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Metabolism of the Persistent Plasticizer Chemical Bis(2-ethylhexyl) Phthalate in Cell Suspension Cultures of Wheat (*Triticum aestivum* L.). Discrepancy from the Intact Plant

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Cell suspension cultures of wheat (*Triticum aestivum* L.) metabolized the persistent plasticizer chemical bis(2-ethylhexyl) phthalate (DEHP; 1 ppm) predominantly to β-D-glucosyl conjugates. After incubation for 48 h at 27 °C, 23% of the applied radioactively labeled chemical was recovered in the total polar metabolite fraction. Prior heat treatment or freeze-thawing of the wheat cells abolished conjugate formation and led to mono(2-ethylhexyl) phthalate (MEHP) as the predominant metabolite (up to 10% conversion). Direct feeding of MEHP to native wheat cells led to 93% conversion to polar metabolites, again consisting largely of β-D-glucosyl conjugates. This suggested that MEHP was a metabolic intermediate and that DEHP esterase activity was rate limiting in DEHP metabolism. The rate of cellular DEHP metabolism in fact agreed with the rate of the DEHP esterase reaction determined in crude cell-free extracts. Therefore, no significant permeation barrier between the intracellular enzyme and external DEHP appeared to exist in cell suspension cultures. In contrast, the DEHP esterase activity of intact leaves has previously been found to be inaccessible to external DEHP.

INTRODUCTION

The use of plant cell cultures offers a rapid, reproducible, and inexpensive way to study the metabolism of environmental chemicals without interference by abiotic or microbial degradation reactions (Mumma and Hamilton, 1979; Mumma and Davidonis, 1983; Sandermann et al., 1977, 1984). It is widely assumed that cultured plant cells can serve as a metabolic model of the intact plant. In fact, a considerable similarity between metabolism in cell cultures and in the intact plant has been observed for example for 2,4-D (Mumma and Hamilton, 1979; Bristol et al., 1977; Scheel and Sandermann, 1981), diphenamide (Davis et al., 1978), cisanilide (Frear and Swanson, 1975), fluorodifen (Locke and Baron, 1972), carbaryl (Locke et al., 1976), diclofopmethyl (Dusky et al., 1980, 1982), EL-494 (Abdelmonem and Mumma, 1982), fenvalerate (Davidonis and Mumma, 1984), fluoroimide (Ohori and Aizawa, 1983), baythroid (Preiss et al., 1984), 4-chloro-2-methylphenoxyacetic acid (Cole and Loughman, 1983), and pentachlorophenol (Scheel et al., 1984).

The extrapolation from cell cultures to the intact plant became, however, questionable when the metabolism of certain nonpolar chemicals that are known as ubiquitous persistent contaminants of natural vegetation was studied in plant cell suspension cultures. In spite of their known persistence, DDT (Scheel and Sandermann, 1977; Arjmand and Sandermann, 1985), DDE (Arjmand and Sandermann, 1985), benzo[*a*]pyrene (von der Trenck and Sandermann, 1978; Harms, 1983), hexachlorobenzene (Sandermann et al., 1984), and bis(2-ethylhexyl) phthalate (DEHP; Sandermann et al., 1984) were all found to be partially converted to polar metabolites. In the case of DEHP (Figure 1) a unique esterase catalyzing a cleavage of the plasticizer chemical has been discovered in cultured wheat cells. This enzyme has been purified to electrophoretic homogeneity and characterized (Krell and Sandermann, 1984). This esterase has also been purified 10-fold from intact wheat plants, where the enzyme was, however, shown to be inaccessible for external DEHP (Krell and Sandermann, 1985). The main purpose of the present study was to analyze the role of the esterase in DEHP metabolism by cultured wheat cells.

EXPERIMENTAL SECTION

Materials. [*Carboxyl*-^{14}C]-DEHP and [*carboxyl*-^{14}C]-MEHP were obtained and purified as previously described (Krell and Sandermann, 1984). [*Carboxyl*-^{14}C]-MEHP-β-D-glucosyl tetraacetate was prepared from [*carbonyl*-^{14}C]-MEHP and tetra-*O*-acetylglucosyl bromide by the procedure described for abscisic acid β-D-glucosyl tetraacetate (Neill et al., 1983). The product (retention time 24.4 min) was separated from unreacted MEHP (retention time 19.6 min) by reversed-phase HPLC (see below). Characterization was by chemical ionization mass spectroscopy (reagent gas ammonia) that showed in particular the molecular ion (+18) at m/z 626.

The cell suspension culture of wheat (*Triticum aestivum* L., var. Koga II) was maintained on B5 medium as previously described (Sandermann et al., 1984; Scheel et al., 1984).

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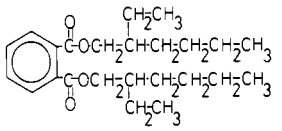

Figure 1. Chemical structure of bis(2-ethylhexyl) phthalate (DEHP).

Study of Metabolism. At day 12 of culture, 40 μg of [^{14}C]-DEHP (0.2 μCi) or [^{14}C]-MEHP (0.5 μCi) was administered to 40 mL of wheat cultures as solutions in 40 μL of methanol. Incubation was for 48 h in the dark. The cells were than harvested by filtration, and the cells and culture fluids were extracted with chloroform/methanol as described (Sandermann et al., 1984; Scheel et al., 1984). Usually, five to seven parallel incubations were carried out, with two additional, autoclaved cultures (121 °C, 20 min) serving as controls. Sterility controls were also performed (cf. Sandermann et al., 1984). The parent chemicals were differentiated from metabolites present in the various extract phases by TLC in solvent system A (see below). Radioactivity associated with the "insoluble" residues was determined after combustion. All procedures and the evaluation of data were as previously described (Sandermann et al., 1984; Scheel et al., 1984).

Mild Heat Treatment. Wheat cell cultures (12-day old, 40 mL) were placed for 10 min into an 80 °C oven. During this time, the culture fluid reached about 40–50 °C. The cultures were then cooled to the usual incubation temperature of 27 °C, and [^{14}C]-DEHP was added as described above.

Freeze–Thaw Treatment. Wheat cultures (12-day old, 40 mL) were frozen in liquid nitrogen, followed by thawing in a 40 °C water bath and a 3-s sonication period in a Model 220 sonic bath (Branson). This treatment was repeated four times. The cultures were then brought to the usual incubation temperature of 27 °C, and [^{14}C]-DEHP was added as described above.

Derivatization and Cleavage Reactions. Methylation with diazomethane and acetylation with acetic anhydride in pyridine were carried out as described (Arjmand and Sandermann, 1985). Hydrolysis with 2 N HCl (2 h, 100 °C) and 1 N NaOH (24 h, 37 °C) as well as β-glucosidase treatment were also carried out by previous procedures (Scheel and Sandermann, 1981; Arjmand and Sandermann, 1985).

Thin-Layer Chromatography (TLC). Precoated silica gel G plates (Merck No. 5554) were used with the following solvent system: A, petroleum ether (40–60 °C)/diethyl ether/acetic acid, 16:4:1 (v/v/v); B, ethyl acetate/acetic acid/water, 3:1:1 (v/v/v). R_f values in solvent systems A and B, respectively, were as follows: DEHP, 0.85/0.96; MEHP, 0.51/0.96; phthalic acid, 0.05/0.76; phthalic anhydride, 0.42/0.76.

High-Performance Liquid Chromatography (HPLC). The set-up consisted of two Model 110A pumps, a Model 420 controller, and a Model 332 gradient mixer, all from Beckman (Munich). Elution was followed with a Model LB 503 radiomonitor (Berthold, Wildbad). The samples were filtered through a 0.2-μm Schleicher–Schüll RC 58 filter and applied to a 4.6 × 250 mm Ultrosphere ODS (5-μm) column for separation. The gradient programs used are given in the Results section.

Paper Electrophoresis. High-voltage paper electrophoresis was carried out with a Model 64 Pherograph instrument (Hormuth-Vetter, Heidelberg). The buffer was 0.2 M ammonium acetate adjusted to pH 5.8 with acetic acid. Macherey Nagel No. 214 paper was used with picric acid as the visible standard.

Table I. Metabolism of MEHP and DEHP in Wheat Cell Suspension Cultures

	MEHP (n = 6)	DEHP (n = 7)[a]
wet wt cells[b]	4.8 ± 0.6	4.6 ± 0.2
total rec of [^{14}C][c]	97.0 ± 4.4	91.9 ± 4.2
[^{14}C] in growth med[d]	12.4 ± 1.9	6.7 ± 0.6
unchanged parent chem	<0.5	60.1 ± 13.5
polar metab[e]	92.7 ± 4.5	23.3 ± 11.3
nonpolar metab[f]	<0.5	1.7 ± 0.8
insol res	4.2 ± 0.7	3.7 ± 0.7

[a] Taken from Sandermann et al. (1984). [b] Values given in grams ± standard deviation. [c] Includes measured losses of [^{14}C] by adsorption to glass walls, filters, etc. Values are given as percent of applied radioactivity in this and subsequent lines of the table. [d] [^{14}C] in the medium of the heat-inactivated control cultures was 62.2 ± 4.1% (MEHP) and 3.4 ± 0.4% (DEHP), respectively. [e] Polar metabolites remained at the origin upon TLC in solvent system A. [f] Nonpolar metabolites migrated faster than the parent chemical upon TLC in solvent system A.

Mass Spectroscopy. Electron-impact mass spectra were obtained with a Finnigan 4000 instrument at 70 eV using a direct sample inlet. Chemical ionization mass spectra were obtained with ammonia as the reagent gas on a Finnigan MAT 44S instrument with an ionization energy of 170 eV.

RESULTS

Metabolism of DEHP and MEHP in Wheat Cell Cultures. The metabolism of [^{14}C]-DEHP and its more polar derivative [^{14}C]-MEHP was studied in wheat cell suspension cultures under the previous standard conditions (Sandermann et al., 1984; Scheel et al., 1984). The results obtained are summarized in Table I. The total metabolic conversion rates during 48 h were 28.7% in the case of DEHP and 96.9% in the case of MEHP. In both cases, most of the metabolites consisted of polar soluble metabolites remaining at the origin upon TLC in solvent system A.

In the case of DEHP, 1.7% of the applied radioactivity was associated to about equal proportions with seven chloroform-extracted nonpolar metabolites that had the following R_f values in solvent system A: 0.27, 0.32, 0.40, 0.48, 0.55, 0.63, 0.74. Similar R_f values and amounts of these nonpolar metabolites were also obtained with soybean cell suspension cultures. These possibly hydroxylated metabolites appeared to be derived from DEHP rather than MEHP because they were immobile upon high-voltage paper electrophoresis at pH 10.4 (Scheel and Sandermann, 1982).

The "insoluble" residues amounted to 4.2% (MEHP) and 3.7% (DEHP) of the applied radioactivity.

The standard deviations observed were in the same range as previously observed with other xenobiotics studied under the same conditions (Sandermann et al., 1984; Scheel and Sandermann, 1981; Scheel et al., 1984).

As previously reported (Sandermann et al., 1984), MEHP and free phthalic acid were not detectable (<0.2%) as DEHP metabolites. However, when the cell cultures were subjected to mild heat treatment or freeze–thawing prior to incubation with DEHP, MEHP became the predominant metabolite, appearing as a prominent peak at R_f 0.51 (TLC solvent system A) and 0.96 (TLC solvent system B). The corresponding results are summarized in Table II. When the cell cultures were autoclaved (10 min, 121 °C) prior to incubation with DEHP or MEHP, only the unchanged parent compounds were isolated in ≥98% recovery.

Cellular DEHP Esterase Activity. The total DEHP conversion rate of 28.7% (Table I) means that 29 nmol of the applied 102 nmol of DEHP had been metabolized.

Table II. Effect of Pretreatments on DEHP Metabolism by Cultured Wheat Cells[a]

	native cells	heat treatment	freeze–thawing
wet wt of cell matl, g	4.6	3.4	2.2
total rec of [^{14}C][b]	91.9	101.3	103.5
total [^{14}C] in med	6.7	4.0	18.8
unchanged DEHP			
cell-assoc	58.7	95.8	62.2
in med	1.4	1.3	10.7
isolated MEHP			
cell-assoc	<0.1	<0.5	3.6
in med	<0.1	2.8	7.7
other polar metab			
cell-assoc	18.0	<0.2	0.4
in med	5.3	<0.2	0.4
insol res	3.7	1.3	4.3

[a] The average values given were derived from seven parallel experiments (native cells same experiment as in Table I) and two parallel experiments each in the case of heat treatment and freeze–thawing, respectively. [b] Footnote *c* of Table I applies.

Since 4 g of wheat cells contain about 100 mg of soluble protein (Krell and Sandermann, 1984), the specific metabolic rate was 29 nmol/100 mg of protein × 48 h, which is equivalent to 1.6 nkat/kg of protein. The standard kat unit refers to 1 mol of product formed per second. The DEHP concentration in the experiments of Table I (2.5 μM) corresponded to 50% of the apparent K_m value determined for the DEHP esterase from cultured wheat cells (Krell and Sandermann, 1984). The in vivo metabolic rate thus leads to an extrapolated in vitro rate of roughly 6 nkat/kg of protein. This value is within the range of experimentally determined specific DEHP esterase activities that are between 4 and 14 nkat/kg of protein in crude extracts from cultured wheat cells (Krell and Sandermann, 1984).

Comparison of Metabolites Formed from DEHP and MEHP. The predominance of MEHP in the DEHP metabolic experiments of Table II suggested an intermediary role of MEHP in DEHP metabolism. Therefore, the polar soluble metabolite fractions formed from DEHP and MEHP, respectively, were compared. The polar metabolite fractions gave in both cases two equally strong peaks with R_f values of 0.53 and 0.69 upon TLC in solvent system B. In addition to this heterogeneity with regard to polarity, there was also heterogeneity with regard to electrical charge. High-voltage paper electrophoresis at pH 5.8 led to about equal amounts of noncharged material with $R_{picrate}$ = 0 and of material with one negative charge migrating near authentic MEHP ($R_{picrate}$ = 0.7). Free phthalic acid migrated to $R_{picrate}$ = 1.8.

The wheat polar metabolite fraction was also studied by gel permeation chromatography on Biogel P-2. An apparent heterogeneity with regard to size was observed since two peaks of radioactivity were eluted. About 60% of the polar DEHP metabolites appeared near the exclusion volume, the remainder being included (Scheel and Sandermann, 1982).

Acid or alkaline hydrolysis of the polar metabolite fractions from DEHP as well as MEHP led to complete cleavage and to release of only free phthalic acid as shown by TLC in solvent systems A (R_f 0.05) and B (R_f 0.76) and by high-voltage paper electrophoresis ($R_{picrate}$ = 1.8).

The extent of cleavage by β-glucosidase (about 50%) was the same for the polar metabolite fractions formed from either DEHP and MEHP. The released aglycones differed from free phthalic acid as well as MEHP (see below).

In summary, the polar metabolite fractions derived from either DEHP or MEHP were heterogeneous. Although these fractions were shown to be similar in several respects, chemical identity of the components has not been established. Because of the higher conversion rate, further characterization was carried out with the polar metabolite fraction formed from [^{14}C]-MEHP.

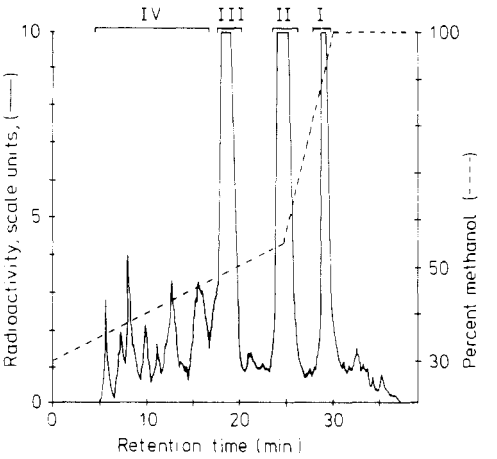

Figure 2. HPLC fractionation of the soluble polar metabolite fraction formed from [^{14}C]-MEHP. Column elution was with the following gradient (---), a linear gradient between 30% and 55% aqueous methanol for 25 min, followed by a linear gradient between 55% and 100% methanol for 5 min, and finally isocratic elution with methanol. The flow rate was 1 mL/min, and the elution profile (—) was determined with a Berthold Model LB 503 radiomonitor. The eluted fractions were pooled as indicated to give subfractions I–IV, respectively.

Table III. HPLC Retention Times of DEHP Metabolites and Some Reference Compounds [Column Elution with a Linear Gradient from 50% to 100% Methanol (20 min), followed by Isocratic Elution with Methanol; Flow Rate 1 mL/min; Elution followed with a Radiomonitor; All Phthalates Studied [*Carboxyl*-^{14}C] Labeled]

	retentn time,[a] min		
sample	underivatized	acetylated[b]	
metab I	10.8	20.8	16.0
metab II	9.2	20.1	14.8
metab III	7.2	20.8	11.2
phthalic acid	4.2	11.6	
MEHP	19.6	25.0	
MEHP glucoside tetraacetate		24.4	
DEHP	31.9		

[a] Values derived from three or more independent determinations in each case. [b] Acetylation performed after a prior routine methylation treatment.

Preliminary Characterization of MEHP Metabolites. Reversed-phase HPLC resolved the polar metabolite fraction derived from MEHP into three defined and an additional heterogeneous subfraction, as shown in Figure 2. These subfractions were numbered I–IV in the reverse order of their elution. When examined by TLC in solvent system B, subfraction I had R_f 0.53, subfraction II had R_f 0.69, and subfractions III and IV were each resolved into two major components with R_f values of 0.53 and 0.69, respectively. Treatment with β-glucosidase led to 18%, 100%, 4%, and no detectable cleavage for subfractions I–IV, respectively. The aglycones released from subfractions I and II had R_f values of ~0 and 0.20 in solvent system A and of 0.74 and 0.91 in solvent system B, respectively. The aglycones were thereby differentiated from free phthalic acid and MEHP, but remained unidentified.

Prior to further characterization, subfractions I–III were rechromatographed. The HPLC retention times of the metabolites and of some reference compounds are listed

in Table III. As a preliminary test for the presence of a free carboxyl group, subfractions I and II were treated with diazomethane. This treatment did not lead to a change in HPLC retention time whereas methylation led to considerable shifts of MEHP and free phthalic acid (Table III). The metabolite subfractions were then acetylated and again studied by HPLC. Two about equally strong product peaks were observed. Their retention times are listed in Table III. The reason for the occurrence of two acetylation products has remained unidentified although the same observation has previously been made after acetylation of a metribuzin β-D-glucoside conjugate (Frear et al., 1983). Synthetic MEHP-β-D-glucosyl tetraacetate had a HPLC retention time of 24.4 min so that this compound did not appear to be present in any of the metabolite subfractions. Attempts to obtain electron-impact or chemical ionization mass spectra of the purified acetylated subfractions were largely unsuccessful. Broad evaporation curves were obtained, pointing to decomposition or heterogeneity of the samples. In the chemical ionization mass spectra (reagent gas ammonia) distinct fragments at m/z 408 (up to 50% relative abundance) and at m/z 696 (up to 40% relative abundance) were diagnostic for the presence of peracetylated mono- and dihexosides, respectively (Dougherty et al., 1974; Horton et al., 1974). Apparently, these sugars had been present in the purified metabolites in a labile linkage that was at least partially broken in the acetylation step performed at 40 °C. The electron-impact mass spectra were complex but did contain fragments typical for acetylated hexoses (m/z 331, 271, 169, 127, 109) as well as phthalates (m/z 181, 163, 149, 83).

DISCUSSION

MEHP as a Primary DEHP Metabolite. After incubation of DEHP with native wheat cells, MEHP could not be detected as a metabolite. However, the following results nevertheless indicated MEHP to be the primary metabolic intermediate: (i) Mild heat treatment or freeze–thawing of the wheat cells led to MEHP as the predominant metabolite. Apparently, the rather stable DEHP esterase reaction (Krell and Sandermann, 1984) had remained largely unaffected while subsequent metabolic reactions were inhibited. (ii) Feeding of MEHP instead of DEHP led to a much higher yield of polar metabolites. (iii) The polar metabolite fraction formed from MEHP resembled that formed from DEHP by a number of criteria. While the above results identified MEHP as the primary DEHP metabolite, the structures of the secondary metabolites formed were not elucidated. The analytical and mass spectral data, as well as the results of β-glucosidase treatment, tentatively suggested that MEHP was mainly converted to hexose conjugates. It remained open to which extent glycosyl transfer occurred to the free carboxyl group of MEHP, or to the side chain on the aromatic ring, after some hydroxylation reaction. The simple acyl-β-D-glucosyl conjugate of MEHP was apparently not present.

The minor nonpolar DEHP metabolites could have resulted from direct hydroxylation of DEHP. Interestingly, ω-hydroxylated derivatives of dibutyl phthalate have been isolated from the common house plant *Sanseviera trifasciata*, as presumed endogeneous plant components (Paré, 1981).

Role of the DEHP Esterase Reaction. The agreement between the cellular transformation rate of DEHP and the in vitro DEHP esterase activity of crude extracts suggested that the esterase was freely accessible to external DEHP. The essentially complete transformation of external MEHP to polar metabolites resembling those formed from DEHP furthermore suggested that the DEHP esterase reaction was rate limiting in DEHP metabolism by cultured wheat cells.

Comparison with the Intact Plant. After application to wheat leaves, DEHP was not metabolized even though metabolic enzymes, in particular DEHP esterase, were shown to be present (Krell and Sandermann, 1985). The inaccessibility (crypticity) of the metabolic enzymes has been attributed to partitioning and/or adsorption of external DEHP to cuticles, triglyceride droplets, and cell wall components (Krell and Sandermann, 1985). In contrast, DEHP was significantly metabolized by wheat cell suspension cultures, and intracellular DEHP esterase activity rather than the permeation step appeared to be rate limiting. This drastic discrepancy from the intact plant may also exist for other nonpolar xenobiotics so that in these cases cultured plant cells cannot serve as a metabolic model of the intact plant. This conclusion is in contrast to the metabolic similarities described for a considerable number of relatively polar xenobiotics (see Introduction).

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Isolation and Detection of Dialkyl Phthalates from Pork

Janis Cerbulis* and D. Michael Byler

Thin-layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR), and gas chromatography–mass spectrometry (GC–MS) indicate that lipid extracts from pork fat taken from commercial cuts of meat contain, as minor constituents, dialkyl phthalates (DAP). GC–MS revealed further that the most abundant phthalate component was a dioctyl ester. This class of compounds migrates on TLC plates with the neutral lipids, but when chromatographed on a silicic acid column these species appear in the glycolipid fraction. Examination of extracts from the plastic with which the meat was wrapped showed no aromatic esters. Thus, these substances either were accidentally introduced during processing or were already present in the live animal.

Phthalates are widely used by chemical manufacturers as plasticizers. The most commonly used of these is bis-(2-ethylhexyl) phthalate, commercially known as dioctyl phthalate or DOP (Bemis et al., 1982). These esters have been detected in a variety of animal tissues, including eggs layed by hens to which DOP was directly administered (Ishida et al., 1981) and in samples of bovine milk (Cerbulis and Ard, 1967). In some instances phthalate esters may be introduced during food processing and handling due to contact with specific plastic products; in other cases, the phthalates may have already been present in the animal prior to slaughter.

During a study of the composition of hydrocarbons and sphingolipid bases from pork, a minor, previously unreported constituent was found in the lipid fraction isolated from the fatty tissues. Thin-layer chromatography (TLC), Fourier transform infrared (FTIR) spectroscopy, and gas chromatography–mass spectrometry (GC–MS) indicate that this newly detected material consists principally of a mixture of dialkyl phthalates (DAP). The most abundant component observed by GC–MS was a dioctyl phthalate ester.

MATERIAL AND METHODS

Solvents and Column Packing Materials. Nanograde solvents were used throughout this study. Evaporation of these materials showed no traces of phthalates or other organic contaminants detectable by TLC. Silicic acid was washed with ether and chloroform–methanol (2:1) prior to use.

Pork. To obtain a representative sample of pork fat, pork products were bought from different supermarkets. The following principal cuts of pork were used: fresh ham, fresh picknic shoulder, and feet. After the plastic wrap was removed from the pork, all further contact with plastics was scrupulously avoided. The tissue was chopped in a meat chopper and then thoroughly mixed to make a homogeneous sample. Prior to handling the pork products, all containers and the chopper were washed with chloroform.

Lipid Extraction. The lipids were obtained by four subsequent extractions of the homogeneous pork sample described above with chloroform–methanol (2:1, v/v), as reported previously (Cerbulis, 1967) with a ratio of 20 mL of solvent/1 g of sample. The extracts were evaporated to dryness; then, the residue was taken up with chloroform–methanol (2:1) and washed with 0.2 volume of water. The $CHCl_3$ layer was evaporated to dryness and analyzed by TLC and column chromatography.

TLC. Sybron/Brinkmann Sil G-25 precoated TLC plates were used. Developing solvents were petroleum ether–diethyl ether–acetic acid (90:10:1, v/v/v) for neutral lipids and chlorofrom–methanol–water (65:25:4, v/v/v) for polar lipids (glycolipids, phospholipids). Reagent-grade ferric chloride–sulfuric acid was used to visualize the separated lipids (Cerbulis et al., 1984).

Silicic Acid Column Separation of Pork Fat. Initial separation of the components in the lipid extract was carried out on Unisil columns (2.5 × 25 cm) as described previously (Cerbulis et al., 1983): fraction A, eluted with chloroform, contained neutral lipids; fraction B, eluted with acetone, contained glycolipids; fraction C, eluted with methanol, contained phosphatidyl lipids and sphingomyelin. Each fraction was analyzed by TLC using both solvent systems. Fraction B was subjected to preparative TLC using chloroform–methanol–water solvent described above. The TLC spot subsequently identified as DAP (see Results) was removed and redissolved in chloroform to obtain samples for examination by FTIR spectroscopy and GC–MS.

Extraction of Plastic (Wrapping Material). The plastic with which the pork was wrapped was also analyzed

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