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New Cyclopentenyl Fatty Acids in Flacourtiaceae. Straight-Chain Fatty Acids and Cyclic Fatty Acids in Lipids during Maturation of the Seeds†

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ABSTRACT: In the course of a study on the biosynthesis and metabolism of cyclopentenyl fatty acids in Flacourtiaceae the structures of the unsaturated straight-chain fatty acids occurring in the seed lipids of *Caloncoba echinata* and *Hydnocarpus anthelminthica* were determined. The monounsaturated straight-chain fatty acids were found to be mixtures of positional isomers whereas the polyunsaturated straight-chain fatty acids were almost exclusively linoleic and α-linolenic acids. A structural relationship between unsaturated straight-chain and cyclopentenyl fatty acids could not be recognized. Oxygenated fatty acids were not detected. Several new cyclopentenyl fatty acids were isolated and their structures were elucidated. In *C. echinata*, *H. anthelminthica*, and *H. wightiana*, 15-(2-cyclopenten-1-yl)pentadecanoic acid, "hormelic acid," and 15-(2-cyclopenten-1-yl)-8-pentadecenoic acid, "oncobic acid," were detected in addition to 13-(2-cyclopenten-1-yl)-9-tridecenoic acid, an isomer of gorlic acid. In *C. echinata* only, 11-(2-cyclopenten-1-yl)-6-undecenoic acid, "manoaic acid," and the Δ^4 and Δ^9 isomers of this acid were found. Cyclopentenyl fatty acids were predominantly found in triacylglycerols; however, they occurred also in the phospholipids and glycolipids. In all lipid classes, the proportions of cyclopentenyl fatty acids increased during maturation of the seeds. Most of the cyclopentenyl fatty acids were synthesized during the last 3–4 months of maturation. The results of this work suggest that cyclopentenyl fatty acids are not formed from straight-chain fatty acids.

The tribes *Oncobeae* and *Pangieae* of Flacourtiaceae, plants indigenous to the tropical zone, are known to contain cyclopentenyl fatty acids (Hegnauer, 1966). These unusual fatty acids are found as constituents of triacylglycerols, which are deposited in oil droplets in the parenchymatous cells of the endosperm. The oils extracted from the seeds, some of them known as "chaulmoogra oil" and "gorli oil," have been used for centuries in the treatment of leprosy (Schlossberger, 1938).

The most prominent cyclopentenyl fatty acids are hydnocarpic acid, 11-(2-cyclopenten-1-yl)undecanoic acid, chaulmoogric acid, 13-(2-cyclopenten-1-yl)tridecanoic acid, and gorlic acid, a C_{18}-cyclopentenyl fatty acid having a double bond in Δ6 position of the aliphatic chain (Paget, 1937). In addition, several cyclopentenyl fatty acids of shorter chain lengths, aleprolic, aleprestic, aleprylic, and alepric acids, are known (Cole and Cardoso, 1939). The correct structure of the cyclopentenyl fatty acids has been established by Shriner and Adams (1925) and the first total synthesis of a naturally occurring acid of this type, *i.e.*, *d*-chaulmoogric acid, has been achieved by Mislow and Steinberg (1955), who have also determined its absolute configuration.

Nothing is known about the biosynthesis and metabolism of the various cyclopentenyl fatty acids. One may assume that unsaturated straight-chain fatty acids or oxygenated fatty acids of unusual structures are precursors of cyclopentenyl fatty acids in Flacourtiaceae.

The aim of the present investigation was to elucidate structural relationships between unsaturated straight-chain fatty acids and cyclopentenyl fatty acids occurring in the seed lipids of *Caloncoba echinata* and *Hydnocarpus anthelminthica* (P.). The seed lipids of *C. echinata* are characterized by the occurrence of large amounts of chaulmoogric and gorlic acids (André and Jouatte, 1928) whereas the seed lipids of *H. anthelminthica* are known for the predominance of hydnocarpic acid (Power and Barrowcliff, 1905).

We have found that the monounsaturated straight-chain fatty acids occurring in the lipids of *C. echinata* and *H. anthelminthica* seeds are mixtures of positional isomers whereas the polyunsaturated straight-chain fatty acids are those commonly encountered in the plant kingdom. We could not detect oxygenated fatty acids of unusual structures.

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In the course of the present study we have found several new monounsaturated cyclopentenyl fatty acids. Methyl esters of these acids were degraded by ozonolysis and the resulting trihydric alcohols were converted to 4-methylalkanes. This method for the elucidation of the structures of cyclopentenyl fatty acids is illustrated in Figure 1 using methyl hormelate as an example. We have also isolated new polyunsaturated cyclopentenyl fatty acids and have determined their structures. These acids, including "gorlic acid," were found to be mixtures of isomers. The distribution of the straight-chain fatty acids and cyclopentenyl fatty acids in various lipid classes occurring in seeds has also been determined at different stages of maturity.

Materials and Methods

Seeds of *Caloncoba echinata* and *Hydnocarpus anthelminthica* (*P.*) were received from Honolulu Botanical Gardens and from the Harold L. Lyon Arboretum of the University of Hawaii, Honolulu, Hawaii. Seed oil of *Hydnocarpus wightiana* was obtained from the Ernakulam Trading Company, Ltd., Ernakulam, India. Pure reference compounds were purchased from Nu Chek Prep, Elysian, Minn. Cyclopentenyl fatty acids were isolated by solid–liquid countercurrent distribution with urea (Sumerwell, 1957), followed by liquid–liquid countercurrent distribution (Ahrens and Craig, 1952).

Lipids were separated on layers of Silica Gel H (E. Merck AG, Darmstadt, Germany) of various thickness. When working on a preparative scale, lipid fractions were detected in diffused light or were made visible by spraying the plate with a 2′,7′-dichlorofluorescein solution and viewing under uv light. After elution of lipids from the adsorbent with diethyl ether or mixtures of $CHCl_3$–MeOH–H_2O, the dye was removed by filtering the lipid solutions through glass wool. In analytical thin-layer chromatography (tlc), plates were sprayed with 50% H_2SO_4, the reagent solution of Dittmer and Lester (1964), or the α-naphthol reagent of Siakotos and Rouser (1965).

Gas chromatography was carried out on an Hewlett-Packard instrument, Model 5750 G, equipped with a flame ionization detector. Methyl esters were analyzed on both polar and nonpolar stationary phases as indicated in Table II. Peaks were identified by comparison with known standards and peak areas were measured by triangulation or directly calculated by an integrator linked to the recorder. In preparative work the gas chromatograph was equipped with a stream splitter.

Mass spectra were recorded on a Varian CH7 instrument (Varian Mat GmbH, Bremen, Germany). Spectra were obtained at ionization potentials of 70 and 25 eV, the source temperature was 200°, and the inlet temperature 50°. Infrared spectra were taken on a Perkin-Elmer spectrophotometer, Model 21, using CCl_4 as solvent. Optical rotations were measured in $CHCl_3$ using a Perkin-Elmer polarimeter. Melting points were taken on a Kofler heating stage and are uncorrected. Elemental analyses were carried out by Mikroanalytisches Laboratorium I. Beetz, Kronach, Germany.

Determination of Structures. Methyl esters of straight-chain fatty acids and of cyclopentenyl fatty acids were derived from total seed oils or isolated from fractions of methyl esters enriched by solid–liquid countercurrent distribution with urea or by distillation.

Samples of methyl esters were fractionated on a column, 40 cm × 2 cm, of silver nitrate impregnated Florisil (Carroll, 1963; Willner, 1965). About 900 mg of methyl esters, dissolved in hexane, was fractionated with 500 ml of hexane, 1000 ml, each of 0.5, 1, and 2% Et_2O in hexane, followed by 1000 ml of Et_2O. Fractions of 10 ml were collected and monitored by argentation–tlc as well as by gas–liquid chromatography (glc). Corresponding fractions were combined and further purified by argentation–tlc using hexane–Et_2O (75:25) as developing solvent. Pure methyl esters were isolated from these fractions by means of glc on a column containing 3% of OV-1 on Supelcoport (Supelco, Inc., Bellefonte, Pa.) as stationary phase.

Straight-Chain Fatty Acids. The structures of unsaturated straight-chain fatty acids were determined by reductive ozonolysis of their methyl esters (Privett *et al.*, 1963; Stein and Nicolaides, 1962). The aldehydes and aldesters formed were analyzed by temperature-programmed glc (5 min at 80°, then 6°/min to 190°, then 10 min at 190°) using 15% DEGS on Anakrom D (Analabs, Inc., North Haven, Conn.) and isothermally, at 130°, using 3% OV-1 on Supelcoport. Identification was made by comparison with standards obtained by ozonolysis of methyl Δ^{10}-undecenoate, petroselinate, vaccenate, erucate, linolenate, and arachidonate.

Cyclopentenyl Fatty Acids. The structures of cyclopentenyl fatty acids were determined as follows (see Figure 1). The esters were ozonized in pentane solution at −60° (Privett *et al.*, 1963), and the ozonides were reduced with $LiAlH_4$ in dry Et_2O (Sousa and Bluhm, 1960). The α,ω-dihydroxy-4-hydroxymethylalkanes formed were recrystallized from Et_2O at low temperatures. The purity of the triols was checked by tlc using $CHCl_3$–MeOH (90:10) as solvent. The triols were converted to the corresponding mesylates and subsequently reduced to the 4-methylalkanes by means of $LiAlH_4$ in dry Et_2O (Baumann *et al.*, 1966). The branched-chain hydrocarbons were purified by tlc using pentane as solvent. The mass spectra of these compounds exhibited pronounced fragments at *m/e* 71 ($^+CH(CH_3)(CH_2)_2CH_3$) and at *m/e* 43 + 14*n* ($^+CH(CH_3)(CH_2)_nCH_3$), thus establishing the 4-methyl branch, as well as the molecular peaks. The structures of cyclopentenyl fatty acids having a double bond in the aliphatic chain were substantiated by gas chromatographic analysis of the aldesters formed by ozonolysis, as described above.

Synthesis of Hormelic Acid.[1] 13-(2-Cyclopenten-1-yl)tridecanol (700 mg, 2.6 mmol), obtained from methyl chaulmoograte by reduction with $LiAlH_4$, was reacted with 500 mg (4.2 mmol) of methanesulfonyl chloride in pyridine according to the procedure of Baumann and Mangold (1964). Two crystallizations from absolute ethanol, at room temperature, afforded 485 mg (54%) of pure 13-(2-cyclopenten-1-yl)tridecyl methanesulfonate, mp 62–63°, $[\alpha]_D^{23}$ +47.3° (*c* 5.7, $CHCl_3$). *Anal.* Calcd for $C_{19}H_{36}O_3S$ (344.56): C, 66.22; H, 10.54; S, 9.31. Found: C, 66.38; H, 10.40; S, 9.21. The ir spectrum exhibited the bands characteristic for long-chain methanesulfonates (Spener, 1973), in addition to two weak bands at 3048 and 1612 cm^{-1} indicating the presence of a cyclopentene ring, and a band at 920 cm^{-1} which was barely visible as a shoulder.

To a cold suspension of sodium (63 mg, 2.7 mmol) in 40 ml

[1] This work was initiated at the Hormel Institute of the University of Minnesota, Austin, Minn., during the authors' affiliation with this institute. By naming one of the acids "hormelic acid" the authors wish to express their gratitude toward the Hormel Institute and the Hormel Foundation. "Oncobic acid" relates to the tribe *Oncobeae* of Flacourtiaceae and "manoaic acid" to the Manoa valley near Honolulu, Hawaii, place of the Harold L. Lyon Arboretum of the University of Hawaii.

of xylene, 500 mg (3.1 mmol) of diethyl malonate was added at once and allowed to react overnight. Then, the mixture was stirred and heated to 110°, and 404 mg (1.2 mmol) of the methanesulfonate in 10 ml of dry xylene was added during 30 min, heating and stirring being continued over a period of 4 hr. Upon cooling the solution, the alkyl malonic ester formed was saponified and the dicarboxylic acid decarboxylated following established procedures (Spener and Mangold, 1973). Crystallization first from acetone at 4°, then from chloroform at −11°, yielded 298 mg (81%) of pure 15-(2-cyclopenten-1-yl)pentadecanoic acid (hormelic acid), mp 74.5–75.0°, lit. 72–73° (Vandyke and Adams, 1926), $[\alpha]_D^{24}$ +52.0° (*c* 11.0, $CHCl_3$). *Anal.* Calcd for $C_{20}H_{36}O_2$ (308.51): C, 77.86; H, 11.77; O, 10.37. Found: C, 77.85; H, 11.68; O, 10.44. Methyl hormelate was obtained by reacting hormelic acid with diazomethane; $[\alpha]_D^{24}$ +49.3° (*c* 10.7, $CHCl_3$); its purity was assessed by tlc using hexane–Et_2O (90:10) as developing solvent, and by glc on 3% OV-1 as stationary phase.

Isolation of Individual Lipid Classes. The lipids in seeds of *Caloncoba echinata* and *Hydnocarpus anthelminthica* of different stages of maturity were extracted with 2-propanol and $CHCl_3$–MeOH (Nichols, 1964). The yellowish oil obtained was dissolved in hexane and applied to layers of silica gel, 0.5 g of lipids per 20 × 40 cm plate (Schmid *et al.*, 1967), and fractionated with hexane–Et_2O (85:15). Four fractions were scraped off, and the lipids were recovered. The hydrocarbons, the sterol esters, and the triacylglycerols were each extracted with wet diethyl ether, whereas ionic and other polar lipids in the fourth fraction were extracted with $CHCl_3$–MeOH–H_2O (3:5:2) (Slotta, 1966). In order to isolate residual triacylglycerols as well as diacylglycerols, free fatty acids, and sterols, the latter fraction was separated further using hexane–Et_2O–AcOH (70:30:1) as solvent. The following lipid classes were purified further and isolated for analyses of their constituent fatty acids: triacylglycerols, diacylglycerols, free fatty acids, ethanolamine phosphoglycerides, choline phosphoglycerides, inositol phosphoglycerides, phosphatidic acids, monogalactosyl diacylglycerols, digalactosyl diacylglycerols, and esterified sterol glycosides.

Triacylglycerols. The fractions of triacylglycerols obtained by chromatography of the total lipid extracts were combined with the residual triacylglycerols recovered from the lowermost fractions. The total triacylglycerols were isolated after chromatography on 1-mm layers of Silica Gel H, using the solvent hexane–Et_2O (90:10, twice). Methyl esters were obtained by methanolysis with MeOH–HCl (Stoffel *et al.*, 1959).

Diacylglycerols and Free Fatty Acids. Fatty acids were isolated after tlc on Silica Gel H using hexane–Et_2O–AcOH (50:50:1) and converted to methyl esters. The diacylglycerols and sterols, which were not resolved in this system, were subjected to methanolysis without prior elution from the adsorbent. The resulting methyl esters were separated from the sterols by chromatography on Silica Gel H with hexane–Et_2O (60:40, twice). A fraction tentatively identified as long-chain alcohols was detected in trace amounts.

Phospholipids and Glycolipids. The fractions of ionic and other polar lipids isolated from total lipids were separated by two-dimensional tlc, using $CHCl_3$–MeOH–7 N ammonia (65:25:4) in the first direction and $CHCl_3$–MeOH–AcOH–H_2O (85:12.5:12.5:2) in the second (Nichols, 1964; Galliard, 1968). Between the two runs, the plates were kept *in vacuo* for 15 min. After the second run, plates were sprayed with 2′,7′-dichlorofluorescein solution and again were kept in high vacuum to remove residual solvents. Spots were marked under uv light and scraped into screw-capped tubes. Methyl esters were obtained after methanolysis with MeOH–concentrated H_2SO_4–benzene (86:4:10) (Chalvardjian, 1964).

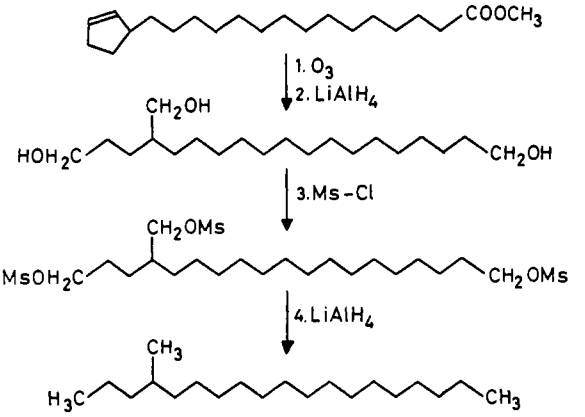

FIGURE 1: Degradation scheme of cyclopentenyl fatty acids; Ms = $-SO_2CH_3$.

Analysis of Methyl Esters. Samples of methyl esters, dissolved in hexane, were analyzed by glc on both polar and nonpolar stationary phases (Zeman and Pokorny, 1963).

As methyl esters of straight-chain fatty acids and of cyclopentenyl fatty acids are not completely resolved on either polar and nonpolar phases, such mixtures were prefractionated by argentation chromatography (Bandi and Mangold, 1969). About 15 mg of methyl esters was applied to a 20 × 20 cm plate, coated with Silica Gel G containing 10% $AgNO_3$, and developed with hexane–Et_2O (75:25). Fractions were made visible by spraying the layers with 2′,7′-dichlorofluorescein solution and viewing under uv light. Fractions were scraped off and eluted with Et_2O saturated with 1% hydrochloric acid. The fractions of saturated straight-chain, monounsaturated straight-chain, diunsaturated cyclic, and triunsaturated straight-chain methyl esters were each recovered and analyzed by glc on both phases.

Results

Unsaturated Straight-Chain Fatty Acids. Methyl esters of these acids were subjected to reductive ozonolysis, followed by glc of the fragments formed. Results of these structural analyses are presented in Table I.

The monoenoic straight-chain fatty acids showed a prevalence of Δ^9 isomers in chains up to 18 carbon atoms. The hexadecenoic acids also contained Δ^6 and Δ^7 isomers, the octadecenoic acids included Δ^8 and Δ^{11} isomers, whereas the eicosenoic acids consisted to 90% of the Δ^{11} isomer, with small amounts of Δ^8, Δ^9, and Δ^{10} isomers being present.

The 18:2 and 18:3 acids were almost exclusively the $\Delta^{9,12}$ and $\Delta^{9,12,15}$ isomers, respectively, *i.e.*, linoleic and α-linolenic acids; only 2.7% of the 18:3 acids were the $\Delta^{6,9,12}$ isomer, *i.e.*, γ-linolenic acid.

According to their behavior in argentation–tlc and glc, all of the straight-chain fatty acids occurred in the cis configuration.

Oxygenated Fatty Acids. Small amounts of α-hydroxy fatty acids were detected whereas oxygenated fatty acids of unusual structures could not be found.

Cyclopentenyl Fatty Acids. The methyl esters of new cyclopentenyl fatty acids, tentatively identified by their retention times in analytical glc, were isolated by argentation–tlc followed by preparative glc. The structures of these acids were ascertained by chemical degradation and by mass spectrom-

TABLE I: Isomeric Unsaturated Fatty Acids in Triacylglycerols of *Caloncoba echinata* Seeds.[a]

	Positions of Double Bonds[b]							
	Δ^4	Δ^5	Δ^6	Δ^7	Δ^8	Δ^9	Δ^{10}	Δ^{11}
Monounsaturated straight chain fatty acids[c]								
16:1			1.8	5.5		92.7		
18:1					1.3	94.8		3.9
20:1					3.3	3.2	3.5	90.0
Polyunsaturated straight chain fatty acids[c]								
18:2						100.0		
18:3			2.7			97.3		
Polyunsaturated cyclopentenyl fatty acids[c]								
16:2cy	17.3		63.6			19.1		
18:2cy			85.5			14.5		
20:2cy					81.7	18.3		

[a] Expressed in mol %. [b] For polyunsaturated fatty acids the position of the double bond closest to the carboxyl group is given. [c] See footnote *c* in Table II.

etry of the methyl esters, and by comparison of the physical properties of the natural substances with those of synthetic compounds. Results are presented in Table I.

15-(2-Cyclopenten-1-yl)pentadecanoic acid (hormelic acid) was isolated from the seed oils of *Caloncoba echinata*, *Hydnocarpus anthelminthica*, and *Hydnocarpus wightiana*. Chemical degradation of the methyl ester according to Figure 1 yielded 4-methylnonadecane. Hormelic acid was synthesized by chain elongation of chaulmoogric acid. The natural product and the synthetic preparation behaved identically in several chromatographic systems and exhibited identical fragmentation patterns in mass spectrometry: a base peak at *m/e* 67 (cyclopentene), prominent peaks at *m/e* 82 (at 25 eV base peak), 191, 209 ($CH_2{=}CH(CH_2)_{11}CO$), 241 ($^+(CH_2)_{13}COOCH_3$), 290 (M − 32), and the molecular peak at *m/e* 322 with an abundancy of 12%.

A mixture of isomeric 11-(2-cyclopenten-1-yl)undecenoic acids was isolated from the seed oil of *C. echinata*. Ozonolysis of the methyl esters followed by glc of the resulting fragments showed that the major isomer was 11-(2-cyclopenten-1-yl)-6-undecenoic acid (manoaic acid); the Δ^4 and Δ^9 isomers were also present.

The "gorlic acid" isolated from *C. echinata*, *H. anthelminthica*, and *H. wightiana* proved to consist of a mixture of 13-(2-cyclopenten-1-yl)-6-tridecenoic acid, which had been characterized by Paget (1937), and the Δ^9 isomer, which had not been found previously.

A mixture of isomeric 15-(2-cyclopenten-1-yl)pentadecenoic acids was isolated from *C. echinata*, *H. anthelminthica*, and *H. wightiana*. Ozonolysis of the methyl esters followed by glc of the resulting fragments showed that the major isomer was 15-(2-cyclopenten-1-yl)-8-pentadecenoic acid (oncobic acid), and the minor constituent was the Δ^9 isomer.

Methyl esters of the naturally occurring mono- and diunsaturated cyclic C_{20} acids were hydrogenated and compared with a sample of methyl 15-cyclopentylpentadecanoate that had been prepared by catalytic hydrogenation of synthetic hormelate. The three samples behaved identically in glc and yielded identical mass spectra: a base peak at *m/e* 74 (McLafferty rearrangement), prominent peaks at *m/e* 87, 101, 129, 143, 185, 199, 255 ($^+(CH_2)_nCOOCH_3$ for n = 2, 3, 5, 6, 9, 10, 14), 69 (cyclopentane), 281 (M − 43), and the molecular ion at *m/e* 324 with an abundancy of 63%. A peak, characteristic for this compound, was found at *m/e* 225 (presumably M − $C_8H_{15}^+$). The interpretation of these data is in agreement with the conclusions reached by Christie *et al.* (1969) who had investigated the fragmentation patterns of methyl 11-cyclopentylundecanoate and methyl 13-cyclopentyltridecanoate.

Lipid Classes. Seeds of *Caloncoba echinata* and *Hydnocarpus anthelminthica* were investigated at different stages of maturity. Around 3 months before maturity, the oil content in both *C. echinata* and *H. anthelminthica* averaged 1.5% and increased to over 2.1% during a 3-week period; at full maturity it rose to over 40%; that of shelled seeds of *H. anthelminthica* varied from 35 to 64%. The oil content of dried seeds of *C. echinata* was 52%.

The lipids from immature seeds of *H. anthelminthica* consisted of 80.6% triacylglycerols, 16.1% phospholipids and glycolipids, and 3.3% of others, as determined gravimetrically. The percentage of triacylglycerols in mature seeds, 3 months later, rose to over 98%. Similar results were obtained with seeds from *C. echinata*.

Neutral lipids identified were hydrocarbons, sterol esters, triacylglycerols, diacylglycerols, fatty acids, and, possibly, traces of long-chain alcohols.

The ionic and other polar lipids included ethanolamine phosphoglycerides, choline phosphoglycerides, inositol phosphoglycerides, phosphatidic acids, and glycerol phosphoglycerides whereas ethanolamine lysophosphoglycerides and choline lysophosphoglycerides were assigned only tentatively. Monogalactosyl diacylglycerols, digalactosyl diacylglycerols, sulfoquinovosyl diacylglycerols, sterol glycosides, esterified sterol glycosides, and two fractions of cerebrosides were present; however, the constituent carbohydrate and sterol moieties were not determined separately. Choline phosphoglyceride was the major polar lipid, with lesser amounts of ethanolamine phosphoglycerides, inositol phosphoglycerides, and phosphatidic acids and small amounts of glycerol phosphoglycerides. The major glycolipids were monogalactosyl diacylglycerols and esterified sterol glycosides, their amounts approximately corresponding to those of ethanolamine phosphoglycerides and inositol phosphoglycerides. Sulfoquinovosyl diacylglycerols were found in minor amounts only. The relative amounts of polar lipid classes were roughly estimated by comparison of charred spots after spraying the plates with chromic sulfuric acid. No major differences were observed between *C. echinata* and *H. anthelminthica* and no

dramatic changes in relative amounts occurred during maturation of the seeds.

Acyl Moieties in Lipids. Seeds of *C. echinata* and *H. anthelminthica* were collected 11 weeks prior to maturity; their lipid content was between 2.1 and 2.5% of the fresh weight. The distribution of straight-chain and cyclopentenyl fatty acids in lipids of these seeds, whose total phospholipid and glycolipid content amounted to 12% of either oil, is presented (Tables II and III), because in mature seeds phospholipids and glycolipids were found as minor constituents (total of 1.5%) only.

The 16:0 and 18:1 acids were the predominant straight-chain fatty acids, with smaller percentages of 16:1, 18:2, and 18:3 acids. Cyclopentenyl fatty acids were the prevalent constituents of triacylglycerols in these seeds, the C_{18} acids being predominant in *C. echinata* and characteristic for gorli oil (André and Jouatte, 1928), whereas the C_{16} homolog abounded in *H. anthelminthica* (Power and Barrowcliff, 1905).

The percentage of cyclic acids in diacylglycerols and free fatty acids was between 40 and 45%, roughly half of that in triacylglycerols. Compared to triacylglycerols, 18:1 and 18:2 were augmented, as well as saturated acids, especially palmitic acid. Stearic acid, however, was considerably increased in the free fatty acid fractions. Long-chain alcohols, found in trace amounts only, exhibited a predominance of even-chain saturated compounds, accompanied by small proportions of hexadecenyl and octadecenyl alcohols.

Palmitic acid was the major saturated fatty acid, in polar lipids, whereas stearic acid occurred at a rather low level. With the exception of monogalactosyl diacylglycerols and digalactosyl diacylglycerols, high levels of 18:1 and 18:2 were found in polar lipids, particularly in ethanolamine phosphoglycerides, choline phosphoglycerides, and phosphatidic acids of both *C. echinata* and *H. anthelminthica*. In monogalactosyl diacylglycerols and digalactosyl diacylglycerols, 18:2 and 18:3 predominated.

Cyclopentenyl fatty acids were distributed in phospholipids and glycolipids of *C. echinata* and *H. anthelminthica*. The occurrence of chaulmoogric and gorlic acids was more pronounced in samples of *C. echinata*. In addition, the newly detected hormelic acid and oncobic acid were present in small amounts in almost all polar lipids investigated. Total cyclic acid content averaged between 10 and 20%, an exception for a lower content being phosphatidic acids, for higher contents (up to 28%) being some glycolipids. An increase in cyclopentenyl fatty acids in individual polar lipids, concomitantly with the greater availability of cyclic acids during ripening, was observed (F. Spener, unpublished).

When the fatty acid compositions of triacylglycerols at different stages of maturity are compared, information about the time of synthesis of individual fatty acids can be gained. The compositions of the major fatty acids in triacylglycerols of *C. echinata*, representing 95% of total fatty acids, are shown in Figure 2.

Already 14 weeks before maturity chaulmoogric acid constituted 48% of total fatty acids and increased to 62%; a concomitant loss of unsaturated straight-chain fatty acids was observed. The gorlic acid content did not exhibit any dramatic change. Analyses of fatty acids of phospholipid and glycolipid classes at the same stages of maturity also showed an increase in cyclic acids. It has been mentioned earlier that 3–4 months before maturity the percentage of triacylglycerols in seed lipids was 80% and rose to over 98% in mature seeds. Considering this heavy increase of the oil content, with a concomitant increase of chaulmoogric acid, the most active and almost exclusive synthesis of cyclic acids is occurring during the last 14 weeks before maturity. In *H. anthelminthica*, which belongs to the other tribe producing cyclopentenyl fatty acids, a similar time span was observed, the main product, in this case, being hydnocarpic acid.

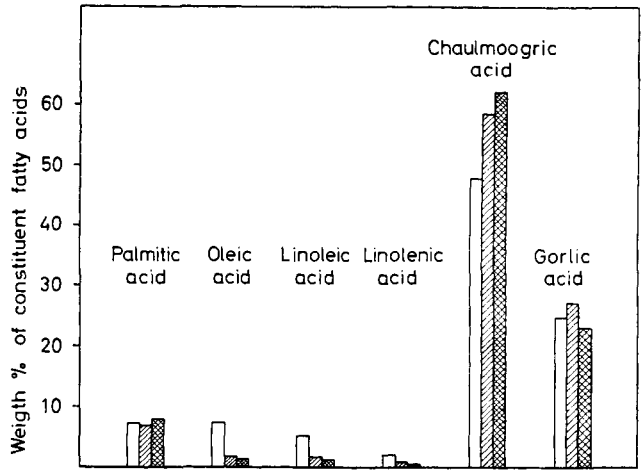

FIGURE 2: Major constituent fatty acids of triacylglycerols in seeds of *Caloncoba echinata:* white bars, 14 weeks before maturity; hatched bars, 11 weeks before maturity; cross-hatched bars, full maturity.

Discussion

The monunsaturated straight-chain fatty acids in lipids of *C. echinata* and *H. anthelminthica* seeds, as described in the present communication, are the same as those found in leaves (F. Spener and H. K. Mangold, submitted for publication) and tissue cultures (Spener *et al.*, 1974) of these plants. Regardless of the source, the monounsaturated straight-chain fatty acids having chain lengths of 16, 18, and 20 carbon atoms consist of mixtures of positional isomers. The polyunsaturated straight-chain fatty acids in lipids of *C. echinata* and *H. anthelminthica* seeds as well as in leaves (F. Spener and H. K. Mangold, submitted for publication) and tissue cultures (Spener *et al.*, 1974) have almost exclusively chain lengths of 18 carbon atoms. With the exception of small proportions of γ-linolenic acid, which were detected in seeds of *C. echinata*, the polyunsaturated fatty acids comprise only linoleic and α-linolenic acids. Obviously, the desaturases active in seeds, leaves, and tissue cultures of *C. echinata* and *H. anthelminthica* exhibit similar specificities.

The structures of the unsaturated straight-chain fatty acids do not reveal a relationship to the cyclopentenyl fatty acids that would indicate a possible precursor role of the former acids. Oxygenated fatty acids of unusual structures which could also be considered as precursors of cyclopentenyl fatty acids were not detected. Although we cannot, at present, exclude that some unsaturated or oxygenated straight-chain fatty acids having high turnover rates are precursors of cyclopentenyl fatty acids, it appears likely that other compounds function as intermediates. Thus, an idea of Stumpf (1963) should be considered which involves an intramolecular condensation of adipoyl-diCoA to a CoA derivative of a five-membered ring which in turn is elongated to the various cyclopentenyl fatty acids.

The polyunsaturated cyclopentenyl fatty acids having 16, 18, and 20 carbon atoms are mixtures of positional isomers. Only the Δ^9 isomers occur in all three chain lengths; it is striking that they are present in similar proportions.

Analyses of the constituent fatty acids in the various lipid

TABLE II: Constituent Fatty Acids of Lipids in *Caloncoba echinata* Seeds.[a,b]

Chain Length: No. of Double Bonds[c]	Triacyl-glycerols	Diacyl-glycerols	Free Fatty Acids	Ethanolamine Phospho-glycerides	Choline Phospho-glycerides	Inositol Phospho-glycerides	Glycerol Phospho-glycerides	Phosphatidic Acids	Monogalactosyl Diacyl-glycerols	Digalactosyl Diacylglycerols	Esterified Sterol Glycosides
12:0	tr	0.3	1.4	1.5	0.8	1.1	0.6	2.7	0.6	0.1	1.6
12:1cy	tr										
13:0	tr	tr	tr								
14:0	tr	0.2	2.0	1.1	0.7	0.7	1.8	2.0	0.5	0.6	1.2
14:1cy	tr										
15:0	tr	0.1	0.6	0.3	0.4	0.3	0.4	0.3	0.1	0.2	0.4
15:1	tr	tr	0.3	0.2	0.2	tr	0.2	0.2	0.1	0.1	0.1
16:0	6.7	12.1	15.6	19.3	14.6	26.6	36.1	15.4	8.3	10.6	21.8
16:1	1.5	2.2	3.8	1.8	1.7	1.6	2.8	2.1	1.3	1.7	1.8
16:1cy	1.2	1.0	2.2	0.1	1.2	0.7	0.7	1.3	0.2	0.1	0.9
17:0		0.3	0.6	0.5	0.6	0.6	0.5	0.4	0.2	0.5	0.6
17:1		0.3	0.6	0.7	0.6	0.4	1.1	0.5	0.4	0.7	0.4
18:0	0.1	1.2	5.9	4.0	3.2	3.5	5.4	1.3	2.8	7.6	8.2
18:1	1.7	8.0	14.1	11.8	21.2	11.0	12.3	19.8	16.7	12.9	11.1
18:2	1.6	19.0	10.1	39.3	35.0	27.1	16.2	38.6	30.1	22.6	21.1
18:3	0.6	1.0	0.7	3.4	2.4	3.2	2.2	7.0	27.0	28.5	2.3
18:1cy	58.6	35.1	28.1	10.5	9.2	16.3	14.1	5.0	3.6	5.0	18.7
18:2cy	27.2	17.7	14.0	3.2	4.7	5.1	4.5	2.5	5.7	5.7	7.0
20:0		tr			0.5	0.2			0.1	0.2	0.3
20:1	0.1			0.6	1.1	0.5	0.9	0.4	0.7	1.4	0.6
20:1cy	0.2	0.5	tr	1.0	0.7	0.5	0.2	0.4	0.7	0.8	1.2
20:2cy	0.5	1.0		0.7	1.2	0.6	tr	tr	0.9	0.7	0.8

[a] Expressed in wt %; tr, trace (<0.1%). [b] Analyzed as methyl esters at 177°, on a 6 ft × 1/8 in. column filled with 15% DEGS on Anakrom D, 100–120 mesh, carrier gas N_2, 20 ml/min; and at 182° on a 6 ft × 1/8 in. column, filled with 3% OV-1 on Supelcoport, 80–100 mesh, carrier gas N_2, 15 ml/min. [c] The affix "cy" denotes the cyclopentene structure of the acid.

TABLE III: Constituent Fatty Acids of Lipids in *Hydnocarpus anthelminthica* Seeds.[a,b]

Chain Length: No. of Double Bonds[c]	Triacyl-glycerols	Diacyl-glycerols	Free Fatty Acids	Ethanolamine Phospho-glycerides	Choline Phospho-glycerides	Inositol Phospho-glycerides	Glycerol Phospho-glycerides	Phosphatidic Acids	Monogalactosyl Diacyl-glycerols	Digalactosyl Diacyl-glycerols	Esterified Sterol Glycosides
12:0	0.1	0.3	0.3								
12:1cy	tr										
13:0		tr	tr								
14:0	0.2	0.2	0.4	3.2	0.4	0.5	0.6	0.4	1.7	3.4	3.9
14:1cy	0.7										

15:0	0.1	0.1	0.2	0.5	0.1	0.2	0.6	0.3	0.7	1.2	0.8
15:1	tr	0.1	0.1	0.5	0.1	0.2	0.6	0.3	0.4	0.6	0.6
16:0	4.8	9.9	16.5	22.9	21.2	27.5	25.9	19.3	12.2	17.1	26.3
16:1	1.2	3.7	3.1	3.6	2.2	1.9	3.3	1.9	5.5	4.2	5.4
16:1cy	64.9	27.6	18.7		3.7	9.6		tr	0.2	4.2	9.3
17:0	0.1	0.4	0.4	2.1	0.5	0.5	1.3	0.7	0.7	9.4	2.2
17:1	0.2	0.8	0.6	1.9	1.2	0.8	2.5	2.0	2.0	0.8	1.5
18:0	0.1	0.7	1.3	8.1	2.5	3.4	11.4	5.1	4.7	9.3	7.8
18:1	2.1	22.2	20.9	16.1	30.8	16.7	27.4	25.6	19.5	10.8	15.9
18:2	2.2	15.6	20.7	22.9	26.9	21.6	14.4	34.9	18.8	9.2	11.1
18:3	0.9	1.5	2.7	2.2	3.4	5.4	4.3	6.1	24.5	5.6	8.9
18:1cy	18.3	11.6	9.0	5.7	3.8	9.7	6.2	2.5	1.8	15.2	3.7
18:2cy	3.5	3.6	3.1	3.0	0.7	0.6	1.5	1.0	5.3	8.9	0.8
20:0			tr								
20:1cy	0.4	0.5	0.7	6.0	0.5	1.0					1.2
20:2cy	0.2	1.1	1.3	1.3	2.0	0.4					0.6

[a–c] See footnotes in Table II.

classes showed that the cyclopentenyl fatty acids predominated in the triacylglycerols of the seeds. Already 11 weeks prior to maturation, when the lipid content of the seeds was only 2.5%, the fatty acid distribution of the triacylglycerols resembled that of the mature seeds. In the following weeks, the amounts of triacylglycerols increased to a total oil content of 40% at maturity. It is evident from Figure 2 that during this time saturated straight-chain fatty acids and monounsaturated cyclopentenyl fatty acids were formed at a rapid rate while the synthesis of other fatty acids proceeded at a lower rate.

In immature seeds of both *C. echinata* and *H. anthelminthica* the proportions of cyclopentenyl fatty acids in diacylglycerols were about half of those found in the triacylglycerols. This finding suggests that a single diacylglycerol pool is available for the synthesis of acyl lipids. On the one hand, transacylases catalyze the esterification of diacylglycerols to triacylglycerols utilizing preferentially the most abundant CoA derivatives of cyclopentenyl fatty acids, and, on the other hand, the pool of diacylglycerols is available also for the formation of phospholipids and glycolipids. The occurrence of cyclopentenyl fatty acids in the various phospholipid and glycolipid classes of seeds as well as leaves of *C. echinata* and *H. anthelminthica* suggests that these acids do not play a special role in the lipid metabolism of these plants. Obviously, cyclopentenyl fatty acids do not impair the functions of phospholipids and glycolipids in the cell. It appears that both immature seeds and leaves of *C. echinata* and *H. anthelminthica* are suitable systems for studying the biosynthesis and metabolism of the cyclopentenyl fatty acids.

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The Mechanism of Interfacial Activation of Phospholipase A_2†

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ABSTRACT: The activating effect of substrate aggregation on the activity of *Crotalus adamanteus* phospholipase A_2 was investigated. Kinetic analyses were carried out using dibutyryl-, dihexanoyl-, and dioctanoyllecithin at 45° both below and above the critical micelle concentration. The major source of the enhanced rate of hydrolysis of the aggregated substrates lies in a much lower entropy of activation. It is suggested that the origin of this entropy difference probably arises from the fact that in the aggregated state, only the reactive end of the substrate molecule is presented to the enzyme during collisions. An additional factor might arise from conformational constraints placed on the glycerophosphoryl group of the substrate in the aggregate state. Data are presented which suggested that a negative charge is present at the surface of the aggregate which markedly influences the pH dependence of the reaction and the apparent kinetic mechanism. These effects are sensitive to the addition of salts such as KCl or $MgCl_2$, which influence the surface charge. The validity of the steady-state kinetic analysis of aggregate substrates was investigated and conditions were defined under which meaningful kinetic data can be collected. The meaning of K_m and V_m for these systems has also been explored. No evidence for hydrophobic interaction of the enzyme with the interface could be found, and it is suggested that the activating effect of the interface is not related to irreversible adsorption to the interface.

It is well known that the physical properties of the substrate can markedly influence the activity of phospholipase A_2 (EC 3.1.1.4) (see Wells (1972) for references to earlier studies). Recent studies (deHaas *et al.*, 1971; Wells, 1972; Pieterson, 1973) have shown that the aggregated form of short-chain lecithins is a considerably better substrate than the monomeric form.

The origin of this activation by substrate aggregation is unknown. Verger *et al.* (1973), studying pancreatic phospholipase A_2, have proposed that the enzyme penetrates between the lecithin molecules in the interface, but it is not clear how this penetration could lead to enhanced activity. Pieterson (1973) has proposed an "anchoring" site on the enzyme which somehow can account for interfacial activation. At present the nature of this "anchoring" site remains vague, although in a kinetic sense it can account for enhanced rates in the presence of aggregated substrates.

It has been suggested that substrate aggregation might alter the apparent kinetic mechanism of the enzyme (Wells, 1972). To date the only detailed kinetic analysis of monomeric substrates has been performed using the *Crotalus adamanteus* (Eastern Diamondback Rattlesnake) enzyme (Wells, 1972), while the only detailed kinetic analysis of aggregated substrates has been performed using the pancreatic enzyme (deHaas *et al.*, 1971).

The purpose of this study was to explore possible mechanisms of the interfacial activation of phospholipase A_2 and to compare the apparent kinetic mechanism of the hydrolysis of monomeric and aggregated substrates using *C. adamanteus* phospholipase A_2.

Materials and Methods

Materials. Enzyme purification and substrate (dibutyryl-, dihexanoyl-, and dioctanoyllecithins) preparation have been described previously (Wells and Hanahan, 1969; Wells, 1972; Misiorowski and Wells, 1973). Ammonium purpurate (murexide) was purchased from K and K Laboratories (Plainview, N. Y.), Phenol Red from J. T. Baker Co. (Phillipsburg, N. J.), 8-anilino-1-naphthalenesulfonic acid (ANS)[1] was purchased

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonic acid; cmc, critical micelle concentration.