labelled moiety of the triacylglycerol molecule as well as for a determination of its molecular association with any other labelled or unlabelled moieties of the total molecule. The latter requirement places further demands upon the analytical system, and the resulting problems are discussed in the concluding part of this series (34).

ACKNOWLEDGMENT

The studies by the authors and their collaborators were supported by funds from the Medical Research Council of Canada, Ottawa, Ontario; the Ontario Heart Foundation, Toronto, Ontario, and the Hospital for Sick Children Foundation, Toronto, Ontario.

REFERENCES

1. Kuksis, A., J.J. Myher and L. Marai. JAOCS 61:1582 (1984).
2. Brockerhoff, H.J. Lipid Res. 1:10 (1965).
3. Christie, W.W. Lipid Analysis, Pergamon Press, Oxford, Great Britain. (1973).
4. Myher, J.J., and A. Kuksis. Can. J. Biochem. 57:117 (1979).
5. Kuksis, A., in Lipid Chromatographic Analysis (Marinetti, G.V., ed.), Marcel Dekker, New York, NY. (1967) pp. 239-337.
6. Myher, J.J., A. Kuksis, W.C. Breckenridge and J.A. Little. Lipids 19:683 (1984).
7. Myher, J.J., A. Kuksis and G. Steiner. Ibid. 19:673 (1984).
8. Myher, J.J., A. Kuksis, S.C. Vasdev and K.J. Kako. Can. J. Biochem. 57:1315 (1979).
9. Kuksis, A., L. Marai and D.A. Gornall. J. Lipid Res. 8:352 (1967).
10. Perkins, E.G., and P.V. Johnston. Lipids 4:301 (1969).
11. Myher, J.J., and A. Kuksis. Can. J. Biochem. 60:638 (1982).
12. Myher, J.J., and A. Kuksis. Can. J. Biochem. Cell Biol. 62:352 (1984).
13. Yurkowski, M., and H. Brockerhoff. Biochim. Biophys. Acta 125 :55 (1966).
14. Manganaro, F., J.J. Myher, A. Kuksis and D. Kritchevsky. Lipids 16:508 (1981).
15. Myher, J.J., A. Kuksis, W.C. Breckenridge, V. McGuire and J.A. Little. Ibid. 20:90 (1985).
16. Pind, S., A. Kuksis and J.J. Myher. (1983) (Unpublished results).
17. Bugaut, M., J.J. Myher, A. Kuksis and A.G.D. Hoffman. Biochim. Biophys. Acta 792:254 (1984).
18. Kuksis, A., L. Marai and J.J. Myher. J. Chromatogr. Biomed. Applic. 273:43 (1983).
19. Brockerhoff, H. Lipids 6:942-956 (1971).
20. Myher, J.J., L. Marai, A. Kuksis and D. Kritchevsky. Ibid. 12:775 (1977).
21. Morley, N.H., and A. Kuksis. J. Biol. Chem. 247:6389 (1972).
22. Paltauf, F., F. Esfandi and A. Holasek. FEBS Letters 40:119 (1974).
23. Snyder, F., in Ether Lipids (Snyder, F., ed.) Academic Press, New York, NY, (1972) pp. 273-295.
24. Warner, H.R., and W.E.M. Lands. JAOCS 85:60 (1963).
25. Polheim, D., J.S.K. David, M. Schultz, M.B. Wylie and J.M. Johnston. J. Lipid Res. 14:415 (1973).
26. Bugaut, M., A. Kuksis and J.J. Myher. (1984) Unpublished results.
27. Paltauf, F., and E. Wagner. Biochim. Biophys. Acta 431:359 (1976).
28. Litchfield, C. Analysis of Triglycerides, Academic Press, New York, NY (1972).
29. Kuksis, A. Progress Chem. Phys. Fats Other Lipids 12:1 (1972).
30. Holub, B.J., and A. Kuksis. Adv. Lipid Res. 16:1 (1978).
31. Mills, S.C., L.J. Cook, T.W. Scott and P.J. Nestel. Lipids 11:49 (1975).
32. Bezard, J.A., and M.A. Ouedraogo. J. Chromatogr. 196:279 (1980).
33. Manganaro, F., J.J. Myher and A. Kuksis. JAOCS 60:734, Abs. No. 225 (1983).
34. Kuksis, A., J.J. Myher and L. Marai. JAOCS 62:767 (1985).

[Received July 6, 1984]

♣Lipid Methodology – Chromatography and Beyond. Part III. Analyses of Natural and ^{2}H-Labeled Glycerolipids by GC/MS and LC/MS with Specific Enzymic Hydrolyses

A. KUKSIS, J.J. MYHER and **L. MARAI,** Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

In the concluding part of this series, the authors review recent attempts to adopt combinations of chromatography with other complementary analytical techniques to the study of stable isotope-labeled molecules as tracers of glycerolipid metabolism. It is shown that LC/MS in combination with specific enzymic hydrolyses has special advantages for this purpose. Using deuterium-labeled non-lipid precursors, effective labeling of both newly synthesized fatty acids and glycerol has been obtained and their molecular association and positional distribution (fatty acids) in the newly formed glycerolipid molecules has been determined as an indicator of the metabolic pathways involved. The above experimental routines extend the analytical lipid methodology beyond the capabilities of chromatography and radio-chromatography with or without complementary enzymatic analyses.

The studies reviewed in the present part and in the previous two parts of this series provide outlines for a potential practical assessment of the various metabolic pathways of glycerolipids, including the identification and quantitation of the true precursor and product pools involved in specific biosynthetic or degradative transformations. Such investigations have not been possible in the past by chromatographic or radio-chromatographic means. Some of the problems that remain may be subject to solution by means of GC/MS/MS and LC/MS/MS. It is hoped that the new and improved methodology will be matched in the future by comparable advances in the sampling of plasma and cellular components, in selected isotope labeling of the de novo products, and by improvements in the overall design of the metabolic experiments. Although the extension of lipid methodology beyond chromatography has greatly simplified the demands of the experimental design, it is obvious that improved experimental design and sampling techniques will result in further advances in the quality of the observations and in the understanding of lipid metabolism.

*To whom correspondence should be addressed.

INTRODUCTION

In Parts I (1) and II(2) of this series we reviewed the application of GC/MS and LC/MS to the resolution and quantitation of molecular species of both diacyl- and triacylglycerols. Furthermore, in Part II (2) we discussed how an identification and quantitation of positional isomers and enantiomers becomes possible when the above techniques are combined with specific enzymic hydrolysis. In Part III, which is the concluding article in this series, we wish to

point out the suitability of the above combination of analytical techniques for the separation, identification and quantitation of natural and deuterium-labeled glycerolipids, which thus far has remained beyond the capability of chromatographic techniques.

The measurement of deuterated and natural glycerolipid molecules has been used as a means of identification of newly synthesized and old molecular species in a metabolic system (3,4). Thus, when the biosynthesis of lipids is allowed to proceed in the presence of deuterium oxide (5,6) or deuterated ethanol (7,8), the glycerol and the fatty acids synthesized de novo become labeled with deuterium and are incorporated into the newly formed glycerolipids, which can be distinguished from the already existing species by their deuterium content. Since the newly synthesized fatty acids and glycerol can be combined with old or unlabeled glycerol and fatty acids in various proportions, a determination of the distribution of the deuterium-labeled moieties of the glycerolipid molecule provides information about the biochemical pathways involved in their formation. Furthermore, an imposition of a physiological or pharmacological stimulus upon the metabolic system, resulting in an alteration of the distribution pattern of the label, can provide information about the metabolic control of the transformations (6). In the past, qualitative and quantitative presence of newly synthesized molecular species of glycerolipids in a tissue usually has been demonstrated by means of incorporation of radioactive tracers into the various biosynthetic products (9). Since radioactive molecules are present in only trace amounts, specific radioactivity has been determined as a means of measuring the relative rates of turnover of different molecular species. While this technique is satisfactory for comparing the relative ratios of incorporation of certain singly labeled metabolites into the lipid molecules, it is not suitable for studies where more than one radio-isotopic atom can occur in the same molecule, and radio-isotopes cannot generally be used for identifying newly formed and old molecules. Finally, a determination of the intramolecular distribution of radio-activity usually is impossible without extensive chemical degradation. Metabolic studies with stable isotope-labeled markers eliminate many of these difficulties. In the following it is shown that chromatography and mass spectrometry in combination with specific enzymic analysis can provide practical routines for obtaining analytical information of an unprecedented detail about the metabolic transformations of glycerolipids in normal and stimulated cells, which ought to lead to better understanding of lipid metabolism and its role in the structure and function of cell membranes.

Theoretical Considerations

Glycerophospholipids constitute the major lipid components of all membranes and display a great diversity of structure and function, while the triacylglycerols are the most important storage forms of energy in animals and influence the chemical and physical properties of lipoproteins (10). The general pathways of glycerolipid biosynthesis and degradation are well established, and for several glycerolipid classes even the turnover times of individual molecular species have been determined (11,12). However, the role of the different glycerolipid classes and of the various fatty acid patterns of the glycerolipids in the membrane structure and function has not been established. Even in those instances where a clear-cut requirement for a specific glycerolipid class and/or molecular species has been shown (e.g. lung surfactant), the specific mechanism of action of the lipid is uncertain (12). The significance of the detailed pairing of the fatty acids in the glycerophospholipid molecules of cell membranes and its metabolic control has remained obscure. Furthermore, much of the in vitro work has been compromised by the detergent properties of the substrates and by the membrane association of the enzymes involved (13). As a result, there has been a need to place greater emphasis upon in vivo experiments, which has further complicated the analytical problem.

Figure 1 summarizes the major pathways for the biosynthesis of phosphatidic acid from glucose, glycerol and monoacylglycerols, while Figure 2 summarizes the formation of the glycerophospholipids and triacylglycerols from phospatidic acid and diacylglycerols. When the biosynthesis is performed in the presence of deuterium oxide (5,6,14), the glycerol derived from glucose can be distinguished from the old glycerol derived from diet or from degradation of preformed glycerolipids in the tissues. Likewise, unlabeled diacylglycerol could be contributed by the mono- and diacylglycerols arising from degradation of preformed glycerolipids in the tissues or from diet. Neither Figure 1 nor Figure 2 gives any indication of the source of the fatty acids. The saturated acids could arise from de novo synthesis, degradation of preformed tissue lipids, or from diet. Biosynthesis followed by desaturation and chain elongation also could have yielded the monoenoic and some oligoenoic acids. There is no linoleic acid formed in the body, but it can be subject to chain elongation and further desaturation to arachidonic acid. Any one or all of the acids could have come from the diet or have been released from preformed tissue lipids. Thus, for any class of glycerophospholipids, one or both fatty acids may be newly synthesized and combined with newly synthesized glycerol, and one or both newly synthesized acids may be combined with old glycerol; also, both old fatty acids may be combined with new or old glycerol (3,4). The combinations become much more complex when different types of newly synthesized fatty acids are considered. The acids arising from desaturation of newly synthesized acids and chain elongations of old acids can be combined with newly formed or with old glycerol as well as be paired with old or new fatty acids. Additional variations can arise from utilization of glycerol from different sources, some of which can be distinguished by appropriate stable isotope-labeling. Finally, the glycerolipid transformations are known to involve the alkyl and alkenyl ethers of glycerol, although at lower rates and on a

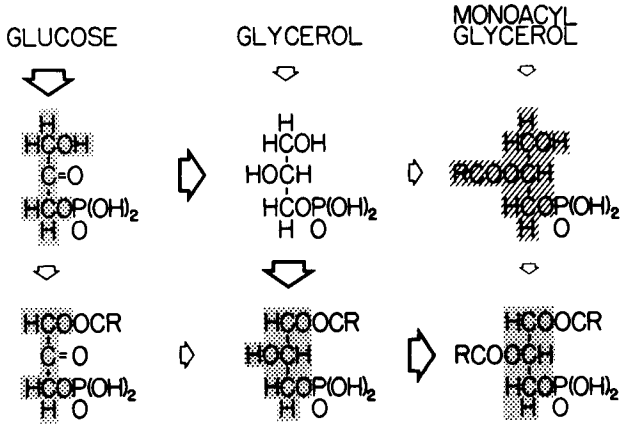

FIG. 1. Summary of theoretically possible pathways of phosphatidic acid biosynthesis from glucose, glycerol and 2-monoacylglycerol. The figure represents the conversion of glucose into dihydroxyacetone-phosphate, sn-glycerol-3-phosphate, lysophosphatdic acid and phosphatidic acid as a major pathway (indicated by heavy arrows). Lesser contributions to appropriate intermediates of this pathway are made through acylation of dihydroxyacetonephosphate and glycerolphosphate, and by phosphorylation of glycerol and 2-monoacylglycerol. Light shading indicates glycerol moieties derived from glucose, while heavy shading identifies the glycerol moieties derived from exogenous 2-monoacylglycerol.

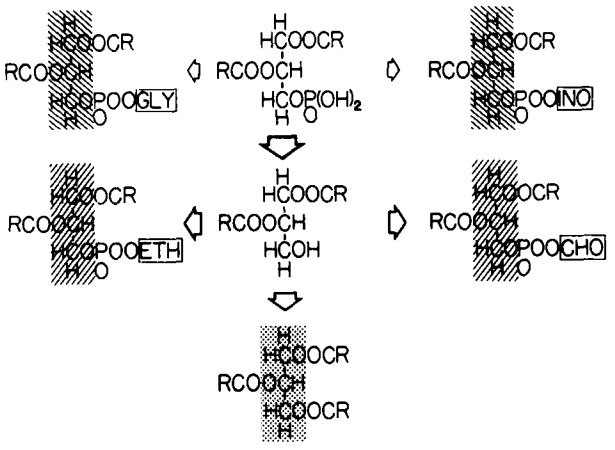

FIG. 2. Summary of theoretically possible pathways of formation of glycerophospholipids and triacylglycerols from phospatidic acid and diacylglycerols. The upper part of the figure depicts the formation of phosphatidylglycerol (GLY) and phosphatidylinositol (INO) from phosphatidic acid via the CDP-diacylglycerol pathway. The middle part shows the formation of phosphatidylethanolamine (ETH) and phosphatidylcholine (CHO) via the CDP-nitrogenous base pathway, which utilizes free diacylglycerols as the acceptors of the phosphorylated bases. The bottom part of the figure indicates the acylation of diacylglycerols to triacylglycerols.

much smaller mass scale. The theoretical complexity becomes bewildering when it is realized that the different molecular species are converted into the glycerophospholipids and triacylglycerols at different rates, and that the final products are subject to further acyl exchange (11,12). Nevertheless, work with deuterium labeled ethanol (7,15) and deuterium oxide (5,6,14) has shown that many of these products can be recognized and quantitated and their rates of formation determined by GC/MS. Even more promising appears to be the LC/MS (8) approach.

GC/MS of Deuterium-Labeled Glycerolipids

During the de novo synthesis of palmitic acid by isolated rat liver in 75% deuterium oxide, approximately 50% of the hydrogens in the fatty chain become replaced by deuterium, resulting in an accumulation of 10 or more deuterium atoms per molecule, which are distributed statistically among the newly formed molecules (5,6). Likewise, heavy labeling is obtained for stearic and myristic acids, which are minor products of fatty acid biosynthesis, as well as for their immediate desaturation products. The products of chain elongation can be recognized by the presence of an excess of deuterium near the carboxyl-end of the chain (6,16). The newly synthesized glycerol contains up to 5 deuterium atoms per molecule (14) which are presumably introduced by exchange of the activated hydrogens in the phosphorylated intermediate as well as by the reduction of the aldehyde or keto groups of the glycerophosphates. As a result it is possible to calculate the pool size and turnover rate of each glycerolipid species, provided appropriate time-course curves are determined.

Figure 3 shows a GC/MS spectrum obtained for the tertiary-butyldimethylsilyl (t-BDMS) ethers of the palmitoyloleoylglycerol species recovered from the free diacylglycerol fraction isolated from a rat liver after one hr perfusion with buffers containing 75% deuterium oxide (4). The newly synthesized glycerol in the molecule can be recognized from the ions at m/z 172-175. Myher et al. (17) have shown that the m/z 171 fragment contains four glycerol hydrogens and no acyl hydrogens, and thus can be used to measure total isotope incorporation into glycerol.

The ion intensities of m/z 172-175 are indeed elevated in relation to the m/z 171 ion. The labeling of the palmitic acid portion of the molecule is best seen by monitoring the

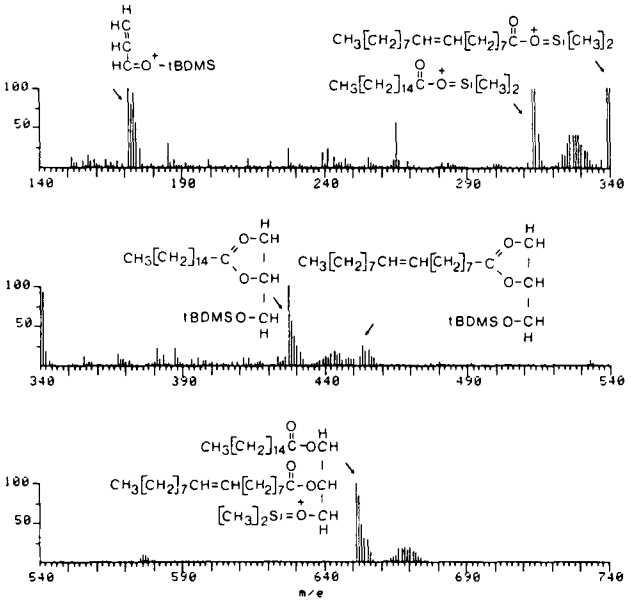

FIG. 3. GC/MS spectrum of the t-BDMS ether of the monoenoic diacylglycerols isolated from free diacylglycerol fraction of rat liver perfused for 1 hr with buffer containing 75% deuterium oxide. GC/MS conditions as given elsewhere (4).

ions at m/z 320-335, which represent the RCO + 74 fragments. In this case m/z 313 represents unlabeled palmitic acid and m/z 320-335 are the corresponding ions of species having from 7 to 22 deuterium atoms per palmitoyl residue. The ions at m/z 428-433 represent unlabeled palmitic acid residues in combination with labeled glycerol, while the ions at m/z 439-445 represent newly synthesized palmitic acid residues in combination with or without newly synthesized glycerol. The ions at m/z 453-460 represent combinations of newly synthesized glycerol with old oleic acid. The fragments at m/z 652-656 indicate the diacylglycerol molecules having labeled glycerol but not labeled fatty acids, while ions at m/z 658-675 indicate molecules with labeled palmitate plus unlabeled oleate plus labeled and unlabeled glycerol. The completely unlabeled molecule gives a fragment at m/z 651, which is due to the M-57 ion. By subtracting the amount of labeled glycerol that was found with unlabeled fatty acid from the amount of total labeled glycerol, it was possible to demonstrate that virtually all the labeled palmitic acid was coupled with labeled glycerol (14).

Figure 4 shows the mass chromatograms obtained for the t-BDMS ethers of the monoenoic diacylglycerols from the above liver perfusion (Fig. 3). The total ion current indicates the relative proportions of the diacylglycerol species ranging from 16:0 16:1 through 16:0 18:1 to 18:0 18:1. The ion at m/z 669 represents the species containing labeled palmitic acid (e.g. 15 deuteriums) plus labeled glycerol (e.g. 3 deuteriums) and unlabeled oleic acid, while the ion at m/z 651 represents the species containing unlabeled palmitic acid in combination with unlabeled oleic acid and unlabeled glycerol. The ion at m/z 427 indicates all the monoenoic diacylglycerol species which contain unlabeled palmitic acid in combination with unlabeled glycerol, while the ion at m/z 328 identifies all the monoenoic diacylglycerol species, which contain labeled palmitic acid (e.g. 15 deuteriums) as part of the molecule. The scans at m/z 174 and 171 represent the species containing labeled (e.g. 3 deuteriums) and unlabeled glycerol as part of the monoenoic diacylglycerol molecules, respectively. Following appropriate calibration, the ion yields can be quantitated and the data used to establish the time course of formation of each of the glycerolipid species (14).

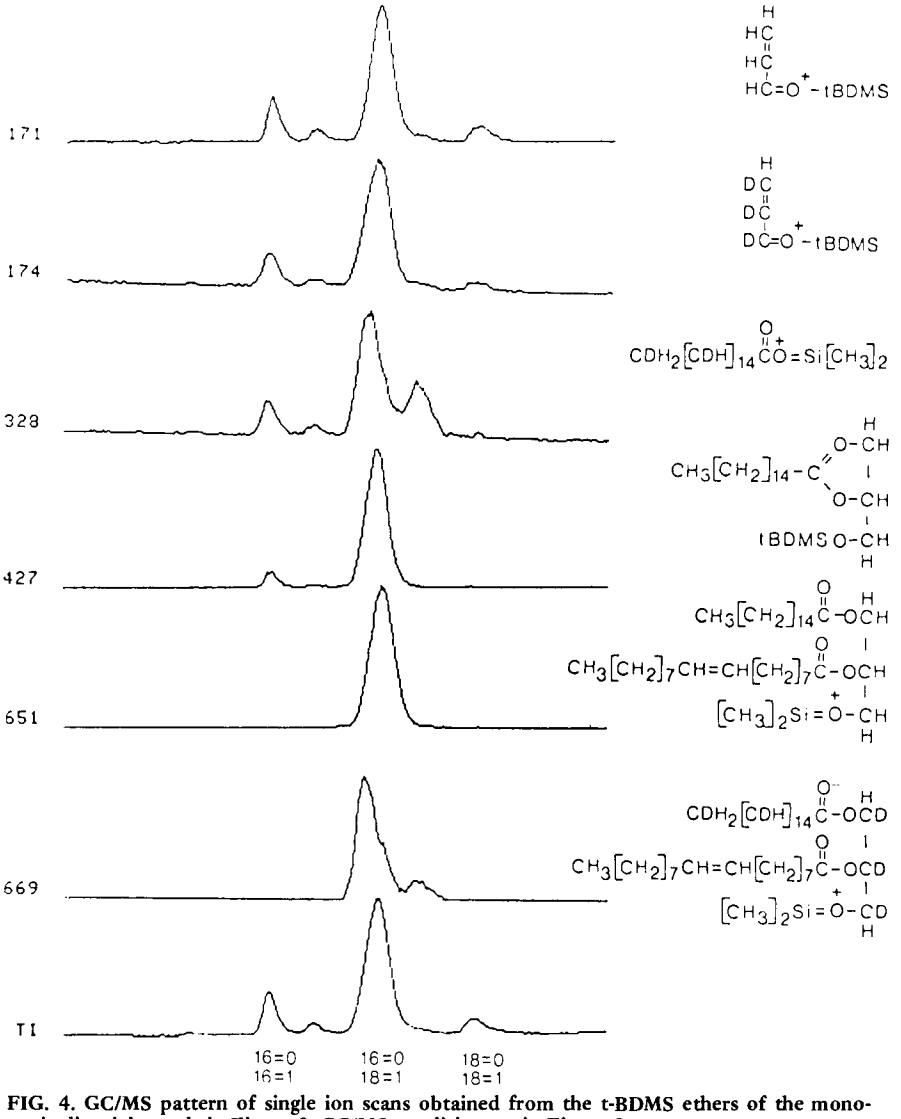

FIG. 4. GC/MS pattern of single ion scans obtained from the t-BDMS ethers of the monoenoic diacylglycerols in Figure 3. GC/MS conditions as in Figure 3.

This approach is equally well suited for the examination of the positional distribution and molecular association of the deuterium labeled fatty acids in the sn-1,2- and sn-2,3-diacylglycerol moieties of triacylglycerols, as obtained by Grignard degradation and phospholipase C hydrolysis of the rac-phosphatidylcholines prepared as intermediates (18). Since the sn-1,2- and sn-2,3-diacylglycerols share a common glycerol residue, there would be no difference in the deuterium content of the glycerol moieties of the two enantiomers. There have been no previous studies of the GC/MS spectra of deuterium labeled triacylglycerols.

LC/MS of Deuterium-Labeled Glycerolipids

The LC/MS spectra (CI mode, using eluting solvent as reagent gas) of the t-BDMS ethers of the sn-1,2-diacylglycerol moieties of the glycerophospholipids and of the sn-1,2- and sn-2,3-diacylglycerol moieties of the triacylglycerols were obtained following reversed phase resolution as described in Part I of this series (1). Figure 5 shows the total ion current profile recorded for the sn-1,2-diacylglycerol moieties of the phosphatidylcholines of the liver of a rat receiving deuterated ethanol (8), along with the complete mass spectrum of Peak 11 of the HPLC elution sequence. The peak is due to the presence of 16:0 18:2 species as a major component. The presence of deuterium is seen from the extra masses accompanying the protonated molecular ion (m/z 707) and the ions containing palmitic (m/z 427) and linoleic (m/z 451) acid in combination with glycerol. The 16:0 18:2 overlaps in part with 18:1 18:2, which also contains some deuterium as seen from the extra masses accompanying the protonated molecular ion (m/z 733) and the $[MH-132]^+$ ion (m/z 601). Figure 6 shows the mass chromatograms constructed to obtain the distribution of the molecular species containing different amounts of deuterium in the palmitic acid and glycerol moieties. It is seen that the different species contain palmitate and glycerol moieties with a comparable deuterium content. The deuterium content of the glycerol moiety can be seen from the [MH-palmitic acid]$^+$ ion, which is not shown here. Using the LC/MS technique, we have determined the deuterium content of all the molecular species of the major phospholipids of rat liver microsomes and have demonstrated that the different species of the phosphatidylethanolamine are degraded at the same rate by the endogenous phospholipases (8). In comparison to the GC/MS spectra, the chemical ionization spectra obtained by LC/MS possessed higher intensities of the ions needed for the determination of deuterium content of the total molecules and of specific parts of the molecules. The LC/MS analyses can be performed similarly to determine the positional distribution and molecular association of the deuterated fatty acids in the sn-1,2- and sn-2,3-diacylglycerol moieties derived from triacylglycerols by stereospecific degradation (18). As a result, comparisons can be made between the deuterium content of the palmitic acids in the sn-1- and the sn-3-position of the triacylglycerol molecule.

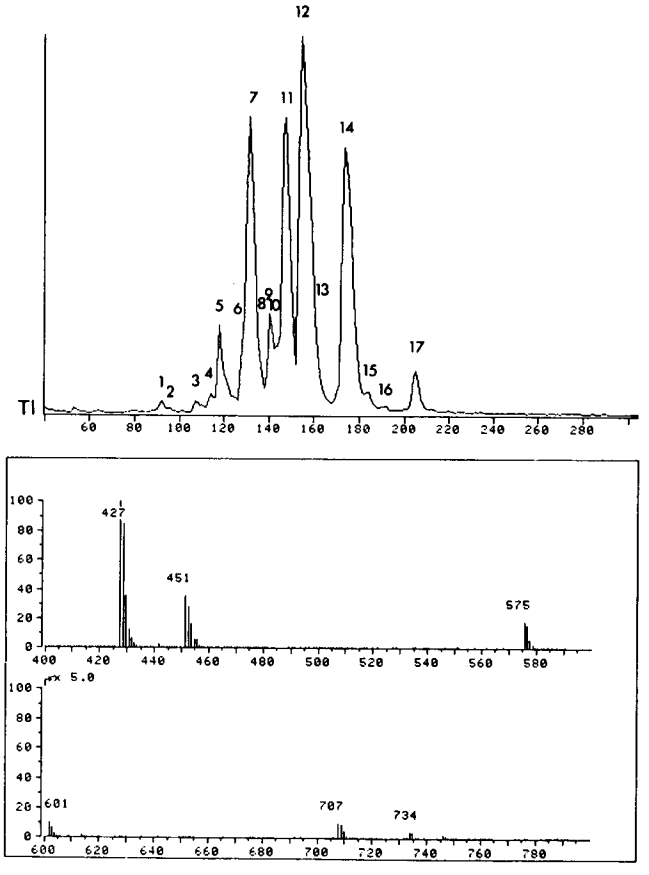

FIG. 5. LC/CIMS profile of the t-BDMS ethers of the sn-1,2-diacylglycerol moieties of the phosphatidylcholine of liver of a rat after infusion of deuterated ethanol over a period of 20 hrs (A) and the mass spectrum of Peak 11 (B). Major Peaks: 5, 16:0 22:6; 7, 16:0 20:4; 9, 18:0 22:6; 11, 16:0 18:2; 12, 18:0 20:4; 14, 16:0 18:1 + 18:0 18:2 + 18:1 18:1; 17, 18:0 18:1. LC/MS conditions as given elsewhere (19).

The obtained data can then be used to support appropriate hypotheses about the nature of the fatty acid pools serving as source of substrate during triacylglycerol biosynthesis.

Figure 7 gives the LC/MS profile of rat liver triacylglycerols along with the full mass spectra of two major triacylglycerol peaks from the elution pattern containing palmitic acid, as obtained from animals receiving perdeuterated ethanol overnight (8). The HPLC elution patterns of the deuterated triacylglycerols are similar to those obtained for the natural or non-deuterated triacylglycerols of the liver of a rat receiving non-deuterated ethanol (19). The upper spectrum in Figure 7 represents 16:0 16:0 18:2, as indicated by the $[MH\text{-}RCOOH]^+$ ions at m/z 551 (16:0 16:0) and 575 (16:0 18:2), and the $[MH]^+$ ion at m/z 831. In addition, the mass spectrum indicates the presence of 16:0 18:1 18:2 species, which is eluted just ahead of the 16:0 16:0 18:2 species. The presence of the other species is obvious from the $[MH\text{-}RCOOH]^+$ ions at m/z 601 (18:1 18:2), as well as the $[MH]^+$ ion at m/z 857, and the common ions at m/z 575 (16:0 18:2) and 577 (16:0 18:1). The lower mass spectrum represents mainly 16:0 18:1 18:1 species. The $[MH\text{-}RCOOH]^+$ ions at m/z 577 (16:0 18:1) and 603 (18:1 18:1) along with the $[MH]^+$ ion at m/z 859 attest to it. The $[MH\text{-}RCOOH]^+$ ion at m/z 603 and the $[MH]^+$ ion at m/z 885 are due to 18:1 18:1 18:1. The overlapping triacylglycerols represent critical pairs, which are only partially resolved in the present LC/MS system. The ions containing palmitic acid as well as those containing other fatty acids contain up to 5 deuterium atoms per fragment. This indicates that the deuterium is confined largely to the glycerol moiety of the triacylglycerol molecule in this sample. The presence of both deuterated palmitic acid and deuterated glycerol would be seen in other peaks. In those instances where the triacylglycerol peaks overlap too closely for a reliable assignment of the fragment ions of the deuterated and undeuterated molecules, it may be necessary to effect a preliminary resolution by argenta-

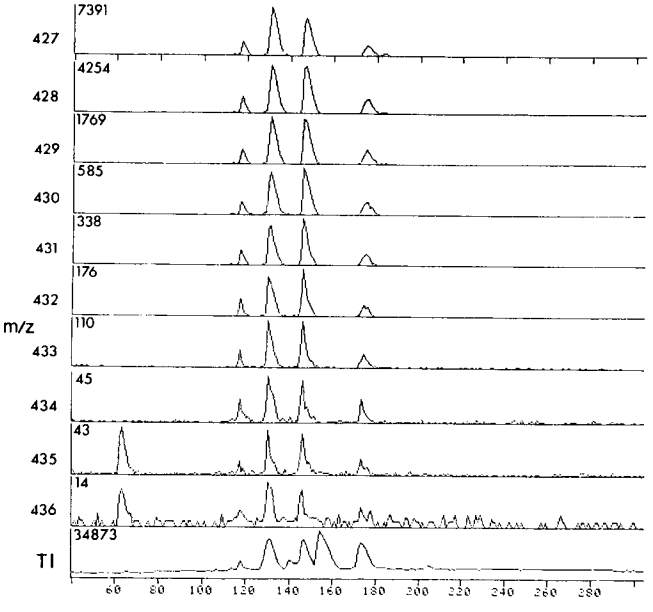

FIG. 6. Mass chromatograms of $[MH\text{-}RCOOH]^+$ ions representing the palmitoylglycerol moiety of the diacylglycerols derived from rat liver phosphatidylcholines in Figure 5. m/z 427-436, intensities of $[MH\text{-}RCOOH]^+$ ions of increasing deuterium content. II, total ion current. Other LC/CIMS conditions as in Figure 5. The relative intensity values given in the upper left hand corner of the ion chromatograms refer to the major peak in each elution pattern.

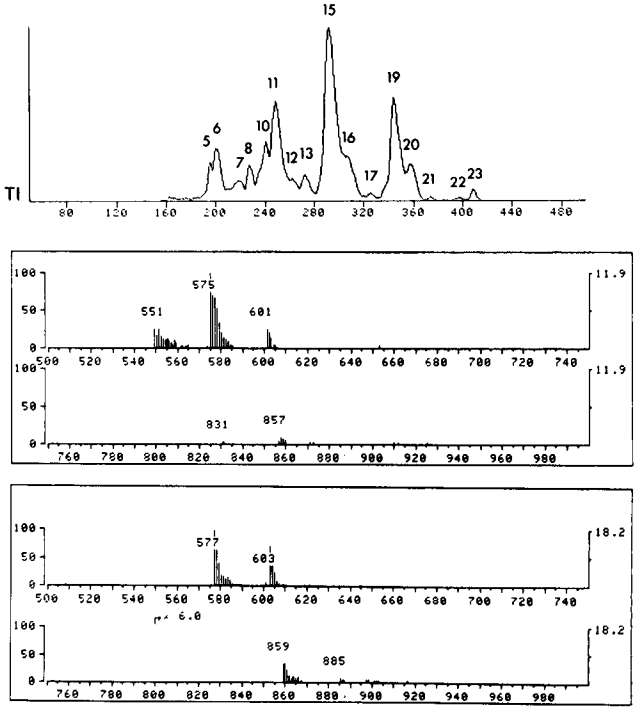

FIG. 7. LC/CIMS profile of the triacylglycerols of the liver of a rat infused with deuterated ethanol for 20 hrs (A) and the mass spectra of two of the major peaks (Peak 16 and 19) containing palmitic acid. Instrument and operating conditions as in Figure 5.

tion TLC. Figure 8 shows the LC/MS elution pattern of the diene fraction of the above triacylglycerol mixture, along with the full mass spectra for the 16:0 16:0 18:2 and 16:0 18:1 18:1 species. It is now seen that the contaminating species have been removed revealing mass spectra characteristic of single molecular species of triacylglycerols in each peak, which was not the case for the corresponding peaks shown in Figure 7.

Following a stereospecific degradation to the sn-1,2- and sn-2,3-diacylglycerols, it is also possible to compare the per cent replacement of deuterium into the palmitic acid residues of the 16:0 16:0 and of the 16:0 18:2 moieties of the triacylglycerol shown in Figures 7 and 8. In order to obtain the distribution of the palmitic acid in the reverse isomers, it is necessary to subject the sn-1,2- and sn-2,3-diacylglycerol t-BDMS ethers to a partial hydrolysis with pancreatic lipase. A comparison of the LC/MS spectra of the parent diacylglycerol and of the residual monoacylglycerol yields the desired information as explained in Part II of this series (2).

From the mass spectra in Figure 8 it is seen that the $[MH\text{-}RCOOH]^+$ ions corresponding to the 16:0 16:0 and the 16:0 18:2 moieties arising from the dienoic triacylglycerols contain significant amounts of deuterium, presumably due to the deuterated glycerol in the ion products. Separate estimates of the deuterium content of the palmitic and glycerol residues are obtained by a stereospecific degradation of the diene fraction to the sn-1,2- and the sn-2,3-diacylglycerols. Upon subsequent LC/MS analysis of the isolated sn-1,2- and sn-2,3-diacylglycerols, the 16:0 18:2 peak yields an $[MH\text{-}RCOOH]^+$ ion, which represents a combination of glycerol with linoleic acid from which an estimate of the deuterium content of the glycerol can be obtained. There is no deuterium associated with the linoleic acid.

It must be noted that a simple GC/MS or LC/MS analysis of the deuterated acylglycerols leaves certain ambiguities about the exact origin of each "monoacylglycerol" and "diacylglycerol" fragment. Instead of subjecting the entire mixture of deuterated and undeuterated acylglycerols of a particular molecular species to the fragmentation process, therefore, it would be desirable to fragment separately each mass of the parent molecules. This is theoretically possible by means of GC/MS/MS and LC/MS/MS, where the parent molecules are ionized and resolved in the first mass spectrometer followed by a fragmentation of each ionized molecule in a second mass spectrometer (20). Such analysis would allow a direct relation of the deuterium content of a specific parent molecule to the deuterium content of the resulting acylglycerol fragments. Since these analyses have not yet been performed with acylglycerol molecules, the MS/MS extension of chromatographic analyses will not be discussed further at the present time.

By means of the LC/MS analysis of the rat liver triacylglycerols labeled endogenously with deuterium, we have demonstrated that the deuterated palmitate and stearate and the deuterated glycerol are fully equilibrated with the non-deuterated counterparts throughout the molecular species of triacylglycerols at the end of a 20-hr period of infusion of deuterated ethanol (19). This conclusion is consistent with the known rapid rates of turnover of the hepatic triacylglycerol pools (21). In another study we have shown (8) that the endogenous deuterium-labeled phosphatidylethanolamines of rat liver microsomes undergo a rapid random degradation of the molecular species by endogenous lipases upon incubation in vitro in the presence of Ca^{++} ions, while the molecular species of phosphatidylcholine remain unaffected.

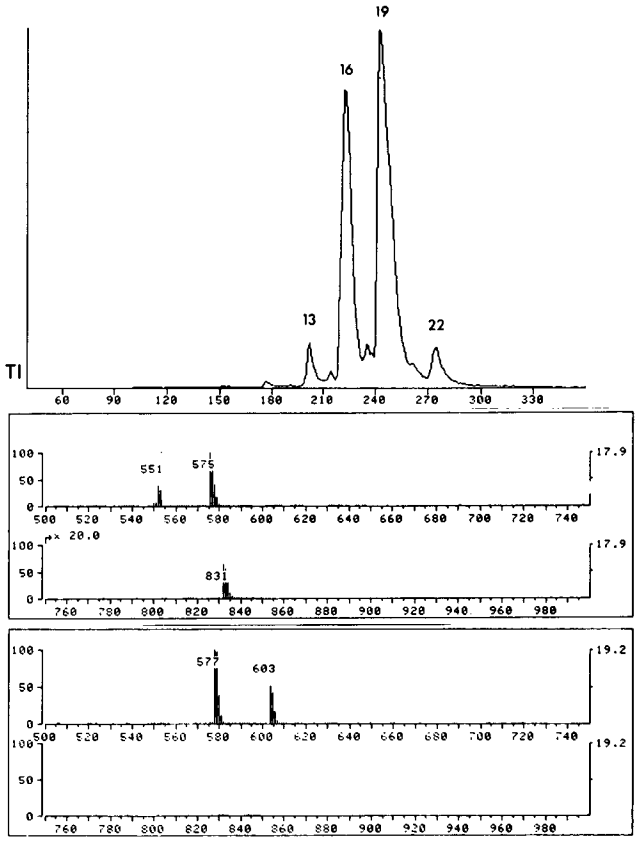

FIG. 8. LC/CIMS elution profile of the dienoic fraction of deuterated rat liver triacylglycerols (A) and the full mass spectrum for Peak 16 (B) and Peak 19 (C). Instrument and operating conditions as in Figure 5.

ACKNOWLEDGMENTS

The study was supported by funds from the Medical Research Council of Canada, Ottawa, Ontario; the Ontario Heart Foundation, Toronto, Ontario, and the Hospital for Sick Children Foundation, Toronto, Ontario.

REFERENCES

1. Kuksis, A., J.J. Myher and L. Marai. JAOCS 61:1582 (1984).
2. Kuksis, A., J.J. Myher and L. Marai. JAOCS 62:762 (1985).
3. Kuksis, A., and J.J. Myher, in Lipids, Vol. 1 (Paoletti, R., G. Porcellati and G. Jacini, eds.), Raven Press, New York, NY. (1976) pp. 23-38.
4. Kuksis, A., and J.J. Myher, in Membrane Fluidity (Kates, M., and A. Kuksis, eds.), Humana Press, Clifton, NJ, (1980) pp. 3-22.
5. Kuksis, A., J.J. Myher, L. Marai, S.K.F. Yeung, I. Steiman and S. Mookerjea. Can J. Biochem. 53:509 (1975).
6. Tanaka, Y., and S. Ando. Biochem. Res. 2:404 (1981).
7. Curstedt, T., and J. Sjovall. Biochim. Biophys. Acta. 369:173 (1974).
8. Pind, S., A. Kuksis, J.J. Myher and L. Marai. Can. J. Biochem. Cell Biol. (1985). In Press.
9. Chase, G.D., and J.L. Rabinowitz. Principles of Radioisotope Methodology, 2nd ed., Burges, Minneapolis, MN (1962).
10. Morrisett, J.D., H.J. Pawnall, R.L. Jackson, R. Segura, A.M. Gotto, Jr. and O.D. Taunton, in Polyunsaturated Fatty Acids (Kunau, W-H., and R.T. Holman, eds.) American Oil Chemists' Society, Champaign, IL, (1977) pp. 139-161.
11. Holub, B.J., and A. Kuksis. Adv. Lipid Res. 16:1 (1978).
12. Ohno, K., T. Akino and T. Fujiwara. Revs. Perinatal Medicine, 2:227 (1978).
13. Bell, R.M., L.M. Ballas and R.A. Coleman. J. Lipid Res. 22:391 (1982).

14. Steiman, I., J.J. Myher, A.G.D. Hoffman and A. Kuksis. Proc. Can. Fed. Biol. Soc. 24:182. Abstr. No. 328 (1981).
15. Curstedt, T. Biochim. Biophys. Acta 398:265 (1975).
16. Wadke, M., H. Brunengraber, J.M. Lowenstein, J.J. Dolhun and P. Arsenault. Biochemistry 12:2619 (1973).
17. Myher, J.J., A. Kuksis, L. Marai and S.K.F. Yeung. Anal. Chem. 50:557 (1978).
18. Bugaut, M., J.J. Myher, A. Kuksis and A.G.D. Hoffman. Biochim. Biophys. Acta 792:254 (1984).
19. Pind, S., A. Kuksis, J.J. Myher and L. Marai. Can. J. Biochem. Cell Biol. Submitted (1985).
20. Voyksher, R.D., J.R. Hass and M.M. Bursey. Anal. Chem. 54:2465 (1982).
21. Glaumann, H., A. Bergstand and J.L.E. Ericsen. J. Cell. Biol. 64:356 (1975).

[Received July 6, 1984]

Fatty Acid Development in a Soybean Mutant with High Stearic Acid[1]

G.L. GRAEF, Department of Agronomy; **L.A. MILLER**, Department of Food Technology; **W.R. FEHR**, Department of Agronomy, and **E.G. HAMMOND**, Department of Food Technology, Iowa State University, Ames, Iowa 50011

ABSTRACT

The fatty acid composition of developing soybean (*Glycine max* [L.] Merrill) seeds was evaluated in the mutant line, A6, and its parent, FA8077. Seeds of both lines were harvested at 2-day intervals from 15 to 39 days after flowering (DAF) and at 4-day intervals from 39 DAF until maturity. Significant differences between the two lines were observed for stearic and oleic acid percentages at 19 DAF. The maximum difference between the lines was at 25 DAF, when A6 had 45.4% and FA8077 had 4.1% stearic acid. The increase in stearic acid percentage in A6 was accompanied by a decrease in oleic acid to 16.8% at 25 DAF, compared with 53.7% oleic acid for FA8077. The two lines did not differ in development of palmitic, linoleic and linolenic acids. The protein and oil content of mature seeds were similar for the two lines.

INTRODUCTION

A soybean (*Glycine max* [L.] Merrill) mutant with high stearic acid was identified at Iowa State University in 1981. It was an M_2 plant selection from sodium azide (NaN_3) treatment of seeds from the line FA8077. The mutant, designated A6, showed a fatty acid composition never before reported for soybean (1). The average stearic acid content of soybean oil is 4.0%, with a range from 2.2% to 7.2% for genotypes available in the world collection (2). A6 contained about 28% stearic acid in its seed oil. Graef et al. (5) determined from the cross of A6 × FA8077 that the high stearic acid in A6 was controlled by a recessive allele at one locus.

In previous studies of developing soybean seeds, the stearic acid percentage of the oil seemed to remain relatively constant (3,4). Fehr et al. (3) reported that stearic acid percentages remained more or less constant during seed development, from 26 days after flowering (DAF) to maturity. Rubel et al. (4) observed a decrease in stearic acid percentage from 8% at 24 DAF to 3% at 32 DAF. The stearic acid percentage remained constant from 32 DAF until maturity. Palmitic and linolenic acid decreased in percentage during seed development, while oleic and linoleic acid percentages increased (3,4).

The objective of our study was to determine the stage in seed development at which the deviation in stearic acid synthesis for A6 is initiated and to examine the effect of high stearic acid percentage on the development of other fatty acids.

PROCEDURES

A6 and FA8077 were grown in 1.5-m rows spaced 1 m apart at the Agronomy Research Center near Ames, Iowa in May 1982. Two hundred and forty pods were tagged at the third, fourth or fifth node from the top of the main stem of plants of both lines (6). The age of seeds in each pod was expressed as DAF, which was the number of days between fertilization of the flower and harvest of the pod.

Seeds were harvested at 2-day intervals from 15 to 39 DAF and at 4-day intervals from 39 DAF to maturity (51 DAF). On each of the 16 sampling dates, 15 pods of each line were removed from the plants and taken to the laboratory. The seeds were removed from the pods and the weights of three 10-seed samples of each line were determined. Each 10-seed sample was placed in a test tube and frozen at -29 C until all samples from the 16 dates had been collected.

At the time of fatty acid analysis, each 10-seed sample was freeze-dried for 20 hr, and the dry weight of the samples was recorded. The beans were ground to a fine powder in a mortar with 20 ml/g dry weight of chloroform-methanol (2:1, v/v), then centrifuged for 15 min. A volume of water equal to 1/5 the volume of the chloroform-methanol solution was added to the supernatant. This caused the solvent to separate into two layers. The lower chloroform layer was collected, and the chloroform evaporated. The residual lipids were dissolved in hexane (10 mg/ml), and 1 ml of the hexane solution was transferred to a 2-ml reaction vial and evaporated to about 0.2 ml. Then 0.5-ml of 1.0 M sodium methoxide in methanol was added to the vials and allowed to react for 2 hr to convert the oil to methyl esters. Next, 1 ml of distilled water was added, and one hr later a few drops of distilled hexane were added to dissolve the fatty acids. The top layer of the solution (fatty acids in hexane) was removed and placed in a 2-ml glass vial. Approximately 1.5 µl of each sample were injected into a Beckman GC-5 gas chromatograph (Fullerton, California) fitted with hydrogen flame detectors. The column was 2 m long and 3.2 mm in diameter, packed with 15% EGSSX on Chromosorb W 100/120 mesh and maintained at 185 C. The N flow was 40 ml/min, H flow was 50 ml/min and air flow was 300 ml/min. Standard ester mixtures by Nucheck (Elysian, Minnesota) were run on a regular basis for calibration. A PET 2001 computer (Commodore Business Machines, Santa Clara, California) was used to control injection, integrate peak areas and calculate percentages of palmitic, stearic, oleic, linoleic and linolenic acids.

The oil samples were analyzed in three replications of a randomized complete-block design. A replication consisted of one 10-seed sample from each of the 16 harvesting periods for the two lines. Standard statistical procedures were used for data analysis. For the analysis of variance, lines and sampling periods were considered to be fixed effects.

[1]Journal Paper No. J-11444 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Projects 2475 and 2493.