

# Chloroacetamides Affect the Plasma Membrane

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In the present study membrane fatty acids were analyzed to find a link between the biosynthesis inhibition of very-long-chain fatty acids and the phytotoxic effects of herbicidal chloroacetamides. Accordingly, we have isolated membranes of cucumber seedlings (*Cucumis sativus*) by two-phase partitioning and analyzed their fatty acid content. Saturated VLCFAs ranging from C20 to C26 were found in high amounts (22%) in the plasma membrane fraction. Non-modified VLCFAs were predominantly present in phospholipids, while saturated 2-hydroxylated VLCFAs were identified in cerebrosides. Treatment of intact seedlings with chloroacetamides markedly reduced the VLCFA content in the plasma membrane. This result could be specified by fatty-acid labeling using [<sup>14</sup>C]malonate as a substrate for fatty acid elongation. *De novo* incorporation of VLCFAs into the plasma membrane and into microsomal membranes, respectively, was severely impaired by chloroacetamides with  $I_{50}$  values between 10 to 100 nm. These results confirm the previous finding that chloroacetamides inhibit VLCFA biosynthesis localized in the microsomes (Böger *et al.*, Pest Manage. Sci. **56**, 497–508, 2000). The direct consequence of this inhibition is a strong decrease of VLCFAs required as constituents of the plasma membrane and the substitution by shorter acyl chains. Apparently, physical properties and function of the plasma membrane are affected eventually leading to death of the plant.

## Introduction

Very-long-chain fatty acids (VLCFAs) with a 20 to 26 carbon chain are minor, but ubiquitous fatty acid components in higher plants found in epicuticular waxes, seed storage lipids and membrane lipids as well (for review see Domergue *et al.*, 1998). The biosynthesis of these compounds could be attributed to the endoplasmatic reticulum (ER) and the Golgi apparatus, where they are formed by successive elongation of a C18 fatty acyl precursor by two carbons originating from malonyl-CoA. After subsequent lipid formation the VLCFA-containing lipids are transported to cellular membranes and secreted to produce epicuticular waxes.

In eukaryotic cells VLCFAs are predominantly found in sphingolipids, a membrane lipid structurally similar to glycerolipids. In plants, typical sphingolipids are made up by the long-chain sphingosine backbone which is glycosylated and amide-linked to an usually hydroxylated (very-)long-chain fatty acid, called cerebroside (Fig. 1). Apparently, cerebrosides are not equally distributed throughout all cellular membranes, but are predominantly found in the plasma membrane of

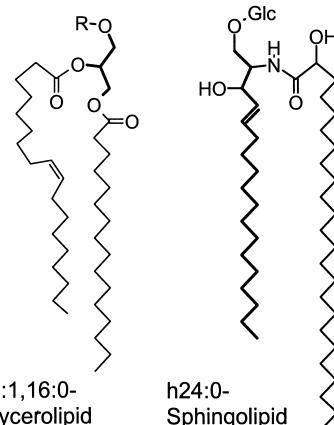


Fig. 1. Structures of typical membrane lipids found in plant plasma membranes.

Left: a typical glycerophospholipid with a glycerol backbone (printed in bold, R means phosphatidylcholine, -ethanolamine etc.), esterified to two C16–C18 fatty acids, usually one of them being unsaturated. Right: a typical sphingolipid (cerebroside) consisting of a C18 amino alcohol glucosylated at carbon no. 1 and *N*-amide-linked (carbon no. 2) to a hydroxy fatty acid (for example h24:0).

plants and yeast (Rochester *et al.*, 1987; Dickson and Lester, 1998). Cerebrosides are essential constituents of the plasma membrane involved in various physiological functions including signaling, exocytosis, anchoring of proteins, and vesicular protein transport (for review see e.g. Schneiter, 1999).

Chloroacetamide herbicides have previously been found to strongly inhibit microsomal fatty acid elongation resulting in a strong decrease of VLCFAs in plant and algal cells (for review see Böger *et al.*, 2000). Although the target enzyme of chloroacetamide-type inhibitors was disclosed as the microsomal fatty acid elongase, the subsequent secondary effects caused by inhibition of VLCFA biosynthesis are yet to be clarified. Various physiological effects reported for chloroacetamide treatment indicate a malfunction of cellular membranes (Ebert, 1980; Mellis *et al.*, 1982). The present study provides evidence for a link between typically observed secondary effects of chloroacetamides and inhibition of VLCFA biosynthesis.

## Materials and Methods

### Plant material, chemicals

Seeds of *Cucumis sativus* var. *Delikatess* were obtained from Gawaz (Bergheim, Germany), [ $2\text{-}^{14}\text{C}$ ]malonate (2.1 GBq mol $^{-1}$ ) was from Hartmann Analytics (Hannover, Germany). Lipid and fatty acid standards, monensin, derivatisation reagents and other chemicals were from Sigma (Deisenhofen, Germany). Solvents used for lipid analysis were at least of analytical grade purchased from Sigma and Merck (Darmstadt, Germany).

### Incubation and labeling of cucumber seedlings

Seeds germinated on a grid allowing the roots to be supplied with tap water applied in plastic pots. After three days metazachlor was added as an ethanolic stock solution (0.1%, v/v) giving a final concentration of 1 to 100  $\mu\text{M}$  herbicide. The seedlings grew for another three days in a 16 h/8 h day/night regimen at 28 °C and 80  $\mu\text{Einstein m}^{-2}\text{s}^{-1}$  and were harvested without roots.

For labeling experiments, seedlings were grown on vermiculite and tap water under the same light and temperature conditions for 7 days. Samples of cotyledon slices (2.1 g) were incubated in petri

dishes containing nutrient (20 mM KNO $^3$ , 0.2 mM CaSO $_4$ , 1  $\mu\text{M}$  Na $_2\text{MO}_4$ ), herbicide (0.01–1  $\mu\text{M}$ ), and [ $^{14}\text{C}$ ]malonate (14 Mdpm, 16  $\mu\text{M}$ ) for 24 h at 20 °C in continuous light (15  $\mu\text{Einstein m}^{-2}\text{s}^{-1}$ ).

### Plasma membrane isolation

All subsequent operations were performed at 4 °C. Seedlings (30 g fresh weight) were homogenized in a blender (4  $\times$  20 s) with 100 ml of 50 mM 3-(N-morpholino)propanesulfonic acid (Mops-KOH, pH 7.5) containing 0.2% (w/v) casein, 2 mM dithiothreitol (DTT), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.6% polyvinyl-polypyrrolidone and 330 mM sucrose. The homogenate was filtered (miracloth 100  $\mu\text{m}$ ) and separated from the plastids by centrifugation (10,000  $\times g$ , 20 min). The microsomal fraction, obtained as a pellet by centrifugation (35,000  $\times g$ , 1 h), was resuspended in 4 ml of 5 mM phosphate buffer (pH 7.8) including 330 mM sucrose and 2 mM DTT, 5 mM EDTA.

Plasma membrane, cytosolic membranes, and thylakoids were obtained by adapting the two-phase partitioning method (Kjelbom and Larsson, 1984) to cucumber (Larsson, pers. commun.). The two-phase system (12 g weight) was composed of dextran 500 and polyethylene glycol 3350 (each 6.1%, w/w), 330 mM sucrose, 5 mM phosphate buffer (pH 7.8), 1 mM KCl, 0.1 mM EDTA, 1 mM DTT. Only the purest membrane fractions obtained after additional partitioning were washed by pelleting (100,000 g, 1 h), resuspended in phosphate buffer (see above), and stored in liquid nitrogen. Protein was estimated using a dye concentrate from Bio-Rad Laboratories (Munich, Germany).

For labeled tissue (2.1 g fresh weight), the procedure was downscaled (30 ml homogenization buffer) to obtain a 400- $\mu\text{l}$  microsomal fraction which was purified twice using a two-phase system of 2.1 g weight. The labeled membranes of the upper and lower phase were collected and immediately subjected to fatty acid analysis.

### Analysis of fatty acids

Fatty acids were released from membrane phospholipids and cerebrosides considering their different chemical properties. To achieve gentle saponification of phospholipid species, alkaline

hydrolysis was performed with 10% KOH/ MeOH (v/v) for 1 h at 70 °C. Resulting fatty acids were extracted twice with *n*-hexane and transmethylated using BF<sub>3</sub>/ MeOH (details see Matthes *et al.*, 1998). For analysis of *N*-amide-linked cerebroside fatty acids, acid transmethylation was performed at 80 °C using either 3 M HCl in 80% methanol (v/v) or 14% BF<sub>3</sub>/MeOH (w/w). The methyl ester products were extracted with *n*-hexane as described above. Reaction time was varied from 1 to 20 h and the recovery of resulting fatty acid methyl esters was calculated using standard gluco- and galactocerebrosides and cerebrosides isolated from cucumber lipids (see *Lipid analysis*).

Hydroxy fatty acids were transformed to trimethylsilyl derivatives by gentle heating (60 °C) of completely dried samples with *N,O*-bis(trimethylsilyl)acetamide. Fatty acid samples were analyzed by gas chromatography (Shimadzu 15A) on a 30 × 0.25 mm × 20 µm capillary column (SP-2330, Supelco, Deissenhofen, Germany) equipped with a flame ionization detector. The initial oven temperature was 140 °C for 1 min followed by an increase of 5 °C/min to 270 °C.

Fatty acids were identified by mass spectrometry and authentic standards purchased from Supelco, Deissenhofen, Germany (C16-C30 fatty acids) and Matreya, Pleasant Gap, PA, USA (C16-C24 hydroxy fatty acids). The GC/MS system (Hewlett-Packard HP-5MS) was equipped with the same capillary column as above. Characteristic ions of non-modified fatty acid methyl esters were M<sup>+</sup> and (M-15)<sup>+</sup>, trimethylsilyl ethers of hydroxy fatty acid methyl esters were identified by (M-59)<sup>+</sup> and (M-15)<sup>+</sup>.

Radioactivity of the labeled membrane fractions was measured by liquid scintillation counting (LKB 1215 Rackbeta II, Pharmacia) and labeled fatty acid methyl esters were analyzed by radio-HPLC (Ramona, Raytest, Straubenhart, Germany) using a 250 mm × 4.6 mm × 4 µm RP-18 column (Macherey-Nagel, Düren, Germany) and a linear solvent gradient (rate 1.5 ml/min) of initially acetonitrile:methanol (9:1, v/v) increased to pure acetonitrile after 15 min. Labeled non-modified fatty acid methyl esters were identified by standards synthesized from [<sup>14</sup>C]methanol (Matthes *et al.*, 1998).

### Lipid analysis

Lipids of membrane fractions were extracted by a modified procedure from Bligh and Dyer (1959). Sample aliquots (0.5 ml) were extracted at 70 °C with methanol (2.5 ml) for 15 min to separate the supernatant (5000 g, 5 min). The pellet was reextracted by ultrasonic immersion with 2 ml chloroform:methanol (2:1, v/v). Combined supernatants were diluted with 2.5 ml aqueous NaCl (10%, w/v) and the lipids recovered from the chloroform phase.

Aliquots of lipids redissolved in chloroform were separated on TLC plates (Silica Gel 60, Merck, Germany) using a solvent system of chloroform:methanol:water (65:25:4, v/v). Lipids were visualized by staining with iodine or 10% H<sub>2</sub>SO<sub>4</sub> (v/v) followed by heating (120 °C, 30 min). Main components were identified by co-migration with standards and determined densitometrically (Software ImageQuant, Molecular Dynamics, Sunnyvale, CA, USA).

For analysis of individual lipid fatty acids, corresponding spots were scraped off the plate, eluted into solvent (2 × 1 ml) by ultrasonication (2 × 5 min) and dried under nitrogen. Fatty acid samples were analyzed as described above.

### Immunoblot analysis

Membrane fractions were resuspended in Mops (3-(*N*-morpholino)propanesulfonic acid), pH 7.5, denatured at 40 °C for 10 min in the presence of 10% (w/v) sodium dodecylsulfate (SDS) and separated by a 7.5% (w/w) SDS-PAGE. Proteins were blotted on an Immobilon-P membrane (Millipore), and blocked with 5% non-fat milk powder solubilized in phosphate buffer (4.1 mM NaH<sub>2</sub>PO<sub>4</sub>, 3.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 140 mM NaCl, 0.05% (w/v) Tween 20, pH 6.8).

H<sup>+</sup>-ATPase, a marker enzyme of the plasma membrane, was detected by a monoclonal mouse antibody (clone no. 46E5B11C10) raised against maize H<sup>+</sup>-ATPase. The antiserum incubation (1:10,000) was carried out overnight at 4 °C in phosphate buffer of above, and the antigen-antibody complex was detected by a peroxidase-conjugated secondary mouse antibody (Bio-Rad, Munich, Germany) using 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) pH 7.5 including 5 mM EDTA, 150 mM NaCl, 0.05% Triton

X-100. The blots were developed with an ECL Western blot chemiluminescence detection kit and Hyperfilm ECL films (Amersham, Germany) followed by densitometric analysis (see above). Proteins localized on the transfer membrane were stained with 0.1% (w/v) aqueous amidoschwarz (Merck) in water:isopropanol:acetic acid (73:20:7, v/v).

#### Data reliability

The experiments were repeated at least three times and representative experiments are shown. When error bars or values are given, data represent means  $\pm$  SE of three to six independent experiments. Reliability of fatty acid and lipid analyses were confirmed by controls using buffer instead of membrane fractions.

### Results and Discussion

#### Plasma membrane purity and lipid analysis

Light-grown cucumber seedlings were analyzed following homogenisation, separation of plastids, and subfractionation of a microsomal fraction by two-phase partition, a procedure modified for green cucumber tissue. Phase separation yielded an almost colourless plasma membrane fraction, practically free of chlorophyll, indicative of very little contamination by plastid residues. Accordingly, the remaining microsomal membranes (intracellular membranes) mainly consist of endoplasmatic reticulum, Golgi apparatus, mitochondria, residual plastid envelope and thylakoid membranes (Widell and Larsson, 1990).

Immunoreaction of the plasma membrane H<sup>+</sup>-ATPase isoenzyme with a monoclonal antibody was almost exclusively observed with protein from the plasma membrane preparation suggesting a 95% enrichment (Table I). In addition, analysis of the lipid and fatty acid pattern clearly corresponded to the typical composition of plasma membrane lipids, exhibiting a higher content of sterols, cerebrosides, and VLCFAs (Moreau *et al.*, 1988; Larsson *et al.*, 1990).

In contrast to the conventional method for analysis of cerebroside fatty acids using an overnight hydrolysis (see e.g. Cahoon and Lynch, 1991), we could recover 95% of *N*-amide-linked fatty acids by a 5-hour transmethylation procedure using methanolic boron trifluoride. The recovery of fatty acids did not depend significantly on their structure in terms of hydroxylation and content of double bonds.

#### Membrane-derived very-long-chain fatty acids

Fatty acid determination was performed with whole seedlings and membrane fractions. Table II outlines the fatty acid pattern of various cellular membranes including plastids, intracellular membranes and the plasma membrane.

Our results show the highest level of total VLCFAs in the plasma membrane (21.8%), followed by the intracellular membranes (12.0%), whereas plastids contained only minor amounts (1.6%) of VLCFAs (see Table II, data in bold). In principle, VLCFAs were concentrated at the expense of unsaturated C18 fatty acids. Especially the level of linolenic acid (18:3) was lowest in the

Table I. Purification of the cucumber plasma membrane.

Biochemical data showing some characteristics of the purest fractions of intracellular membranes and the plasma membrane of *Cucumis sativus* seedlings obtained after two-phase partitioning of the microsomal fraction. Lipid-bound fatty acids were determined by combination of TLC and GC analysis.

|  | Plasma membrane               | Intracellular membranes      |
|--|-------------------------------|------------------------------|
| Protein (mg/g fresh weight)                          | 0.11 $\pm$ 0.03               | 0.42 $\pm$ 0.14              |
| Presence of H <sup>+</sup> -ATPase (% distribution)  | > 95                          | < 5                          |
| Sterols (% distribution)                             | 64 $\pm$ 17                   | 36 $\pm$ 11                  |
| Chlorophyll content (% distribution)                 | 3 $\pm$ 2                     | 97 $\pm$ 3                   |
| Phospholipid-bound fatty acids (%)                   | 64 $\pm$ 5                    | 70 $\pm$ 6                   |
| <b>Cerebroside-bound fatty acids (%)<sup>a</sup></b> | <b>30 <math>\pm</math> 11</b> | <b>11 <math>\pm</math> 1</b> |
| Non-estimated fatty acids (%) <sup>a</sup>           | 6 $\pm$ 2                     | 19 $\pm$ 5                   |

<sup>a</sup> Including free fatty acids and plastidic galactolipids.

Table II. Fatty acid composition of cellular membranes.

Lipids present in different cellular fractions were completely hydrolyzed and the resulting fatty acids determined by gas chromatography. Fatty acids (FA) are termed x:y, according to the number of carbon chain length (x) and the number of double bonds (y). Non-modified very-long-chain fatty acids (VLCFA) were found ranging from C20 to C26, 2-hydroxy fatty acids (hFA) ranged from C16 to C26. Further abbreviations: SFA, saturated fatty acids; UFA, unsaturated fatty acids including 18:1, 18:2; 18:3; LCFA, C16-C18 long-chain fatty acids.

| Tissue                  | Total fatty acids<br>μmol g <sup>-1</sup> fresh weight | 16:0 | 18:0 | UFA  | Hydroxy<br>FA | Non-<br>modified<br>VLCFA | Total<br>VLCFA | Ratio:<br>LCFA<br>VLCFA | Ratio:<br>UFA<br>SFA |
|-------------------------|--|------|------|------|---------------|---------------------------|----------------|-------------------------|----------------------|
|                         |  | mol% | mol% | mol% | mol%          | mol%                      | mol%           |                         |                      |
| Whole seedlings         | 9.70 ± 0.57  | 15.1 | 4.5  | 79.0 | n.d.          | 1.4                       | <b>1.4</b>     | n.d.                    | 0.27                 |
| Plastids                | 5.67 ± 1.18  | 16.2 | 5.4  | 75.8 | 2.3           | 0.3                       | <b>1.6</b>     | 63                      | 0.32                 |
| Intracellular membranes | 0.41 ± 0.20  | 20.5 | 6.3  | 58.6 | 12.0          | 2.6                       | <b>12.0</b>    | 7.0                     | 0.70                 |
| Plasma membrane         | 0.31 ± 0.08  | 27.7 | 8.0  | 37.3 | 20.7          | 6.3                       | <b>21.8</b>    | 3.6                     | 1.70                 |

plasma membrane, whereas in intracellular membranes and in plastid membranes it was the main fatty acid (17%, 37%, 58%, respectively, not shown in the Table). The accumulation of VLCFAs in the plasma membrane is also reflected by a higher proportion of saturated acyl chains (6fold as much as in whole seedlings, see Table II). Fatty acids linked to plasma membrane lipids contain about one carbon atom more than intracellular and plastidic membranes (calculated from data of Table II). This contributes to the extraordinary thickness of the plasma membrane (Schneiter *et al.*, 1999) creating the most hydrophobic lipid bilayer examined in this study.

VLCFAs are minor components relating to the total fatty acids in cucumber. This is due to the very low VLCFA level in plastids, since thylakoids and plastid envelopes contain about 90% of the total fatty acids in green cells (Moreau *et al.*, 1998). The lower amount found in the cucumber plastids (Table II) is due to incomplete recovery of the plastids by the multi-step preparation procedure used.

In this study, we focused on the presence and significance of VLCFAs within the cell in order to elucidate the primary effect of the elongase inhibition on the actual VLCFA level. Since little is known about the function of VLCFAs, we focused on the estimation of VLCFAs in different types of membranes, considering the principal type of the lipid backbone (glycerol- or sphingolipid), the carbon chain length, and the fatty acid modification (hydroxylation). Lipid classes were not analyzed in detail.

The plasma membrane of cucumber included saturated VLCFAs with even- or odd chain lengths ranging from C20 to C26. We have also found 2-hydroxylated species with a carbon chain length of C16 to C26 (Fig. 2 and Fig. 3). Hydroxy fatty acids were preferably present in plasma membrane cerebrosides with about 40% of the acyl chains longer than C18. Phospholipids contained lower levels (5–10%) of VLCFAs present

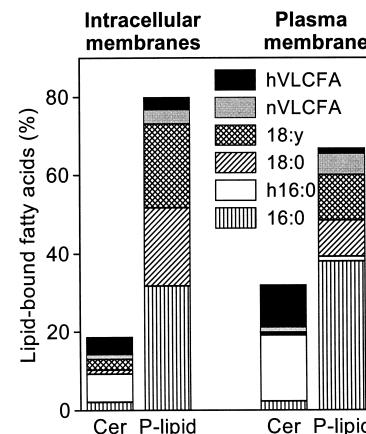


Fig. 2. Fatty acid composition of membrane lipids purified from 6-day old cucumber seedlings. Intracellular and plasma membranes were obtained by two-phase partitioning of microsomes. Cerebroside (Cer) and phospholipid (P-lipid) fatty acids were quantified following TLC separation, hydrolysis and gas chromatographic analysis. Fatty acids (x:y) are termed according to the number of carbons (x) and double bonds (y). Very-long-chain fatty acids (VLCFA) are separated into non-modified (n-) and 2-hydroxylated species (h-). The main hydroxy species were C16 and C24.

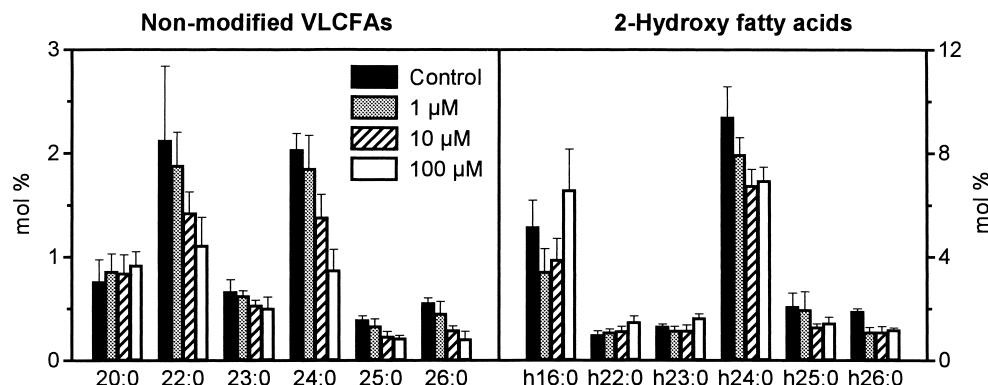


Fig. 3. Content of non-modified VLCFAs and 2-hydroxy fatty acids in the plasma membrane of *Cucumis sativus*. Fatty acids (x:y) are indicated by the number of carbons and double bonds, hydroxy fatty acids are labeled with h. Seedlings were grown for 3 days followed by a 3-day treatment with 1, 10, and 100  $\mu$ M metazachlor. Membrane fatty acids were determined following two-phase partitioning of microsomal membranes, lipid hydrolysis and gas chromatographic analysis.

in a non-modified and hydroxylated state as well (Fig. 2). These results correspond to earlier findings with various plant species (Cahoon and Lynch, 1991; Bohn *et al.*, 2001).

In intracellular membranes we found a three-fold lower level of cerebroside-linked fatty acids than in the plasma membrane, accompanied by a generally lower level of VLCFAs (Fig. 2). The proportion of phospholipids was about equal in either membranes (Table I) exhibiting a more similar fatty acid profile than was the case with cerebrosides (Fig. 2).

#### Effect of metazachlor on plasma membrane fatty acids

Using the purified plasma membrane of metazachlor-treated cucumber seedlings, analysis of the plasma membrane content revealed that metazachlor changes the composition of fatty acids (Fig. 3).

The total amount of lipid fatty acids was not significantly altered as reported earlier by Wu *et al.* (2000). Additionally, we found no effect on fatty acid desaturation: The level of unsaturated C18 fatty acids was not affected by up to 100  $\mu$ M metazachlor (data not shown). In higher plants some chloroacetamide-induced effects on C18 fatty acid desaturation cannot be generally excluded (Möllers and Albrecht, 1994; Couderchet *et al.*, 1993). This may be due to a secondary cellular re-

sponse caused by higher herbicide concentrations used in the experiments.

Chloroacetamides severely reduced the plasma membrane VLCFAs (Fig. 3). This was true for non-modified as well as hydroxy-VLCFAs. The maximum concentration of metazachlor (100  $\mu$ M) diminished the overall VLCFA content of the plasma membrane by 30%. The level of the most abundant non-modified VLCFAs (C22 and C24) dropped by 48% and 58%, respectively. The content of the main hydroxylated fatty acid (C24) was reduced by 28%.

Relating to inhibition of VLCFA biosynthesis and subsequent incorporation into membrane lipids, some trends have to be pointed out: The longer the acyl chain of the VLCFA species, the higher the inhibition by metazachlor (Fig. 3). This finding corresponds to previous analyses of VLCFA formation in cucumber and leek seedlings *in vivo* and *in vitro* (Matthes *et al.*, 1998; Schmalfuß *et al.*, 2000).

Precursors of VLCFAs accumulated, namely C16-hydroxy fatty acid (Fig. 3). Surprisingly, also some hydroxy-VLCFAs tended to accumulate (details see Fig. 3). This effect may reflect an antagonism between metazachlor-induced inhibition and feedback of non-processed intermediates.

The decrease of hydroxy-VLCFAs in the plasma membrane indicates that their biosynthesis depends on preformed VLCFAs. Fatty acid hydroxylation was reported to occur after elongation

with cytochrome P450 enzymes (Caballo-Hurtado *et al.*, 1998). Accordingly, only 2-hydroxylated VLCFAs are usually found in plants (see e.g. Bohn *et al.*, 2001; Cahoon and Lynch, 1991). On the other hand, an elongation of hydroxy-C18 fatty acid was recently found in *Lesquerella* seeds (Moon *et al.*, 2001).

The inhibition rate (50% with about 100  $\mu\text{M}$  metazachlor) was low compared with reduction of labeled VLCFAs in whole cucumber seedlings (Matthes *et al.*, 1998) or in the plasma membrane (see next section). This is a consequence of the herbicide application technique. To avoid the uncertain analysis of morphologically altered seedlings, the seeds first germinated for three days without herbicide. After that, the seedlings were treated with metazachlor for another three days. Accordingly, an unknown background of pre-formed VLCFAs was present before the onset of herbicide-induced inhibition of VLCFA synthesis. Therefore in such experiments the susceptibility to the inhibitor appears to be low.

#### Effect of metazachlor on VLCFA de novo synthesis

To demonstrate the inhibitor effect on the levels of membrane VLCFAs, we firstly measured the *de novo* incorporation of [ $^{14}\text{C}$ ]malonate firstly into lipids of the intracellular membranes, which essentially accounts for the inhibition of VLCFA biosynthesis in ER and Golgi, and secondly into plasma membrane lipids being supplied with freshly synthesized VLCFAs (Moreau *et al.*, 1988). Using radio-HPLC, we found labeled plasma membrane fatty acids in the range of C16-C24. Since HPLC separation of the fatty acids was performed according to increasing chain length, non-modified and hydroxy fatty acids could not be completely separated (Fig. 4). However, with 1  $\mu\text{M}$  metazachlor present, the incorporation of any VLCFA was practically abolished whereas formation of C16-C18 fatty acids was not affected.

Analysis of the incorporation of radiolabel into intracellular membranes and the plasma membrane revealed a comparable reduction of labeled VLCFAs in both compartments (Fig. 5). Apparently, the inhibition is somewhat higher (using  $10^{-8}\text{ M}$  metazachlor, see Fig. 5) in the intracellular membranes which is due to the site of VLCFA formation localized in ER and Golgi. As we have

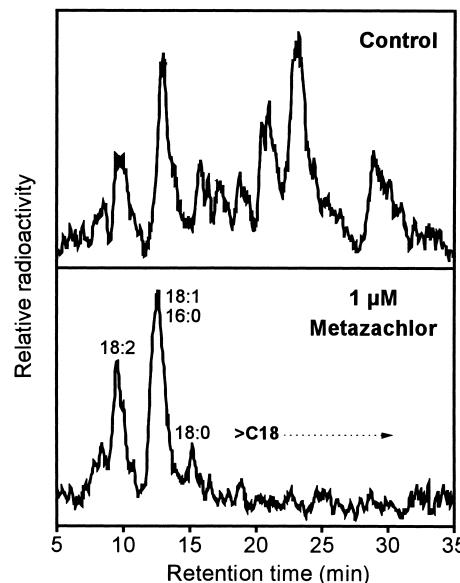


Fig. 4. Radio-HPLC analysis of  $^{14}\text{C}$ -labeled fatty acids derived from the plasma membrane of *Cucumis sativus*. 6-day-old leaf discs were incubated for 24 h with 1  $\mu\text{M}$  metazachlor and 16  $\mu\text{M}$  [ $^{14}\text{C}$ ]malonate. Modified and non-modified fatty acids coeluted under the conditions used. Very-long-chain species longer than C18 were identified by authentic standards.

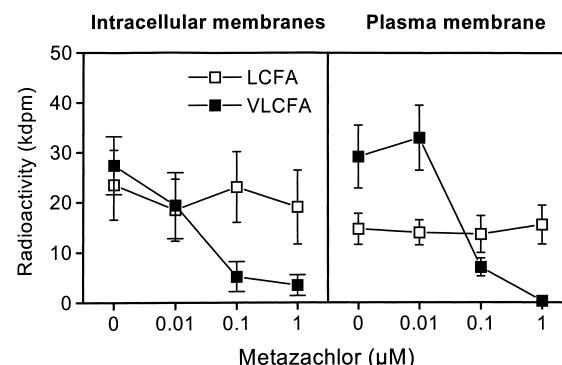


Fig. 5. Labeling of fatty acids obtained from intracellular and plasma membranes of cucumber seedlings. 6-day old cotyledon slices were incubated for 24 h in petri dishes with 16  $\mu\text{M}$  [ $^{14}\text{C}$ ]malonate and increasing concentrations of metazachlor. Fatty acids were separated by radio-HPLC into long-chain fatty acids (LCFA, C16-C18) and very-long-chain fatty acids (VLCFA, C20-C24).

found recently, the first and rate-limiting step of VLCFA biosynthesis catalyzed by 3-ketoacyl synthase is inhibited by chloroacetamides (unpubl. results).

No elongase activity was found in the plasma membrane. Consequently, incorporation of VLCFA-containing lipids into the plasma membrane requires lipid transport. Since monensin is known as an inhibitor interfering with the *transport* of VLCFA-containing lipids (Bertho *et al.*, 1991), we compared the activity of this compound with that of chloroacetamides. Applied to cucumber leaf discs, 1  $\mu\text{M}$  monensin exhibited no significant effect (Fig. 6). Consequently, chloroacetamide-induced lack of VLCFAs originates from inhibition of biosynthesis rather than transport of VLCFA-containing lipids.

A decreased level of VLCFAs in the plasma membrane was not only observed with chloroacetamides. Besides the chloroacetamide herbicides metazachlor and *S*-metolachlor, several chemically different inhibitors like fentrazamide (a tetrazolinone) and cafenstrole (a triazole) were strong inhibitors of VLCFA supply (Fig. 6). Remarkably, in our assay, the *S*-isomer of metolachlor was the phytotoxic and the active compound as well. The opposite was true for the *R*-isomer. This discrep-

ancy was also shown with the inhibition of VLCFA biosynthesis *in vivo* and *in vitro* (Matthes *et al.*, 1998; Schmalfuß *et al.*, 2000) and with the field activity of metolachlor (O'Connel *et al.*, 1998). A correlation between VLCFA biosynthesis and phytotoxic activity of these compounds was reported by Couderchet *et al.* (1998).

#### Concluding remarks on the phytotoxic effects

Metazachlor (1 to 10  $\mu\text{M}$ ) applied to cucumber during germination results in stunted seedlings with thickened stems and leaves as was reported for weeds (Fuerst *et al.*, 1987). Electron microscopic studies with sorghum showed that metolachlor affects the *de novo* formation of membranes of the plastid, mitochondria, and endoplasmic reticulum (Ebert, 1980). Metolachlor also impairs cell division shown at the level of longitudinal growth. These findings resemble chloroacetamide-treated alga showing swollen enlarged cells incapable to proliferate (Weishaar and Böger, 1994).

More recently, VLCFAs have been assumed to be required for curved membrane structures in transgenic *Arabidopsis* (Millar *et al.*, 2000). We speculate that curving may be based on an asymmetric distribution of VLCFA-containing lipids (as assumed for mammalian cells) within the bilayer (Schneiter, 1999). Furthermore, the putative target enzyme of chloroacetamides, 3-ketoacyl synthase, was suggested to be responsible for the "fiddlehead" mutation in *Arabidopsis* (Pruitt *et al.*, 2000). The authors suggested an altered composition of VLCFA-linked lipids to be responsible for processes of vegetative development. Morphological characteristics of the phenotype reported are similar to those of chloroacetamide-treated plants (Lechelt-Kunze *et al.*, 2002).

Cerebrosides represent a major lipid class of the plasma membrane (30%, see Table I) and are a prominent source of VLCFAs (Fig. 2). They are thought to be involved in various physiological functions. They are instrumental for raft formation, and subsequent anchoring and transport of proteins (Simons and Ikonen, 1999). Keeping in mind that the formation of membrane domains is triggered by the level of cerebrosides and sterols (Xu *et al.*, 2001), a decisive role of the bound (very-long-chain) fatty acid has to be assumed. It

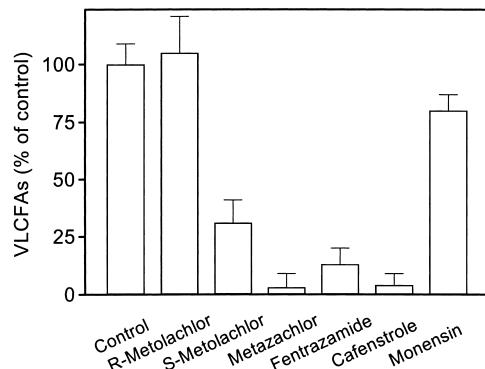


Fig. 6. Inhibition of VLCFA incorporation into the plasma membrane of *Cucumis sativus* by different herbicides and monensin. Fatty acid elongation was monitored by incorporation of 16  $\mu\text{M}$  [ $^{14}\text{C}$ ]malonate with 1  $\mu\text{M}$  herbicide present for 24 h. The content of labeled VLCFAs was determined by radio-HPLC.

Metolachlor, (*RS*)-2-chloro-6'-ethyl-*N*-(2-methoxy-1-methylethyl)acetoo-*o*-toluidide; metazachlor, 2-chloro-*N*-(pyrazol-1-ylmethyl)acet-2',6'-xylidide; fentrazamide, 1-(ethylcyclohexylcarbamoyl)-4-(2-chlorophenyl)tetrazolinone; cafenstrole, *N,N*-diethyl-3-mesitylsulfonyl-1*H*-1,2,4-triazole-1-carboxamide.

For structures see Böger *et al.* (2000), for monensin see Merck-Index, 10th edition, Merck and Comp., Rahway, USA.

is known that lipid accumulation in membrane domains is due to the special physical properties of cerebrosides essentially determined by van der Waals interactions and hydrogen bonds (for review see Schneiter, 1999). These properties are determined by both, the long chain (C18) sphingosine backbone and the long extending saturated acyl chain found in cucumber cerebrosides of the plasma membrane (Fig. 1).

The increasing chain length of fatty acids enhances the accumulation of cerebrosides in rafts and enables tight lipid packing (Wang *et al.*, 2001). Analyzing the arrangement of acyl chains, Boggs and Koshy (1994) found both, partly interdigitation and protrusion of acyl chains dependent on the distinct fatty acid pattern. Schneiter *et al.* (1999) examined lipid sorting with membrane lipids of yeast and suggested that the precise chain length of VLCFAs present determines the composition of neighbouring lipids. Thus, a minor shift of the fatty acid pattern may result in critical changes of the lipid layer leading to death as was shown with yeast cells (Ho *et al.*, 1997).

In summary, there is strong evidence that the very long acyl chains present in cerebrosides and

phospholipids are instrumental for physical properties and metabolic function of the plasma membrane. As documented by our data, metazachlor decreases the ratio of VLCFA/ C16-C18 fatty acids from about 1:3 to 1:5 or even smaller ratios. This should change the membrane properties markedly. Accordingly, depletion of plasma membrane VLCFAs represents the first physiological consequence of chloroacetamide action, followed by a cascade of secondary effects eventually leading to plant death.

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