CHCl_3$ soluble products, showed that all of the ^{3}H was contained in hexadecanetriol. Thus, 10,16-dihydroxypalmitic acid was directly incorporated into a polymer. In order to test whether this incorporation represented synthesis of cutin or a nonspecific acylation of some other cellular polymer, the biosynthetically labeled residue was sequentially treated with several hydrolytic enzymes (Table I). Cellulase, pectinase, and Pronase treatments resulted in the loss of about 50% of the residue and only 25% of the radioactivity. Since some loss of material was unavoidable during the handling of the very small amount of powdered residue, radioactivity released into the true soluble phase by these treatments was most probably much less than 25%. On the other hand, treatment of the remaining residue with a fungal "cutinase" preparation (Purdy and Kolattukudy, 1973) solubilized only 3% of the total residue but released over 60% of the radioactivity. The radioactivity released was contained exclusively in 10,16-dihydroxypalmitic acid, as shown by thin-layer chromatography. Thus, the bulk of the label incorporated into the insoluble material was contained in cutin.

The biosynthetically labeled residue derived from [G-^{3}H]-

TABLE I: Sequential Enzyme Treatment and Chemical Depolymerization of the Insoluble Residue Derived from [G-^{3}H]-10,16-Dihydroxypalmitic Acid.

Treatment[a]	% Residue Solubilized: Weight	% Residue Solubilized: Radioactivity	Identity of Soluble Product
Cellulase + pectinase	41	21	ND
Pronase	6	4	ND
Cutinase	3	61	10,16-Dihydroxypalmitic acid
$LiAlH_4$	42	95	1,7,16-Hexadecanetriol
$LiBH_4$	35	96	1,7,16-Hexadecanetriol
$NaBH_4$	9	7	*b*
KOH in ethanol	47	94	10,16-Dihydroxypalmitic acid
BF_3 in methanol	41	95	Methyl 10,16-dihydroxypalmitate

[a] For sequential enzyme treatment, an 11.5-mg portion of insoluble residue containing 42,500 cpm was treated in the order noted above. For each chemical treatment, a 16-mg portion of insoluble residue containing 856,000 dpm was employed. Treatment conditions and assay procedures are described in the Experimental Section. [b] Traces of radioactivity were noted in the hexadecanetriol region of the thin-layer chromatogram and in a component less polar than hexadecanetriol. ND = not determined.

10,16-dihydroxypalmitic acid was also depolymerized by a variety of chemical techniques in order to determine the type of bond involved in linking the monomer substrate to the cutin polymer (Table I). $NaBH_4$ treatment was ineffective in depolymerizing the residue, and this result is consistent with the inability of $NaBH_4$ to reduce oxygen ester linkages. Treatment of the labeled cutin with $LiAlH_4$, $LiBH_4$, alcoholic KOH or BF_3 in methanol resulted in extensive depolymerization of the radioactive residue. The resulting soluble lipids were subjected to thin-layer chromatographic analysis. The only radioactive product released by $LiBH_4$ treatment of cutin was hexadecanetriol. Since $LiBH_4$ is known to reduce esterified carboxyls but not free carboxyl groups, this result indicates that the carboxyl group of the labeled monomer substrate was esterified. Furthermore, as dihydroxypalmitic acid could not be detected in the products obtained by $LiBH_4$ treatment of the labeled residue, the possibility that the labeled monomer was attached to the polymer solely by the hydroxyl group of the monomer can be ruled out. Treatment of the residue with $LiAlH_4$ released hexadecanetriol as the only radioactive product, while hydrolysis and methanolysis of the residue released radioactive 10,16-dihydroxypalmitic acid and its methyl ester, respectively (Table I). Thus, in each case both the degree of depolymerization of the insoluble residue and the structure of the resulting soluble products were consistent with an ester linkage between the carboxyl group of the substrate and free hydroxyl groups of the cutin polymer.

Cofactor Requirements. In the absence of added cofactors, the rate of incorporation of 10,16-dihydroxypalmitic acid into cutin was negligible. With either ATP or CoA alone, the rate of incorporation was 7 and 5%, respectively, of the rate obtained with both cofactors. With both cofactors and boiled enzyme, the rate was 5% of that with active enzyme (Croteau and Kolattukudy, 1973). Thus, there was an absolute requirement for ATP and CoA suggesting that the CoA ester was the true substrate for the esterifying enzyme. Therefore, the hydroxyacyl chain was probably esterified to the cutin polymer by a transacylase (acyl-CoA–cutin transacylase). This demonstration of the requirement for activation of the carboxyl group prior to esterification helps to explain the efficient esterification process observed at the very low monomer concentrations that are found in tissues which synthesize cutin (Kolattukudy and Walton, 1972b; Kolattukudy *et al.*, 1973). These results, together with the observation that activation of the carboxyl group is required for the C-10 hydroxylation of 16-hydroxypalmitic acid (Walton and Kolattukudy, 1972b), strongly support the hypothesis that a palmitoyl thioester undergoes sequential hydroxylations after which the products are transferred directly to the growing polymer without involving free monomers.

Localization of Enzymatic Activity. The 3000*g* supernatant fraction, either alone, with boiled pellet, or with added purified *V. faba* cutin, was devoid of transacylase activity with all of the substrates employed. The 3000*g* pellet did not incorporate 10,16-dihydroxypalmitic acid, or other substrates, into soluble polymers as determined by thin-layer chromatographic analysis of the soluble lipids. Therefore, it appeared that both the thiokinase and the transacylase, as well as the polymer which presumably functions as the primer, were contained in the particulate preparation.

Time Course and Effect of Protein Concentration. The rate of incorporation of 10,16-dihydroxypalmitic acid into cutin was linear with respect to protein concentration up to about 2.5 mg/ml for a 3-hr assay, and fairly linear rates of incorporation of 10,16-dihydroxypalmitic acid were observed up to 4 hr at 1.4 mg/ml protein level (Croteau and Kolattukudy, 1973). In most studies, 3- or 4-hr incubation periods were used with protein levels from 0.5 to 1.5 mg/ml so that all measurements were within the linear portion of the time-course curve.

pH Dependence. Preliminary studies of the effect of pH on the incorporation of 10,16-dihydroxypalmitic acid into cutin revealed a bimodal pH curve with maxima at about pH 7.0 and about pH 8.5 (Croteau and Kolattukudy, 1973). The bimodal curve suggests different pH requirements for activation and transacylation steps. In order to further test this possibility, the effect of pH on the incorporation of palmitic acid and palmitoyl-CoA into cutin was determined. As was the case with 10,16-dihydroxypalmitic acid, palmitic acid showed a bimodal curve with similar pH maxima. When palmitoyl-CoA was the substrate, the pH curve showed a single optimal pH near 8.0. Thus, the pH optimum near 7.0 probably represents activation to acyl-CoA, while that at pH 8.0–8.5 represents transfer of the acyl moiety from the CoA derivative to cutin.

Effect of Monomer Concentration. There was a linear increase in the rate of incorporation of 10,16-dihydroxypalmitic acid into cutin as the concentration of acid was increased, giving rise to a typical substrate saturation curve (Figure 1) with an apparent K_m of 1.1×10^{-4} M with respect to 10,16-dihydroxypalmitic acid and a V_{max} of 0.87 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}. Since the substrate was dispersed with a detergent, the terms, concentrations, and apparent K_m should be taken with the usual precautions.

Palmitic acid and 16-hydroxypalmitic acid are significant components of *V. faba* cutin, and both of these acids can function as substrates for the CoA-dependent cutin synthesizing system (Croteau and Kolattukudy, 1973). Reductive depolym-

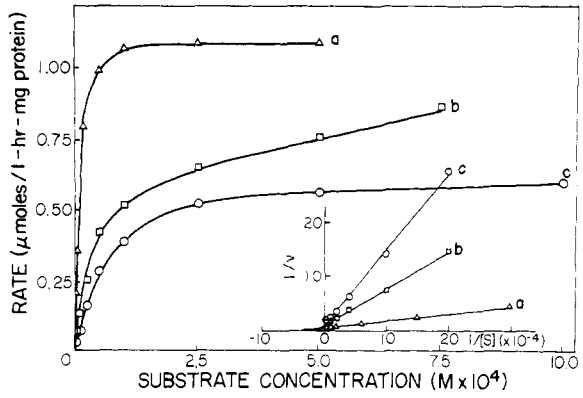

FIGURE 1: Effect of substrate concentration on its rate of incorporation into cutin by the particulate preparation from *V. faba* epidermis: (a) palmitic acid, (b) 16-hydroxypalmitic acid, (c) 10,16-dihydroxypalmitic acid. Reaction mixture contained 3.3×10^{-3} M ATP, 2.5×10^{-4} M CoA, substrate as indicated, and 1.5 ml of enzyme suspension containing (a) 1.33 mg of protein, (b) 1.0 mg of protein, and (c) 0.95 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Incubation was anaerobic at 30° for 4 hr. Incorporation into the cutin polymer was determined as described in the Experimental Section.

erization ($LiAlH_4$) of the radioactive residue derived from [1-^{14}C]palmitic acid yielded only [^{14}C]hexadecanol. Reductive depolymerization of the radioactive residue derived from [G-^{3}H]-16-hydroxypalmitic acid yielded only [^{3}H]hexadecane-1,16-diol. Thus, both substrates were incorporated, as such, without further modification of the carbon chains. Linear increases in the rates of incorporation were observed when the concentration of either of these substrates was increased, giving rise to typical substrate saturation curves (Figure 1). Apparent K_m for palmitic acid was 2×10^{-5} M with a V_{max} of 1.3 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}, while apparent K_m for 16-hydroxypalmitic acid was 6.7×10^{-5} M with a V_{max} of 1.0 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}, under the present experimental conditions. These results show that at moderately low concentrations of the monomer fairly good rates of incorporation into the polymer occurred. The higher K_m's and the lower rates observed for 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid should be taken with caution because of the limitations noted above and the rather crude nature of the preparation used. Even more importantly, the transacylase enzyme system involved here may never be exposed to free acids *in vivo* because the hydroxylation steps probably occur on a CoA ester. Thus, the relative rates and K_m's may be a reflection of only the activation step, which may be rate limiting under our experimental conditions. It is probable that the hydroxylated acyl chains are not readily activated.

Administration of labeled palmitic acid, 16-hydroxypalmitic acid, or 10,16-dihydroxypalmitic acid with various combinations and concentrations of the other two unlabeled substrates resulted in inhibition of incorporation of radioactivity in all cases (data not shown), indicating that there was direct competition between substrates at either the activation or transacylation step or both. Thus, the enzyme system did not appear to function as a true "polymerase" in that it did not require the presence of all monomers for polymerization to occur. In this respect, the cutin synthesizing enzyme is analogous to the transglycosylases involved in carbohydrate polymer synthesis rather than to the nucleic acid polymerases.

Substrate Analogs and Derivatives. In order to determine some of the structural requirements of the substrate, several analogs and derivatives of palmitic acid, 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid were tested for their incorporation into cutin by the particulate preparation (Table II). Reduction of the carboxyl group of each of the substrate acids to an alcohol function prevented incorporation into the polymer, again indicating that it is the carboxyl group of the incoming monomer that is esterified to the free hydroxyl functions of cutin. As might be expected, methyl esters were poor substrates compared to their parent acids. Furthermore, the small amount of activity observed may well have resulted from hydrolysis of the methyl esters before incorporation into the polymer. Acetates of hydroxy acids were incorporated with 60–70% of the efficiency of the parent acids. The acetylated substrates would be expected to show somewhat lower incorporation rates than would the hydroxy acids because esterification of an acetylated hydroxy acid to a free hydroxyl group on the polymer terminates that point of growth of the polymer, whereas in the normal case the hydroxyl group(s) of the most recently incorporated monomer could accept more monomers. Although under the present experimental conditions some hydrolysis of the acetates could have taken place before incorporation, the results provide further evidence against the possibility that the hydroxyl group of the incoming monomer is the primary point of attachment to the polymer.

TABLE II: Incorporation of Substrate Analogs and Derivatives into Cutin by the Particulate Preparation from *V. faba* Epidermis.[a]

Substrate	Rel Rate
Palmitic acid	100
Methyl palmitate	31
Hexadecanol	10
16-Hydroxypalmitic acid	100
Methyl 16-hydroxypalmitate	21
Hexadecane-1,16-diol	6
16-Acetoxypalmitic acid	73
10,16-Dihydroxypalmitic acid	100
Methyl 10,16-dihydroxypalmitate	19
Hexadecane-1,7,16-triol	3
10,16-Diacetoxypalmitic acid	56

[a] Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M substrate, and 1 ml of enzyme suspension containing 0.62 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Incubation was anaerobic at 30° for 4 hr. Absolute rates are: palmitic acid, 1.04 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}; 16-hydroxypalmitic acid, 0.35 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}; 10,16-dihydroxypalmitic acid, 0.28 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}. Incorporation into the cutin polymer was determined as described in the Experimental Section.

Substrate Specificity. Vicia faba cutin contains, almost exclusively, members of the C_{16} family of monomer acids (Kolattukudy and Walton, 1972b). Availability of the various chain lengths to the enzyme system *in vivo*, or the specificity of the enzyme, could determine the chain lengths of the monomer acids found in cutin. The results summarized in Table III suggest that the cell-free preparation prefers C_{16} acids. Thus, the specificity of the enzyme system appears to be in agreement with the types of chains found in *V. faba* cutin. The chain length preference appears to diminish with increasing hydroxyl

TABLE III: Incorporation of Various Substrates into Cutin by the Particulate Preparation from *V. faba* Epidermis.[a]

Substrate	Rel Rate
Capric acid	10
Lauric acid	43
Myristic acid	97
Palmitic acid	100
Stearic acid	49
16-Hydroxypalmitic acid	53
18-Hydroxystearic acid	29
10,16-Dihydroxypalmitic acid	30
9- or 10,18-Dihydroxystearic acid	27
9,10,18-Trihydroxystearic acid	7
9- or 10-Hydroxypalmitic acid	83
9,10-Dihydroxypalmitic acid	59
Hexadecane-1,16-dioic acid	13

[a] Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M substrate, and 1 ml of enzyme suspension containing 0.66 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Incubation was anaerobic at 30° for 4 hr. The rate for palmitic acid = 1.32 μmol $l.^{-1}$ hr^{-1} mg of $protein^{-1}$. Incorporation into the cutin polymer was determined as described in the Experimental Section.

TABLE IV: Effect of Inhibitors on the Incorporation of Palmitoyl-CoA into Cutin by the Particulate Preparation from *V. faba* Epidermis.[a]

Inhibitor[b]	Concn (M)	Rel Rate
None		100
Cl-HgBzO	5×10^{-4}	99
Cl-HgBzO	10^{-3}	150
MalNEt	10^{-3}	110
IAA	10^{-3}	111
Dip-F	10^{-4}	50
Dip-F	5×10^{-4}	44
Dip-F	10^{-3}	33
TPCK	10^{-3}	90
$PhCH_2SO_2F$	10^{-3}	83

[a] Reaction mixture contained inhibitor, 2×10^{-5} M [1-^{14}C]-palmitoyl-CoA, and 0.5 ml of enzyme suspension containing 0.23 mg of protein, in a total volume of 3 ml of 0.05 M Tris-Cl buffer (pH 8.0) containing 0.25 M sucrose and 10^{-3} M $MgCl_2$. Incubation was anaerobic at 30° for 2 hr. Base rate for control = 2.06 μmol $l.^{-1}$ hr^{-1} mg of $protein^{-1}$. Incorporation into the cutin polymer was determined as described in the Experimental Section. The reaction mixtures were preincubated for 30 min before the addition of substrate. [b] Abbreviations used are: Cl-HgBzO, *p*-chloromercuribenzoate; MalNEt, *N*-ethylmaleimide; IAA, iodoacetamide; Dip-F, diisopropyl fluorophosphate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

substitution. For example, 16-hydroxypalmitic acid was incorporated at a rate 83% faster than that observed with 18-hydroxystearic acid, while 10,16-dihydroxypalmitic acid was incorporated only 11% faster than 9,18- or 10,18-dihydroxystearic acid (Table III). Since polyhydroxy acids are probably not readily activated, the decrease in chain-length discrimination of the enzyme system with increasing chain substitution is not surprising. Because of this limitation, the true chain-length specificity of the transacylase cannot be ascertained from the present data. The observation that hydroxy C_{16} acids not found in cutin (9- or 10-hydroxypalmitic acid and 9,10-dihydroxypalmitic acid) are good substrates does suggest, however, that the transacylase has fairly broad specificity within the C_{16} family. The in-chain hydroxy C_{16} acids appear to be somewhat better substrates than their terminally hydroxylated analogs. Hexadecane-1,16-dioic acid is a minor component of *V. faba* cutin (Kolattukudy and Walton, 1972b) and it is a relatively poor substrate for the enzyme system.

Inhibitors. Sulfhydryl reagents, at 5×10^{-4} M, had little or no effect on palmitoyl-CoA incorporation into cutin (Table IV, data for only *p*-chloromercuribenzoate is included). However, addition of *N*-ethylmaleimide, iodoacetamide, and *p*-chloromercuribenzoate at 10^{-3} M resulted in some stimulation of activity, probably because of inhibition of endogenous thioesterases. Diisopropyl fluorophosphate effectively inhibited the incorporation of palmitoyl-CoA into cutin (50% inhibition at 10^{-4} M), while L-1-tosylamido-2-phenylethyl chloromethyl ketone and phenylmethanesulfonyl fluoride were less effective. Phenylmethanesulfonyl fluoride is known to inhibit palmitoyl-CoA hydrolysis by pigeon and rat liver fatty acid synthetase (Kumar, 1973) and thus might also function by inhibiting competing reactions for palmitoyl-CoA.

The transacylase is probably extracellular and it is most likely situated in close association with cutin itself. The observation that relatively high levels of inhibitors were required to produce an effect might reflect the relative inaccessibility of the enzyme in this particulate system. In any event, the results suggest that the transacylase contains an essential serine residue.

Effect of Leaf Maturity on Transacylase Activity. Incorporation of palmitic acid into the hydroxy acids of cutin in *V. faba* leaves ceased when the leaves reached full size, indicating that cutin synthesis is under strict physiological control (Kolattukudy, 1970b). In order to determine if this control resides at the transacylase level, the cutin synthesizing activity described here was measured in particulate preparations from mature, fully expanded leaves (6 × 9 cm in size). In these leaves, the transacylase activity was less than 12% of that in young, rapidly expanding leaves. Thus, regulation of cutin synthesis also involved regulation of the transacylase.

Esterification of 10,16-Dihydroxypalmitic Acid to Cutin by a Primer-Dependent Enzyme System. Although the particulate preparation was useful in defining the reaction and in determining several characteristics of the enzyme system, it did not respond to added boiled pellet or purified cutin, indicating that endogenous primers were utilized. Thus, the nature of the primer and its effects on transacylation could not be determined. Attempts were therefore made to prepare a primer-requiring enzyme system. Mild sonication of the particulate preparation followed by centrifugation at 3000*g* provided a supernatant preparation which, when incubated with [G-^{3}H]-10,16-dihydroxypalmitic acid and appropriate cofactors, showed transacylase activity only in the presence of exogenous boiled pellet or purified cutin (Table V). As shown in Table V, purified cutin was a far more efficient primer for the transacylation than was boiled pellet (at a 3000*g* supernatant to pellet ratio of 1:2). Cellulose was not effective as a primer (Table V),

TABLE V: Primer Dependence of the Incorporation of [G-^{3}H]-10,16-Dihydroxypalmitic Acid by the Sonicated 3000*g* Supernatant Preparation from *V. faba* Epidermis.[a]

Primer	Rate of Incorporation (μmol l.$^{-1}$ hr^{-1} mg of protein^{-1})
None	0.017
Cellulose	0.025
Boiled pellet	0.061
V. faba cutin	0.180

[a] Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M [G-^{3}H]-10,16-dihydroxypalmitic acid, primer as indicated, and 1 ml of enzyme (sonicated 3000*g* supernatant) containing 0.3 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing 0.25 M sucrose, 2.5×10^{-3} M dithioerythritol, and 10^{-3} M $MgCl_2$. *V. faba* cutin and cellulose were used at a 2 mg/ml level. Incubation was anaerobic at 30° for 4 hr. Incorporation was determined as described in the Experimental Section.

TABLE VI: Cofactor Requirements for the Primer-Dependent Transacylase System from *V. faba* Epidermis and the Relative Rates of Incorporation of Monomer Acids.[a]

Additions	Substrate	Rate of Incorporation (μmol l.$^{-1}$ hr^{-1} mg of protein^{-1})
None	10,16-Dihydroxypalmitic acid	0.067
ATP	10,16-Dihydroxypalmitic acid	0.111
CoA	10,16-Dihydroxypalmitic acid	0.115
ATP, CoA	10,16-Dihydroxypalmitic acid	0.180
Boiled enzyme control	10,16-Dihydroxypalmitic acid	0.027
ATP, CoA	16-Hydroxypalmitic acid	0.326
ATP, CoA	Palmitic acid	1.57
None	Palmitoyl-CoA	2.54

[a] Reaction mixture contained 3.3×10^{-4} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M substrate, *V. faba* cutin primer (2 mg/ml), and 1 ml of enzyme (sonicated 3000*g* supernatant) containing 0.3 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Incubation was anaerobic at 30° for 4 hr. Incorporation was determined as described in the Experimental Section.

and 10,16-dihydroxypalmitic acid was not esterified to glycerol, hexadecanol, cholesterol, or polyethylene glycol (mol wt 200) to any detectable extent when these substances were tested as soluble acyl acceptors (data not shown). Thus, the incorporation demonstrated appeared to represent cutin synthesis and was not a nonspecific acylation.

The 3000*g* supernatant obtained after sonication contained roughly 25% of the transacylase activity and 30% of the protein of the original particulate preparation. Attempts to liberate higher levels of activity from the particulate preparation by sonication in the presence of Triton X-100 were unsuccessful, although the detergent itself was only slightly inhibitory to the primer-dependent enzyme. Centrifugation of the 3000*g* supernatant preparation at 105,000*g* for 90 min provided a supernatant fraction which contained approximately 40% of the activity and 50% of the protein of the original 3000*g* supernatant. The 105,000*g* pellet contained a detectable amount of cutin as shown by $LiAlH_4$ reduction of the solid, followed by gas–liquid chromatography of the $CHCl_3$ soluble products (analyzed as the trimethylsilyl ethers). Thus, although the 3000*g* supernatant preparation did contain a detectable level of endogenous cutin, this system did show an absolute requirement for exogenous primer. The ultrasonic treatment must have disrupted the interaction between the transacylase enzyme and the natural primer matrix, thereby allowing exogenous cutin to function as the primer. The 3000*g* supernatant preparation was therefore employed to study the nature of the primer requirement.

Cofactor Requirements and Substrates Utilized. The cofactors required by the primer-dependent enzyme system were the same as those required by the particulate system, and all members of the C_{16} family of cutin acids tested as substrates could also function with the primer-dependent system (Table VI). Thus, the primer-dependent system appeared to be quite similar to the particulate system, except that exogenous cutin primer was required. The rate of incorporation of 10,16-dihydroxypalmitic acid was lower with the primer-dependent preparation than that obtained with the particulate preparation, and the relative rates of incorporation of the various substrates differed somewhat. However, such differences could easily result from different enzyme levels or activities (*i.e.*, thiokinase, transacylase, thioesterase) in the two preparations, or could reflect the inability to achieve optimum substrate–enzyme–primer interaction in the primer-dependent preparation.

Effect of Cutin Level on Transacylase Activity. The effect of cutin level on the incorporation of 10,16-dihydroxypalmitic acid into cutin by the primer-dependent system is shown in Figure 2. Incorporation of this acid into the polymer increased with increasing cutin level up to 3 mg/ml. Higher levels of cutin were difficult to suspend in the assay medium, even with very vigorous shaking, and at cutin levels beyond 3 mg/ml the

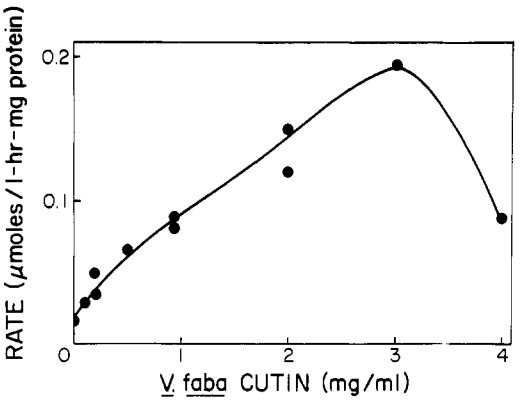

FIGURE 2: Effect of cutin level on the rate of incorporation of 10,16-dihydroxypalmitic acid into cutin by the primer-dependent system from *V. faba* epidermis. Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M [G-^{3}H]-10,16-dihydroxypalmitic acid, cutin primer as indicated, and 1 ml of enzyme (sonicated 3000*g* supernatant) containing 0.27 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Incubation was anaerobic at 30° for 4 hr. Incorporation into the cutin polymer was determined as described in the Experimental Section and rates were corrected for boiled controls which were run at each cutin level.

TABLE VII: Effect of Primers from Different Plant Sources on Transacylase Activity of the Primer-Dependent System from *V. faba* Epidermis.[a]

Source of Cutin	Type of Cutin and Major Component(s)	Hydroxyl Value (μmol/mg)	Rel Rate of Incorporation
Vicia faba leaf	C_{16} family, 10,16-dihydroxypalmitic acid	1.63	100
Papaya fruit	C_{16} family, 9,16-dihydroxypalmitic acid	1.94	47
Tomato fruit	C_{16} family, dihydroxypalmitic acid (mixed isomers)	1.84	26
Senecio odoris leaf	C_{16}–C_{18} family, intermediate, epoxy C_{18} acids	1.58	35
Grape fruit	C_{18} family, epoxy C_{18} acids	1.63	35
Apple fruit	C_{18} family, trihydroxy C_{18} acids	2.04	38

[a] Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M [G-^{3}H]-10,16-dihydroxypalmitic acid, cutin primer, and 1 ml of enzyme (sonicated 3000*g* supernatant) containing 0.23 mg of protein, in a total volume of 4 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Cutin, prepared from mature tissue, was used at a 2 mg/ml level. Incubation was anaerobic at 30° for 4 hr. Rate of incorporation for *V. faba* cutin = 0.22 μmol $l.^{-1}$ hr^{-1} mg of $protein^{-1}$. Hydroxyl values were determined by acetylation with [1-^{14}C]acetic anhydride, and incorporation into the cutin polymer was determined as described in the Experimental Section.

TABLE VIII: Effect of Chemical Modification of Cutin on Its Priming Activity.[a]

Primer	Treatment	Hydroxyl Value (μmol/mg)	Rel Rate of Incorporation
V. faba	None	1.63	100
V. faba	BF_3–CH_3OH	1.56	99
V. faba	Acetic anhydride	0.32	16
Apple	None	1.82	40
Apple	OsO_4	2.58	85
Apple	KIO_4, $NaBH_4$	1.70	64

[a] Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M [G-^{3}H]-10,16-dihydroxypalmitic acid, cutin primer, and 1 ml of enzyme (sonicated 3000*g* supernatant) containing 0.40 mg of protein in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Primer level was, in all cases, equivalent to 2 mg of control cutin/ml. Incubation was anaerobic at 30° for 4 hr. Rate of incorporation for *V. faba* control = 0.15 μmol $l.^{-1}$ hr^{-1} mg of $protein^{-1}$. Treatment conditions and assay procedures are described in the Experimental Section. Control cutins were untreated, but were subjected to identical washing and preparation procedures as treated cutins.

rate of incorporation decreased as shown by both the gel-suspension and the combustion assay techniques. Most assays were thus carried out at the 1–2 mg/ml cutin level. Preincubation of the enzyme in the presence of primer to promote enzyme-primer contact had no effect on the activity.

Effect of Primers from Different Plant Sources. Table VII shows the effect of cutins from a variety of different plant sources on the transacylase activity using 10,16-dihydroxypalmitic acid as the substrate. The cutins used encompass a wide range of chemical types and hydroxyl values (a measure of free hydroxyl functions), yet, the *V. faba* enzyme system showed a fairly strong preference for *V. faba* cutin as primer. Furthermore, no correlation was observed between the priming activity and the chemical nature of the cutin, or the hydroxyl value of the cutin. For example, tomato cutin and *V. faba* cutin have very similar monomer compositions, yet *V. faba* cutin was almost four times more efficient as a primer than was tomato cutin. Apple cutin, which has the highest hydroxyl value of any cutin examined, had only a slightly higher priming efficiency than did *S. odoris* cutin, which has the lowest hydroxyl value. Thus, the enzyme must recognize some other features of the cutin structure, possibly the three-dimensional architecture of the polymer.

Effects of Structural Modification of Cutin on Priming Efficiency. The response of the primer-dependent system to several chemically modified primers is presented in Table VIII. Methylation of the free carboxyl groups of *V. faba* cutin had virtually no effect on the priming activity (the slight decrease in hydroxyl value and rate is most likely due to some loss of acyl monomers *via* a small amount of transesterification which was unavoidable in the methylation procedure). Acetylation of the free hydroxyl groups of *V. faba* cutin, on the other hand, greatly diminished priming activity (84% decrease), presumably by decreasing the number of sites available for substrate esterification (80% decrease in hydroxyl value). These results strongly support the conclusion arrived at earlier that free carboxyl groups of cutin are not involved in the esterification process, and are consistent with the conclusion that the carboxyl group of the incoming monomer becomes esterified to a free hydroxyl group of the primer.

In order to test whether increasing the hydroxyl value of cutin would increase priming activity, apple cutin, which contains a significant proportion of unsaturated monomer acids (Walton and Kolattukudy, 1972a), was treated with OsO_4 to convert double bonds to vicinal diol functions. The hydroxyl value of the OsO_4 treated cutin was 40% higher than that of the control, while the priming activity was approximately doubled (Table VIII). Thus, chemically increasing the number of hydroxyl groups of a given cutin increases the priming efficiency of that cutin. However, even the OsO_4 treated apple cutin was not as efficient a primer as was *V. faba* cutin.

Another chemical modification of the cutin primer was also attempted. Apple cutin contains a substantial proportion of trihydroxy C_{18} monomer acids which have a 9,10-vicinal diol function (Walton and Kolattukudy, 1972a). Treatment of apple cutin with KIO_4 followed by $NaBH_4$ reduction results in the replacement of each *vic*-diol function by two primary alcohol functions. Such a modification would be expected to open up the cutin matrix making the priming sites more accessible to the enzyme. The results in Table VIII indicate that such a treatment increased priming efficiency by 60% compared to

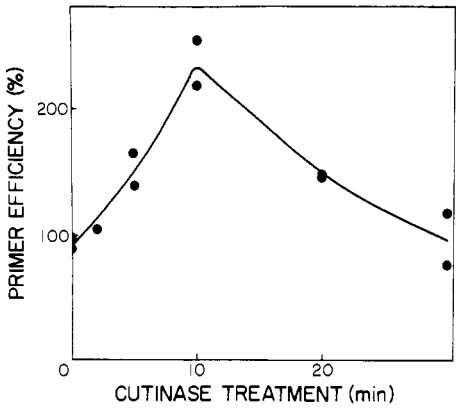

FIGURE 3: Effect of cutinase treatment on priming activity with the primer-dependent system from *V. faba* epidermis. Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M [G-^{3}H]-10,16-dihydroxypalmitic acid, treated *V. faba* cutin at 1 mg (original weight) per ml level, and 1 ml of enzyme (sonicated 3000*g* supernatant) containing 0.24 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Incubation was anaerobic at 30° for 4 hr. Preparation of cutinase-treated cutin is described in the Experimental Section. Incorporation into the cutin polymer was determined as described in the Experimental Section and rates were corrected for boiled controls which were run at each time period. Rate obtained with untreated cutin = 0.093 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}. Treatment of the *V. faba* cutin with boiled cutinase for 10 min did not change its priming efficiency.

that of the untreated control. The slight decrease in hydroxyl value which resulted from the KIO_4–$NaBH_4$ treatment was probably due to the loss of unbonded terminal portions of the trihydroxyacyl chains during periodate cleavage.

Partial hydrolysis of the cutin polyester could provide a means of increasing the number of starting points for polymer synthesis, thus increasing its activity as a primer. To test this possibility, the effect of cutinase treatment on the priming efficiency of *V. faba* cutin was determined (Figure 3). The priming ability of *V. faba* cutin increased with the duration of treatment with cutinase up to about 10 min. However, longer periods of treatment resulted in a decrease in priming efficiency of the cutin, presumably because of extensive hydrolysis of the polymer. Even a 10-min cutinase treatment of *V. faba* cutin removed a sufficient number of hydroxyacyl monomers to decrease the hydroxyl value by 10%. Thus, the results presented in Figure 4 probably represent the balance between the two opposing effects: increase in the availability of priming sites *vs.* net loss of priming sites.

Effect of Cutin Maturity on Priming Efficiency. Cutin membranes from young leaf or fruit tissue are very fragile, presumably because they are not as extensively polymerized as is the cutin from mature tissue. It is possible that such developing cutin would function as a more efficient primer than would mature cutin. Cutin prepared from tissues of various ages were thus tested for priming efficiency (Table IX). In the case of apple, the hydroxyl value of cutin from young fruit was slightly less than that of cutin from mature fruit, but the former was a much more efficient primer than the latter. In the case of *V. faba* cutin, both hydroxyl value and priming efficiency decreased with maturity of the leaf from which the cutin was obtained. Thus, the exceptional priming efficiency of cutin from very young *V. faba* leaves could be due to the relatively high hydroxyl number, to the openness of the immature polymer, or to a combination of these factors. The standard *V. faba* cutin preparation used in almost all other experiments was prepared by harvesting all of the leaves from fully grown plants and, therefore, this preparation represents a wide range of physiological ages with a predominance of the more rugged cutin membranes from mature leaves. The hydroxyl value of this cutin is relatively low, reflecting the high proportion of cutin from old leaves in this preparation, but its priming efficiency is fairly high, most probably due to the presence of sufficient cutin from young or very young leaves.

TABLE IX: Effect of Maturity of the Cutin Source on Priming Activity.[a]

Primer	Hydroxyl Value (μmol/mg)	Rel Rate of Incorporation
Apple fruit (young)	1.96	51
Apple fruit (mature)	2.04	36
V. faba leaf (very young)	1.91	149
V. faba leaf (young)	1.74	102
V. faba leaf (mature)	1.70	94
V. faba leaf (standard)	1.63	100

[a] Reaction mixture contained 3.3×10^{-3} M ATP, 1.2×10^{-4} M CoA, 10^{-4} M [G-^{3}H]-10,16-dihydroxypalmitic acid, cutin primer, and 1 ml of enzyme (sonicated 3000*g* supernatant) containing 0.4 mg of protein, in a total volume of 5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing sucrose, dithioerythritol, and $MgCl_2$ as specified in the Experimental Section. Incubation was anaerobic at 30° for 4 hr. Rate of incorporation of *V. faba* (standard) = 0.15 μmol l.$^{-1}$ hr^{-1} mg of protein^{-1}. Primers were used at a 2 mg/ml level and were prepared from the following tissues: *V. faba* (very young), from unopened leaves, average size 3×1.7 cm; (young), from partially opened leaves, average size 5×3 cm; (mature), from fully opened leaves, average size 9×6 cm; (standard), from all leaves of mature plants; apple (young), average diameter of fruit 3 cm; (mature), average diameter 8 cm. Hydroxyl values were determined by acetylation with [1-^{14}C]acetic anhydride, and incorporation into the cutin polymer was determined as described in the Experimental Section.

In order to determine whether cutin would specifically bind the transacylase, enzyme preparations were preincubated with various types of cutin. After centrifugation to remove the cutin, the protein level and palmitoyl-CoA–cutin transacylase activity remaining in the supernatant were determined. Although protein binding increased with hydroxyl value, transacylase binding did not correlate to either the hydroxyl value or the chemical nature of the cutin involved.

The results presented in this paper show, for the first time, that monomer acids are incorporated into the cutin polymer *via* an acyl-CoA–cutin transacylase. Specificity studies indicated that the particulate enzyme system prefers the C_{16} family of monomer acids which are native to *V. faba* cutin, and that the primer-dependent enzyme system prefers *V. faba* cutin to all other primers tested. Thus, the specificity involves recognition of both the monomers and the cutin primer of this species. Enzyme systems similar to those reported here were also prepared from the petals of *V. faba* flower buds and from the epidermis of *Senecio odoris* leaves. The transacylase described in this paper is unique in that it catalyzes the formation of a hydroxy-fatty acid polymer.

Acknowledgments

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CORRECTIONS

"Properties and Activity of the Lipopolysaccharide–Receptor from Human Erythrocytes," by Georg F. Springer,* James C. Adye, Anatoly Bezkorovainy, and Bruno Jirgensons, Volume 13, Number 7, March 26, 1974, page 1379.

In the Abstract: right-hand column, line 1, delete hyphen; insert comma after "inactivated."

In Figure 1: the coordination of the legends with the figures is incorrect: 1b is lower left and 1c is above.

In the references: Lüderitz, O., *et al.*, must be S17, not 517; Springer, G. F., *et al.*, must be S202, not 5202.

"Influence of Globin Structure on the State of the Heme. I. Human Deoxyhemoglobin," by Max F. Perutz,* Jane E. Ladner, Sanford R. Simon, and Chien Ho, Volume 13, Number 10, May 7, 1974, page 2163.

Table I as printed is uncorrected and incomplete. The correct table appears below.

TABLE I: Salt Bridges and Hydrogen Bonds Specifically Stabilizing the T or R Structures.

Interactions within Subunits	Interactions between Subunits	Loss of Interactions Sufficient to Tip Equil. of Unliganded Hb Fully to R
	T Structure	
1. Tyr-140α-OH----OC-Val-93α	6. Lys-127α_1-$NH_3^+\cdots{}^-OOC$ \ Arg-141α_2	1, 6, 7. Des-Arg-141α-Tyr-140α
2. Tyr-145β-OH----OC-Val-98β		2, 4, 5. Stripped Hb Bethesda (Tyr-145$\beta\rightarrow$His)
3. His-146β-$Im^+\cdots{}^-OOC$-Asp-94β	7. Asp-126α_1-$COO^-\cdots{}^+$Gua /	
4. $^+NH_3$-Val-1β \ HPO_4^{2-}	8. Lys-40α_1-$NH_3^+\cdots{}^-OOC$-His-146β_2	3, 6, 7, 8. NES-des-Arg-141α or des-Arg-141α-His-146β
	9. Val-1β-NH_3^+ /	
5. $^+NH_3$-Lys-82β	10. Lys-82β-NH_3^+ - - 2,3-DPG[a]	13. Kempsey (Asp-99$\beta\rightarrow$Asn)
	11. His-2β-Im^+ - - /	
	12. His-143β-Im^+ - - /	
	13. Tyr-42α_1-OH----OOC-Asp-99β_2	
	R Structure	
None detected so far	14. Lys-127α_1-$NH_3^+\cdots{}^-OOC$-Arg-141α_2[b]	Loss of interaction sufficient to tip allosteric equilibrium of liganded Hb fully to the T structure
	15. Val-1β_1-$NH_3^+\cdots{}^-OOC$-His-146β_2[b]	
	16. Asp-94α_1-COO^-----NH_2-Asn-102β_2	16. Kansas (Asn-102β-Thr)

[a] DPG = diphosphoglycerate. [b] Probably very weak salt bridges.