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EXAMINATION OF THE ESTERIFIED FATTY ACIDS FROM MOUSE ERYTHROCYTE AND SYNAPTOSOMAL MEMBRANE PHOSPHOLIPIDS AND THEIR DISTRIBUTION BETWEEN THE VARIOUS PHOSPHOLIPID TYPES

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SUMMARY

Esterified fatty acids from mouse erythrocyte and synaptosomal membranes were characterised by fused-silica capillary gas–liquid chromatography and gas chromatography–mass spectrometry. Structural information was obtained from the mass spectra of a number of derivatives including trimethylsilyl (TMS), methyl and picolinyl esters together with the TMS ethers of glycols derived from the unsaturated acids. In addition to previously characterised acids, small concentrations of several acids previously unreported from these membranes were identified. These included branched chain acids and several unsaturated acids.

INTRODUCTION

Recent studies on the effects of ethanol on the composition of cell membranes have revealed changes in the relative concentrations of the major constituent fatty acids of the phospholipids[1–6] and in the cholesterol content[7–9] in response to repeated administration of the drug. In several studies, the changes were thought to contribute to the development of tolerance by making the membrane less sensitive to the fluidizing effects of ethanol. To date, studies on the phospholipids have concentrated on changes in the relative concentrations of the major esterified fatty acids in the bulk phospholipid fraction. However, for a full understanding of the biochemical mechanisms leading to the observed differences in composition it is important to measure changes occurring in the concentration of the minor constituents as these often represent intermediates in the biochemical pathways leading to the major acids. Studies of the effects of ethanol on membranes so far carried out in our laboratory[4,6] have included mouse erythrocyte and synaptosomal membranes. It has been shown that, in erythrocytes, changes in the relative proportions of the major saturated and unsaturated fatty acids in the bulk phospholipid fraction are such that, irrespective

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of any variations shown by different groups of control animals, the ratios of the various esterified acids tend towards constant values following treatment by the drug. In an extension of this work to the minor fatty acids using capillary gas–liquid chromatography (GLC), it has been found that several of the observed compounds appeared not to have been reported as constituents of these membranes. This paper, therefore, reports the results of a study on the composition of the esterified fatty acids in the mouse erythrocyte and synaptosomal membrane, chosen as examples of membranes with contrasting functions.

EXPERIMENTAL

Materials

Reference fatty acids were obtained from Sigma (London, U.K.). Solvents were obtained from BDH and were distilled twice.

Preparation of membranes

Male Charles River CD-1 mice (24–29 g) which had been maintained on Charles River diet No. 22RF were killed by cervical dislocation and bled from the neck. Blood from 8 mice was collected into heparinized tubes at 4°C and the erythrocyte membranes were isolated by the method described by Hanahan and Ekholm[10]. The brains were removed and synaptosomal membranes were isolated as described in our earlier paper[4]. The quality of the membranes was checked by electron microscopy and by assay of marker enzymes. The protein content was determined by the method of Lowry *et al.*[11].

Isolation of lipids

The membranes were suspended in methanol using two 40-s periods of sonication and the lipids were extracted into chloroform–methanol (2:1, v/v) using the procedure of Folch *et al.*[12]. The lipids were then separated by silicic acid column chromatography[13] into neutral (chloroform eluate), glycolipid (acetone eluate), and phospholipid (methanol eluate) fractions. Phospholipids were assayed by measurement of the phosphorus content by the method of Morrison[14] and half of the sample was hydrolysed by heating with dilute (1 *M*) methanolic sodium hydroxide solution for 1 h at 80°C. For quantitative measurements of the major fatty acids, the internal standard, eicosadienoic acid, was added to the phospholipid mixture prior to hydrolysis. Quantitation of the fatty acids as their methyl esters was performed by GLC as described previously[4]. For qualitative analysis, samples were converted into various derivatives as described below and examined by combined gas chromatography–mass spectrometry (GC–MS).

Phospholipid classes present in the other half of the phospholipid fraction were separated by thin-layer chromatography (TLC) and examined by GLC and GC–MS as described below.

TLC

Individual phospholipid types were separated by one-dimensional TLC using glass plates coated with a 0.5-mm layer of a 4:1 (w/w) mixture of silica gel HF_{254} (av. particle size 15 μm) and silica gel G (particle size 10–40 μm) (E. Merck, Darm-

stadt, F.R.G.). Plates were pre-washed with the developing solvent and activated at 100°C. Approximately 100 μg of the phospholipid mixture in 60 μl methanol–chloroform (2:1, v/v) were applied as a streak to the plates together with reference phospholipids and additional spots of the sample mixture for later location of the separated compounds. The plate was dried in a desiccator for 15 min before being developed in chloroform–methanol–acetic acid–water (25:15:4:2, v/v/v/v)[15]. The reference compounds were visualised with iodine vapour contained in a perspex restriction box in order to locate the areas occupied by the separated membrane phospholipids. Because of the similar R_F values for phosphatidylserine (PS) and phosphatidylinositol (PI) and the relatively small amounts of each present in the membrane sample, these phospholipids were combined. The regions of the plate occupied by PS + PI, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were scraped off and the phospholipids were eluted from the gel with the above solvent mixture by shaking twice at 40°C for 20 min. For quantitivative determinations of the fatty acids, the internal standard, eicosadienoic acid was added at this stage.

Preparation of derivatives

Methyl esters. These were prepared by heating the acids with methanol–hydrochloride acid (0.3 ml, prepared from anhydrous methanol and acetyl chloride, Applied Science Labs. State College, PA, U.S.A.) for 10 min at 60°C and then removing the reagents with a stream of nitrogen. To avoid loss of the more volatile components, care was taken to ensure that the nitrogen stream was turned off as soon as all the methanol had been removed. For examination by GLC, the methyl esters were redissolved in methyl acetate.

Trimethylsilyl (TMS) esters. TMS esters were prepared by heating the acids (equivalent to about 10% of the total fraction) with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 10 μl) containing 1% trimethylchlorosilane (TMCS) for 10 min at 60°C.

[$^2H_9$]TMS derivatives[16]. These were prepared as above using [$^2H_{18}$]bis(trimethylsilyl)acetamide in place of BSTFA.

Picolinyl esters[17,18]. The acids (from 10% of the sample) were converted into their chlorides by reaction with thionyl chloride for 1 min at room temperature and these were reacted with 3-pyridylcarbinol in acetonitrile (10% solution) as described previously[18]. BSTFA (10 μl) was added to derivatize unreacted 3-pyridylcarbinol before these derivatives were examined by GLC and GC–MS.

Glycol-TMS ethers. The unsaturated fatty acids in the mixture were converted into glycols by a modification of the method described by Capella and Zorzut[19]. Thus the fatty acid methyl esters in pyridine–dioxane (1:8, v/v) were treated with an excess of osmium tetroxide in dioxane and left at room temperature overnight. The products were extracted as described[19] and converted into either TMS or [$^2H_9$]TMS derivatives as described above.

Gas chromatography

Samples were examined at various times by gas chromatography using several systems: (a) a Varian 2440 gas chromatograph fitted with a flame ionization detector and a 2 m × 2 mm glass column packed with either 3% SE-30 on 100–120 mesh Gas Chrom Q (Applied Science) (methyl and TMS esters) or 10% SP-2330 on

100–120 mesh Chromosorb W AW (Supelco) (methyl esters). The injector and detector temperatures were 300°C and the column oven was temperature programmed from 150 to 300°C at 4°C min^{-1} (SE-30 column) or from 140 to 240°C at 4°C min^{-1} (SP-2330 column). The carrier gas was nitrogen at 30 ml min^{-1}. (b) In addition, the methyl esters, TMS esters and picolinyl esters were examined with a 50 m × 0.32 mm OV-1 (film thickness 0.52 μm) fused-silica capillary column (Hewlett-Packard) using on-column injection (SGE OCI-2 system). The carrier gas was helium at 2 ml min^{-1} and the detector temperature was 300°C. Samples were injected at room temperature, the temperature was raised to 150°C and then programmed at 2°C to 300°C. The output was recorded with a Hewlett Packard 3390A recording integrator. (c) The same column was used in a Hewlett-Packard 5890A gas chromatograph under similar conditions. Samples were injected via the split inlet using a split ratio of 10:1.

GC–MS

Packed column GC-MS data of the picolinyl esters were recorded with a VG Micromass 12B mass spectrometer interfaced to a VG 2050 data system and via a glass jet separator to a Varian 2440 gas chromatograph containing a 2 m × 2 mm glass column packed with 3% SE-30 on 100–120 mesh Gas Chrom Q (Applied Science). The column oven was temperature programmed from 150 to 300°C at 4°C min^{-1} and the transfer line, separator, and ion source temperatures were 300°C, 300°C and 260°C respectively. Other operating conditions were: trap current, 100 μA; electron energy, 25 eV; accelerating voltage, 2.4 kV and scan, 3 s $decade^{-1}$.

Capillary column GC–MS data for all derivatives were recorded with a VG Micromass 70/70F mass spectrometer interfaced to the VG 2050 data system and to a Varian 2440 gas chromatograph. The capillary column and injector were as described above. The outlet of the capillary column was taken to a short length (50 mm) of glass capillary tube which led to the ion source. The column oven was temperature programmed as described above and the transfer line was maintained at 300°C. Other operating conditions were: ion source temperature, 260°C; electron energy, 70 eV; trap current, 1 mA; accelerating voltage, 4 kV and the scan speed was 0.3 s $decade^{-1}$. Single-ion traces of m/z 74 for the methyl esters were obtained by tuning the magnet to this mass and recording the chromatogram from the main amplifier.

Fast atom bombartment mass spectrometry

Fast atom bombartment (FAB) spectra of the intact phosphatidylcholine fraction were recorded with a VG ZAB-1F mass spectrometer using argon atoms and a glycerol matrix.

RESULTS

Identification of acids

Fig. 1 shows a separation of the fatty acids from the total phospholipid fraction of mouse erythrocyte membranes as their methyl esters on a 50-m fused-silica capillary column and Table I lists the identified compounds. Peaks not listed in the table were not produced by fatty acids. Acids were identified where possible (peaks 1, 7, 10, 11, 14–17, 19, 24, 26 and 34) by comparison of the retention times and mass

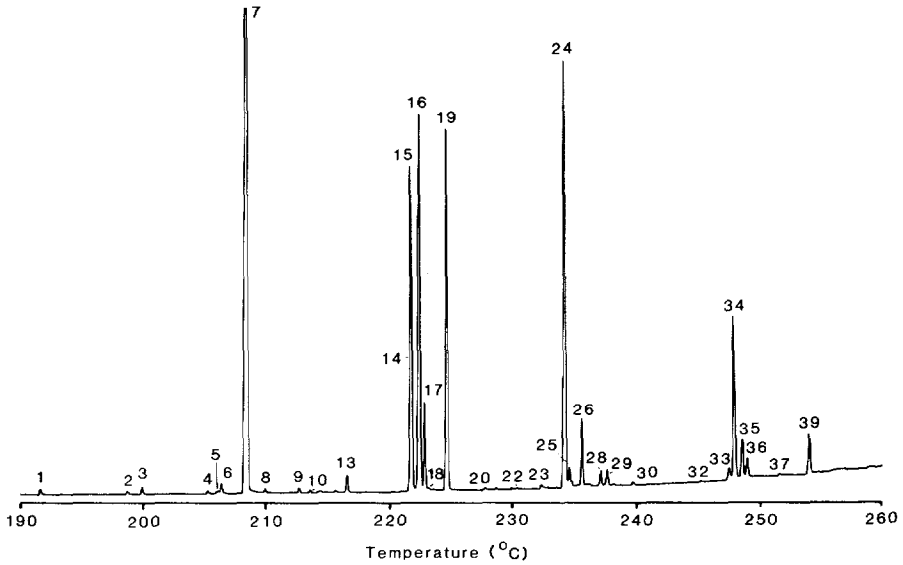

Fig. 1. Separation of fatty acid methyl esters derived from mouse erythrocyte membrane phospholipids on a 50 m × 0.32 mm fused-silica capillary column (film thickness 0.52 μm) using the Varian 2440 gas chromatograph. Other conditions are given in the Experimental section.

spectra with those of authentic standards. Structures of the other acids were determined from the mass spectra of the TMS and picolinyl derivatives[17]. Monounsaturated fatty acids were additionally identified by the mass spectra of the TMS ethers of the glycols formed by their reaction with osmium tetroxide.

For the structural determination of the poly-unsaturated fatty acids, the glycol-TMS derivatives were unsuitable on account of their high molecular weight and failure to elute from the column. The picolinyl esters, on the other hand, were very satisfactory; their mass spectra contained prominent molecular ions and fragment ions diagnostic of the double bond position[18]. The acids producing peaks 24 and 34 were identified as arachidonic (20:4) and docosahexaenoic acid (22:6) respectively by use of these derivatives and by comparison of GC–MS data with those of authentic standards. These are well characterised constituents of mammalian cell membranes. The acid producing peak 26 was identified as 20:3, delta-8,11,14, the biochemical precursor of arachidonic acid, by the mass spectrum of its picolinyl ester and by comparison of its GC–MS properties with those of an authentic standard. The acid producing peak 28 was identified as 20:2, delta-11,14 by the spectrum of its picolinyl ester and by comparison with a reference sample. Its biosynthesis was probably by chain elongation of linoleic acid.

Three poly-unsaturated C_{22} fatty acids were identified in addition to 22:6, delta-4,7,10,13,16,19. Peak 35 (Fig. 1) was produced by 22:4, delta-7,10,13,16 as shown by abundant ions at m/z 423 ($M^{\ddagger}$), 352, 312 and 272 in the spectrum of its picolinyl ester[18]. Corresponding fragment ions in the spectrum of the derivative of 22:6 were 2 mass units lower, reflecting the presence of the additional delta-4 double bond. The absence of this double bond in 22:4 was additionally indicated by the presence of the ion at m/z 164 (ref. 18). This compound appeared to have been formed

TABLE I

FATTY ACIDS FROM MOUSE ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS

Peak (Fig. 1)	*Acid*	*Composition (%)*			
		Total	*PC*	*PE*	*PS/PI*
1	14:0	< 0.01	0.26	< 0.01	< 0.01
3	15:0	< 0.01	0.26	0.32	0.17
5	16:1-delta-7	< 0.01	< 0.01	< 0.01	< 0.01
6	16:1-delta-9	0.88	0.75	0.54	0.44
7	16:0	37.10	48.71	18.30	17.36
10	*i*-17:0	< 0.01	< 0.01	< 0.01	< 0.01
11	*anteiso*-17:0	< 0.01	*	*	*
13	17:0	0.51	0.54	0.51	1.27
14	18:3 (linolenic) (minor)	6.14	6.83	4.06	3.07
15	18:2 (linoleic)				
16	18:1-delta-9	19.13	13.29	33.26	23.77
17	18:1-delta-11	3.23	4.73	3.36	3.73
19	18:0	17.47	15.86	14.13	30.98
23	19:0	< 0.01	< 0.01	< 0.01	< 0.01
24	20:4	5.89	2.06	8.17	11.43
26	20:3	0.87	0.79	0.88	0.60
28	20:2	0.28	*	*	*
29	20:1-delta-11	2.36	0.49	4.37	2.93
29a	20:1-delta-13	< 0.01	< 0.01	< 0.01	< 0.01
30	20:0	0.48	*	0.53	0.16
	21:1-delta-13	0.09	*	*	0.59
34	22:6	3.18	0.61	5.05	2.35
35	22:4	1.43	< 0.01	3.27	0.85
36	22:5	< 0.01	< 0.01	< 0.01	< 0.01
37	22:1-delta-13	< 0.01	*	*	*
38	22:1-delta-15	< 0.01	*	*	*
39	22:0	**	0.36	0.66	2.02

* Compound not detected.

** Not measured because of the presence of a contaminant.

by chain elongation of arachidonic acid. The compound producing peak 36 was a 22:5 acid (M^+ of its picolinyl ester was at *m/z* 421) and in the absence of a clean spectrum (the spectrum contained ions from 22:4 which eluted in the previous scan) was tentatively assigned the structure 22:5, delta-4,7,10,13,16. This was based on the relatively high abundance of the fragment ions at *m/z* 244, 260, 310 and 360 which were also found in the spectrum of the picolinyl derivative of 22:6 and the absence of *m/z* 390 which would have been produced by cleavage adjacent to the delta-19 double bond. The remaining C_{22} acid was 22:3, delta-10,13,16 identified by major ions at *m/z* 425 ($M^{\ddagger}$), 314 and 274 in the spectrum of its picolinyl derivative. Chain elongation of 20:3, delta-8,11,14 would appear to be the obvious biosynthetic pathway to this compound.

TABLE II

FATTY ACIDS FROM MOUSE SYNAPTOSOMAL MEMBRANE PHOSPHOLIPIDS

Acid	*Composition (%)*			
	Total	*PC*	*PE*	*PS/PI*
14:0	0.02	*	*	*
15:0	*	0.01	0.39	0.09
16:1-delta-7	< 0.01	< 0.01	< 0.01	< 0.01
16:1-delta-9	0.57	0.82	0.36	2.21
16:0	27.00	42.38	9.36	14.64
i-17:0	< 0.01	< 0.01	< 0.01	< 0.01
anteiso-17:0	< 0.01	*	*	*
17:0	0.11	0.06	0.46	0.38
18:2 (linoleic)	0.42	*	*	0.27
18:1-delta-9	15.89	23.49	10.07	13.49
18:1-delta-11	5.14	6.99	2.45	1.43
18:0	21.65	17.75	36.69	43.37
19:0	< 0.01	< 0.01	< 0.01	< 0.01
20:4	10.75	4.56	13.26	14.25
20:3	0.37	0.13	0.41	*
20:1-delta-11	0.57	0.86	0.62	1.20
20:1-delta-13	0.09	0.13	< 0.01	< 0.01
20:0	*	*	0.81	0.44
21:1-delta-13	0.02	*	*	*
22:6	17.30	2.90	22.13	8.21
22:4	*	*	2.73	*
22:1-delta-13	< 0.01	0.03	0.24	0.14
22:1-delta-15	< 0.01	*	*	*
22:0	0.12	*	0.15	1.46

* Compound not detected.

Quantitative studies

Erythrocyte membranes contained 0.327 mg of phospholipid per 1 mg of membrane protein.

The relative concentrations of the fatty acids in the total erythrocyte membrane were determined by capillary column GLC and are listed in Table I. Those from the

TABLE III

ANALYSIS OF THE MAJOR DIACYL SPECIES OF MOUSE ERYTHROCYTE PHOSPHATIDYLCHOLINE BY FAB MASS SPECTROMETRY

Mass	*% of total*	*Carbon number*	*Double bond number*	*Most probable acids*
758	20.4	34	2	16:0 18:2
760	40.7	34	1	16:0 18:1
772	4.5	35	2	17:0 18:2
782	7.7	36	4	16:0 20:4
786	10.4	36	2	18:0 18:2
788	11.8	36	1	18:0 18:1
810	4.5	38	4	18:0 20:4

synaptosomal membranes are listed in Table II. Some small variation in composition was seen between samples from different batches of mice as can be seen by comparing the results shown in Fig. 1 with those presented in the total lipid column of Table I which were recorded from a different set of animals. Phospholipid types were then separated by TLC and the fatty acid profiles were again determined by GLC. Results are also listed in Tables I and II. The phosphatidylcholine fraction from the mouse erythrocyte membranes was further examined by FAB mass spectrometry in glycerol to identify the individual molecular species. Strong $[M + H]^+$ ions were formed which, in combination with the data in Table I, allowed the individual major compounds to be identified. Results are listed in Table III. Because of the relatively high noise level and the presence of both $[M + H]^+$ and $[M + Na]^+$ ions, it was difficult to place significance on peaks below about 5% even after several scans. These minor peaks are thus omitted from Table III.

DISCUSSION

All of the major peaks identified in the mouse erythrocyte membranes are well documented constituents and require no further comment. However, some of the minor constituents do not appear to have been fully characterised in these membranes. These include the two branched-chain acids (peaks 10 and 11). Although of low relative abundance, these compounds appear to be true membrane constituents and may contribute to the 17:0, 18:2 species found in the membranes by FAB analysis. They are not thought to have been introduced by contamination from the most likely sources of branched-chain acids, namely bacteria or skin-surface lipids because of the conditions under which the samples had been processed and because the major acid from human skin-surface lipids, 16:1, delta-6 (ref. 20) was not detected. Branched-chain acids of this type, although major constituents of meibomian[21–23] and sebaceous gland[20] secretions, are not common constituents of mammalian tissues[24]. They have, however, been reported to occur in adipose tissue[25,26], other lipid extracts[27] and, recently in human erythrocyte membranes[28].

The presence of substantial quantities of *cis*-vaccenic acid (*cis*-18:1, delta-11) in mammalian membrane phospholipids does not appear to have been well recognised although studies with bulk lipid extracts from several mammalian tissues have revealed its presence. Thus Baufeld and Luther[29] in 1961 reported its presence in human erythrocytes and Holloway and Wakil[30] in 1964 reported that it was a normal constituent of the fatty cells of the rat where up to 50% of the 18:1 fatty acid fraction was found to be *cis*-vaccenic acid. Elovson reported its presence in the phospholipids of rat liver in the following year[31] and in 1971 Lecerf and Bezard[32] demonstrated its formation from palmitoleic (*cis*-16:1, delta-9) and incorporation into phospholipids of rat lung. The occurrence of this acid and eighteen other isomeric monoenoic acids in rat liver lipids was demonstrated by Schmitz *et al.*[33] in 1977 by GC–MS using glycol-TMS derivatives.

These functionally different membranes from the mouse thus appear to have qualitatively similar compositions. The compositions of the erythrocyte membranes appeared to be very similar to those of human erythrocyte membranes recently reported by Alexander *et al.*[28]. Although the membranes studied by these authors appeared to contain more of the minor acids, including branched chain acids, this,

to some extent, may have reflected these authors' use of a more polar column. The polyunsaturated acids had the same distribution of double bonds in both species.

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REFERENCES

1 J. M. Littleton and G. John, *J. Pharm. Sci.*, 29 (1977) 579.
2 G. Y. Sun and A. Y. Sun, *Res. Commun. Chem. Pathol. Pharmacol.*, 24 (1979) 405.
3 A. J. Waring, H. Rottenberg, T. Ohnishi and E. Rubin, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 2582.
4 D. R. Wing, D. J. Harvey, J. Hughes, P. G. Dunbar, K. A. McPherson and W. D. M. Paton, *Biochem. Pharmacol.*, 31 (1982) 3431.
5 C. Alling, S. Liljequist and J. Engel, *Med. Biol.*, 60 (1982) 149.
6 D. R. Wing, D. J. Harvey, S. Belcher and W. D. M. Paton, *Biochem. Pharmacol.*, 33 (1984) 1625.
7 J. H. Chin, L. M. Parsons and D. B. Goldstein, *Biochim. Biophys. Acta*, 513 (1978) 358.
8 T. L. Smith and M. J. Gerhart, *Life Sci.*, 31 (1982) 1419.
9 L. M. Parsons, E. J. Gallaher and D. B. Goldstein, *J. Pharmacol. Exp. Ther.*, 223 (1982) 472.
10 D. J. Hanahan and J. E. Ekholm, *Methods Enzymol.*, 31 (1974) 168.
11 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
12 J. Folch, M. Lees and G. H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
13 G. Rousner, G. Kritchevsky and A. Yamamoto, in G. V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Vol. 3, Marcel Dekker, New York, 1976, p. 713.
14 W. R. Morrison, *Anal. Biochem.*, 7 (1964) 218.
15 V. P. Skipski, R. F. Peterson and M. Barclay, *Biochem. J.*, 90 (1964) 374.
16 J. A. McCloskey, R. N. Stillwell and A. M. Lawson, *Anal. Chem.*, 40 (1968) 233.
17 D. J. Harvey, *Biomed. Mass Spectrom.*, 9 (1982) 33.
18 D. J. Harvey, *Biomed. Mass Spectrom.*, 11 (1984) 340.
19 P. Capella and C. M. Zorzut, *Anal. Chem.*, 40 (1968) 1458.
20 N. Nicolaides, *Science (Washington, D.C.)*, 186 (1974) 19.
21 C. Baron and H. A. Blough, *J. Lipid Res.*, 17 (1976) 373.
22 J. M. Tiffany, *Exp. Eye Res.*, 29 (1979) 195.
23 N. Nicolaides, J. K. Kaitaranta, T. H. Rawdah, J. I. Macy, F. M. Boswell, III, and R. E. Smith, *Invest. Ophthalmol. Vis. Sci.*, 20 (1981) 522.
24 N. Polger, in F. D. Gunstone (Editor), *Topics in Lipid Chemistry*, Vol. 2, Wiley, New York, (1971) 207.
25 R. G. Ackman, S. N. Hooper and R. P. Hansen, *Lipids*, 7 (1972) 683.
26 A. Smith, A. G. Calder, A. K. Lough and W. R. H. Duncan, *Lipids*, 14 (1979) 953.
27 A. Smith, A. G. Calder, E. R. Morrison and G. A. Garton, *Biomed. Mass Spectrom.*, 6 (1979) 345.
28 L. R. Alexander, J. B. Justice, Jr. and J. Madden, *J. Chromatogr.*, 342 (1985) 1.
29 H. Baufeld and P. Luther, *Klin. Wochschr.*, 39 (1961) 444.
30 P. W. Holloway and S. J. Wakil, *J. Biol. Chem.*, 239 (1964) 2489.
31 J. Elovson, *Biochim. Biophys. Acta*, 106 (1965) 291.
32 J. Lecerf and J. Bezard, *Arch. Sci. Physiol.*, 25 (1971) 431.
33 B. Schmitz, U. Murawski, M. Pflueger and H. Egge, *Lipids*, 12 (1977) 307.