Plant Physiol. (1996) 110: 403–411

Changing the Dimensions of Suberin Lamellae of Green Cotton Fibers with a Specific Inhibitor of the Endoplasmic Reticulum-Associated Fatty Acid Elongases[1]

Alain Schmutz, Antony J. Buchala, and Ulrich Ryser*

Institut für Botanische Biologie, Universität Freiburg, A. Gockelstrasse 3, CH-1700 Freiburg, Switzerland

The fibers of the green lint mutant of cotton (*Gossypium hirsutum* L.) contain large amounts of wax and are suberized. More than 96% of the bifunctional aliphatic suberin monomers (α,ω-alkanedioic acids and ω-hydroxyalkanoic acids) have chain lengths of C_{22} and C_{24} in green cotton fiber suberin. In fibers grown in the presence of *S*-ethyl-*N,N*-dipropylthiocarbamate (EPTC), a specific inhibitor of the endoplasmic reticulum-associated fatty acid elongases, the aliphatic suberin monomers were shortened to chain lengths of C_{16} and C_{18}. Whereas the amounts of most suberin monomers were not negatively affected by the inhibitor treatment, the amounts of α,ω-alkanedioic acids and of glycerol were reduced by more than 80%. Analysis in the transmission electron microscope showed a reduction in suberin content after EPTC treatment. The suberin layers were discontinuous and consisted of fewer lamellae than in the controls. A small proportion (up to 22%) of the electron-translucent suberin lamellae were thinner after EPTC treatment, probably because of the shortening of the aliphatic suberin monomers. A larger proportion of the electron-translucent lamellae were thicker than the lamellae in the controls. Possible explanations for this observation are discussed.

Color mutants of cotton (*Gossypium hirsutum* L.) fibers have been known for a long time, and their genetics are well established (Endrizzi et al., 1985). In the last few years, colored cotton fibers have also been used to a small extent for the production of textiles. The fibers of the *Lg* mutant contain high amounts of wax (Conrad, 1941), and their secondary walls are suberized (Ryser et al., 1983; Yatsu et al., 1983; Ryser and Holloway, 1985). The suberin is deposited in several concentric cell-wall layers alternating with cellulose. The cell walls of cotton fibers and other seed coat epidermal cells are, in addition to suberin, covered by a thin cuticle (Yatsu et al., 1983; Ryser, 1985). White cotton fibers may be suberized to varying degrees at the fiber base (Ryser, 1992). The epidermal cells of the seed coat not forming fibers are suberized in all cotton species examined so far (Ryser and Holloway, 1985), and suberin and associated wax probably function as constitutive defensive layers against soil-borne pathogens (Schmutz et al., 1994a, 1994b).

Suberin is an important but poorly understood biopolymer. Despite the well-known suberin model of Kolattukudy (1980), the monomer composition of suberin and interunit-bonding patterns remain essentially unknown (Davin and Lewis, 1992). In particular, little evidence has been found for the postulated lignin-like, polyaromatic suberin domain (Zimmermann et al., 1985; Borg-Olivier and Monties, 1989, 1993). Bernards et al. (1995) have shown that the polyaromatic domain of suberin is composed mainly of hydroxycinnamic acids rather than of the corresponding alcohols as in lignins. Recently, it was shown that glycerol could be liberated from the cell walls of exhaustively extracted green cotton fibers by treatments cleaving ester bonds, and glycerol was proposed as a new suberin monomer (Schmutz et al., 1993). The immediate significance of this finding is obvious: the aliphatic monomers of cotton fiber suberin can form only linear polymers on their own, whereas glycerol allows the formation of a three-dimensionally cross-linked network. The three hydroxyl groups of glycerol seem to be ideally suited to link and stabilize the three axes of a lamellated structure.

The presence of glycerol and fatty acids in suberin is reminiscent of the situation in biological membranes and led us to speculate that fatty acids of suberin might be oriented perpendicularly to the lamellation of the polymer and that the lengths of these fatty acids could define the thickness of the electron-translucent suberin lamellae (Schmutz et al., 1993). This speculation is supported to some extent by the classical work of Falk and El Hadidi (1961). These authors compared the lamellated ultrastructure of the suberin of potato and *Acacia seyal* and found the electron-translucent suberin lamellae to have a rather constant thickness of about 3 nm, whereas the thickness of the electron-opaque lamellae showed a much higher interspecies- and intraspecies-specific variation. Replacement of the long-chain fatty acids of suberin with fatty acids of shorter chain length should therefore reduce the thickness of the electron-translucent suberin lamellae. We decided to test this hypothesis by using EPTC to reduce the chain length of fatty acids in suberin. EPTC is a specific inhibitor of the ER-associated fatty acid elongases that produce very-long-chain fatty acids from C_{16} and C_{18} fatty acid precursors synthesized in plastids (Harwood, 1991). Green cotton fibers were used as the experimental material because

[1] This work was supported by the Swiss National Science Foundation (grant No. 31–39648.93 to U.R.).

* Corresponding author; e-mail ulrich.ryser@unifr.ch; fax 41–37–29–97–40.

Abbreviations: AIP, 2-aminoindan-2-phosphonic acid; EPTC, *S*-ethyl-*N,N*-dipropylthiocarbamate; *Lg*, green lint.

about 96% of the aliphatic suberin monomers with two functional groups (α,ω-alkanedioic acids and ω-hydroxyalkanoic acids) have chain lengths of C_{22} and C_{24} in this material. The aliphatic monomers of potato suberin with two functional groups, in comparison, are composed of about 95% C_{18} ω-hydroxyalkenoic and α,ω-alkenedioic acid monomers (Kolattukudy and Agrawal, 1974). It will be shown that EPTC-treated green cotton fibers produce suberin with a qualitatively and quantitatively different monomer composition. These changes in monomer composition are also reflected in the ultrastructure of suberin. The results support our hypothesis that the thickness of the electron-translucent suberin lamellae is determined by the length of fatty acids and that glycerol is a suberin monomer.

MATERIALS AND METHODS

Plant Material

An *Lg* genotype of cotton (*Gossypium hirsutum* L., obtained from Gerdat, Bouaké, Ivory Coast) plants was grown in a greenhouse at 25 to 30°C during the day and 18 to 22°C at night. One day after anthesis, the ovules were removed from the ovaries and cultured in vitro according to the method of Beasley and Ting (1973) in a medium containing 5 μM IAA, 5 μM GA_3, and 120 mM Glc. To inhibit callus formation, silver thiosulfate (50 μM) was added to the culture medium (Vain et al., 1989). Thirty days after anthesis, the ovules and the attached fibers were frozen in liquid N_2, and the frozen fibers were detached with tweezers and ground to a fine powder in a mortar and pestle. The cell-wall powder was washed with distilled water on glass-fiber filters, lyophilized, and weighed.

Inhibition of Fatty Acid Elongation

The thiocarbamate herbicide EPTC was dissolved in 100 μL of 50% (v/v) ethanol and added to the culture medium to different final concentrations (1, 10, and 100 μM) 11 or 18 d after anthesis. An equivalent amount of ethanol was added to the controls.

Extraction of the Purified Cell Walls

The lyophilized cell walls were extracted three times with hexane at about 40°C, three times with chloroform: methanol (2:1, v/v) at about 60°C, and finally two times with methanol at about 70°C in closed Pyrex tubes. In one experiment, an additional extraction with aqueous SDS (1%, w/v) was performed.

Depolymerization Procedure and GC-MS

The hexane and chloroform-methanol extracts and the exhaustively extracted cell-wall residues were depolymerized by acid-catalyzed transesterification with 5% (w/v) HCl in methanol for 16 h (extracts) or for 40 h (cell-wall residues) at 50°C. After addition of 5% (w/v) aqueous NaCl solution, the esters were extracted with CH_2Cl_2, the organic layer was dried over Na_2SO_4, the solvent was evaporated, and the resulting components were converted to their corresponding trimethyl silyl ethers. GC analysis was performed using N_2 as carrier gas at 1 mL min^{-1} on an SE 52 Permabond (Macherey-Nagel, Oensingen, Switzerland) column (25 m, i.d. 0.33 mm, film thickness 0.5 μm) at a temperature of 140°C for 2 min, which was then elevated to 280°C at 4° min^{-1}. The ionizing energy of the MS was 70 electron volts. In one experiment, 1-pentadecanol was added as an internal standard to the samples before transesterification. A mixture of standards contained *trans*-caffeic acid, C_{16} α,ω-alkanedioic acid, C_{22} α,ω-alkanedioic acid, C_{16} ω-hydroxyalkanoic acid, and 1-pentadecanol (internal standard).

Determination of Glycerol and Glc

The chloroform-methanol extracts and the exhaustively extracted cell-wall residues were hydrolyzed with 5% (w/v) KOH in methanol at about 70°C for 2 and 24 h, respectively. The hydrolyzed cell-wall residues were washed on glass-fiber filters with methanol, lyophilized, and weighed. The filtrates and the hydrolyzed chloroform-methanol extracts were neutralized with 2 N H_2SO_4, and salt precipitates were removed by filtration. The neutralized hydrolysates were extracted three times with hexane to remove fatty acids. Glycerol was quantitated with a commercial test combination (Boehringer Mannheim). The hydrolyzed and washed cell-wall residues were solubilized and hydrolyzed with sulfuric acid (Saeman et al., 1945), and the Glc content was determined with the Glc-oxidase test (Boehringer Mannheim).

Transmission EM

Ultrathin sections with gray interference colors were cut at a microtome setting of 35 nm and stained with lead citrate for 15 min. The thin sections were mounted on Formvar-coated Ni grids and examined at 80 kV in a Philips CM 100 electron microscope equipped with a BioTwin high-contrast lens, and a computerized, five-axis controlled goniometer, the Compustage.

For the measurement of the thickness of suberin lamellae, well-oriented suberin layers (those with good visibility of the lamellation) were moved with a rotation holder into a position parallel to the tilt axis. The layers were then tilted until they were optimally oriented. In this position, a micrograph was taken at a magnification of about ×160,000 after a lens normalization step. Two additional micrographs were taken at tilt angles of ±2.5°, to allow us to define exactly the places of optimal orientation of the suberin lamellae on the prints. The prints were enlarged 2.15 times and the thickness of the suberin lamellae was measured with a calibrated magnifying lens (×10). For the calibration of the final magnification, the 8.75-nm spacing of catalase crystals (BAL-TEC, Balzers, Switzerland) was used. The mean value of the spacings of five crystals was determined by measuring each crystal at four different places.

Statistical Analysis

The measurements of lamellar thickness were subjected to statistical analysis. Because some histograms of EPTC-treated samples did not necessarily show a normal frequency distribution, the significance of the difference of mean values was determined with the nonparametric two-group test of Wilcoxon. Very similar P values were obtained with a two-group *t* test.

RESULTS

Development of Cotton Ovule Cultures in the Presence of EPTC

Ovules of the *Lg* mutant of cotton were excised from the ovaries 1 d after anthesis and cultured in vitro. If EPTC (final concentration 10 μM) had already been added to the culture medium at the beginning of the culture period, subsequent growth and development of ovules and fibers were completely inhibited. If the inhibitor was added 10 d later, at the beginning of secondary wall formation in the fibers, secondary walls of approximately normal thickness developed, as estimated by transmission EM. The higher inhibitor concentrations (10 and 100 μM EPTC) reduced the intensity of the green color of isolated and lyophilized cell walls, whereas 1 μM EPTC had no visible effect. These differences were still clearly visible after solvent extraction of the cell walls.

To obtain a quantitative estimate of secondary wall formation, cell-wall-bound Glc was determined in purified cell walls after treatment of the ovules with different inhibitor concentrations. The results are shown in Table I. With increasing inhibitor concentrations, the dry weight of the fibers, as well as the Glc content of the cell walls, increased steadily, indicating that cellulose biosynthesis was not negatively affected by the inhibitor treatment. The increase in the relative Glc content of the purified cell walls (after exhaustive extraction and hydrolysis with methanolic KOH) may indicate that with increasing EPTC concentration thicker secondary walls are formed. In a parallel experiment (Schmutz et al., 1993) with AIP, a specific and potent inhibitor of Phe-ammonia-lyase, the fiber dry weight and the Glc content of the cell walls were not affected by increasing inhibitor concentrations. The corresponding fiber dry weights per 100 ovules were 108 mg (control), 98 mg (3 μM AIP), 106 mg (10 μM AIP), and 116 mg (30 μM AIP).

Table I. *Cell-wall-bound Glc in fibers from ovules cultured in vitro with different concentrations of EPTC*

Fibers of about 100 ovules from 8 to 10 ovaries were pooled for Glc determination. Values were calculated for 100 ovules in each treatment.

Treatment	Fiber Dry Wt in mg	Yield of Extracted and Alkali-Hydrolyzed Fibers in mg (Percent of fiber dry wt)	Glc Content in mg (Percent of extracted and alkali-hydrolyzed fibers)
Control	104.7	69.6 (66.5)	48.0 (69.0)
1 μM EPTC	124.5	75.3 (60.5)	56.8 (75.4)
10 μM EPTC	160.5	100.7 (62.8)	78.3 (77.8)
100 μM EPTC	187.4	119.2 (63.6)	98.5 (82.7)

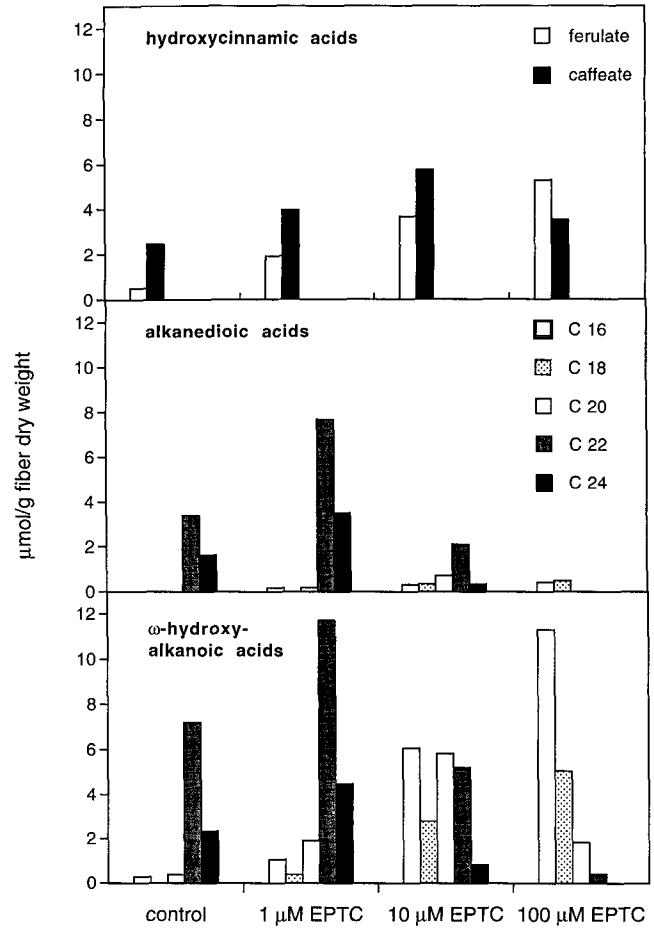

Figure 1. Effect of EPTC on the cell-wall-bound hydroxycinnamic acids, α,ω-alkanedioic acids, and ω-hydroxyalkanoic acids of green cotton fibers. EPTC was added to ovules cultured in vitro at the beginning of secondary wall formation (11 d after anthesis).

Effect of EPTC on Fatty Acid and Hydroxycinnamic Acid Composition of Suberin

Purified and exhaustively extracted cell walls of green cotton fibers were depolymerized by acid-catalyzed transesterification, and the solubilized aliphatic and aromatic monomers were identified as their trimethyl silyl ethers by GC-MS. With increasing inhibitor concentration, ω-hydroxyalkanoic acids and α,ω-alkanedioic acids of increasingly shorter chain length could be identified. At 100 μM EPTC, C_{16} and, to a lesser extent, C_{18} acids were the predominant suberin monomers, whereas in the controls and at 1 μM EPTC, C_{22} and C_{24} monomers dominated (Fig. 1). The absolute amounts of polymer-bound alkanoic acids were not negatively affected by the inhibitor treatment. However, an increase of the ω-hydroxyalkanoic acids and a strong reduction of α,ω-alkanedioic acids were observed (Table II). These effects were observed in three independent experiments.

The main hydroxycinnamic acid liberated by acid-catalyzed transesterification from the cell-wall residues was caffeate, followed by ferulate and traces of *p*-coumarate. The caffeate content of the cell walls was not negatively

affected by the inhibitor treatment, but it is interesting that a strong increase in wall-bound ferulate was observed with increasing inhibitor concentrations (Fig. 1).

Effect of EPTC on Fatty Acid and Hydroxycinnamic Acid Composition of the Wax

The chloroform-methanol extract of green cotton fibers is yellow-green and fluorescent and contains the bulk of the extractives of green cotton fibers. A large portion of this so-called "wax" fraction is composed of oligomeric materials consisting mainly of a colorless but fluorescent compound and several yellow compounds containing fatty acids, glycerol, and unknown cinnamic acid derivatives (Ryser and Holloway, 1985; Schmutz et al., 1993, 1994b). After treatment with EPTC, the amount of extractives of the green cotton fiber cell walls was reduced from 11.1% (of fiber dry weight) in the control to 8.2% (1 μM EPTC), 3.5% (10 μM EPTC), and 2.1% (100 μM EPTC) in the EPTC-treated samples. The chloroform-methanol extracts were dried and depolymerized, and the aliphatic and aromatic components were analyzed by GC-MS. Hydroxycinnamic acids, ω-hydroxyalkanoic acids, and α,ω-alkanedioic acids clearly decreased with increasing inhibitor concentration (Fig. 2). For the fatty acids, the expected decrease in acids with longer chain lengths (C_{20} to C_{24}) was observed. In the controls, larger amounts of all classes of compounds were found in the wax than in the cell-wall residue (Tables II and III). After treatment with 100 μM EPTC, the wax was strongly depleted in these compounds, whereas the cell-wall residue now contained higher amounts of all of the components.

Glycerol Content of Suberin and Wax after EPTC Treatment

The glycerol content of the wax and of the exhaustively extracted cell-wall residue was determined enzymatically after alkaline hydrolysis (Tables II and III). It should be noted that glycerol and fatty acids were not determined in the same experiments. In addition, a variation by a factor of 4 in the contents of glycerol and other suberin monomers was observed in different batches of ovule cultures. In the wax and in the cell-wall residue, the glycerol content decreased with increasing inhibitor concentration, the decrease being much more pronounced in the wax. From the wax and the cell-wall residue, 2 and 19%, respectively, of the levels of glycerol in the control samples could be recovered after treatment with 100 μM EPTC.

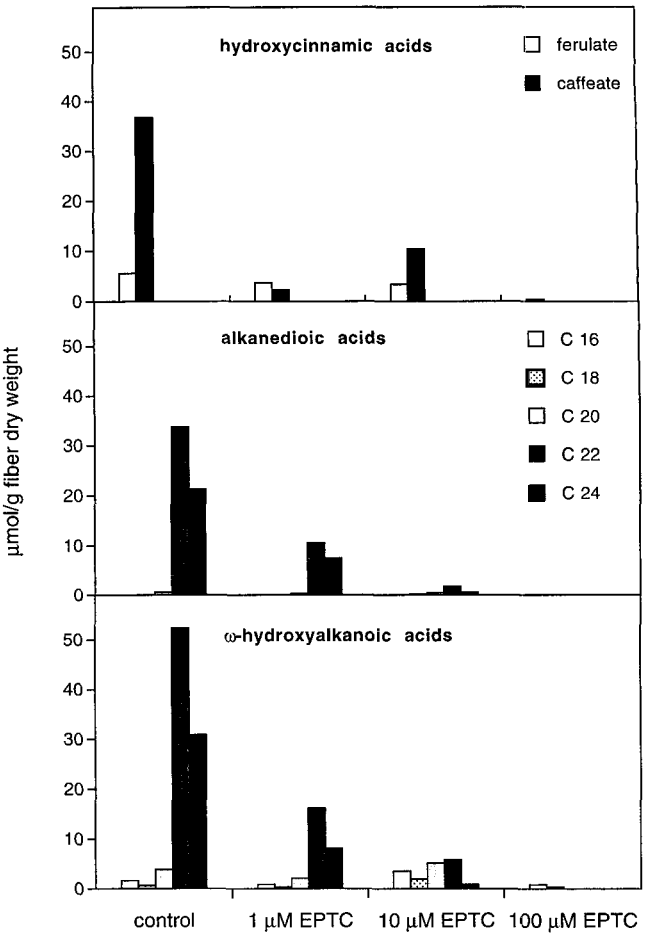

Figure 2. Effect of EPTC on the hydroxycinnamic acids, α,ω-alkanedioic acids, and ω-hydroxyalkanoic acids in the wax of green cotton fibers. EPTC was added to ovules cultured in vitro at the beginning of secondary wall formation (11 d after anthesis).

Ultrastructure of the Suberin Layers after EPTC Treatment

Fibers of ovules treated with 100 and 10 μM EPTC were analyzed in the transmission electron microscope. After

Table II. *Content of glycerol and aliphatic components in exhaustively extracted fibers from ovules cultured in vitro with different concentrations of EPTC*

The values shown are the results of the experiment performed with 1-pentadecanol as an internal standard. The experiment was repeated at least twice with similar results.

Treatment	Glycerol[a]	1-Alkanols	Alkanoic Acids	ω-Hydroxyalkanoic Acids	α,ω-Alkanedioic Acids
			μmol/g fiber dry wt (% control)		
Control	53.5 (100)	1.21 (100)	2.09 (100)	10.2 (100)	5.02 (100)
1 μM EPTC	54.2 (101)	1.63 (135)	4.09 (196)	19.6 (192)	11.6 (231)
10 μM EPTC	24.9 (46)	1.16 (96)	2.55 (122)	20.6 (202)	3.81 (76)
100 μM EPTC	9.88 (19)	0.49 (40)	2.30 (110)	18.5 (181)	0.89 (18)

[a] The glycerol content was not determined in the same experiment.

Table III. *Content of glycerol and aliphatic components in chloroform-methanol extracts of fibers from ovules cultured in vitro with different concentrations of EPTC*

The values shown are the results of the experiment performed with 1-pentadecanol as an internal standard. The experiment was repeated at least twice with similar results.

Treatment	Glycerol[a]	1-Alkanols	Alkanoic Acids	ω-Hydroxyalkanoic Acids	α,ω-Alkanedioic Acids
			μmol/g fiber dry wt (% control)		
Control	127.6 (100)	7.83 (100)	25.0 (100)	89.5 (100)	55.4 (100)
1 μM EPTC	86.4 (68)	2.64 (34)	11.2 (45)	27.7 (31)	18.1 (33)
10 μM EPTC	10.0 (7.8)	1.07 (14)	8.56 (34)	17.4 (19)	2.84 (5.1)
100 μM EPTC	2.61 (2.0)	0 (0)	1.81 (7.2)	1.12 (1.3)	0 (0)

[a] The glycerol content was not determined in the same experiment.

treatment of the ovules with 100 μM EPTC at the beginning of secondary wall formation (11 d after anthesis), only a small amount of suberin could be detected in the secondary walls; the suberin layers were short and did not form continuous, concentric layers as in the controls. In addition, the suberin layers were composed of only one or two lamellae, whereas in the controls usually layers with several lamellae predominated. The lamellae also appeared to be much thicker and more variable in size after the 100 μM EPTC treatment, as compared to the controls. In a second experiment, the inhibitor was added during active secondary wall formation (18 d after anthesis). Therefore, in the same fiber normal suberin layers were initially formed, and this was followed by formation of the lamellae in the presence of the inhibitor (Fig. 3). The differences between control and EPTC treatment can again be seen clearly. Likewise, after treatment of the ovules with 10 μM EPTC, less suberin was deposited than in the controls. The suberin layers were, however, longer than after treatment with 100 μM EPTC and often contained more than two lamellae. It is most interesting that thin and thick lamellae could often be observed in the same field of view (Fig. 4).

The periodicity of suberin lamellae in green cotton fibers of plants grown in the greenhouse, determined earlier (Ryser et al., 1983), was 4.2 ± 0.4 nm (±SD, n = 15). The periodicity determined in this study for fibers of ovules cultured in vitro was 4.3 ± 0.3 nm (±SD, n = 147). The thickness of the electron-translucent lamellae was 3.4 ± 0.3 nm, and the thickness of the electron-opaque lamellae was calculated to correspond to 1.0 nm. It can be concluded that the periodicities of suberin lamellae of fibers derived from ovule cultures and from intact plants are very similar, if not identical. The effects of EPTC on the thickness of suberin lamellae are summarized in Table IV. After treatment with 100 μM EPTC, the suberin lamellae were significantly thicker than in the controls ($P \leq 0.0001$). After treatment with 10 μM EPTC, no statistically significant differences were detected in comparison to the controls ($P = 0.21$ and 0.26). Additional interesting information can be obtained from histograms that show the distribution of lamellar thickness in controls and EPTC-treated fibers. After treatment with 100 μM EPTC, 9% of the lamellae were thinner than the control lamellae. Only about 16% of the lamellae fall into the size class of the controls, the majority of the lamellae (75%) being thicker (Fig. 5). It is interesting that fluctuations in the number of counts per size class are observed that are close to multiples of the length of esterified C_{16} ω-hydroxyalkanoic acids (about 2.2 nm). For the electron-translucent lamellae (Fig. 5A), a first calculated multiple would be situated at about 4.8 nm; for the combined electron-translucent and electron-opaque lamellae (Fig. 5B), the value would be at about 5.4 nm. Similar results were obtained after treatment with 10 μM EPTC (results not shown). At this inhibitor concentration the thinnest lamellae were found in the same size class as after treat-

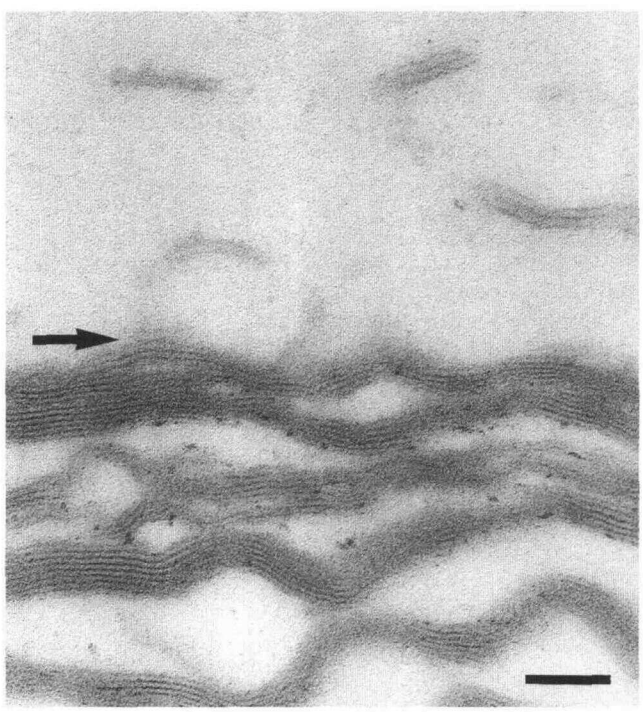

Figure 3. Effect of 100 μM EPTC on the ultrastructure of cotton fiber suberin. The inhibitor was added to ovules cultured in vitro during secondary wall formation (18 d after anthesis). The suberin at the top of the micrograph (above arrow) was deposited in the presence of EPTC, and the suberin below the arrow was deposited in the absence of EPTC. The following effects of EPTC are visible: (a) greatly reduced amounts of suberin deposits, (b) the formation of discontinuous suberin layers, (c) a reduction in the number of lamellae per suberin layer, and (d) an increased thickness of the suberin lamellae. Bar = 50 nm.

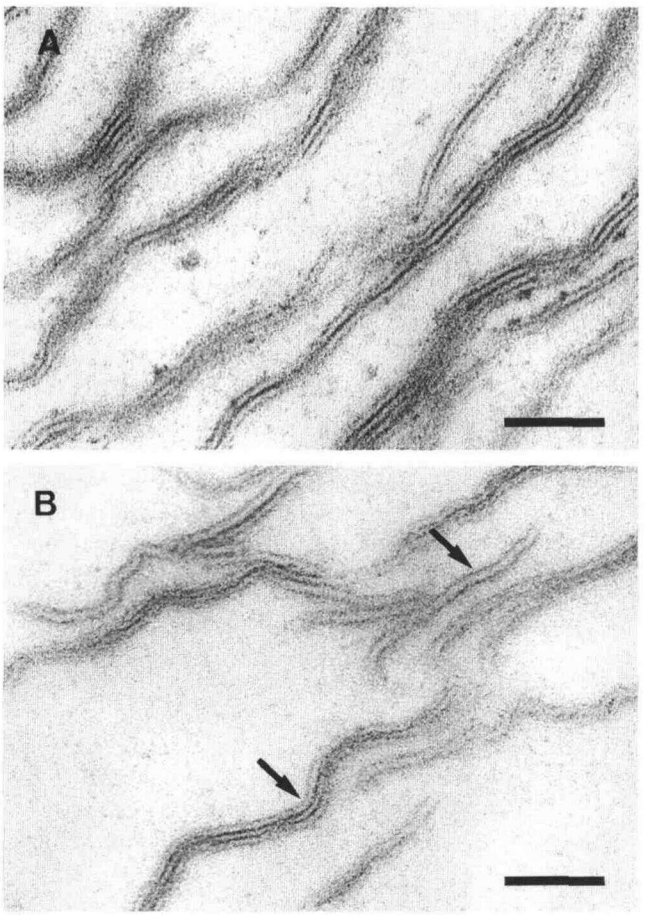

Figure 4. Effect of 10 μM EPTC on the ultrastructure of cotton fiber suberin. A, Control, suberin deposited in the absence of inhibitor. B, Inhibitor added at the beginning of secondary wall formation (11 d after anthesis). Similar but less drastic effects are observed than those occurring after the addition of 100 μM EPTC. The deposition of very thin suberin lamellae in addition to thicker ones in the same field of view is remarkable. Arrows indicate well-oriented thin and thick suberin lamellaes. Bars = 50 nm.

ment with 100 μM EPTC and made up 22% of the total, whereas 55% of the lamellae fell into the size class of the controls and 24% were thicker than the controls.

DISCUSSION

The monomer composition of the suberin in green cotton fibers developing on excised ovules cultured in vitro has been determined to our knowledge for the first time in six different experiments and was found to be qualitatively very similar to the monomer composition of fibers growing on plants (Schmutz et al., 1993, 1994b; A. Schmutz, A.J. Buchala, and U. Ryser, unpublished results). The amounts of suberin monomers, however, varied by a factor of about 4, the lower values corresponding to fibers from plants in the greenhouse. The larger variation in the amounts of suberin monomers of fibers from in vitro cultures probably depends on the hormone status of the cotton plants at the time of excision of the ovules.

The ratio of α,ω-alkanedioic acids to ω-hydroxyalkanoic acids (0.5) reported in this paper is considerably higher than those found by Yatsu et al. (1983) and Ryser and Holloway (1985) for the suberins of fibers grown on plants (0.35 and 0.19, respectively). According to our unpublished results, these differences depend on the different methods of suberin depolymerization and do not reflect real differences between the suberins of fibers grown in vitro or in vivo.

EPTC and other thiocarbamate herbicides selectively inhibit the formation of very-long-chain fatty acids. The active compounds are probably the sulfoxide metabolites of the thiocarbamates (Abulnaja and Harwood, 1991). The exact mechanism of inhibition is not known, but it is possible that the elongases involved in the biosynthesis of very-long-chain fatty acids are an important target for the metabolites of the thiocarbamate herbicides (Harwood, 1991). Thiocarbamates caused changes in the composition of cutin, suberin, and associated wax that contain long-chain fatty acids and their derivatives (Kolattukudy and Brown, 1974; Bolton and Harwood, 1976). In this paper we report to our knowledge the first application of a thiocarbamate herbicide to a system producing suberin composed mainly of very-long-chain fatty acids, and we compare the changes in suberin monomer composition with the changes in the ultrastructure of suberin.

A very striking observation was the complete inhibition of ovule growth when the inhibitor was added at the beginning of ovule culture. When added toward the end of fiber elongation growth, the inhibitor did not, however, interfere negatively with the metabolic processes necessary for the biosynthesis of cellulose, C_{16} and C_{18} fatty acids, and hydroxycinnamic acids. These observations strongly argue against the hypothesis that the thiocarbamate herbicides interfere generally with enzymes, coenzymes, or metabolic intermediates containing sulfhydryl groups (Fuerst, 1987) but rather agrees with the proposals that the inhibitors interfere more specifically with the elongation of fatty acids (Harwood, 1991).

As expected, EPTC inhibited the synthesis of very-long-chain alkanols, alkanoic acids, ω-hydroxyalkanoic acids, and α,ω-alkanedioic acids in green cotton fibers. The long-chain monomers were essentially replaced by similar amounts of homologous monomers of shorter chain length in suberin, with the exception of the α,ω-alkanedioic acids. Only small amounts of α,ω-alkanedioic acids (about 18% of the controls) could be recovered from suberin after treatment with 100 μM EPTC. The reasons for this observation are not known, but the effect is probably the result of the chain-length specificity of certain enzymes. A possible candidate is ω-hydroxyalkanoic acid dehydrogenase, the first enzyme involved in the oxidation of ω-hydroxyalkanoic acids to α,ω-alkanedioic acids. The corresponding potato enzyme was purified to homogeneity and a model of the substrate-binding site was presented. Evidence was ob-

Table IV. *Effect of EPTC on the thickness of suberin lamellae*

EPTC Concentration	No. of Measurements	Administration of EPTC	Thickness of Lamellae				
			Translucent and opaque (±SE)	P value	Translucent (± SE)	P value	Opaque[a]
μM		*d after anthesis*	*nm*		*nm*		*nm*
0	34	11	4.30 ± 0.04		3.39 ± 0.05		0.9
100	34	11	5.90 ± 0.30	<0.0001	4.31 ± 0.18	0.0001	1.6
0	11	18	4.05 ± 0.06		3.26 ± 0.08		0.8
100	11	18	5.53 ± 0.17	0.005	4.41 ± 0.15	0.005	1.1
0	55	11	4.36 ± 0.04		3.36 ± 0.04		1.0
10	55	11	4.31 ±0. 08	0.21	3.47 ± 0.08	0.26	0.8

[a] Calculated values.

tained for a hydrophobic binding region and a binding site for the distal carboxyl group (Agrawal and Kolattukudy, 1978a, 1978b). The potato enzyme is not able to oxidize C_{22} ω-hydroxyalkanoic acids. The cotton enzyme, not purified to date, may, on the other hand, have a low affinity for shorter ω-hydroxyalkanoic acids. This hypothesis is supported by the accumulation of ω-hydroxyalkanoic acids by a factor of about 1.8 after treatment with 100 μM EPTC and by the observation that at this EPTC concentration about equal amounts of C_{18} and C_{16} α,ω-alkanedioic acids were released from the suberin polymer upon depolymerization, whereas C_{16} was clearly the predominant chain length of the ω-hydroxyalkanoic acids released.

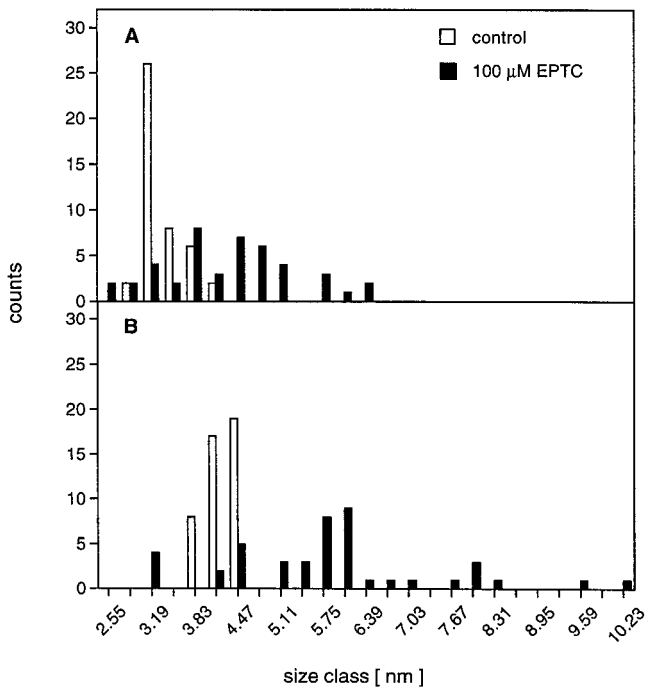

Figure 5. Histogram showing the thickness of suberin lamellae after treatment with 100 μM EPTC and in controls. A, Electron-translucent lamellae only. B, Electron-translucent and electron-opaque lamellae combined. Definition of size classes: Bars include lamellae with a thickness of $x \pm 0.16$ nm.

The glycerol content of the exhaustively extracted cell walls was strongly reduced by the treatments with EPTC. At 100 μM EPTC, only 19% of polymer-bound glycerol could be recovered from the cell walls. The α,ω-alkanedioic acid content was similarly reduced to 18%. Other suberin monomers were only slightly affected. Probably glycerol and α,ω-alkanedioic acids are secreted together as part of a suberin oligomer into the cell-wall space. According to this interpretation the effect of EPTC on the glycerol content of suberin is indirect and depends on the availability of α,ω-alkanedioic acids. We conclude that, after treatment with EPTC, α,ω-alkanedioic acids and glycerol were the limiting factors for the deposition of the multilamellated suberin layers, and lack of these acids was essentially responsible for the changed ultrastructure of suberin. Thus, indirect evidence for a structural role of glycerol in cotton fiber suberin was obtained.

It is interesting to compare the effects of EPTC on suberin and associated waxes of green cotton fibers with the effects obtained after treatment with AIP, a specific and potent inhibitor of Phe-ammonia-lyase. Added to in vitro cultures of cotton ovules throughout the period of secondary wall formation in the fibers, AIP completely inhibited the development of the colored compounds as well as the deposition of the multilamellated suberin layers (Schmutz et al., 1993). Less than 10% of glycerol and of suberin monomers could be recovered from the AIP-treated fibers (Schmutz et al., 1994b), clearly implicating cinnamic acid derivatives in the deposition of the suberin layers in green cotton fibers. The total amounts of wax were not reduced by AIP treatment. For reasons that are not yet understood, the proportion of ω-hydroxyalkanoic acids decreased in favor of higher amounts of α,ω-alkanedioic acids.

The relationship between the wax and the suberin polymer is not really understood. It is possible that the polymer is deposited first and serves as a scaffold for the deposition of the wax, as was proposed earlier (Schmutz et al., 1993). Alternatively, it might be postulated that the wax is first deposited and then crystallizes, and finally polymerization

takes place at the borders of the crystalline regions, as proposed for cutin polymerization (Riederer, 1991). Our data do not provide a direct solution to this problem. In fact, we were rather surprised by the close similarity in monomer composition of the wax and the polymer, as well as by the almost identical distribution of chain lengths of fatty acids in the two fractions. After EPTC treatment, an increased depletion in absolute and relative amounts of all measured compounds was observed in the wax as compared to the suberin polymer. This observation indicates that, after EPTC treatment, the available suberin monomers are preferentially incorporated into the polymer and not into the waxes.

The shortening of the fatty acids at the higher inhibitor concentrations and the significantly altered monomer composition led to a change in the ultrastructure of the suberin layers. Relatively short suberin layers were deposited, containing only one or two lamellar periods. The distribution of the thickness of the suberin lamellae was very broad at the higher inhibitor concentrations compared to the controls. It is interesting that the size distribution of the lamellae showed a periodicity of about 2.2 nm, corresponding approximately to the length of esterified C_{16} ω-hydroxyalkanoic acids. This result strongly suggests that the length of the fatty acids determines the thickness of the electron-translucent suberin lamellae, according to our working hypothesis. This is even more clearly demonstrated by the significant numbers of very thin lamellae in the inhibitor-treated samples, i.e. 22% after treatment with 10 μM EPTC and 9% after treatment with 100 μM EPTC. The distance between two carbon atoms in the longitudinal direction of an extended aliphatic chain is 0.126 nm. A reduction in chain length from C_{22} to C_{18} or C_{16} should, therefore, lead to a reduction of 0.5 or 0.75 nm in the thickness of each electron-translucent lamella. The thin lamellae are approximately two size classes thinner than the mean values of the control lamellae (Fig. 5). This difference corresponds to 0.64 nm and is very close to the theoretically expected difference of 0.5 to 0.75 nm. Taking into account the multilamellated structure of cotton fiber suberin we expected to be able to measure a clear-cut decrease in the thickness of the electron-translucent lamellae after the inhibitor treatments. This goal was only partially achieved, because of the strong diminution of the α,ω-alkanedioic acid and glycerol contents in the inhibitor-treated samples.

The observation that, at the highest inhibitor concentration, lamellae of fairly constant thickness also occur indicates that some cross-linking is maintained throughout the suberin layers. However, the cross-links occurring in native suberin are probably replaced to a large part by mono- di- and oligomers of esterified ω-hydroxyalkanoic acids. It is interesting that in the controls and in the inhibitor-treated samples the electron-translucent lamellae were always found to be about 0.3 nm thicker than the extended fatty acids defining their length (Fig. 5), leaving some space for the carboxylic group of hydroxycinnamic acids and glycerol. This observation is consistent with the hypothesis that the electron-translucent and the electron-opaque lamellae are composed of aliphatic and aromatic suberin constituents, respectively. The function of glycerol could be to cross-link the two types of lamellae.

ACKNOWLEDGMENTS

We thank N. Amrhein (Eidgenössische Technische Hochschule, Zürich, Switzerland) and H. Chancy (Cermav, Centre National de la Recherche Scientifique, Grenoble, France) for helpful discussions; N. Amrhein for critical reading of the manuscript; O. Schafer (University of Freiburg, Chemistry Department) for modeling and length determinations of organic molecules; M. Schorderet for excellent technical assistance with thin sectioning, EM, and measuring of the suberin lamellae; L. Vincent for cultivating the cotton plants; and E. Kreuz (Ciba-Geigy, Basel, Switzerland) for the gift of EPTC.

Received August 2, 1995; accepted October 23, 1995.

LITERATURE CITED

Abulnaja KO, Harwood JL (1991) Interaction of thiocarbamate herbicides with fatty acid synthesis in germinating peas and their microsomal fractions. Phytochemistry **30:** 2883–2887

Agrawal VP, Kolattukudy PE (1978a) Purification and characterization of a wound-induced ω-hydroxyfatty acid:NADP oxidoreductase from potato tuber disks (*Solanum tuberosum* L.). Arch Biochem Biophys **191:** 452–465

Agrawal VP, Kolattukudy PE (1978b) Mechanism of action of a wound-induced ω-hydroxyfatty acid:NADP oxidoreductase isolated from potato tubers (*Solanum tuberosum* L.). Arch Biochem Biophys **191:** 466–478

Beasley CA, Ting IP (1973) The effects of plant growth substances on *in vitro* fiber development from fertilized cotton ovules. Am J Bot **60:** 130–139

Bernards MA, Lopez ML, Zajicek J, Lewis NG (1995) Hydroxycinnamic acid-derived polymers constitute the polyaromatic domain of suberin. J Biol Chem **270:** 7382–7386

Bolton P, Harwood JL (1976) Effect of thiocarbamate herbicides on fatty acid synthesis by potato. Phytochemistry **15:** 1507–1509

Borg-Olivier O, Monties B (1989) Caractérisation des lignines, acides phénoliques et tyramine dans les tissues subérisés du périderme naturel et du périderme de blessure de tubercule de pomme de terre. CR Acad Sci Ser III Sci Vie **308:** 141–147

Borg-Olivier O, Monties B (1993) Lignin, suberin, phenolic acids and tyramine in the suberized, wound-induced potato periderm. Phytochemistry **32:** 601–606

Conrad CM (1941) The high wax content of green lint cotton. Science **94:** 113

Davin LB, Lewis NG (1992) Phenylpropanoid metabolism: biosynthesis and monolignols, lignans and neolignans, lignins and suberins. *In* HA Stafford, RK Ibrahim, eds, Phenolic Metabolism in Plants. Plenum Press, New York, pp 325–375

Endrizzi JE, Turcotte EL, Kohel RJ (1985) Genetics, cytology, and evolution of *Gossypium*. Adv Genet **23:** 271–375

Falk H, El Hadidi MN (1961) Der Feinbau der Suberinschichten verkorkter Zellwände. Z Naturforsch **16b:** 134–137

Fuerst EP (1987) Understanding the mode of action of the chloroacetamide and thiocarbamate herbicides. Weed Technol **1:** 270–277

Harwood JL (1991) Lipid synthesis. *In* AR Katritzky, GJ Sabongi, eds, Topics in Applied Chemistry, Target Sites for Herbicide Action. Plenum Press, New York, pp 57–94

Kolattukudy PE (1980) Biopolyester membranes of plants: cutin and suberin. Science **208:** 990-1000

Kolattukudy PE, Agrawal VP (1974) Structure and composition of aliphatic constituents of potato tuber skin (suberin). Lipids **9:** 682–691

Kolattukudy PE, Brown L (1974) Inhibition of cuticular lipid biosynthesis in *Pisum sativum* by thiocarbamates. Plant Physiol **53:** 903–906

Riederer M (1991) The cuticle as barrier between terrestrial plants and the atmosphere–the significance of the wax structure for cuticular permeability. Naturwissenschaften **78:** 201–208

Ryser U (1985) Cell wall biosynthesis in differentiating cotton fibres. Eur J Cell Biol **39:** 236–256

Ryser U (1992) Ultrastructure of the epidermis of developing cotton (*Gossypium*) seeds: suberin, pits, plasmodesmata, and their implication for assimilate transport into cotton fibers. Am J Bot **79:** 14–22

Ryser U, Holloway PJ (1985) Ultrastructure and chemistry of soluble and polymeric lipids in cell walls from seed coats and fibres of *Gossypium* species. Planta **163:** 151–163

Ryser U, Meier H, Holloway PJ (1983) Identification and localization of suberin in the cell walls of green cotton fibres (*Gossypium hirsutum* L., var. green lint). Protoplasma **117:** 196–205

Saeman JF, Buhl JL, Harris EF (1945) Quantitative saccharification of wood and cellulose. Ind Eng Chem Anal Ed **17:** 35–37

Schmutz A, Buchala A, Jenny T, Ryser U (1994a) The phenols in the wax and in the suberin polymer of green cotton fibres and their functions. Acta Hortic **381:** 269–275

Schmutz A, Jenny T, Amrhein N, Ryser U (1993) Caffeic acid and glycerol are constituents of the suberin layers in green cotton fibres. Planta **189:** 453–460

Schmutz A, Jenny T, Ryser U (1994b) A caffeoyl-fatty acid-glycerol ester from wax associated with green cotton fibre suberin. Phytochemistry **36:** 1343–1346

Vain P, Flament P, Sudain P (1989) Role of ethylene in embryogenic callus initiation and regeneration in *Zea mays* L. J Plant Physiol **135:** 537–540

Yatsu LY, Espelie KE, Kolattukudy PE (1983) Ultrastructural and chemical evidence that the cell wall of green cotton fiber is suberized. Plant Physiol **73:** 521–524

Zimmermann W, Nimz H, Seemüller E (1985) ^{1}H and ^{13}C NMR spectroscopic study of extracts from corks of *Rubus idaeus*, *Solanum tuberosum*, and *Quercus suber*. Holzforschung **39:** 45–49