The amt of phospholipid lost in the initial aqueous phase of the Folch wash was also investigated (29). First, some visible remaining drops of lower phase at the bottom of the flasks were removed by Pasteur pipet. The aqueous layers were taken to dryness under vacuum at 37C. The residues were dissolved in 10 ml distilled water and dialysed in the cold overnight against 2 liters of distilled water. Dialysis was continued for about 3½ hr longer with 2 liters of fresh distilled water. The material remaining in the dialysis bag was taken to dryness, the residue dissolved and washed into tubes for phosphorus assay with about 3 ml C:M 2:1. Less than 1% of the lipid phosphorus recovered from the entire extraction was found in the undialysable portion of the first aqueous phase.

Qualitatively, by TLC, the lipids in the granules and membranes were identical, but when the particles as such were tested in clotting systems, the membranes were more active than the granules. On the other hand, lipids extracted from both the granules and membranes behaved similarly in various clotting systems. It appeared that the lipid (or lipoprotein) in the platelet membrane was more "available" for interaction with the plasma clotting factors than was the lipid from the granules. In addition, the granules appeared to be the major site of acid phosphatase, beta-glucuronidase, and cathepsin activity, which was consistent with the hypothesis that they may be lysosomes. Some of the granules are mitochondria. As shown by Fleischer (30), these are rich in phospholipid, but we would suggest that such phospholipid does not ordinarily play a role in blood coagulation.

An approach to the study of platelet lipids and their role in in vitro coagulation has been described. It is stressed that correlations with in vivo coagulation mechanisms be made with the realization that these situations may not be comparable.

ACKNOWLEDGMENT

Supported by Grant HE09070 of the National Heart Institute, NIH, USPHS, the New York Heart Association, and USPHS 2G-297, A. M. 01431.

REFERENCES

1. Macfarlane, R. G., Nature *202*, 498 (1964).
2. Spaet, T. H., Lipids, in "Progress in the Chemistry of Fats and Other Lipids," R. T. Holman et al., eds., Vol. VI, Pergamon Press, New York, 1963, p. 171.
3. Mustard, J. F., E. A. Murphy, H. C. Rowsell and H. G. Downie, J. Atheroscler. Res. *4*, 1 (1964).
4. Merskey, C., and Marcus, A. J., Ann. Rev. Med. *14*, 323 (1963).
5. Marcus, A. J., and Zucker-Franklin, D., J. Clin. Invest. *43*, 1241 (1964) (Abstract).
6. Marcus, A. J., and T. H. Spaet, J. Clin. Invest. *37*, 1836 (1958).
7. Bell, W. N., and H. G. Alton, Nature *174*, 880 (1954).
8. Marcus, A. J., H. L. Ullman, L. D. Safier and H. S. Ballard, J. Clin. Invest. *41*, 2198 (1962).
9. Hirsch, J., and E. H. Ahrens, Jr., J. Biol. Chem. *233*, 311 (1958).
10. Marcus, A. J., H. L. Ullman and M. Wolfman, J. Lipid Res. *1*, 179 (1960).
11. Lea, C. H., D. N. Rhodes and R. D. Stoll, Biochem. J. *60*, 353 (1955).
12. Rouser, G., J. O'Brien and D. Heller, JAOCS *38*, 14 (1961).
13. Rouser, G., A. J. Bauman, G. Kritchevsky and D. Heller, JAOCS *38*, 544 (1961).
14. Marinetti, G. V., J. Lipid Res. *3*, 1 (1962).
15. Skipski, V. P., R. F. Peterson and M. Barclay, J. Lipid Res. *3*, 467 (1962).
16. Skipski, V. P., R. F. Peterson and M. Barclay, J. Lipid Res. *4*, 227 (1963).
17. Skipski, V. P., R. F. Peterson and B. Barclay, Biochem. J. *90*, 374 (1964).
18. Farquhar, J. W., Biochim. Biophys. Acta *60*, 80 (1962).
19. Stoffel, W., F. Chu and E. H. Ahrens, Jr., Anal. Chem. *31*, 307 (1959).
20. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel and E. H. Ahrens, Nutr. Rev. *17*, Suppl. 1 (1959).
21. Lipsky, S. R., and Landowne, R. A., in Colowick, S. P., and Kaplan, N. O., eds.: Methods in Enzymology, Vol. VI. Academic Press, New York, 1963, p. 513.
22. James, A. T., in Glick, D., ed.: Methods of Biochemical Analysis, vol. VIII, Interscience, New York, 1960, p. 1.
23. Zilversmit, R. D., A. J. Marcus and H. L. Ullman, J. Biol. Chem. *236*, 47 (1961).
24. Silver, M. J., D. L. Turner, I. Rodalewicz, N. Girodano, R. Holburn, S. F. Herb and F. E. Luddy, Thromb. Diath. Haemorrh. *10*, 164 (1963).
25. Ferguson, J. H., A. J. Marcus and A. J. Robinson, Blood *22*, 19 (1963).
26. Woodside, E. E., D. G. Therriault and W. Kocholaty, Blood *24*, 76 (1964).
27. Marcus, A. J., unpublished observations.
28. Marcus, A. J., and Zucker-Franklin, D., Blood *23*, 389 (1964).
29. Folch, J., M. Lees and G. H. Sloane-Stanley, J. Biol. Chem. *226*, 497 (1957).
30. Fleischer, S., G. Brierly, H. Klouwen and D. B. Slautterback, J. Biol. Chem. *237*, 3264 (1964).
31. Davie, E. W., and Ratnoff, O. D. Science *145*, 1310 (1964).

Arachidonic, 5, 11, 14, 17-Eicosatetraenoic and Related Acids in Plants—Identification of Unsaturated Fatty Acids

HERMANN SCHLENK and JOANNE L. GELLERMAN, The Hormel Institute, University of Minnesota, Austin, Minnesota

Abstract

Arachidonic and related fatty acids which normally are found only in animals or microorganisms have been isolated and identified from several mosses and ferns. Fatty acids with a double bond in position 5, separated by more than one methylene group from other double bonds, have been found in *Ginkgo biloba* and *Equisetum*. Analyses of fatty acids from numerous plants, in particular their chlorophyll containing parts, are listed according to components.

The experimental part gives details on structure determination of the usual methylene-interrupted fatty acids by ozonization-hydrogenation-GLC. Alkaline isomerization combined with these procedures was applied to determine the unusual double bond structures. The method permits positional identification of an internal double bond.

Introduction

TENTATIVE IDENTIFICATIONS of unsaturated fatty acid methyl esters are often made by comparison of retention times in gas-liquid chromatography (GLC) of the unknown with an authentic ester. Preferably, their equivalent chain lengths (ECL) (38,63) in GLC over different phases are compared. Interpolation of ECL may be helpful in characterizing a sample when an authentic ester of the expected structure is not accessible but when similar type esters of different chain length are available. Obviously, esters of different chain length cannot have identical double bond structures. Systematic studies have been reported by Ackman (1–5) where relative retention times are correlated with the location of methylene-interrupted double bond systems. As such data are accumulated, they contribute further to tentative identifications of fatty esters with a minimum of effort and substance.

The composition of mixtures may be such that resolution by GLC is unsatisfactory, that coordination of peaks from different phases is uncertain, or that ECL's merely indicate the presence of esters which are different from any reference compound. Another reason for isolation and investigation of structure by chemical means is to verify the tentative identification of an ester that is well known but is unexpected in

the particular material. Such cases are exemplified here.

GLC indicated the presence of arachidonic acid in amts of 10–40% of the total fatty acids in moss and fern (22). Fatty esters from Ginkgo (21) and other plants contained components which behaved in GLC quite differently from any of the common unsaturated straight-chain esters. In both cases, the mixtures were fractionated to enable chemical study of the unexpected or novel compounds.

The experimental section gives details on (a) the identification of fatty acids with methylene-interrupted double bonds and (b) procedures for determining double bonds which are interrupted by more than one methylene group. It is followed by analyses of fatty acids from plants belonging to a variety of botanical divisions and by a discussion of the results.

Experiments

Arachidonic Acid in Plants

Mixed mosses, mainly *Brachythecium* and *Mnium*, were washed free of soil and blotted dry. About 1 kg of this material was extracted with $CHCl_3$ + MeOH, 2:1, in a large Waring blendor with plastic parts which are resistant to the solvent. The lipids were saponified, the unsaponifiables removed by extraction and the fatty acids esterified with CH_2N_2 (50). The esters were freed from polar pigments by chromatography on a silicic acid column (SiO_2 + Chromosorb, 2:1) with petroleum ether (Skellysolve B) + diethyl ether, 95:5 (23). About 1.3 g of purified esters were obtained and GLC (52) showed the peak, 34% of total areas, which had already been encountered in smaller samples from other mosses and had been tentatively assigned to arachidonate.

The mixed esters were subjected to liquid-liquid chromatography (LLC) (23,51) on silicone oil as the stationary phase. The recovered esters contained small amts of silicone oil and were purified from this contaminant by alembic distillation (23). The fractions contained the esters of 16:0 + 18:1; 16:1 + 18:2 + 20:3; 18:3 + 20:4; and 16:3 + 18:4 + 20:5. GLC (53) afforded separation of the esters which were superimposed in the LLC bands. A Beckman GC-2 apparatus was used with a 3/8 in × 12 ft aluminum column which contained about 100 g of 20% β-cyclodextrin (β-CDX) acetate on Gas-Chrom P, 30–60 mesh; the temperature was 236C and the flow rate of He was 150 ml/min. Individual esters or mixtures of unsaturated isomers were obtained in sufficient amt for detailed analyses of structures. GLC and hydrogenation-GLC of the original mixture had indicated the presence of some 22:0 and 24:0 but they were not further verified.

Structure Determination of Fatty Acids with Methylene-Interrupted Double Bonds by Ozonization-Hydrogenation-GLC. Samples of 2-10 mg were ozonized. The esters were dissolved in 2 ml of purified methylene chloride or methyl acetate in a rimless test tube, 125 × 15 mm, which fit into the ozonization vessel of the Bonner semimicro ozonizer (11). The apparatus was placed in a hood since the vessel was removed and exchanged while the ozone-generator was in operation.

The ozonizer was flushed with O_2 for 1 hr at a flow rate of 80 ml/min before turning on the high voltage. Sample and outer tube were cooled in an ice-salt mixture to −10C in a transparent Dewar vessel. The tubes were attached to the ozonizer after it had been equilibrated for more than 5 min. The appropriate time of ozonization was found empirically. For example, 24 sec suffices, for complete ozonization of 2 mg of methyl arachidonate or, in more general terms, about 1 sec/microequivalent of double bond is needed. The sample tube was removed and excess O_3 was immediately carried away by N_2 at 0C. About 100 mg of Pd catalyst on $CaCO_3$, poisoned with lead salt according to Lindlar (35,42), was added. H_2 was bubbled through the suspension for 10 min at 0C and the catalyst was removed by filtration.

TABLE I

Fatty Acids of *Brachythecium* + *Mnium*

Component		GLC area, %	Isolated mg	% Composition of isomers
	14:0	0.1		
	16:0	12.1	120	
7–				8
9–	16:1	2.5	30	31
11–				61
7,10,13–	16:3	2.6	2	
	18:0	trace		
9–				65
11–	18:1	2.8	28	35
9,12–	18:2	14.4	92	
6,9,12–				3
9,12,15–	18:3	14.7	88	97
6,9,12,15–	18:4	trace	2	
	20:0	0.3		
	20:1	0.3		
	20:2	trace		
8,11,14–				78
11,14,17–	20:3	trace	10	22
5,8,11,14–	20:4	33.8	175	
5,8,11,14,17–	20:5	5.6	36	
	22:0	1.7		
	24:0	2.3		

The procedures require 10–15 min and it is advisable to begin identification of the aldehydic fragments by GLC within the next half hour. Aldehyde-esters were identified with a Beckman GC-2 apparatus with H_2-flame detector, using a 1/4 in. × 12 ft. aluminum column packed with 20% β-CDX acetate on Gas-Chrom P, 30–60 mesh, at 220C. Aldehydes with more than 5 C atoms can be found in the same chromatograms but the low boiling solvent obscures the short chain aldehydes in GLC. Therefore, the terminal double bond is located by repeating the ozonolysis and subsequent procedures in ethyl caprate (59). GLC is then carried out with a 1/4 in × 10 ft column packed with 20% Carbowax 4000 on Chromosorb W, 60–100 mesh, at 160C. Authentic unsaturated esters were the source of aldehyde-esters and aldehydes in reference chromatograms.

Table I lists the unsaturated acids of the mosses, *Brachythecium* and *Mnium*, as they were identified by ozonization and gives their quantification by GLC. More than 40% of the original esters were recovered after analytical and separation procedures. Isomers were not separated before ozonization. Their relative composition was determined from GLC areas of the aldehyde-esters (54,47).

Eicosapentaenoate, arachidonate and octadecatrienoate (mostly linolenate) from the mosses were also subjected to alkaline isomerization (27) and yielded the proper UV spectra.

Arachidonic acid in lipids from several ferns was indicated by GLC of the mixed fatty esters on β-CDX acetate and propionate. Its identity was ascertained with a sample of esters which had been prepared from *Adiantum pedatum* in the manner described above. However, the amt available was less than in the foregoing experiment and, therefore, the component in question was isolated from the mixture by GLC without prefractionation (23,53). About 50 mg esters was applied and yielded 5 mg of the surmised arachidonate. With such an amt, the column was overloaded for separation of peaks in close sequence. However, the desired ester of this sample

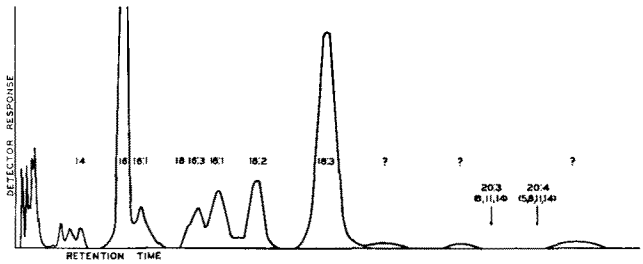

FIG. 1. Gas-liquid chromatogram of ginkgo leaf methyl esters.

emerged well apart from other esters so that it was collected in purity.

Hydrogenation-GLC proved that the ester is a straight-chain C_{20} compound. Ozonization-hydrogenation-GLC as above yielded C_5 aldehyde-ester and C_6 aldehyde. These results, together with the ECL's of the ester, establish the identity of arachidonic acid from the fern.

In general, structures of unsaturated fatty acids with the normal methylene-interrupted double bond system can be determined by fragmentation at the double bonds and identification of the proximal (carboxyl—first double bond) and terminal (last double bond—methyl) moieties. The chain length of the normal ester must be known. The total number of C atoms of the identified fragments is subtracted from it and the difference must be a multiple of 3 C atoms. These data, in combination with ECL or UV absorption before and after alkaline isomerization, afford unambiguous determination of the double bond structure so that identification of the internal fragments is superfluous. The three-carbon units may be indicated after ozonization-reduction as malondialdehyde in GLC (42), or they are found as malonic acid after ozonization-oxidation by LLC (31). Their quantification is not satisfactory.

The above procedures for structure determination are unambiguous only with the usual methylene-interrupted structures. Discrepancies arise when other structures are encountered.

Polyunsaturated Acids of Unusual Structure in Plants

Methyl esters obtained from lipids of leaves or nuts of *Ginkgo biloba* gave gas-liquid chromatograms as exemplified in Figure 1. They have several peaks which cannot be assigned by GLC over different phases to any polyunsaturated esters of common type. Hydrogenation-GLC of the mixture did not show any unusual peaks. Isolation of the esters was undertaken for further investigation of their structure (21).

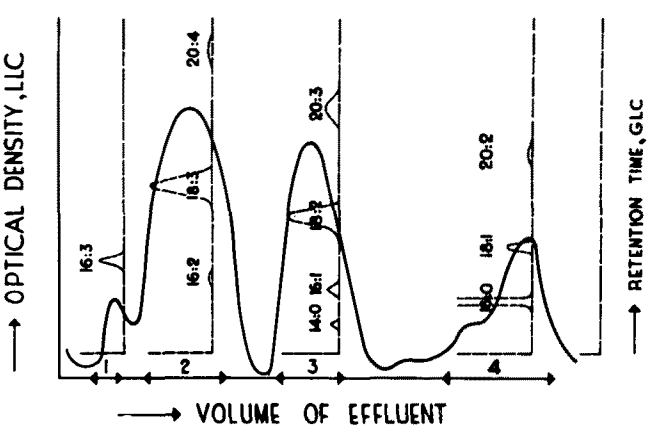

FIG. 2. Liquid-liquid chromatogram of ginkgo leaf methyl esters (solid line) and analysis of the four fractions by GLC (broken line).

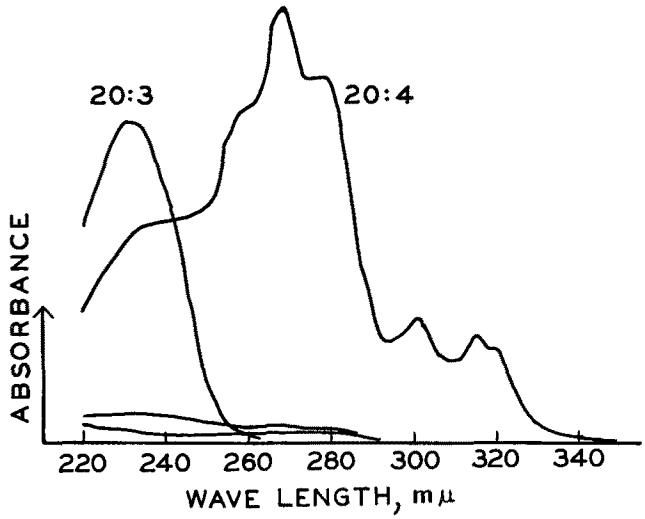

FIG. 3. UV absorbance of 20:4 and 20:3 esters after alkaline isomerization. Samples: 0.1 mg in 10 ml ethanol; quantification was not attempted. The lower curves show absorbance of nonisomerized samples in equal concn.

About 0.8 g of purified mixed methyl esters was prepared from fresh Ginkgo leaves by procedures outlined above and subjected to LLC. In Figure 2 the pooling of LLC fractions is indicated by arrows in the abscissa and their composition is shown as GLC recordings. The peaks of unknown esters are designated as 20:4, 20:3 and 20:2 since hydrogenation-GLC showed C_{20} in these fractions. The number of double bonds was anticipated according to the rules for superposition of fatty esters in LLC (48). The subsequent work proved this to be correct.

Further fractionation was accomplished by GLC and yielded between 1 mg and 200 mg of esters. They were uniform in chain length but some of the unsaturated esters contained isomers.

A sample of eicosatrienoate which had been prepared similarly from Ginkgo nuts contained less than 5% *trans* double bond according to IR spectroscopy. After change of solvent, this sample was hydrogenated analytically (46) and consumed 91% of the amount of H_2 calculated for 3 double bonds. The hydrogenated ester was methyl arachidate.

The UV spectra in Figure 3 of 20:4 and 20:3, before and after alkaline isomerization (27,39), show that one of their anticipated double bonds does not conjugate. The prominent maximum of 20:4 at 268 mμ is due to 3 conjugated double bonds while the less pronounced maxima at 300 to 315 mμ are caused by a compound with 4 conjugated double bonds. The

I. O_3–H_2 determines position of extreme double bonds:

$CH_3OOC\ C\ C\ C\ C{=}C\ .\ .\ .\ C{=}C\ .\ .\ .\ C{=}C\ C\ C\ C\ C\ C$

C_5 Aldehyde Ester, by GLC on β-CDX ac. — C_6 Aldehyde, by GLC on Carbowax

II. O_3–H_2O_2–CH_2N_2 determines length of internal fragment:

$CH_3OOC\ C\ C\ C\ C{=}$ $=C\ C\ C\ C\ C\ C{=}C\ C\ C{=}$ or $=C\ C\ C{=}C\ C\ C\ C\ C\ C{=}$ $=C\ C\ C\ C\ C\ C$

C_5 Di-ester, C_6 Di-ester, by GLC on β-CDX ac., C_6 Ester,

III. Alk. Isom.–O_3–H_2 determines position of internal double bond:

$CH_3OOC\ C\ C\ C\ C{=}C\ C\ C\ C\ C\ C{=}C\ C\ C{=}C\ C\ C\ C\ C\ C$

Alk. ↓ Isom.

$CH_3OOC\ C\ C\ C\ C{=}C\ C\ C\ C\ C\ C{=}C\ C{=}C\ C\ C\ C\ C\ C\ C$

+

$CH_3OOC\ C\ C\ C\ C{=}C\ C\ C\ C\ C\ C\ C{=}C\ C{=}C\ C\ C\ C\ C\ C$

C_5 Aldehyde Ester, by GLC on β CDX ac. — C_6 + C_7 Aldehyde, by GLC on Carbowax

FIG. 4. Scheme for identifying 5,11,14–20:3 methyl ester (portions identified are underlined).

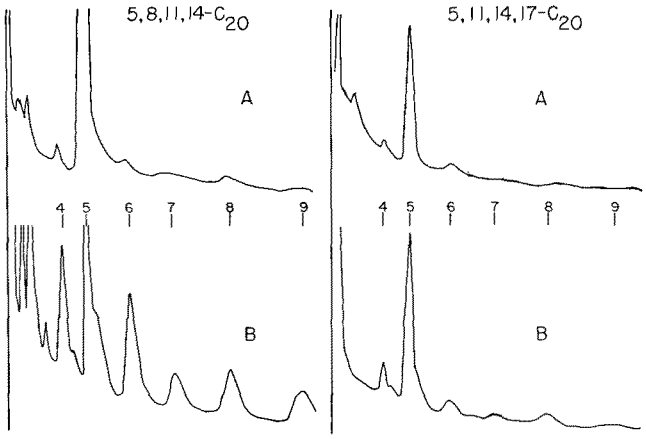

FIG. 5. Aldehyde esters resulting from ozonization of polyunsaturated esters (A) before and (B) after alkaline isomerization.

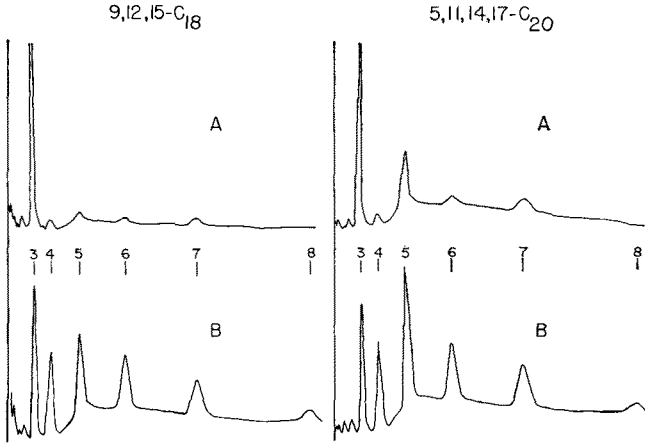

FIG. 6. Aldehydes resulting from ozonization of polyunsaturated esters (A) before and (B) after alkaline isomerization.

relative heights of maxima 268, 300 and 315 mμ indicate that the conjugated tetraenoate represents only a minor amt. The spectrum of 20:3 is clearer, inasmuch as only two double bonds are conjugated by alkali.

Structure Determination by Ozonization Combined with Alkaline Isomerization. The positions of the first and the last of the three double bonds in 20:3 were established by ozonization-hydrogenation-GLC and C_5 aldehyde-ester and C_6 aldehyde were the only fragments detected besides the peak which arises from malondialdehyde. Without any further data (see end of section (a)), the ester would be designated as arachidonate. However, this is not acceptable in view of accumulated evidence for three double bonds, only two of which can conjugate. When cyclohexene and cyclopentene were ozonized, GLC failed to show any peak at all. Apparently, the missing link which must be a higher homolog of malondialdehyde is lost under our GLC conditions.

With two double bonds amenable to alkaline conjugation, the alternative structures are 5,8,14–20:3 and 5,11,14–20:3. Fragmentation at the double bonds should yield from both structures an internal fragment of 6 C atoms and it was desirable to verify this. Ozonization followed by oxidation with H_2O_2 (31) and esterification of the resulting acids revealed, by GLC, C_5 diester, C_6 monoester and C_6 diester. The former two compounds represent the already established proximal and terminal parts of the molecule. C_6 diester is the internal fragment which had not been found by ozonization-hydrogenation-GLC.

It remained to decide if the internal double bond is located in position 8 or 11. Resort was taken to alkaline isomerization under the following considerations.

Standard conditions of alkaline isomerization supposedly conjugate double bonds only when they are interrupted by not more than one methylene group (19,41). Conjugation of two double bonds takes a twofold course so that oxidative splitting of the isomerized mixture yields the novel fragments R′–C–C and R″–C–C (underlined).

$$\begin{array}{c} R'\text{–}CH\text{=}CH\text{–}CH_2\text{–}CH\text{=}CH\text{–}R'' \\ \text{Alkal.} \downarrow \text{Isom.} \\ R'\text{–}CH\text{=}CH\text{–}CH\text{=}\underline{CH\text{–}CH_2\text{–}R''} \\ + \\ \underline{R'\text{–}CH_2\text{–}CH}\text{=}CH\text{–}CH\text{=}CH\text{–}R'' \end{array}$$

The nonconjugating double bond of the 20:3 ester must be at the end of the whole double bond system, either in position 5 or 14. Accordingly, only one new homolog, C_6 aldehyde-ester or C_7 aldehyde, will be found by splitting the ester after isomerization, provided the isolated double bond remains unchanged.

This reasoning was checked by subjecting oleate and linoleate to isomerization, re-esterification and ozonization-hydrogenation-GLC. The double bond of oleate did not shift markedly and linoleate gave equivalent amounts of C_9 and C_{10} aldehyde-esters and of C_6 and C_7 aldehydes. Fortunately, these prerequisites are also fulfilled when the isolated and the methylene-interrupted double bonds are in one molecule. The same procedures were applied to 5,?, 14–20:3 and yielded C_5 aldehyde-ester, C_6 aldehyde and C_7 aldehyde. Since the latter compound appears only after isomerization, it is concluded that 20:3 from Ginkgo is 5,11,14–eicosatrienoic acid and, according to IR spectroscopy, it is the all-*cis* compound. The procedures are summarized in Figure 4.

Conjugation by the alkali-high temperature method is assumed to occur only with methylene-interrupted double bonds. Although our experiments with 5,11, 14–20:3 confirm this, the general validity of the rule is not too well substantiated (19,41). Besides, it is obvious from the literature that the isomerization products of linolenic are considerably more complex than those of linoleic acid (58). It is seen from the chromatograms on the lower left of Figures 5 and 6 that ozonization of isomerized arachidonate and linolenate does not permit any conclusions as to the original structures. Aldehyde esters and aldehydes appear at any chain length between C_3 and C_9. However, this is not so with 20:4 acid from Ginkgo.

Analyses of the preparation used for the subsequent procedures showed 20:4 to be >85% pure (see below). Ozonization revealed C_5 aldehyde ester and C_3 aldehyde as major proximal and terminal moieties. Since

TABLE II

Fatty Acid Composition of *Ginkgo biloba* (GLC Area %)

Component	Leaf		Nut	
	%	Position of double bonds and rel. amts of isomers	%	Position of double bonds and rel. amts of isomers
14:0	1.0		2.6	
16:0	22.3		11.3	
16:1	3.1	7 <9	6.2	9 >11
16:2	+	7,10 >9,12	+	7,10 <9,12
16:3	4.3	7,10,13 >9,12,15		
18:0	0.7			
18:1	6.8	9 >11	30.0	9 >11
18:2	20.8	9,12 major	42.3	5,11 <9,12
18:3	32.0	9,12,15 major	1.6	5,11,14≅ 9,12,15>> 11,14,17
20:2	+	5,11>11,14 >14,17	0.9	5,11 >11,14
20:3	6.3	5,11,14 major	4.1	5,11,14 major
20:4	1.7	5,11,14,17	+	5,11,14,17

TABLE III

Composition of Fatty Acids from Plants Containing Arachidonic Acid (GLC area %)

Item No.	Division[a]	Systematic name or family	Comon name	14:0	16:0	16:1	16:3	18:0	18:1	18:2	18:3 6,9,12–9,12,15–	20:0	20:3 5,8,11,14–	20:4 5,8,11,14–	20:5 5,8,11,14,17–	22:0	24:0	Others
1	4. Charophyta	*N.tella*[b]	Algae	1.4	21.4	1.6	7.2	+	2.3	31.2		17.0		6.3				
2		*Polytrichum juniperinum*	Hair cap moss	5.8	9.9	1.2		2.0	7.1	11.6		12.9	9.9	9.3		16.2		16:2, 8.6, 20:2, 0.9
3		*Hedwigia ciliata*	Moss	1.4	10.0	1.1		2.1	9.6	15.0	+	24.4	2.4	14.9		9.7	13.4	
4	15. Bryophyta	*Hylocomium splendens*	Moss	0.6	9.3	1.4		2.4	8.5	17.9		20.6	1.0	13.2	10.2	4.1	8.3	18:4, +
5		*Sphagnum*	Peat moss	1.1	14.5	7.8	3.1	1.9	11.8	16.2	+	31.4	+	9.6		2.1	5.0	15:1, 5.3; 20:1, +
6a		leafy parts		0.7	20.6	1.4	2.2	1.6	25.6	19.5	1.6	12.6	+	10.2	+	+	+	22:3, +
6b		*Mnium cuspidatum*, sporophytes	Moss	2.8	33.6	1.8	3.6	3.6	11.4	14.5	3.8	9.1	+	11.1	+	+	+	22:3, +
7	17. Microphyllophyta	*Lycopodium*	Club moss	1.4	21.4	2.3	5.0	+	10.3	13.1	+	39.2	+	2.2		1.0		5,8,11–20:3 likely
8		*Adiantum pedatum*	Maidenhair fern	0.9	19.2	2.2	4.0	0.6	7.1	7.2		37.4	0.8	10.8	2.7			16:2, 0.5; 18:4, +
9		*Onoclea sensibilis*	Sensitive fern	0.4	21.1	6.8	5.3		4.8	5.4		47.9		8.0	+			16:2, 1.0; 20:2, 0.3; 22:3, 2.4
10	19. Pterophyta	*Osmunda Claytoniana*	Interrupted fern	0.7	24.1	3.9	8.1		4.7	7.0		44.6		6.9	+			
11		*Matteucia struthiopteris*[c]	Ostrich fern	2.9	17.6	3.0	4.1		3.2	6.8		54.2		8.2	+			22:3, +

[a] Classification according to H. C. Bold, 1956 (9,10).
[b] Obtained from General Biological Supply, Chicago, Ill.
[c] Arachidonic acid was also found in the fruiting leaf.

a triene is prominent after conjugation, the structure of 20:4 should be 5,8,11,17–20:4 or 5,11,14,17–20:4. Ozonization of the isomerized material led to C_5 aldehyde ester of a purity which compares well with that obtained from the nonisomerized ester and which is in sharp contrast to the aldehyde esters obtained from arachidonate after isomerization. The fixed double bond is in position 5, whereas the three conjugating double bonds are accumulated towards position 17. In agreement with this, the mixture of aldehydes arising by isomerization-ozonization closely resembles that from linolenate. It gives further proof that the unusual tetraenoic acid of Ginkgo is 5,11, 14,17–20:4. The chromatograms which lead to this conclusion are shown in Figures 5 and 6.

GLC of the 20:4 ester did not show any impurity, but 7% C_{18} was detected by hydrogenation-GLC of the sample. IR and UV spectroscopy revealed 10–15% *trans* and conjugated double bonds, i.e., somewhat more than the sample of Figure 3. Peroxide was not present. It is seen from Figure 6 (upper right and left) that C_5 aldehyde resulted to a greater extent from the sample of 5,11,14,17–20:4 than any of the extraneous aldehydes from linolenate which had been prepared by bromination-debromination. Isomerization of 20:4 led to a small amt of tetraene conjugation. Although these data do not make questionable the double bond structure of the major component, they are too pronounced for declaring the contaminants to be artifacts.

Conjugated esters like β-eleostearate cannot be chromatographed under our GLC conditions. Possibly the C_{18} contaminant is a conjugated polyenoic compound which is found by GLC only after hydrogenation.

The occurrence of *trans* double bond is probably correlated with the conjugated contaminant. A *trans* double bond in the major component would require IR absorption of twice the value found. Therefore, it appears most likely that 5,11,14,17–20:4 is, like 5,11,14–20:3, the all-*cis* compound. However, *trans* Δ^5 acids have been found from natural sources (see below, Table V). Apparently the uniformity of *cis* configuration which prevails with methylene-interrupted fatty acids of biological origin does not apply to the special group of Δ^5 acids.

Table II exemplifies complete analyses of ginkgo leaf and nut fatty acids. Leaves were harvested at different seasons of the year but startling changes in the fatty acid composition were not encountered.

Polyunsaturated fatty acids with a "remote" double bond in position 5 have been found in recent years from other natural sources and their structures have been elucidated by a variety of methods. A 20:3 acid from *Podocarpus nagi* (17,34) was identified by Takagi (61) as 5,11,14–20:3 which is the same as one of the acids from ginkgo. He applied oxidative degradation ($KMnO_4$-periodate) to the fatty acid and to the fatty alcohol obtained by $LiAlH_4$ reduction. The trienoic acid was also treated with N_2H_4 and the fragments obtained by oxidation of the resulting mono- and dienoic acids were in accord with the above structure. Bagby et al. (8) subjected a 20:2 acid to the sequences oxidation (performic acid)–splitting (periodic acid)–selective reduction of aldehyde groups ($NaBH_4$)–oxidation of alcoholic groups ($KMnO_4$). From the fragments he concluded that the structure was 5,13–20:2.

The double bonds of an 18:3 acid were located in positions 5, 9 and 12 by similar procedures (7). How-

TABLE IV
Composition of Fatty Acids from Plants Containing Members of the Δ^5 Group (GLC Area %)

Division	Systematic name	Common name	14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2 5,11–	18:2 9,12–	18:3 9,12,15–	20:2	20:3 5,11,14–	20:4 5,11,14,17–	24:0
18. Arthrophyta	*Equisetum hyemale*	Scouring rush	0.3	24.2	0.6	1.0	4.2		0.5		15.1	48.6		3.6	1.6	
	Equisetum arvense	Field horsetail		23.2	4.4	1.8	9.4		4.0		11.5	43.0		0.6	2.1	
21. Ginkgophyta	*Ginkgo biloba* (leaves)	Maidenhair tree, Ginkgo	1.5	19.3	1.5	0.3	4.9	1.6	4.8	1.0	7.2	48.2	1.2	1.7	3.4	3.3

ever, IR absorption had revealed that one of them is a *trans* double bond. The acid was treated with lipoxidase (36,37) and yielded diene conjugation. Since the enzyme is specific for *cis,cis* methylene-interrupted double bonds (26,43), the *trans* configuration must be at position 5.

Davidoff and Korn (13,14,15) oxidized ($KMnO_4$-periodate) an 18:2 acid, isomeric to linoleic, as methyl and as ethyl ester, and found it to be 5,11–18:2. The ethyl ester was used to distinguish the dicarboxylic acids which arise from proximal and internal moieties. They were identified (GLC) as methyl-ethyl and dimethyl esters after methylation of the carboxyl groups which had been created by oxidation of the double bonds.

Partial fragmentation (OsO_4 followed by hydrogenation and periodate) has been applied by de Jong and van der Wel (29) to identify 11,15–18:2 and other isolinoleic acids which do not isomerize. The method may also be helpful with the Δ^5 acids discussed here, and with other nonconjugating systems.

Some of these procedures are involved and not easily adaptable to small amts. In our hands, cleavage by ozonization-hydrogenation brings about fewer side products than ozonization-oxidation or $KMnO_4$-periodate. The scheme of Figure 4 was carried out with less than 20 mg of trienoic and tetraenoic esters. Step III is necessary only with these highly unsaturated esters.

Additional Analytical Results

Tables III, IV and VI give data on fatty acids which had been obtained mainly from the green parts of plants collected in Minnesota in 1963. GLC conditions were similar to those described previously (52). Quantifications are according to area percentages since correction factors for the unusual esters have not been determined. Positional identification of mono- and dienoic acids was not attempted and isomers as in Table I are likely to occur. Conventional isomeric trienes such as α- and γ-linolenate are better separated by GLC than isomers with fewer double bonds so that their tentative structures are specified.

Members of the Δ^5 series (Table IV) can be easily distinguished from their conventional isomers by GLC. Their identity was concluded by GLC comparisons with the authentic compounds which were available and had been identified as described in the foregoing. GLC data on the novel esters will be incorporated by Holman into a comprehensive publication from this institute on ECL of polyunsaturated esters. It suffices here to state qualitatively that among isomers with identical terminal group, the one having the shortest proximal moiety is the first to emerge on polar phases in GLC; e.g., ECL 5,11,14–20:3 < 8, 11,14–20:3. On the other hand, with identical proximal group, the compound having the shortest terminal group emerges last; e.g., ECL 5,8,11,14–20:4 < 5,11, 14,17–20:4. This is seen also from Figure 1.

Table V lists Δ^5 acids which have been reported by other authors. Analyses of some of the plant fatty acids listed in the other tables are found in earlier literature but numerous source materials here are novel or modern methods have been applied for the first time. Correlation with earlier reports was not attempted and, quite obviously, much of the data given here should be considered exploratory rather than final (see Introduction).

Discussion

The occurrence of eicosatetraenoic and, more specifically, arachidonic acid in plant lipids has been reported several times in earlier literature (28). These findings are open to doubt since, at best, they are based on alkaline isomerization. Similar technical deficiency must be held against some of the reports during the last decade (6,12,30,40,60).

The vast majority of knowledge on plant fatty acids refers to plants of highest evolutionary level, i.e., angiospermae, and arachidonic acid was not found in them. Our data (see Table V) are in agreement with this. These samples do not contain arachidonic acid or, more cautiously, this acid is not present in an amt that could be detected by GLC without preceding enrichment. However, the complete absence of arachidonic acid in members of this division should not be taken for granted. Tetraenoic acids are potential components in higher plants as exemplified by parinaric acid (from *Parinarium* and *Impatiens*) and, more significantly, all-*cis*-6,9,12,15-octadecatetraenoic acid (from *Boraginaceae*) (57,62).

The apparent absence of arachidonic acid in angiospermae led to the generally accepted opinion that it does not occur in the plant kingdom (16,25,55). The concept was weakened first when arachidonic acid was found in *Ochromonas danica* (23,24) and *malhamensis* (49), algae (32), and *Euglena* (18,33,44,45). These organisms may be classified as plants or animals, partly depending on the botanists' or zoologists' needs. The occurrence of arachidonic acid in mosses and ferns is a more drastic break from the general rule since they are indisputably assigned to the plant kingdom. Although their lipid content is low, particularly in mosses, the percentage of arachidonic in total fatty acids is rather high and in the order of that found in normal rat liver fatty acids.

TABLE V
Other Occurrences of Δ^5 Acids in Plants

Division	Systematic name	Common name	Structure of Δ^5 acids	Author
9. Schizomycota	*Bac. megaterium*		5-16:1; 5-18:1	Fulco, Levy and Bloch (20)
10. Myxomycota	*Dictyostelium discoideum*	Slime mold	5,9-16:2; 5,9-18:2; 5,11-18:2	Davidoff and Korn (13,14)
22. Coniferophyta	*Podocarpus Nagi*	Japanese podocarpus	5,11-20:2— 5,11,14-20:3	Takagi (61)
24. Anthophyta	*Thalictrum polycarpum*	Sierra meadowrue	*trans*-5-18:1; *trans*-5,*cis*-9,*cis*-12-18:3	Bagby et al. (7)
	Limnanthes Douglasii	Meadow foam	5-20:1; 5-22:1; 5,11-22:2	Bagby et al. (8,56)

TABLE VI
Composition of Fatty Acids from Other Plants
(GLC Area %)

Item No.	Division	Systematic name or family	Common name	14:0	15:0	16:0	16:1	16:2	16:3	17:0	18:0	18:1	18:2	18:3 9,12,15–	20:0	22:0	Others
1	Combination of	*Evernia Mesomorpha*	Lichen	1.2		7.8	0.7				2.2	13.5	42.6	3.2			
2	low divisions	*Parmelia*	Lichen	3.8	0.4	13.5	1.9			1.4	3.3	18.6	51.3	2.6			20:2, +; 27.4
3	20. Cycadophyta	*Cycas revoluta*	Cycad	0.7		22.8	0.6	1.1	3.7		+	3.7	31.0	36.5	+	+	20:2, +; 20:3, +
4	22. Coniferophyta	*Picea*	Blue spruce	1.7		16.1	5.6				1.4	14.5	16.7	32.4	0.7	4.2	18:4, +; 20:3, +
5		*Magnoliaceae*	Magnolia			18.5	1.2	0.5			2.1	3.7	25.5	41.9			14:1, 1.2; 20:2, 1.0
6		*Aceraceae*	Maple	0.7	1.0	19.8	1.6		0.5			5.3	10.1	59.5			20:2, 3.9
7		*Ulmaceae*	Elm	0.5		15.5	1.8		0.6			3.4	11.9	64.3			18:4, +
8a			plant	1.6	0.8	15.7	0.4			2.3	2.2	25.4	12.5	16.9		12.0	15:1, 0.2; 17:1, 0.9
		Carnegia gigantea[a]	Saguaro cactus														
8b			seed	+		17.2	+				2.5	36.5	43.2		0.4	+	
9		*Lactua saliva*	Head lettuce, seed	0.1		9.1	0.5				3.3	25.6	56.3		5.0		20:1, +
10		*Iridaceae*	Iris	4.0		17.6	1.6		2.2			2.8	15.3	52.4			
11		*Labiatae*	Coleus	0.6		19.2	3.8	0.4	2.4			1.6	12.3	59.8			
12		*Arisaema triphyllum*	Jack-in-the-pulpit	0.5	0.2	17.8	0.4	1.1			1.5	3.3	12.6	58.6			18:4, 4.0
13	23. Anthophyta	*Impatiens biflora*	Touch-me-not	2.7	0.1	22.5	5.5				1.9	8.4	8.1	48.2		2.8	
14		*Typha latifolia*	Common cat-tail	0.2		16.3	4.2		1.8	0.1		0.6	17.8	56.3		2.5	15:1, 0.2
15		*Sagittaria*	Arrowhead plant	0.5		17.5	2.1		5.9			1.6	8.1	48.0			?, 16.0
16		*Nuphar*	Cow-lily	0.3	0.1	18.7	3.7	1.0	2.6			3.9	16.2	53.6			
17		*Lemna*	Duckweed	1.4	0.9	21.7	3.3	0.2			1.4	2.8	17.9	45.8		1.5	20:1, +
18		*Potamogeton*	Pondweed	1.2	0.1	21.6	6.3	4.4	2.4	0.3		3.8	11.8	44.8			14:1, 0.5; 18:4, 2.9
19		*Scirpus*	Bulrush	0.7	0.1	20.7	4.8	0.2		1.6	0.9	2.1	15.0	50.8		2.8	15:1, 0.2
20		*Myriophyllum*	Water-milfoil	2.2	0.4	17.3	17.6			0.4	0.9	3.0	23.4	36.6			
21		*Anacharis occidentalis*	Water-weed	0.7	0.1	20.0	5.6				3.0	4.4	17.4	48.9			14:1, +; 18:4, 0.2

[a] Collected in Arizona.

The number of species among Thallophyta, Bryophyta, and Pteridophyta (divisions 1–19 according to Bold (9,10)) is estimated to be 95,000. The fatty acids of perhaps 25 of these species have been investigated by up-to-date methods and they are the basis for taxonomic and phylogenetic hypotheses in current publications. The data here add a few more examples but broaden the foundation for such speculations only slightly. Although the loss, or repression, of the animal-type conversion of polyenoic fatty acids in higher plants is obvious, it is still quite uncertain where a line of characteristic difference should be drawn. Δ^5 and arachidonic acids appear sporadically in lower and higher divisions, presently without any recognizable pattern.

Double bonds in positions 5 and 11 are characteristic for the unusual acids of ginkgo and horsetail but there is no reason to assume that the spacing of 4 methylene groups originates from a methylene-interrupted system by selective hydrogenation. A $\Delta^{5,9}$ acid was indicated as a contaminant of ginkgo acids by some ozonizations but is has not yet been completely identified. Δ^5 monoenoic acids occur in other materials and polyenoic acids with $\Delta^{5,9}$, $\Delta^{5,11}$ and $\Delta^{5,13}$ have been found (see Table V). The remote double bond in position 5 and other double bonds are formed independently. The introduction of the remote Δ^5 seems to be steered by the carboxyl group.

The following observation deserves further attention. An acid derived from Δ^5 by chain elongation, i.e., an acid with a remote double bond in Δ^7, has neither been found in this laboratory nor has it been reported by other authors. Similarly, C_{22} acids derived from 5,8,11,14–20:4 or 5,8,11,17–20:5 acids have not been found in mosses or ferns although these potential precursors are present. Korn mentions the same point with *Acanthamoeba* which "convert 9,12–18:2 to 5,8,11,14–20:4, but there is no evidence of further elongation and desaturation" (33). Elongation of the chain in these plants and organisms may be blocked once a double bond appears in position 5 regardless of whether the other double bonds line up with it in conventional structure or are separate from it. This is in contrast to vertebrates where the conversion of arachidonic or 5,8,11,14,17–20:5 acid into more highly unsaturated C_{22} acids is well known. A search for 22:4 and 22:5 and identification of 22:3 in plants (see Tables III and IV) may fortify the point of distinction.

It is characteristic for animal lipids that 6,9,12–18:3 and 8,11,14–20:3 are found only in very small amts although they are intermediates in the conversion of linoleic into arachidonic acid. Similarly in plants, the amt of these acids is much smaller than that of arachidonic acid (Table III).

ACKNOWLEDGMENTS

Support for USPHS, NIH (Grant AM 05165) and The Hormel Foundation. Botanical samples, identifications and discussions, T. H. Haines, H. Hayes, H. W. Kircher, R. McLeester, W. Olson, C. Van der Schans, L. H. Weinstein, and C. J. Weiser; spectroscopy by G. Mizuno and J. R. Chipault; laboratory assistance from J. A. Finbraaten.

REFERENCES

1. Ackman, R. G., Nature *194*, 970–971 (1962).
2. Ackman, R. G., JAOCS *40*, 558–564; 564–567 (1963).
3. Ackman, R. G., and R. D. Burgher, J. Chromatog. *11*, 185–194 (1962).
4. Ackman, R. G., R. D. Burgher and F. M. Jangaard, Can. J. Biochem. Physiol. *41*, 1627–1641 (1963).
5. Ackman, R. G., JAOCS *40*, 744–747 (1963).
6. Arpino, A., Riv. Ital. Sostanze Grasse *38*, 275–276 (1961); C. A. *56*, 1544b (1962).
7. Bagby, M. O., C. R. Smith, Jr., K. L. Mikolajczak and I. A. Wolff, Biochemistry *1*, 632–639 (1962).
8. Bagby, M. O., C. R. Smith, Jr., R. K. Miwa, R. L. Lohmar and I. A. Wolff, J. Org. Chem. *26*, 1261–1265 (1961).
9. Bold, H. C., "Morphology of Plants," Harper & Row, New York, 1957, pp. 615–620.

10. Bold, H. C., "The Plant Kingdom," 2nd ed., Prentice Hall, Inc., Englewood Cliffs, N.J., 1964.
Inc., Englewood Cliffs, N.J., 1964.
11. Bonner, W. A., J. Chem. Educ. 30, 452–453 (1953).
12. Colakoglu, M., Ankara Univ., Ziraat Fak., Yayinlari No. *116*, 1–71 (1957); C. A., *54*, 8111h (1960).
13. Davidoff, F., and E. D. Korn, Biochem. Biophys. Res. Commun. *9*, 54–58 (1962).
14. Davidoff, F., and E. D. Korn, J. Biol. Chem. *238*, 3199–3209 (1963).
15. Davidoff, F., and E. D. Korn, *Ibid.* 3210–3215 (1963).
16. Deuel, H. J., Jr., "The Lipids," Vol. III, Interscience Publishers, New York, 1957, p. 328.
17. Earle, F. R., C. A. Glass, G. C. Geisinger and I. A. Wolff, JAOCS *37*, 440–447 (1960).
18. Erwin, J., and K. Bloch, Biochem. Z. *338*, 496–511 (1963).
19. Farmer, E. H., Trans. Faraday Soc. *38*, 358 (1942).
20. Fulco, A. J., R. Levy and K. Bloch, J. Biol. Chem. *239*, 998–1003 (1964).
21. Gellerman, J. L., and H. Schlenk, Experientia *19*, 522–523 (1963).
22. Gellerman, J. L., and H. Schlenk, Experientia, *20*, 426-427 (1964).
23. Gellerman, J. L., and H. Schlenk, J. Protozool., in press.
24. Haines, T. H., S. Aaronson, J. L. Gellerman and H. Schlenk, Nature *194*, 1282–1283 (1962).
25. Hilditch, T. P., "The Chemical Constitution of Natural Fats," 3rd ed., Chapman & Hall, London, 1956, p. 146 and subsequent.
26. Holman, R. T., and G. O. Burr, Arch. Biochem. *7*, 47–54 (1945).
27. Holman, R. T., and H. Hayes, Anal. Chem. *30*, 1422–1425 (1958).
28. Human Nutrition Research Division, Agricultural Research Service, U.S.D.A., May 1959, "Fatty Acids in Animal and Plant Products," especially Table III (leading reference to literature up to 1955).
29. de Jong, K., and H. van der Wel, Nature *202*, 553–555 (1964).
30. Kato, A., Yukagaku *10*, 174–177 (1961); C. A. *55*, 26481h (1961).
31. Klenk, E., and W. Bongard, Hoppe-Seyler's Z. Physiol. Chem. *290*, 181–198 (1952); E. Klenk and G. Kremer, *Ibid.* *320*, 111–125 (1960).
32. Klenk, E., W. Knippreth, D. Eberhagen and H. P. Koof, Hoppe-Seylers Z. Physiol. Chem. *334*, 44–59 (1963).
33. Korn, E. D., J. Lipid Res. *5*, 352–362 (1964).
34. Koyama, Y., and Y. Toyama, J. Chem. Soc. Japan, Pure Chem. Sect. *78*, 1223–1224 (1957); abstract, JAOCS *36*, 265 (1959); C. A. *53*, 20844g (1959).
35. Lindlar, H., Helv. Chim. Acta *35*, 446–450 (1952).
36. MacGee, J., Anal. Chem. *31*, 298–302 (1959).
37. Mattson, F. H., in "Chemistry of Lipids as Related to Atherolsclerosis," (Symposium, 1957) ed. I. H. Page, Charles C. Thomas, Publishers, Springfield, Ill., 1958, pp. 39–40.
38. Miwa, T. K., K. L. Mikolajczak, E. R. Fontaine and I. A. Wolff, Anal. Chem. *32*, 1739–1742 (1960).
39. Montag, W., E. Klenk, H. Hayes and R. T. Holman, J. Biol. Chem. *227*, 53–60 (1957).
40. Pearl, J. A., and P. F. McCoy, J. Org. Chem *26*, 550–552 (1961).
41. Pitt, G. A. Y., and R. A. Morton, "Progress in the Chemistry of Fats and Other Lipid," Vol. 4, p. 227–278, specifically p. 244 and 245 (1957).
42. Privett, O. S., and C. Nickell, JAOCS *39*, 414–419 (1962).
43. Privett, O. S., C. Nickell and W. O. Lundberg, *Ibid.* *32*, 505–511 (1955).
44. Rosenberg, A., Biochemistry *2*, 1148–1154 (1963).
45. Rosenberg, A., and M. Pecker, *Ibid.* *3*, 254–258 (1964).
46. Roth, H., in "Methoden der Organischen Chemie," ed. E. Müller, Thieme Vlg. Stuttgart, vol. II, p. 292–294 (1953).
47. Sand, D. M., and H. Schlenk, JAOCS, this Symposium.
48. Schlenk, H., in "Fatty Acids," vol. III, ed. K. S. Markley, Interscience, New York, 1964, p. 2175 and subsequent.
49. Schlenk, H., and co-workers, unpublished data.
50. Schlenk, H., and J. L. Gellerman, Anal. Chem. *32*, 1412–1414 (1960).
51. Schlenk, H., and J. L. Gellerman, JAOCS *38*, 555–562 (1961).
52. Schlenk, H., J. L. Gellerman and D. M. Sand, Anal. Chem. *34*, 1529–1532 (1962).
53. Schlenk, H., and D. M. Sand, Anal. Chem. *34*, 1676 (1962).
54. Sen, N., and H. Schlenk, JAOCS *41*, 241–247 (1964).
55. Shorland, F. B., in "Comparative Biochemistry," Vol. III, eds. M. Florkin and H. S. Mason, Academic Press, New York, 1962, p. 1 and subsequent.
56. Smith, C. R., Jr., M. O. Bagby, T. K. Miwa, R. L. Lohmar and I. A. Wolff, J. Org. Chem. *25*, 1770–1774 (1960).
57. Smith, C. R., Jr., J. W. Hagemann and I. A. Wolff, JAOCS *41*, 290–291 (1964).
58. Sontag, N. O. V., in "Fatty Acids," ed. K. S. Markely, 2nd edition, part 2, pp. 1040–1043, Interscience Publishers, New York, 1961.
59. Stein, R. A., JAOCS *38*, 636–640 (1961).
60. Stine, C. A., and J. B. Doughty, Forest Products J., *11*, 530–535 (1961); C. A. *56*, 3705c (1962).
61. Takagi, T., JAOCS *41*, 516–519 (1964).
62. Wagner, H., and H. König, Biochem. Z. 339, 212–218 (1963).
63. Woodford, F. P., and C. M. van Gent, J. Lipid Res. *1*, 188–190 (1960).

Positional Isomerism of Unsaturated Fatty Acids in the Rat Quantification of Isomeric Mixtures

D. SAND, N. SEN and H. SCHLENK, The Hormel Institute, Austin, Minnesota

Abstract

Mono- and dienoic acids of lipids from rat milk, rat sucklings, and rats on a fat-deficient diet were investigated. The percentage of uncommon isomers of palmitoleic acid was highest in milk, the newborn and the suckling rats, but receded after weaning. Isomers of linoleic acid were found only in traces in sucklings but became pronounced in rats on diets lacking essential fatty acids. The proportion of 8,11-diene among octadecadienoic acids increased markedly under such conditions within one week and two additional isomers became prominent after longer periods of fat-deficient diet.

A supplement of hydrogenated coconut fat did not influence the occurrence of these isomers.

Dietary petroselinic acid is incorporated by the rat into tissue lipids. 4-Hexadecenoic and a small amt of 8-eicosenoic acid arise from it.

Quantification of isomeric mixtures by ozonization-hydrogenation and subsequent gas-liquid chromatography is discussed in detail.

Introduction

IT IS KNOWN THAT LIPIDS of rats reared on a fat-deficient diet contain unsaturated acids which are isomeric with those in rats on a normal diet (9,18,22,24). In particular, the fatty acids of rats in an advanced state of essential fatty acid (EFA) deficiency have been investigated and 5,8,11–20:3 was found prominent among polyunsaturated acids. Quantifications of this acid relative to arachidonic has served as a parameter for defining the degree of EFA deficiency (16). In most of these studies the acids from liver or liver phosphatides were analyzed since there the level of polyunsaturated acids is relatively high regardless of the nutritional state of the animal (5).

The present report concerns mainly the isomers of monoenoic and dienoic acids found in the total lipids of the rat and the progressive change which these acids undergo during early life and in the course of dietary regimens. In an exploratory experiment, various diets were fed for 3 weeks to rats 25 days old. Isomers of palmitoleic acid present at the beginning of this period had diminished, regardless of the dietary treatment. Within the same time, the amt of isomers of linoleic acid had become significant in rats on a fat-free diet. On the basis of these results a more elaborate experiment was designed.

The dietary period was extended to 6 months and fatty acid analyses were made at time intervals. Analyses were extended to the feed and milk of dams and the progeny during the first 3 weeks after birth. A fat-free diet and the same diet supplemented with either hydrogenated fat or corn oil were used. The data of the preliminary experiment were closely verified by the results of the comprehensive experiment; therefore, only the latter are reported in detail.

Positional isomers of monoenoic esters are not separated well by common gas-liquid chromatography (GLC) procedures and this difficulty is also often en-